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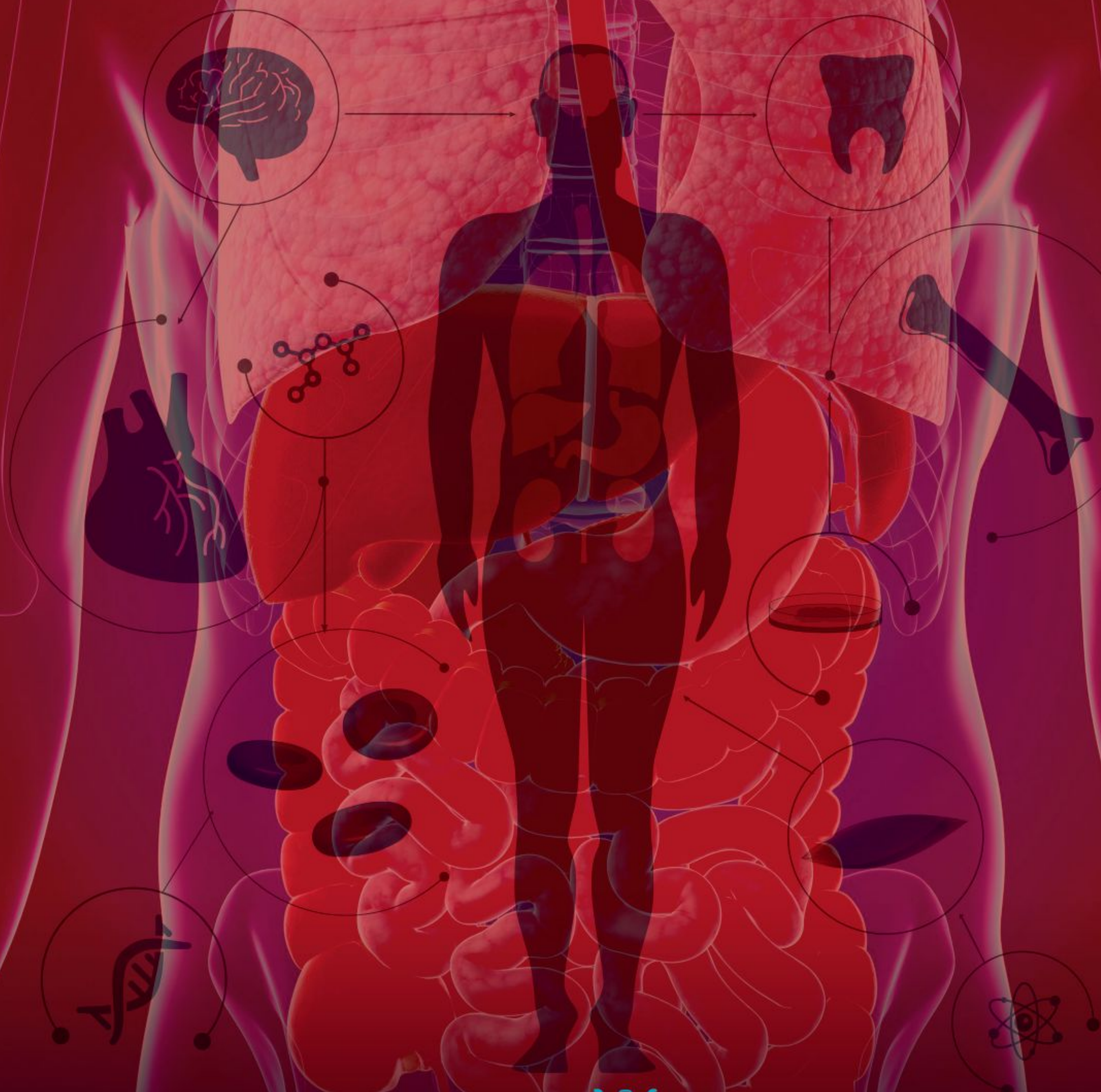
Reprint supplement

MSCs: Biology and Therapeutics

Reviews and research on MSC biology and prospects for therapeutic application.



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Foreword

We are pleased to introduce the latest edition of *Cell Press Selections*. These editorially curated reprint collections highlight a particular area of life science by bringing together articles from the Cell Press journal portfolio. In this selection, we present recent insights into the biology and therapeutic potential of mesenchymal stem/stromal cells (MSCs). We have chosen a set of Review, Perspective, and Forum articles that illustrate a broad range of current topics in this area and have paired them with three research articles that investigate contributions of MSCs to human disease.

Research into MSCs has been expanding at a rapid pace in recent years, and there is a growing appreciation of the clinical potential of MSCs. Advances in our understanding of how MSCs can contribute to tissue homeostasis, regeneration, and disease have yielded insights into their physiology and their dynamic interplay with cells in various tissues and contexts, including cancer and the immune system. These insights are informing strategies for harnessing MSC modulatory properties in a therapeutic setting. Despite this progress, many aspects of MSC biology and mechanisms of action remain unclear, and there is active debate about approaches to clinical investigation. The articles in this selection outline the current status and future prospects in all these different areas.

We hope that you will enjoy reading this collection of articles and will visit www.cell.com to find other high-quality research and review articles across the entire spectrum of the stem cell field.

Finally, we are grateful for the generosity of Rainbow Scientific and Biological Industries, who helped to make this reprint collection possible.

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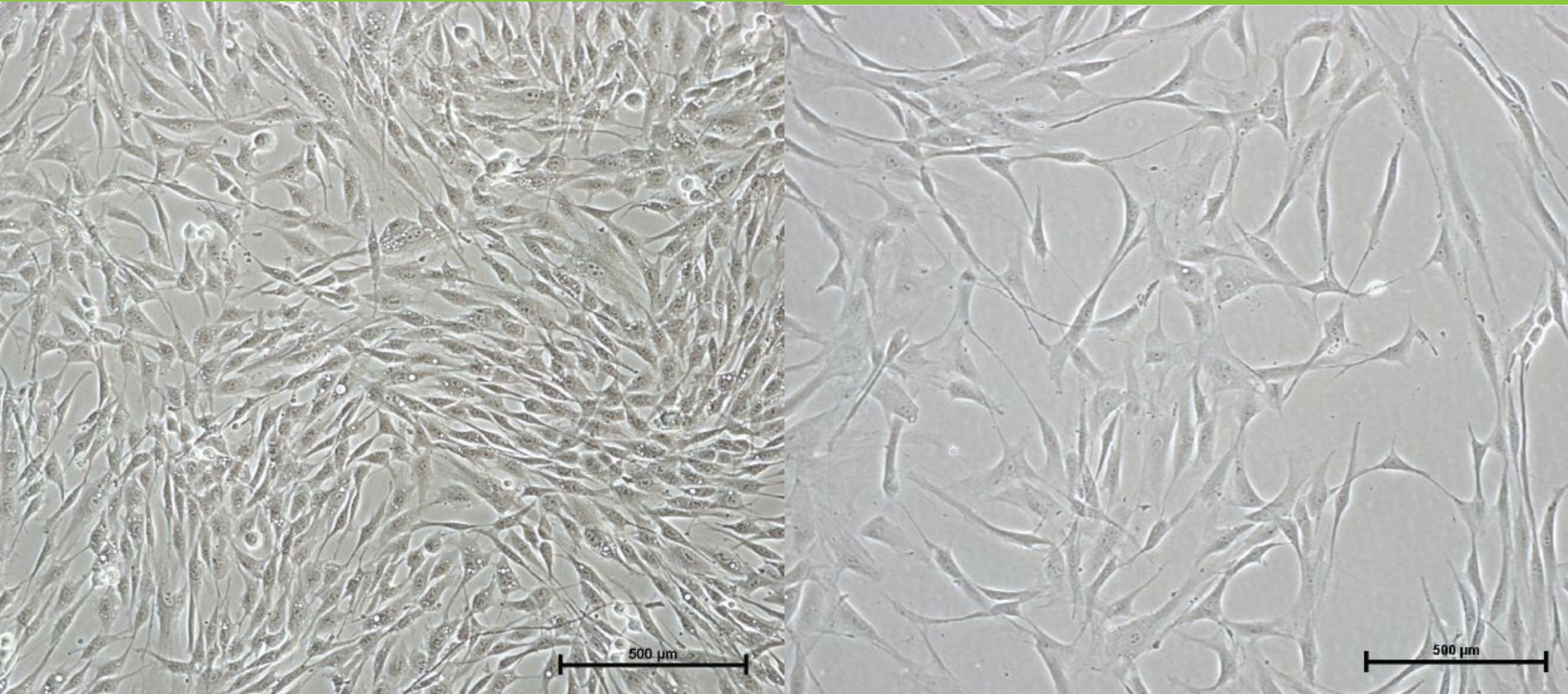

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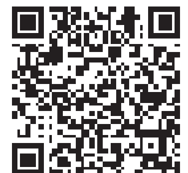

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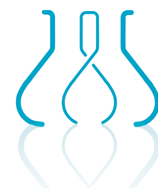

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MSCs: Biology and Therapeutics

Forum

MSC-Based Product Characterization for Clinical Trials: An FDA Perspective — *Michael Mendicino, Alexander M. Bailey, Keith Wonnacott, Raj K. Puri, and Steven R. Bauer*

Perspective

Mesenchymal Stromal Cells: New Directions — *Armand Keating*

Reviews

Mesenchymal Stromal Cells: Sensors and Switchers of Inflammation — *Maria Ester Bernardo and Willem E. Fibbe*

Harnessing the Mesenchymal Stem Cell Secretome for the Treatment of Cardiovascular Disease — *Sudhir H. Ranganath, Oren Levy, Maneesha S. Inamdar, and Jeffrey M. Karp*

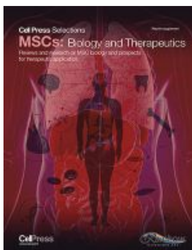
Toward *in situ* Tissue Engineering: Chemokine-Guided Stem Cell Recruitment — *Kristin Andreas, Michael Sittinger, and Jochen Ringe*

Articles

MSC-Regulated MicroRNAs Converge on the Transcription Factor FOXP2 and Promote Breast Cancer Metastasis — *Benjamin G. Cuiffo, Antoine Campagne, George W. Bell, Antonio Lembo, Francesca Orso, Evan C. Lien, Manoj K. Bhasin, Monica Raimo, Summer E. Hanson, Andriy Marusyk, Dorraya El-Ashry, Peiman Hematti, Kornelia Polyak, Fatima Mechta-Grigoriou, Odette Mariani, Stefano Volinia, Anne Vincent-Salomon, Daniela Taverna, and Antoine E. Karnoub*

Myelodysplastic Cells in Patients Reprogram Mesenchymal Stromal Cells to Establish a Transplantable Stem Cell Niche Disease Unit — *Hind Medyouf, Maximilian Mossner, Johann-Christoph Jann, Florian Nolte, Simon Raffel, Carl Herrmann, Amelie Lier, Christian Eisen, Verena Nowak, Bettina Zens, Katja Müdder, Corinna Klein, Julia Obländer, Stephanie Fey, Jovita Vogler, Alice Fabarius, Eva Riedl, Henning Roehl, Alexander Kohlmann, Marita Staller, Claudia Haferlach, Nadine Müller, Thilo John, Uwe Platzbecker, Georgia Metzgeroth, Wolf-Karsten Hofmann, Andreas Trumpp, and Daniel Nowak*

Glioblastoma Stem Cells Generate Vascular Pericytes to Support Vessel Function and Tumor Growth — *Lin Cheng, Zhi Huang, Wenchao Zhou, Qiulian Wu, Shannon Donnola, James K. Liu, Xiaoguang Fang, Andrew E. Sloan, Yubin Mao, Justin D. Lathia, Wang Min, Roger E. McLendon, Jeremy N. Rich, and Shideng Bao*



On the cover: Research into MSCs has been expanding at a rapid pace in recent years, and there is a growing appreciation of the therapeutic potential of MSCs. The cover image depicts the variety of contexts in which MSCs are being investigated for their contributions to tissue homeostasis, regeneration, and disease.

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MSC-Based Product Characterization for Clinical Trials: An FDA Perspective

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Proposals submitted to the FDA for MSC-based products are undergoing a rapid expansion that is characterized by increased variability in donor and tissue sources, manufacturing processes, proposed functional mechanisms, and characterization methods. Here we discuss the diversity in MSC-based clinical trial product proposals and highlight potential challenges for clinical translation.

Introduction

MSCs, commonly referred to as mesenchymal stem cells or mesenchymal stromal cells, are a diverse population of cells with a wide range of potential therapeutic applications. Despite considerable interest and effort, there are currently no FDA-approved Biologics License Applications (BLAs) for any MSC-based products. There is also no consistent nomenclature or definition of MSCs (Keating, 2012). In 2006, the International Society for Cellular Therapy (ISCT) proposed a set of minimal criteria to characterize MSCs (such as cell surface marker expression). More recently, researchers in the MSC field have shown that these commonly described markers are not distinctive and therefore may not be sufficient for defining the cellular composition and biological function or functions of an MSC-based product. Although not an FDA requirement, stakeholder efforts toward generating consensus on MSC definitions would be a useful development for the field, which would allow comparison across multiple studies and could facilitate potential clinical use. In order to better understand the composition, phenotype, and range of bioactivity for MSCs, it may be useful to be able to correlate MSC surface and/or secreted protein markers with their in vitro and in vivo bioactivity. However, incomplete knowledge about MSC mechanisms-of-action (MOAs) and how these may relate to outcomes for different clinical indications and routes of administration make such comparisons especially difficult. It is important to note that the FDA reviews each regulatory submission based on its own merits, and nomenclature is not a regulatory concern during early clinical development. Closer to licensure, however, nomenclature assumes greater significance for regulatory requirements, such as product labeling.

In order to track emerging trends in this rapidly expanding field, we assessed initial filings of 66 Investigational New Drug (IND) submissions to the FDA for MSC-based products and MSC-related information from worldwide clinical trial registries (as of December, 2012). We limited our assessment to MSC-based products that were used in similar ways to avoid potential confusion that would arise from making less biologically relevant comparisons. For example, we excluded whole bone marrow mononuclear preparations even when the sponsor described their product using MSC terminology. We also excluded trials that only used MSCs during manufacturing of a final cell therapy product (e.g., MSCs used for ex vivo culture only) in which MSCs were not administered to patients. Our analysis revealed a high degree of variability in terms of MSC sources, manufacturing processes, and in vitro and in vivo product characterization. This lack of consensus highlights potential challenges to the clinical translation of MSC-based products.

Donor and Tissue Source Diversity in MSC-Based Product INDs

There was an approximately 3-fold increase in the number of MSC-based product IND submissions to the FDA between 2006 and 2012 in the set we assessed. In this period, there was also a substantial increase in registered MSC clinical trials initiated worldwide (246 trials; source: http://www.clinicaltrials.gov, "Mesenchymal Stem OR Mesenchymal Stromal," queried in January, 2013). Despite this rapid expansion in clinical trials and calls for revised nomenclature, the original terminology (Mesenchymal Stem Cell) still appears to predominate. We found that the term "Mesenchymal Stem Cell" was used to describe 72% of MSC-based product registered clinical trials worldwide, and 76% of MSC-based products in original IND submissions prior to 2013. As the number of registered clinical trials worldwide and MSC-based product IND submissions increased, the diversity in donor and tissue source increased as well. Donor source diversity refers to whether the cells were isolated from an autologous (self) or allogeneic (non-self) donor. It also refers to the variability observed between donors, which could be related to age and health of the donor among other factors. Almost all MSC-based product INDs prior to 2008 were sourced from allogeneic donor bone marrow (e.g., 100% in 2006). Since then, MSC donor and tissue source diversity has significantly increased. For example, the percentage of allogeneic MSC-based products being evaluated under IND decreased to 42% in 2011 (the only year the percentage dropped below 50% prior to 2013) and increased to

73% allogeneic MSC-based product INDs in 2012.

One parameter that significantly affects how MSCs are described according to the literature is tissue source (Phinney and Sensebé, 2013). Strikingly, the proportion of the 66 IND submissions that evaluated MSC-based products derived from bone marrow was 100% through 2007 but decreased to ∼55% by 2012 (Figure 1). Prior to 2013, the second most common source under IND was umbilical cord or placental tissue; the third most common was adipose tissue. The number of adipose-derived MSC-based product INDs has increased significantly since 2011 (there was a 3-fold increase between 2011 and 2012 alone). This shift is consistent with clinical trials registered worldwide, where less than half of the MSC-based product trials registered in 2012 used bone marrow as a source.

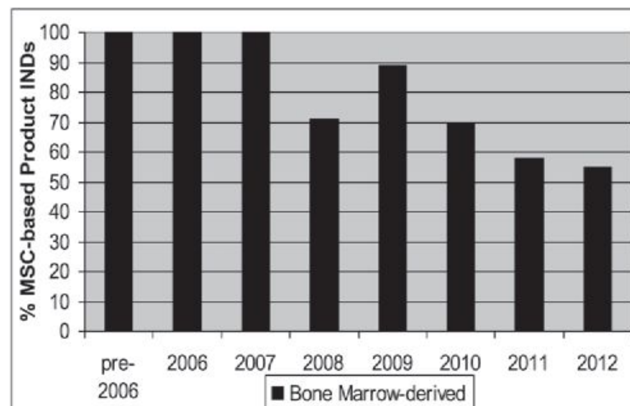

Figure 1. The Tissue Source for MSC-Based Product INDs Has Become Diverse over Time
Data from original IND submissions were compiled (n = 66). The percentage of bone-marrow-derived MSC-based product INDs is displayed per year.

Manufacturing Diversity in MSC-Based Product INDs

MSC researchers and manufacturers use a wide range of protocols to manufacture their MSC-based products. Of these manufacturing differences, we assessed four prominent parameters that have the potential to influence product characteristics: (1) fetal bovine serum (FBS), (2) atmospheric oxygen (∼21%), (3) cryopreservation of the final product, and (4) cell banking (working or master cell bank). The majority of regulatory submissions (over 80%) describe the use of FBS during manufacturing. The range of FBS concentration in media ranges from approximately 2%–20%, with 10% FBS the most common concentration. Different percentages of FBS result in different amounts of growth factors present in a culture, thus some MSC manufacturers emphasize the importance of qualifying FBS lots to facilitate product comparability between manufacturing runs. However, it may be challenging to determine which tests and product characteristics (i.e., critical quality attributes) are the most appropriate to select for comparison. The choice of assays and/or markers tested can be just as challenging for implementing manufacturing process changes with respect to demonstrating final product comparability (Carmen et al., 2012). The most common alternative for FBS described in regulatory submissions is human platelet lysate, where performing qualification and demonstrating comparability are similarly important. Many submissions describe the use of growth factors in addition to serum (∼25%). Importantly, many sponsors describe process development to include replacing the use of animal-derived serum during their manufacturing process.

Most (∼90%) MSC-based product submissions describe the use of atmospheric oxygen during cell culture. Some groups have described the utility of more physiological oxygen conditions for MSC manufacture (e.g. low oxygen tension, such as 5% O_2).

The majority of MSC-based product regulatory submissions (over 80%) also describe the use of cryopreservation to store and transport their final product, which is usually thawed within a few hours of patient infusion. Recently, MSC researchers have described challenges inherent in assessing the potential functionality of MSCs after thawing immediately before infusion, especially when bioactivity assays are often performed on MSCs prior to or without cryopreservation, or following culture rescue (Galipeau, 2013). A common postthaw test described in an MSC-based product regulatory submission is viability, expected to exceed 70% for intravenously administered MSCs. However, it is unclear how relevant viability is for MSC functionality immediately postthaw especially when considering the potential for delayed cytotoxicity (e.g., 24–72 hr). For example, there is no well-documented evidence that MSCs have the ability to produce and secrete factors produced de novo in response to microenvironmental cues immediately postthaw.

A smaller proportion (35%) of regulatory submissions described the use of cell banking systems. When cell banking is employed, a multitiered system (e.g., master cell bank and working cell bank) is described about one-third of the time. If a cell banking system is described, we have found that MSCs, in general, are grown to a higher range of passage numbers or population doublings to manufacture the final product. Even when no banking system is employed, there is a wide range of passages or population doublings described to manufacture the final product. For example, passage numbers approaching ten have been noted. Although population doubling data is more informative, it is often not described. Given the logistical requirements of a cell banking system, all banked MSCs are cryopreserved at one or more stages of manufacturing.

Cell Surface Marker Characterization Proposed in MSC-Based Product INDs

We found substantial variability in the panel of cell surface markers proposed for characterization of MSC-based products in FDA regulatory submissions in terms of frequencies and ranges of expression (Table 1). The number of MSC markers used for characterization at different stages (i.e., in-process and/or lot release testing) is also variable (Table S1 available online). In general, even in cases where many markers are studied "for information purposes only," only a select few markers are proposed for lot release criteria. Although there is variability in the proposed marker criteria described in a given MSC-based product regulatory submission, seven of the nine initial ISCT-proposed markers (Dominici

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Table 1. MSC-Based Product Phenotypic Marker Expression Proposed in MSC-Based Product INDs

Usage RANK	Common Product Marker Name	% Usage	Range Described RANK	% with Range Described	Av. Min. % ± SD	Av. Max. % ± SD
1	*CD45*	*91*	*2*	*58*	*0 ± 0*	*7 ± 6.84*
2	**CD105**	**73**	**1**	**67**	**88 ± 7.54**	**100 ± 0**
3	**CD90**	**61**	**3**	**36**	**87 ± 7.17**	**100 ± 0**
4	**CD73**	**52**	**4**	**29**	**86 ± 7.24**	**100 ± 0**
5	*CD34*	*48*	*7*	*21*	*0 ± 0*	*9 ± 6.56*
6	*CD14*	*47*	*6*	*24*	*0 ± 0*	*7 ± 7.00*
7	*HLA Class II*	*44*	*5*	*27*	*0 ± 0*	*9 ± 7.15*
8	**CD44**	**30**	—	—	—	—
9	**HLA Class I**	**26**	**10**	**14**	**74 ± 18.60**	**100 ± 0**
10	**CD29**	**24**	—	—	—	—
11	**CD106**	**23**	—	—	—	—
12	*CD19*	*21*	—	—	—	—
13	*CD80*	*21*	—	—	—	—
14	*CD86*	*21*	—	—	—	—
15	**CD166**	**20**	**8**	**18**	**92 ± 4.52**	**100 ± 0**
16	**CD10**	**18**	—	—		
17	**CD146**	**15**	**9**	**15**	**67 ± 4.83**	**100 ± 0**
18	*CD40*	*15*	—	—	—	—
19	*CD11b*	*14*	—	—	—	—
20	**CD200**	**12**	—	—	—	—

The top 20 product markers are displayed and ranked as aggregate data in terms of percent usage across all original MSC submissions analyzed (n = 66). "% Usage" refers to how often a particular marker is qualitatively proposed at any stage of product characterization across all MSC submissions analyzed (i.e., positive or negative). In addition, the top 10 product markers, described using quantitative ranges of expression proposed, are displayed and ranked as aggregate data in terms of percent usage across all original MSC submissions analyzed (n = 66). "% with range described" refers to how often a particular marker was proposed with a quantitative range of expression. "Av. Min. ± SD" refers to the average minimum value in the range of expression, plus or minus the standard deviation. "Av. Max. ± SD" refers to the average maximum value in the range of expression, plus or minus the standard deviation. Positive markers are in bold and negative markers are in italics. All percentages are rounded to the nearest whole percent value, and standard deviations are to two decimal places.

et al., 2006) are ranked at the top of the list (Table 1). The same top seven markers were specifically utilized for lot release criteria, such as for sponsor-proposed identity and purity (see FDA's guidance document for more information on identity and purity at http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm092705.pdf). We found that most of the MSC-based product IND submissions propose some subset of seven of the initial ISCT-proposed marker criteria (CD105, CD73, CD90, CD45, CD34, CD14, and HLA class II), albeit with more loosely defined ranges of expression. For example, instead of CD105 expression levels at 95% or greater as per ISCT-proposed criteria, MSC-based submissions propose CD105 expression levels as low as ~80%. It is unclear whether the quantitative difference in expression levels proposed is relevant in terms of overall MSC product characterization. The same seven markers are arguably the most commonly described in the literature as well (Mafi et al., 2011).

MSC-Based Product Bioactivity Characterization In Vitro and In Vivo

We also found significant heterogeneity in descriptions of MSC bioactivity characterization in situations where a candidate marker for a given assay has been defined. "Candidate marker" refers to a molecular marker that may be correlated with bioactivity. Examples include a secreted factor, or expression of proteins on the surface of either the MSCs or target cells (e.g., T cells) that may be related to a given biological activity. Such candidate markers are often proposed by the sponsor as the potential basis for a potency assay during clinical development (an industry perspective on cell therapy potency is reviewed in Bravery et al., 2013); also, the FDA has published a guidance document on cell therapy potency (see FDA website at http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM243392.pdf). Our survey found that less than half of the initial MSC-based product IND submissions describe marker-based bioactivity assays. Of the regulatory submissions that did contain such descriptions, most were submitted by commercial sponsors using MSCs from allogeneic donors. The described markers of bioactivity include factors secreted from MSCs, such as proangiogenic growth factors or anti-inflammatory/Th2 cytokines, as well as markers tested in MSC-leukocyte coculture proliferation assays. Another consideration that can be taken into account is emerging evidence that MSC tissue source may impact bioactivity. In one example, adipose-derived MSCs were found to have a greater immunosuppressive capacity on

T cells and monocytes via increased expression/secretion of anti-inflammatory factors in comparison to bone-marrow-derived MSCs (Melief et al., 2013).

MSC bioactivity may also be dependent on product-specific factors, clinical indication, and route of administration. Many groups have shown the influence of the microenvironment on cell bioactivity. Given the responsiveness of MSC characteristics to microenvironmental cues, we looked at target clinical indications for MSC-based product regulatory submissions and proposed routes of administration. We found that MSCs-based products are being investigated for a wide range of clinical indications. Cardiovascular and neurological targets are the most common, with orthopedic targets not far behind (out of the same 66 MSC-based product INDs prior to 2013). In addition, nearly one-fourth of MSC-based product regulatory submissions propose to investigate indications characterized primarily by immune-mediated disease attributes, such as for treatment of Graft versus Host Disease (GvHD). Multiple routes of administration are proposed. Just over half of submissions surveyed propose intravenous, while the other half proposes a range of routes, including but not limited to injection directly into the heart (often via a custom delivery device), intramuscular injection, and topical application. The wide-ranging clinical indications and routes of administration were also observed in MSC clinical trials registered worldwide. The varying understanding of how MSCs may exert beneficial effects, and the often unknown influence of clinical condition and route of administration on an MSC-based product's bioactivity, leaves many unanswered questions for the scientific community to address.

We conducted an analysis of proof-of-concept animal studies submitted to the FDA that describe MSC-based product characterization in vivo (48 MSC-based product INDs submitted from 2007–2011, a subset of the 66 submissions assessed here). The majority of MSC-based products are evaluated in proof-of-concept animal studies. Many of the study designs incorporated a combined evaluation of the cellular product's (1) phenotype, (2) proliferative ability, (3) distribution, and (4) survival/persistence postadministration, which may be useful for determining a product's MOA. While no MSC-based product was evaluated for all four of these parameters, 23% of MSC-based products were evaluated for three, 37% of products were evaluated for two, and 14% of products were evaluated for one of these parameters. About one-fourth of submissions did not report an evaluation of any of these parameters in their proof-of-concept animal studies. Specifically, when MSC-based products were evaluated in animal studies, proliferation and phenotype postadministration were described for less than 20% of the products, distribution was described for nearly two-thirds of the products, and persistence was always described for the products. The contribution of assessing each of these four parameters to better understand what an MSC population contains and how the cells exert biological activity is yet to be determined.

Concluding Comments

Many stakeholders portray MSCs as well understood, homogeneous cell types with predictable properties. However, there is significant diversity in how sponsors have defined, manufactured, and described MSCs in regulatory submissions to the FDA. This diversity is apparent for tissue sourcing, product manufacturing, cell surface marker expression, and other in vitro and in vivo MSC-based product characteristics reported by sponsors. However, a subset of seven cell surface markers are routinely identified in MSC-based product IND submissions (CD105, CD73, CD90, CD45, CD34, CD14, and HLA class II), which is consistent with those markers most commonly described in the literature. It remains unclear which particular set of markers will be sufficient to describe this complex and heterogeneous product class. In their 2006 position paper, ISCT emphasized that their proposed identifying criteria were not to be confused with final product lot release specifications developed for clinical trials (Dominici et al., 2006). Interestingly, literature and regulatory submission descriptions appear to indicate that many researchers believe otherwise. In addition, the fraction of "stem-like" cells in a population of MSCs appears to be relatively rare, quite heterogeneous, and can vary in proportion depending on donor and tissue source (i.e., interpopulation heterogeneity). Variation can be found even when the same donor and tissue source is utilized (i.e., in cases of intrapopulation heterogeneity), which can further vary based on manufacturing conditions. The assumption that the most commonly described marker set is sufficient to characterize MSCs in order to understand what an MSC population contains and what its potential functions are may pose a challenge for clinical translation (and ultimately licensure) to be addressed by the scientific community.

Further characterization of MSC-based products to better understand the existence, phenotype, and impact of MSC subpopulations may also be important for advancing MSC-based therapies. This is especially true for non-bone-marrow-derived MSC-based products, where even less information is often described by sponsors. Early development of assays and screening for MSC-specific markers, an approach taken by the FDA MSC Consortium (Lo Surdo et al., 2013; Mindaye et al., 2013), contributes to our understanding of the composition of MSC-based products and their in vitro and in vivo bioactivity. Markers that can predict potential therapeutic benefit may inform correlation of more MSC characterization data with clinical data as it becomes available. The analyses described herein are not meant to imply that there is a defined set of characterization markers that are required for FDA approval. Rather, our goal is to highlight potential challenges to the clinical translation of MSC-based products in an effort to inform research efforts supporting development of MSC-based cellular therapies.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes one table and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.01.013.

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REFERENCES

Bravery, C.A., Carmen, J., Fong, T., Oprea, W., Hoogendoorn, K.H., Woda, J., Burger, S.R., Rowley, J.A., Bonyhadi, M.L., and Van't Hof, W. (2013). Cytotherapy *15*, 9–19.

Carmen, J., Burger, S.R., McCaman, M., and Rowley, J.A. (2012). Regen. Med. *7*, 85–100.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, Dj., and Horwitz, E. (2006). Cytotherapy *8*, 315–317.

Galipeau, J. (2013). Cytotherapy *15*, 2–8.

Keating, A. (2012). Cell Stem Cell *10*, 709–716.

Lo Surdo, J.L., Millis, B.A., and Bauer, S.R. (2013). Cytotherapy *15*, 1527–1540.

Melief, S.M., Zwaginga, J.J., Fibbe, W.E., and Roelofs, H. (2013). Stem Cells Transl Med *2*, 455–463.

Mindaye, S.T., Ra, M., Lo Surdo, J.L., Bauer, S.R., and Alterman, M.A. (2013). Stem Cell Res. (Amst.) *11*, 793–805.

Mafi, P., Hindocha, S., Mafi, R., Griffen, M., and Khan, W.S. (2011). Open Orthop J *5* (*Suppl 2*), 253–260.

Phinney, D.G., and Sensebé, L. (2013). Cytotherapy *15*, 140–145.

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Mesenchymal Stromal Cells: New Directions

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Research into mesenchymal stromal/stem cells (MSCs) has been particularly exciting in the past five years. Our understanding of mechanisms of MSC-mediated tissue regeneration has undergone considerable evolution. Recent investigation of the primary in situ counterpart of cultured MSCs has led to fresh insights into MSC physiology and its role in the immune system. At the same time, the clinical application of MSCs continues to increase markedly. Taken together, a reappraisal of the definition of MSCs, a review of current research directions, and a reassessment of the approach to clinical investigation are timely and prudent.

Introduction

Few cell types have captivated so many biomedical researchers over the last 10 years as have mesenchymal stromal/stem cells (MSCs). PubMed, in 2012, identifies over 17,000 references for "mesenchymal stem cells" and more than 4,500 for "mesenchymal stromal cells." There have been several comprehensive recent reviews on MSCs (Uccelli et al., 2008; Bianco et al., 2008; Tolar et al., 2010; Ranganath et al., 2012). Hence, rather than cover all of the work in this field, in this perspective I will focus on some areas that have seen notable advance in the past 5 years and others that warrant further investigation to improve our insight into the properties and potential of this intriguing cell population.

First, a case can be made to revisit the nomenclature and definition of MSCs, not as a semantic exercise, but to better define the direction of research. The availability of new molecular tools makes the need for rigorous definitions increasingly important. Moreover, the differences between MSC populations derived from different tissues are becoming more apparent, presenting an additional challenge to devising a universal definition. MSCs as currently defined are a phenomenon of in vitro culture, suggesting that extrapolating the function of these cells to activity in vivo must be done with caution. This limitation highlights the need for direct in vivo studies with endogenous MSCs or an equivalent physiological population as an essential next step in establishing their true biological role. It is encouraging in this regard that recent studies have employed transgenic animal models to enable the tracking and assessment of MSC-like cells in vivo. The mechanisms underlying tissue regeneration and immune modulation by therapeutic doses of MSCs also require further elucidation, particularly the extent to which the two processes intersect. The more recent appreciation that MSCs may not mediate tissue regeneration by direct cell replacement is also likely to redirect investigation into more fruitful directions. Finally, in view of the extraordinarily rapid and extensive use of MSCs clinically, a reappraisal of the approach to the development of clinical protocols based on confirmed laboratory and preclinical observations would be timely and helpful.

Background

MSCs were initially identified as a subpopulation of bone marrow cells with osteogenic potential as shown by heterotopic transplantation and subsequently were confirmed to contain clonal, plastic adherent bone-marrow derived nonhematopoietic cells in the mouse and guinea pig (Friedenstein et al., 1968, 1970, 1976). An in vitro colony assay developed by Friedenstein and coworkers to detect the clonogenic cell among this population (the colony-forming unit-fibroblast [CFU-F]) was also adapted for human marrow (Castro-Malaspina et al., 1980). Subsequent studies in the 1980s focused on the role of a similar population of bone marrow stromal cells derived from the adherent layers of long-term bone marrow cultures in supporting hematopoiesis (Dexter et al., 1977, reviewed in Clark and Keating, 1995). Caplan's proposal that these cells were mesenchymal "stem" cells (Caplan, 1991) capable of differentiation to all cells of mesodermal lineage stimulated investigation into their role in mediating tissue regeneration. Although the multilineage differentiation potential of MSCs was later shown (Pittenger et al., 1999), in vivo demonstration that these cells possess the hallmark stem cell characteristics of self-renewal and differentiation had not been accomplished.

Confusion arising from the definition of the MSC population made comparisons among published studies in the 1990s and 2000s problematic and led to the proposal of new terminology and criteria by the International Society for Cellular Therapy (ISCT) (Horwitz et al., 2005; Dominici et al., 2006). According to these widely adopted proposals, the cells were more appropriately considered mesenchymal stromal cells given that not all were stem cells (Horwitz et al., 2005). The minimum criteria for MSCs included plastic adherence and in vitro trilineage differentiation to adipogenic, chondrogenic, and osteogenic cells (Dominici et al., 2006). Additional requirements included cell surface expression of CD105 (endoglin, SH2), CD73 (ecto-5′-nucleotidase), and CD90 (Thy1) and the absence of the hematopoietic markers, CD45, CD19, CD19 or CD79, CD14 or CD11b, and HLA-DR. A particular challenge for the field has been the absence of a specific marker to define MSCs, although a large number of different determinants have been associated, albeit not exclusively, with them (reviewed by Lindner et al., 2010 for human MSCs), including CD271 (low-affinity nerve growth factor receptor) (Jones et al., 2002) and CD146 (Sacchetti et al., 2007). MSCs are also highly active metabolically, secreting not only components of the extracellular matrix (Wight et al., 1986) but also a vast array of cytokines (reviewed by Horwitz and Dominici, 2008). More recent work has documented

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extensively the secretome and proteome of MSCs (Ranganath et al., 2012).

In addition to bone marrow, MSC populations can be obtained readily from adipose tissue (Zuk et al., 2002) and also from a variety of tissues including placenta (In ’t Anker et al., 2004), skin (Shih et al., 2005), umbilical cord blood (Erices et al., 2000), umbilical cord perivascular cells (Sarugaser et al., 2005), umbilical cord Wharton’s jelly (Wang et al., 2004), dental pulp (Gronthos et al., 2000), amniotic fluid (Nadri and Soleimani, 2007), synovial membrane (De Bari et al., 2001), and breast milk (Patki et al., 2010).

Revisiting the Definition of MSCs

The minimum criteria for defining MSCs established earlier (Horwitz et al., 2005; Dominici et al., 2006) may now be unduly constraining for a number of reasons. First, the characteristics of MSCs may vary according to the source of tissue. In an effort to define an MSC-like product, scientific entrepreneurs and biotechnology companies have focused on differences in surface marker profile to optimize intellectual property protection of relatively similar cell types. The recognition of species-specific differences in cell characteristics and generation of a variety of transcriptional and secretomic signatures for the cells also indicate diversity. Moreover, panels of reagents (especially antibodies) equivalent to those available for characterizing human MSCs are still not in place for a number of other species, so the criteria recommended by the ISCT (Dominici et al., 2006) may be difficult to meet.

The challenge is to devise an appropriate definition without losing the benefit that the current criteria provide in enabling evaluation of different studies of similar, if not identical, cell populations. A major hurdle is the absence of a single characteristic or marker with which to define MSCs. Nonetheless, a re-evaluation is timely and will require consensus among leading investigators in the field. In addition to standard methods of cell characterization of which surface marker profile and differentiation potential are the mainstays, the relative benefits of more advanced molecular tools including assessments of the cell transcriptome, proteome, and secretome (Ranganath et al., 2012) should be evaluated in creating this new definition. Moreover, the need to demonstrate trilineage differentiation, especially toward the chondrogenic lineage by MSCs derived from tissues other than bone marrow, also requires reassessment.

It is possible that a global definition of MSCs may now be overly simplistic or unnecessary. Specific definitions of particular MSC subsets may suffice, provided that they accurately and reproducibly define the cells under study. For example, the so-called stromal vascular fraction (SVF) of adipose-derived cells represents a highly heterogenous cell population and contains cells that express CD90 but not CD105 until they become plastic adherent (Yoshimura et al., 2006). Nonetheless, the cells have been considered to be MSC like. This issue is of additional significance because SVF cells have been extensively applied in clinical settings, despite a paucity of reported trials. It is unclear whether these cell products are uniformly defined prior to clinical administration.

Some general concepts of a new approach to the nomenclature, definition, and characterization of MSCs may provide a framework for discussion. The rationale is to help inform the investigation of these cells rather than to serve merely as a classification:

(1) The general population of MSCs should continue to be identified as mesenchymal stromal cells, although this is not an ideal term.
(2) The term ‘‘mesenchymal stem cell’’ should be used to specifically describe a cell with documented self-renewal and differentiation characteristics.
(3) MSCs should be categorized as cultured or primary—this is an important distinction (see below) because the characteristics are likely to be different and should avoid confusion when comparisons are made between studies.
(4) The source of MSCs should be specified (e.g., adipose, BM, cord blood, etc.); differences in cell characteristics are likely to be encountered.
(5) Species should be identified—this information is not always explicitly stated in the text of publications (except in the Methods section) and has led to confusion in the past.
(6) Minimum criteria for a surface marker profile need to be revisited and are likely to vary among species.
(7) The need to document the in vitro differentiation potential of the cells should be re-examined.
(8) The in vitro clonogenic capacity of MSCs should be enumerated.
(9) The reproducible representation of transcriptome, proteome, and secretome of MSCs should be evaluated and the major factors influencing the signatures should be identified and specified.
(10) Consideration should be given to characterizing the cells according to tissue specificity (e.g., the differentiation potential of human umbilical cord perivascular cells is more extensive than for BM MSCs).

Stem Cell Properties of Cultured MSCs

Despite numerous reviews attesting to the stem cell nature of MSCs from their ability to undergo differentiation along at least three lineages, there appear to be only three studies that can lay claim to identifying stem cells among human cultured MSCs, on the basis of rigorous clonal analysis. Muraglia et al. (2000) showed by limiting cell dilution that clones arising from single cells of bone marrow stromal cultures displayed multilineage differentiation potential and exhibited self-renewal. These authors proposed a hierarchical model in which there was sequential loss of lineage potential from the most primitive osteo-chondroadipogenic to osteo-chondrogenic, and finally to osteogenic precursors. Notably, osteo-adipogenic and chondro-adipogenic precusors were not detected, nor were purely chondrogenic or adipogenic clones. Lee et al. (2010) conducted single-cell studies of GFP-marked human MSCs (using irradiated stromal feeder layers to facilitate growth) and demonstrated that a minor subpopulation with high proliferative potential exhibited differentiation along osteogenic, chondrogenic, and adipogenic lineages and could self-renew from colony replating assays.

Analyzing the clonogenic differentiation capacity of another MSC population, human umbilical cord perivascular cells

(HUCPVCs), Sarugaser et al. (2009) documented the self-renewal and multipotent capacity of an infrequent mesenchymal stem cell able to differentiate to myogenic, osteogenic, chondrogenic, adipogenic, and fibroblastic lineages and proposed a hierarchical stem cell lineage relationship for these cells. These examples highlight the differences in differentiation potential between cells obtained from different tissues. This is an important area of investigation because as in the case of hematopoietic stem cell lineage relationships, much can be learned from studies of MSC clones that may be lost by an investigation of a heterogeneous MSC population, even one enriched for clonogenic cells.

Immunomodulatory Properties of Cultured MSCs

At this point, there is a considerable body of literature documenting the pleotropic effects of MSCs on the immune system. MSCs act on both the adaptive and innate immune systems by suppressing T cells, suppressing dendritic cell maturation, reducing B cell activation and proliferation, inhibiting proliferation and cytotoxicity of NK cells, and promoting the generation of regulatory T cells via an IL-10 mechanism. The role of MSCs in mediating these processes by affecting the expression of inflammatory cytokines is well established. This topic has been covered extensively in several reviews (Nauta and Fibbe, 2007; Le Blanc and Ringdén, 2007; Uccelli et al., 2008; Tolar et al., 2010; Chen et al., 2011, among others), and I will therefore focus on drawing attention to a few key issues.

One major area of MSC-mediated activity is T cell suppression (Yang et al., 2009). Several recent studies have identified pathways that are involved, including downregulation of NF-κB signaling and cell cycle arrest at G0/G1 (Jones et al., 2007; Choi et al., 2011). However, it is still somewhat unclear to what extent these pathways will have physiological significance. Some of the confusion in the literature in this area may be alleviated by the appreciation that there are major differences in the mechanisms of T cell suppression among species. For example, in humans and Rhesus monkeys, indoleamine 2,3- dioxygenase (IDO) is predominantly involved in T cell suppression, whereas nitric oxide is the main mediator in mice (Ren et al., 2008; Ren et al., 2009).

One emerging area of investigation involves studies of Toll-like receptors (TLRs) on MSCs and their contribution to immune modulation. These receptors respond to so-called danger signals consisting of molecules released by injured tissue or microbial invasion (e.g., endotoxin, LPS, dsRNA, and heat shock proteins). At least ten human TLRs are known and are expressed on innate immune effector cells (Kawai and Akira, 2011). Surprisingly, functional TLR3 and TLR4 are abundantly expressed on human BM-derived MSCs. Ligation of these TLRs induces activation of proinflammatory signals and prevents the suppression of T cell proliferation, possibly by MSC-mediated downregulation of Notch ligand (Liotta et al., 2008; Tomchuck et al., 2008). MSC-associated TLR signaling appears to not only involve a direct immune stress response but also the promotion of MSC migration (with TLR3 ligation). Interestingly, TLR3 and TLR4 stimulation does not appear to suppress IDO activity or PGE2 levels that decrease inflammatory responses (Liotta et al., 2008) and raises important implications for the role of MSCs in host defense. These observations suggest that activation of the TLRs on MSCs may maintain antiviral host defense.

TLR-mediated proinflammatory responses by MSCs could potentially have additional functional implications. On the basis of the divergent patterns of TLR3 versus TLR4 ligation in a short-term assay with respect to cytokine and chemokine secretion, cell migration capabilities, TGF-β secretion, and expression of the downstream effectors, SMAD3/SMAD7, Waterman et al. (2010) proposed a novel paradigm for MSC action. In their model, MSCs can polarize to a proinflammatory MSC1 type (TLR4-primed) or an immunosuppressive MSC2 (TLR3-primed) phenotype, analogous to the action of M1 versus M2 monocyte/macrophages (Dayan et al., 2011). Thus, the classical monocyte/macrophage responses to injury are reprised with the MSC1 (response to acute injury)/MSC2 (anti-inflammatory/healing) model (Figure 1).

It is also possible that through TLR signaling, MSCs play a pivotal role in both initiating the clearance of pathogens and promoting the repair of injured tissue, raising the possibility that MSCs could be employed clinically to augment host defense (Auletta et al., 2012). For future applications, the challenge will be to discover the key factors that contribute to achieving a balance that functions effectively in the best interests of the host. The next steps include confirmatory studies using different assays for further testing in animal models. In that regard, investigators will need to deal with the additional level of complexity from MSC-mediated augmentation of IL-10 production by macrophages via TLR4 ligation (Németh et al., 2009).

As is true for most studies of MSCs, the bulk of these immune modulation experiments were conducted with cultured MSCs. Data generated in vivo from putatively equivalent primary MSCs (MSCs in situ) remain lacking. Unfortunately, an assessment of immune interactions of uncultured MSCs in vivo has the same limitations as those for other MSC studies: the low frequency of primary MSCs in vivo, a lack of appropriate animal models, and interspecies variation in mechanisms of action (Ren et al., 2009). Differing results may also be reconciled by taking into account opposing mechanisms to maintain immune homeostasis. Alternative explanations include differences in cell dose, assay methodology, and MSC source. Given these limitations, an attempt to extrapolate in vitro data by Uccelli et al. (2008) is laudable and possibly amenable to testing. These authors provide several intriguing potential explanations for effects in vivo. For example, the effect of infused cultured MSCs on NK and dendritic cells may result in potentially opposite interactions that eventually will be resolved by predominant microenvironmental cytokine levels.

Evolving Concepts of Tissue Regeneration by MSCs

Over the past decade, there has been considerable evolution in our understanding of the mechanisms underlying tissue regeneration from MSCs. Progress may have been limited to some extent by the concept of the mesenchymal "stem" cell and the implicit idea that the objective was cell "replacement" therapy. For example, the concept of transdifferentiation of hematopoietic progenitors into cardiac cells was difficult to dislodge, despite rigorous studies failing to support the idea (Murry et al., 2004; Balsam et al., 2004). It was interesting that this notion was displaced by the phenomenon of cell fusion, another

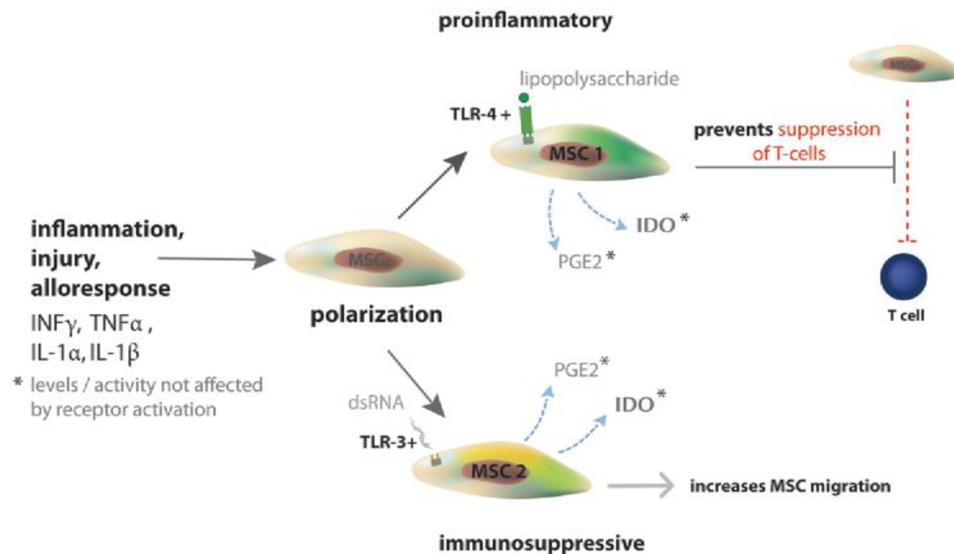

Figure 1. Proposed Immunomodulatory Mechanisms of Cultured MSCs
MSC-mediated immune interactions shown here include a proposed polarization of MSCs into MSC1 and MSC2 cells as a result of activation of Toll-like receptors (based on work by Waterman et al., 2010). Activation of MSC-resident TLR4 leads to a MSC1 or M1 type cell with a proinflammatory response, whereas activation of TLR3 gives rise to a M2 type MSC with an anti-inflammatory/immunosuppressive response. Overall outcome will depend on the balance between the cytokines/chemokines released into the microenvironment.

biological process also unlikely to account for documented improvements in preclinical models of cell treatment of injured tissue (if only because of its very low frequency).

However, the possibility that partial cellular reprogramming, leading to the acquisition of some characteristics of the desired lineage, could contribute to the tissue regeneration capacity of MSCs (Rose et al., 2008) remains to be investigated. A recent example of high throughput screening using human MSCs to identify small molecules that promote chondrogenic differentiation suggests an approach that may be more fruitful (Johnson et al., 2012). These investigators showed that the small molecule kartogenin induces chondrocyte differentiation of MSCs, protects articular cartilage in vitro, and promotes cartilage repair after intra-articular injection in an osteoarthritis animal model. Whether the administration of exogenous MSC-derived chrondrocytic cells will be superior to local treatment with the heterocyclic molecule alone is not yet known. Nonetheless, a more extensive drug discovery approach to identify molecules that mediate the differentiation/reprogramming of MSCs along mesodermal lineages is an exciting prospect.

Other explanations for the varying degrees of efficacy mediated by MSCs have been extensively reviewed elsewhere and are often characterized as ‘‘paracrine’’ effects. The cells are perceived to exert their effects by the release of factors that stimulate tissue recovery on many potential levels, including stimulation of endogenous stem/progenitor cells, suppression of apoptosis of vulnerable cells, remodeling of extracellular matrix, and stimulation of new blood vessel formation. Investigating MSCs as cytokine ‘‘factories’’ will likely uncover new mechanisms and identify compounds that may in some cases supplant the cells themselves (Ranganath et al., 2012). For example, tumor necrosis factor-inducible gene 6 protein (TSG6) is an immunosuppressive molecule produced by MSCs that partially recapitulates the hemodynamic improvement after intravenous infusion of the cells following experimental acute myocardial infarction in mice (Lee et al., 2009). This study serves to further underscore the shift toward the importance of the immunomodulatory properties of MSCs in regenerating injured tissue. Another example is the association between cardiac improvement and an MSC-mediated switch in macrophages/monocytes infiltrating ischemic tissue from the M1 to M2 phenotype (Dayan et al., 2011). Of interest, the switch was observed among circulating monocytes but not in the bone marrow, raising the possibility of a potentially useful distinction between more commonly accepted paracrine phenomena versus an allocrine effect produced by exogenous cells in a remote location.

How MSCs communicate with endogenous cells requires further study and the contribution by which cell-cell contact mediates the biological effects needs further clarification. In this regard, exploring the role of exosomes, secreted vesicles potentially involved in intercellular communication may provide novel insights (Lai et al., 2011).

Physiological Role of Primary In Situ MSCs

The study of culture-expanded MSCs is unlikely to help establish the physiological role of native in vivo cells. Progress in dealing with this limitation has initially been slow, partly because potentially useful experimental tools have been employed only recently and the frequency of putative native MSCs is very low. However, momentum is growing as the importance of these studies becomes more evident.

McGonagle and others have shown that the in vivo counterpart of MSCs has the following immunophenotype: $CD45^{-}$ or low, $CD271^{+}$ (Jones et al., 2002). More recent data show that the cells within this population have greater transcriptional activity than cultured MSCs or dermal fibroblasts, reflecting

broader differentiation potential and a marked increase in the transcription of osteogenic and Wnt-related genes (Churchman et al., 2012). $CD105^+$ cells can also be isolated in situ from human bone marrow and exhibit high levels of CFU-F activity, generate $CD105^+$ $CD90^+$, and $CD106^+$ cells that undergo trilineage differentiation (adipogenic, chondorgenic and osteogenic lineages) after culture, and differentiate into osteoblasts in vivo in response to BMP-2 (Aslan et al., 2006).

Other evidence indicates that the human in situ MSC in vivo is $CD146^+$, gives rise to CFU-F, and exhibits self-renewal in vivo. These cells are also capable of forming both bone and heterotopic hematopoiesis-associated MSCs from single clones in immune-deficient murine experiments. The $CD146^+CD45^-$ cells are subendothelial and localize in vivo as adventitial reticular cells (Sacchetti et al., 2007). More recent work from another group has confirmed that CFU-F activity resides exclusively in the $CD271^+$ cell population enriched directly from human marrow cells and shown that both $CD271^+CD146^+$ or $CD271^+CD146(^-)$ cells can give rise to stromal clones that form bone ossicles and hematopoiesis-associated stromal cells (Tormin et al., 2011). The Frenette group has shown that a small proportion of MSCs are $Nestin^+$, can self-renew in vivo, contain all the CFU-F activity of the bone marrow, and undergo osteogenic, chondrogenic, and adipogenic differentiation (Méndez-Ferrer et al., 2010). The relationship of these mesenchymal stem cells and CXCL12-abundant reticular (CAR) cells (Sugiyama et al., 2006), which also have osteoprogenitor capacity, requires further investigation. However, short-term ablation of CAR cells in vivo impaired the ability of BM cells to undergo adipogenic and osteogenic differentiation (Omatsu et al., 2010). The Scadden group has further examined osteolineage progenitors in the MSC pool. Their recent elegant study of bone maintenance and repair (Park et al., 2012) highlights the importance of genetic tools that better define the in vivo role of BM MSCs. They showed that a subset of $Nestin^+$ osteolineage-restricted MSCs present in vivo are able to replace short-lived mature osteoblasts to maintain homeostasis and respond to bone injury (Park et al., 2012).

Taking an innovative approach involving phage display and cell sorting, Daquinag et al. (2011) screened combinatorial libraries for peptides that target adipose stromal cells in vivo in the mouse based on the immunophenotype profile, $CD34^-CD31^-CD45^-$. They found a cell surface marker, the N-terminally truncated proteoglycan, δ-decorin highly expressed on the cells in vivo and identified resistin, a known protein adipokine, as its endogenous ligand. They hypothesized that signaling by resistin via the δ-decorin receptor regulates the fate of adipose stromal cells. Although observed almost in passing, the authors note that the δ-decorin is localized on the cell surface that faces away from blood vessels, suggesting an opportunity to interact with extracellular matrix components. In addition, they found that culturing the stromal vascular fraction (SVF) of adipose cells under standard conditions for generating MSCs led to loss of cell surface δ-decorin. These data underscore the challenges associated with identifying unique cell markers on cultured MSCs. Nonetheless, a similar approach for identifying an analogous receptor/ligand on bone marrow-derived MSCs may also yield valuable information regarding the nature and biology of the native MSC in vivo.

Clinical Application

At this point, there is extensive clinical activity involving MSCs, and many available treatments are outside the oversight of national regulatory bodies or clinical trial sites such as ClinicalTrials.gov. Moreover, the outcomes of a large number of these treatments are not documented in peer-reviewed journals. Unfortunately, the rationale for the clinical application of MSCs, particularly in regenerative medicine, has lagged behind laboratory observations. It is important to optimize the design of MSC trials based on the most current preclinical observations to maximize their scientific rigor. Several protocols involving systemic administration of MSCs to treat injured tissue are still in progress because of the notion of cell replacement therapy rather than on the more recently accepted paracrine and anti-inflammatory effects of these cells. The study outcomes are unlikely to be optimal if the major effect is actually an anti-inflammatory one and may arise from number of factors including inappropriate dose, scheduling, or route of administration. Furthermore, the coadministration of anti-inflammatory agents may be a confounding factor.

A second issue is the difficulty in fully evaluating completed clinical trials for which the results have not been formally published in international peer-reviewed journals. Valuable insights into trials design, patient selection, underlying rationale, and potential improvements would be gained by rigorous peer review.

Nevertheless, the results of several phase II trials with MSCs show promise. Le Blanc's phase II trial using MSCs to treat steroid-resistant aGvHD (Le Blanc et al., 2008) indicates that a multicenter randomized controlled trial should be conducted. Because several transplant centers already routinely employ MSCs for that indication on the basis of only the phase II data, the need for a randomized controlled trial seems quite urgent.

MSCs were also tested for their ability to support kidney transplantation on the basis of the promising data treating aGvHD. In an open label prospective trial, 159 patients undergoing a living related donor kidney transplant were registered for randomization to receive IL-2 receptor antibody induction therapy versus autologous BM-MSCs to assess rejection rate (Tan et al., 2012). Although patient and graft survival were similar, patients receiving MSCs had a lower incidence of acute rejection, decreased probability of opportunistic infections, and better kidney function 1 year later.

In addition, preclinical data have suggested that MSCs may have a role in the management of acute myocardial infarction. An industry-sponsored randomized double-blind placebo-controlled dose escalation study of systemically administered MSCs after acute myocardial infarction in 53 patients provided safety and preliminary efficacy data (Hare et al., 2009). Adverse events were similar between the test and placebo groups over a 6 month period. Ventricular arrhythmias were reduced ($p = 0.025$) and pulmonary function improved ($p = 0.003$) in patients receiving MSCs. In a subset analysis, patients with an anterior acute myocardial infarct had improved ventricular function (ejection fraction) compared with the placebo cohort. These are encouraging data and a prospective randomized trial with clinically significant endpoints is awaited with interest.

Well-designed clinical trials will be critical for determining whether MSCs can be effective in treating tissue injury or

immune disorders. Success is more likely if clinician investigators work very closely with laboratory researchers to design better clinical trials. Particular attention should be paid to factors that may be overlooked but could affect the efficacy of MSCs, including culture conditions/medium, oxygen tension, time from thawing after cryopreservation to administration, the tissue source of the MSCs, priming or activation prior to administration, the use of gene engineered versus unmodified cells, MSC subsets, and autologous versus allogeneic cells. Given the serious limitations associated with testing human MSCs in animal models, detailed analysis of immune and other perturbations in patients participating in prospective trials should be undertaken to help optimize subsequent protocols. Two other areas also require further attention. The importance of cell tracking and persistence of the exogenous MSCs in vivo in affecting clinical outcome can only be addressed when the cells are safely and effectively labeled and monitored. Although several studies have looked at the persistence of MSCs in animal models, similar correlative studies are required in human study recipients. Currently, the only viable option is superparamagnetic iron nanoparticles and magnetic resonance imaging, but even this approach has very limited availability. Finally, given the underreporting of MSC treatments and the paucity of publications describing long-term follow-up after MSC administration, a convincing case can be made for establishing a database registry of cell therapy recipients to track treatment outcomes and monitor for long-term adverse events. Two additional aspects suggest that clinical correlative studies are warranted. Although the recent observation of the acquisition of chromosomal aberrations in cultured human adult stem cells (Ben-David et al., 2011) is of uncertain significance (Sensebé et al., 2012; Prockop and Keating, 2012), it will be important to correlate assays of genetic instability prospectively with clinical outcome. The other aspect relates to the interaction of MSCs with cancer. Although there is a growing body of literature in this area (Djouad et al., 2003), the outcomes of experimental models appear to be conflicting. A spectrum of responses has been observed with different tumor models, from tumor suppression to stimulation. Clinical correlation with studies of the signaling pathways involved in stromal-tumor interactions is an important goal that should also accompany the establishment of a cell therapy patient registry.

Conclusions

In summary, the past 5 years have been remarkably active for MSC studies. Several initiatives can be undertaken to further accelerate the process of enhancing our understanding of MSC biology and improve access to well-designed clinical trials. Current definitions of this cell population need to be revisited given the wide range of tissue sources and the recognition of subpopulations with specific properties. Extending the availability of an international reference MSC repository for access to all investigators is also a priority. Additional animal models need to be developed to better identify and study the biology of primary in situ MSCs. The design of optimal clinical trials requires the close cooperation of laboratory and clinical investigators. Future studies need to be designed that also include assaying perturbations in patients in vivo and are therefore best positioned to overcome some of the inherent limitations of xenogeneic animal models with human MSCs.

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REFERENCES

Aslan, H., Zilberman, Y., Kandel, L., Liebergall, M., Oskouian, R.J., Gazit, D., and Gazit, Z. (2006). Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells. Stem Cells *24*, 1728–1737.

Auletta, J.J., Deans, R.J., and Bartholomew, A.M. (2012). Emerging roles for multipotent, bone marrow-derived stromal cells in host defense. Blood *119*, 1801–1809.

Balsam, L.B., Wagers, A.J., Christensen, J.L., Kofidis, T., Weissman, I.L., and Robbins, R.C. (2004). Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature *428*, 668–673.

Ben-David, U., Mayshar, Y., and Benvenisty, N. (2011). Large-scale analysis reveals acquisition of lineage-specific chromosomal aberrations in human adult stem cells. Cell Stem Cell *9*, 97–102.

Bianco, P., Robey, P.G., and Simmons, P.J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell *2*, 313–319.

Caplan, A.I. (1991). Mesenchymal stem cells. J. Orthop. Res. *9*, 641–650.

Castro-Malaspina, H., Gay, R.E., Resnick, G., Kapoor, N., Meyers, P., Chiarieri, D., McKenzie, S., Broxmeyer, H.E., and Moore, M.A. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood *56*, 289–301.

Chen, P.-M., Yen, M.-L., Liu, K.-J., Sytwu, H.-K., and Yen, B.-L. (2011). Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. J. Biomed. Sci. *18*, 49.

Choi, H., Lee, R.H., Bazhanov, N., Oh, J.Y., and Prockop, D.J. (2011). Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. Blood *118*, 330–338.

Churchman, S.M., Ponchel, F., Boxall, S.A., Cuthbert, R., Kouroupis, D., Roshdy, T., Giannoudis, P.V., Emery, P., McGonagle, D., and Jones, E.A. (2012). Native CD271(+) multipotential stromal cells (MSCs) have a transcript profile indicative of multiple fates with prominent osteogenic and Wnt pathway signalling activity. Arthritis Rheum. Published online February 29, 2012. 10.1002/art.34434.

Clark, B.R., and Keating, A. (1995). Biology of bone marrow stroma. Ann. N Y Acad. Sci. *770*, 70–78.

Daquinag, A.C., Zhang, Y., Amaya-Manzanares, F., Simmons, P.J., and Kolonin, M.G. (2011). An isoform of decorin is a resistin receptor on the surface of adipose progenitor cells. Cell Stem Cell *9*, 74–86.

Dayan, V., Yannarelli, G., Billia, F., Filomeno, P., Wang, X.-H., Davies, J.E., and Keating, A. (2011). Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Basic Res. Cardiol. *106*, 1299–1310.

De Bari, C., Dell'Accio, F., Tylzanowski, P., and Luyten, F.P. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. *44*, 1928–1942.

Dexter, T.M., Allen, T.D., and Lajtha, L.G. (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell. Physiol. *91*, 335–344.

Djouad, F., Plence, P., Bony, C., Tropel, P., Apparailly, F., Sany, J., Noël, D., and Jorgensen, C. (2003). Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood *102*, 3837–3844.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, Dj., and Horwitz, E.M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells.

The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315–317.

Erices, A., Conget, P., and Minguell, J.J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. Br. J. Haematol. *109*, 235–242.

Friedenstein, A.J., Petrakova, K.V., Kurolesova, A.I., and Frolova, G.P. (1968). Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation *6*, 230–247.

Friedenstein, A.J., Chailakhjan, R.K., and Lalykina, K.S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. *3*, 393–403.

Friedenstein, A.J., Gorskaja, J.F., and Kulagina, N.N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp. Hematol. *4*, 267–274.

Gronthos, S., Mankani, M., Brahim, J., Robey, P.G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl. Acad. Sci. USA *97*, 13625–13630.

Hare, J.M., Traverse, J.H., Henry, T.D., Dib, N., Strumpf, R.K., Schulman, S.P., Gerstenblith, G., DeMaria, A.N., Denktas, A.E., Gammon, R.S., et al. (2009). A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J. Am. Coll. Cardiol. *54*, 2277–2286.

Horwitz, E.M., and Dominici, M. (2008). How do mesenchymal stromal cells exert their therapeutic benefit? Cytotherapy *10*, 771–774.

Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Deans, R.J., Krause, D.S., and Keating, A.; International Society for Cellular Therapy. (2005). Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy *7*, 393–395.

In 't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., de Groot-Swings, G.M., Claas, F.H., Fibbe, W.E., and Kanhai, H.H. (2004). Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells *22*, 1338–1345.

Johnson, K., Zhu, S., Tremblay, M.S., Payette, J.N., Wang, J., Bouchez, L.C., Meeusen, S., Althage, A., Cho, C.Y., Wu, X., and Schultz, P.G. (2012). A Stem Cell-Based Approach to Cartilage Repair. Science. Published online April 5, 2012.

Jones, E.A., Kinsey, S.E., English, A., Jones, R.A., Straszynski, L., Meredith, D.M., Markham, A.F., Jack, A., Emery, P., and McGonagle, D. (2002). Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum. *46*, 3349–3360.

Jones, S., Horwood, N., Cope, A., and Dazzi, F. (2007). The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells. J. Immunol. *179*, 2824–2831.

Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity *34*, 637–650.

Lai, R.C., Chen, T.S., and Lim, S.K. (2011). Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regen. Med. *6*, 481–492.

Le Blanc, K., and Ringdén, O. (2007). Immunomodulation by mesenchymal stem cells and clinical experience. J. Intern. Med. *262*, 509–525.

Le Blanc, K., Frassoni, F., Ball, L., Locatelli, F., Roelofs, H., Lewis, I., Lanino, E., Sundberg, B., Bernardo, M.E., Remberger, M., et al; Developmental Committee of the European Group for Blood and Marrow Transplantation. (2008). Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet *371*, 1579–1586.

Lee, R.H., Pulin, A.A., Seo, M.J., Kota, D.J., Ylostalo, J., Larson, B.L., Semprun-Prieto, L., Delafontaine, P., and Prockop, D.J. (2009). Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell *5*, 54–63.

Lee, C.C., Christensen, J.E., Yoder, M.C., and Tarantal, A.F. (2010). Clonal analysis and hierarchy of human bone marrow mesenchymal stem and progenitor cells. Exp. Hematol. *38*, 46–54.

Lindner, U., Kramer, J., Rohwedel, J., and Schlenke, P. (2010). Mesenchymal Stem or Stromal Cells: Toward a Better Understanding of Their Biology? Transfus. Med. Hemother. *37*, 75–83. Published online Mar 15, 2010.

Liotta, F., Angeli, R., Cosmi, L., Filì, L., Manuelli, C., Frosali, F., Mazzinghi, B., Maggi, L., Pasini, A., Lisi, V., et al. (2008). Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. Stem Cells *26*, 279–289.

Méndez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature *466*, 829–834.

Muraglia, A., Cancedda, R., and Quarto, R. (2000). Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J. Cell Sci. *113*, 1161–1166.

Murry, C.E., Soonpaa, M.H., Reinecke, H., Nakajima, H., Nakajima, H.O., Rubart, M., Pasumarthi, K.B., Virag, J.I., Bartelmez, S.H., Poppa, V., et al. (2004). Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature *428*, 664–668.

Nadri, S., and Soleimani, M. (2007). Comparative analysis of mesenchymal stromal cells from murine bone marrow and amniotic fluid. Cytotherapy *9*, 729–737.

Nauta, A.J., and Fibbe, W.E. (2007). Immunomodulatory properties of mesenchymal stromal cells. Blood *110*, 3499–3506.

Németh, K., Leelahavanichkul, A., Yuen, P.S.T., Mayer, B., Parmelee, A., Doi, K., Robey, P.G., Leelahavanichkul, K., Koller, B.H., Brown, J.M., et al. (2009). Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat. Med. *15*, 42–49.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., and Nagasawa, T. (2010). The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity *33*, 387–399.

Park, D., Spencer, J.A., Koh, B.I., Kobayashi, T., Fujisaki, J., Clemens, T.L., Lin, C.P., Kronenberg, H.M., and Scadden, D.T. (2012). Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. Cell Stem Cell *10*, 259–272.

Patki, S., Kadam, S., Chandra, V., and Bhonde, R. (2010). Human breast milk is a rich source of multipotent mesenchymal stem cells. Hum. Cell *23*, 35–40.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143–147.

Prockop, D.J., and Keating, A. (2012). Relearning the lessons of genomic stability of human cells during expansion in culture: Implications for clinical research. Stem Cells. Published online April 11, 2012. 10.1002/stem.1103.

Ranganath, S.H., Levy, O., Inamdar, M.S., and Karp, J.M. (2012). Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. Cell Stem Cell *10*, 244–258.

Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A.I., Zhao, R.C., and Shi, Y. (2008). Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell *2*, 141–150.

Ren, G., Su, J., Zhang, L., Zhao, X., Ling, W., L'huillie, A., Zhang, J., Lu, Y., Roberts, A.I., Ji, W., et al. (2009). Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells *27*, 1954–1962.

Rose, R.A., Jiang, H., Wang, X.H., Helke, S., Tsoporis, J.N., Gong, N., Keating, S.C., Parker, T.G., Backx, P.H., and Keating, A. (2008). Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. Stem Cells *26*, 2884–2892.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., and Bianco, P. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324–336.

Sarugaser, R., Lickorish, D., Baksh, D., Hosseini, M.M., and Davies, J.E. (2005). Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells *23*, 220–229.

Sarugaser, R., Hanoun, L., Keating, A., Stanford, W.L., and Davies, J.E. (2009). Human mesenchymal stem cells self-renew and differentiate according to a deterministic hierarchy. PLoS ONE *4*, e6498.

Sensebé, L., Tarte, K., Galipeau, J., Krampera, M., Martin, I., Phinney, D.G., and Shi, Y.; MSC Committee of the International Society for Cellular Therapy. (2012). Limited acquisition of chromosomal aberrations in human adult mesenchymal stromal cells. Cell Stem Cell *10*, 9–10, author reply 10–11.

Shih, D.T., Lee, D.C., Chen, S.C., Tsai, R.Y., Huang, C.T., Tsai, C.C., Shen, E.Y., and Chiu, W.T. (2005). Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. Stem Cells *23*, 1012–1020.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity *25*, 977–988.

Tan, J., Wu, W., Xu, X., Liao, L., Zheng, F., Messinger, S., Sun, X., Chen, J., Yang, S., Cai, J., et al. (2012). Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. JAMA *307*, 1169–1177.

Tolar, J., LeBlanc, K., Keating, A., and Blazar, B.R. (2010). Hitting the right spot with mesenchymal stromal cells (MSCs). Stem Cells *28*, 1446–1455.

Tomchuck, S.L., Zwezdaryk, K.J., Coffelt, S.B., Waterman, R.S., Danka, E.S., and Scandurro, A.B. (2008). Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem Cells *26*, 99–107.

Tormin, A., Li, O., Brune, J.C., Walsh, S., Schütz, B., Ehinger, M., Ditzel, N., Kassem, M., and Scheding, S. (2011). CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. Blood *117*, 5067–5077.

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal stem cells in health and disease. Nat. Rev. Immunol. *8*, 726–736.

Wang, H.S., Hung, S.C., Peng, S.T., Huang, C.C., Wei, H.M., Guo, Y.J., Fu, Y.S., Lai, M.C., and Chen, C.C. (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells *22*, 1330–1337.

Waterman, R.S., Tomchuck, S.L., Henkle, S.L., and Betancourt, A.M. (2010). A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS ONE *5*, e10088.

Wight, T.N., Kinsella, M.G., Keating, A., and Singer, J.W. (1986). Proteoglycans in human long-term bone marrow cultures: biochemical and ultrastructural analyses. Blood *67*, 1333–1343.

Yang, S.H., Park, M.J., Yoon, I.H., Kim, S.Y., Hong, S.H., Shin, J.Y., Nam, H.Y., Kim, Y.H., Kim, B., and Park, C.G. (2009). Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10. Exp. Mol. Med. *41*, 315–324.

Yoshimura, K., Shigeura, T., Matsumoto, D., Sato, T., Takaki, Y., Aiba-Kojima, E., Sato, K., Inoue, K., Nagase, T., Koshima, I., and Gonda, K. (2006). Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J. Cell. Physiol. *208*, 64–76.

Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. (2002). Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell *13*, 4279–4295.

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Mesenchymal Stromal Cells: Sensors and Switchers of Inflammation

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In addition to their stem/progenitor properties, mesenchymal stromal cells (MSCs) possess broad immunoregulatory properties that are being investigated for potential clinical application in treating immune-based disorders. An informed view of the scope of this clinical potential will require a clear understanding of the dynamic interplay between MSCs and the innate and adaptive immune systems. In this Review, we outline current insights into the ways in which MSCs sense and control inflammation, highlighting the central role of macrophage polarization. We also draw attention to functional differences seen between vivo and in vitro contexts and between species. Finally, we discuss progress toward clinical application of MSCs, focusing on GvHD as a case study.

Introduction

Mesenchymal stromal cells (MSCs) are adult, fibroblast-like multipotent cells characterized by the ability to differentiate into tissues of mesodermal origin, such as adipocytes, chondroblasts, and osteoblasts (Friedenstein et al., 1974; Pittenger et al., 1999). First identified and isolated from the bone marrow (BM), MSCs can now be expanded from a variety of other tissues including adipose tissue (AT), umbilical cord blood (UCB), skin, tendon, muscle, and dental pulp (Im et al., 2005; Campagnoli et al., 2001; Kawashima, 2012). MSCs can be isolated based on their ability to adhere to plastic culture dishes, and they are capable of significant expansion by consecutive in vitro passaging (Pittenger et al., 1999).

Historically, a challenge that has faced the field has been the lack of uniform criteria to define MSCs, which has hindered efforts to compare results obtained from different experimental and clinical studies. In response to this challenge, the International Society for Cellular Therapy formulated minimal criteria for defining MSCs in order to create a broader consensus for more uniform characterization of these cells (Dominici et al., 2006). Although there remains much debate over how to define such a broad population of cells, it is clear that some populations of MSCs are capable of exhibiting stem cell function in vivo (Keating, 2012; Sacchetti et al., 2007).

In addition to their stem/progenitor properties, MSCs have also been shown to possess broad immunoregulatory abilities and are capable of influencing both adaptive and innate immune responses. Recent findings have demonstrated that MSCs actively interact with components of the innate immune system and that, through these interactions, they display both anti-inflammatory and proinflammatory effects (Keating, 2012; Le Blanc and Mougiakakos, 2012; Prockop and Oh, 2012). This ability of MSCs to adopt a different phenotype in response to sensing an inflammatory environment is not captured in assays that are commonly used to characterize these cells, but it is crucial for understanding their therapeutic potential in immune-mediated disorders. Much of the characterization of these properties has been conducted in vitro, and there are outstanding questions about the degree to which they represent activities that are functionally relevant for endogenous and/or transplanted cells in vivo. The putative role of stromal cells in maintaining tissue homeostasis serves as the basis for their application in disorders resulting from autogeneic or allogeneic immune responses, including Graft-versus-Host Disease (GvHD) and autoimmune disorders (Le Blanc et al., 2008; Duijvestein et al., 2010) and can be referred to as "stromal cell therapy." The application of MSCs in these inflammatory disease settings suggests that the stem cell properties of MSCs, including their ability to engraft, may be independent from their ability to regulate tissue homeostasis.

Animal models are of critical importance for translating in vitro immune regulatory properties of MSCs into therapeutic application and dissecting mechanisms of efficacy. Although murine and human MSCs share properties such as multilineage differentiation capacity, they are also distinct with respect to other properties. A notable example of this divergence is the susceptibility of murine BM-derived MSCs to transform upon culture expansion. In addition, murine and human MSCs employ different effector molecules (i.e., nitric oxide [NO] and indolamine 2,3 dioxygenase [IDO], respectively) for immune regulation, for example during suppression of T cell proliferation (Ren et al., 2008; François et al., 2012). In some studies, human MSCs have been applied in mouse models of either immune competent or immune deficient mice. These differences should be taken into consideration when interpreting in vivo effects of murine MSCs, especially in light of efforts to look at clinical application of MSCs.

In this Review we discuss the regulatory properties of MSCs with respect to their ability to modify tissue homeostasis and inflammation. MSCs are sensors of inflammation and are able to adopt a proinflammatory or anti-inflammatory phenotype by interfering with innate and adaptive immune responses both in vitro and in vivo. For the sake of clarity, these integrated responses are discussed separately. In addition, a comparison between murine and human MSCs will be also be covered. Finally, the clinical application of MSCs in the setting of acute GvHD treatment and biomarker development will be reviewed.

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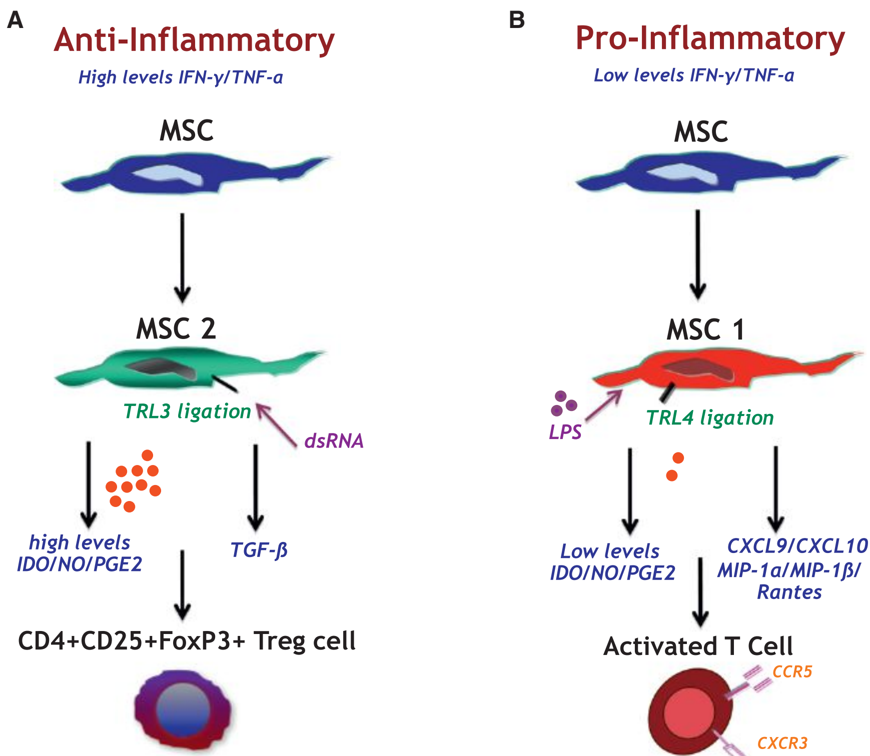

Figure 1. Polarization of MSCs into a Proinflammatory and Anti-Inflammatory Phenotype

(A) In the presence of an inflammatory environment (high levels of TNF-α and IFN-γ), MSCs become activated and adopt an immune-suppressive phenotype (MSC2) by secreting high levels of soluble factors, including IDO, PGE2, NO, TGF-β, Hepatocyte Growth Factor (HGF), and hemoxygenase (HO), that suppress T cell proliferation. The switch toward MSC1 or MSC2 type may also depend on MSC stimulation through Toll-like receptors (TLRs) expressed on their surface (Waterman et al., 2010). Triggering through double stranded RNA (dsRNA) derived from viruses stimulates TLR3 on the surface of MSCs and may induce the polarization into the MSC2 anti-inflammatory type. Together with constitutive secretion of TGF-β by MSCs, emergence of regulatory T cells (Tregs) is favored.

(B) In the absence of an inflammatory environment (low levels of TNF-α and IFN-γ), MSCs may adopt a proinflammatory phenotype (MSC1) and enhance T cell responses by secreting chemokines that recruit lymphocytes to sites of inflammation (e.g, MIP-1α and MIP-1β, RANTES, CXCL9, and CXCL10) (Ren et al., 2008; Li et al., 2012). These chemokines bind to receptors present on T cells, i.e., CCR5 and CXCR3. Polarization to a proinflammatory MSC1 phenotype can also be influenced by activation of TLR4 by low levels of lipopolysaccharide (LPS) derived from Gram-negative bacteria. The levels of immune-suppressive mediators, such as IDO and NO, are low when the MSC1 phenotype is adopted. The balance between these opposing pathways may serve to promote host defense on one hand and at the same time create a loop that prevents excessive tissue damage and promotes repair.

MSCs as Sensors of the Inflammatory Microenvironment: Impact of Innate Immunity

Inflammation serves as a localized or systemic protective response elicited by infection, injury, or tissue destruction and serves to eliminate pathogens and preserve host integrity. Within hours after the onset of an inflammatory response, molecules expressed by pathogens or associated with tissue injury are recognized by Toll-like receptors (TLRs) present on innate effector cells. TLR ligation triggers phagocytosis and the release of inflammatory mediators, which may initiate innate immune responses that provide a first line of nonspecific defense, mainly through the activation of phagocytic cells, including macrophages and neutrophils (Gordon and Mantovani, 2011). TLR ligation may not only activate phagocytic cells but also stromal cells, including MSCs, thus creating an inflammatory environment (Mantovani et al., 2013; Waterman et al., 2010).

MSCs Induce Macrophage Polarization in an Inflammatory Environment: Contribution of In Vitro Studies

Much of what is known about immunmodulatory properties of MSCs has been discovered through cocultures of MSCs and immune cells. Human and mouse MSCs dynamically express a number of distinct and overlapping TLRs in culture, and in vitro stimulation of specific TLRs affects the subsequent immune modulating responses of MSCs (Nemeth et al., 2010; Tomchuck et al., 2008; Delarosa et al., 2012). Under hypoxic culture conditions, stimulation of MSCs with the proinflammatory cytokines IFN-γ, TNF, IFN-α, and IL-1β upregulates expression of a subset of TLRs, thus increasing the sensitivity of MSCs to the inflammatory milieu (Raicevic et al., 2010). However, prolonged stimulation with TLR ligands causes downregulation of TLR2 and TLR4 (Mo et al., 2008), most likely as a self-regulatory mechanism to prevent overactive skewing of the immune response.

To direct appropriate immune responses to a diversity of pathogenic insults, the different TLRs are activated by specific endogenous or pathogen-associated molecules, including lipopolysaccharide (LPS) from Gram-negative bacteria (TLR4) and double strand RNA (dsRNA) carried by some viruses (TLR3). Waterman et al. (2010) have suggested that MSCs may polarize into two distinctly acting phenotypes following specific TLR stimulation, resulting in different immune modulatory effects and distinct secretomes. The TLR4-primed MSC population exhibits a proinflammatory profile (MSC1) and the TLR3-primed MSC population delivers anti-inflammatory signals (MSC2) (Figure 1). Although the molecular pathways that promote a proinflammatory or anti-inflammatory secretome following TLR ligation remain unclear, the concept of MSC polarization into proinflammatory and anti-inflammatory cells provides an attractive model to explain and interrogate the apparently contradictory roles of MSCs in inflammation.

Within the innate immune system, it is well established that macrophages are key players in initiating and controlling inflammation (Mantovani et al., 2013), and MSCs can influence macrophage function depending on the inflammatory context (see sections below). Monocytes arriving at an inflammatory environment can develop into activated M1 macrophages or convert into alternatively activated M2 macrophages depending on microenvironmental cues. While M1 macrophages stimulate

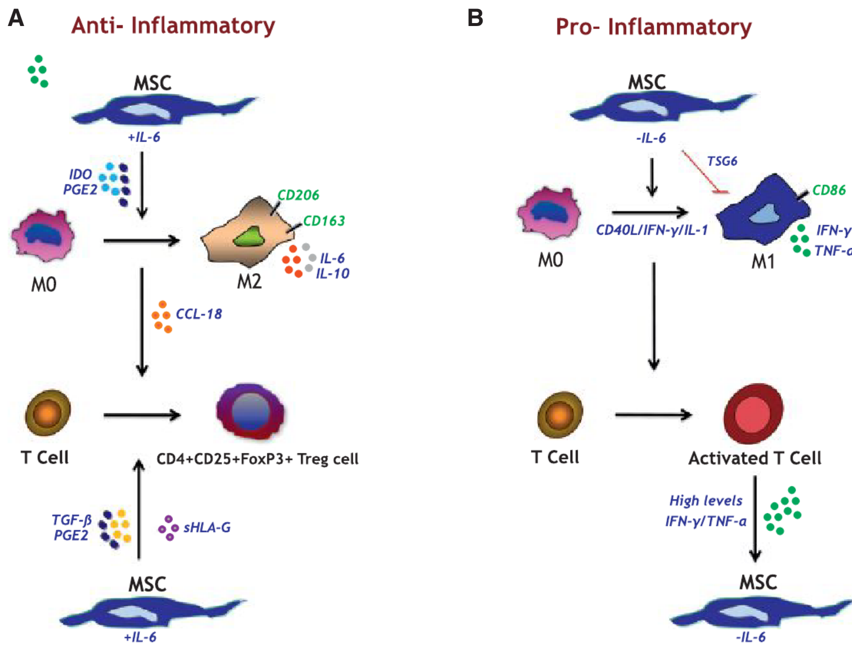

Figure 2. Interactions between MSCs and Monocytes: MSCs Balance the Polarization of Monocytes (M0) toward M1 and M2 Macrophages

(A) MSCs constitutively produce IL-6, which polarizes monocytes (M0) toward anti-inflammatory IL-10-producing M2 macrophages (Eggenhofer and Hoogduijn, 2012). This polarization is initiated by and dependent on a combination of cell-cell contact mechanisms and the secretion of soluble factors, including IDO and PGE2. The polarizing effect of MSCs on M2 macrophages is closely linked to their ability to favor the emergence of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs). The emergence of Tregs can be supported by MSCs by indirect and direct mechanisms: indirectly via the production of CCL18 by MSC-induced M2 macrophages and directly via the production of TGF-β by MSCs (Melief et al., 2013a). Other molecules involved in the MSC-mediated generation of Tregs are Prostaglandin E2 (PGE2) and soluble HLA-G (sHLA-G).

(B) In the absence of IL-6, MSCs induce the polarization of M0 toward proinflammatory M1 macrophages through secretion of IFN-γ and IL-1 and surface expression of CD40L. M1 macrophages secrete IFN-γ and TNF-α and express costimulatory molecules on their surface that promote T cell activation. High levels of proinflammatory signals, including TNF-α and IFN-γ produced by activated T cells or proinflammatory M1 macrophages, act as a feedback mechanism and induce the anti-inflammatory pathway shown in (A). The balance between anti- and proinflammatory pathways is crucial in controlling host defense and inflammation and preventing excessive tissue damage.

local inflammation by releasing proinflammatory cytokines, M2 macrophages secrete a combination of cytokines (including high levels of IL-10 and TGF-β1 and low levels of IL-1, IL-6, TNF-α, and IFN-γ) that together exert an anti-inflammatory effect and allow tissue regeneration following inflammation (Mantovani et al., 2013).

MSCs actively interact with components of the innate immune system and influence their subsequent immunoregulatory and regenerative behavior (Keating, 2012; Le Blanc and Mougiakakos, 2012). The production of proinflammatory cytokines by M1 macrophages or activated T cells may activate MSCs and trigger the release of mediators that skew the differentiation of monocytes toward an anti-inflammatory profile and ultimately toward M2 macrophages (Le Blanc and Mougiakakos, 2012) (Figure 2). In addition to polarization of MSCs, macrophage polarization provides a supplementary mechanism to maintain balance between proinflammatory and anti-inflammatory effects. This dynamic regulatory feedback between MSCs and macrophages generates a profound sensitivity to the surrounding microenvironment that is displayed through the ability to switch between proinflammatory and anti-inflammatory activities. In both cases, switching mechanisms rely on the production of soluble mediators, including the immunosuppressive factors inducible NO synthase (iNOS, for mice) and IDO (for humans), which are induced by proinflammatory cytokines. The concentrations of these factors may be critical in triggering the switch between proinflammatory and anti-inflammatory MSCs and, thereby, also between M1 and M2 macrophages (Ren et al., 2008).

MSCs Enhance Immune Responses during Early-Stage Inflammation

The proinflammatory activities of MSCs may be beneficial in the early phase of inflammation and help in mounting a proper immune response. During the acute phase of inflammation, neutrophils migrate toward the site of inflammation where they accumulate within minutes and act mainly through phagocytosis (Kolaczkowska and Kubes, 2013). In mice, the recognition of microbial molecules by tissue-resident MSCs results in increased production of growth factors, such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage migration inhibitory factor (MIF), that recruit neutrophils and enhance their proinflammatory activity (Brandau et al., 2010). Moreover, TLR3-activated human BM-MSCs (MSC2) promote the in vitro survival of resting and activated neutrophils in an IL-6-, IFN-β-, and GM-CSF-dependent manner (Cassatella et al., 2011).

In addition to neutrophils, immune responses may be enhanced by MSCs through the production of chemokines that recruit lymphocytes to sites of inflammation. Human MSCs produce the chemokines CXCL-9, CXCL-10, and CXCL-11 upon stimulation with proinflammatory cytokines. In vitro studies with murine and human MSCs suggest that these stimulatory effects only occur when MSCs are exposed to insufficient levels of proinflammatory cytokines, such as TNF-α and IFN-γ. Under these immune-enhancing conditions, murine MSCs elicit insufficient levels of NO to inhibit T cell proliferation. Indeed, inhibition of iNOS or its genetic ablation resulted in strong enhancement of T cell proliferation by murine MSCs (Ren et al., 2008; Li et al., 2012; Shi et al., 2012). Under similar conditions, human MSCs produce insufficient IDO (rather than iNOS) to suppress T cell proliferation (Figure 1). These data suggest that iNOS for murine cells or IDO for human cells may serve as a molecular switch between immune-suppressive to immune-enhancing effects of MSCs.

MSCs Suppress Immune Responses and Inflammation to Promote Tissue Homeostasis

When exposed to sufficient levels of proinflammatory cytokines, MSCs may respond by adopting an immune-suppressive MSC

phenotype to dampen inflammation and promote tissue homeostasis through polarization toward anti-inflammatory cells and M2 macrophages in vitro. Coculture of monocytes with human or mouse BM-MSCs promotes the formation of M2 macrophages (Eggenhofer and Hoogduijn, 2012) (Figure 2) and this is dependent on both cellular contact and soluble factors, including prostaglandin E2 (PGE2) and catabolites of IDO activity such as kynurenine (Németh et al., 2009; Eggenhofer and Hoogduijn, 2012). Moreover, activation of MSCs with IFN-γ, TNF-α, and LPS increases the expression of cyclooxygenase 2 (COX2) and IDO in BM-MSCs, thereby further promoting a homeostatic response toward M2 macrophage polarization (Németh et al., 2009; François et al., 2012). Through the release of chemokine (C-C motif) ligands CCL2, CCL3, and CCL12, human and mouse BM-MSCs can recruit monocytes and macrophages into inflamed tissues and promote wound repair (Chen et al., 2008).

This polarizing effect of MSCs on M2 macrophages is closely linked with the ability of MSCs to favor the emergence of regulatory T cells (Tregs), which are involved in immunosuppression. TGF-β is a factor that is constitutively produced by MSCs and that directly induces Tregs in a monocyte-dependent manner. M2 polarized macrophages also produce IL-10, which is directly immune suppressive. In addition, M2 macrophages produce CCL18, a factor that in conjunction with TGF-β promotes the generation of Tregs (Melief et al., 2013a) (Figure 2). The MSC-derived factors that induce the differentiation of monocytes toward M2 macrophages have not been identified.

These data underline the importance of the interactions between MSCs and the innate immune system in balancing proinflammatory and anti-inflammatory responses in order to preserve tissue integrity. The central role of macrophages in the induction of the anti-inflammatory effect of MSCs is depicted in Figure 2.

Role of MSCs in Orchestrating Adaptive Immune Responses

The adaptive immune system is antigen-specific and allows the development of immunological memory. It comprises $CD4^+$ T-helper and $CD8^+$ cytotoxic T lymphocytes that deliver a tailored antigen-specific immune response following antigen processing and presentation by antigen-presenting cells (APCs). T helper cells comprise a subpopulation of cells, Tregs, which are specialized in suppression of T cell-mediated immune responses. The innate immune system plays a crucial part in the initiation and subsequent direction of adaptive immune responses, as well as in the removal of pathogens that have been targeted by an adaptive immune response (Yamane and Paul, 2013; Gratz et al., 2013).

MSC and Effector T Cell Immunity

As with innate immunity, much of what is known about the interaction of MSCs with the adaptive immune system is through in vitro studies. MSCs are able to suppress in vitro T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli (Di Nicola et al., 2002) through the secretion of soluble factors that include TGF-β, Hepatocyte Growth Factor (HGF), PGE2, IDO, NO, and hemoxygenase (HO) (Stagg and Galipeau 2013). The release of these suppressive factors is enhanced following stimulation of MSCs with TNF-α and IFN-γ, although unstimulated MSCs also produce these mediators. In human cells IDO promotes the degradation of tryptophan into kynurenine and other catabolites that have been shown to not only suppress T cell proliferation, but also induce Treg differentiation. The suppression of T cell proliferation involves both $CD4^+$ and $CD8^+$ T cells; IFN-γ production and cytotoxicity are also inhibited in a dose-dependent manner (Krampera et al., 2003; Aggarwal and Pittenger, 2005). In murine cells, the critical role of NO in suppressing T cell proliferation is also supported by the observation that in vitro proliferation of murine T cells is boosted by the addition of the NO inhibitor L-NMMA (Li et al., 2012). Similarly, addition of $iNOS^{-/-}$ MSCs induces a dramatic increase in T cell proliferation in coculture assays. While MSCs directly produce soluble factors that suppress T cell proliferation, it has also been suggested that the ability of MSCs to suppress T cell proliferation in vitro is monocyte dependent, since MSCs show a reduced inhibitory action on T cells in the absence of monocytes (Cutler et al., 2010; François et al., 2012).

MSCs and Regulatory T Cells

Several studies have documented the ability of MSCs to polarize T cells toward a regulatory phenotype (Burr et al., 2013) that serves as an important mechanism by which MSCs dampen inflammation. Tregs comprise a subpopulation of T helper cells, are specialized in suppression of T cell-mediated immune responses, and characteristically express the forkhead box P3 (FoxP3) transcription factor. There are two main subsets of Tregs including a population of $FoxP3^+$ natural Tregs, which are thymus derived and specific for self antigens and induced or adaptive Tregs that are derived from mature $CD4^+CD25^-Foxp3^-$ precursors in the periphery following inflammatory stimuli (Chaudhry and Rudensky, 2013).

In vitro coincubation of human MSCs with peripheral blood mononuclear cells (PBMCs) induced the differentiation of $CD4^+$ T cells into $CD25^+FoxP3^+$-expressing regulatory T cells (induced Tregs), a process involving direct MSC contact with helper T cells and PGE2 and TGF-β secretion (Maccario et al., 2005; English et al., 2009). The generation of Tregs was reported to be monocyte dependent and was not observed in cocultures of MSCs and purified $CD4^+$ T cells or monocyte-depleted PBMCs, but it could be restored by the addition of monocytes (Melief et al., 2013a).

Following addition of mitogen-stimulated T cells, MSC-induced Tregs potently suppressed the T cell proliferative response (English et al., 2009). Secretion of HLA-G5 by MSCs has also been shown to promote MSC-induced Treg generation. Blocking experiments using neutralizing anti-HLA-G antibody demonstrated that HLA-G5 contributes first to the suppression of allogeneic T cell proliferation and then to the expansion of $CD4^+CD25^+FoxP3^+$ Tregs (Selmani et al., 2008). In this process, both the activation state of CD4+ T cells and the cytokine milieu may play a role. By exerting inhibitory effects on APCs that process antigens and present them to T cells, MSCs can generate regulatory APCs characterized by their Treg-promoting activity.

All together, these studies indicate that MSCs are able to recalibrate the balance between inflammatory effector T cells and anti-inflammatory Tregs. This process is tightly related to polarization of monocytes toward anti-inflammatory (M2) macrophages (Figure 2). By linking cytokine-mediated immune

suppression (i.e., IL-10 production) and the induction of Treg cells, an amplification of the anti-inflammatory response is obtained.

In Vivo Regulation of Inflammation by MSCs

Ex-Vivo-Expanded MSCs Influence Macrophage Polarization and Immune Responses In Vivo

While the concept of macrophage polarization may explain the apparent discrepancy between proinflammatory and anti-inflammatory activities of MSCs, the biological relevance of these findings remains unclear. In several recent studies, the ability of MSCs to polarize macrophages has also been investigated in vivo. In a sepsis model, the administration of mouse BM-MSCs decreased lethality; however, this effect was not observed after macrophage depletion or after administration of IL-10-specific neutralizing antibodies, suggesting that MSC-induced macrophage polarization also occurs in vivo and may result in reduced tissue damage (Németh et al., 2009). It has been recently observed that Nes^+ MSCs respond to TLR4 ligation by upregulating monocyte chemotactic protein-1 (MCP1) expression, which induces CCR2-dependent migration of monocytes from the BM into the circulation, which may serve as a mechanism to further promote this process (Shi et al., 2011).

In the same model, MSCs improved survival of mice by enhancing the ability of neutrophils to phagocytize bacteria and promote bacterial clearance (Hall et al., 2013; Németh et al., 2009). It was also shown that MSCs, through the induction of IL-10 by monocytes and macrophages, can prevent neutrophils from migrating into tissues, thereby preventing oxidative damage. The inhibition of neutrophil migration into tissues is associated with a higher neutrophil count in the blood, allowing more efficient bacterial clearance and, at the same time, preventing excessive tissue damage. These data suggest that during the early phase of inflammation, MSCs may play a role in promoting neutrophil migration and activation in order to enhance innate immune responses, but during later stages, they may switch toward an inhibitory phenotype resulting in inhibition of migration to protect tissues against oxidative injury.

Similar observations were made in a model of endotoxin-induced lung injury, in which intrapulmonary delivery of mouse BM-MSCs decreased the production of TNF-α and chemokine ligand CXCL2 and increased the production of IL-10 by alveolar macrophages (Gupta et al., 2007). On the same line, in a zymosan-induced peritonitis model, infusion of human BM-MSCs resulted in secretion of TNF-stimulated gene 6 (TSG6), a molecule that interferes with TLR2 nuclear factor-κB (NF-κB) signaling in peritoneal macrophages, thereby attenuating their activation (Choi et al., 2011). In this model the therapeutic effects of MSCs seem to be mediated by endocrine rather than paracrine mechanisms, suggesting that homing to a site of injury is not necessarily required for therapeutic efficacy.

The role of macrophages in MSC-induced Treg formation has been recently confirmed in mouse models of fibrillin-mutated systemic sclerosis and experimental colitis. Infusion of murine BM-MSCs induced transient T cell apoptosis, which triggered macrophages to produce high levels of TGF-β, eventually enhancing $CD4^+CD25^+FoxP3^+$Treg generation. This effect translated into amelioration of the disease phenotype (Akiyama et al., 2012). The polarization of T cells toward a Treg phenotype has been also shown in other experimental models of autoimmune and inflammatory diseases, such as SLE, diabetes, and colitis (Choi et al., 2012; Madec et al., 2009; Duijvestein et al., 2011).

Injection of $iNOS^{-/-}$ MSCs into the footpad of mice generated an aggravated response to ovalbumin-induced Delayed Type Hypersensitivity (DTH) as measured by an increase in footpad thickness and enhanced leukocyte infiltration. Conversely, administration of unmodified MSCs reduced footpad thickness and leukocyte infiltration. These results confirm the dual regulatory role of NO in enhancing or suppressing T cell immunity (Ren et al., 2008; Li et al., 2012).

Insufficient homing of systemically delivered MSCs is considered a major limitation of MSC-based therapies, caused in part by inadequate expression of cell surface adhesion receptors. The modification of human MSC surface with a construct containing sialyl Lewis(x) that is found on the surface of leukocytes and mediates cell rolling within inflamed tissue has been shown to allow rolling of MSCs on inflamed endothelium in vivo in mice and homing to inflamed tissue with higher efficiency compared with native MSCs (Sarkar et al., 2010). This model has been taken one step further by the overexpression of IL-10 in engineered MSCs. The systemic administration of these cells in an ear-inflammation model resulted in a superior anti-inflammatory effect in vivo that was dependent on rapid migration to the inflamed ear. In spite of the rapid clearance of MSCs from the circulation following systemic injection (Lee et al., 2009), these results show that MSCs can be successfully used for the targeted delivery of immune-suppressive molecules to distant sites of inflammation (Levy et al., 2013).

Taken together, these in vivo results indicate that MSCs actively interact with cells of the innate immune system and modulate their function to establish a fine balance between pathogen elimination and repair processes, aiming at controlling inflammation, preventing organ failure, and preserving tissue homeostasis. The further elucidation of mechanisms that trigger a functional switch between MSC phenotypes remains an important research goal for future studies.

MSCs Are Responsive to the Host Microenvironment and Participate in Immune Surveillance

Several reports have indicated that MSCs are not constitutively inhibitory but need to be activated by an inflammatory environment in the host in order to have their immunoregulatory effect mediated (Krampera, 2011). This notion was based on the observation that anti-IFN-γ receptor antibodies can block the suppressive effect of MSCs. The simultaneous presence of other inflammatory cytokines can influence the immunosuppressive effect of MSCs as well as induce changes in their immunophenotype. IFN-γ, TNF-α, and IL-1β are able to induce the upregulation of HLA-class I, ICAM-1, and VCAM-1 on the surface of MSCs, while IFN-γ alone can induce the activity of IDO (Ren et al., 2008). Inflammatory stimuli induce MSCs to secrete molecules involved in the regulation of tissue homeostasis, including NO, IDO, PGE2, HO-1, TSG6, CCL2 chemokine, IL-10, and galectins (Shi et al., 2012).

According to the activation model, MSCs are most effective when administered after the onset of an inflammatory response. In a mouse GvHD model, MSC administration on the same day of bone marrow transplantation (BMT) had no protective effect

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(Sudres et al., 2006), whereas administration 3, 8, or 20 days after BMT significantly suppressed the progression of GvHD and abrogated the related symptoms (Polchert et al., 2008). It has been proposed that pretreatment of MSCs with inflammatory cytokines may mimic the inflammatory environment and may enhance their potential therapeutic efficacy. In support of this theory, administration of IFN-γ-pretreated MSCs protected mice from GvHD-induced death (Polchert et al., 2008). Other studies have indicated that pretreatment with inflammatory cytokines can amplify the therapeutic effect of MSCs in animal models of colitis and acute myocardial ischemia/reperfusion injury (Duijvestein et al., 2011; Luo et al., 2012). These data indicate the importance of the local inflammatory conditions in regulating the anti-inflammatory effects of MSCs. Further dissection of the molecular mechanisms involved in these interactions will be crucial for the development of novel MSC-based therapies.

There is ample evidence that administration of ex vivo expanded MSCs may exert immune-suppressive properties in vivo, for instance by inducing macrophage polarization. However, it is still unclear to what extent primary MSCs in the host play a similar regulatory role. As part of the BM niche, MSCs support hematopoiesis and restore the differentiated compartment of osteoblasts and adipocytes during tissue growth and turnover (Sacchetti et al., 2007). Park et al. (2012) showed that a subset of $Nestin^+$ MSCs present in vivo are able to replace short-lived mature osteoblasts to maintain homeostasis and respond to bone injury.

MSCs may also be involved in tumor progression in a wide range of cancers. Through the release of soluble factors, tumor cells may recruit myeloid cells from the BM to the tumor microenvironment, where they subsequently promote tumor progression by conversion into potent immune suppressive cells, including M2-like Tumor Associated Macrophages (TAMs). TAMs promote tumor growth by producing proangiogenic vascular endothelial growth factor (VGEF) and immune-suppressive factors (PGE2, TGF-β) and by releasing chemo-attracting factors (CCL22) that recruit Tregs (Gabrilovich et al., 2013). Recent evidence indicates that BM-derived MSCs can also be targeted to the tumor microenvironment by factors such as stromal cell-derived factor 1 (SDF-1), platelet-derived growth factor a (PDGF-a), and VEGF (Gabrilovich et al., 2013; Ke et al., 2013; Ren et al., 2012). In the tumor microenvironment MSCs may be conditioned into tumor-resident MSCs that acquire functions that are distinct from those of normal tissue MSCs. One of the mechanisms through which tumor-resident MSCs promote tumor growth involves the production of CCL2, the major chemokine for monocyte trafficking, which results in the recruitment of immune-suppressive macrophages. The chemokine profile and the tumor-promoting properties of tumor-resident MSCs can be mimicked by stimulating normal BM-derived MSCs with TNF-α, suggesting that inflammation drives tumorigenesis by establishing a link between MSCs and monocytes and macrophages (Ren et al., 2012; Guilloton et al., 2012). While their tumor-promoting properties have been firmly established, other reports suggest that preactivation of human MSCs with TNF-α may result in tumor-suppressing activity mediated by upregulation of TRAIL on MSCs and by induction of TRAIL-dependent apoptosis of tumor cells (Lee et al., 2012). The apparent discrepancy between these reports may be explained by the use of adoptively transferred cells by Lee et al. that do not necessarily represent the physiological reality (Mantovani, 2012). In addition, Lee et al. have used a xeno transplant model (NOD-SCID), in which adaptive immune (suppressive) and tumor-promoting responses are lacking. Although mechanisms through which MSCs may promote or suppress tumor progression are not fully clarified, the possible tumor-promoting activity of MSCs should be carefully considered in choosing MSCs for application in cancer patients.

It has been suggested that MSCs play a role in maintaining fetal maternal tolerance in the placenta and that they express molecules known to be involved in this process. Both UCB- and BM-derived MSCs express HLA-G, either in its soluble form or as a surface antigen (Selmani et al., 2008). It is conceivable that HLA-G expression at the feto-maternal interface is one of the factors protecting the fetus from maternal immune attack (Carosella et al., 2008). The expression of HLA-G by MSCs could contribute to their ability to blunt excessive immune responses in a specific environment and to control inflammation and maintain homeostasis. IDO represents another molecule involved in the maintenance of fetal maternal tolerance and may also be produced by MSCs.

Different Biological and Functional Properties of MSCs: Mouse and Man

While animal models may play a crucial role in dissecting efficacy, it is important to note that murine MSCs are intrinsically different from human cells (Table 1). Ex vivo expansion with murine cells is slower than with human cells, and murine MSCs require weeks before entering a linear growth rate (Phinney et al., 1999). At this stage, murine MSCs undergo transformation and immortalization in culture. Several reports have indicated that transformed murine MSCs have an increased proliferation rate, display an altered morphology, carry cytogenetic abnormalities, and form tumors following injection into syngeneic mice. Murine BM-derived MSCs in long-term culture gradually exhibit increased telomerase activity and proceed to a malignant state, resulting in sarcoma formation in vivo (Miura et al., 2006; Tolar et al., 2007). This susceptibility to malignant transformation may be attributed to the high degree of chromosomal instability in genetically unstable inbred mice, characterized by the development of both structural and numerical aberrations even at early culture passages. Therefore, culture-expanded murine MSCs should be regarded as transformed cells, even in the absence of a malignant phenotype. In contrast with these findings, (malignant) transformation of human MSCs has not been directly demonstrated and attempts to induce a malignant phenotype by long-term ex vivo expansion have been unsuccessful (Bernardo et al., 2007). In a recent report, its likelihood has been estimated to be $<10^{-9}$ (Prockop et al., 2010) (Table 1).

Many effector molecules that are thought to be involved in the induction of MSC-mediated immunosuppression are divergent between mice and humans, although some similarities can be found. Release of IFN-γ by target cells induces the release of IDO by human MSCs, which is responsible for the inhibition of T cell proliferation (Krampera, 2011). In mice, IFN-γ and TNF-α stimulates chemokine production by MSCs, resulting in T cell attraction and increased iNOS, which subsequently produces NO for inhibition of T cell proliferation (Ren et al., 2008). Not all immunoinhibitory molecules are divergent between mice and

Table 1. Similarities and Differences between Human and Murine MSCs

	Human MSCs	Murine MSCs
Frequency in BM-MNCs	1:100,000; 1:24,000	1:100,0000; (?)
Growth pattern	linear growth	lag phase followed by linear growth (Phinney et al., 1999)
Presence of chromosomal abnormalities	rare[a] (Tarte et al., 2010; Ben-David et al., 2011)	invariable (Miura et al., 2006; Tolar et al., 2007)
Transformation and immortalization	not reported (Bernardo et al., 2007; Prockop et al., 2010)	frequent (Miura et al., 2006; Tolar et al., 2007)
Expression of MHC class I/II molecules	100%/<10%[b]; up to 80%–100%[c]	100%/<10%[b]; up to 80%–100%[c]
Expression of costimulatory molecules	ICAM-1; VCAM-1	ICAM-1; VCAM-1
Expression of chemokines, cytokines	SDF-1; IL-6; TGF-β; PGE2; LIF; HLA-G; galectins	SDF-1; IL-6; TGF-β; PGE2; galectins
Expression of chemokine/cytokine receptors	IL-1; TNF-α; TLRs; IFN-γ; TGF-β	IL-1; TNF-α; TLRs; IFN-γ; TGF-β
Common effector molecules for immune regulation	IL-6; PGE2; galectins; IL-10; IL-12 (Najar et al., 2010; Ghannam et al., 2010)	IL-6; PGE2; galectins; IL-10; IL-12 (Németh et al., 2009)
Differences in effector molecules for immune regulation	IDO, HLA-G (Krampera, 2011)	NO (Ren et al., 2008)
Key molecules for induction of immune regulation	IFN-γ (induction of IDO) (Krampera, 2011)	IFN-γ and TNF-α (induction of iNOS/NO) (Ren et al., 2008)
Cross-species reactivity	+ murine TNF-α; − murine IFN-γ; + murine NO/IDO	+ human TNF-α; − human IFN-γ; + human IDO/NO

BM-MNCs, bone marrow-mononuclear cells; MHC, major histocompatibility complex; ICAM-1, Intercellular Adhesion Molecule 1; VCAM-1, vascular cell adhesion molecule 1; SDF-1, stromal cell-derived factor 1; IL-6, interleukin-6; TGF-β, transforming growth factor-β; PGE2, prostaglandin E2; LIF, leukemia inhibitory factor; HLA-G, human leukocyte antigen_G; TNF-α, tunor necrosis factor- α; TLRs, Toll-like receptors; IFN-γ, interferon-γ; IDO, indolamine 2,3 dioxygenase; iNOS, inducible NO synthase; NO, nitric oxide.

[a]The presence of chromosomal abnormalities in human MSCs has been rarely reported. Tarte et al. (2010) reported the presence of aneuploidy in MSC preparations for clinical use that were found to be related to senescence of the cells and not to transformation. Prockop et al. (2010) estimated the likelihood of malignant transformation in MSCs to be $<10^{-9}$. Ben-David et al. (2011) reported that 4% of the MSC samples that they analyzed harbored recurrent chromosomal aberrations [3], but the biological significance of these observations was not addressed.

[b]Unstimulated MSCs.

[c]After MSC activation with cytokines (IFN-γ) in the case of human MSCs and dependent on high cell density in the case of murine MSCs.

humans, and PGE2 represents a molecule with a conserved role in MSC-mediated immunoregulation in both humans and mice. While human PGE2 and other factors produced by human MSCs have been shown to participate in the inhibition of T cell proliferative responses in vitro (Najar et al., 2010), PGE2, in conjunction with NO, has been reported to induce an inhibitory IL-10-secreting macrophage phenotype in LPS-containing cocultures in mice (Németh et al., 2009). Moreover, PGE2 and IL-6 produced by human MSCs are, at least in part, responsible for the shift of M0 macrophages into IL-10-producing cells in vitro (Melief et al., 2013b; Ghannam et al., 2010).

The dissimilarities between MSCs isolated from murine and human species require a careful evaluation when choosing animal models to test MSCs in preclinical studies. The contribution of murine models employing murine/human cells to the development of MSC therapy in humans may be limited by the species differences, as shown for the prevention and treatment of GvHD. In this context, several animal studies have addressed the suppressive effect of MSCs, with conflicting results. In one study, human AT-derived MSCs have been infused systemically in mice early after transplantation of haploidentical hematopoietic stem cells (HSCs) and were able to rescue the animals from lethal GvHD (Yañez et al., 2006). Sudres et al. (2006) have reported that a single dose of murine C57BL/6 BM-derived MSCs at time of allogeneic BM transplantation did not affect the incidence and severity of GvHD in BALB/c mice, whereas human UCB-derived MSCs administered at weekly intervals were able to prevent GvHD development after allogeneic transplantation of human PBMCs in NOD/SCID mice (Tisato et al., 2007). The same cells were not effective when administered prophylactically immediately after PBMC infusion, nor when they were infused late in the course of GvHD development. Polchert et al. (2008) tested the ability of MSCs to prevent GvHD by administering a single dose of BALB/c BM MSCs into C57BL/6 mice at different time points. A significant increase in survival of the recipient mice was only observed if MSCs were injected at day +2 or +20 after the allograft. At these time points the levels of IFN-γ were found to be high in the animals, supporting the notion that MSCs need to be activated by inflammatory cytokines to deliver their immunosuppressive effect.

The mixed results of MSC treatment on GvHD prevention and the little effect of MSC infusion on established GvHD reported in these studies remain unexplained. These discrepancies may be related to differences between humans and mice in the pathogenesis of GvHD. In addition, they may be caused by differences in the biological and functional properties of MSCs or by subtle differences in the inflammatory status of the host, resulting in a proinflammatory or anti-inflammatory MSC secretome. Finally, they could also be affected by differences in the experimental models employed. Most data have been derived from

MHC-mismatched models that do not fully reflect human allotransplantation. Indeed, reports in murine models contrast with observations in clinical trials, where MSC treatment has been effective in suppressing established GvHD (Le Blanc et al., 2008) but has little effect on GvHD (Lazarus et al., 2005). These differences may relate to the use of different immune effector molecules between mice and human MSCs (i.e., IDO versus NO) but may also result from lack of cross-species reactivity of cytokines. IFN-γ is species specific and, therefore, human MSCs cannot be activated in vivo by mouse IFN-γ, but can still be stimulated by TNF-α since it is not species specific. In spite of the crucial differences in the use of effector molecules and the lack of cross-species reactivity of key cytokines such as IFN-γ, human MSCs have shown therapeutic effects in mouse models of GvHD (Tisato et al., 2007). However, with the exception of TSG-6 (Lee et al., 2009), the mechanisms of efficacy in these models remain as yet unclear.

Although MSCs have been applied in a variety of disease models, including experimental autoimmune encephalomyelitis (EAE), colitis, retinitis, and myocardial infarction, results are sometimes difficult to reproduce. Strain-specific induced disease models may suffer from experimental fine-tuning in order to arrive at an anticipated outcome. Therefore, there remains a need for robust animal models to test the in vivo modulatory properties of MSCs, and data derived from one model in a single strain may not be sufficient.

Anti-Inflammatory Effects of MSCs in the Clinic: Treatment of GvHD as a Case Study

Stromal therapy in patients with steroid-refractory acute GvHD (aGvHD) occurring after allogeneic HSCT and/or donor lymphocyte infusion is one of the most extensively investigated potential clinical applications of MSCs. Following the first report on a pediatric patient experiencing grade IV treatment refractory aGvHD who was rescued by the infusion of BM-derived MSCs (Le Blanc et al., 2004), a multitude of pilot studies have been performed. A phase II, multicenter clinical trial showed a clinical response in the majority of patients (55 adults and children) with steroid-resistant aGvHD treated with intravenous infusion(s) of allogeneic MSCs. This response translated into a significant difference in survival between complete responders and partial/nonresponding patients (Le Blanc et al., 2008). These results have been extended in a cohort of 37 pediatric patients treated with multiple infusions of MSCs (Ball et al., 2013). Similar results have been reported in a smaller cohort of pediatric patients treated with platelet-lysate (PL)-expanded MSCs (Lucchini et al., 2010). Clearly, these findings need to be confirmed in prospective randomized studies.

The identification of biomarkers that enable evaluation and quantification of MSC efficacy is of paramount importance for the development of MSC therapy. Unfortunately, clinical studies regarding efficacy of MSC treatment have only rarely been used to identify biomarkers predicting response to MSCs. One approach could be that of analyzing clinical samples from GvHD patients treated with MSC infusion(s) to understand the events underlying patient response in vivo. Dander et al. (2012) analyzed plasma levels of two biomarkers for aGvHD, i.e., interleukin 2 receptor alpha (IL-2Rα) and tumor necrosis factor receptor (TNFR) I, in a group of patients with aGvHD before and after MSC treatment. While the levels of the two factors were elevated before MSC infusion, they persistently decreased in responder patients, suggesting that these phenomena were related. Interestingly, the same authors observed that one of the patients responding to MSC treatment showed a decrease in the biomarker concentrations. Thereafter the patient developed chronic GVHD (cGvHD) that did not respond to an additional infusion of MSCs, and the patient's IL-2Rα and TNFRI levels remained stable or even increased after the infusion. This observation is in line with several studies indicating that MSCs need to be activated by an inflammatory environment to deliver their therapeutic effect (Krampera, 2011). This environment may be more frequently present during established aGvHD than in cGvHD.

The available evidence suggests that responses to MSC treatment may be independent of the MSC donor or dose of the immune-suppressive treatment employed. This heterogeneity in response might be related to the presence or absence of the appropriate environment in the patient capable of activating MSCs. Strategies to understand the ongoing patient inflammatory status at the time of MSC infusion could, therefore, allow the development of relevant biomarkers. It is conceivable that heterogeneity in responses could be mainly related to host factors, including an appropriate proinflammatory microenvironment, rather than a result of product-related factors. The consequence of this possibility is that product-related potency assays may be of relatively little value.

Conclusions and Future Directions

We have reviewed here the regulatory properties of MSCs in immune-mediated or inflammatory conditions, emphasizing the central role of the innate immune system in the modulatory effects of MSCs. In particular, we have highlighted the prominent role of monocytes/macrophages in orchestrating both proinflammatory and anti-inflammatory responses (see Figure 2).

While this model is supported by in vitro and animal studies (Dazzi et al., 2012; Le Blanc and Mougiakakos, 2012), it should be noted that it remains to be demonstrated to what extent these pathways are operational in vivo. There are many outstanding questions about the physiological role of MSC-based immune modulation that will need to be addressed to support further development of their clinical application. While prospective randomized clinical trials aiming at demonstrating efficacy and safety are warranted, an adequate understanding of the underlying mechanisms is required to realize their therapeutic potential. An important consequence of the polarization concept is that immunomodulatory effects of MSCs will be largely determined by local inflammatory conditions in the host. Timing and route of delivery of MSC treatment may, therefore, be critical in determining the treatment responses in patients. Biomarkers predictive for response are not yet available, but the notion that early treatment with MSCs for steroid-resistant aGvHD may be more effective than treatment initiated later in the course of GvHD is in accordance with this hypothesis (Ball et al., 2013).

There is a clear need to develop animal models that appropriately address the complex interplay between the "MSC product" and the host microenvironment where these cells execute their regulatory function. Insight into the in vitro modulatory networks that result in the generation of anti-inflammatory

cells (M2 macrophages, Tregs) may help to design relevant models to address these issues.

Regarding their therapeutic effect, MSCs may serve as drug delivery vehicles at local sites of inflammation. Novel molecular tools aimed at defining the MSC secretome, proteome, and transcriptome are being employed to more precisely define the soluble factors that mediate MSC function (Ranganath et al., 2012). These tools include MSC-derived microvesicles or exosomes that can mediate intercellular communication between MSCs and other cells (Biancone et al., 2012). As far as the endocrine effects of MSCs are concerned, it is conceivable that identification of relevant effector molecules could lead to novel treatment modalities that might eventually replace cellular therapy with MSCs. However, the effector functions of MSCs may also depend on paracrine mechanisms that are mediated by the concerted interaction between different molecules that are delivered locally through the directed migration of cells to a site of injury. This complexity must be considered when designing novel therapeutic strategies with MSCs. The latter strategy would open the possibility to direct migration and engineer MSCs in order to deliver effector molecules at particular (tissue-specific) sites (Sarkar et al., 2010; Levy et al., 2013).

MSC therapy has entered the clinic in a variety of applications related to tissue repair and alloimmune or autoimmune disorders (Le Blanc et al., 2008; Duijvestein et al., 2010). According to the clinical trial registry at the National Institute of Health, over 350 clinical trials are currently being conducted with these cells (http://clinicaltrials.gov). Other potentially promising indications include the use of MSCs in solid organ transplantation with the aim of reducing the use of immune-suppressive drugs or treating chronic rejection (Casiraghi et al., 2013). The potential use of MSCs in tolerance induction in organ transplantation relates to their ability to skew the balance between effector T cells and regulatory T cells. In autoimmune disorders, the use of MSC therapy in luminal Crohn's disease or Crohn's fistulas is currently under study (Ciccocioppo et al., 2011).

MSC therapy represents an emerging modality of alternative treatment with the capacity to provide site-specific immunoregulation to control pathogenic T cell responses that drive autoimmunity and allograft rejection. Prospective randomized studies are needed to determine the true scope of the therapeutic potential and provide clear evidence of reproducible efficacy. Nevertheless, this promising property of MSCs, independent of their HSC-supporting capacity, warrants extensive further study.

REFERENCES

Aggarwal, S., and Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood *105*, 1815–1822.

Akiyama, K., Chen, C., Wang, D., Xu, X., Qu, C., Yamaza, T., Cai, T., Chen, W., Sun, L., and Shi, S. (2012). Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. Cell Stem Cell *10*, 544–555.

Ball, L.M., Bernardo, M.E., Roelofs, H., van Tol, M.J., Contoli, B., Zwaginga, J.J., Avanzini, M.A., Conforti, A., Bertaina, A., Giorgiani, G., et al. (2013). Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III-IV acute graft-versus-host disease. Br. J. Haematol., in press. Published online August 31, 2013. http://dx.doi.org/10.1111/bjh.12545.

Ben-David, U., Mayshar, Y., and Benvenisty, N. (2011). Large-scale analysis reveals acquisition of lineage-specific chromosomal aberrations in human adult stem cells. Cell Stem Cell *9*, 97–102.

Bernardo, M.E., Zaffaroni, N., Novara, F., Cometa, A.M., Avanzini, M.A., Moretta, A., Montagna, D., Maccario, R., Villa, R., Daidone, M.G., et al. (2007). Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. Cancer Res. *67*, 9142–9149.

Biancone, L., Bruno, S., Deregibus, M.C., Tetta, C., and Camussi, G. (2012). Therapeutic potential of mesenchymal stem cell-derived microvesicles. Nephrol. Dial. Transplant. *27*, 3037–3042.

Brandau, S., Jakob, M., Hemeda, H., Bruderek, K., Janeschik, S., Bootz, F., and Lang, S. (2010). Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge. J. Leukoc. Biol. *88*, 1005–1015.

Burr, S.P., Dazzi, F., and Garden, O.A. (2013). Mesenchymal stromal cells and regulatory T cells: the Yin and Yang of peripheral tolerance? Immunol. Cell Biol. *91*, 12–18.

Campagnoli, C., Roberts, I.A., Kumar, S., Bennett, P.R., Bellantuono, I., and Fisk, N.M. (2001). Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood *98*, 2396–2402.

Carosella, E.D., Favier, B., Rouas-Freiss, N., Moreau, P., and Lemaoult, J. (2008). Beyond the increasing complexity of the immunomodulatory HLA-G molecule. Blood *111*, 4862–4870.

Casiraghi, F., Perico, N., and Remuzzi, G. (2013). Mesenchymal stromal cells to promote solid organ transplantation tolerance. Curr. Opin. Organ Transplant. *18*, 51–58.

Cassatella, M.A., Mosna, F., Micheletti, A., Lisi, V., Tamassia, N., Cont, C., Calzetti, F., Pelletier, M., Pizzolo, G., and Krampera, M. (2011). Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils. Stem Cells *29*, 1001–1011.

Chaudhry, A., and Rudensky, A.Y. (2013). Control of inflammation by integration of environmental cues by regulatory T cells. J. Clin. Invest. *123*, 939–944.

Chen, L., Tredget, E.E., Wu, P.Y., and Wu, Y. (2008). Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE *3*, e1886.

Choi, H., Lee, R.H., Bazhanov, N., Oh, J.Y., and Prockop, D.J. (2011). Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. Blood *118*, 330–338.

Choi, E.W., Shin, I.S., Park, S.Y., Park, J.H., Kim, J.S., Yoon, E.J., Kang, S.K., Ra, J.C., and Hong, S.H. (2012). Reversal of serologic, immunologic, and histologic dysfunction in mice with systemic lupus erythematosus by long-term serial adipose tissue-derived mesenchymal stem cell transplantation. Arthritis Rheum *64*, 243–253.

Ciccocioppo, R., Bernardo, M.E., Sgarella, A., Maccario, R., Avanzini, M.A., Ubezio, C., Minelli, A., Alvisi, C., Vanoli, A., Calliada, F., et al. (2011). Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. Gut *60*, 788–798.

Cutler, A.J., Limbani, V., Girdlestone, J., and Navarrete, C.V. (2010). Umbilical cord-derived mesenchymal stromal cells modulate monocyte function to suppress T cell proliferation. J. Immunol. *185*, 6617–6623.

Dander, E., Lucchini, G., Vinci, P., Introna, M., Masciocchi, F., Perseghin, P., Balduzzi, A., Bonanomi, S., Longoni, D., Gaipa, G., et al. (2012). Mesenchymal stromal cells for the treatment of graft-versus-host disease: understanding the in vivo biological effect through patient immune monitoring. Leukemia *26*, 1681–1684.

Dazzi, F., Lopes, L., and Weng, L. (2012). Mesenchymal stromal cells: a key player in 'innate tolerance'? Immunology *137*, 206–213.

Delarosa, O., Dalemans, W., and Lombardo, E. (2012). Toll-like receptors as modulators of mesenchymal stem cells. Front Immunol *3*, 182.

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P., Grisanti, S., and Gianni, A.M. (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood *99*, 3838–3843.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, Dj., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315–317.

Duijvestein, M., Vos, A.C., Roelofs, H., Wildenberg, M.E., Wendrich, B.B., Verspaget, H.W., Kooy-Winkelaar, E.M., Koning, F., Zwaginga, J.J., Fidder, H.H., et al. (2010). Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut *59*, 1662–1669.

Duijvestein, M., Wildenberg, M.E., Welling, M.M., Hennink, S., Molendijk, I., van Zuylen, V.L., Bosse, T., Vos, A.C., de Jonge-Muller, E.S., Roelofs, H., et al. (2011). Pretreatment with interferon-γ enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. Stem Cells *29*, 1549–1558.

Eggenhofer, E., and Hoogduijn, M.J. (2012). Mesenchymal stem cell-educated macrophages. Transp. Res. *1*, 12.

English, K., Ryan, J.M., Tobin, L., Murphy, M.J., Barry, F.P., and Mahon, B.P. (2009). Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. Clin. Exp. Immunol. *156*, 149–160.

François, M., Romieu-Mourez, R., Li, M., and Galipeau, J. (2012). Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. Mol. Ther. *20*, 187–195.

Friedenstein, A.J., Deriglasova, U.F., Kulagina, N.N., Panasuk, A.F., Rudakowa, S.F., Luriá, E.A., and Ruadkow, I.A. (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp. Hematol. *2*, 83–92.

Gabrilovich, D.I., Ostrand-Rosenberg, S., and Bronte, V. (2013). Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. *12*, 253–268, http://dx.doi.org/10.1038/nri3175.

Ghannam, S., Pène, J., Torcy-Moquet, G., Jorgensen, C., and Yssel, H. (2010). Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. J. Immunol. *185*, 302–312.

Gordon, S., and Mantovani, A. (2011). Diversity and plasticity of mononuclear phagocytes. Eur. J. Immunol. *41*, 2470–2472.

Gratz, I.K., Rosenblum, M.D., and Abbas, A.K. (2013). The life of regulatory T cells. Ann. N Y Acad. Sci. *1283*, 8–12.

Guilloton, F., Caron, G., Ménard, C., Pangault, C., Amé-Thomas, P., Dulong, J., De Vos, J., Rossille, D., Henry, C., Lamy, T., et al. (2012). Mesenchymal stromal cells orchestrate follicular lymphoma cell niche through the CCL2-dependent recruitment and polarization of monocytes. Blood *119*, 2556–2567.

Gupta, N., Su, X., Popov, B., Lee, J.W., Serikov, V., and Matthay, M.A. (2007). Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. J. Immunol. *179*, 1855–1863.

Hall, S.R., Tsoyi, K., Ith, B., Padera, R.F., Jr., Lederer, J.A., Wang, Z., Liu, X., and Perrella, M.A. (2013). Mesenchymal stromal cells improve survival during sepsis in the absence of heme oxygenase-1: the importance of neutrophils. Stem Cells *31*, 397–407.

Im, G.I., Shin, Y.W., and Lee, K.B. (2005). Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis Cartilage *13*, 845–853.

Kawashima, N. (2012). Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Arch. Oral Biol. *57*, 1439–1458.

Ke, C.-C., Liu, R.-S., Suetsugu, A., Kimura, H., Ho, J.H., Lee, O.K., and Hoffman, R.M. (2013). In vivo fluorescence imaging reveals the promotion of mammary tumorigenesis by mesenchymal stromal cells. PLoS ONE *8*(7), e69658.

Keating, A. (2012). Mesenchymal stromal cells: new directions. Cell Stem Cell *10*, 709–716.

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol. *13*, 159–175.

Krampera, M. (2011). Mesenchymal stromal cell 'licensing': a multistep process. Leukemia *25*, 1408–1414.

Krampera, M., Glennie, S., Dyson, J., Scott, D., Laylor, R., Simpson, E., and Dazzi, F. (2003). Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood *101*, 3722–3729.

Lazarus, H.M., Koc, O.N., Devine, S.M., Curtin, P., Maziarz, R.T., Holland, H.K., Shpall, E.J., McCarthy, P., Atkinson, K., Cooper, B.W., et al. (2005). Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol. Blood Marrow Transplant. *11*, 389–398.

Le Blanc, K., and Mougiakakos, D. (2012). Multipotent mesenchymal stromal cells and the innate immune system. Nat. Rev. Immunol. *12*, 383–396.

Le Blanc, K., Rasmusson, I., Sundberg, B., Götherström, C., Hassan, M., Uzunel, M., and Ringdén, O. (2004). Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet *363*, 1439–1441.

Le Blanc, K., Frassoni, F., Ball, L., Locatelli, F., Roelofs, H., Lewis, I., Lanino, E., Sundberg, B., Bernardo, M.E., Remberger, M., et al.; Developmental Committee of the European Group for Blood and Marrow Transplantation. (2008). Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet *371*, 1579–1586.

Lee, R.H., Pulin, A.A., Seo, M.J., Kota, D.J., Ylostalo, J., Larson, B.L., Semprun-Prieto, L., Delafontaine, P., and Prockop, D.J. (2009). Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell *5*, 54–63.

Lee, R.H., Yoon, N., Reneau, J.C., and Prockop, D.J. (2012). Preactivation of human MSCs with TNF-α enhances tumor-suppressive activity. Cell Stem Cell *11*, 825–835.

Levy, O., Zhao, W., Mortensen, L.J., Leblanc, S., Tsang, K., Fu, M., Phillips, J.A., Sagar, V., Anandakumaran, P., Ngai, J., et al. (2013). mRNA-engineered mesenchymal stem cells for targeted delivery of interleukin-10 to sites of inflammation. Blood, in press. Published online August 26, 2013. http://dx.doi.org/10.1182/blood-2013-04-495119.

Li, W., Ren, G., Huang, Y., Su, J., Han, Y., Li, J., Chen, X., Cao, K., Chen, Q., Shou, P., et al. (2012). Mesenchymal stem cells: a double-edged sword in regulating immune responses. Cell Death Differ. *19*, 1505–1513.

Lucchini, G., Introna, M., Dander, E., Rovelli, A., Balduzzi, A., Bonanomi, S., Salvadè, A., Capelli, C., Belotti, D., Gaipa, G., et al. (2010). Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. Biol. Blood Marrow Transplant. *16*, 1293–1301.

Luo, Y., Wang, Y., Poynter, J.A., Manukyan, M.C., Herrmann, J.L., Abarbanell, A.M., Weil, B.R., and Meldrum, D.R. (2012). Pretreating mesenchymal stem cells with interleukin-1β and transforming growth factor-β synergistically increases vascular endothelial growth factor production and improves mesenchymal stem cell-mediated myocardial protection after acute ischemia. Surgery *151*, 353–363.

Maccario, R., Podestà, M., Moretta, A., Cometa, A., Comoli, P., Montagna, D., Daudt, L., Ibatici, A., Piaggio, G., Pozzi, S., et al. (2005). Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica *90*, 516–525.

Madec, A.M., Mallone, R., Afonso, G., Abou Mrad, E., Mesnier, A., Eljaafari, A., and Thivolet, C. (2009). Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. Diabetologia *52*, 1391–1399.

Mantovani, A. (2012). MSCs, macrophages, and cancer: a dangerous ménage-à-trois. Cell Stem Cell *11*, 730–732.

Mantovani, A., Biswas, S.K., Galdiero, M.R., Sica, A., and Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. J. Pathol. *229*, 176–185.

Melief, S.M., Schrama, C.L.M., Brugman, M.H., Tiemessen, M.M., Hoogduijn, M.J., Fibbe, W.E., and Roelofs, H. (2013a). Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of

monocytes towards anti-inflammatory macrophages. Stem Cells, in press. Published online May 27, 2013. http://dx.doi.org/10.1002/stem.1432.

Melief, S.M., Geutskens, S.B., Fibbe, W., and Roelofs, H. (2013b). Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. Haematologica *98*, 888–895.

Miura, M., Miura, Y., Padilla-Nash, H.M., Molinolo, A.A., Fu, B., Patel, V., Seo, B.M., Sonoyama, W., Zheng, J.J., Baker, C.C., et al. (2006). Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells *24*, 1095–1103.

Mo, I.F., Yip, K.H., Chan, W.K., Law, H.K., Lau, Y.L., and Chan, G.C. (2008). Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors. BMC Cell Biol. *9*, 52.

Najar, M., Raicevic, G., Boufker, H.I., Fayyad Kazan, H., De Bruyn, C., Meuleman, N., Bron, D., Toungouz, M., and Lagneaux, L. (2010). Mesenchymal stromal cells use PGE2 to modulate activation and proliferation of lymphocyte subsets: Combined comparison of adipose tissue, Wharton's Jelly and bone marrow sources. Cell. Immunol. *264*, 171–179.

Németh, K., Leelahavanichkul, A., Yuen, P.S., Mayer, B., Parmelee, A., Doi, K., Robey, P.G., Leelahavanichkul, K., Koller, B.H., Brown, J.M., et al. (2009). Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat. Med. *15*, 42–49.

Nemeth, K., Mayer, B., and Mezey, E. (2010). Modulation of bone marrow stromal cell functions in infectious diseases by toll-like receptor ligands. J. Mol. Med. *88*, 5–10.

Park, D., Spencer, J.A., Koh, B.I., Kobayashi, T., Fujisaki, J., Clemens, T.L., Lin, C.P., Kronenberg, H.M., and Scadden, D.T. (2012). Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. Cell Stem Cell *10*, 259–272.

Phinney, D.G., Kopen, G., Isaacson, R.L., and Prockop, D.J. (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J. Cell. Biochem. *72*, 570–585.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143–147.

Polchert, D., Sobinsky, J., Douglas, G., Kidd, M., Moadsiri, A., Reina, E., Genrich, K., Mehrotra, S., Setty, S., Smith, B., and Bartholomew, A. (2008). IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur. J. Immunol. *38*, 1745–1755.

Prockop, D.J., and Oh, J.Y. (2012). Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol. Ther. *20*, 14–20.

Prockop, D.J., Brenner, M., Fibbe, W.E., Horwitz, E., Le Blanc, K., Phinney, D.G., Simmons, P.J., Sensebe, L., and Keating, A. (2010). Defining the risks of mesenchymal stromal cell therapy. Cytotherapy *12*, 576–578.

Raicevic, G., Rouas, R., Najar, M., Stordeur, P., Boufker, H.I., Bron, D., Martiat, P., Goldman, M., Nevessignsky, M.T., and Lagneaux, L. (2010). Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells. Hum. Immunol. *71*, 235–244.

Ranganath, S.H., Levy, O., Inamdar, M.S., and Karp, J.M. (2012). Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. Cell Stem Cell *10*, 244–258.

Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A.I., Zhao, R.C., and Shi, Y. (2008). Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell *2*, 141–150.

Ren, G., Zhao, X., Wang, Y., Zhang, X., Chen, X., Xu, C., Yuan, Z.R., Roberts, A.I., Zhang, L., Zheng, B., et al. (2012). CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFα. Cell Stem Cell *11*, 812–824.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., and Bianco, P. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324–336.

Sarkar, D., Vemula, P.K., Zhao, W., Gupta, A., Karnik, R., and Karp, J.M. (2010). Engineered mesenchymal stem cells with self-assembled vesicles for systemic cell targeting. Biomaterials *31*, 5266–5274.

Selmani, Z., Naji, A., Zidi, I., Favier, B., Gaiffe, E., Obert, L., Borg, C., Saas, P., Tiberghien, P., Rouas-Freiss, N., et al. (2008). Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells *26*, 212–222.

Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T.M., Serbina, N.V., Lipuma, L., Leiner, I., Li, M.O., Frenette, P.S., and Pamer, E.G. (2011). Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. Immunity *34*, 590–601.

Shi, Y., Su, J., Roberts, A.I., Shou, P., Rabson, A.B., and Ren, G. (2012). How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. *33*, 136–143.

Stagg, J., and Galipeau, J. (2013). Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. Curr. Mol. Med. *13*, 856–867.

Sudres, M., Norol, F., Trenado, A., Grégoire, S., Charlotte, F., Levacher, B., Lataillade, J.J., Bourin, P., Holy, X., Vernant, J.P., et al. (2006). Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. J. Immunol. *176*, 7761–7767.

Tarte, K., Gaillard, J., Lataillade, J.J., Fouillard, L., Becker, M., Mossafa, H., Tchirkov, A., Rouard, H., Henry, C., Splingard, M., et al.; Société Française de Greffe de Moelle et Thérapie Cellulaire. (2010). Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. Blood *115*, 1549–1553.

Tisato, V., Naresh, K., Girdlestone, J., Navarrete, C., and Dazzi, F. (2007). Mesenchymal stem cells of cord blood origin are effective at preventing but not treating graft-versus-host disease. Leukemia *21*, 1992–1999.

Tolar, J., Nauta, A.J., Osborn, M.J., Panoskaltsis Mortari, A., McElmurry, R.T., Bell, S., Xia, L., Zhou, N., Riddle, M., Schroeder, T.M., et al. (2007). Sarcoma derived from cultured mesenchymal stem cells. Stem Cells *25*, 371–379.

Tomchuck, S.L., Zwezdaryk, K.J., Coffelt, S.B., Waterman, R.S., Danka, E.S., and Scandurro, A.B. (2008). Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem Cells *26*, 99–107.

Waterman, R.S., Tomchuck, S.L., Henkle, S.L., and Betancourt, A.M. (2010). A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS ONE *5*, e10088.

Yamane, H., and Paul, W.E. (2013). Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. Immunol. Rev. *252*, 12–23.

Yañez, R., Lamana, M.L., García-Castro, J., Colmenero, I., Ramírez, M., and Bueren, J.A. (2006). Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells *24*, 2582–2591.

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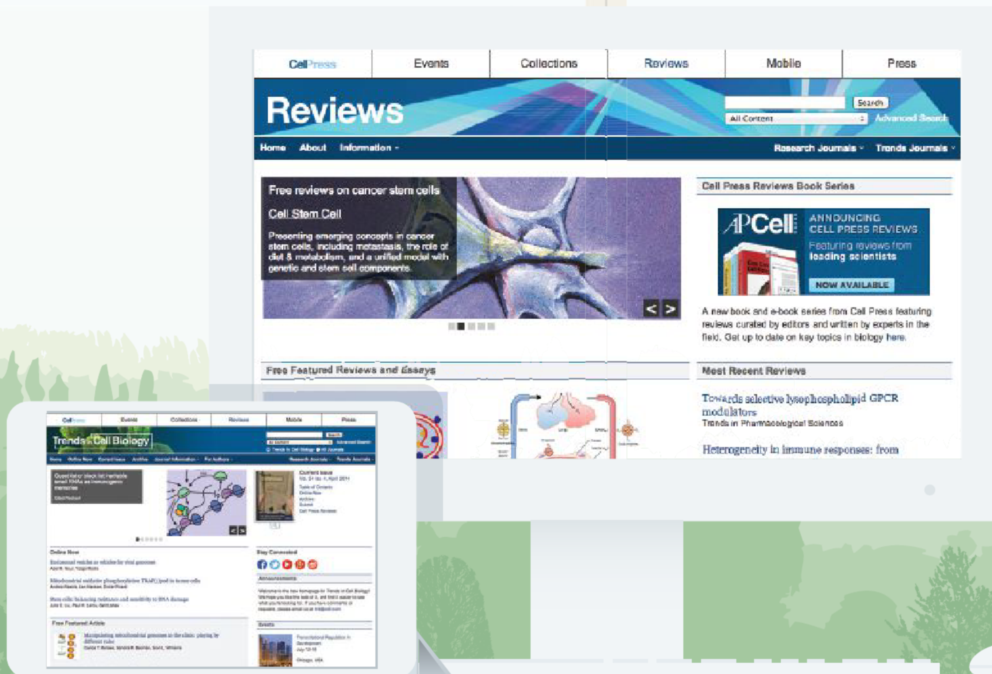

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Review

Harnessing the Mesenchymal Stem Cell Secretome for the Treatment of Cardiovascular Disease

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The broad repertoire of secreted trophic and immunomodulatory cytokines produced by mesenchymal stem cells (MSCs), generally referred to as the MSC secretome, has considerable potential for the treatment of cardiovascular disease. However, harnessing this MSC secretome for meaningful therapeutic outcomes is challenging due to the limited control of cytokine production following their transplantation. This review outlines the current understanding of the MSC secretome as a therapeutic for treatment of ischemic heart disease. We discuss ongoing investigative directions aimed at improving cellular activity and characterizing the secretome and its regulation in greater detail. Finally, we provide insights on and perspectives for future development of the MSC secretome as a therapeutic tool.

Introduction

Ischemic heart disease is the leading cause of human mortality globally, resulting in about 7.25 million deaths each year (World Health Organization, 2011). Acute myocardial infarction (AMI) is the most common cause of heart failure. AMI triggers a series of cellular and molecular changes leading to apoptosis, necrosis, and hypertrophy of cardiomyocytes; impaired neovascularization; interstitial fibrosis and inflammation; reduced contractility; and pathological remodeling. Current therapies have failed to address the devastating aftermath of AMI. Most clinically approved therapeutics focus on modulating hemodynamics to reduce early mortality but do not facilitate cardiac repair in the way that would be needed to reduce the incidence of heart failure (Velagaleti et al., 2008). It is now widely accepted that treatment of the complex pathology resulting from AMI will require taking approaches designed to enhance tissue regeneration via cell transplantation or co-opting local mechanisms that promote healing and inhibit pathological remodeling (Wollert and Drexler, 2010).

Regeneration of an infarcted heart necessitates massive cell replenishment, possibly in the order of a billion cardiomyocytes, and functional integration together with supporting cell types (Laflamme and Murry, 2005). While the search for cardiac-progenitor cells (CPCs) that can readily engraft within damaged tissue and differentiate into functioning cardiomyocytes continues (Xu et al., 2011), regenerative therapy using bone-marrow-derived mononuclear cells (BM-MNCs) and mesenchymal stem cells (MSCs) has shown considerable promise in preclinical studies (Chavakis et al., 2010; Mirotsou et al., 2011). The first stem-cell-based clinical trials for MI (initiated between 2002 and 2005) used unfractionated, easily accessible, and highly heterogeneous adult BM-MNCs. Despite initial positive results indicating safety of BM-MNC transplantation and improved cardiac function, the differences in trial design, treatment methods, outcome evaluation, and cell isolation have prevented general conclusions, and all of these studies require long-term follow-up analysis (Wollert and Drexler, 2010).

Recent clinical trials have looked at relatively homogenous MSCs expanded in culture after isolation from bone marrow (containing 0.001%–0.01% MSCs) as potential cell therapy candidates for AMI owing to their immunomodulatory properties, ready availability, and cardiac stem cell (CSC) niche-regulatory ability. The first clinical trial for AMI using human MSCs (hMSCs) demonstrated the safety of hMSC transplantation and provisional efficacy (Hare et al., 2009). However, the improved cardiac function observed in preclinical studies is without long-term MSC engraftment (Iso et al., 2007), and, in animal studies, systemically administered MSCs exhibit low (~2%) engraftment levels and limited capacity for transdifferentiation into cardiomyocytes posttransplantation (Leiker et al., 2008). Thus, it seems unlikely that MSCs contribute directly to replenishing cardiomyocyte populations in the heart, and this notion motivated MSC-induced immunomodulatory and remodeling effects to be proposed as mechanisms of cardiovascular repair. Although the trophic and immunomodulatory properties of MSCs represent a primary mechanism of therapeutic action that is referred to in many current clinical trials (Ankrum and Karp, 2010; Wollert and Drexler, 2010), it is important to note that these functions of MSCs have not yet been optimized in preclinical models to maximize their therapeutic potential.

The spectrum of regulatory and trophic factors secreted by MSCs, including growth factors, cytokines, and chemokines, is broadly defined as the MSC secretome. A thorough in vivo examination of this MSC secretome and strategies to modulate it are still lacking, but seem essential for rational therapy design and improvement of existing therapies. Despite the absence of

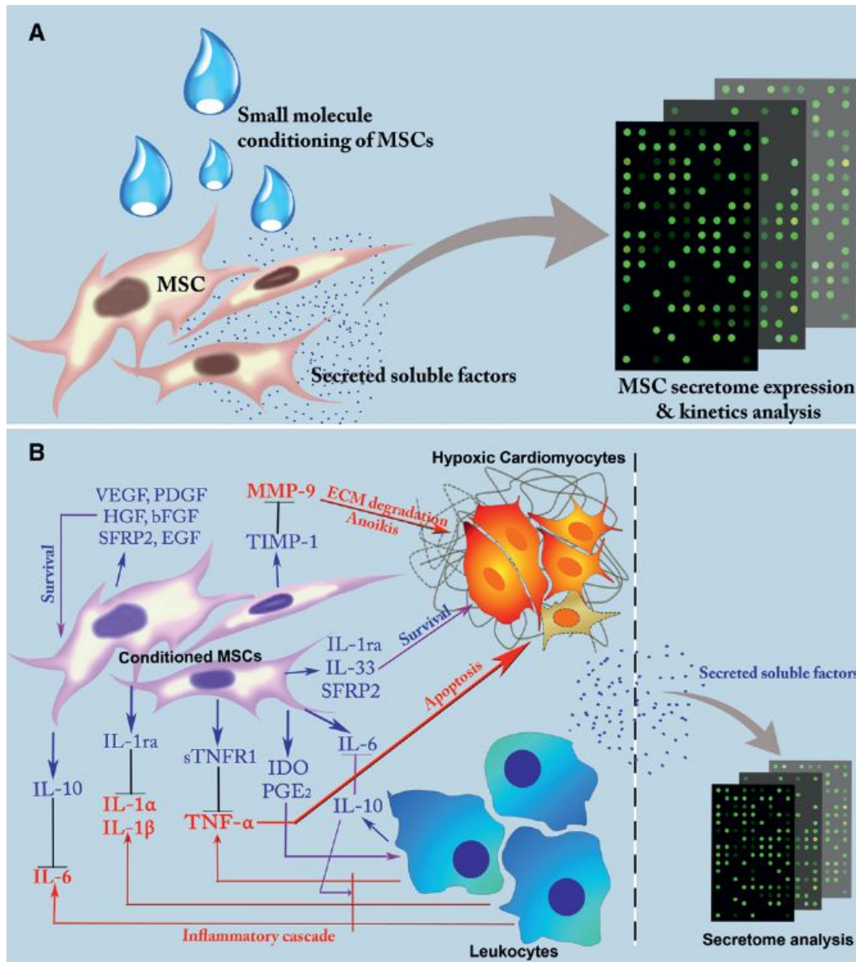

Figure 1. A Proposed Approach for Small-Molecule-Mediated Regulation of the MSC Secretome

(A) Conditioning MSCs with small molecules can stimulate the production of a customized secretome that can be optimized and characterized in vitro.

(B) The effect of small molecule conditioning of MSCs can be tested under highly dynamic, simulated conditions, mimicking microenvironments before and after the onset of MI, and this may include coculture assays with hypoxic cardiomyocytes and inflammatory cells. Proinflammatory cytokines such as TNF-α and IL-6 can be introduced to an MSC culture, for example, and MSC-secreted factors (in blue) such as sTNFR1 and IL-10 can be tracked, as can their ability to modulate the release of inflammatory cytokines from activated leukocytes. MSCs may secrete molecules such as IDO and PGE_2 that induce leukocytes to produce anti-inflammatory cytokines such as IL-10 that attenuate the effects of activated leukocytes and inhibit the proinflammatory activity of constitutively secreted IL-6 (from MSCs) and/or IL-6 already expressed in the myocardium. Likewise, to attenuate pathological remodeling, one can test if small molecules can boost MSC secretion of TIMP-1 to inhibit ECM-degrading proteases such as MMP-9. In addition to paracrine effects, conditioned MSCs may act through autocrine signaling to improve cell survival in hypoxic conditions.

such in vivo data, current MSC-based approaches have shown some promise in preclinical models. In these cases the secretome was modulated by physiological (hypoxic or anoxic), pharmacological (small molecule), cytokine, or growth factor preconditioning and/or genetic manipulations (Afzal et al., 2010; Kamota et al., 2009; Shi et al., 2009; Tang et al., 2010) prior to transplantation. Nevertheless, several questions regarding MSC secretome function and regulation remain unanswered, including the following: (1) what are the most effective approaches to study the MSC secretome in vivo, and are new technologies required to achieve this? (2) How do the properties of the MSC secretome (composition and sustainability) change in vitro and after transplantation, and how does it evolve as a function of the dynamic local microenvironment? (3) What are the best methods to achieve sustainability of the secretome and control over its composition posttransplantation?

Here we discuss current understanding of the MSC secretome and put in perspective its application to cardiovascular therapy. We also review tools for MSC secretome profiling and current preconditioning strategies that aim to transiently control the secretome posttransplantation. Finally, we suggest approaches that could exploit the MSC secretome for cardiovascular therapy.

Profiling the MSC Secretome: Tools and Critical Parameters

To define the specific roles of MSC-secreted factors in cardiovascular regeneration, one should start with biomolecular profiling or secretome analysis of cultured primary MSCs (Figure 1A). A typical MSC secretion profile comprises growth factors, cytokines, extracellular matrix (ECM) proteases, hormones, and lipid mediators (typically in low abundance). Thus, MSC in vitro secretome analysis must consider the effect of serum, which contains many overlapping components and can interfere with detection. To circumvent this problem, MSCs can be cultured for a short time frame in serum-free medium or medium with defined serum replacements. It is critical to consider that secretome expression in vitro is likely very different from what would be expected in vivo where cells within different microenvironments would exhibit unique secretome expression profiles. Conversely, as microenvironments are highly dynamic, they would in turn impact the kinetics of secretome expression. Thus, moving forward it will be critical to examine secretome expression in vitro under conditions that model several relevant in vivo microenvironments.

The tools available for studying secretome expression in vitro include multiplex antibody-based techniques, such as antibody arrays that offer high sensitivity (typically 1–10 pg/ml) as well as high specificity, reproducibility across a broad range of concentrations, and the potential for massively parallel experimentation. High-throughput analysis of the hMSC secretome using a human cytokine antibody array, for example, identified at least 40 proteins with high expression levels varying from 10% to 110% spot intensity relative to the negative control and normalized to

a positive control (Parekkadan et al., 2007). Antibody arrays have also been employed to assess the contribution of MSC-derived factors such as VEGF, TIMP-1, TIMP-2, and TSP-1 in cardiac improvement in swine MI models (Nguyen et al., 2010). The impact of hMSC tissue origin on secretome characteristics (bone marrow versus umbilical cord) has also been examined (Park et al., 2009) using antibody arrays. IL-8 was secreted at higher concentrations in umbilical-cord-blood-derived-MSCs (UCB-MSCs), while IGBP class cytokines were specific to UCB-MSCs compared with BM-MSCs, indicating a potential origin-specific hMSC secretome.

In addition to antibody-based approaches, Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) is useful for characterizing the secretome profile. For example, preconditioning of human adipose-tissue-derived MSCs with TNF-α had a profound impact on the secretome detected using LC-MS/MS (Lee et al., 2010), and led to increased expression of cytokines and chemokines such as IL-6, IL-8, MCP-1, MMPs, PTX3, and Cathepsin L. However, LC-MS/MS was unable to detect many cytokines and growth factors that were present in low concentrations. A more systematic integrated approach for hMSC secretome analysis included LC-MS/MS detection, antibody arrays, microarrays, and bioinformatics (Sze et al., 2007), and identified 201 unique proteins (132 using LC-MS/MS and 72 using antibody arrays). Importantly, Sze et al. used computational analysis to predict the roles of the secretome components in metabolism, immune response, and development.

While current techniques have been useful to identify factors expressed at high levels such as IL-6, IL-8, TIMP-2, VEGF, and MCP-1, suggesting constitutive secretion from BM-hMSCs (Park et al., 2009), a complete list of constitutively expressed MSC secretome factors remains to be generated. Despite recent advances in the characterization of the MSC secretome, current techniques suffer from multiple deficiencies. Gel-based and LC-MS/MS techniques have limited sensitivity to molecules in low concentrations (10–20 fmol), and antibody-based techniques (e.g., ELISA and antibody arrays) are limited by the availability of antibodies to detect secreted proteins. Hence, comprehensive in vitro secretome profiling requires an integrated approach employing multiple techniques. Although determining the mechanism regulating the expression of the secretome is important, the task is made more challenging given that some of the proteins are released during cell death (Skalnikova et al., 2011). Perhaps the most important goal will be to move toward methods to profile the secretome in vivo that can distinguish between factors released from the host versus those secreted by the transplanted MSCs. Reaching this point will require the development of new techniques that can directly quantify the dynamic expression profile of MSC-secreted factors both locally and systemically.

Close to the Heart? Relevance of MSC Paracrine Signaling to Cardiovascular Therapy

Recent studies have suggested four potential mechanisms for how exogenous-culture-expanded MSCs may contribute to cardiovascular repair: MSC transdifferentiation into cardiomyocytes (Hatzistergos et al., 2010), fusion of MSCs with native cells (Noiseux et al., 2006), MSC-induced stimulation of endogenous CSCs via direct cell-cell interaction (Mazhari and Hare, 2007), and MSC-paracrine (or endocrine) signaling (Gnecchi et al., 2005; Lee et al., 2009). MSC transdifferentiation into contractile cardiomyocytes is inefficient at best (Toma et al., 2002) and occurs only in the presence of native cardiomyocytes (Hatzistergos et al., 2010; Loffredo et al., 2011; Mazhari and Hare, 2007). Cell fusion is a rare event, which rules out substantial involvement in MSC-mediated cardiovascular regeneration (Loffredo et al., 2011). Nevertheless, there is strong evidence emerging that rat BM-derived MSCs (rMSCs) secrete trophic factors that may induce activation and proliferation of endogenous CPCs in vitro (Nakanishi et al., 2008). Although it is possible that resident CSCs may differentiate into mature and functional cardiomyocytes upon interaction with transplanted MSCs (Hatzistergos et al., 2010), evidence suggests that CSCs possess only a limited capacity to differentiate into fully mature cardiomyocytes with an adult phenotype (Beltrami et al., 2003; Urbanek et al., 2005). Despite evidence of preferential accumulation of MSCs at sites of myocardial ischemia (Williams and Hare, 2011), exogenously administered MSCs show poor survival and do not persist at the site of AMI (Iso et al., 2007; Terrovitis et al., 2010), probably because of the harsh ischemic microenvironment, characterized by oxidative stress, inflammation, cytotoxic cytokines, and in some instances an absence of ECM for MSC attachment (Rodrigues et al., 2010; Song et al., 2010). Such a hostile microenvironment could hinder the interaction of MSCs with endogenous CSCs.

A more plausible explanation for MSC-mediated cardiovascular repair is an effect on host cells and the microenvironment via MSC-secreted growth factors, cytokines, and other signaling molecules. This proposal is supported by recent preclinical studies (Kanki et al., 2011; Timmers et al., 2011) that demonstrated improved cardiac function upon infusion of cytokines or MSC-conditioned medium (without cell transplantation) (Beohar et al., 2010). Therefore, identifying key MSC-secreted factors and their functional roles in cardiovascular therapies seems a useful approach for rational design of next-generation MSC-based therapeutics.

Effects of the MSC Secretome on Cardiovascular Repair

The functional roles reported for MSC-secreted factors are both impressive and confusing. MSCs are known to be the source of multiple immunomodulatory agents plus trophic factors involved in repair and regenerative processes (Nauta and Fibbe, 2007; van Poll et al., 2008). This broad array of secreted factors suggests possible stress response regulatory roles for MSCs, such as homing of c-kit^{+} cells to injured myocardium (Tang et al., 2010). It is not known whether cytokines released from stressed or dying MSCs make a therapeutic contribution. The hMSC secretome includes multiple factors (Lee et al., 2010; Parekkadan et al., 2007; Park et al., 2009; Sze et al., 2007) known to promote cardiovascular repair (Table S1 available online) and factors that negatively modulate cardiomyocyte apoptosis, inflammation, and pathological remodeling (Table S2). Although several factors in the MSC secretome have shown utility for influencing cardiac repair when delivered exogenously in the absence of MSCs (factors listed in Tables S1 and S2 not marked by an [*]), it is still critical to demonstrate the direct functionality of such factors when secreted from MSCs and the potential synergy that may exist with other secreted factors.

In the context of cardiovascular repair, the array of potential therapeutic mechanisms offered by MSC secretome components spans tissue preservation (antiapoptotic and promitotic), neovascularization, cardiac remodeling (ECM alteration and strengthening of the infarct scar), anti-inflammatory responses (antifibrosis and suppression of inflammatory cells), and the highly contentious endogenous regeneration (activation of CPCs and CSCs). MSCs induce myocardial protection by promoting cardiomyocyte survival and preventing apoptosis through activation of PKC, PI3K/Akt, NF-κB, and STAT3 signaling (Gnecchi et al., 2008; Mirotsou et al., 2011). In ischemic animal models, MSCs mediate neovascularization via paracrine signaling (Kinnaird et al., 2004a, 2004b; Matsumoto et al., 2005; Tang et al., 2005) and have antiapoptotic, anti-inflammatory, and antifibrotic effects on cardiomyocytes and endothelial cells (Bartosh et al., 2010; Berry et al., 2006; Iso et al., 2007; Lee et al., 2009; Shabbir et al., 2009). MSC-induced immunomodulation and antiapoptosis of cardiomyocytes that has been observed in inflammatory heart diseases such as acute myocarditis in mice (Van Linthout et al., 2011) and sepsis in rats (Weil et al., 2010) are likely mediated via paracrine effects. In addition, MSCs exert immunomodulatory effects by inducing neighboring cells to secrete relevant cytokines (Aggarwal and Pittenger, 2005; François et al., 2012; Németh et al., 2009; Prockop and Oh, 2012), which may be useful in inhibiting excessive inflammation and pathological remodeling under MI settings.

MSC Homing to the Injured Myocardium: The Role of MSC Secretome

There is significant debate about whether MSCs need to engraft at the target site of injury or can exert their effects systemically. Engraftment at the target site would in principle seem beneficial due to the potential for cell-cell contact and increased concentrations of immunomodulatory and trophic factors. In the context of cell homing following systemic infusion, sites of MI exhibit increased expression and secretion of selective chemokines, cytokines, and cell adhesion molecules, including ICAM-1, IL-6, SDF-1, VCAM-1, and FN-1 (Ip et al., 2007). However, culture-expanded MSCs exhibit limited homing capacity, probably because of poor expression of receptors for chemokines and adhesion ligands such as CXCR4 and CCR1. As the number of transplanted MSCs homing to the infarcted heart rapidly declines following intravenous infusion (Assis et al., 2010) due to entrapment in the microvasculature, there is a significant need to improve circulation times and homing efficiency of systemically administered cells (Karp and Leng Teo, 2009). For instance, genetic engineering of MSCs has been employed to overexpress key chemokine receptors such as CXCR4 (Cheng et al., 2008) and CCR1 (Huang et al., 2010), and growth factor preconditioning has been used (Hahn et al., 2008; Son et al., 2006) to increase MSC homing to injured myocardium and improve cardiac performance. In addition, bioengineering approaches offer significant potential for chemically modifying the hMSC surface to improve homing to sites of inflammation (Sarkar et al., 2011b). Interestingly, MSCs secrete mobilizing factors such as HGF, LIF, SDF-1, SCF, and VE-Cadherin (Table S1) and thus, optimizing the transplanted MSC secretome could also be beneficial for mobilization and homing of host MSCs.

Striking a Balance between Positive and Negative Factors

Some factors in the MSC secretome, depending on the concentration and release kinetics, may exert inhibitory effects on the cardiac microenvironment, such as apoptosis of cardiomyocytes, inflammation, pathological remodeling, or scar formation. For instance, the TGF-β class of cytokines secreted from poly(I:C)-treated, TLR3-primed hMSCs (Waterman et al., 2010) are known to mediate pathological remodeling during MI and their repressed secretion likely results in decreased collagen deposition. MMP-2, a factor known to mediate ECM degradation during MI (Matsumura et al., 2005) resulting in pathological remodeling via cardiomyocyte anoikis and macrophage infiltration, is endogenously secreted by hMSCs, and the activity of hMSC-secreted MMP-2 can be inhibited by treating hMSCs with TNF-α or hypoxia (Lozito and Tuan, 2011). Additionally, MSC-secreted factors such as MMP-9 and IL-6, responsible for pathological remodeling and proinflammatory responses, respectively, should ideally be maintained at minimum levels because these factors are upregulated in the myocardium during MI (Biswas et al., 2010; Liu et al., 2011). Inhibition of negative factors using antagonists (produced by MSCs) such as TIMP-1 (for MMP-9) and IL-10 (for IL-6) via either intracellular or extracellular targets is one possible strategy for alleviating these effects. Hence, it seems important to not only consider upregulating anti-inflammatory or proangiogenic factors, but also to strive to achieve an appropriate balance between stimulatory and inhibitory factors produced by MSCs as depicted in Figure 1B. In addition to achieving such a balance through iterative in vitro experiments, ultimately the response will need to be preserved following in vivo transplantation, perhaps through bioengineering approaches (Sarkar et al., 2011a) and strategies illustrated in Figure 2.

Bench to Bedside: Practical Considerations for Harnessing the MSC Secretome in Clinical Settings

The first clinical trial for AMI using hMSCs was a randomized, double-blinded, placebo-controlled, dose-escalation study of allogenic hMSCs (Prochymal, Osiris Therapeutics, Inc., Baltimore, MD) (Hare et al., 2009). This study demonstrated the safety of intravenous hMSC transplantation and provisional efficacy (increased left ventricular ejection fraction [LVEF], reduced cardiac arrhythmias, and reverse remodeling compared to placebo) in AMI patients. Results from a phase II multicenter, randomized, double-blind, placebo-controlled study to evaluate Prochymal for safety and efficacy are anticipated in the near future. The ongoing MSC-based trials for treatment of cardiovascular diseases listed in Table 1 reveal an interesting trend in trial designs, in which MSC paracrine mechanisms for improving angiogenesis, cardio-myogenesis, stimulating endogenous cardiac progenitors, and inhibiting remodeling have been highlighted as the primary modes of action. The interim follow-up of two ongoing trials (NCT00677222 and NCT00721045) has reported significant improvement in cardiac functions such as LVEF and stroke volume, and a reduced number of patients with major adverse cardiac events. Nevertheless, the performance of MSCs in these clinical trials has not uniformly met expectations, because positive results and statistical significance were not achieved for all output measures, the

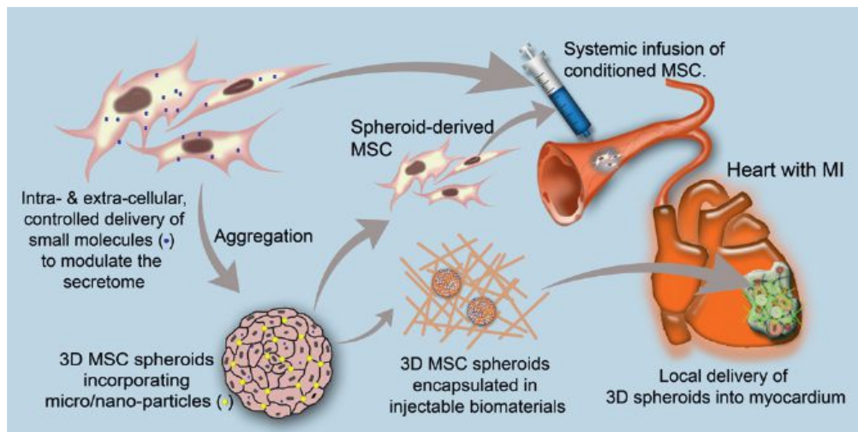

Figure 2. A Proposed Engineering Solution for Sustaining a Customized MSC Secretome In Vivo and Facilitating Cardiovascular Repair
Bioengineering strategies may be employed to control and sustain the expression of the customized MSC secretome through smart-biomaterials-based, intra- and/or extra-cellular, controlled release of stimulating molecules. MSCs, either as single cells or aggregates, may be systemically infused or locally transplanted to facilitate cardiovascular repair with greater control over cell fate and function.

mechanism of action is not fully understood, and the MSC formulations are not fully optimized in terms of delivery methods, secretome composition, cell survival/persistence, and engraftment efficiency. Furthermore, little is known regarding the effect of time of MSC administration on the prevention of cardiomyocyte necrosis/apoptosis, which develops rapidly within 30 min to 12 hr following the onset of MI (Schoen, 2007).

Direct involvement of factors secreted by MSCs in cardiac functional improvement in humans is difficult to demonstrate. Although identification and quantification of the myocardial tissue concentrations of paracrine factors is not feasible, their plasma levels could be indicative of their presence. Future clinical trials should therefore incorporate systematic analysis of the patient plasma not only to elucidate the presence/absence of MSC-secreted paracrine factors but also to investigate whether the impact on host tissue is sustained after elimination of the transplanted MSCs. Although it is challenging to determine whether paracrine factors originate from host cells or transplanted MSCs and to characterize their impact on regulating cytokine expression from host cells (new techniques may be required), a comparative analysis of the patient plasma before and after MSC treatment may provide some insight (and be useful for establishing biomarkers for MSC therapy). Approaches for upregulating specific paracrine factors may help to elucidate (indirectly) mechanisms responsible for the MSC-mediated clinical outcomes (Mirotsou et al., 2007). Although some studies have shown that endocrine activity of dying MSCs can promote regeneration of distant ischemic tissues (Lee et al., 2009), the impact of the MSC secretome on cardiovascular repair can likely be improved through enhancing the survival of the transplanted cells and improving their homing to the target site (Karp and Leng Teo, 2009). Hence, MSC modifications that lead to improved survival and facilitate a sustained and regulated secretome should be considered.

"Cell-free" Therapy: An Alternate to Using MSCs?

Several clinical trials have investigated cytokine therapy approaches for treating cardiovascular diseases (Beohar et al., 2010), and this is further motivated by the improvement in cardiac function seen in preclinical studies from administration of MSC-conditioned medium (Gnecchi et al., 2006). For example, VEGF protein delivery has been shown to improve angiogenesis in coronary artery disease patients (Henry et al., 2003). G-CSF, a cytokine known to mobilize progenitor cells from the bone marrow, was subsequently explored in a series of AMI clinical trials. Despite evidence of safety and feasibility of G-CSF administration in MI patients (Valgimigli et al., 2005), treatment with G-CSF with 5 or 10 μg/kg/day via subcutaneous injection 5 to 6 days after percutaneous coronary intervention (PCI) did not yield any significant increase in LVEF (Engelmann et al., 2006; Ripa et al., 2006). Other cytokines such as GM-CSF, EPO, and IGF-1 have also been tested in clinical trials of cardiovascular diseases (Beohar et al., 2010). To date, single cytokine therapy trials have not met expectations, and there are several possible explanations for why this is the case. Multiple cytokines/growth factors may need to be administered simultaneously at different concentrations and time points to act synergistically to achieve a therapeutic effect. Side effects due to high doses of certain cytokine/growth factors, which may be required due to challenges in protein delivery, can lead to the formation of aberrant and leaky vessels (Carmeliet, 2005), hypotension (Henry et al., 2001), and tumor angiogenesis (Epstein et al., 2001). Controlling the local levels of exogenously delivered cytokines is critical given limitations of pharmacokinetics and stability of proteins in vivo. For instance, intramyocardially delivered, protease-resistant SDF-1 was undetectable after 1 day, yet controlled-release, protease-resistant SDF-1 tethered to self-assembling peptide nanofibers was retained within the myocardial tissue even at day 7 in a rat MI model, and this persistence translated into significant improvement in capillary density and LVEF. Controlled local release of SDF-1 also led to a substantial increase in c-kit$^+$ cell recruitment into the myocardium (Segers et al., 2007). Recombinant periostin exhibited enhanced tissue distribution and persistence via controlled local delivery from Gelfoam patches in a rat MI model (Kühn et al., 2007). Compared to the delivery of single growth factors or cytokines, the use of cells such as MSCs to supply these agents offers significant potential for sustained pharmacokinetics, synergy from multiple factors, and an opportunity for systemic infusion, which is less invasive than local injection and thus amenable to repeated dosing.

Secrets of the MSC Secretome: Underlying Signaling Pathways

Elucidation of the molecular pathways mediating MSC secretome expression is a crucial step toward improving our understanding of the secreted factor profile and its clinical utility.

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Table 1. Ongoing MSC-Based Clinical Trials for Cardiovascular Diseases Registered at clinicaltrials.gov

Clinical Trial ID	Phase	Condition	No. of Patients	Outcome Measure	Cell Delivery Route	Basis of Trial Design
NCT01394432	III	AMI	50	LVSV	endocardial	reduction in scar formation and increased reverse remodeling
NCT00877903	II	MI	220	ESV, LVEF, infarct size	intravenous	improvement in myocardial remodeling and reduction in incidence of CHF
NCT00790764	II	SCI	60	safety	intracoronary and transendocardial	development of mature and stable vessels and improved cardiac function via combinatorial effect of BM-MNCs and MSCs
NCT00555828	I/II	MI	25	safety, feasibility	transendocardial	transdifferentiation of mesenchymal precursor cells (MPCs) into cardiomyocytes
NCT00677222	I	AMI	28	safety, efficacy	space surrounding target vessel (perivascular)	improvement in cardiac function via MSC paracrine actions
NCT01291329	II	AMI	160	myocardial metabolism, perfusion, LVEF	intracoronary	transdifferentiation of MSCs into cardiomyocytes
NCT00768066 (TAC-HFT)	I / II	IHF	60	safety	transendocardial	stimulation of endogenous cardiac stem cells by the transplanted MSCs
NCT00644410	I / II	CHF	60	LVEF	intramyocardial	development of new myocardium and blood vessels
NCT00587990 (PROMETHEUS)	I / II	LVD	45	safety, LVEF, infarct size, ESV	intramyocardial	combinatorial effects of bypass surgery and MSC transplantation
NCT00721045	II	HF	60	safety, efficacy	transendocardial	MPC-induced large blood vessel formation and cardiac repair
NCT00418418	II	MI	60	LVEF, safety	intramyocardial	combinatorial effects of bypass surgery and MSC transplantation
NCT00883727	I / II	MI	20	myocardial perfusion, infarct size	intravenous	transdifferentiation of MSCs into cardiomyocytes and production of new blood vessels
NCT01087996 (POSEIDON)	I / II	LVD, MI	30	safety, efficacy	transendocardial	neo-myogenesis induced by transplanted allogenic and autologous MSCs
NCT01076920 (MESAMI)	I / II	MI, LVD	10	safety, efficacy	transendocardial	transdifferentiation of MSCs to produce new blood vessels
NCT01449032	II	CMI	60	safety, efficacy	not specified	angiogenesis
NCT01442129	II	HF	30	safety, efficacy	intramyocardial	MPC-induced angiogenesis via paracrine signaling combined with LVAD implantation
NCT01392625	I / II	NDC	36	safety, efficacy	transendocardial	neomyogenesis via MSC-CSC interaction
NCT01270139 (NANOM)	I / II	CAD	180	plaque volume	stenting	functional restoration of blood vessels via nanoburning and MSC paracrine effects
NCT01436123 (NANOM2)	I	CAD	120	plaque volume	stenting	reduction of plaque via paracrine signaling in combination with burning effects from Si-Fe NPs

LVSV, left ventricular systolic volume; SCI, severe coronary ischemia; IHF, ischemic heart failure; CHF, congestive heart failure; LVD, left ventricular dysfunction; ESV, end systolic volume; LVEF, left ventricular ejection fraction; CMI, chronic myocardial ischemia; LVAD, left ventricular assist device; NDC, nonischemic dilated cardiomyopathy; CAD, coronary artery disease.

Although further research is required to fully delineate the signaling mechanisms involved in the expression of the MSC secretome, a wide array of signaling pathways have been implicated in paracrine-mediated cardiac repair by MSCs (Gnecchi et al., 2008).

The PI3K/Akt pathway is believed to be involved in the production and secretion of paracrine factors by rMSCs (Gnecchi et al., 2005, 2006). Genetically modifying rMSCs to overexpress the *Akt* gene resulted in the upregulation of the *Akt* target genes *VEGF*, *FGF-2*, *IGF-1*, *HGF*, and *Thrombospondin-4* (Gnecchi et al., 2006). PI3K signaling and ERK1/2 signaling have also been implicated in VEGF production by mMSCs in response to exogenous IL-6 in vitro (Herrmann et al., 2011), although it is unclear whether the effect is solely from the exogenous IL-6 treatment because mMSCs constitutively secrete IL-6. In another study, AngII-stimulated VEGF expression and secretion from rMSCs was mediated by ERK1/2 and the Akt pathway via angiotensin II type 1 (AT_1) receptor in vitro (Shi et al., 2009). Another important signaling pathway is the p38 mitogen-activated protein kinase (*p38 MAPK*), which mediates hMSC

paracrine activity. p38 MAPK was activated as a stress (TNF-α-induced) response, and led to increased in vitro production of VEGF, HGF, and IGF-1 by hMSCs (Wang et al., 2006). The p38 MAPK pathway, along with MEK and PI3K, has also been implicated in mediating TGF-α-induced in vitro HGF production in hMSCs via EGF receptor (EGFR) (Wang et al., 2009). The involvement of p38 MAPK in TGF-α-induced in vitro VEGF production in mMSCs via EGFR (Herrmann et al., 2010) and serum-free-medium-induced in vitro production of IL-6, IL-8, and CXCL1 (Yew et al., 2011) in hMSCs have also been reported.

The JAK-STAT cascade is thought to be a central regulatory pathway in MSC paracrine factor expression. For instance, STAT3 and p38 MAPK were shown to mediate the TNF-α-stimulated VEGF production by mMSCs in vitro (Wang et al., 2007). However, whether VEGF production is independently controlled by p38 MAPK and STAT3 or via a crosstalk between these pathways is not yet clear. STAT3 and MAPK were also activated by treating hMSCs with IL-6, leading to improved in vitro hMSC migratory potential, likely via paracrine activity (Rattigan et al., 2010). In another study, knockout of toll like receptor-4 (*TLR4*) in mMSCs resulted in an increased in vitro secretion of angiogenic factors and chemokines and decreased secretion of inflammatory chemokines via *STAT3* activation (Wang et al., 2010), further highlighting the role of STAT3 signaling in the MSC secretome expression. The transcription factor *GATA-4* has also been implicated in the increased rMSC production of angiogenic paracrine factors (VEGF, IGF-1, and bFGF) and was shown to possess antiapoptotic effects on MSCs under stress via *GATA-4* overexpression (Li et al., 2010).

The transcription factor nuclear factor-κB (NF-κB) is considered a central regulator of stress response and a key mediator of immune responses, regulating the expression of more than 150 target genes (Pahl, 1999) that code for cytokines, chemokines, growth factors, cell adhesion proteins, and cell surface receptors. NF-κB function in hMSCs has been investigated under stress conditions, such as TNF-α, lipopolysaccharide (LPS), and hypoxia (Crisostomo et al., 2008), and its activation was implicated in the increased in vitro production of several growth factors, such as VEGF, FGF-2, and HGF, by hMSCs in response to stress conditions. The involvement of NF-κB via TLR4 receptor activation is demonstrated in the production of prostaglandin E_2 in hMSCs upon treatment with LPS, leading to a reduction in inflammation in a cecal ligation and sepsis (CLP) model in mice (Németh et al., 2009). Recently, more evidence of the involvement of NF-κB signaling in rMSC paracrine factor expression has been reported (Afzal et al., 2010). Diazoxide (DZ), a K_{ATP}-channel-opening small molecule, concomitantly augmented the phosphorylation of PI3K/Akt, glycogen synthase kinase 3β (GSK3β), and NF-κB in rMSCs, resulting in elevated expression levels of growth factors such as IGF, bFGF, HGF, Ang-2, and VEGF in vitro.

Although the mechanistic studies conducted so far have provided some key insights, a more comprehensive understanding of the signaling networks responsible for the unique MSC secretome is still required. The network of signaling pathways involved in constitutive expression of the MSC secretome has not been elucidated yet, but clearly there is a major role for stress signaling that may represent the MSCs' ability to sense and respond to specific stimuli and allow the cells to cope with changing environmental conditions. A comparative study of evolutionarily conserved signaling pathways that mediates MSC secretome expression could be informative. The activation/inhibition of multiple pathways could be essential to obtain an appropriately customized balance of secreted factors. The interaction of kinases with transcription factors is also not well understood. Additionally, two or more transcription factors, for example GATA and STAT, could also interact at the transcriptional level to mediate paracrine secretion (Wang et al., 2005). Considering the complexity of signaling networks, a holistic approach must be used to establish the specific role of receptors, kinases, and transcription factors in the MSC secretome. Such an approach could provide a useful axis for enhanced control over the secretome profile, leading to the development of precisely regulated MSC therapies.

The Secretome Switches: Preconditioning Strategies for Stimulating MSC Paracrine Secretion

A number of preclinical studies have focused on transplanting MSCs into the infarcted heart with the hope that relevant signaling cues from the injury would regulate the MSC secretome (Iso et al., 2007; Lee et al., 2009; Nagaya et al., 2005; Shabbir et al., 2009). However, most of the signaling molecules (such as TNF-α, IL-6, IL-1β, IFN-γ, MCP-1, Fractalkine, and others) secreted by macrophages, monocytes, fibroblasts, and cardiomyocytes during MI are transient. For example, the TNF-α level in the myocardium of rat infarcted hearts peaked at 7–8 days after MI, followed by a decline to basal level in the plasma within 48 hr (Berthonneche et al., 2004; Moro et al., 2007). Thus, even though these signaling molecules could produce a brief extension of MSC survival and an improved response to the highly dynamic and heterogeneous signaling cues during MI, they are unlikely to achieve long-lasting, controlled MSC paracrine action. Hence, other longer-lasting means of improving transplanted MSC function through extending MSC survival or via improved control of the secretome composition have been investigated. Most of these strategies are performed ex vivo and are referred to as preconditioning strategies.

Physiological Preconditioning

Subjecting MSCs to physiological conditions of hypoxia (<5% O_2) and anoxia in vitro and in the ischemic heart has been reported to improve the survival of transplanted MSCs, cardiomyocytes, and endothelial cells via paracrine effects. For example, Kinnaird et al. demonstrated a significant increase (>1.5-fold) in the secretion of several arteriogenic cytokines, including VEGF, bFGF, PlGF, and TGF-β, after subjecting hMSCs to 72 hr hypoxia compared with normoxic conditions (Kinnaird et al., 2004a). However, the increased levels of VEGF and bFGF in hMSC-conditioned medium could only partially account for the improved endothelial cell proliferation response in vitro. A systematic gene expression analysis showed that at least 165 genes, including *vegf*, *egf*, and *mmp-9*, were upregulated >3-fold in rMSCs following 24 hr hypoxic preconditioning (Ohnishi et al., 2007). However, this study did not report secreted protein levels and related functional assays to establish a correlation between secretion levels and function. Moreover, there is considerable variation between studies in terms of the hypoxia exposure time and the resulting secretion levels of paracrine factors. It is also not clear how long hypoxia preconditioning

effects last both in vitro and in vivo. While serum deprivation, yet another in vitro model for ischemia, has also been shown to induce secretion of angiogenic factors by hMSCs (Oskowitz et al., 2011), the observed effect could have been due to differences in cell proliferation rates. In general, physiological preconditioning via hypoxia exposure induces MSCs to activate survival pathways and secrete factors to counteract hypoxic effects. However, given its short duration, it is unclear if this transient response could produce a clinically relevant outcome.

Genetic Manipulation

MSCs can also be engineered with transgenes for conditional gene expression (typically a single gene) with the aim of improving cell survival and controlling the MSC secretome posttransplantation. Transplanting *Akt1*-transfected rMSCs intramyocardially in rat MI models was advantageous and restored a 4-fold increase in myocardial volume (Mangi et al., 2003), but whether this effect was a result of improved rMSC survival or paracrine effects (or both) was not investigated. *Akt*-overexpressing rMSCs showed upregulated transcript levels of cytoprotective genes *vegf*, *bfgf*, *hgf*, *igf1*, and *tb4* in vitro, suggesting their involvement in mediating the early improvement in cardiac function seen in a rat MI model, including significant reduction in infarct size and improved (1.42-fold versus control) ventricular function <72 hr after rMSC transplantation (Gnecchi et al., 2006). Akt overexpression in mMSCs substantially upregulated SFRP2, a paracrine factor that was demonstrated to be responsible for the improved cardiomyocyte survival and reduced infarct size (3-fold versus PBS control) following transplantation (Mirotsou et al., 2007). MSCs have also been genetically modified to overexpress factors such as VEGF (Yang et al., 2010), IGF1 (Haider et al., 2008), and SDF-1 (Tang et al., 2010). When harnessed for cardiovascular applications, these modified MSCs improved angiogenesis, LVEF, c-kit$^+$ and CD31$^+$ cell mobilization, and contractile function, and reduced LV remodeling effects, primarily through paracrine actions. Overexpression of the transcription factor *GATA-4* (Li et al., 2010) and knockout of *TLR4* (Wang et al., 2010) in MSCs resulted in the increased secretion of VEGF. However, these manipulations also resulted in increased (for MSC-*GATA-4*) or decreased (*TLR4*KO-MSC) IGF-1 secretion levels versus wild-type MSCs; the discrepancy is likely due to the genetic targets manipulated and MSC sources (rMSCs for *GATA-4* and mMSCs for *TLR4* knockout). Therefore in these two studies, the role of IGF-1 in the observed cardioprotective effects in rat MI models is not clear. In general, genetic approaches could be harnessed to directly or indirectly upregulate specific MSC paracrine factors via upregulation of established target genes, even though overexpression of certain genes could lead to undesired effects (Fierro et al., 2011). Nonviral modifications should be sought due to the limitations of viral approaches, including the potential for insertional mutagenesis and increased regulatory hurdles. Furthermore, while there have been many attempts to improve MSC function via genetic manipulation, aside from immunomodulatory factors (e.g., IL-10, IDO, and PGE2) and proangiogenic factors (e.g., VEGF), strong candidates worthy of future pursuit have yet to be identified. The lack of such candidates is probably due to an absence of deep understanding of the underlying pathways and a lack of replicated studies by multiple laboratories.

Molecular Preconditioning Using Proteins

Cytokines, chemokines, and growth factors represent key signaling cues during MI (Debrunner et al., 2008) and hence have been used to control MSC paracrine secretion in vitro (Croitoru-Lamoury et al., 2007). Stimulation of MSCs with TNF-α (50 ng/ml) for 24 hr (Wang et al., 2007), SDF-1 (50 ng/ml) for 1 hr (Pasha et al., 2008), or TGF-α (250 ng/ml) and TNF-α (50 ng/ml) for 24 hr (Herrmann et al., 2010) resulted in increased production of VEGF in the conditioned medium compared with unstimulated MSCs. Table 2 highlights the available in vitro data including peak concentration of the secreted paracrine factors as a function of dose and duration of stimulation. So far, however, these studies have not characterized the impact of time and/or dose of stimulation on paracrine factor secretion and cardiac functional improvement in vivo, which would be important for assessing the potential utility in clinical settings.

In an attempt to exploit synergistic effects, cocktails of cytokines, conditioned medium, or serum have been employed to stimulate MSCs. For example, when transplanted into NOD/SCID mice, hMSCs stimulated in vitro by a cytokine cocktail (Flt-3 ligand, SCF, IL-6, HGF, and IL-3) expressed higher levels of *CXCR4* mRNA and showed improved SDF-1-induced migration capacity (>20-fold versus unstimulated MSCs) to the bone marrow 24 hr after transplantation and enhanced homing (>2-fold versus unstimulated MSCs) to the bone marrow of irradiated mice 2–6 months after transplantation (Shi et al., 2007). In another study, hMSCs exposed to LPS-stimulated rat serum for 24 hr responded by secreting higher levels (4.5-fold versus normal serum) of sTNFR1 (Yagi et al., 2010). Importantly, intramuscularly injected serum-stimulated hMSCs attenuated inflammation via paracrine actions of sTNFR1 and other anti-inflammatory cytokines. Another approach that has been tested is coculture of MSCs with other cell types. For example, TSP-1 was upregulated in rMSCs cocultured with retinal ganglion cells (RGCs) (Yu et al., 2008). Block et al. cocultured hMSCs with apoptotic fibroblasts for 48 hr, thus exposing the hMSCs to apoptotic cytokines (Block et al., 2009) and leading the stimulated hMSCs to secrete STC-1, a peptide with antiapoptotic effects on lung epithelial cells. Clearly more comprehensive studies are required to examine the impact of protein-based preconditioning regimens on MSC-based therapeutic approaches, including applications for cardiovascular diseases. A better understanding of how the cytokines expressed in cardiac ischemic or inflammatory microenvironments in vivo modulate MSCs to exert a therapeutic effect could be very helpful for developing more effective protein-based preconditioning approaches.

Pharmacological Preconditioning

Another promising approach for pretreating MSCs prior to transplantation involves small molecules, which have the advantages of ease of synthesis, cost effectiveness, and specific actions on cellular signaling. The availability of small molecule libraries enables high throughput screening to identify molecules for modulating specific cellular functions. However, there is currently no clearly demonstrated evidence of efficient MSC secretome regulation by small molecules. Some studies have suggested that small molecules can increase rMSC survival under ischemic conditions and can bring about a moderate

Table 2. Representative Time- and Dose-Dependent Release Profiles of Paracrine Factors from Preconditioned MSCs In Vitro and Related Functional Improvements

Stimulating Molecule	Dose and Time of Stimulation	Paracrine Factors Upregulated	Peak Concentration[a] (ng/ml)	Fold Change in Concentration versus Nonconditioned MSCs	Functional Improvements	Reference
TNF-α	0.1 0.1, 0.5, 2, 5, *10 ng/ml* for 0, 6, 12, 24 and *48 hr*	IL-6	19.0	15	dose-dependent improvement in monocyte migration upon treatment with varying doses of TNF-α-stimulated, MSC-conditioned medium	Lee et al., 2010
	(as above)	IL-8	38.0	30	(as above)	(as above)
	(as above)	MCP-1	18.0	15	(as above)	(as above)
	(as above)	CXCL6	0.3	30	(as above)	(as above)
TNF-α	100 IU/ml for 6, 24, *72 hr*	IL-8	100.0	6	enhanced migration of preconditioned hMSCs in response to chemokines such as SDF-1	Croitoru-Lamoury et al., 2007
	(as above)	MCP-1	75.0	3.75	(as above)	(as above)
IFN-β	100, 1000, *2000* UI/ml for 6, 24, *72 hr*	MCP-1	10.0	4	(as above)	(as above)
	100, *1000*, 2000 UI/ml for 6, 24, *72 hr*	IP-10	0.42	4	(as above)	(as above)
LPS	0, 0.01, 0.1, *1*, 10 μg/ml for *48 hr*	VEGF	1.35 pg/10^5 cells	2	enhanced VEGF levels in myocardium and improved survival of transplanted mMSCs	Yao et al., 2009

[a]Peak concentrations have resulted from the duration and dose of stimulation (in *italics*) in the respective studies.

improvement in cardiac function in MI models by upregulating intracellular levels of rMSC-expressed paracrine factors (Afzal et al., 2010; Mias et al., 2008; Wisel et al., 2009). In addition to small molecules, LPS treatment enhanced paracrine factor secretion (dose dependently) from mMSCs (Table 2), thereby improving survival of transplanted mMSCs (>1.5-fold versus control) via increased VEGF levels in the myocardium (>2.2-fold versus control) observed 3 weeks after cell transplantation in rat infarct hearts (Yao et al., 2009). If we are looking to apply preconditioning with agents such as LPS that would exert a detrimental systemic impact on the host, it will be important to minimize the concentration delivered with MSCs after preconditioning to prevent pharmacological effects on the host.

Preconditioning through Cell-Cell Interactions and Physical Preconditioning

Promotion of cell-cell interactions between MSCs can also have a profound impact on the MSC secretome. For example, Potapova et al, developed a simple strategy of organizing hMSCs into 3D spheroids of varying sizes using a hanging drop method to increase secretion levels of paracrine factors (Potapova et al., 2007). Using this approach, they observed high concentrations (5–20 times) of IL-11, as well as the proangiogenic cytokines VEGF, bFGF, and angiogenin, in the conditioned medium from hMSC spheroids compared with conditioned medium from hMSC monolayers. The onset of hypoxia in the core of the cell aggregates was proposed to be the driving force for the increased secretion levels. A similar study demonstrated that culturing hMSCs as 3D spheroids restored *CXCR4* functional expression, demonstrated by 35% of the cells derived from day 3 spheroids being *CXCR4*$^+$ (Potapova et al., 2008). In a separate study, hMSC spheroids grown in suspension cultures were found to secrete 60-fold more TSG-6 protein than adherent monolayered hMSCs (Bartosh et al., 2010). More importantly, the size of the spheroid-derived cells was significantly smaller than hMSCs from adherent cultures, allowing them to more readily escape lung entrapment in vivo following systemic infusion. Another approach for increasing secretion of paracrine factors involves culturing monolayers of rat-adipose-tissue-derived MSC sheets, prepared by incubating rMSC monolayers within temperature-responsive dishes at 20°C for about 40 min and detaching them into monolayers of rMSC sheets (Miyahara et al., 2006). Although promising, it is unclear how MSC preconditioned by promoting assembly into 3D aggregates or 2D monolayers will retain their secretion profiles following transplantation as single cells or 2D/3D constructs.

Microenvironmental cues such as shear stress and substrate compliance have also been used to control MSC paracrine activity. For example, human adipose-tissue-derived MSCs subjected to laminar shear stress (10 dyn/cm^2 up to 96 hr) secreted higher amounts of VEGF (2-fold versus static hMSCs) (Bassaneze et al., 2010). In another study, hMSCs grown on hydrogel substrates mimicking hard and soft tissue secreted differential levels of VEGF, IL-8, and uPA for up to 14 days (Seib et al., 2009).

Summary of MSC Preconditioning Strategies

Preconditioning via controlled cell-cell interactions has shown promise for increased secretion of pertinent factors, but may

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Table 3. Unaddressed Critical Issues for Current MSC Preconditioning Strategies

Preconditioning Mode	Issues	Recommended Actions
Physiological	highly varied hypoxia exposure time (4 to 72 hr); nonspecific activation of signaling pathways leading to uncontrolled secretome; duration of preconditioning effects is not well understood	optimize hypoxia exposure time to maximize MSC survival; investigate different modes of hypoxia such as brief exposures mimicking ischemic preconditioning to better understand effect of hypoxia on MSC survival and sustained paracrine action in vivo
Genetic	activates single target gene; gene expression levels do not correlate with the concentration of secreted factors; limited control over local pharmacokinetics of expressed protein; limited understanding of the temporal expression of proteins; safety posttransplantation	multiple gene activation leading to expression/release of a cocktail of proteins that act in synergy; overexpression studies to establish correlation between gene and protein expression levels; optimize the mode of gene and cell delivery (e.g., combine with a biomaterials approach); carefully examine the literature for comparable approaches, perform relevant safety analysis in animal models, and consider nonviral approaches
Protein/cytokine	effects of incubation time and protein concentration on promoting sustained effects are not well understood; high concentrations required to stimulate the MSC due to transport limitations	kinetic studies of pathway activation and factor release in vitro and in vivo; transport limitations could be overcome by intracellularly or extracellularly controlled delivery of proteins/cytokines using polymeric micro/nano-systems
Pharmacological	effects of incubation time and drug concentration on promoting sustained effects are not well understood; agents may exhibit a negative impact on the host	kinetic studies of pathway activation and factor release in vitro and in vivo; identifying highly specific activators/inhibitors; ensuring concentration of agents is minimized in cell suspension prior to transplantation
Cell-cell interaction	the mechanism by which 3D MSC aggregates retain high secretome expression is not clear	perform studies to reveal the involvement of MSC adhesion ligands or ECM in activating specific signaling pathways
Physical	the signaling pathways activated by physical stimuli are not well understood	elucidation of the mechanisms by which physical stimuli influence the MSC secretome; kinetic studies of pathway activation and factor release both in vitro and in vivo

offer less control than other approaches over the components of the secretome. Although physiological and molecular (cytokine/small molecule) preconditioning is simple and more targeted, the impact is transient due to self-regulatory mechanisms, and it is likely that these approaches will have a limited duration of impact posttransplantation. Genetic manipulation has the advantage of a sustained response; however, it is often limited to a single target gene and clinical translation may be challenging due to higher regulatory hurdles if viruses are utilized. Regardless of the preconditioning approach applied, MSCs typically exhibit limited persistence following transplantation, and thus, achieving sustained secretion following a single MSC dose will require new techniques to boost MSC survival. While a proof of concept for this has been achieved using virally transfected MSCs (Mangi et al., 2003; Mirotsou et al., 2007), much-desired nonviral approaches are currently being developed to achieve rapid and safe translation (Yang et al., 2010). Coadministration of drugs such as Atorvastatin with MSCs may also improve the cardiac microenvironment after MI to achieve better survival of the implanted MSCs (Yang et al., 2008). Likewise, immunoselection aimed to enrich the cell population with stromal precursor antigen-1 $(STRO-1)^{+}$ mesenchymal progenitor cells (MPCs) could also improve cardiovascular paracrine activity (Psaltis et al., 2010).

While the majority of preconditioning strategies promote expression/secretion of a narrow class of cytokines that are constitutively secreted by MSCs at basal levels, it would be useful to examine if preconditioning of MSCs could be utilized to express therapeutic factors that are not secreted by MSCs under basal conditions. Despite the promise of several preconditioning strategies, there are critical unmet needs and uncertainties yet to be addressed, and these are listed and discussed in Table 3. However, better control over the MSC secretome posttransplantation could be achieved through customization strategies as depicted in Figure 1, which could be translated into clinics via engineering the MSC secretome using controlled release approaches as depicted in Figure 2.

Summary and Perspectives

Harnessing the MSC secretome for cardiovascular repair seems in principle to have significant clinical potential given the innate immunomodulatory and trophic properties of many of the factors secreted by MSCs. While some groups are directly employing MSC-derived therapeutic paracrine factors in the absence of cells, approaches employing a cocktail of secreted factors will require GMP manufacturing protocols with reproducible batch-to-batch secretome properties (that may be impacted by several factors including the MSC donor), and will require a defined regulatory pathway. Also, in general, cytokine-based approaches have not performed well in clinical trials due to inherent limitations in tissue transport, pharmacokinetics, and protein stability in vivo. These issues can likely be addressed for individual cytokines through the development of appropriate controlled release strategies; however, delivery approaches for complex cocktails of therapeutic agents, such as the isolated MSC secretome, will be a significant challenge.

The majority of MSC-based clinical trials for cardiovascular therapy focus on the potential benefits of the immunomodulatory and trophic properties of MSCs rather than their potential to generate new tissues directly. Although it is still early to draw conclusions, the available trial results are not as promising as has been hoped based on preclinical animal studies. This relative

lack of success is likely due to the use of nonoptimized MSC formulations and poor understanding of how MSCs induce cardiovascular repair. Importantly, signaling pathways mediating the expression and secretion of relevant MSC factors and the mechanism of how they synergistically impact cardiovascular repair are beginning to be elucidated. Nevertheless, the relevance of the MSC secretome to the treatment of cardiovascular disease is still controversial, and hence, identifying and characterizing additional MSC-secreted factors that can either facilitate cardiomyogenesis or activate endogenous CSCs seems crucial. The molecular events responsible for altering the MSC secretome in vivo, as a function of microenvironmental stimuli, remain elusive. Clear understanding of the in vivo MSC secretome and its potential functional benefits is still far from being resolved, and this is a key prerequisite to harnessing this potentially powerful tool for maximal therapeutic benefit.

The currently favored approach for regulating cells after transplantation involves preconditioning MSCs with the aim of improving homing, survival, and secretome control. Clarifying the underlying signaling pathways should enable development of more effective preconditioning regimens to activate/inhibit relevant pathways to maximize the therapeutic effect. As the biology mediating the therapeutic benefit of MSC secretome becomes more defined, targeted preconditioning and genetic manipulation approaches will likely be useful to enhance the therapeutic benefit.

Looking to the future, state-of-the-art bioengineered materials offer the potential for enhanced control of cells and presentation of MSC secretome after transplantation. For example, paracrine factors from hypoxia-conditioned MSCs bound to nano-structured materials have yielded significant hemodynamic functional preservation within an infarcted heart model (Webber et al., 2010), and the transplantation of cells such as CPCs with immobilized IGF-1 on nano-fibers exhibited dual effects from IGF-1-mediated activation of resident cardiac cells and protection of transplanted CPCs (Padin-Iruegas et al., 2009). Cardiac-specific decellularized matrices (Godier-Furnémont et al., 2011; Singelyn and Christman, 2010) and biopolymers (Danoviz et al., 2010) may also serve as injectable biomaterials to deliver MSCs in a more sustainable and effective manner. Recently, we employed a polymer-based controlled drug release strategy to program MSC fate through engineered intracrine-, paracrine-, and endocrine-like mechanisms (Sarkar et al., 2011a). In addition to controlling cell fate, this biomaterials approach provides an opportunity to control the MSC secretome posttransplantation—for example, through sustained intracellular release of small molecules that target specific pathways. As an alternative to transplantation of single-cell suspensions, the MSC secretome may also be exploited through transplantation of engineered MSC spheroids that have shown potential for enhanced paracrine levels in vitro. Given the relatively harsh microenvironment presented at a site of injury or ischemia, it may be of interest to transplant MSCs at a distant site where paracrine factors can reach damaged heart tissue through systemic endocrine effects (Lee et al., 2009). Although challenges remain, harnessing the MSC secretome for meaningful therapeutic outcomes will likely be realized in the near future by capitalizing on customization strategies as depicted in Figure 1 and Figure 2 for controlling and sustaining the MSC secretome posttransplantation.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Tables S1 and S2 and can be found with this article online at doi:10.1016/j.stem.2012.02.005.

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REFERENCES

Afzal, M.R., Haider, H.Kh., Idris, N.M., Jiang, S., Ahmed, R.P., and Ashraf, M. (2010). Preconditioning promotes survival and angiomyogenic potential of mesenchymal stem cells in the infarcted heart via NF-kappaB signaling. Antioxid. Redox Signal. *12*, 693–702.

Aggarwal, S., and Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood *105*, 1815–1822.

Ankrum, J., and Karp, J.M. (2010). Mesenchymal stem cell therapy: Two steps forward, one step back. Trends Mol. Med. *16*, 203–209.

Assis, A.C.M., Carvalho, J.L., Jacoby, B.A., Ferreira, R.L.B., Castanheira, P., Diniz, S.O.F., Cardoso, V.N., Goes, A.M., and Ferreira, A.J. (2010). Time-dependent migration of systemically delivered bone marrow mesenchymal stem cells to the infarcted heart. Cell Transplant. *19*, 219–230.

Bartosh, T.J., Ylöstalo, J.H., Mohammadipoor, A., Bazhanov, N., Coble, K., Claypool, K., Lee, R.H., Choi, H., and Prockop, D.J. (2010). Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. Proc. Natl. Acad. Sci. USA *107*, 13724–13729.

Bassaneze, V., Barauna, V.G., Lavini-Ramos, C., Kalil, J., Schettert, I.T., Miyakawa, A.A., and Krieger, J.E. (2010). Shear stress induces nitric oxide-mediated vascular endothelial growth factor production in human adipose tissue mesenchymal stem cells. Stem Cells Dev. *19*, 371–378.

Beltrami, A.P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., et al. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell *114*, 763–776.

Beohar, N., Rapp, J., Pandya, S., and Losordo, D.W. (2010). Rebuilding the damaged heart: the potential of cytokines and growth factors in the treatment of ischemic heart disease. J. Am. Coll. Cardiol. *56*, 1287–1297.

Berry, M.F., Engler, A.J., Woo, Y.J., Pirolli, T.J., Bish, L.T., Jayasankar, V., Morine, K.J., Gardner, T.J., Discher, D.E., and Sweeney, H.L. (2006). Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am. J. Physiol. Heart Circ. Physiol. *290*, H2196–H2203.

Berthonneche, C., Sulpice, T., Boucher, F., Gouraud, L., de Leiris, J., O'Connor, S.E., Herbert, J.M., and Janiak, P. (2004). New insights into the pathological role of TNF-alpha in early cardiac dysfunction and subsequent heart failure after infarction in rats. Am. J. Physiol. Heart Circ. Physiol. *287*, H340–H350.

Biswas, S., Ghoshal, P.K., Mandal, S.C., and Mandal, N. (2010). Relation of anti- to pro-inflammatory cytokine ratios with acute myocardial infarction. Korean J. Intern. Med. *25*, 44–50.

Block, G.J., Ohkouchi, S., Fung, F., Frenkel, J., Gregory, C., Pochampally, R., DiMattia, G., Sullivan, D.E., and Prockop, D.J. (2009). Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. Stem Cells *27*, 670–681.

Carmeliet, P. (2005). VEGF as a key mediator of angiogenesis in cancer. Oncology *69* (*Suppl 3*), 4–10.

Cell PRESS

Chavakis, E., Koyanagi, M., and Dimmeler, S. (2010). Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back. Circulation *121*, 325–335.

Cheng, Z.K., Ou, L.L., Zhou, X., Li, F., Jia, X.H., Zhang, Y.G., Liu, X.L., Li, Y.M., Ward, C.A., Melo, L.G., and Kong, D. (2008). Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. Mol. Ther. *16*, 571–579.

Crisostomo, P.R., Wang, Y., Markel, T.A., Wang, M., Lahm, T., and Meldrum, D.R. (2008). Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. Am. J. Physiol. Cell Physiol. *294*, C675–C682.

Croitoru-Lamoury, J., Lamoury, F.M.J., Zaunders, J.J., Veas, L.A., and Brew, B.J. (2007). Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone. J. Interferon Cytokine Res. *27*, 53–64.

Danoviz, M.E., Nakamuta, J.S., Marques, F.L.N., dos Santos, L., Alvarenga, E.C., dos Santos, A.A., Antonio, E.L., Schettert, I.T., Tucci, P.J., and Krieger, J.E. (2010). Rat adipose tissue-derived stem cells transplantation attenuates cardiac dysfunction post infarction and biopolymers enhance cell retention. PLoS ONE *5*, e12077.

Debrunner, M., Schuiki, E., Minder, E., Straumann, E., Naegeli, B., Mury, R., Bertel, O., and Frielingsdorf, J. (2008). Proinflammatory cytokines in acute myocardial infarction with and without cardiogenic shock. Clin. Res. Cardiol. *97*, 298–305.

Engelmann, M.G., Theiss, H.D., Hennig-Theiss, C., Huber, A., Wintersperger, B.J., Werle-Ruedinger, A.E., Schoenberg, S.O., Steinbeck, G., and Franz, W.M. (2006). Autologous bone marrow stem cell mobilization induced by granulocyte colony-stimulating factor after subacute ST-segment elevation myocardial infarction undergoing late revascularization: final results from the G-CSF-STEMI (Granulocyte Colony-Stimulating Factor ST-Segment Elevation Myocardial Infarction) trial. J. Am. Coll. Cardiol. *48*, 1712–1721.

Epstein, S.E., Kornowski, R., Fuchs, S., and Dvorak, H.F. (2001). Angiogenesis therapy: amidst the hype, the neglected potential for serious side effects. Circulation *104*, 115–119.

Fierro, F.A., Kalomoiris, S., Sondergaard, C.S., and Nolta, J.A. (2011). Effects on proliferation and differentiation of multipotent bone marrow stromal cells engineered to express growth factors for combined cell and gene therapy. Stem Cells *29*, 1727–1737.

François, M., Romieu-Mourez, R., Li, M., and Galipeau, J. (2012). Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. Mol. Ther. *20*, 187–195.

Gnecchi, M., He, H.M., Liang, O.D., Melo, L.G., Morello, F., Mu, H., Noiseux, N., Zhang, L.N., Pratt, R.E., Ingwall, J.S., and Dzau, V.J. (2005). Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat. Med. *11*, 367–368.

Gnecchi, M., He, H.M., Noiseux, N., Liang, O.D., Zhang, L.M., Morello, F., Mu, H., Melo, L.G., Pratt, R.E., Ingwall, J.S., and Dzau, V.J. (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. *20*, 661–669.

Gnecchi, M., Zhang, Z.P., Ni, A.G., and Dzau, V.J. (2008). Paracrine mechanisms in adult stem cell signaling and therapy. Circ. Res. *103*, 1204–1219.

Godier-Furnémont, A.F.G., Martens, T.P., Koeckert, M.S., Wan, L., Parks, J., Arai, K., Zhang, G.P., Hudson, B., Homma, S., and Vunjak-Novakovic, G. (2011). Composite scaffold provides a cell delivery platform for cardiovascular repair. Proc. Natl. Acad. Sci. USA *108*, 7974–7979.

Hahn, J.Y., Cho, H.J., Kang, H.J., Kim, T.S., Kim, M.H., Chung, J.H., Bae, J.W., Oh, B.H., Park, Y.B., and Kim, H.S. (2008). Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. J. Am. Coll. Cardiol. *51*, 933–943.

Haider, H.Kh., Jiang, S., Idris, N.M., and Ashraf, M. (2008). IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. Circ. Res. *103*, 1300–1308.

Hare, J.M., Traverse, J.H., Henry, T.D., Dib, N., Strumpf, R.K., Schulman, S.P., Gerstenblith, G., DeMaria, A.N., Denktas, A.E., Gammon, R.S., et al. (2009). A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J. Am. Coll. Cardiol. *54*, 2277–2286.

Hatzistergos, K.E., Quevedo, H., Oskouei, B.N., Hu, Q.H., Feigenbaum, G.S., Margitich, I.S., Mazhari, R., Boyle, A.J., Zambrano, J.P., Rodriguez, J.E., et al. (2010). Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ. Res. *107*, 913–922.

Henry, T.D., Rocha-Singh, K., Isner, J.M., Kereiakes, D.J., Giordano, F.J., Simons, M., Losordo, D.W., Hendel, R.C., Bonow, R.O., Eppler, S.M., et al. (2001). Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease. Am. Heart J. *142*, 872–880.

Henry, T.D., Annex, B.H., McKendall, G.R., Azrin, M.A., Lopez, J.J., Giordano, F.J., Shah, P.K., Willerson, J.T., Benza, R.L., Berman, D.S., et al; VIVA Investigators. (2003). The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation *107*, 1359–1365.

Herrmann, J.L., Wang, Y., Abarbanell, A.M., Weil, B.R., Tan, J.N., and Meldrum, D.R. (2010). Preconditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated cardioprotection. Shock *33*, 24–30.

Herrmann, J.L., Weil, B.R., Abarbanell, A.M., Wang, Y., Poynter, J.A., Manukyan, M.C., and Meldrum, D.R. (2011). IL-6 and TGF-α costimulate mesenchymal stem cell vascular endothelial growth factor production by ERK-, JNK-, and PI3K-mediated mechanisms. Shock *35*, 512–516.

Huang, J., Zhang, Z., Guo, J., Ni, A., Deb, A., Zhang, L., Mirotsou, M., Pratt, R.E., and Dzau, V.J. (2010). Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. Circ. Res. *106*, 1753–1762.

Ip, J.E., Wu, Y.J., Huang, J., Zhang, L.N., Pratt, R.E., and Dzau, V.J. (2007). Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. Mol. Biol. Cell *18*, 2873–2882.

Iso, Y., Spees, J.L., Serrano, C., Bakondi, B., Pochampally, R., Song, Y.H., Sobel, B.E., Delafontaine, P., and Prockop, D.J. (2007). Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem. Biophys. Res. Commun. *354*, 700–706.

Kamota, T., Li, T.S., Morikage, N., Murakami, M., Ohshima, M., Kubo, M., Kobayashi, T., Mikamo, A., Ikeda, Y., Matsuzaki, M., and Hamano, K. (2009). Ischemic pre-conditioning enhances the mobilization and recruitment of bone marrow stem cells to protect against ischemia/reperfusion injury in the late phase. J. Am. Coll. Cardiol. *53*, 1814–1822.

Kanki, S., Segers, V.F.M., Wu, W.T., Kakkar, R., Gannon, J., Sys, S.U., Sandrasagra, A., and Lee, R.T. (2011). Stromal cell-derived factor-1 retention and cardioprotection for ischemic myocardium. Circ. Heart Fail *4*, 509–518.

Karp, J.M., and Leng Teo, G.S. (2009). Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell *4*, 206–216.

Kinnaird, T., Stabile, E., Burnett, M.S., Lee, C.W., Barr, S., Fuchs, S., and Epstein, S.E. (2004a). Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ. Res. *94*, 678–685.

Kinnaird, T., Stabile, E., Burnett, M.S., Shou, M., Lee, C.W., Barr, S., Fuchs, S., and Epstein, S.E. (2004b). Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation *109*, 1543–1549.

Kühn, B., del Monte, F., Hajjar, R.J., Chang, Y.S., Lebeche, D., Arab, S., and Keating, M.T. (2007). Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat. Med. *13*, 962–969.

Laflamme, M.A., and Murry, C.E. (2005). Regenerating the heart. Nat. Biotechnol. *23*, 845–856.

Lee, R.H., Pulin, A.A., Seo, M.J., Kota, D.J., Ylostalo, J., Larson, B.L., Semprun-Prieto, L., Delafontaine, P., and Prockop, D.J. (2009). Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell *5*, 54–63.

Lee, M.J., Kim, J., Kim, M.Y., Bae, Y.S., Ryu, S.H., Lee, T.G., and Kim, J.H. (2010). Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells. J. Proteome Res. *9*, 1754–1762.

Leiker, M., Suzuki, G., Iyer, V.S., Canty, J.M., Jr., and Lee, T. (2008). Assessment of a nuclear affinity labeling method for tracking implanted mesenchymal stem cells. Cell Transplant. *17*, 911–922.

Li, H.X., Zuo, S., He, Z.S., Yang, Y.T., Pasha, Z., Wang, Y.G., and Xu, M.F. (2010). Paracrine factors released by GATA-4 overexpressed mesenchymal stem cells increase angiogenesis and cell survival. Am. J. Physiol. Heart Circ. Physiol. *299*, H1772–H1781.

Liu, C.P., Yeh, J.L., Wu, B.N., Chai, C.Y., Chen, I.J., and Lai, W.T. (2011). KMUP-3 attenuates ventricular remodelling after myocardial infarction through eNOS enhancement and restoration of MMP-9/TIMP-1 balance. Br. J. Pharmacol. *162*, 126–135.

Loffredo, F.S., Steinhauser, M.L., Gannon, J., and Lee, R.T. (2011). Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell *8*, 389–398.

Lozito, T.P., and Tuan, R.S. (2011). Mesenchymal stem cells inhibit both endogenous and exogenous MMPs via secreted TIMPs. J. Cell. Physiol. *226*, 385–396.

Mangi, A.A., Noiseux, N., Kong, D.L., He, H.M., Rezvani, M., Ingwall, J.S., and Dzau, V.J. (2003). Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat. Med. *9*, 1195–1201.

Matsumoto, R., Omura, T., Yoshiyama, M., Hayashi, T., Inamoto, S., Koh, K.R., Ohta, K., Izumi, Y., Nakamura, Y., Akioka, K., et al. (2005). Vascular endothelial growth factor-expressing mesenchymal stem cell transplantation for the treatment of acute myocardial infarction. Arterioscler. Thromb. Vasc. Biol. *25*, 1168–1173.

Matsumura, S., Iwanaga, S., Mochizuki, S., Okamoto, H., Ogawa, S., and Okada, Y. (2005). Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. J. Clin. Invest. *115*, 599–609.

Mazhari, R., and Hare, J.M. (2007). Mechanisms of action of mesenchymal stem cells in cardiac repair: potential influences on the cardiac stem cell niche. Nat. Clin. Pract. Cardiovasc. Med. *4* (*Suppl 1*), S21–S26.

Mias, C., Trouche, E., Seguelas, M.H., Calcagno, F., Dignat-George, F., Sabatier, F., Piercecchi-Marti, M.D., Daniel, L., Bianchi, P., Calise, D., et al. (2008). Ex vivo pretreatment with melatonin improves survival, proangiogenic/mitogenic activity, and efficiency of mesenchymal stem cells injected into ischemic kidney. Stem Cells *26*, 1749–1757.

Mirotsou, M., Zhang, Z.Y., Deb, A., Zhang, L.N., Gnecchi, M., Noiseux, N., Mu, H., Pachori, A., and Dzau, V. (2007). Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc. Natl. Acad. Sci. USA *104*, 1643–1648.

Mirotsou, M., Jayawardena, T.M., Schmeckpeper, J., Gnecchi, M., and Dzau, V.J. (2011). Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. J. Mol. Cell. Cardiol. *50*, 280–289.

Miyahara, Y., Nagaya, N., Kataoka, M., Yanagawa, B., Tanaka, K., Hao, H., Ishino, K., Ishida, H., Shimizu, T., Kangawa, K., et al. (2006). Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat. Med. *12*, 459–465.

Moro, C., Jouan, M.G., Rakotovao, A., Toufektsian, M.C., Ormezzano, O., Nagy, N., Tosaki, A., de Leiris, J., and Boucher, F. (2007). Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling. Am. J. Physiol. Heart Circ. Physiol. *293*, H3014–H3019.

Nagaya, N., Kangawa, K., Itoh, T., Iwase, T., Murakami, S., Miyahara, Y., Fujii, T., Uematsu, M., Ohgushi, H., Yamagishi, M., et al. (2005). Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation *112*, 1128–1135.

Nakanishi, C., Yamagishi, M., Yamahara, K., Hagino, I., Mori, H., Sawa, Y., Yagihara, T., Kitamura, S., and Nagaya, N. (2008). Activation of cardiac progenitor cells through paracrine effects of mesenchymal stem cells. Biochem. Biophys. Res. Commun. *374*, 11–16.

Nauta, A.J., and Fibbe, W.E. (2007). Immunomodulatory properties of mesenchymal stromal cells. Blood *110*, 3499–3506.

Németh, K., Leelahavanichkul, A., Yuen, P.S.T., Mayer, B., Parmelee, A., Doi, K., Robey, P.G., Leelahavanichkul, K., Koller, B.H., Brown, J.M., et al. (2009). Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat. Med. *15*, 42–49.

Nguyen, B.K., Maltais, S., Perrault, L.P., Tanguay, J.F., Tardif, J.C., Stevens, L.M., Borie, M., Harel, F., Mansour, S., and Noiseux, N. (2010). Improved function and myocardial repair of infarcted heart by intracoronary injection of mesenchymal stem cell-derived growth factors. J Cardiovasc. Transl. Res. *3*, 547–558.

Noiseux, N., Gnecchi, M., Lopez-Ilasaca, M., Zhang, L., Solomon, S.D., Deb, A., Dzau, V.J., and Pratt, R.E. (2006). Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. Mol. Ther. *14*, 840–850.

Ohnishi, S., Yasuda, T., Kitamura, S., and Nagaya, N. (2007). Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells *25*, 1166–1177.

Oskowitz, A., McFerrin, H., Gutschow, M., Carter, M.L., and Pochampally, R. (2011). Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. Stem Cell Res. (Amst.) *6*, 215–225.

Padin-Iruegas, M.E., Misao, Y., Davis, M.E., Segers, V.F.M., Esposito, G., Tokunou, T., Urbanek, K., Hosoda, T., Rota, M., Anversa, P., et al. (2009). Cardiac progenitor cells and biotinylated insulin-like growth factor-1 nanofibers improve endogenous and exogenous myocardial regeneration after infarction. Circulation *120*, 876–887.

Pahl, H.L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene *18*, 6853–6866.

Parekkadan, B., van Poll, D., Suganuma, K., Carter, E.A., Berthiaume, F., Tilles, A.W., and Yarmush, M.L. (2007). Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS ONE *2*, e941.

Park, C.W., Kim, K.-S., Bae, S., Son, H.K., Myung, P.-K., Hong, H.J., and Kim, H. (2009). Cytokine secretion profiling of human mesenchymal stem cells by antibody array. Int. J Stem Cells *2*, 59–68.

Pasha, Z., Wang, Y., Sheikh, R., Zhang, D., Zhao, T., and Ashraf, M. (2008). Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. Cardiovasc. Res. *77*, 134–142.

Potapova, I.A., Gaudette, G.R., Brink, P.R., Robinson, R.B., Rosen, M.R., Cohen, I.S., and Doronin, S.V. (2007). Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. Stem Cells *25*, 1761–1768.

Potapova, I.A., Brink, P.R., Cohen, I.S., and Doronin, S.V. (2008). Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells. J. Biol. Chem. *283*, 13100–13107.

Prockop, D.J., and Oh, J.Y. (2012). Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol. Ther. *20*, 14–20.

Psaltis, P.J., Paton, S., See, F., Arthur, A., Martin, S., Itescu, S., Worthley, S.G., Gronthos, S., and Zannettino, A.C.W. (2010). Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J. Cell. Physiol. *223*, 530–540.

Rattigan, Y., Hsu, J.M., Mishra, P.J., Glod, J., and Banerjee, D. (2010). Interleukin 6 mediated recruitment of mesenchymal stem cells to the hypoxic tumor milieu. Exp. Cell Res. *316*, 3417–3424.

Ripa, R.S., Jørgensen, E., Wang, Y.Z., Thune, J.J., Nilsson, J.C., Søndergaard, L., Johnsen, H.E., Køber, L., Grande, P., and Kastrup, J. (2006). Stem cell mobilization induced by subcutaneous granulocyte-colony stimulating factor to improve cardiac regeneration after acute ST-elevation myocardial infarction: result of the double-blind, randomized, placebo-controlled stem cells in myocardial infarction (STEMMI) trial. Circulation *113*, 1983–1992.

Rodrigues, M., Griffith, L.G., and Wells, A. (2010). Growth factor regulation of proliferation and survival of multipotential stromal cells. Stem Cell Res. Ther. *1*, 32.

Sarkar, D., Ankrum, J.A., Teo, G.S.L., Carman, C.V., and Karp, J.M. (2011a). Cellular and extracellular programming of cell fate through engineered intracrine-, paracrine-, and endocrine-like mechanisms. Biomaterials *32*, 3053–3061.

Sarkar, D., Spencer, J.A., Phillips, J.A., Zhao, W., Schafer, S., Spelke, D.P., Mortensen, L.J., Ruiz, J.P., Vemula, P.K., Sridharan, R., et al. (2011b). Engineered cell homing. Blood *118*, e184–e191.

Schoen, F.J. (2007). The Heart. In Robbins and Cotran Pathologic Basis of Disease, Seventh Edition, V. Kumar, A. Abbas, and N. Fausto, eds. (New Delhi, India: Reed Elsevier India Pvt Ltd), pp. 577–579.

Segers, V.F.M., Tokunou, T., Higgins, L.J., MacGillivray, C., Gannon, J., and Lee, R.T. (2007). Local delivery of protease-resistant stromal cell derived factor-1 for stem cell recruitment after myocardial infarction. Circulation *116*, 1683–1692.

Seib, F.P., Prewitz, M., Werner, C., and Bornhäuser, M. (2009). Matrix elasticity regulates the secretory profile of human bone marrow-derived multipotent mesenchymal stromal cells (MSCs). Biochem. Biophys. Res. Commun. *389*, 663–667.

Shabbir, A., Zisa, D., Suzuki, G., and Lee, T. (2009). Heart failure therapy mediated by the trophic activities of bone marrow mesenchymal stem cells: a noninvasive therapeutic regimen. Am. J. Physiol. Heart Circ. Physiol. *296*, H1888–H1897.

Shi, M., Li, J., Liao, L., Chen, B., Li, B., Chen, L., Jia, H., and Zhao, R.C. (2007). Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice. Haematologica *92*, 897–904.

Shi, R.Z., Wang, J.C., Huang, S.H., Wang, X.J., and Li, Q.P. (2009). Angiotensin II induces vascular endothelial growth factor synthesis in mesenchymal stem cells. Exp. Cell Res. *315*, 10–15.

Singelyn, J.M., and Christman, K.L. (2010). Injectable materials for the treatment of myocardial infarction and heart failure: the promise of decellularized matrices. J Cardiovasc. Transl. Res. *3*, 478–486.

Skalnikova, H., Motlik, J., Gadher, S.J., and Kovarova, H. (2011). Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. Proteomics *11*, 691–708.

Son, B.R., Marquez-Curtis, L.A., Kucia, M., Wysoczynski, M., Turner, A.R., Ratajczak, J., Ratajczak, M.Z., and Janowska-Wieczorek, A. (2006). Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells *24*, 1254–1264.

Song, H., Cha, M.J., Song, B.W., Kim, I.K., Chang, W., Lim, S., Choi, E.J., Ham, O., Lee, S.Y., Chung, N., et al. (2010). Reactive oxygen species inhibit adhesion of mesenchymal stem cells implanted into ischemic myocardium via interference of focal adhesion complex. Stem Cells *28*, 555–563.

Sze, S.K., de Kleijn, D.P.V., Lai, R.C., Khia Way Tan, E., Zhao, H., Yeo, K.S., Low, T.Y., Lian, Q., Lee, C.N., Mitchell, W., et al. (2007). Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol. Cell. Proteomics *6*, 1680–1689.

Tang, Y.L., Zhao, Q., Qin, X.Y., Shen, L.P., Cheng, L.L., Ge, J.B., and Phillips, M.I. (2005). Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann. Thorac. Surg. *80*, 229–236, discussion 236–237.

Tang, J.M., Wang, J.N., Guo, L.Y., Kong, X., Yang, J.Y., Zheng, F., Zhang, L., and Huang, Y.Z. (2010). Mesenchymal stem cells modified with stromal cell-derived factor 1 alpha improve cardiac remodeling via paracrine activation of hepatocyte growth factor in a rat model of myocardial infarction. Mol. Cells *29*, 9–19.

Terrovitis, J.V., Smith, R.R., and Marbán, E. (2010). Assessment and optimization of cell engraftment after transplantation into the heart. Circ. Res. *106*, 479–494.

Timmers, L., Lim, S.K., Hoefer, I.E., Arslan, F., Lai, R.C., van Oorschot, A.A.M., Goumans, M.J., Strijder, C., Sze, S.K., Choo, A., et al. (2011). Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. Stem Cell Res. (Amst.) *6*, 206–214.

Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J., and Kessler, P.D. (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation *105*, 93–98.

Urbanek, K., Rota, M., Cascapera, S., Bearzi, C., Nascimbene, A., De Angelis, A., Hosoda, T., Chimenti, S., Baker, M., Limana, F., et al. (2005). Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ. Res. *97*, 663–673.

Valgimigli, M., Rigolin, G.M., Cittanti, C., Malagutti, P., Curello, S., Percoco, G., Bugli, A.M., Della Porta, M., Bragotti, L.Z., Ansani, L., et al. (2005). Use of granulocyte-colony stimulating factor during acute myocardial infarction to enhance bone marrow stem cell mobilization in humans: clinical and angiographic safety profile. Eur. Heart J. *26*, 1838–1845.

Van Linthout, S., Savvatis, K., Miteva, K., Peng, J., Ringe, J., Warstat, K., Schmidt-Lucke, C., Sittinger, M., Schultheiss, H.P., and Tschöpe, C. (2011). Mesenchymal stem cells improve murine acute coxsackievirus B3-induced myocarditis. Eur. Heart J. *32*, 2168–2178.

van Poll, D., Parekkadan, B., Borel Rinkes, I.H.M., Tilles, A.W., and Yarmush, M.L. (2008). Mesenchymal stem cell therapy for protection and repair of injured vital organs. Cell Mol. Bioeng. *1*, 42–50.

Velagaleti, R.S., Pencina, M.J., Murabito, J.M., Wang, T.J., Parikh, N.I., D'Agostino, R.B., Levy, D., Kannel, W.B., and Vasan, R.S. (2008). Long-term trends in the incidence of heart failure after myocardial infarction. Circulation *118*, 2057–2062.

Wang, J., Paradis, P., Aries, A., Komati, H., Lefebvre, C., Wang, H., and Nemer, M. (2005). Convergence of protein kinase C and JAK-STAT signaling on transcription factor GATA-4. Mol. Cell. Biol. *25*, 9829–9844.

Wang, M.J., Crisostomo, P.R., Herring, C., Meldrum, K.K., and Meldrum, D.R. (2006). Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism. Am. J. Physiol. Regul. Integr. Comp. Physiol. *291*, R880–R884.

Wang, M.J., Zhang, W.J., Crisostomo, P., Markel, T., Meldrum, K.K., Fu, X.Y., and Meldrum, D.R. (2007). STAT3 mediates bone marrow mesenchymal stem cell VEGF production. J. Mol. Cell. Cardiol. *42*, 1009–1015.

Wang, Y., Weil, B.R., Herrmann, J.L., Abarbanell, A.M., Tan, J.N., Markel, T.A., Kelly, M.L., and Meldrum, D.R. (2009). MEK, p38, and PI-3K mediate cross talk between EGFR and TNFR in enhancing hepatocyte growth factor production from human mesenchymal stem cells. Am. J. Physiol. Cell Physiol. *297*, C1284–C1293.

Wang, Y., Abarbanell, A.M., Herrmann, J.L., Weil, B.R., Manukyan, M.C., Poynter, J.A., and Meldrum, D.R. (2010). TLR4 inhibits mesenchymal stem cell (MSC) STAT3 activation and thereby exerts deleterious effects on MSC-mediated cardioprotection. PLoS ONE *5*, e14206.

Waterman, R.S., Tomchuck, S.L., Henkle, S.L., and Betancourt, A.M. (2010). A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS ONE *5*, e10088.

Webber, M.J., Han, X., Murthy, S.N., Rajangam, K., Stupp, S.I., and Lomasney, J.W. (2010). Capturing the stem cell paracrine effect using heparin-presenting nanofibres to treat cardiovascular diseases. J. Tissue Eng. Regen. Med. *4*, 600–610.

Weil, B.R., Manukyan, M.C., Herrmann, J.L., Wang, Y., Abarbanell, A.M., Poynter, J.A., and Meldrum, D.R. (2010). Mesenchymal stem cells attenuate myocardial functional depression and reduce systemic and myocardial inflammation during endotoxemia. Surgery *148*, 444–452.

Williams, A.R., and Hare, J.M. (2011). Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ. Res. *109*, 923–940.

Wisel, S., Khan, M., Kuppusamy, M.L., Mohan, I.K., Chacko, S.M., Rivera, B.K., Sun, B.C., Hideg, K., and Kuppusamy, P. (2009). Pharmacological preconditioning of mesenchymal stem cells with trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine) protects hypoxic cells against oxidative stress and enhances recovery of myocardial function in infarcted heart through Bcl-2 expression. J. Pharmacol. Exp. Ther. *329*, 543–550.

Wollert, K.C., and Drexler, H. (2010). Cell therapy for the treatment of coronary heart disease: a critical appraisal. Nat. Rev. Cardiol. *7*, 204–215.

World Health Organization (2011). The top 10 causes of death. http://www.who.int/mediacentre/factsheets/fs310/en/index.html.

Xu, H.S., Yi, B.A., and Chien, K.R. (2011). Shortcuts to making cardiomyocytes. Nat. Cell Biol. *13*, 191–193.

Yagi, H., Soto-Gutierrez, A., Navarro-Alvarez, N., Nahmias, Y., Goldwasser, Y., Kitagawa, Y., Tilles, A.W., Tompkins, R.G., Parekkadan, B., and Yarmush, M.L. (2010). Reactive bone marrow stromal cells attenuate systemic inflammation via sTNFR1. Mol. Ther. *18*, 1857–1864.

Yang, Y.J., Qian, H.Y., Huang, J., Geng, Y.J., Gao, R.L., Dou, K.F., Yang, G.S., Li, J.J., Shen, R., He, Z.X., et al. (2008). Atorvastatin treatment improves survival and effects of implanted mesenchymal stem cells in post-infarct swine hearts. Eur. Heart J. *29*, 1578–1590.

Yang, F., Cho, S.W., Son, S.M., Bogatyrev, S.R., Singh, D., Green, J.J., Mei, Y., Park, S., Bhang, S.H., Kim, B.S., et al. (2010). Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles. Proc. Natl. Acad. Sci. USA *107*, 3317–3322.

Yao, Y.W., Zhang, F.M., Wang, L.S., Zhang, G.H., Wang, Z.J., Chen, J.M., and Gao, X. (2009). Lipopolysaccharide preconditioning enhances the efficacy of mesenchymal stem cells transplantation in a rat model of acute myocardial infarction. J. Biomed. Sci. *16*, 74.

Yew, T.-L., Hung, Y.-T., Li, H.-Y., Chen, H.-W., Chen, L.-L., Tsai, K.-S., Chiou, S.-H., Chao, K.-C., Huang, T.-F., Chen, H.-L., and Hung, S.C. (2011). Enhancement of wound healing by human multipotent stromal cell conditioned medium: the paracrine factors and p38 MAPK activation. Cell Transplant. *20*, 693–706.

Yu, K., Ge, J., Summers, J.B., Li, F., Liu, X., Ma, P., Kaminski, J., and Zhuang, J. (2008). TSP-1 secreted by bone marrow stromal cells contributes to retinal ganglion cell neurite outgrowth and survival. PLoS ONE *3*, e2470.

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Toward *in situ* tissue engineering: chemokine-guided stem cell recruitment

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Chemokines are potent stem cell homing and mobilization factors, and artificially increasing the concentrations of specific chemokines at injury sites is an up-to-date strategy to potentiate and prolong the recruitment of endogenous stem cells and to amplify *in situ* tissue regeneration. We briefly outline the latest progress in stem cell recruitment focusing on the interactions of mesenchymal stem cells (MSCs) with chemokines, complement cascade peptides, bioactive lipids, and glycosaminoglycans (GAGs). We present recent advances in state-of-the-art chemokine delivery devices suitable for various applications and critically evaluate the perspectives and challenges of the chemokine-guided *in situ* strategy for translation in regenerative medicine.

In situ tissue engineering

Although the body possesses inherent mechanisms that guide stem cells to sites of tissue damage to promote regeneration, these endogenous processes are often insufficient to achieve full tissue repair. In the past few years, knowledge of the endogenous repair mechanisms of injured tissues has paved the way for future *in situ* strategies that aim to potentiate and prolong the body's own repair capacity. One such possible *in situ* approach relies on the controlled and prolonged delivery of chemoattractants, such as chemokines (see Glossary), at the site of injury to actively enhance the recruitment of endogenous stem cells to the tissue defect and thus actively to amplify intrinsic tissue repair processes.

Although they remain a point of contention (Box 1), MSCs have attracted particular interest in the field of *in situ* tissue engineering and we focus on them because they hold great promise for a multitude of emerging therapies to regenerate injured musculoskeletal tissues (e.g., bone, tendon, cartilage), myocardial infarct tissue, injured renal or liver tissue, and brain or spinal cord injuries [1,2]. The endogenous chemokine-guided MSC homing approach has distinct advantages and disadvantages (Table 1) compared

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Glossary

Cationic antimicrobial peptides: released on activation of the complement cascade from injured tissues and stimulate stem or progenitor cell trafficking.

Chemokine receptors: G protein-coupled seven-transmembrane domain receptors that typically couple heterotrimeric GTP-binding proteins to mediate intracellular signaling cascades. They are named according to the type of chemokine they bind.

Chemokines: a family of small cytokines divided into four subfamilies based on their structural motifs (CC, CXC, CX3C, and XC), each of which is defined by a different spacing of cysteines (C) that form essential disulfide bonds to create the characteristic and highly conserved tertiary structure. Chemokines are involved in various biological processes, including cell trafficking, cell differentiation, angiogenesis, and organogenesis. Chemokines can activate and direct the migration of stem or progenitor cells.

Chemotaxis: directional cell migration along gradients of chemoattractants. The complex chemotaxis process depends on a temporally and spatially coordinated interplay between chemokines, their receptors, and other molecules such as extracellular matrix components, adhesion factors, and proteases.

Extracellular matrix (ECM): a network of extracellular macromolecules that supports various important biological functions, such as structural support, cell adhesion, or migration, and acts as a reservoir for various cytokines and growth factors.

Glycocalyx: a macromolecular GAG coating whose composition is dependent on the cell type and the developmental stage of the cell.

Glycosaminoglycans (GAGs): linear, anionic, and highly heterogeneous carbohydrate polymers comprising disaccharide units. The six major classes are heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, keratin sulfate, and hyaluronic acid. They are important components of the ECM in many different tissues and are expressed as part of the proteoglycans on the cellular surface. GAGs undergo strong interactions with chemokines and are critical for chemotaxis *in vivo*.

Hyaluronic acid: also known as hyaluronan; a GAG comprising repeating units of glucuronic acid and *N*-acetylglucosamine. It is the major component of the ECM in bone marrow.

Ischemic tissue: tissue suffering from oxygen shortage.

Poly(lactic-co-glycolic acid) (PLGA): a biodegradable and biocompatible copolymer derived from L- or D,L-lactic acid and glycolic acid monomer units that are linked by ester bonds. Degradation of PLGA polymers occurs by hydrolysis of the ester bonds to the endogenous monomers that are metabolized in the tricarboxylic acid cycle.

Priming factors: biological molecules that markedly enhance the chemotactic responsiveness of stem or progenitor cells.

Sphingolipids: bioactive components of the cell membrane that act as important intracellular signaling molecules. They are also secreted extracellularly as chemoattractants for stem or progenitor cells.

Stromal cell-derived factor-1 alpha (SDF-1α) (CXCL12): a chemokine that is constitutively expressed at high levels in bone marrow and plays a distinct role in maintaining the quiescent hematopoietic stem cell pool in the marrow. Apart from regulating stem or progenitor cell migration to injured tissues, CXCL12 is involved in many other essential biological processes, including motility of numerous immune cells, hematopoiesis, angiogenesis, embryonic development, HIV/AIDS, apoptosis, and tumor metastasis.

Thymus-expressed chemokine (TECK) (CCL25): a chemokine that induces strong chemotaxis of human immature thymocytes and has recently been identified as a potent chemoattractant for human MSCs.

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Box 1. Mesenchymal stem cells: definition and characteristics

The precise definition and characterization of MSCs continue to be debated [2]. No specific marker is as yet known unambiguously to define and identify MSCs in the *in vivo* niche. Therefore, it is difficult fully to determine their *in vivo* characteristics and potentials for homing and mobilization. Additionally, it is challenging to compare accurately their *in vivo* features with the properties of *in vitro* expanded MSCs [88]. This is further complicated by the absence of standardized protocols for isolating and expanding the cells and by phenotypic changes that can occur during *in vitro* cultivation [10]. In an effort toward standardization, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell Therapy (ISCT) suggested minimal defining marker criteria: (i) plastic adherence; (ii) presence of CD105, CD73, and CD90 and absence of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules; and (iii) *in vitro* trilineage differentiation into osteoblasts, adipocytes, and chondroblasts [89]. Recent studies identified MSCs *in vivo* as Nestin^{+} cells that are associated with hematopoietic stem cells and nerve fibers at perivascular sites in the bone marrow [90]. Among the different terms that are used in the literature, 'mesenchymal stem cells' [91] and 'multipotent mesenchymal stromal cells', as proposed by the ISCT, are the most predominantly used terms to designate these cells. MSCs constitute a key structural and functional component in the marrow and are required for trafficking, proliferation, and differentiation of hematopoietic stem cells [1]. They can facilitate tissue homeostasis and repair by lineage-specific differentiation into cells that are lost or defective, secreting paracrine/anti-inflammatory factors, or by immunomodulation [1,2]. Bone marrow is the main source for the isolation of human MSCs. However, these cells can also be isolated from numerous other tissues.

with transplantation of *ex vivo* expanded MSCs or MSC-derived microvesicles that make use of MSC-associated paracrine factors. We review the background of the endogenous *in situ* mobilization strategy involving stem cells and focus on MSCs as the major cell source and chemokines as the pivotal chemoattractants. Although we emphasize MSCs, the strategies are also likely to apply to other stem cell types. We survey chemokine-triggered mobilization mechanisms, strategies to enhance these processes by means of suitable chemokine delivery devices, and other chemoattractants that act cooperatively or synergistically with chemokines, and conduct a critical examination of the perspectives and challenges of the chemokine-guided *in situ* strategy.

Chemokines are potent MSC recruitment factors

Apart from the decisive role of chemokines in tissue-specific homing of leukocytes in inflammation, distinct homeostatic chemokines were also implicated as guiding cues for directional trafficking of adult stem cells [3]. In this context, several studies have determined a wide set of chemokine receptors of all four subfamilies on the surface of *in vitro* expanded human MSCs: CCR1–11, CXCR1–7, CX_3CR1, and XCR1 [4–9]. However, these data are partly conflicting, because some authors determined receptors as present that other authors found to be absent. This heterogeneity is likely to be due to donor-dependent differences, the absence of standardized isolation and culture expansion protocols, and the pronounced phenotypic changes in MSCs associated with *in vitro* monolayer culture [10]. *In vitro* chemotaxis of human MSCs has been detected toward a wide set of chemokines, including CCL2, CCL3, CCL5, CCL7, CCL17, CCL19–22, CCL25, CCL28, CXCL8, CXCL10–13, CXCL16, and CX_3CL1 [4–9,11,12]. The chemokines CCL20, CCL25, CXCL9, and CXCL16 are also functionally involved in the MSC transendothelial migration cascade [13]. MSC chemokine receptor expression and chemotaxis are further increased by ischemic and proinflammatory preconditioning and on shear stress [8,9,14]. CXCL12, or stromal cell-derived factor-1 alpha (SDF-1α), is probably the most prominent stem or progenitor cell homing factor, attracting MSCs, endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs),

Table 1. Comparison of different options for MSC therapy

	Stem cell-based approach Transplantation of MSCs/MSC–MVs[a]	Chemokine-based approach Endogenous MSC homing
Cell harvest	• Invasive donor tissue biopsy • Transport to GMP facility	• Not required
***Ex vivo* cultivation**	• Expensive and labor-intensive *in vitro* cell expansion under GMP conditions • Impact on stem cell phenotype and function (e.g., reduced migration potential, altered factor secretion) • Further manipulation may be required (e.g., to enhance *in vivo* homing/cell function or large-scale MV secretion)	• Not required
***In vivo* delivery and monitoring**	• Systemic or local cell/MV delivery, if applicable in combination with scaffold/matrix construct • Cell labeling before transplantation allows *in vivo* MSC tracking to monitor biodistribution and engraftment	• Suitable (injectable) chemokine delivery device required that is applied locally to injured tissue • Migration of endogenous MSCs can hardly be monitored *in vivo*; post-mortem histology may identify recruited (stem) cell types
Clinical translation	• Pharmaceutical regulation (transplanted MSCs: ATMP[b]; MVs: classification unclear) • GMP-conforming cell cultivation • Cell-based approach; complicated production, storage, and logistics	• Pharmaceutical regulation • GMP-conforming synthesis of chemokines • Factor-based (cell-free) approach; off-the-shelf products simplify mass production, storage, and logistics
Disadvantages	• Safety concerns: potential contamination, pathogen transmission, tumorigenesis, and immune rejection • High costs due to expensive GMP-conforming cell cultivation • Technically complex procedure	• Safety concerns: potential inflammatory side effects, fibrosis • Unpredictability of required chemokine dosing and release pattern • Need for sophisticated biodegradable release devices

[a]MVs, microvesicles.

[b]ATMP, Advanced Therapy Medicinal Product.

neural stem cells (NSCs), smooth muscle progenitor cells, epithelium progenitor cells, and fibroblast progenitors. CXCL12 signals through two different receptors, CXCR4 and CXCR7, although the latter does not mediate signaling pathways that drive migration [15]. Although MSCs show only low CXCR4 surface expression, the receptor is presented intracellularly to a great degree [6,16] and MSCs migrate *in vitro* [4,16,17] and *in vivo* [18] in response to a CXCL12 gradient. Notably, the *in vitro* CXCL12 recruitment potential for human MSCs is quite low and some authors did not detect any chemotaxis [7]. The chemokine CCL25, or thymus-expressed chemokine (TECK), is a very potent *in vitro* chemoattractant for human MSCs that express the sole counter-receptor CCR9 [11]. Interestingly, the *in vitro* chemotactic response of human MSCs to CCL25 is more than tenfold greater than that to CXCL12. Other chemokine candidates with evidence of high recruitment efficiency of human MSCs include CCL2, CCL3, CCL5, CCL7, CCL21, CCL22, CXCL8, and CX_3CL1 [19–21].

To investigate chemotaxis, there are several static and dynamic (transendothelial) *in vitro* migration assays, as well as methods to track labeled cells in the animal after transplantation, such as *in vivo* microscopy and intravital microscopy, and each has specific advantages and limitations (Box 2). Intravital microscopy can be used to visualize the migration of labeled cells in a certain organ or tissue after surgical dissection; for example, to investigate chemokine-guided interactions of MSCs with the endothelium *in vivo* [8]. In view of the lack of a specific MSC marker, unlabeled transplanted MSCs may be detected *ex vivo* on post-mortem histology by utilizing species or sex mismatch. A discrepancy between *ex vivo* expanded and primary MSCs regarding homing or mobilization mechanisms and efficiency is most likely to be due to culture-related phenotypic or functional differences (e.g., in the expression of chemokine receptors and adhesion molecules [10]). However, there are currently no reliable methods to identify native MSCs *in situ* that would be needed to track their chemotaxis *in vivo*.

Chemokine response of tissues on injury

In a great step forward in understanding the mobilization of endogenous stem cells, CXCL12 was identified as a key player in stem cell homing. CXCL12 is constitutively secreted in the bone marrow stroma to retain bone marrow-derived stem cells in the marrow and is required for homing of circulating stem cells to the marrow (Figure 1A) [15]. The mechanisms underlying the physiological regulation of CXCL12-triggered stem cell trafficking toward ischemically injured tissues [22,23] is based on the finding that reduced oxygen tissue tension *in vivo* is directly proportional to the expression of CXCL12, which is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1), in the ischemic tissue. Secretion of CXCL12 from injured tissues results in reversal of the CXCL12 gradient between the bone marrow and the periphery, thus recruiting CXCR4-positive stem cells to the injured tissue site (Figure 1B). The hypoxia-induced mobilization mechanism was further supported [18,24] by the finding that MSC mobilization efficiency increases during chronic hypoxic conditions and MSC trafficking occurs via the CXCL12–CXCR4 axis. CXCL12 is transiently upregulated in ischemic myocardium [25] and upregulated in ischemic brain lesions [26], ischemic renal tissue [27], injured bone [28], burn wounds [29], and mechanically stretched skin tissue [30], to promote subsequent recruitment of MSCs. Besides CXCL12, few other chemokines have been reported to guide MSC mobilization to injured tissues. CCL2 is secreted on vascular injury and ischemic cerebral injury [31,32], CCL5 is released from degenerative intervertebral discs [33], CCL7 is transiently upregulated in infarcted myocardium [20], CCL21 is expressed in wounded skin [21], and CX3CR1 is released from ischemic brain lesions [34]. Other physiological signaling factors besides chemokines, such as growth factors (e.g., hepatocyte growth factor, platelet-derived growth factor), phospholipids, and ligands for toll-like receptors [19], were also found to direct intrinsic MSCs to injury sites. The exact physiological mechanisms underlying MSC migration along chemotactic gradients are not completely understood. Notably, other resident stem cell populations (e.g., EPCs, HSCs, NSCs) are also naturally mobilized by chemokines in response to injury to drive neovascularization, regeneration of ischemic tissues, or wound healing [3,35]. Additional effects of chemokines on tissue regeneration, such as participation in angiogenesis and stimulating secretion of proteases and trophic factors, also need to be considered.

Cooperative or synergistic factors in chemokine function

Although the important role of the CXCL12–CXCR4 axis in stem cell homing and mobilization is undisputed, cumulative evidence strongly indicates the involvement of other soluble factors and extracellular matrix components acting independently and cooperatively or synergistically with chemokines. In the mobilization of HSCs, CXCL12 gradients are positively primed by cationic antimicrobial peptides (CAMPs), such as LL-37, C1q, or C3a, and bioactive lipids, such as sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P), which are released from injured tissues and foster robust chemotaxis even to sites with very low chemokine concentrations [36–39]. Priming stem cell chemotaxis seems to be a compelling notion because of the highly proteolytic environment of injured tissues. Nonhematopoietic bone marrow-derived stem cells migrate to CXCL12-secreting ischemic myocardium in significantly higher numbers in the presence of such priming factors [40]. CAMP (C1q) and bioactive lipids (S1P and C1P) are also effective chemoattractants for MSCs and C1q was identified as a priming agent for CXCL12-triggered MSC chemotaxis, suggesting an important and previously underappreciated role of priming factors in the MSC mobilization cascade [41–43]. However, the exact molecular mechanisms linking priming factors with chemokine-triggered migration remain to be elucidated.

GAGs also modulate the *in vivo* activity of chemokines by immobilizing chemokines either to the extracellular matrix (ECM), to generate stable haptotactic gradients required for chemotaxis under shear flow, or to cell surfaces, facilitating binding to chemokine receptors. GAGs

Box 2. *In vitro* chemotaxis assays and *in vivo* cell tracking

Several *in vitro* assays can be used to investigate cell chemotaxis and chemokine-triggered cell interactions with the endothelium under static and dynamic conditions (Table I). These *in vitro* assays do not capture all of the complex *in vivo* parameters related to chemotaxis such as chemokine or cell interactions with the ECM and with other cell types (e.g., endothelial cells), chemokine or receptor oligomerization, shear flow, the complex composition of native tissue matrices, and the multiplicity of signaling molecules associated with the microenvironment of injured tissue. Advances in the design of modern microfluidics-based biochips promise better imitations of *in vivo* conditions and greater physiological relevance.

The most widely used imaging techniques for *in vivo* cell tracking of transplanted stem cells are MRI, optical fluorescence imaging (bioluminescence and fluorescence imaging), and positron emission tomography (PET) and single-photon emission CT (SPECT) (Table II). Most commonly, cells are labeled directly by introducing an imaging agent into the cytosol. Direct cell labeling has major limitations: leakage of the labeling agent after donor cell death may give false-positive signals and the labeling agent is diluted during cell division. Although cell-labeling procedures such as passive diffusion, endocytosis, electroporation, and liposome-based incorporation techniques are fairly simple, there is no clear correlation between the labeling signal and cell viability. By contrast, imaging of indirectly labeled cells is based on the physiological activity of the cells. A reporter gene is introduced into a cell's genome and translated into a protein that is used to image only that cell over its entire lifetime as well as the progeny cells. Using luciferase, fluorescent tracers such as GFP, and radiotracer-binding reporter genes, *in vivo* imaging of indirectly labeled cells can be performed by optical fluorescence imaging and PET/SPECT, respectively. However, gene modification is required and reporter gene systems are often difficult to implement.

Table I. Key *in vitro* chemotaxis assays that can be used with stem cells

Assay type	Main features	Advantages Disadvantages
Filter membrane chemotaxis assay	• Chemotaxis is measured through a porous filter membrane (e.g., Boyden chamber/Transwell assay) • Filters can be coated with extracellular proteins or cell monolayers (e.g., endothelial cells)	• Commercially available, quantitative and fast read-out, easy handling, high statistical reliability, suitable for screening multiple factors • End-point determination, static assay, unstable diffusion-driven chemotactic gradient
Hydrogel-based chemotaxis assay	• Cells are embedded above/within a gel and chemotactic gradients are established in the gel • Migratory cell behavior can be visualized continuously via time-lapse video microscopy or confocal laser-scanning microscopy	• Allows interactions with 3D ECM, kinetic determination of detailed migrational parameters • Static assay, complex experimental set-up/read-out and data processing
Capillary chamber chemotaxis assay	• Two chambers linked by a narrow connecting bridge (e.g., Dunn chamber) • Cells migrate in the connecting capillary area, can be monitored continuously via microscopy	• Standardized and commercially available, small sample volume • Static assay, unstable diffusion-driven chemokine gradient, complex read-out and data processing
Microfluidics-based chemotaxis assay	• Microfluidic device contains enclosed microcapillaries to analyze chemotaxis and transendothelial migration continuously by video microscopy • Channels can be coated with extracellular proteins or cell monolayers	• Commercially available, small sample volume, dynamic assay, kinetic determination of detailed migrational parameters, control of stable chemokine gradient and other parameters • Complex experimental set-up/read-out and data processing
Microfluidics-based biochip	• Microelectromechanical system with microfabricated channels in which cell migration is investigated continuously by video microscopy • May combine multiple parameters simultaneously (e.g., continuous flow, 3D ECM, endothelial monolayer and chemokine diffusion gradients)	• Commercially available, small sample volume, dynamic assay, kinetic determination of detailed migrational parameters, control of stable chemokine gradient and other parameters, allows parallel investigations • Complex experimental set-up/read-out and data processing, high cost

Table II. Imaging modalities suitable for *in vivo* stem cell tracking

Parameter	MRI	Optical fluorescence imaging	SPECT and PET
Energy deposition	No ionizing radiation, uses magnetic and radiofrequency fields	No ionizing radiation, uses light absorption and emission	Ionizing radiation (PET: positron emitters; SPECT: γ emitters)
Spatial resolution	High	Limited	Limited
Anatomical information	Detailed 3D anatomical information	Limited anatomical information, small area of observation	Limited anatomical information, interconnection with CT required
Tissue penetration	Depth independence	Limited because light undergoes absorption and scattering	Depth independence
Sensitivity	Limited	High at limited depths	High
Imaging agent	[a]SPIONs, gadolinium chelates	Fluorophores, quantum dots, luciferins	Radiotracers
Imaging time	Weeks to months	Days to weeks	Hours to days
Equipment	Expensive	Small and cheap	Expensive

[a]SPIONs, superparamagnetic iron oxide nanoparticles.

are negatively charged, interact strongly with positively charged chemokines, protect chemokines from proteolysis, and stabilize the formation of a large variety of chemokine oligomers [44]. Interaction of chemokines with GAGs is essential to promote chemotaxis of leukocytes *in vivo* [45]; this is not well known for MSC chemotaxis. For HSCs, however, hyaluronan, a major GAG component of bone marrow ECM, is an *in vivo* priming factor for

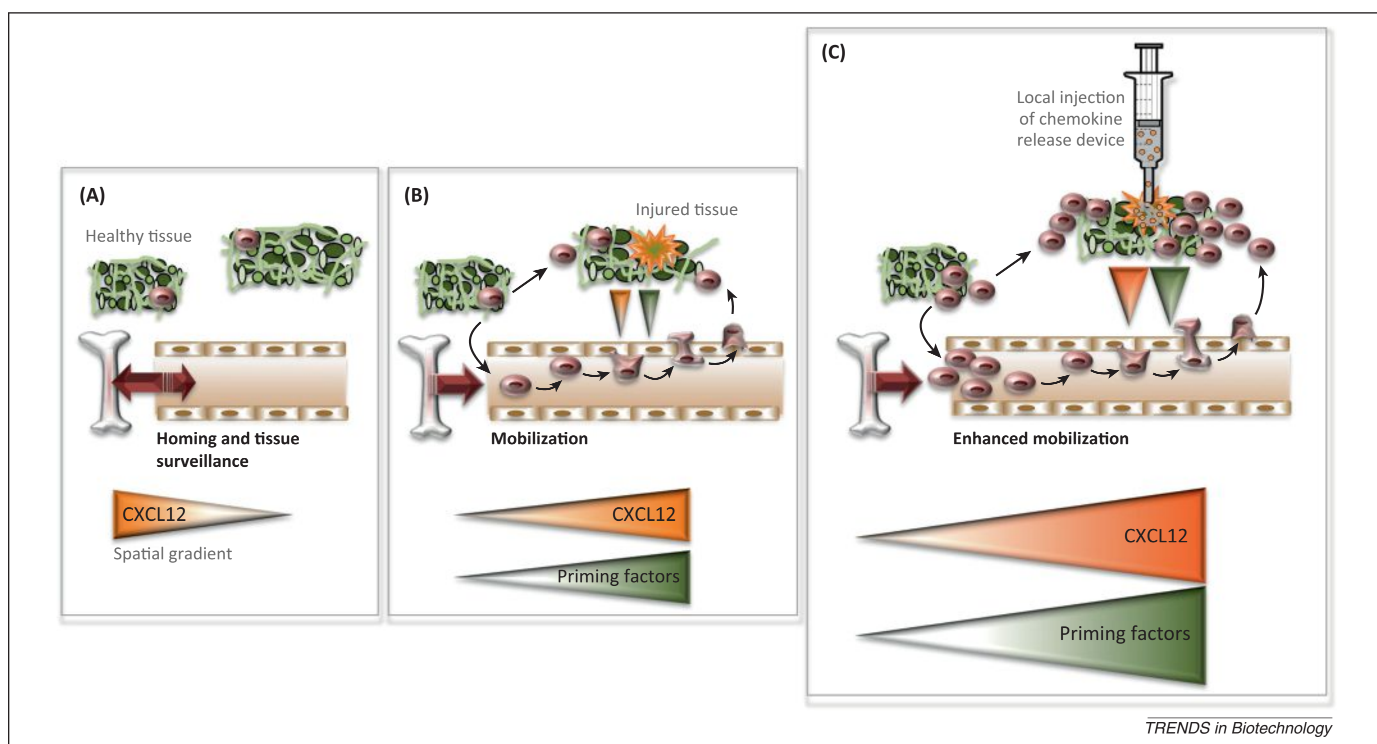

Figure 1. Schematics of stem cell trafficking along injury-inducible factors. **(A)** Homeostasis in healthy tissue. Stem cells shuttle between the bone marrow and the peripheral tissues as needed while ensuring sufficient retention and homing in response to chemokine gradients (orange). **(B)** Natural healing response after acute injury. Stem cells enter then leave the circulation to reach the injured tissue site. Stem cell trafficking is guided along gradients of chemokines (orange) and of other specific injury-inducible priming factors (green). **(C)** Enhanced healing response through an *in situ* regeneration strategy. Local release of additional chemokines at the injury tissue site prolongs and potentiates the recruitment of stem cells to the injury and catalyzes the inherent repair process.

transendothelial migration to sites with low CXCL12 concentrations, suggesting crosstalk between CD44, a hyaluronan receptor, and CXCR4 [46]. Furthermore, CXCL12–GAG interactions are important for retention of HSCs in bone marrow under homeostatic conditions and for stem cell recruitment and appropriate tissue revascularization after acute ischemia [47]. GAGs may also add to the specificity of chemokine function by selective accumulation on the cell glycocalyx. For example, endothelial cells from bone marrow and from umbilical veins differ in the glycocalyx GAG pattern that is required to present chemokines to rolling stem cells during transendothelial migration, which may contribute to specific HSC homing to bone marrow [48].

Toward chemokine-guided *in situ* tissue engineering

Efficient mobilization of stem cells to injured tissues is crucial for proper regeneration. However, there is only a limited time window after injury during which tissues secrete increased chemokine concentrations and during which stem cells are mobilized naturally. For example, CXCL12 expression increases for only a few days in the myocardium [49]. Thus, the extent of the body's inherent tissue repair is often modest, which motivates interest in strategies to maximize and prolong recruitment of regenerative cells to promote or enhance the intrinsic regeneration process. In this regard, local application of additional chemokines at the injured tissue site would be an attractive approach to tissue repair in regenerative medicine (Figure 1C). However, chemokines have several features that make them difficult to work with as therapeutics: they possess short half-lives, are cleaved by proteases, undergo rapid diffusion on bolus injection, and may cause inflammatory side effects [50]. Additionally, chemokines must be distributed in spatially defined gradients at precisely the right times to promote chemotaxis of stem cells. Given these constraints, the use of suitable delivery vehicles is indispensable for the application of chemokines. A large number and variety of chemokine release devices for stem cell recruitment (Table 2) has been applied to CCL19/CCL20/CCL21-guided immunotherapy [51–54] and CXCL12-guided *in situ* tissue regeneration [55–82], including both *in vitro* and *in vivo* trials. Additionally, CXCL12 release devices were used to investigate inflammation-mediated cancer metastasis [83] and CCL2 release devices were used to guide *in situ* tissue regeneration [84].

CXCL12 release systems have been employed *in vivo* to recruit endogenous MSCs, HSCs, EPCs, smooth muscle progenitor cells, and neural progenitor cells for the regeneration of ischemic myocardium [55,56,80], ischemic skeletal muscle [66], tendon [63], bone [58,67,78], partial-thickness cartilage defects [81], periodontal tissues and tooth-like structures [60], and blood vessels [76] and to induce angiogenesis [61,74] and wound healing [62]. The employed release devices mainly comprise synthetic biomaterials, such as poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone), and hydrogels derived from natural sources, such as collagen, alginate, and GAG-based carbohydrate polymers.

The synthetic polymer used most extensively for protein release is PLGA, a biodegradable and biocompatible copolymer approved for certain clinical applications by the US

Table 2. Chemokine release devices for endogenous (stem/progenitor) cell recruitment[a]

Delivery device	Refs
CCL19/CCL20/CCL21-guided immunotherapy	
Ethylene vinyl acetate polymer rods	[51]
PLGA microspheres	[52]
Crosslinked hydrogel comprising dextran vinyl sulfone and reactive PEG[b]	[53]
Alginate hydrogel microspheres	[54]
CXCL12-guided in situ *tissue regeneration*	
Self-assembling peptides	[55]
Covalent binding to PEGylated fibrin patch	[56]
Heparinized collagen scaffold	[57]
Mineralized collagen type 1 scaffold	[58]
Poly(lactide ethylene oxide fumarate) hydrogel	[59]
Poly(ε-caprolactone)–hydroxyapatite scaffold filled with collagen gel	[60]
Gelatin hydrogel	[61]
Heparin-loaded alginate patch	[62]
Collagen gel in silk–collagen sponge scaffold	[63]
Adsorption to PLGA scaffold	[64]
PLGA microspheres	[65]
Alginate microspheres in collagen-based matrix	[66]
Gelatin hydrogel	[67]
Heparin crosslinked with collagen	[68]
starPEG–heparin hydrogel	[69]
Chitosan-poly(γ-glutamic acid) polyelectrolyte complex	[70]
Chitosan/tripolyphosphate/fucoidan nanoparticles	[71]
[c]PLA/gelatin scaffold	[72]
Intrafibrillar-silicified collagen scaffold	[73]
Heparin-based hydrogel	[74]
Crosslinkable hyaluronic acid hydrogel	[75]
Heparin coating	[76]
Glycidyl methacrylated dextran/gelatin microcapsules with thermoresponsive poly(*N*-isopropylacrylamide) pore gates	[77]
Poly(ε-caprolactone)/gelatin membrane	[78]
Gelatin–hydroxyphenylpropionic acid hydrogels and dextran sulfate/chitosan polyelectrolyte complex nanoparticles	[79]
Hyaluronic acid-based hydrogel	[80]
Collagen type 1 scaffold	[81]
Poly(L-lysine)–hyaluronan multilayer film	[82]
CXCL12 involvement in inflammation-mediated cancer metastasis	
PLGA scaffold	[83]
CCL2-guided in situ *tissue regeneration*	
Alginate microparticles	[84]

[a]A PubMed literature search was performed to include studies on chemokine protein delivery devices for cell recruitment using the search criteria 'chemokine', 'delivery', 'release', 'cell recruitment', and/or 'migration'. This search produced 34 publications: four reports on CCL19/CCL20/CCL21 release used for immunotherapy; 28 studies on CXCL12 release for *in situ* tissue regeneration; one study on CXCL12 release to investigate inflammation-mediated cancer metastasis; and one study on CCL2 release for *in situ* tissue regeneration. There may be more published studies on chemokine release devices that did not match our search criteria. Studies using pump systems or genetic (stem/progenitor) cell modifications to achieve sustained chemokine delivery are not included.

[b]PEG, polyethylene glycol.

[c]PLA, poly(lactic acid).

FDA and the European Medicines Agency. PLGA microspheres allow tailored spatiotemporal protein release and can be applied locally with minimal invasiveness by injection (Figure 2) [85]. A promising alternative to synthetic polymers are natural GAG-based biomaterials, which are more hydrated, exploit existing natural interactions with chemokines, and may protect the chemokine from enzymatic degradation [86]. In this context, heparinized collagen scaffolds [57,68], heparin-based hydrogels [69,74], heparin-coating [76], hyaluronic acid-based hydrogels [75,80], and poly(L-lysine)–hyaluronan multilayer films [82] have already been used successfully for chemokine delivery. In particular, hyaluronic acid can prime stem cell migration along low CXCL12 gradients [46,75] and hyaluronic acid-based hydrogels present a promising delivery device for chemokines (Figure 2).

Perspectives and challenges of the chemokine-guided *in situ* strategy

Preclinical *in vivo* proof-of-concept studies with chemokine delivery devices are conducted at an increasing rate and cell-free chemoattractant-based therapies are likely to become more important in the future. A therapeutic product can comprise a syringe filled with a suspension of chemokine-releasing PLGA microspheres or chemokines immobilized on a hyaluronic acid-based hydrogel (Figure 2) that could be injected locally into a multitude of injured tissues (e.g., ischemically injured heart or kidney, muscle, cartilage, bone, tendon). Such a release device could be stored and sold off the shelf, providing a more efficient, convenient, and cost- or labor-saving therapeutic tool in regenerative medicine. Such a device would be simpler to implement than the MSC transplantation or MSC microvesicle approaches; for example, by avoiding the requirement for Good Manufacturing Practice (GMP) *ex vivo* cell cultivation (Table 1). No removal is required when the particles or hyaluronic acid are depleted of the chemokine because the biomaterials employed are biodegradable.

Several aspects have to be considered regarding the potential clinical setting of chemokine release devices. Because distinct priming factors can amplify chemokine-triggered stem cell mobilization [41–43], simultaneous release of chemokines and priming factors or combining local chemokine release with systemic application of priming factors may have additive or even synergistic effects on stem cell recruitment and *in situ* tissue regeneration. Similarly, it is possible that chemokines can potentiate but may not be the sole promoters of stem cell recruitment to injured tissues: they may guide endogenous stem cell trafficking only, when the injured microenvironment provides all other guiding cues. This is the case for stem cell mobilization to healthy heart tissue [25], but it is unclear whether the same could be said of mobilization to damaged tissue. Chemokine release devices could thus be more suitable for treating tissues directly after injury than for preventive treatment. Release devices may thus also be particularly suitable for the acute phase of a disease. However, whether chemokine release treatment can be employed for chronic injuries, such as chronic postischemic heart failure, remains to be determined.

Furthermore, exogenous chemokine application therapy may be complicated by the presence of multiple disease states in a patient. For example, chemokine delivery to injured tissues may be inadequate for cancer patients because chemokine receptors (e.g., CXCR4) are also expressed by various cancer cell types, contributing to tumor progression and metastasis [3].

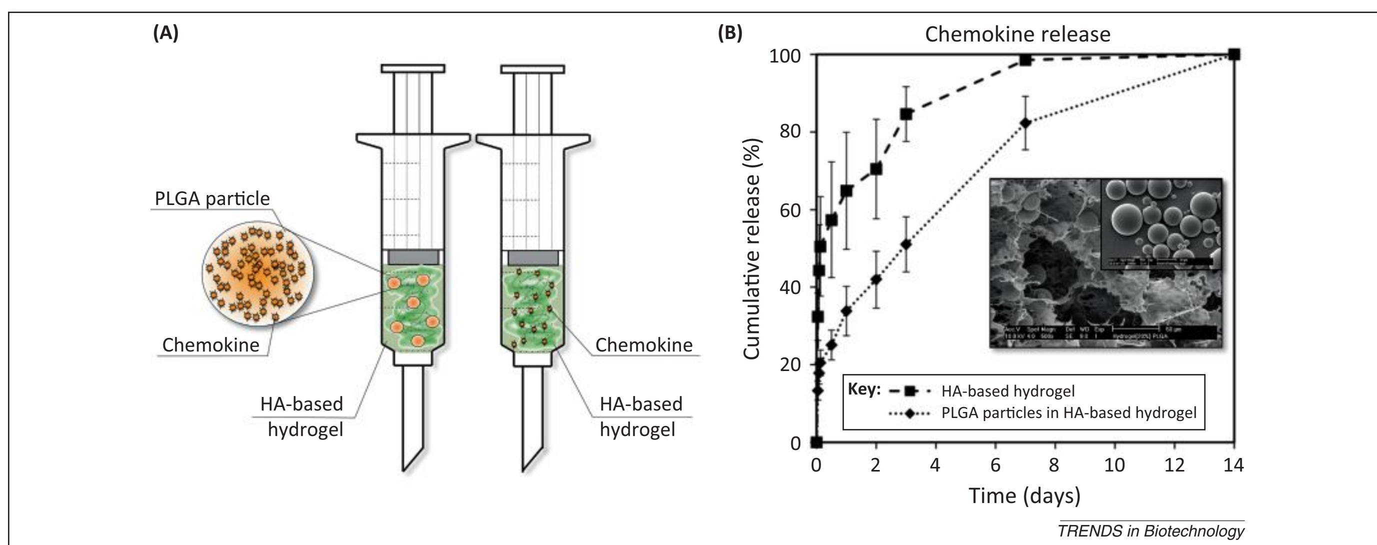

Figure 2. Chemokine release from poly(lactic-co-glycolic acid) (PLGA) microspheres and hyaluronan-based hydrogels. **(A)** Potential *in situ* tissue engineering product comprising a syringe containing a suspension of chemokine-loaded PLGA microspheres (left) or hyaluronic acid (HA)-based hydrogel with immobilized chemokines (right). **(B)** Time course of cumulative release of the chemokine CCL25 from pure HA-based hydrogel (broken line) or PLGA microspheres embedded in a HA-based hydrogel (dotted lines). Representative scanning electron micrographs show CCL25-loaded PLGA microspheres that are integrated in the HA hydrogel (inset).

The success of chemokine application will require careful attention to the biological, chemical, and even physical environment and context of tissue regeneration. For example, additional growth factors released in specific spatiotemporal patterns may be required. Mechanical and structural support of the injured tissue could be a concern. Successful tissue regeneration by recruited endogenous stem cells may require an appropriate local environment of artificial ECM to engraft, proliferate, and/or differentiate efficiently. Developing a biocompatible and biodegradable chemokine release scaffold that is tailored to the injured tissue to provide essential biophysical and chemical cues may be necessary.

Although chemokines appear to have great potential for *in situ* tissue engineering, this approach is in its early days and challenges remain (Table 1). Most importantly, the safety, efficacy, and quality of the tissue repair needs to be evaluated critically in clinical trials. In this context, it is difficult to determine which chemokine concentrations are both safe and effective for stimulating endogenous stem cell recruitment *in vivo*. Chemokine concentrations administered above normal ischemic levels and for prolonged durations may be appropriate, but the ideal *in vivo* dose and duration of chemokine release remains unknown.

It seems that heterogeneous cell populations are mobilized to tissue injury sites in response to chemokines and it may not be possible to recruit a specific resident stem cell type. For example, CXCL12, is known to mobilize multiple CXCR4-expressing stem or progenitor cell types to sites of injury, including MSCs, HSCs, EPCs, NSCs, smooth muscle progenitor cells, and immune cells such as lymphocytes and monocytes [3,35]. The nature of the attracted cell types may depend on the type of tissue or injury and it remains a major challenge to identify mobilized host cell populations. There are options for cell characterization, such as post-mortem immunohistochemical and flow cytometric analyses of harvested tissues or cells. Such methods may be successful with appropriate antibodies against the corresponding chemokine receptor, stem cell-associated markers (e.g., c-kit, Sca-1, CD34), and tissue cell or inflammatory cell-specific markers [55–57,63,64,67,72,79,81]. However, the lack of specific *in situ* stem cell surface markers (as is the case for MSCs), subsequent differentiation of recruited stem cells, or phenotypic changes following mobilization may hamper precise histological identification. Longitudinal tracking of host stem cell migration is a large unmet need in the field of *in situ* tissue engineering. Tracking cell migration would be helpful to correlate the concentration and duration of released chemokines with the number of recruited cells and with functional tissue recovery to better evaluate and optimize chemokine release therapies.

Recruitment of different regenerative cell types can contribute to the delicate and well-coordinated mechanisms of tissue repair. However, recruitment of potentially detrimental cells, such as immune cells (e.g., monocytes, dendritic cells, T cells, macrophages) and fibrocytes, may cause deleterious biological responses such as chronic inflammation and fibrosis. Homeostatic chemokines (e.g., CCL19, CCL21, CCL25, CCL28, CXCL12, CXCL13) are particularly known to be involved in stem cell mobilization, but they also stimulate recruitment of immature immune cells and may foster local maturation of immune effector cells within the target tissue, perhaps promoting immune response [3]. Interestingly, recent *in vivo* studies on CXCL12-guided *in situ* tissue regeneration reported reduced inflammatory and fibrotic responses rather than increased infiltration of inflammatory cells [63,64]. Released CXCL12 may reduce mast cell activation, leading to a subsequent downstream reduction of inflammatory cell response, and fibrosis may be decreased due to participation of recruited stem cells [64]. Furthermore, recruited MSCs may suppress the activity of immune cells *in vivo* – a property that has been shown for *in vitro* expanded MSCs – and thus promote the resolution of inflammation [87]. In addition, CXCL12 reduces inflammatory cytokine secretion from adjacent injured tissues [64,66]. Excessive

recruitment of immune cells could be avoided with devices that release appropriate chemokines (e.g., CXCL12) at low doses in a controlled manner. Codelivery of anti-inflammatory substances or regulatory cytokines that suppress the activity of immune cells could also be considered.

Concluding remarks and future perspectives

The local and sustained delivery of chemokines to injured tissues is an up-to-date therapeutic strategy and the latest *in vivo* proof-of-concept studies are promising. Chemokine release devices present a convenient off-the-shelf product for various clinical applications and offer distinct advantages over stem or progenitor cell or microvesicle transplantation approaches. However, further investigation is required to uncover the molecular and cellular mechanisms of chemokine-guided stem cell recruitment *in vivo* and there are therapeutic issues regarding dose, safety, and efficacy. Most importantly, there is the need to establish suitable animal models to investigate native stem cells *in situ* and track them *in vivo*, to exploit homing mechanisms therapeutically. Because the response of stem cells to chemokines seems to depend on the status of the injured tissue and the inflammatory response, we need deeper knowledge of priming or upstream factors and the underlying signaling cascades of chemokine action. It is also immensely important to gain deeper insights into the specific interactions of GAGs with chemokines during stem cell migration. Glycomics holds great promise here to obtain sequence information about GAGs in tissue ECM and on the cell surface to elucidate interactions with chemokines. Gaining a better understanding of the complex physiological interactions between chemokines, priming factors, and GAGs in tissue homeostasis, injury, and repair is the main challenge to be met in the near future to bring new chemokine-guided therapeutic options forward for *in situ* tissue regeneration.

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References

1 Shi, C. (2012) Recent progress toward understanding the physiological function of bone marrow mesenchymal stem cells. *Immunology* 136, 133–138
2 Keating, A. (2012) Mesenchymal stromal cells: new directions. *Cell Stem Cell* 10, 709–716
3 Anders, H.J. *et al.* (2014) Pathomechanisms: homeostatic chemokines in health, tissue regeneration, and progressive diseases. *Trends Mol. Med.* 20, 154–165
4 Ringe, J. *et al.* (2007) Towards *in situ* tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J. Cell. Biochem.* 101, 135–146
5 Sordi, V. *et al.* (2005) Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 106, 419–427
6 Honczarenko, M. *et al.* (2006) Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells* 24, 1030–1041
7 Von Luttichau, I. *et al.* (2005) Human adult CD34^{-} progenitor cells functionally express the chemokine receptors CCR1, CCR4, CCR7, CXCR5, and CCR10 but not CXCR4. *Stem Cells Dev.* 14, 329–336
8 Rüster, B. *et al.* (2006) Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 108, 3938–3944
9 Ponte, A.L. *et al.* (2007) The *in vitro* migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells* 25, 1737–1745
10 Bara, J.J. *et al.* (2014) Bone marrow-derived mesenchymal stem cells change phenotype following *in vitro* culture: implications for basic research and the clinic. *Stem Cells* 32, 1713–1723
11 Binger, T. *et al.* (2009) Migration potential and gene expression profile of human mesenchymal stem cells induced by CCL25. *Exp. Cell Res.* 315, 1468–1479
12 Kalwitz, G. *et al.* (2010) Chemokine profile of human serum from whole blood: migratory effects of CXCL-10 and CXCL-11 on human mesenchymal stem cells. *Connect. Tissue Res.* 51, 113–122
13 Chamberlain, G. *et al.* (2011) Mesenchymal stem cells exhibit firm adhesion, crawling, spreading and transmigration across aortic endothelial cells: effects of chemokines and shear. *PLoS ONE* 6, e25663
14 Liu, H. *et al.* (2010) Hypoxic preconditioning advances CXCR4 and CXCR7 expression by activating HIF-1alpha in MSCs. *Biochem. Biophys. Res. Commun.* 401, 509–515
15 Rankin, S.M. (2012) Chemokines and adult bone marrow stem cells. *Immunol. Lett.* 145, 47–54
16 Wynn, R.F. *et al.* (2004) A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 104, 2643–2645
17 Stich, S. *et al.* (2009) Gene expression profiling of human mesenchymal stem cells chemotactically induced with CXCL12. *Cell Tissue Res.* 336, 225–236
18 Liu, L. *et al.* (2011) Hypoxia-inducible factor-1alpha is essential for hypoxia-induced mesenchymal stem cell mobilization into the peripheral blood. *Stem Cells Dev.* 20, 1961–1971
19 Vanden Berg-Foels, W.S. (2014) *In situ* tissue regeneration: chemoattractants for endogenous stem cell recruitment. *Tissue Eng. Part B: Rev.* 20, 28–39
20 Schenk, S. *et al.* (2007) Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* 25, 245–251
21 Sasaki, M. *et al.* (2008) Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J. Immunol.* 180, 2581–2587
22 Ceradini, D.J. *et al.* (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat. Med.* 10, 858–864
23 Ceradini, D.J. and Gurtner, G.C. (2005) Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. *Trends Cardiovasc. Med.* 15, 57–63
24 Rochefort, G.Y. *et al.* (2006) Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. *Stem Cells* 24, 2202–2208
25 Abbott, J.D. *et al.* (2004) Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation* 110, 3300–3305
26 Wang, Y. *et al.* (2008) SDF-1alpha/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. *Brain Res.* 1195, 104–112
27 Togel, F. *et al.* (2005) Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. *Kidney Int.* 67, 1772–1784
28 Kitaori, T. *et al.* (2009) Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum.* 60, 813–823

29 Hu, C. *et al.* (2013) CXCL12/CXCR4 axis promotes mesenchymal stem cell mobilization to burn wounds and contributes to wound repair. *J. Surg. Res.* 183, 427–434
30 Zhou, S.B. *et al.* (2013) Mechanical stretch upregulates SDF-1alpha in skin tissue and induces migration of circulating bone marrow-derived stem cells into the expanded skin. *Stem Cells* 31, 2703–2713
31 Zhang, F. *et al.* (2009) Transforming growth factor-beta promotes recruitment of bone marrow cells and bone marrow-derived mesenchymal stem cells through stimulation of MCP-1 production in vascular smooth muscle cells. *J. Biol. Chem.* 284, 17564–17574
32 Wang, L. *et al.* (2002) Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. *Exp. Hematol.* 30, 831–836
33 Pattappa, G. *et al.* (2014) CCL5/RANTES is a key chemoattractant released by degenerative intervertebral discs in organ culture. *Eur. Cell Mater.* 27, 124–136 discussion 136
34 Zhu, J. *et al.* (2009) Fractalkine and CX3CR1 are involved in the migration of intravenously grafted human bone marrow stromal cells toward ischemic brain lesion in rats. *Brain Res.* 1287, 173–183
35 Lau, T.T. and Wang, D.A. (2011) Stromal cell-derived factor-1 (SDF-1): homing factor for engineered regenerative medicine. *Expert Opin. Biol. Ther.* 11, 189–197
36 Ratajczak, M.Z. *et al.* (2012) A novel perspective on stem cell homing and mobilization: review on bioactive lipids as potent chemoattractants and cationic peptides as underappreciated modulators of responsiveness to SDF-1 gradients. *Leukemia* 26, 63–72
37 Jalili, A. *et al.* (2010) Complement C1q enhances homing-related responses of hematopoietic stem/progenitor cells. *Transfusion* 50, 2002–2010
38 Wu, W. *et al.* (2012) The bone marrow-expressed antimicrobial cationic peptide LL-37 enhances the responsiveness of hematopoietic stem progenitor cells to an SDF-1 gradient and accelerates their engraftment after transplantation. *Leukemia* 26, 736–745
39 Ratajczak, M.Z. *et al.* (2014) The role of sphingosine-1 phosphate and ceramide-1 phosphate in trafficking of normal stem cells and cancer cells. *Expert Opin. Ther. Targets* 18, 95–107
40 Karapetyan, A.V. *et al.* (2013) Bioactive lipids and cationic antimicrobial peptides as new potential regulators for trafficking of bone marrow-derived stem cells in patients with acute myocardial infarction. *Stem Cells Dev.* 22, 1645–1656
41 Kim, C. *et al.* (2013) Ceramide-1-phosphate regulates migration of multipotent stromal cells and endothelial progenitor cells – implications for tissue regeneration. *Stem Cells* 31, 500–510
42 Li, C. *et al.* (2009) Homing of bone marrow mesenchymal stem cells mediated by sphingosine 1-phosphate contributes to liver fibrosis. *J. Hepatol.* 50, 1174–1183
43 Qiu, Y. *et al.* (2012) Mesenchymal stromal cells derived from umbilical cord blood migrate in response to complement C1q. *Cytotherapy* 14, 285–295
44 Salanga, C.L. and Handel, T.M. (2011) Chemokine oligomerization and interactions with receptors and glycosaminoglycans: the role of structural dynamics in function. *Exp. Cell Res.* 317, 590–601
45 Proudfoot, A.E. *et al.* (2003) Glycosaminoglycan binding and oligomerization are essential for the *in vivo* activity of certain chemokines. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1885–1890
46 Avigdor, A. *et al.* (2004) CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood* 103, 2981–2989
47 Rueda, P. *et al.* (2012) Homeostatic and tissue reparation defaults in mice carrying selective genetic invalidation of CXCL12/proteoglycan interactions. *Circulation* 126, 1882–1895
48 Netelenbos, T. *et al.* (2001) Differences in sulfation patterns of heparan sulfate derived from human bone marrow and umbilical vein endothelial cells. *Exp. Hematol.* 29, 884–893
49 Askari, A.T. *et al.* (2003) Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* 362, 697–703
50 Chen, F.M. *et al.* (2011) Homing of endogenous stem/progenitor cells for *in situ* tissue regeneration: promises, strategies, and translational perspectives. *Biomaterials* 32, 3189–3209
51 Kumamoto, T. *et al.* (2002) Induction of tumor-specific protective immunity by *in situ* Langerhans cell vaccine. *Nat. Biotechnol.* 20, 64–69
52 Zhao, X. *et al.* (2005) Directed cell migration via chemoattractants released from degradable microspheres. *Biomaterials* 26, 5048–5063
53 Singh, A. *et al.* (2009) *In situ* crosslinking hydrogels for combinatorial delivery of chemokines and siRNA–DNA carrying microparticles to dendritic cells. *Biomaterials* 30, 5187–5200
54 Wang, Y. and Irvine, D.J. (2011) Engineering chemoattractant gradients using chemokine-releasing polysaccharide microspheres. *Biomaterials* 32, 4903–4913
55 Segers, V.F. *et al.* (2007) Local delivery of protease-resistant stromal cell derived factor-1 for stem cell recruitment after myocardial infarction. *Circulation* 116, 1683–1692
56 Zhang, G. *et al.* (2007) Controlled release of stromal cell-derived factor-1 alpha *in situ* increases c-kit+ cell homing to the infarcted heart. *Tissue Eng.* 13, 2063–2071
57 Bladergroen, B.A. *et al.* (2009) *In vivo* recruitment of hematopoietic cells using stromal cell-derived factor 1 alpha-loaded heparinized three-dimensional collagen scaffolds. *Tissue Eng. Part A* 15, 1591–1599
58 Thieme, S. *et al.* (2009) Stromal cell-derived factor-1alpha-directed chemoattraction of transiently CXCR4-overexpressing bone marrow stromal cells into functionalized three-dimensional biomimetic scaffolds. *Tissue Eng. Part C: Methods* 15, 687–696
59 He, X. *et al.* (2010) Migration of marrow stromal cells in response to sustained release of stromal-derived factor-1alpha from poly(lactide ethylene oxide fumarate) hydrogels. *Int. J. Pharm.* 390, 107–116
60 Kim, K. *et al.* (2010) Anatomically shaped tooth and periodontal regeneration by cell homing. *J. Dent. Res.* 89, 842–847
61 Kimura, Y. and Tabata, Y. (2010) Controlled release of stromal-cell-derived factor-1 from gelatin hydrogels enhances angiogenesis. *J. Biomater. Sci. Polym. Ed.* 21, 37–51
62 Rabbany, S.Y. *et al.* (2010) Continuous delivery of stromal cell-derived factor-1 from alginate scaffolds accelerates wound healing. *Cell Transplant.* 19, 399–408
63 Shen, W. *et al.* (2010) The effect of incorporation of exogenous stromal cell-derived factor-1 alpha within a knitted silk–collagen sponge scaffold on tendon regeneration. *Biomaterials* 31, 7239–7249
64 Thevenot, P.T. *et al.* (2010) The effect of incorporation of SDF-1alpha into PLGA scaffolds on stem cell recruitment and the inflammatory response. *Biomaterials* 31, 3997–4008
65 Cross, D.P. and Wang, C. (2011) Stromal-derived factor-1 alpha-loaded PLGA microspheres for stem cell recruitment. *Pharm. Res.* 28, 2477–2489
66 Kuraitis, D. *et al.* (2011) A stromal cell-derived factor-1 releasing matrix enhances the progenitor cell response and blood vessel growth in ischaemic skeletal muscle. *Eur. Cell. Mater.* 22, 109–123
67 Ratanavaraporn, J. *et al.* (2011) Synergistic effects of the dual release of stromal cell-derived factor-1 and bone morphogenetic protein-2 from hydrogels on bone regeneration. *Biomaterials* 32, 2797–2811
68 Sobkow, L. *et al.* (2011) Prolonged transendothelial migration of human haematopoietic stem and progenitor cells (HSPCs) towards hydrogel-released SDF1. *Ann. Hematol.* 90, 865–871
69 Baumann, L. *et al.* (2012) A novel, biased-like SDF-1 derivative acts synergistically with starPEG-based heparin hydrogels and improves eEPC migration *in vitro*. *J. Control. Release* 162, 68–75
70 Goncalves, R.M. *et al.* (2012) Mesenchymal stem cell recruitment by stromal derived factor-1-delivery systems based on chitosan/poly(gamma-glutamic acid) polyelectrolyte complexes. *Eur. Cell. Mater.* 23, 249–260 discussion 260–261
71 Huang, Y.C. and Liu, T.J. (2012) Mobilization of mesenchymal stem cells by stromal cell-derived factor-1 released from chitosan/tripolyphosphate/fucoidan nanoparticles. *Acta Biomater.* 8, 1048–1056
72 Ko, I.K. *et al.* (2012) Combined systemic and local delivery of stem cell inducing/recruiting factors for *in situ* tissue regeneration. *FASEB J.* 26, 158–168
73 Niu, L.N. *et al.* (2012) Intrafibrillar silicification of collagen scaffolds for sustained release of stem cell homing chemokine in hard tissue regeneration. *FASEB J.* 26, 4517–4529
74 Prokoph, S. *et al.* (2012) Sustained delivery of SDF-1alpha from heparin-based hydrogels to attract circulating pro-angiogenic cells. *Biomaterials* 33, 4792–4800
75 Purcell, B.P. *et al.* (2012) Synergistic effects of SDF-1alpha chemokine and hyaluronic acid release from degradable hydrogels on directing

bone marrow derived cell homing to the myocardium. *Biomaterials* 33, 7849–7857

76 Yu, J. *et al.* (2012) The effect of stromal cell-derived factor-1alpha/heparin coating of biodegradable vascular grafts on the recruitment of both endothelial and smooth muscle progenitor cells for accelerated regeneration. *Biomaterials* 33, 8062–8074

77 Chen, F.M. *et al.* (2013) Surface-engineering of glycidyl methacrylated dextran/gelatin microcapsules with thermo-responsive poly(*N*-isopropylacrylamide) gates for controlled delivery of stromal cell-derived factor-1alpha. *Biomaterials* 34, 6515–6527

78 Ji, W. *et al.* (2013) Incorporation of stromal cell-derived factor-1alpha in PCL/gelatin electrospun membranes for guided bone regeneration. *Biomaterials* 34, 735–745

79 Lim, T.C. *et al.* (2013) Chemotactic recruitment of adult neural progenitor cells into multifunctional hydrogels providing sustained SDF-1alpha release and compatible structural support. *FASEB J.* 27, 1023–1033

80 MacArthur, J.W., Jr *et al.* (2013) Sustained release of engineered stromal cell-derived factor 1-alpha from injectable hydrogels effectively recruits endothelial progenitor cells and preserves ventricular function after myocardial infarction. *Circulation* 128, S79–S86

81 Zhang, W. *et al.* (2013) The use of type 1 collagen scaffold containing stromal cell-derived factor-1 to create a matrix environment conducive to partial-thickness cartilage defects repair. *Biomaterials* 34, 713–723

82 Dalonneau, F. *et al.* (2014) The effect of delivering the chemokine SDF-1alpha in a matrix-bound manner on myogenesis. *Biomaterials* 35, 4525–4535

83 Ko, C.Y. *et al.* (2012) The use of chemokine-releasing tissue engineering scaffolds in a model of inflammatory response-mediated melanoma cancer metastasis. *Biomaterials* 33, 876–885

84 Jay, S.M. *et al.* (2010) Dual delivery of VEGF and MCP-1 to support endothelial cell transplantation for therapeutic vascularization. *Biomaterials* 31, 3054–3062

85 Fredenberg, S. *et al.* (2011) The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems – a review. *Int. J. Pharm.* 415, 34–52

86 Sadir, R. *et al.* (2004) Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *J. Biol. Chem.* 279, 43854–43860

87 Shi, Y. *et al.* (2012) How mesenchymal stem cells interact with tissue immune responses. *Trends Immunol.* 33, 136–143

88 Karp, J.M. and Leng Teo, G.S. (2009) Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 4, 206–216

89 Dominici, M. *et al.* (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317

90 Mendez-Ferrer, S. *et al.* (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829–834

91 Caplan, A.I. (1991) Mesenchymal stem cells. *J. Orthop. Res.* 9, 641–650

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MSC-Regulated MicroRNAs Converge on the Transcription Factor FOXP2 and Promote Breast Cancer Metastasis

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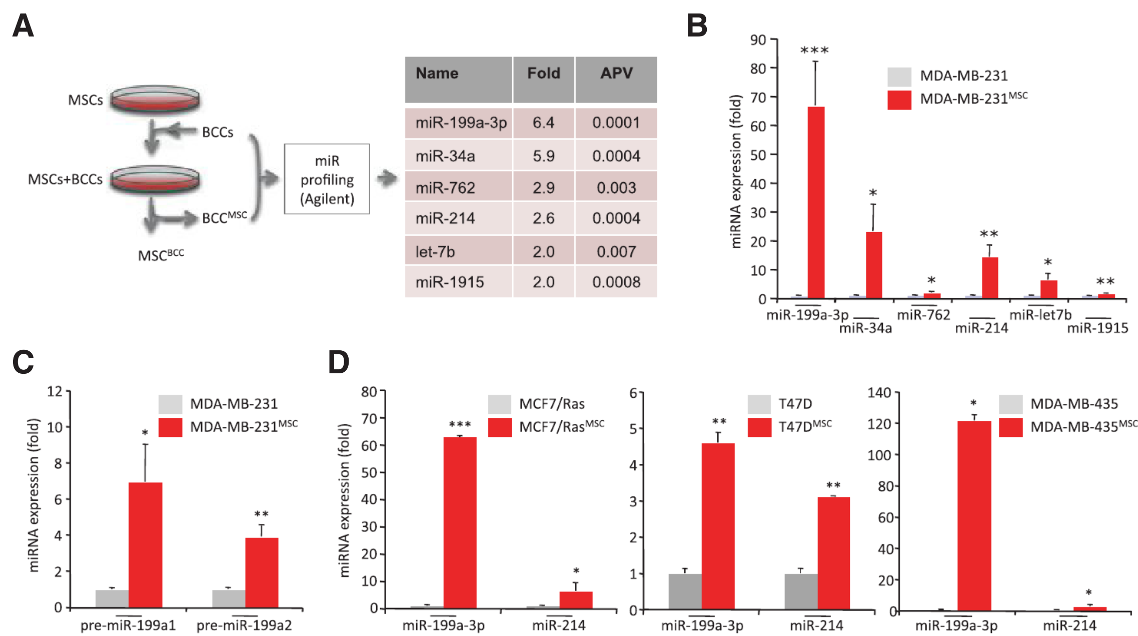
SUMMARY

Mesenchymal stem/stromal cells (MSCs) are progenitor cells shown to participate in breast tumor stroma formation and to promote metastasis. Despite expanding knowledge of their contributions to breast malignancy, the underlying molecular responses of breast cancer cells (BCCs) to MSC influences remain incompletely understood. Here, we show that MSCs cause aberrant expression of microRNAs, which, led by microRNA-199a, provide BCCs with enhanced cancer stem cell (CSC) properties. We demonstrate that such MSC-deregulated microRNAs constitute a network that converges on and represses the expression of *FOXP2*, a forkhead transcription factor tightly associated with speech and language development. FOXP2 knockdown in BCCs was sufficient in promoting CSC propagation, tumor initiation, and metastasis. Importantly, elevated microRNA-199a and depressed *FOXP2* expression levels are prominent features of malignant clinical breast cancer and are associated significantly with poor survival. Our results identify molecular determinants of cancer progression of potential utility in the prognosis and therapy of breast cancer.

INTRODUCTION

Cancer cells within breast carcinomas coexist with a heterogeneous milieu of stromal cells that collectively constitute the tumor-associated microenvironment. Numerous studies have provided substantial evidence that the interactions between cancer cells and components of the tumor stroma are pivotal in breast cancer pathogenesis (Barcellos-Hoff et al., 2013). In particular, such interactions—which co-opt mechanisms of wound healing, tissue maintenance, or development—appear to induce changes in cancer cells that are sufficient to actuate metastatic progression (Quail and Joyce, 2013). On this front, our group and others have shown that contextual mechanisms instigated in the cancer cells by the tumor microenvironment can cause dramatic increases in cancer malignancy via ostensibly reversible processes, such as epithelial-to-mesenchymal transitions (EMTs) (e.g., El-Haibi et al., 2012; Polyak and Weinberg, 2009). Indeed, cancer cells appear to be highly responsive to promalignant signals originating from the tumor microenvironment, providing attractive new avenues for the development of therapeutic approaches based on the inhibition of tumor-stroma crosstalk.

Mesenchymal stem cells (MSCs; also known as mesenchymal stromal cells) are a heterogeneous class of stromal progenitor cells that participate in tissue maintenance under normal homeostasis and are likewise closely associated with pathologic stromal responses to tissue injury and neoplasia (Cuiffo and Karnoub, 2012; Prockop et al., 2010). In the context of developing breast carcinomas, tumor-proximal MSCs have been shown to serve as active catalysts of cancer progression, robustly promoting breast cancer cell (BCC) invasion and metastasis (Karnoub et al., 2007; Goldstein et al., 2010; Liu et al., 2011; El-Haibi et al., 2012; Chaturvedi et al., 2013). MSCs have also been described to play similar roles in other cancer contexts, including lung cancer (Suzuki et al., 2011), prostate cancer (Prantl et al., 2010), and colon cancer (Shinagawa et al., 2010), suggestive of general promalignant activities for MSCs recruited into epithelial tumors. Indeed, MSCs recovered from human breast,



Name	Fold	APV
miR-199a-3p	6.4	0.0001
miR-34a	5.9	0.0004
miR-762	2.9	0.003
miR-214	2.6	0.0004
let-7b	2.0	0.007
miR-1915	2.0	0.0008

Figure 1. Induction of miR-199a and miR-214 in MSC-Primed BCCs

(A) Schematic of MSC+BCC coculture and sorting of BCCs for miRNA profiling. Six miRNAs were enriched ≥2.0-fold in MSC-activated BCCs (BCC^{MSC}) compared to resting BCCs (APV is adjusted p value, n = 4).

(B) Semiquantitative real-time PCR (rtPCR-ΔΔct) validation of the mature miRNAs identified in (A).

(C) rtPCR-ΔΔct probing precursor stem-loops derived from miR-199a1 and miR-199a2 (n = 3).

(D) rtPCR-ΔΔct probing miR-199a2-derived mature miRNAs in MSC-activated MCF7/Ras, T47D, and MDA-MB-435 cells; $MCF7/Ras^{MSC}$; $T47D^{MSC}$; and $MDA\text{-}MB\text{-}435^{MSC}$ (n = 3).

All rtPCR-ΔΔct panels display mean fold enrichment ± SEM. *p < 0.05; **p < 0.01, ***p < 0.001 in two-tailed Student's t test. See also Figure S1.

prostate, or ovarian tumors display promalignant phenotypes indicative of their crucial supportive functions in the progression of these tumors (McLean et al., 2011; Yan et al., 2012; Jung et al., 2013). However, at present, the molecular mechanisms underlying MSC contributions to tumor pathogenesis remain incompletely understood.

MicroRNAs (miRNAs, or miRs) are short noncoding RNAs that regulate gene expression via hybridization to complementary sequences of mRNAs, resulting in either translational inhibition or degradation of the target sequences (Bartel, 2009). miRNAs may affect the expression of hundreds of targets, thereby serving to define cellular identity and differentiation state through large-scale regulation of gene expression programs (Miyoshi et al., 2011). Importantly, miRNAs play critical functions in cancer pathogenesis and an expanding body of evidence has cataloged their deregulation in multiple aspects of tumor development, including invasion and metastasis (Ma et al., 2007; Tavazoie et al., 2008). To date, however, promalignant alterations to the cancer cell miRNA landscape as derived from their interactions with stromal cells have not been comprehensively characterized.

In the present work, we sought to identify potential miRNA deregulations associated with breast cancer malignancy instigated by prometastatic MSCs. We found that MSCs trigger a select set of miRNAs in BCCs, which, spearheaded by miR-199a, converge on and repress the expression of the transcription factor forkhead-box P2 (FOXP2). FOXP2 is a transcriptional repressor that has been primarily implicated in regulating speech and language development, as well as developmental neurogenesis, in humans (Fisher and Scharff, 2009; Tsui et al., 2013; Vernes et al., 2011). Aside from serving functions in the differentiation of tissues such as lung and esophagus (Shu et al., 2007), little is known regarding the roles of FOXP2 in nonneuronal contexts. We report here that miR-199a overexpression, or *FOXP2* silencing, endows BCCs with cancer-stem-cell (CSC)-like traits, enhances their tumor-initiating capabilities, and fosters their metastatic propensities. We describe miR-199a upregulation and *FOXP2* repression as prominent features of aggressive clinical breast cancers, and we found that they represent independent prognostic parameters for overall patient survival, indicative of their critical roles in breast tumor pathogenesis. Our work implicates a causal role for the speech gene *FOXP2* in breast cancer metastasis and elucidates elements of its tumor-stroma-initiated miRNA regulatory network.

RESULTS

MSC Priming Induces miR-199a-3p and miR-214 in BCCs

To characterize the miRNA alterations exhibited by MSC-primed BCCs, we cultured GFP-labeled MDA-MB-231 BCCs together with human bone-marrow-derived MSCs (BCC:MSC ratio of 1:3) for 3 days. GFP-BCCs were then isolated by fluorescence-activated cell sorting (FACS; $MDA\text{-}MB\text{-}231^{MSC}$), and their mature miRNAs were profiled by subtractive miRNA arrays (Agilent) using RNA derived from resting GFP-BCCs cultured alone as control (Figure 1A). These analyses revealed that only six

miRNAs were significantly enriched ($\geq$2-fold; adjusted $p < 0.05$) in MDA-MB-231^{MSC} when compared to control BCCs (Figure 1A): miR-199a-3p, miR-34a, miR-762, miR-214, miR-let-7b, and miR-1915. Semiquantitative real-time PCR (rtPCR-$\Delta\Delta$ct) was used to validate the microarray findings, and it confirmed a multifold induction of these miRNAs in MSC-activated BCCs (Figure 1B).

Among this set, miR-199a-3p particularly attracted our attention. Noticeably, miR-199a-3p levels exhibited the highest levels of enrichment in MDA-MB-231^{MSC} compared to the other MSC-triggered miRs, rising more than $\sim$65-fold over those of controls (Figure 1B). Interestingly, miR-199a-3p upregulation, while not previously functionally correlated with human breast carcinoma development, has been observed in other cancer contexts, such as esophageal (Feber et al., 2011), gastric (Brenner et al., 2011), or lung (Sakurai et al., 2011) carcinomas, suggesting a potential role for miR-199a-3p in breast cancer pathogenesis.

We observed that the MDA-MB-231^{MSC} population continued to produce high levels of miR-199a-3p even after separation from MSCs (Figure S1A available online), suggesting that its induction was sustained and intrinsic to BCC^{MSC}. In this regard, miR-199a-3p derives from two separate genomic regions in humans: one located on chromosome 19 and the other on chromosome 1, comprising the miR-199a1 (A1) and miR-199a2 (A2) loci, respectively. We proceeded to determine the relative contributions of A1 or A2 to the levels of mature miR-199a-3p present in MDA-MB-231^{MSC} by assessing the levels of the distinct locus-specific miRNA precursor stem-loops (pri-miRs) using rtPCR-$\Delta\Delta$ct. These experiments revealed that both pri-miR-199a1 and pri-miR-199a2 were significantly increased in MDA-MB-231^{MSC}, with a slightly elevated contribution of A1 ($\sim$60%) compared to A2 ($\sim$40%) (Figure 1C). Interestingly, miR-199a2 is transcribed from an intronic sequence of the *DNM3OS* gene, which also encodes for miR-214. miR-214 was similarly identified in our profiling arrays as induced by $\sim$15-fold in MDA-MB-231^{MSC} (Figure 1B) and likewise maintained elevated levels 3 days after separation from MSCs (Figure S1A). These observations suggest that miR-199a-3p (produced from the A1 and A2 loci) and miR-214 (produced form the A2 locus) are functionally coregulated in MDA-MB-231^{MSC}.

To probe whether the ability of MSCs to trigger miR-199a-3p and miR-214 upregulation in BCCs is idiosyncratic to the MDA-MB-231 model, we tested the response of other BCCs to MSCs. Indeed, admixture of MSCs to MCF7/Ras, T47D, and MDA-MB-435 cells caused significant upregulation of both miR-199a-3p and miR-214, albeit to different extents with differing ratios of miR-199a-3p/miR-214 (Figure 1D), likely a consequence of the relative contributions of A1 versus A2 loci in these systems.

Of note is that robust induction of miR-199a-3p and miR-214 by bone-marrow-derived MSCs required cell-cell contact between BCCs and MSCs (Figure S1B) and did not occur upon the contact of BCCs with phenotypically similar fibroblastic cells, such as WI-38 cells or panniculus-derived MSCs (ad-MSCs; Figure S1C). Interestingly, the contact of BCCs with human-breast-derived MSCs (Br-MSCs; derived from reduction mammoplasty) or activated fibroblasts (CAFs; derived from human breast tumors) resulted in only miR-199a-3p upregulation, and not that of miR-214 (Figure S1C). Together, these observations suggest that bone-marrow-derived MSCs may be uniquely capable of activating both A1 and A2 loci, while highlighting miR-199a-3p activation as a potential common element of the BCC response to activated stroma in multiple settings.

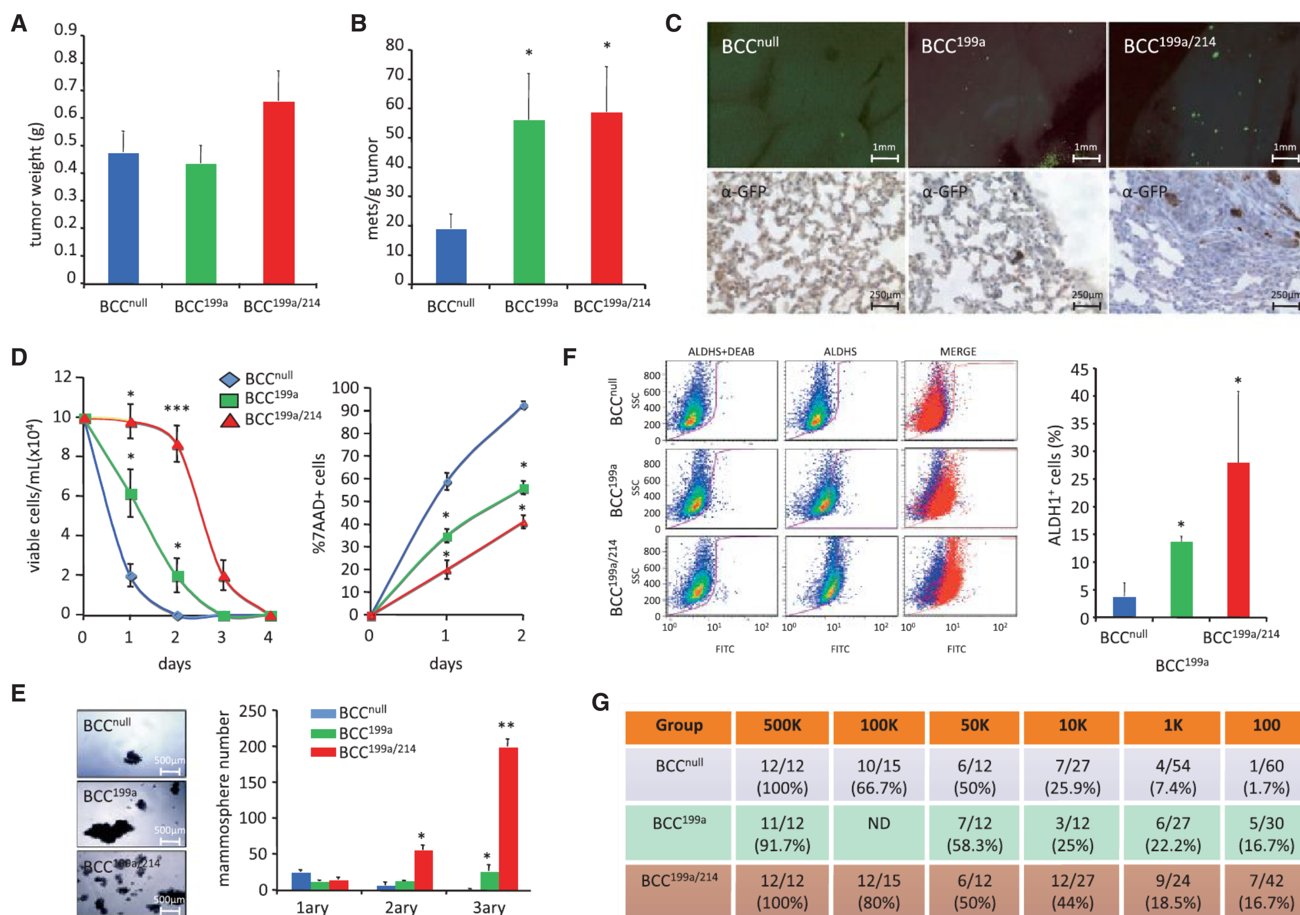
miR-199a and miR-214 Promote Metastasis and CSC Phenotypes

Because our miRNA profiling studies were conducted on whole BCC^{MSC} populations, we could not rule out the possibility that certain BCCs within the total population express both miR-199a-3p and miR-214 (i.e., where both loci, or the A2 locus alone, is active), while others express only miR-199a-3p (i.e., when A1 locus alone is active). Accordingly, we proceeded to model both possibilities, stably expressing exogenous miR-199a, or both miR-199a and miR-214, in MDA-MB-231 cells (BCC^{199a} and BCC$^{199a/214}$, respectively). The expression levels of the respective miRNAs in BCC^{199a} and BCC$^{199a/214}$, verified by rtPCR-$\Delta\Delta$ct and compared to control counterparts harboring an empty vector (BCC^{null}), showed >40-fold and >3-fold upregulation in miR-199a-3p and miR-214 levels, respectively (Figure S2A).

We first explored the malignant potential of BCC^{199a} and BCC$^{199a/214}$ by examining their tumorigenic and metastatic abilities compared to BCC^{null}. For this purpose, equal numbers of cells of each group were implanted subcutaneously into athymic Nude mice and allowed to form tumors for 10–14 weeks. While BCC^{199a} and BCC$^{199a/214}$ tumors did not differ from BCC^{null} tumors in average weight at the time of tissue harvest ($\sim$0.5–0.65 g; Figure 2A), enumeration of the GFP-positive BCC colonies in the lungs of the respective animals using fluorescence microscopy revealed that mice implanted with either BCC^{199a} or BCC$^{199a/214}$ had $\sim$3-fold the average of lung metastases per gram of tumor when compared with BCC^{null} controls (Figures 2B and 2C). These experiments demonstrated an enhanced malignancy of BCC^{199a} and BCC$^{199a/214}$ in vivo and prompted us to further characterize their metastasis-associated phenotypic attributes in vitro.

In these regards, BCC^{199a} and BCC$^{199a/214}$ exhibited no proliferative advantage over their control counterparts in 2D culture conditions (Figure S2B). Furthermore, these cells did not display increased expression of mesenchymal markers, such as vimentin, N-cadherin, smooth muscle actin (SMA), or lysyl oxidase (LOX) at the mRNA and/or protein levels, and they exhibited some resurgence in E-cadherin mRNA expression (Figures S2C and S2D). In addition, BCC^{199a} and BCC$^{199a/214}$ manifested an $\sim$50% reduced intrinsic motility compared to BCC^{null} in Boyden chamber motility assays (Figure S2E). These observations suggested that the increased metastasis observed in BCC^{199a} and BCC$^{199a/214}$ is manifested through pathways distinct from those governing proliferation, invasion, and motility.

Our group and others have previously demonstrated that MSC activation of BCCs increases the population of putative CSCs as demonstrated by multifold upregulation in ALDH1 positivity and mammosphere-forming capacities (El-Haibi et al., 2012; Liu et al., 2011). CSCs are characterized by their distinctive capacity for tumor initiation, a trait that is thought to be integral to metastatic colonization because disseminated cancer cells engender new growths at distant sites (Malanchi et al., 2012). Supporting



Group	500K	100K	50K	10K	1K	100
BCCnull	12/12 (100%)	10/15 (66.7%)	6/12 (50%)	7/27 (25.9%)	4/54 (7.4%)	1/60 (1.7%)
BCC199a	11/12 (91.7%)	ND	7/12 (58.3%)	3/12 (25%)	6/27 (22.2%)	5/30 (16.7%)
BCC199a/214	12/12 (100%)	12/15 (80%)	6/12 (50%)	12/27 (44%)	9/24 (18.5%)	7/42 (16.7%)

Figure 2. miR-199a and miR-214 Expression Causes Propagation of CSC Traits and Metastasis

(A) Mean weights (gram) ± SEM of matched tumors isolated from mice 8–14 weeks after implantation of BCC^{null} (n = 77), BCC^{199a} (n = 53), and $BCC^{199a/214}$ (n = 81).

(B) Metastatic index. Mean number of GFP-positive lung metastases ± SEM per gram of primary tumor burden per mouse. BCC^{null} (n = 37), BCC^{199a} (n = 27), and $BCC^{199a/214}$ (n = 37).

(C) Representative images of lung GFP-positive colonies in mice in (B). Upper panels: fluorescence microscopy; lower panels: anti-GFP immunohistochemistry.

(D) Minimal serum tumbling assay. Left: mean numbers of viable cells ± SEM, determined by trypan exclusion (n = 3). Right: percentage of apoptotic cells from left assessed by 7AAD staining/FACS analysis (n = 3).

(E) Sphere formation assay. Representative mean number of spheres in primary (1ary), secondary (2ary), and tertiary (3ary) passaged cultures ± SEM (n = 3).

(F) Left: representative ALDEFLUOR analyses of the indicated cells. DEAB, a specific inhibitor of ALDH1, was used as a control. Right: quantification of ALDEFLUOR assays (± SEM; n = 3).

(G) Tumor-initiation assay. Table indicating number of tumors initiated (>0.05 g) and total number of BCC injections per cell dilution (#cells/injection) is shown. p values calculated using ELDA (see Experimental Procedures) for BCC^{199a} and $BCC^{199a/214}$, respectively, are: 500K cell group: 0.232, 1; 100K cell group: 0.407; 50K cell group: 1,1; 10K cell group: 0.004, 0.152; 1K cell group: 0.064, 0.046; 100 cell group: 0.009, 0.005. *p < 0.05; **p < 0.01, ***p < 0.001 in two-tailed Student's t test.

See also Figure S2.

this notion, we observed that highly metastatic BCC^{MSC} exhibited increased tumorigenic properties in limiting dilution tumor-initiation analyses, forming subcutaneous tumors in Nude mice ~2.5 times more frequently than in controls, with as little as 100 cells per injection (Figure S2F).

With this information, we proceeded to determine whether the increased metastasis of BCC^{199a} and $BCC^{199a/214}$ correlated with their acquisition of CSC characteristics. Interestingly, BCC^{199a} and $BCC^{199a/214}$ displayed enhanced resistance to suspension-induced cell death in a minimal serum tumbling assay, exhibiting >50% survival rates after 24 hr of suspension, and a corresponding >50% reduction in their apoptosis rates as measured by 7-aminoactinomycin D (7AAD; Figure 2D). In addition, BCC^{199a} and $BCC^{199a/214}$ displayed increased abilities to grow in low-attachment mammosphere growth conditions after serial passages (Figure 2E), and they showed multifold increases in the expression levels of the CSC-associated marker ALDH1 (Ginestier et al., 2007), as determined by rtPCR (Figure S2G) and by ALDEFLUOR-based FACS assays (Figures 2F). Most importantly, however, BCC^{199a} and $BCC^{199a/214}$ possessed markedly enhanced tumor-initiating capabilities in limiting-dilution tumor assays in Nude mice, forming tumors at 100 cells per injection at ~2–3 times the rate of their BCC^{null} controls (Figure 2G). These observations suggested that the enhanced

metastasis of BCC^{199a} and $BCC^{199a/214}$ correlated with their acquisition of CSC-like traits.

We found that the enhanced metastasis-related phenotypes of BCC^{199a} were largely similar or identical to those of $BCC^{199a/214}$, suggesting that the promalignant activities of $BCC^{199a/214}$ rested largely on the actions of miR-199a. To distinguish between the effects of mature miR-199a-3p and miR-199a-5p, both of which are produced by stable expression of pre-miR-199a from our vector, we transfected individual RNA duplexes—offset to allow generation of only a specific single mature miRNA—coding for either *199a-3p* or *199a-5p* into MDA-MB-231 cells. While transient expression of *199a-3p* led to a significant ~2.5-fold increase in ALDH1 positivity, *199a-5p* expression did not (Figure S2H), despite substantial expression levels of *199a-5p* in these cells (Figure S2I). This suggested that miR-199a-3p is the critical miRNA produced from miR-199a in enhancing the observed population of putative CSCs.

In these regards, BCC^{199a} exhibited marked increases in the expression levels of additional breast CSC-associated markers, such as MYC (Figure S2J), GD2S, and POSTN (Figure S2K; (Liu et al., 2009; Malanchi et al., 2012; Battula et al., 2012; Nair et al., 2014). Furthermore, stable overexpression of miR-199a in other BCC lines, such as MCF7/Ras, T47D, or MDA-MB-435 cells, led to ~10-, ~6-, and ~4-fold increases, respectively, in their ALDH1 positivity (Figure S2L). Similarly, such expression caused upregulation of POSTN (Figure S2M) and the $CD44^{high}/CD24^{low}$ population (Figure S2N) in T47D cells and increased MYC expression in MCF7/Ras cells (Figure S2J), suggesting that the ability of miR-199a-3p to regulate CSC phenotypes is not idiosyncratic of MDA-MB-231 cells. Notably, >65% of mice tail-vein-injected with BCC^{199a} exhibited lung metastases at limiting conditions where BCC^{null} controls exhibited none (Figure S2O), suggesting that the induction of CSC traits by miR-199a contributes to secondary tissue colonization.

Downregulation of the Speech Gene *FOXP2*, Observed in BCC^{199a} and $BCC^{199a/214}$, Promotes CSC Traits and Metastasis

We next aimed to elucidate the mechanistic details underlying the malignancy of BCC^{199a} and $BCC^{199a/214}$. For this purpose, we probed BCC^{199a} and $BCC^{199a/214}$ for the expression levels of >20 published targets for miR-199a-3p (or miR-214), but we did not find consistent downregulation of such targets in both BCC^{199a} and $BCC^{199a/214}$ as compared to BCC^{null} (Figures S3A and S3B; see Supplemental Information), underscoring the importance of cellular context in determining miRNA functions.

To identify potential miRNA effectors in BCC^{199a} and $BCC^{199a/214}$, we proceeded to utilize array-based approaches, focusing upon those that have been correlated with the acquisition or maintenance of stem-like properties. In these regards, we carried out a targeted rtPCR-ΔΔct array screen of 84 genes associated with stem cell maintenance or differentiation (QIAGEN), evaluating their relative mRNA levels in BCC^{199a} and $BCC^{199a/214}$ as compared to BCC^{null}. These analyses indeed identified a number of stem-cell-associated genes that were significantly upregulated in BCC^{199a} and/or $BCC^{199a/214}$, including GATA-binding protein-6/*GATA6* (Zhang et al., 2008), lin-28 homolog B/Lin28B (Zhou et al., 2013), homeobox C9/ *HOXC9* (Okamoto et al., 2007), and msh homeobox 2/*MSX2* (Dorado et al., 2011). Surprisingly, only forkhead box P2/ *FOXP2* was found to be significantly (≥2-fold; $p < 0.005$) downregulated in both cell types, exhibiting 7- and 13-fold reductions in its expression levels in BCC^{199a} and $BCC^{199a/214}$, respectively (Figure S3B).

FOXP2 is a member of the forkhead family of transcription factors (Myatt and Lam, 2007). It has been described to act as a transcriptional repressor, primarily in the context of neural development and function (Spiteri et al., 2007). Its functions have been shown to be essential for developmental neurogenesis (Tsui et al., 2013), for neuronal plasticity, and for the capacity for human speech (Fisher and Scharff, 2009). FOXP2 has likewise been shown to serve an essential role in the development and differentiation of nonneuronal tissues, such as lung and esophagus (Shu et al., 2007; Shu et al., 2001). However, a causal role for FOXP2 deregulation in breast cancer pathogenesis has not been established. We validated the array results regarding *FOXP2* using independent rtPCR-ΔΔct analyses (Figure 3A), and we further found that its protein levels were severely repressed in both BCC^{199a} and $BCC^{199a/214}$ (Figure 3B), as well as in BCC^{MSC} (Figure 3C). Because loss of FOXP2 was shown to preserve progenitor cell identity and block differentiation in the abovementioned contexts, we hypothesized that FOXP2 downregulation might play a role in defining the CSC-like phenotypes of BCC^{199a}, $BCC^{199a/214}$, and BCC^{MSC}.

We tested this possibility first by probing for *FOXP2* levels in the FACS-enriched ALDH1-positive fractions of MDA-MB-231^{MSC}. Indeed, ALDH1-positive cells, which possess tumor-initiating capacities (forming 60% tumors at 10,000 cells per injection compared to ALDH1-negative counterparts; Figure S3C), exhibited a striking >95% reduction in their *FOXP2* content (Figure 3D). Furthermore, expression of miR-199a in T47D cells, which enhanced their CSC-like characteristics (Figures S2L–S2N), also prompted a marked downregulation of FOXP2 (Figure 3E). These findings correlated FOXP2 downregulation with the propagation of CSC-like traits.

To determine the functional consequences of FOXP2 inhibition on the induction of CSC-like phenotypes and breast cancer progression, eight different retroviral plasmids expressing various short-hairpin RNAs designed for knockdown of *FOXP2* (shFOXP2) were stably expressed in MDA-MB-231 cells ($BCC^{shFOXP2}$). The functionality of each hairpin was verified by western blot analyses, and this revealed two efficient hairpins, shFOXP2-1.5 and shFOXP2-2.2, that precipitated an ~70% reduction of FOXP2 protein levels (Figure 4A). When probed with ALDEFLUOR, $BCC^{shFOXP2-1.5}$ and $BCC^{shFOXP2-2.2}$ exhibited significant (>40- and >10-fold, respectively) upregulation in their ALDH1 positivity compared to control cells expressing a scrambled hairpin control ($BCC^{shSCRAM}$; Figures 4B and S4A). In addition to increases in their ALDH1 positivity, shFOXP2-expressing cells also exhibited increases in *OCT4* (Figure S4B) and c-Myc (Figures 4C and S4C) and a 2-fold increase in their $CD44^{high}/CD24^{low}$ populations (Figure S4D). Furthermore, shFOXP2 expression enhanced mammosphere colony formation in suspension by an average of ~2.5-fold (Figure 4D) and provided BCCs with enhanced abilities to resist anoikis (Figure S4E). Most importantly, $BCC^{shFOXP2-2.2}$ initiated subcutaneous tumors in Nude mice at a frequency of 28% and with as little as 100 cells per injection, a dilution prohibitive for $BCC^{shSCRAM}$ (Figure 4E).

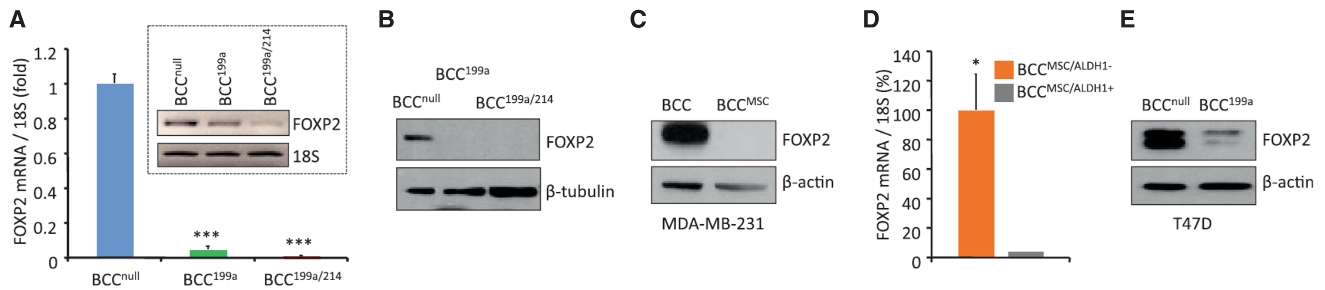


Figure 3. A Targeted PCR Screen Identified FOXP2 as a Putative Target in BCC^{199a} and BCC$^{199a/214}$

(A) Representative rtPCR-ΔΔct probing *FOXP2* in the indicated cell lines (± SEM; n = 3). Inset: DNA gel from rtPCR-ΔΔct.

(B) Western blot probing for FOXP2 in whole lysates as indicated. β-tubulin was used as a loading control (n = 3).

(C) Western blot for FOXP2 in whole-cell lysates of resting or MDA-MB-231^{MSC} cells. β-actin was used as a loading control (n = 3).

(D) rtPCR-ΔΔct analysis showing relative abundance of *FOXP2* mRNA in FACS-fractionated ALDH1-positive versus ALDH1-negative MDA-MB-231^{MSC} cells (± SEM; n = 3).

(E) FOXP2 western blot in the indicated whole T47D lysates (n = 2). β-actin was used as a loading control.

*$p < 0.05$; ***$p < 0.001$ in two-tailed Student's t test. See also Figure S3.

These results indicated that FOXP2 inhibition produces cells with in vitro and in vivo phenotypes consistent with those of CSCs.

Mirroring the phenotypes of BCC$^{199a/214}$, BCC^{shFOXP2} did not exhibit enhanced proliferation in vitro (Figure S4F) or enhanced tumor growth in vivo (Figure 4F). However, mice bearing BCC^{shFOXP2} tumors exhibited a dramatic ~9-fold increase in the numbers of metastatic lung foci per gram of primary tumor as compared to controls bearing BCC^{shSCRAM} tumors (Figures 4G and 4H). Altogether, these results demonstrate that FOXP2 inhibition is sufficient to promote the propagation of CSC-like phenotypes in BCCs, paralleling an observed marked enhancement of metastasis.

FOXP2 Downregulation and miR-199a-3p Upregulation Are Prominent Features of Aggressive Clinical Breast Cancer

We sought to determine the clinical relevance of our findings, first concentrating on whether *FOXP2* downregulation represents a feature of clinical breast cancer. For this purpose, we mined the recently published breast cancer sequencing data made available by the Cancer Genome Atlas Network (2012), and we found *FOXP2* to be downregulated ~4-fold in primary breast tumors as compared to normal tissues (Figure S5A). Importantly, we found that *FOXP2* levels were repressed ~2-fold in invasive ductal breast carcinomas (IDCs) as compared to benign lesions (Figure S5B; Chen et al., 2010). To explore whether *FOXP2* depression is associated with particular breast cancer subtypes, we interrogated publicly available luminal A, luminal B, HER2-enriched, and basal-like breast tumor expression data sets (Pawitan et al., 2005; Dedeurwaerder et al., 2011; Sabatier et al., 2011). These analyses revealed *FOXP2* downregulation as a common feature of all these subtypes (Figures 5A, S5C, and S5D) and were mirrored by our own rtPCR-ΔΔct analysis on RNA derived from macrodissected breast cancers, in which we observed a striking ~80% *FOXP2* repression across tumor samples (n = 74) when compared to RNA derived from normal tissues (n = 5; Figure 5B). Importantly, we found that low FOXP2 expression inversely correlated with overall disease-free survival (Figure 5C), as well as distant metastasis-free survival (Figure 5D) in independent cohorts, suggesting that *FOXP2* repression is indicative of increased malignancy in clinical breast cancer.

Similarly, we found miR-199a-3p levels to be elevated in IDC samples compared to in situ ductal cancers (Figure S5E; Farazi et al., 2011; Volinia et al., 2012). Within IDC, miR-199a-3p levels correlated with disease progression and associated significantly with lymph node positivity (N1 or N2) in a study of >600 breast cancer patients (Figure 5E; Cancer Genome Atlas Network, 2012). Most interestingly, miR-199a-3p levels were significantly elevated in primary tumors of patients who exhibited relapse (Figure 5F) and also correlated with decreased patient survival over 5- and 10-year intervals (Figures 5G). Collectively, these observations suggested that deregulation of FOXP2 and miR-199a-3p are common features of breast cancer progression and highlight important prognostic values for these players in breast cancer pathogenesis.

FOXP2 Is a Common Target for a Converging and Interrelated Set of MSC-Regulated miRNAs

We aimed to gain molecular insight into the regulation of *FOXP2* by MSC-induced miR-199a-3p. We analyzed the proximal (~1 kb) *FOXP2* 3′ UTR for consensus miRNA seed sites using in silico miRNA target prediction algorithms, such as RNAhybrid, miRWalk, Targetscan, and Pictar, but we were unable to find strong consensus seed sites for miR-199a-3p with the predicted free energy cutoff of ≤ -25 kcal/mol. However, these approaches did reveal putative target sites for each of the other miRNAs induced in BCC^{MSC}, namely miR-762, miR-let-7b, miR-34a, and miR-1915 (Figures S6A and S6B). For this reason, we tested the capability of these particular miRNAs in repressing *FOXP2* mRNA expression. Indeed, stable expression of miR-762 or miR-1915, or transient expression of miRNA mimics for miR-let-7b or miR-34a (Figure S6C), brought about a significant reduction in FOXP2 levels in MDA-MB-231 cells (Figures 6A and 6B). Consistent with FOXP2 downregulation, these cells exhibited an increase in ALDH1 positivity as determined by ALDEFLUOR assays, displaying ~20-, ~75-, ~30-, and ~30-fold increases by miR-let-7b, miR-34a, miR-762, and miR-1915, respectively (Figure 6C).

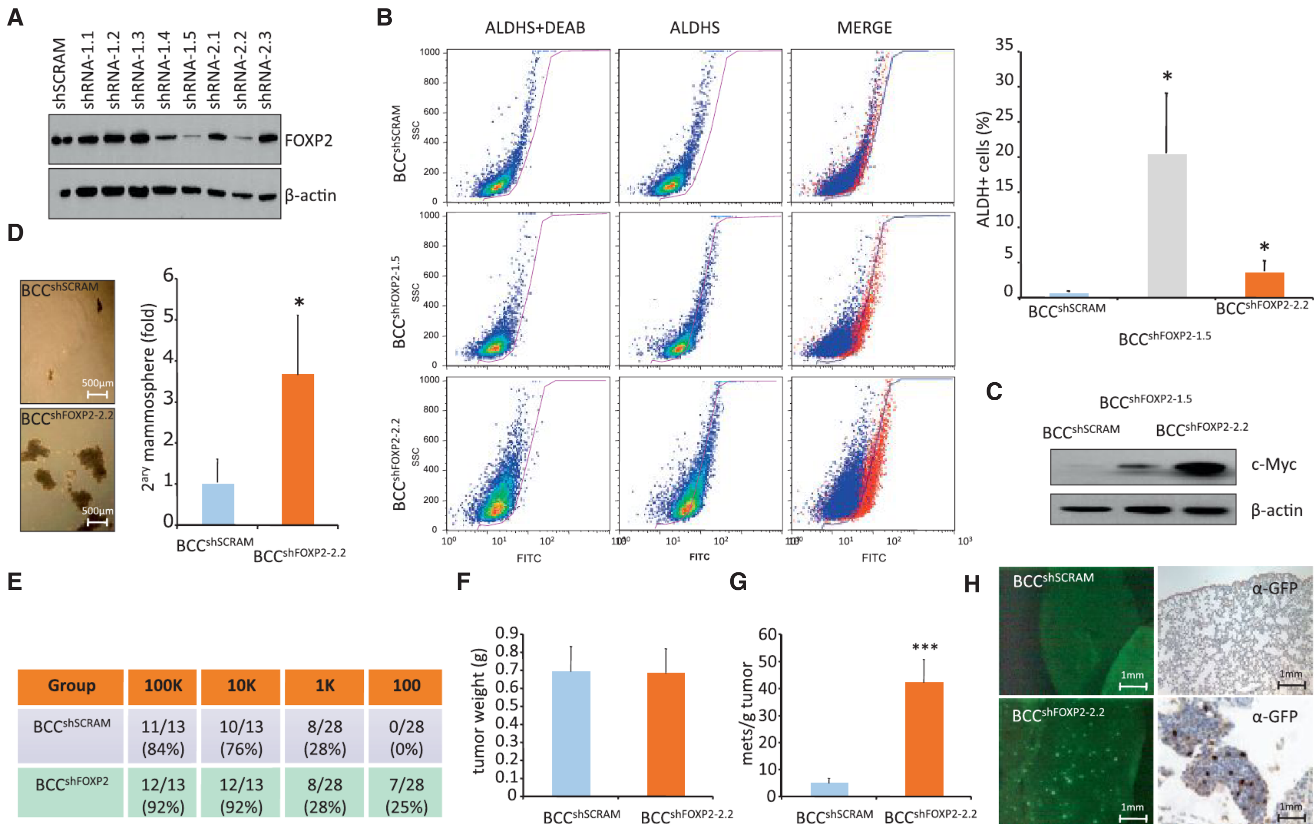


Figure 4. FOXP2 Downregulation Drives CSC Phenotypes and Metastasis

(A) FOXP2 western blot after knockdown by the indicated shRNAs.

(B) Representative ALDEFLUOR analyses of the indicated cell lines (n = 4). DEAB was used as a control.

(C) Representative western blot for c-Myc in whole-cell lysates of the indicated MDA-MB-231 cells. β-actin was used as a loading control (n = 3).

(D) Sphere formation assay. Representative images and quantification of secondary spheres (n = 3).

(E) Tumor-initiation assay. Table indicates the number of tumors initiated (>0.05 g) and the total number of BCC injections for each cell dilution (#cells/injection). Respective p values for $BCC^{shSCRAM}$ and $BCC^{shFOXP2}$ calculated by ELDA were as follows: 100K cells group: 0.536; 10K cells group: 1; 1K cells group: 0.736; 100 cells group: 0.0375.

(F) Mean weight (grams) ± SEM of matched subcutaneous primary tumors derived from Nude mice after 8–14 weeks of $BCC^{shSCRAM}$ (n = 30) or $BCC^{shFOXP2}$ (n = 46) injections.

(G) Metastatic index. Mean numbers of GFP-positive lung metastases ± SEM per gram of primary tumor burden per mouse are shown; n = 16 for $BCC^{shSCRAM}$ and n = 25 for $BCC^{shFOXP2-2.2}$.

(H) Representative images of GFP-positive colonies and anti-GFP IHC in the lungs of $BCC^{shSCRAM}$ or $BCC^{shFOXP2-2.2}$ mice in (G).

*p < 0.05; ***p < 0.001 in two-tailed Student's t test. See also Figure S4.

Based on these results and the fact that miR-199a-3p was not predicted to target the proximal 3′ UTR of *FOXP2*, we asked if miR-199a might repress *FOXP2* through the actions of these four miRNAs. Indeed, we found that the expression levels of miR-let-7b, miR-34a, miR-762, and miR-1915 were all elevated ~7-, ~4.5-, ~5-, and ~4.5-fold, respectively, in $BCC^{199a/214}$ (Figure 6D), suggesting a coregulatory relationship gathering these miRs with miR-199a-3p. In support of this notion, and using targeted qPCR assays, we found that the expression levels of miR-199a-3p in clinical breast cancer specimens correlated with the expression levels of miR-let-7b, miR-34a, and miR-1915 (Figure 6E; we were unable to test miR-762 because the sensitivity of the assay necessitated the use of prohibitively large amounts of primary RNA material). These findings are consistent with the existence of an operational crosstalk between MSC-induced miRNAs in BCCs and highlight one mechanism through which miR-199a-3p represses FOXP2 expression. Of translational importance, high concerted expression levels of miR-199a-3p, miR-let-7b, miR-34a, and miR-1915 were indicative of overall poorer survival in breast cancer patients as assessed by Kaplan-Meier analyses (Figure 6F) and Cox multivariate analysis (Figure S6D), suggesting that the described miR network represents a powerful and significant prognostic indicator in clinical breast cancer. These results reveal functional cooperation between members of an interrelated regulatory network of miRNAs, led by miR-199a, which converge to inhibit the expression of *FOXP2* and thereby promote tumor initiation and metastasis (Figure 6G).

DISCUSSION

By probing for BCC miRNAs deregulated by MSC stimulation, we identified a set of interrelated miRs whose actions converge

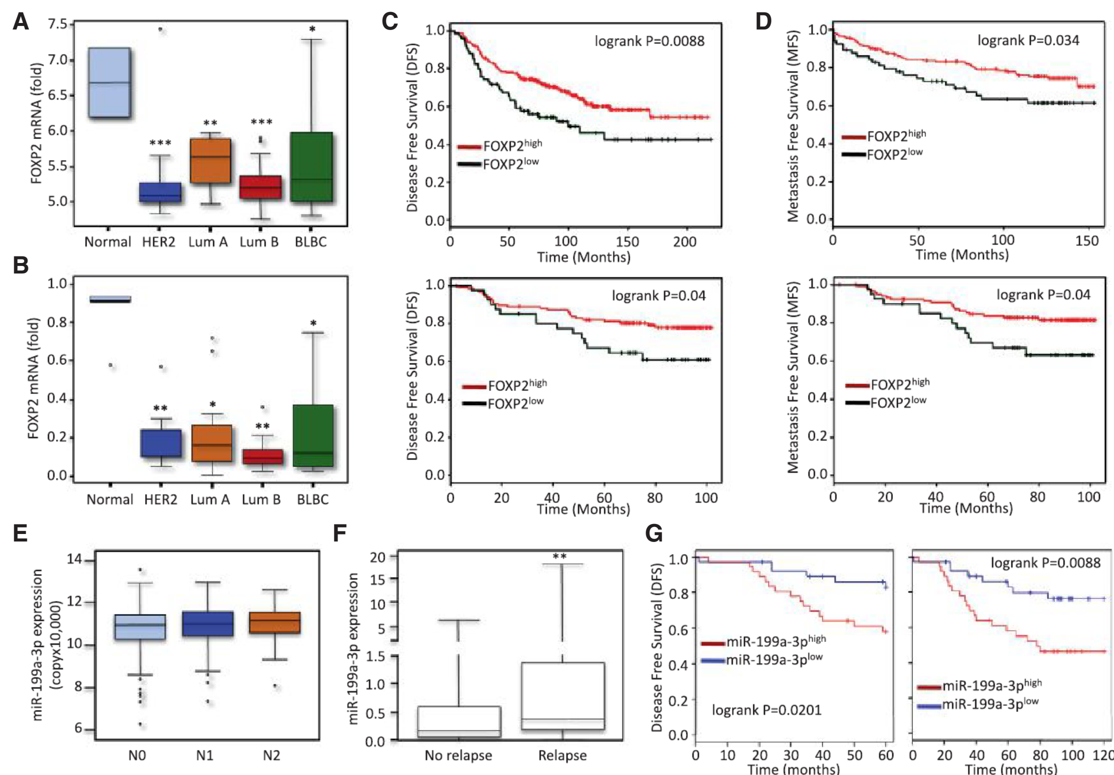


Figure 5. Upregulation of miR-199a and Downregulation of FOXP2 in Clinical Breast Cancer

(A) *FOXP2* levels (Log2) in normal versus the indicated breast cancer subtypes in GSE20711. p values for normal (n = 2) versus HER2 (n = 26), Lum A (n = 13), Lum B (n = 22), and BLBC (n = 27) were 0.0005325, 0.001527, 4.573 × e^{-6}, and 0.03824, respectively. Significance was determined using unpaired Student's t test.

(B) *FOXP2* rtPCR-ΔΔct on macrodissected breast cancer specimens derived from HER2 (n = 12), Lum A (n = 25), Lum B (n = 25), and BLBC (n = 12) subgroups versus normal (n = 5). Significance was determined using unpaired Student's t test.

(C) Upper: disease-free survival of high- and low-*FOXP2* expression groups from van de Vijver (2002) NKI platform (n = 221 and 74, respectively). Reporter Contig used was 35884_RC, HR = 1.7(1.1 – 2.6), and Chi-square p = 0.016. Lower: disease-free survival analyses performed on high- and low-*FOXP2* expression groups in Pawitan et al. (2005) platform HG-U133B (n = 119 and 40, respectively). Reporter used was 243278_at, HR = 1.9(1.0 – 3.7), and Chi-square p = 0.041.

(D) Upper: metastasis-free survival analyses from Miller (2005) using platform HG_U133B (n = 195 and 65, respectively). Reporter used was 235201_at, HR = 1.7(1.0 – 2.8), and Chi-square p = 0.046. Lower: disease-free survival analyses performed on high- and low-*FOXP2* expression groups in Pawitan et al. (2005), platform HG-U133B (n = 119 and 40, respectively). Reporter used was 243278_at, HR = 2.2(1.1 – 4.2), and Chi-square p = 0.025.

(E) miR-199a-3p expression in IDC patients (n = 664; Cancer Genome Atlas Network, 2012) with or without lymph node positivity. N0 geometric mean RPM is 1780.7 (n = 301), N1 geometric mean is 1951.8 (n = 223), and N2 geometric mean is 2141.3 (n = 82). Spearman correlation test N0 < N1 < N2, p = 0.013.

(F) Normalized miR-199a-3p levels (rtPCR-ΔΔct) on primary-tumor-derived RNA in relapse-free breast cancer patients (n = 34) versus relapsed patients (n = 40) in Cimino et al. (2013). Mann-Whitney p = 0.029 and Wilcoxon p = 0.0193.

(G) miRNA-199a-3p median fold change (FC) stratified the independent populations in Cimino et al. (2013) into two groups, which were significantly different in their survival probability (n = 73).

See also Figure S5.

to downregulate the developmental transcription factor FOXP2. We demonstrated that expression of these miRs or knockdown of FOXP2 was sufficient to increase breast CSC-like traits, fostering increased tumor-initiating abilities and enhancing tumor metastasis. These results incriminate miRNA-regulated pathways in breast CSC propagation and metastasis and describe an involvement of the speech-associated transcription factor FOXP2 in regulating breast cancer malignancy, thereby providing mechanistic insights into breast cancer pathogenesis.

A series of studies have described the enrichment of A1 and A2 loci miRNAs in cancer tissues. Indeed, miR-199a expression has been found to be elevated in a number of solid malignancies, such as lung cancer (Mascaux et al., 2009), colorectal cancer (Wan et al., 2013), ovarian cancer (Iorio et al., 2007), and melanoma (Pencheva et al., 2012). Similarly, upregulated levels of miR-214 have been reported in ovarian (Yang et al., 2008), pancreatic (Zhang et al., 2010), and oral (Scapoli et al., 2010) cancers. In breast, two reports have described increased levels of miR-199a-3p in malignant myoepithelioma of the breast (Bockmeyer et al., 2011) and elevated miR-214 levels in the blood of patients diagnosed with malignant breast tumors (Schwarzenbach et al., 2012). Despite these intriguing studies, the functional contributions of miR-199a and miR-214 to breast cancer progression were unknown. Recently, miR-199a has been shown to target apolipoprotein E (ApoE) and the heatshock factor DNAJA4 in the context of melanoma, which was shown to relieve the inhibitory influence of ApoE on endothelial

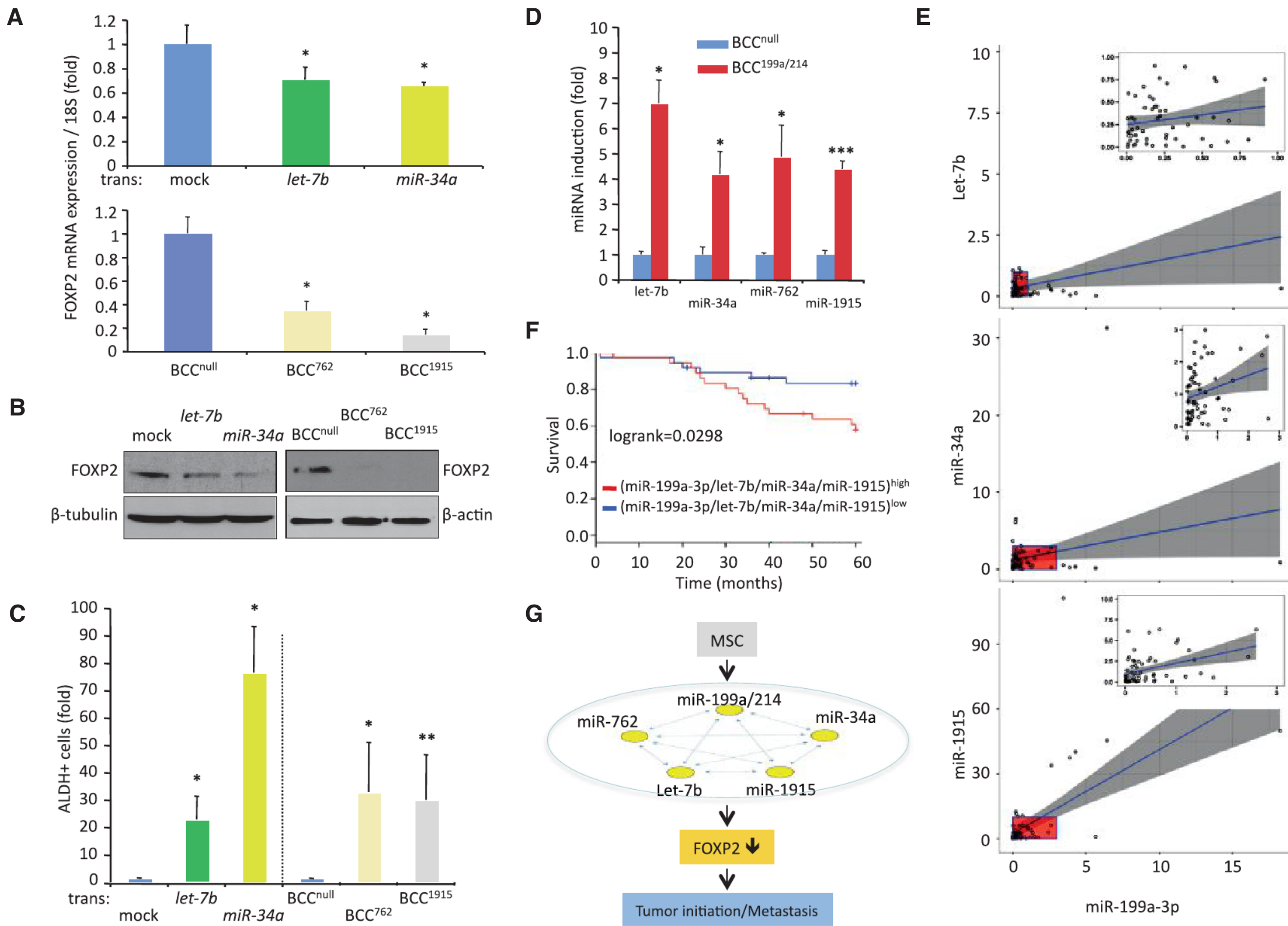


Figure 6. MSC-Induced miRNAs Converge on FOXP2

(A) Upper: rtPCR-ΔΔct for FOXP2 mRNA levels in MDA-MB-231 cells transiently transfected with miR-let-7b or miR-34a mimics compared to mock. (± SEM; n = 3). Lower: rtPCR-ΔΔct of *FOXP2* in MDA-MB-231 cells stably expressing miR-762 (BCC^{762}), miR-1915 (BCC^{1915}), or controls (BCC^{null}) (± SEM; n = 3).

(B) Western blots showing FOXP2 levels in MDA-MB-231 whole lysates of BCC^{null}, BCC^{762}, or BCC^{1915} (left) or in BCCs transiently transfected with miR-let-7b or miR-34a mimics (right) (n = 3).

(C) Relative fold ALDH1-positive cells compared to controls in the indicated groups in (B) (± SEM; n = 3).

(D) rtPCR-ΔΔct for the indicated miR in BCC^{null} and $BCC^{199a/214}$ (± SEM; n = 3).

(E) Pearson coefficient of miRNA fold changes shows significant correlations between expression levels of miR-199a-3p and miR-1915, miR-let-7b, and miR-34a, as determined by rtPCR-ΔΔct on clinical samples from Cimino et al. (2013).

(F) Median fold change (FC) of the combined expression levels of miR-199a-3p, miR-34a, miR-let-7b, and miR-1915 stratify the population in Cimino et al. (2013) into two groups with different 5 year survival probability (n = 73).

(G) Model: MSC stimulation of BCCs induces a set of miRNAs, led my miR-199a/214, which converge on FOXP2. The downregulation of FOXP2 is sufficient to promote tumor initiation and metastasis.

*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ in two-tailed Student's t test. See also Figure S6.

recruitment, thereby affording cancer colonies with enhanced vascularization (Pencheva et al., 2012). While we cannot rule out similar paracrine actions of miR-199a/214 in our models, we did not find *ApoE* and *DNAJA4* to be downregulated in MDA-MB-231 stably expressing these miRNAs, underscoring the importance of cell context in miRNA targeting of complementary mRNAs. Here, we have elucidated an ostensibly autocrine mechanism of action for A1 and A2 loci miRNAs in breast carcinoma pathogenesis.

To determine how BCC^{199a} and $BCC^{199a/214}$ acquire their malignant phenotypes, we tested the expression levels of a large subset of published targets for miR-199a, but we were unable to verify consistent target downregulation across BCC^{199a}, $BCC^{199a/214}$, and BCC^{MSC}. Influenced by the observations that BCC^{199a}, $BCC^{199a/214}$, and BCC^{MSC} acquired CSC-like traits, we conducted stem-cell-factor-focused screens and identified FOXP2 as a factor that was significantly and consistently repressed in all three conditions.

FOXP2 is a transcription factor that has been tightly linked to nervous system development, encompassing activities that range from neuronal maturation to axonal guidance and speech regulation (Fisher and Scharff, 2009; Tsui et al., 2013; Vernes et al., 2011). On the molecular level, FOXP2 functions in transcriptional repressor complexes, which downregulate the

expression of a multitude of targets involved in lineage determination (Shu et al., 2001; Li et al., 2004; Shu et al., 2007; Konopka et al., 2009). However, to our knowledge, functions for FOXP2 in breast cancer development have not been previously reported. We found FOXP2 downregulation to be sufficient for enhanced tumor-initiating and metastatic abilities of cancer cells in animals, and we further observed *FOXP2* to be significantly downregulated across multiple clinical subtypes of breast cancers. Particularly, we observed that HER2-enriched tumors exhibited a reproducible and statistically significant 4-fold downregulation of *FOXP2* (and ~2.5-fold increase in miR-199a-3p) when compared to the BLBC subtype (Figure S5F), suggestive of a preferential enrichment for this pathway in HER2 breast cancer. Interestingly, HER2 tumors displayed increased SMA-positive fibroblastic cell infiltrates (consistent with CAF/MSC-like cells) when compared to BLBC (Toullec et al., 2010; data not shown), which draws a strong association between CAF/MSC tumor content and a depression of *FOXP2* expression in clinical breast cancer. Furthermore, we found that expression of a UTR-free *FOXP2* cDNA in BCCs in the context of miR-199a overexpression or in the background of MSC stimulation significantly inhibited both lung colonization (Figure S4G) and ALDH1 positivity (Figure S4H). Together, these observations strongly suggest that FOXP2 plays a critical role in breast cancer pathogenesis. It remains unclear as to whether FOXP2 exerts these activities via transcriptional repression and whether its downregulation in clinical breast cancer is an event that occurs early or late in tumor development. Because of its involvement in tumor initiation and metastasis, we hypothesize that FOXP2 may serve dual roles, both in tumorigenesis and in tumor progression to metastasis. Efforts to decipher the molecular mode of action of FOXP2 in breast cancer initiation, maintenance, and progression are currently underway.

The present work also highlights pathways utilized by tumor-associated MSCs to foster the malignancy of BCCs. Previous work from our group and others has shown that the contact between MSCs and BCCs resulted in gene expression changes in both cell types, favoring the development of a microenvironment that is conducive to metastatic progression. Indeed, BCC-activated MSCs primarily produce the chemokine CCL5, which acts back on the neighboring BCCs in a paracrine fashion and through CCR5, fostering their invasive migration and increased lung colonization (Karnoub et al., 2007; Chaturvedi et al., 2013). Similarly, we previously reported that MSC-activated BCCs are most enriched in EMT markers and phenotypes, mechanisms predominantly mediated by LOX via a CD44-Twist signaling axis (El-Haibi et al., 2012). Importantly, we observed that neither CCL5 (Figures S1D and S1E) nor LOX (Figure S1F) was sufficient in triggering CSC-associated miR-199a-3p/miR-214 expression, consistent with our previous results that CCL5 and LOX do not foster CSC-like traits in cancer cells (data not shown; El-Haibi et al., 2012). Intriguingly, and despite these observations, we found that TWIST1 expression in the cancer cells was sufficient to promote miR-199a-3p and miR-214 expression (Figure S1G) as well as FOXP2 repression (Figure S1H) and that it was critically required for the induction of the A1/A2 loci by MSCs (Figure S1I). These results are indicative of a complex crosstalk operating between MSC-driven EMT and CSC machineries and are suggestive of independent outside-in signaling axes regulating miR-199a in multiple BCC^{MSC} (e.g., Figure S1J).

Metastatic progression requires cancer cells to overcome dissimilar obstacles related to loss of adhesion and local invasion, intra/extravasation, and reacquisition of adhesion and proliferative capacities for colonization of inhospitable secondary tissues. It is plausible to reason that negotiating these sequential steps would require reversible shifts in gene expression programs. For this reason, studying the steady state stable transcriptional profiles of metastatic nodules may not provide a comprehensive understanding of the otherwise obligatorily plastic pathways that contribute to the establishment of secondary cancer colonies. In these regards, the MSC-induced model of tumor metastasis, which has gained increased attention over the past few years, possesses distinct advantages that enable the discovery of temporal, stroma-instigated pathways that permit cancer cells to execute the multiple steps of the metastasis cascade.

EXPERIMENTAL PROCEDURES

Detailed procedures can be found in the Supplemental Information.

Cells

BCCs were cultured using standard protocols described elsewhere (Karnoub et al., 2007; El-Haibi et al., 2012). Primary BCCs DT22 and DT28 are described elsewhere (Drews-Elger et al., 2014). Primary human bone marrow MSCs (BM-MSCs), primary ad-MSCs, Br-MSCs, and WI-38 human embryonic lung fibroblasts were propagated as previously described (El-Haibi et al., 2012; Hanson et al., 2013) and utilized before passage 5. CAFs were described previously (Hu et al., 2008).

Cocultures and Sorting

For direct cocultures, MSCs, CAFs, or WI-38 cells were cocultured with GFP-BCCs (at 3:1 ratio) for 72 hr (e.g., El-Haibi et al., 2012). All cells were cultured individually in parallel as controls. GFP-BCCs were recovered by FACS and processed as described below. For indirect cocultures, MSCs and BCCs were grown for 72 hr across 0.4 μm membrane in a Boyden chamber setup, and BCCs were collected and processed for RT-qPCR determinations as described below.

Agilent Arrays

An Agilent oligonucleotide microarray system (miRNA AMADID 025987, Agilent Technologies) was used to detect miRNA gene variation in MDA-MB-231 stimulated with BM-MSCs as compared to controls.

rtPCR-ΔΔct Analysis and Primers

Total RNA was extracted using miRNeasy kit (QIAGEN) and was processed for reverse transcriptase with a miScript II RT kit and via qPCR using standard procotols. Miscript primers used and primer sequences are listed in the Supplemental Information.

Constructs

pRRL3-GFP-BCCs were previously described (El-Haibi et al., 2012). For stable miRNA overexpression, GFP-BCCs were transfected with pEGP-miR Null, pEGP-miR-199a-2 (Cell Biolabs), or pcDNA3.2/V5 hsa-mir-214 (D. Bartel) and stable transfectants were selected with puromycin and/or G418. pEGP-miR-1915 and pEGP-miR-762 were generated by PCR amplification of precursor stem-loops from human genomic DNA and were subcloned into a miRNA Select pEGP-miR (Cell Biolabs). QIAGEN miScript miRNA Mimics #MSY0000232 and # MSY0000231 were used for expression of miR-199a-3p and miR-199a-5p. #MSY0000063 and #MSY0000255 RNA duplexes (QIAGEN) were used for expression of miR-let-7b-5p and miR-34a-5p, respectively. pLKO.1 FOXP2 shRNAs and shSCRAM constructs (Dana-Farber Cancer Institute) were stably expressed in MDA-MB-231 as standard. The long-form (variant 2) of FOXP2 was purchased from Origene.

Proliferation Assays, Western Blotting, Transwell Motility, Anoikis, ALDEFLUOR, and Sphere Formation Assays

These assays were conducted using standard procedures described in detail in the Supplemental Information.

Tumor Initiation and Metastasis Analyses

Female athymic Nude mice (Charles River Laboratories #490) were subjected to 200 μl (2:1 complete DMEM/cells:Reduced Growth Factor Matrigel; BD Biosciences) subcutaneous injections of BCCs. Tumorigenesis was assessed via palpation and confirmed by fluorescence microscopy after excision. Statistical analyses were performed utilizing ELDA: Extreme Limiting Dilution Analysis software (Hu and Smyth, 2009). Fluorescence microscopy was used to assess lung metastasis.

PCR Profiling Arrays

BCC-derived RNA was analyzed using Human Stem Cell PCR Array (QIAGEN #PAHS-501Z), and data was analyzed using RT^2 Profiler PCR Array Data Analysis software (http://www.sabiosciences.com/pcrarraydataanalysis.php).

Clinical Analyses

FOXP2 determinations were derived from ROCK (Cancer Genome Atlas Network, 2012; Chen et al., 2010); from GSE20711, GSE21653, and GSE1456; or from tumors collected under approved Curie IRB protocols. miRNA determinations were derived from Farazi et al. (2011), from Cancer Genome Atlas Network (2012), from breast cancer samples in Cimino et al. (2013), or from the Curie set.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.10.001.

AUTHOR CONTRIBUTIONS

B.G.C. and A.E.K. designed research; and B.G.C., A.C., G.W.B., A.L., F.O., E.C.L., M.K.B., M.R., F.M.G., S.V., A.V.S., D.T., and A.E.K. performed research and collected and analyzed data. S.E.H., A.M., D.E.A., P.H., and K.P. provided primary cells; O.M. provided clinical specimens; B.G.C. and A.E.K. wrote and edited the paper; and A.E.K. directed and supervised research.

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REFERENCES

Barcellos-Hoff, M.H., Lyden, D., and Wang, T.C. (2013). The evolution of the cancer niche during multistage carcinogenesis. Nat. Rev. Cancer *13*, 511–518.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215–233.

Battula, V.L., Shi, Y., Evans, K.W., Wang, R.Y., Spaeth, E.L., Jacamo, R.O., Guerra, R., Sahin, A.A., Marini, F.C., Hortobagyi, G., et al. (2012). Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. J. Clin. Invest. *122*, 2066–2078.

Bockmeyer, C.L., Christgen, M., Müller, M., Fischer, S., Ahrens, P., Länger, F., Kreipe, H., and Lehmann, U. (2011). MicroRNA profiles of healthy basal and luminal mammary epithelial cells are distinct and reflected in different breast cancer subtypes. Breast Cancer Res. Treat. *130*, 735–745.

Brenner, B., Hoshen, M.B., Purim, O., David, M.B., Ashkenazi, K., Marshak, G., Kundel, Y., Brenner, R., Morgenstern, S., Halpern, M., et al. (2011). MicroRNAs as a potential prognostic factor in gastric cancer. World J. Gastroenterol. *17*, 3976–3985.

Cancer Genome Atlas Network (2012). Comprehensive molecular portraits of human breast tumours. Nature *490*, 61–70.

Chaturvedi, P., Gilkes, D.M., Wong, C.C., Kshitiz, Luo, W., Zhang, H., Wei, H., Takano, N., Schito, L., Levchenko, A., and Semenza, G.L. (2013). Hypoxia-inducible factor-dependent breast cancer-mesenchymal stem cell bidirectional signaling promotes metastasis. J. Clin. Invest. *123*, 189–205.

Chen, D.T., Nasir, A., Culhane, A., Venkataramu, C., Fulp, W., Rubio, R., Wang, T., Agrawal, D., McCarthy, S.M., Gruidl, M., et al. (2010). Proliferative genes dominate malignancy-risk gene signature in histologically-normal breast tissue. Breast Cancer Res. Treat. *119*, 335–346.

Cimino, D., De Pitta, C., Orso, F., Zampini, M., Casara, S., Penna, E., Quaglino, E., Forni, M., Damasco, C., Pinatel, E., et al. (2013). miR148b is a major coordinator of breast cancer progression in a relapse-associated microRNA signature by targeting ITGA5, ROCK1, PIK3CA, NRAS, and CSF1. FASEB J *27*, 1223–1235.

Cuiffo, B.G., and Karnoub, A.E. (2012). Mesenchymal stem cells in tumor development: emerging roles and concepts. Cell Adhes. Migr. *6*, 220–230.

Dedeurwaerder, S., Desmedt, C., Calonne, E., Singhal, S.K., Haibe-Kains, B., Defrance, M., Michiels, S., Volkmar, M., Deplus, R., Luciani, J., et al. (2011). DNA methylation profiling reveals a predominant immune component in breast cancers. EMBO Mol Med *3*, 726–741.

Dorado, J., Lonardo, E., Miranda-Lorenzo, I., and Heeschen, C. (2011). Pancreatic cancer stem cells: new insights and perspectives. J. Gastroenterol. *46*, 966–973.

Drews-Elger, K., Brinkman, J.A., Miller, P., Shah, S.H., Harrell, J.C., da Silva, T.G., Ao, Z., Schlater, A., Azzam, D.J., Diehl, K., et al. (2014). Primary breast tumor-derived cellular models: characterization of tumorigenic, metastatic, and cancer-associated fibroblasts in dissociated tumor (DT) cultures. Breast Cancer Res. Treat. *144*, 503–517.

El-Haibi, C.P., Bell, G.W., Zhang, J., Collmann, A.Y., Wood, D., Scherber, C.M., Csizmadia, E., Mariani, O., Zhu, C., Campagne, A., et al. (2012). Critical role for lysyl oxidase in mesenchymal stem cell-driven breast cancer malignancy. Proc. Natl. Acad. Sci. USA *109*, 17460–17465.

Farazi, T.A., Horlings, H.M., Ten Hoeve, J.J., Mihailovic, A., Halfwerk, H., Morozov, P., Brown, M., Hafner, M., Reyal, F., van Kouwenhove, M., et al. (2011). MicroRNA sequence and expression analysis in breast tumors by deep sequencing. Cancer Res. *71*, 4443–4453.

Feber, A., Xi, L., Pennathur, A., Gooding, W.E., Bandla, S., Wu, M., Luketich, J.D., Godfrey, T.E., and Litle, V.R. (2011). MicroRNA prognostic signature for nodal metastases and survival in esophageal adenocarcinoma. Ann. Thorac. Surg. *91*, 1523–1530.

Fisher, S.E., and Scharff, C. (2009). FOXP2 as a molecular window into speech and language. Trends Genet. *25*, 166–177.

Ginestier, C., Hur, M.H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C.G., Liu, S., et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell *1*, 555–567.

Goldstein, R.H., Reagan, M.R., Anderson, K., Kaplan, D.L., and Rosenblatt, M. (2010). Human bone marrow-derived MSCs can home to orthotopic breast cancer tumors and promote bone metastasis. Cancer Res. *70*, 10044–10050.

Hanson, S.E., Kim, J., and Hematti, P. (2013). Comparative analysis of adipose-derived mesenchymal stem cells isolated from abdominal and breast tissue. Aesthet. Surg. J. *33*, 888–898.

Hu, Y., and Smyth, G.K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods *347*, 70–78.

Hu, M., Yao, J., Carroll, D.K., Weremowicz, S., Chen, H., Carrasco, D., Richardson, A., Violette, S., Nikolskaya, T., Nikolsky, Y., et al. (2008). Regulation of in situ to invasive breast carcinoma transition. Cancer Cell *13*, 394–406.

Iorio, M.V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C.G., Alder, H., et al. (2007). MicroRNA signatures in human ovarian cancer. Cancer Res. *67*, 8699–8707.

Jung, Y., Kim, J.K., Shiozawa, Y., Wang, J., Mishra, A., Joseph, J., Berry, J.E., McGee, S., Lee, E., Sun, H., et al. (2013). Recruitment of mesenchymal stem cells into prostate tumours promotes metastasis. Nat Commun *4*, 1795.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R., and Weinberg, R.A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature *449*, 557–563.

Konopka, G., Bomar, J.M., Winden, K., Coppola, G., Jonsson, Z.O., Gao, F., Peng, S., Preuss, T.M., Wohlschlegel, J.A., and Geschwind, D.H. (2009). Human-specific transcriptional regulation of CNS development genes by FOXP2. Nature *462*, 213–217.

Li, S., Weidenfeld, J., and Morrisey, E.E. (2004). Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. Mol. Cell. Biol. *24*, 809–822.

Liu, M., Casimiro, M.C., Wang, C., Shirley, L.A., Jiao, X., Katiyar, S., Ju, X., Li, Z., Yu, Z., Zhou, J., et al. (2009). p21CIP1 attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo. Proc. Natl. Acad. Sci. USA *106*, 19035–19039.

Liu, S., Ginestier, C., Ou, S.J., Clouthier, S.G., Patel, S.H., Monville, F., Korkaya, H., Heath, A., Dutcher, J., Kleer, C.G., et al. (2011). Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. Cancer Res. *71*, 614–624.

Ma, L., Teruya-Feldstein, J., and Weinberg, R.A. (2007). Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature *449*, 682–688.

Malanchi, I., Santamaria-Martínez, A., Susanto, E., Peng, H., Lehr, H.A., Delaloye, J.F., and Huelsken, J. (2012). Interactions between cancer stem cells and their niche govern metastatic colonization. Nature *481*, 85–89.

Mascaux, C., Laes, J.F., Anthoine, G., Haller, A., Ninane, V., Burny, A., and Sculier, J.P. (2009). Evolution of microRNA expression during human bronchial squamous carcinogenesis. Eur. Respir. J. *33*, 352–359.

McLean, K., Gong, Y., Choi, Y., Deng, N., Yang, K., Bai, S., Cabrera, L., Keller, E., McCauley, L., Cho, K.R., and Buckanovich, R.J. (2011). Human ovarian carcinoma–associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. J. Clin. Invest. *121*, 3206–3219.

Miller, L.D., Smeds, J., George, J., Vega, V.B., Vergara, L., Ploner, A., Pawitan, Y., Hall, P., Klaar, S., Liu, E.T., and Bergh, J. (2005). An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc. Natl. Acad. Sci. USA, *102*, 13550–13555.

Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D.L., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., et al. (2011). Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell *8*, 633–638.

Myatt, S.S., and Lam, E.W. (2007). The emerging roles of forkhead box (Fox) proteins in cancer. Nat. Rev. Cancer *7*, 847–859.

Nair, R., Roden, D.L., Teo, W.S., McFarland, A., Junankar, S., Ye, S., Nguyen, A., Yang, J., Nikolic, I., Hui, M., et al. (2014). c-Myc and Her2 cooperate to drive a stem-like phenotype with poor prognosis in breast cancer. Oncogene *33*, 3992–4002.

Okamoto, O.K., Oba-Shinjo, S.M., Lopes, L., and Nagahashi Marie, S.K. (2007). Expression of HOXC9 and E2F2 are up-regulated in CD133(+) cells isolated from human astrocytomas and associate with transformation of human astrocytes. Biochim. Biophys. Acta *1769*, 437–442.

Pawitan, Y., Bjöhle, J., Amler, L., Borg, A.L., Egyhazi, S., Hall, P., Han, X., Holmberg, L., Huang, F., Klaar, S., et al. (2005). Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. Breast Cancer Res. *7*, R953–R964.

Pencheva, N., Tran, H., Buss, C., Huh, D., Drobnjak, M., Busam, K., and Tavazoie, S.F. (2012). Convergent multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent melanoma metastasis and angiogenesis. Cell *151*, 1068–1082.

Polyak, K., and Weinberg, R.A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat. Rev. Cancer *9*, 265–273.

Prantl, L., Muehlberg, F., Navone, N.M., Song, Y.H., Vykoukal, J., Logothetis, C.J., and Alt, E.U. (2010). Adipose tissue-derived stem cells promote prostate tumor growth. Prostate *70*, 1709–1715.

Prockop, D.J., Kota, D.J., Bazhanov, N., and Reger, R.L. (2010). Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). J. Cell. Mol. Med. *14*, 2190–2199.

Quail, D.F., and Joyce, J.A. (2013). Microenvironmental regulation of tumor progression and metastasis. Nat. Med. *19*, 1423–1437.

Sabatier, R., Finetti, P., Cervera, N., Lambaudie, E., Esterni, B., Mamessier, E., Tallet, A., Chabannon, C., Extra, J.M., Jacquemier, J., et al. (2011). A gene expression signature identifies two prognostic subgroups of basal breast cancer. Breast Cancer Res. Treat. *126*, 407–420.

Sakurai, K., Furukawa, C., Haraguchi, T., Inada, K., Shiogama, K., Tagawa, T., Fujita, S., Ueno, Y., Ogata, A., Ito, M., et al. (2011). MicroRNAs miR-199a-5p and -3p target the Brm subunit of SWI/SNF to generate a double-negative feedback loop in a variety of human cancers. Cancer Res. *71*, 1680–1689.

Scapoli, L., Palmieri, A., Lo Muzio, L., Pezzetti, F., Rubini, C., Girardi, A., Farinella, F., Mazzotta, M., and Carinci, F. (2010). MicroRNA expression profiling of oral carcinoma identifies new markers of tumor progression. Int. J. Immunopathol. Pharmacol. *23*, 1229–1234.

Schwarzenbach, H., Milde-Langosch, K., Steinbach, B., Müller, V., and Pantel, K. (2012). Diagnostic potential of PTEN-targeting miR-214 in the blood of breast cancer patients. Breast Cancer Res. Treat. *134*, 933–941.

Shinagawa, K., Kitadai, Y., Tanaka, M., Sumida, T., Kodama, M., Higashi, Y., Tanaka, S., Yasui, W., and Chayama, K. (2010). Mesenchymal stem cells enhance growth and metastasis of colon cancer. Int. J. Cancer *127*, 2323–2333.

Shu, W., Yang, H., Zhang, L., Lu, M.M., and Morrisey, E.E. (2001). Characterization of a new subfamily of winged-helix/forkhead (Fox) genes that are expressed in the lung and act as transcriptional repressors. J. Biol. Chem. *276*, 27488–27497.

Shu, W., Lu, M.M., Zhang, Y., Tucker, P.W., Zhou, D., and Morrisey, E.E. (2007). Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. Development *134*, 1991–2000.

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., Vernes, S.C., Fisher, S.E., Ren, B., and Geschwind, D.H. (2007). Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. Am. J. Hum. Genet. *81*, 1144–1157.

Suzuki, K., Sun, R., Origuchi, M., Kanehira, M., Takahata, T., Itoh, J., Umezawa, A., Kijima, H., Fukuda, S., and Saijo, Y. (2011). Mesenchymal stromal cells promote tumor growth through the enhancement of neovascularization. Mol. Med. *17*, 579–587.

Tavazoie, S.F., Alarcón, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P.D., Gerald, W.L., and Massagué, J. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. Nature *451*, 147–152.

Toullec, A., Gerald, D., Despouy, G., Bourachot, B., Cardon, M., Lefort, S., Richardson, M., Rigaill, G., Parrini, M.C., Lucchesi, C., et al. (2010). Oxidative stress promotes myofibroblast differentiation and tumour spreading. EMBO Mol Med *2*, 211–230.

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Tsui, D., Vessey, J.P., Tomita, H., Kaplan, D.R., and Miller, F.D. (2013). FoxP2 regulates neurogenesis during embryonic cortical development. J. Neurosci. *33*, 244–258.

van de Vijver, M.J., He, Y.D., van't Veer, L.J., Dai, H., Hart, A.A., Voskuil, D.W., Schreiber, G.J., Peterse, J.L., Roberts, C., et al. (2002). A gene-expression signature as a predictor of survival in breast cancer. N. Engl. J. Med. *347*, 1999–2009.

Vernes, S.C., Oliver, P.L., Spiteri, E., Lockstone, H.E., Puliyadi, R., Taylor, J.M., Ho, J., Mombereau, C., Brewer, A., Lowy, E., et al. (2011). Foxp2 regulates gene networks implicated in neurite outgrowth in the developing brain. PLoS Genet. *7*, e1002145.

Volinia, S., Galasso, M., Sana, M.E., Wise, T.F., Palatini, J., Huebner, K., and Croce, C.M. (2012). Breast cancer signatures for invasiveness and prognosis defined by deep sequencing of microRNA. Proc. Natl. Acad. Sci. USA *109*, 3024–3029.

Wan, D., He, S., Xie, B., Xu, G., Gu, W., Shen, C., Hu, Y., Wang, X., Zhi, Q., and Wang, L. (2013). Aberrant expression of miR-199a-3p and its clinical significance in colorectal cancers. Med. Oncol. *30*, 378.

Yan, X.L., Fu, C.J., Chen, L., Qin, J.H., Zeng, Q., Yuan, H.F., Nan, X., Chen, H.X., Zhou, J.N., Lin, Y.L., et al. (2012). Mesenchymal stem cells from primary breast cancer tissue promote cancer proliferation and enhance mammosphere formation partially via EGF/EGFR/Akt pathway. Breast Cancer Res. Treat. *132*, 153–164.

Yang, H., Kong, W., He, L., Zhao, J.J., O'Donnell, J.D., Wang, J., Wenham, R.M., Coppola, D., Kruk, P.A., Nicosia, S.V., and Cheng, J.Q. (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res. *68*, 425–433.

Zhang, Y., Goss, A.M., Cohen, E.D., Kadzik, R., Lepore, J.J., Muthukumaraswamy, K., Yang, J., DeMayo, F.J., Whitsett, J.A., Parmacek, M.S., and Morrisey, E.E. (2008). A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. Nat. Genet. *40*, 862–870.

Zhang, X.J., Ye, H., Zeng, C.W., He, B., Zhang, H., and Chen, Y.Q. (2010). Dysregulation of miR-15a and miR-214 in human pancreatic cancer. J Hematol Oncol *3*, 46.

Zhou, J., Ng, S.B., and Chng, W.J. (2013). LIN28/LIN28B: an emerging oncogenic driver in cancer stem cells. Int. J. Biochem. Cell Biol. *45*, 973–978.

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Myelodysplastic Cells in Patients Reprogram Mesenchymal Stromal Cells to Establish a Transplantable Stem Cell Niche Disease Unit

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SUMMARY

Myelodysplastic syndromes (MDSs) are a heterogeneous group of myeloid neoplasms with defects in hematopoietic stem and progenitor cells (HSPCs) and possibly the HSPC niche. Here, we show that patient-derived mesenchymal stromal cells (MDS MSCs) display a disturbed differentiation program and are essential for the propagation of MDS-initiating $Lin^{-}CD34^{+}CD38^{-}$ stem cells in orthotopic xenografts. Overproduction of niche factors such as CDH2 (N-Cadherin), IGFBP2, VEGFA, and LIF is associated with the ability of MDS MSCs to enhance MDS expansion. These factors represent putative therapeutic targets in order to disrupt critical hematopoietic-stromal interactions in MDS. Finally, healthy MSCs adopt MDS MSC-like molecular features when exposed to hematopoietic MDS cells, indicative of an instructive remodeling of the microenvironment. Therefore, this patient-derived xenograft model provides functional and molecular evidence that MDS is a complex disease that involves both the hematopoietic and stromal compartments. The resulting deregulated expression of niche factors may well also be a feature of other hematopoietic malignancies.

INTRODUCTION

Myelodysplastic syndromes (MDSs) are a heterogeneous group of malignant clonal diseases that affect older individuals (median age 68–75 years) with an incidence in the range of 3–10/100,000. MDSs are characterized by ineffective hematopoiesis and the presence of dysplastic cells in the bone marrow as well as peripheral cytopenias. Clinically, patients present with symptoms such as anemia, bleeding, or infection. Classification of MDS is carried out according to risk-score systems such as the World Health Organization (WHO) classification or the international prognostic scoring systems (IPSS and IPSS-R). These scoring systems allow the accurate segregation of patients according to prognosis and are used to adapt therapeutic options to individual patients (Garcia-Manero, 2012). Treatment options for MDS range from best supportive care, hematopoietic growth factors, or immunomodulatory drugs such as lenalidomide in lower-risk patients to treatment with DNA demethylating agents, cytotoxic chemotherapy, or hematopoietic stem cell (HSC) transplantation with curative intent for patients in higher-risk subgroups (Garcia-Manero, 2012). Genome-wide discovery approaches recently revealed a number of genetic lesions in patients with MDS that provide valuable insights into the underlying biology of MDS (Haferlach et al., 2014; Bejar et al., 2011, 2012; Papaemmanuil et al., 2013; Walter et al., 2012). This knowledge has been successfully used to generate genetic mouse models of MDS (Abdel-Wahab et al., 2013; Muto et al., 2013). However, it is expected

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that no single model can recapitulate the disease heterogeneity and complexity seen in patients.

Several attempts to generate a robust xenograft model in immunodeficient mice have been undertaken, but these have demonstrated inconsistent, transient, and low levels of engraftment, particularly with regard to samples taken from lower-risk MDS patients (Martin et al., 2010; Muguruma et al., 2011; Thanopoulou et al., 2004). In addition, distinguishing normal HSC from MDS stem cell (MDS HSC) engraftment was difficult, given that large cytogenetic lesions that allow easy tracking of the malignant clone are only present in about half of the MDS patients and that no distinguishing cell-surface markers have been identified to date (Martin et al., 2010; Muguruma et al., 2011; Thanopoulou et al., 2004). Most importantly, patient samples that did engraft in these studies represented higher-risk MDS, which are closer to acute myeloid leukemia (AML) (Pang et al., 2013). Recently, several studies suggested that alterations in the bone marrow niche influence the development of myeloid neoplasms (reviewed by Raaijmakers, 2012). Mice deficient for retinoic acid receptor γ (*RAR*γ) develop myeloproliferative syndromes induced solely by the RARγ-deficient microenvironment (Walkley et al., 2007). More recently, MDS could efficiently be induced in mice in which *DICER*, a gene encoding a microRNA processing enzyme, was deleted in osteoprogenitor cells (Raaijmakers et al., 2010), whereas expression of an activated form of β-catenin in osteoblasts alters the differentiation of hematopoietic progenitors, leading to the development of AML (Kode et al., 2014). Finally, in chronic myeloid leukemia (CML), niche cells have been shown to exert a protective role in the response to Imatinib in vitro (Zhang et al., 2013). Altogether, these reports strongly support the hypothesis that abnormal niche environment provides "fertile soil" for the expansion of the neoplastic cells in vivo.

RESULTS

Cotransplantation of CD34$^+$ Cells with Patient-Derived MSCs Allows Efficient and Long-Term MDS Reinstallment in NSG Mice

Our study is based on the analysis of 31 MDS patients who were classified as follows: IPSS low risk (n = 7), intermediate-1 risk (n = 24), and WHO 2008 classifications MDS 5q (n = 7), MDS RCMD (n = 14), MDS RAEB I (n = 6), MDS-U (n = 2), and MDS RARS (n = 2; Table S1). Among these, 24 were tested for their ability to propagate MDS in a xenograft setting (low risk, n = 5; intermediate-1 risk, n = 19). Based on the hypothesis that disease-propagating cells (DPCs) in lower-risk MDS form a functional unit with their stromal niche cells, we decided to compare the engraftment of MDS-derived CD34$^+$ cells injected either alone or in combination with their corresponding in vitro expanded mesenchymal stromal cells into the bone marrow cavity of sublethally irradiated NOD/LtSz-scid IL2Rγc$^{-/-}$ (NSG) mice (Figure 1A). We opted for the intrabone injection because of the hypothesis that hematopoietic and stromal cells may require physical interaction. Then, mice were analyzed for human chimerism (human CD45 [hCD45] expression) at 16–28 weeks posttransplantation. Of the cases transplanted with CD34$^+$ cells alone, only one of seven samples (MDS14) showed engraftment above the 1% threshold we set for this study (one of three mice engrafted; Figure 1B, left). In contrast, coinjection of CD34$^+$ cells with MDS MSCs resulted in a significantly higher engraftment in 70% of the patient samples analyzed (14 of 20 patients; range = 1%–22%; p = 0.026; Figure 1B). A direct comparison with the identical CD34$^+$ MDS samples was possible for four patients: MDS14, MDS17, MDS18, and MDS19 (Figure 1B). MDS17 did not engraft in either condition. Importantly, the remaining three samples showed an enhanced engraftment with MDS MSCs, further validating our finding.

Abnormal Lineage Distribution and Clonal Tracking of MDS Cells in the Xenograft

Bone marrow cells from engrafted mice were further analyzed with lineage-specific antibodies (CD19 for B cells and CD33 for myeloid cells). When compared to mice repopulated with age-matched healthy old CD34$^+$ cells, most recipients of MDS cells had a significant disproportionate output of myeloid cells (MDS, 70.3% ± 5.3%; healthy, 10.7% ± 1.4%; $p < 0.0001$; Figure 1C). Four patients (MDS12, MDS24, MDS38, and MDS52) showed an increased B lymphoid output, which may indicate the engraftment of healthy stem cells as opposed to an MDS-derived clone (Figure 1C). To ascertain the origin of the xenografted cells (MDS or healthy), we used several methods to molecularly track the lesions that were initially identified in the patients. Primary hematopoietic cells from each patient were analyzed with chromosomal banding and SNP array (SNP-A) as well as targeted next-generation sequencing (NGS), interrogating acquired mutations in 17 frequently mutated genes described in MDS (Table S2). This strategy allowed us to identify at least one traceable lesion for all patients included in this study with the exception of MDS19 (Table S1). Therefore, bone marrow from patient 19 was subjected to whole-exome sequencing, which revealed the presence of a *BCORL1* mutation in 58% of the bone marrow cells (corresponding to a mutational allele frequency of 29%). Importantly this mutation was not detected in MSCs isolated from the same patient, thereby excluding a germline origin (data not shown). For each engrafted mouse, hCD45$^+$ cells were purified by fluorescence-activated cell sorting (FACS) and analyzed with SNP-A or interphase fluorescence in situ hybridization (FISH) analysis for the detection and quantification of del(5q) or del(21q) (Figures 1D–1F and Table S3) as well as by employing specific pyrosequencing assays in order to track and quantify the mutated alleles (Figure 1G). Table S3 provides a comprehensive summary of the mutational allele frequencies detected in human cells isolated from all xenografted mice displayed in Figure 1B. In the case of cytogenetic aberrations, the numbers refer to the percentage of analyzed cells that scored positive for the lesion, whereas, for heterozygous point mutations, these numbers refer to the allele frequency of the mutated variant. These data confirm that these cells carried the same lesion(s) as the one(s) identified in the original patient samples. Furthermore, we could even observe a significant expansion of an MDS clone carrying both an *SF3B1* mutation and a del(5q) in the mouse engrafted with patient MDS11 cells (Figures 1D and 1E). Even though human cells isolated from MDS16 did not carry any lesion, they exhibited the typical marked myeloid bias observed in most other MDS samples that were validated by molecular analysis (Figure 1C). The predominant B cell population isolated from xenografts of patients

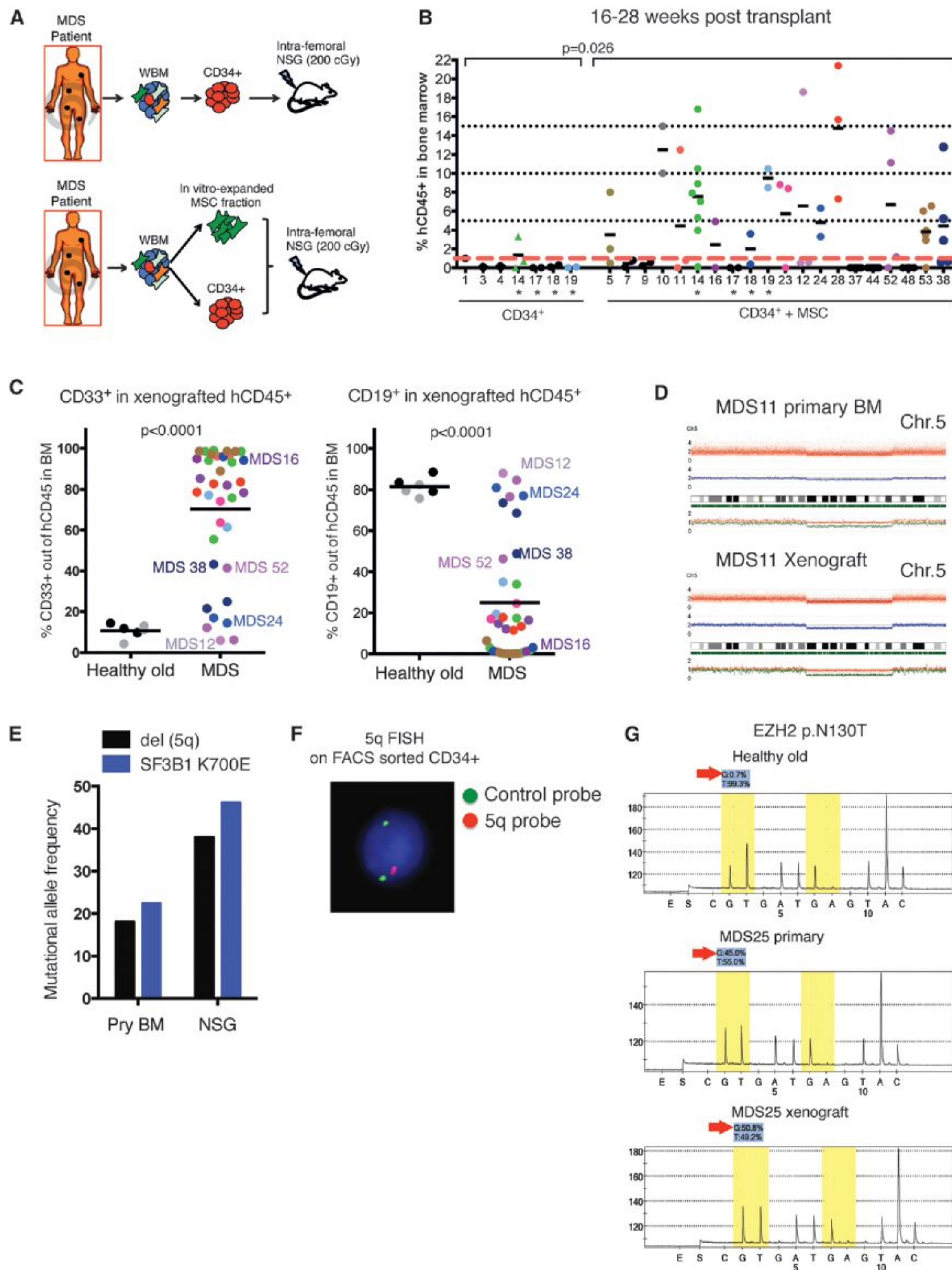


Figure 1. Enhanced Engraftment of Lower-Risk MDS by Cotransplantation of Patient-Derived MSCs

(A) Schematic experimental setup. MDS $CD34^+$ were injected in the bone marrow cavity of sublethally irradiated NSG mice either alone ($CD34^+$) or in combination with MDS-derived MSCs ($CD34^+$ + MSC).

(B) Percentage of $hCD45^+$ cells in the bone marrow of xenografted mice (%$hCD45^+$) analyzed 16–28 weeks posttransplantation. The red dotted line indicates the 1% threshold used in this study to define positive engraftment. Numbers on the x axis are patient IDs. Each dot represents one mouse. Mean engraftment values were compared in the two cohorts with a Mann-Whitney test (p = 0.026). Asterisks indicate paired patient samples analyzed side by side.

(legend continued on next page)

MDS12 and 24 did not carry the mutations present in the patient; however, the CD33$^+$ fraction did (Table S3), indicating the coengraftment of a healthy and an MDS stem cell. Patient 38 engrafted three mice, one of which showed a significant MDS engraftment, whereas the two others mostly gave rise to a B lymphoid-biased graft with cells that did not carry the lesion seen in the original patient.

Human MDS-Initiating Cells display Long-Term Self-Renewal Activity in Serial Transplantation Assays

As expected, human cells with stem cell (CD34$^+$CD38$^-$) and progenitor (CD34$^+$CD38$^+$) phenotypes were detectable in all xenografts with MDS MSCs, albeit at varying frequencies (Figure 2A and data not shown). In addition, analysis of myeloid, and in some cases B lymphoid, cells isolated from NSG xenografts showed that they carried the molecular lesion found in the primary patient (Figures 2B and 2C and data not shown). Because cells with a hematopoietic stem and progenitor cell (HSPC) phenotype were readily detectable in primary xenografts, we addressed whether they could be serially transplanted into secondary recipients. Cells with an hCD45$^+$CD34$^+$ phenotype were isolated with FACS from a mouse previously engrafted with CD34$^+$ cells of patient MDS14 (Figure 2D). The presence of the chromosome 5q deletion was quantified by FISH analysis of FACS-purified human cells (Figure 2E). Then, 10 weeks post-transplantation, two of three recipients engrafted with as little as 6,500 transplanted CD45$^+$CD34$^+$ cells along with MDS MSCs (Figure 2F). Even though the contribution was below 1%, these cells were positive for del(5q), demonstrating their origin from MDS14 (Figure 2E). Similar results were obtained with serial transplantation of CD45$^+$ or CD45$^+$CD34$^+$ cells from patients 10 and 23, respectively (Figure S1A). These data demonstrate that lower-risk MDS stem cells can harbor long-term self-renewal activity.

Higher MDS Engraftment and Typical Signs of Dysplasia in NSGS Recipients

In vitro studies suggest that MSCs and growth factors have synergistic effects on the expansion of HSPCs (Walenda et al., 2011). However, it is well known that a subset of murine growth factors exhibit limited cross-reactivity with the human orthologs of their receptors, which could limit the growth of human stem cells in a murine host (Wunderlich et al., 2010). Therefore, we attempted to further improve human MDS engraftment by using the recently described NSGS mouse strain that constitutively expresses the human cytokines IL3, GM-CSF, and stem cell factor (SCF). These mice have been shown to improve the engraftment of primary human AML (Wunderlich et al., 2010). More recently, investigators demonstrated enhanced normal human myelopoiesis after injection of CD34$^+$ human cord blood cells in this strain (Miller et al., 2013). We compared the engraftment of four patient samples side by side in age-matched NSG and NSGS mice also coinjected with MDS MSCs. This analysis revealed an augmented human chimerism in NSGS in comparison to NSG mice (Figure 3A), which increases over time in the NSGS mice, as indicated by an illustrative example (Figure S1B). Similar to the NSG model, characteristic molecular lesions present in the samples from the patients were also found in the xenografted cells (Figures S1C–S1G). These data are summarized in Figure 3A, in which the mean frequency of MDS cells (hCD45$^+$ cells carrying an MDS lesion) in the bone marrow of NSG or NSGS mice are displayed. These results show a consistently enhanced MDS disease burden in the NSGS strain.

Given that the presence of dysplastic cells is one of the main clinical features of MDS, we performed Pappenheim staining of bone marrow smears in order to analyze the morphology of engrafted cells. This analysis revealed readily detectable dysplastic cells exclusively in mice engrafted with MDS-derived cells (Figure 3B). As shown in Figure 3B, typical signs of dysplastic erythropoiesis with megaloblastic and vacuolized proerythroblasts in both the primary patient bone marrow sample (top right) and the corresponding xenograft (Figure 3B, bottom right) is apparent. Importantly, these dysplastic signs were absent in unmanipulated NSGS mice (Figure 3B, top left) as well as mice engrafted with healthy age-matched CD34$^+$ cells (Figure 3B, bottom left). Altogether, these data indicate that expression of human cytokines further improves the engraftment and growth of human MDS cells in mice.

MDS Initiating and Propagating Cells Have a Lin$^-$CD34$^+$CD38$^-$ Phenotype, Retain Multipotency, and Display Variegated Clonality

The identification of the DPC in human MDS has so far been hampered by the lack of a transplantation assay for this disease. Molecular alterations described in MDS are rarely found in lymphoid compartments, raising the possibility that the MDS-DPC might be a myeloid restricted progenitor rather than an early stem cell harboring both myeloid and lymphoid potential. Alternatively, genetic and/or epigenetic changes in MDS stem cells might prevent their ability to commit to the lymphoid lineage. To address this issue, erythroid (CD235a$^+$ CD71$^+$), myeloid (CD33$^+$), and lymphoid (CD3$^+$CD19$^+$) cells from five primary samples from MDS patients were isolated

(C) Immunophenotyping of hCD45$^+$ cells isolated from the bone marrow of mice engrafted with either MDS (n = 12 patients displayed in B and transplanted in a total of 33 mice) or healthy age-matched CD34$^+$ cells (n = 2 healthy donors each transplanted in three mice) both injected with MDS MSCs. MDS xenografts showed a significant skewing toward myeloid output (unpaired Student's t test, $p < 0.0001$).

(D) High-density SNP array analysis. The shown profile depicts a heterozygous deletion of chromosome 5q in patient MDS11 bone marrow (top) and its corresponding xenograft (bottom).

(E) Molecular analysis of MDS11 primary patient sample (Pry BM) and its corresponding xenograft in an NSG mouse (NSG). Data show the presence of a *SF3B1* mutation (allelic frequency = 22%) and a deletion of chromosome 5q (35% of cells positive; i.e., 17.5% relative allelic frequency displayed in the graph) in the primary patient sample. In the xenograft, MDS cells carrying these lesions expand to allelic frequencies of >40% for *SF3B1* and >35% for del(5q).

(F) Interphase FISH for tracking the chromosome 5q deletion on FACS-sorted human CD34$^+$ cells from a mouse engrafted with patient sample MDS14 cells.

(G) Example of a quantitative pyrosequencing assay designed to track and quantify mutations in primary samples as well as xenografted fractions. The burden of the mutated allele is indicated with a red arrow. Bone marrow from healthy old donors (>60 years) was used as a control.

See also Tables S1, S2, and S3.

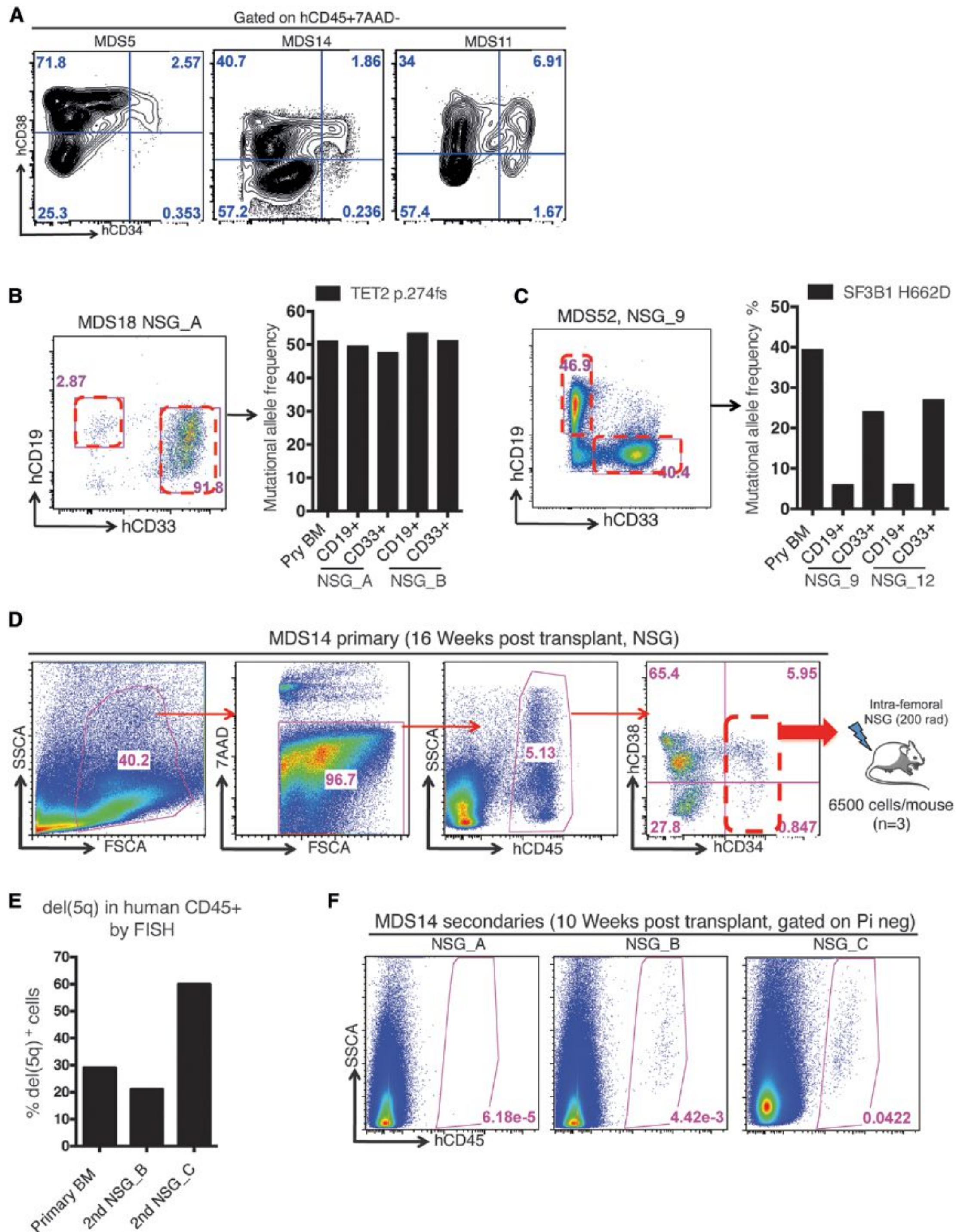


Figure 2. MDS Cells Sustain Long-Term Multilineage Hematopoiesis in NSG Mice

(A) Representative FACS plots showing that human cells with progenitor ($CD34^+CD38^+$) and stem cell ($CD34^+CD38^-$) phenotypes are readily detectable in the xenografts with MDS MSCs 16–28 weeks posttransplantation.

(B) The indicated fractions were purified by FACS and subjected to mutational allele frequency quantification by pyrosequencing.

(B and C) Gating scheme used to sort B lymphoid ($CD19^+$) and myeloid cells ($CD33^+$) form the bone marrow of mice engrafted with cells from patients MDS18 and MDS52.

(D) Workflow of serially transplanting a primary xenografted sample from MDS14 into three secondary recipients 16 weeks after the initial transplant.

(E and F) Secondary xenografts, which were generated by transplanting as little as 6,500 $CD34^+$ cells from the primary mouse retain the primary del(5q) molecular lesion as detected by FISH analysis of FACS-sorted fractions depicted in (F). In all FACS experiments, dead cells were excluded with 7AAD.

See also Figure S1.

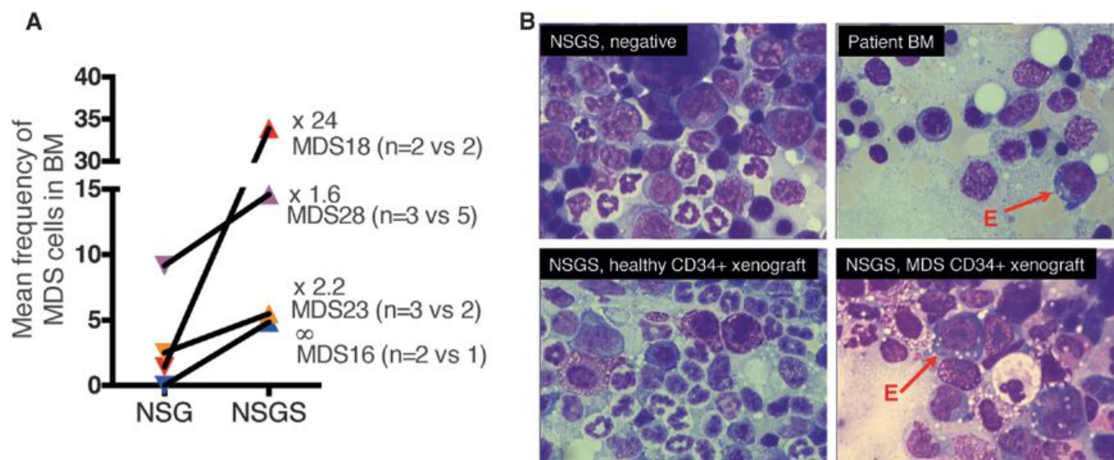


Figure 3. NSGS Mice Further Enhance the Engraftment of Dysplastic MDS Cells

(A) Side by side comparison of engraftment levels in NSG and NSGS mice injected with CD34$^+$ + MDS MSCs derived from four patients (MDS16, MDS18, MDS23, and MDS28). Data show the mean frequency of MDS cells as determined by multiplication of the fraction of hCD45$^+$ cells carrying the MDS-specific molecular lesion by the percentage of hCD45$^+$ cells in the bone marrow of engrafted mice.

(B) Representative example of a Pappenheim staining of bone marrow smears of an untreated native NSGS mouse (upper left), primary MDS patient bone marrow (upper right), NSGS mouse xenotransplanted with CD34$^+$ cells from a healthy donor (lower left), and NSGS mouse xenotransplanted with CD34$^+$ cells from the corresponding MDS patient (lower right). The primary patient bone marrow showed pronounced signs of dysplasia most predominant in the erythropoietic compartment with megaloblastic and vacuolized proerythroblasts (red arrows with "E"). These can also be readily detected in the xenografted NSGS mice from this patient (lower right, red arrow with "E").

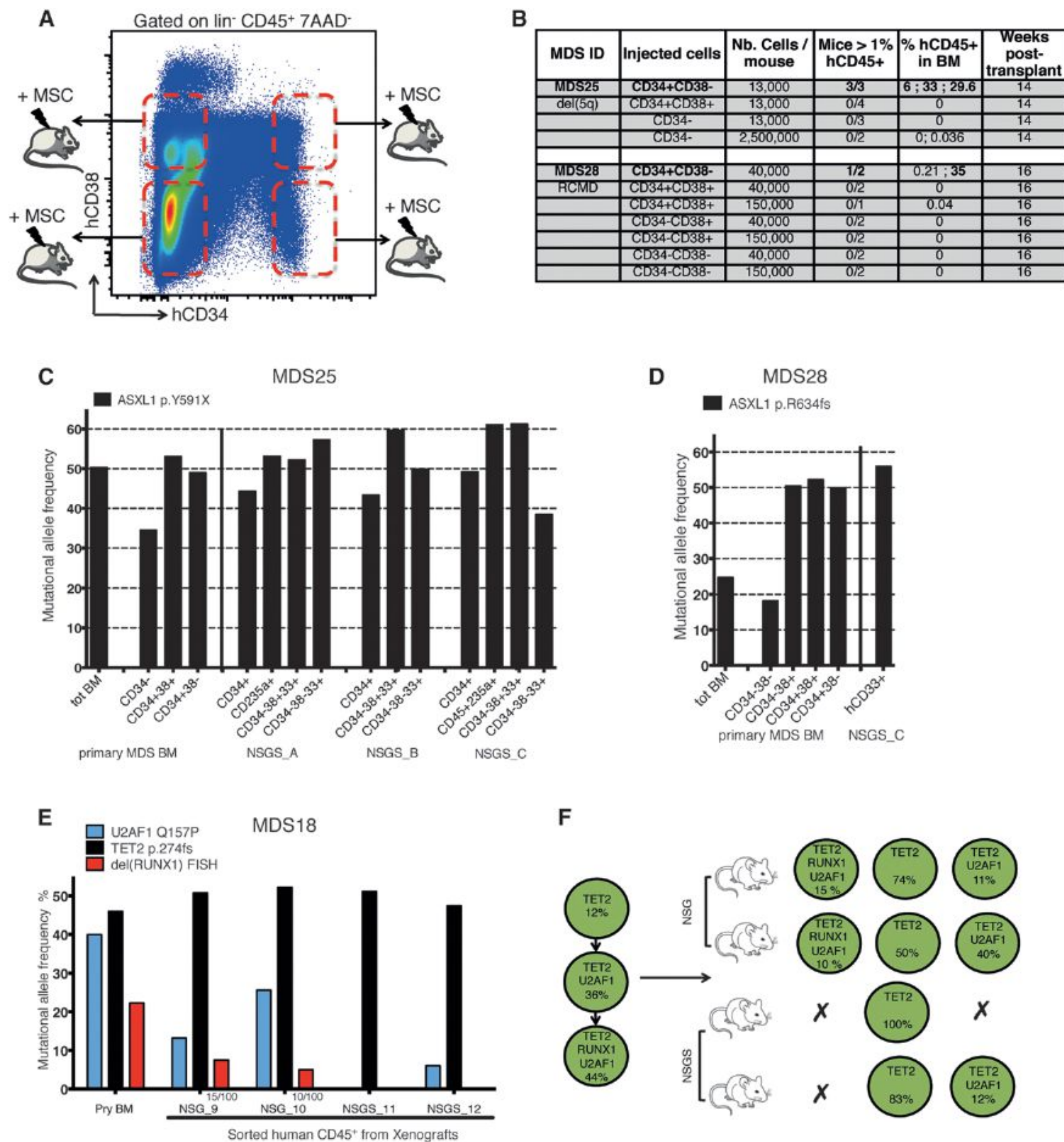
See also Figures S1B–S1G and Table S3.

and analyzed for the presence of mutations. These data show that myeloid and erythroid cells are consistently derived from the MDS cells (Figures S2A–S2E). Interestingly, contribution to the lymphoid lineage could also be readily detected in two of the five patients (MDS25 and MDS16). To experimentally identify the stem cell origin of the DPC in lower-risk MDS, we FACS purified stem and progenitor populations of two patients on the basis of lineage negativity (CD235a, CD19, CD4, CD8, and CD20), CD45 positivity, and CD34 and CD38 expression and tested their ability to propagate the MDS cells in NSGS mice (Figure 4A). Each of the four FACS-sorted populations were injected into two or four mice (depending on the number of primary cells recovered) and analyzed after 14–16 weeks posttransplantation for human cell chimerism. As outlined in Figure 4B, engraftment was achieved exclusively in mice that had received the Lin$^-$CD34$^+$CD38$^-$ stem cell fraction but not any other cell population. Mutational tracking confirmed the patient origin of the engrafted MDS cells (Figures 4C and 4D).

Analysis of primary bone marrow cells isolated from patient 18 revealed the presence of three mutations (*TET2*, *U2AF1*, and del(*RUNX1*)) that we could track with our established workflow in both NSG and NSGS mice (Figures 3A and 4E–4F). A heterozygous *TET2* mutation was the most frequent genetic alteration, and 92% of the cells carried this lesion, as evidenced by a mutational allele frequency of 46%, indicating that this is likely to be the founder clone in this patient. The other lesions, (*U2AF1* and del(*RUNX1*)) were detected in 80% and 44% of the cells, respectively (*U2AF1* mutational allele frequency = 40%; del(*RUNX1*) allele frequency = 22%). These data indicate that these two lesions are co-occurring and present in subclones that may have evolved from the founder clone in a linear fashion (Figures 4E and 4F). Subsequent analysis of hCD45$^+$ cells isolated from xenografted mice (two NSG and two NSGS) showed that del(*RUNX1*)-bearing cells were not detectable in the NSGS model (NSGS11 and NSGS12). Similarly, the *U2AF1*-bearing clone is largely outcompeted in this model by the founder *TET2*-only-bearing clone. In contrast NSG mice display engraftment of all three clones detected in the original patient bone marrow, albeit at different frequencies (Figures 4E and 4F). Altogether, these data show that we observe simultaneous engraftment of independent clones in the mouse when more than one clone is present in the patient, therefore closely mimicking the patient situation (NSG9 and NSG10).

MDS Patient-Derived MSCs Differ from Healthy MSCs at the Functional Level

The data in Figure 1B demonstrate that coinjection of MDS MSCs enhances the engraftment of MDS HSCs in NSG mice. The in vitro expanded MSCs used in this study fulfill the criteria established by the International Society of Stem Cell Therapy in terms of surface phenotype (CD45$^-$HLA-DR$^-$CD105$^+$CD73$^+$CD90$^+$CD44$^+$CD146$^+$; Figure S3A) and were devoid of hematopoietic cells, including macrophages, as demonstrated by the lack of CD45 and CD14 expression as well as the undetectable expression of myeloid specific genes such as cathepsin G (*CTSG*), proteinase 3 (*PRTN3*), and matrix metallopeptidase 9 (*MMP9*) in our RNA sequencing data (Figure S3B and data not shown). In addition, MSCs were functional, given that they were able to form an ectopic bone marrow niche (ossicle) when coinjected subcutaneously with hydroxyapatite tricalcium phosphate particles (HA-TCP), a resorbable bone substitute (Bianco et al., 2013) (Figure S3C). To directly test whether a specific supporting effect is exerted by MDS patient-derived MSCs, the identical patient



B

MDS ID	Injected cells	Nb. Cells / mouse	Mice > 1% hCD45+	% hCD45+ in BM	Weeks post-transplant
MDS25	**CD34+CD38-**	13,000	**3/3**	**6 ; 33 ; 29.6**	14
del(5q)	CD34+CD38+	13,000	0/4	0	14
	CD34-	13,000	0/3	0	14
	CD34-	2,500,000	0/2	0; 0.036	14
MDS28	**CD34+CD38-**	40,000	**1/2**	0.21 ; **35**	16
RCMD	CD34+CD38+	40,000	0/2	0	16
	CD34+CD38+	150,000	0/1	0.04	16
	CD34-CD38+	40,000	0/2	0	16
	CD34-CD38+	150,000	0/2	0	16
	CD34-CD38-	40,000	0/2	0	16
	CD34-CD38-	150,000	0/2	0	16

Figure 4. Disease-Propagating Stem Cells in Lower-Risk MDS Are Restricted to the Lin⁻CD34⁺CD38⁻ Cell Subset and Show Variegated Clonality

(A) Gating scheme of FACS-sorted populations used for xenotransplantation into NSGS mice. Four fractions were sorted on the basis of CD34 and CD38 expression pregated on live cells (7AAD⁻), lineage negativity (CD19, CD4, CD8, CD20, and CD235a), and positivity for CD45.

(B) Number of injected cells and engraftment results 14–16 weeks posttransplantation.

(C and D) Mutation tracking and quantification by pyrosequencing in all sorted subfractions of the primary patient as well as subpopulations of the human cells isolated from the engrafted NSGS mice.

(E and F) Detection of variegated clonality by molecular analysis of primary bone marrow cells isolated from patient MDS18 (Pry BM) and hCD45⁺ cells from the corresponding xenografted mice. The numbers below the red bars represent the number of del(*RUNX1*)⁺ cells scored by interphase FISH over the total number of cells analyzed. For the primary bone marrow sample, del(*RUNX1*) was evaluated by SNP array. Data show that the primary MDS patient sample is composed of three different clones containing successively accumulated mutations in the following order: *TET2*, *U2AF1*, and a genomic deletion of *RUNX1*.

See also Tables S1, S2, and S3.

sample was either cotransplanted with MDS MSCs or age-matched healthy MSCs obtained from patients undergoing hip replacement surgery. Our data show that MDS MSCs provided CD34⁺ MDS cells with significantly enhanced engraftment capacity in all five patients tested ($p = 0.03$; Figures 5A and 5B)

MDS Patient-Derived MSCs Exhibit Specific Key Molecular Features, which Can Be Directly Induced by MDS Cells

To determine whether MDS-derived MSCs are altered, we compared their transcriptomes with age-matched healthy

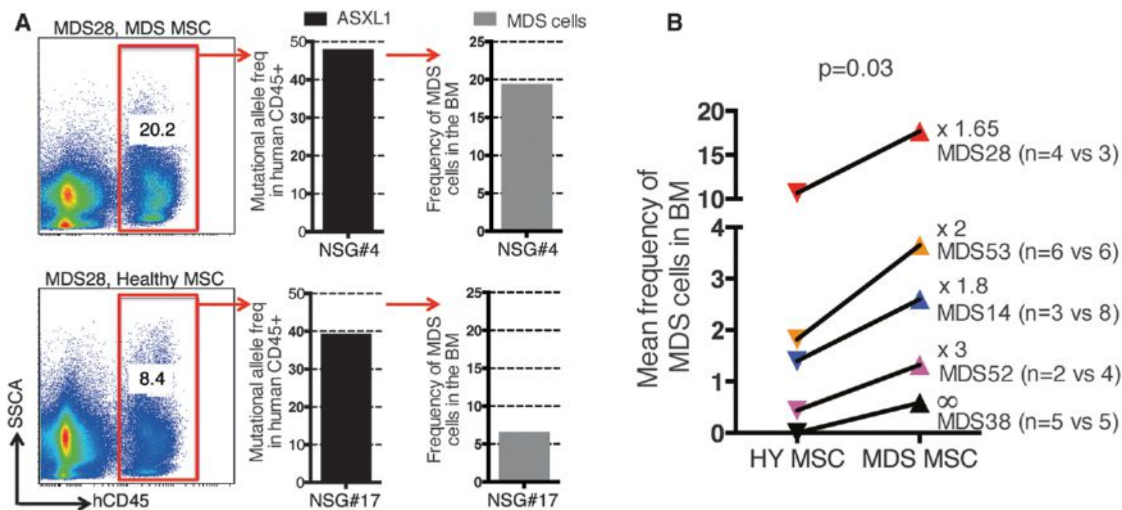


Figure 5. Comparison of MDS Engraftment with MDS MSCs as Compared to Age-Matched Healthy MSCs

(A) Exemplary FACS plots depicting differential MDS engraftment of MDS28 patient sample in NSG mice cotransplanted with either MDS MSCs or healthy MSCs derived from an age-matched donor (left) and inference of the frequency of engrafted MDS cells as determined by multiplication of the fraction of $hCD45^{+}$ cells carrying the MDS-specific molecular lesion (here, *ASXL1*) by the percentage of $hCD45^{+}$ cells in the bone marrow of engrafted mice (graphs on the right).

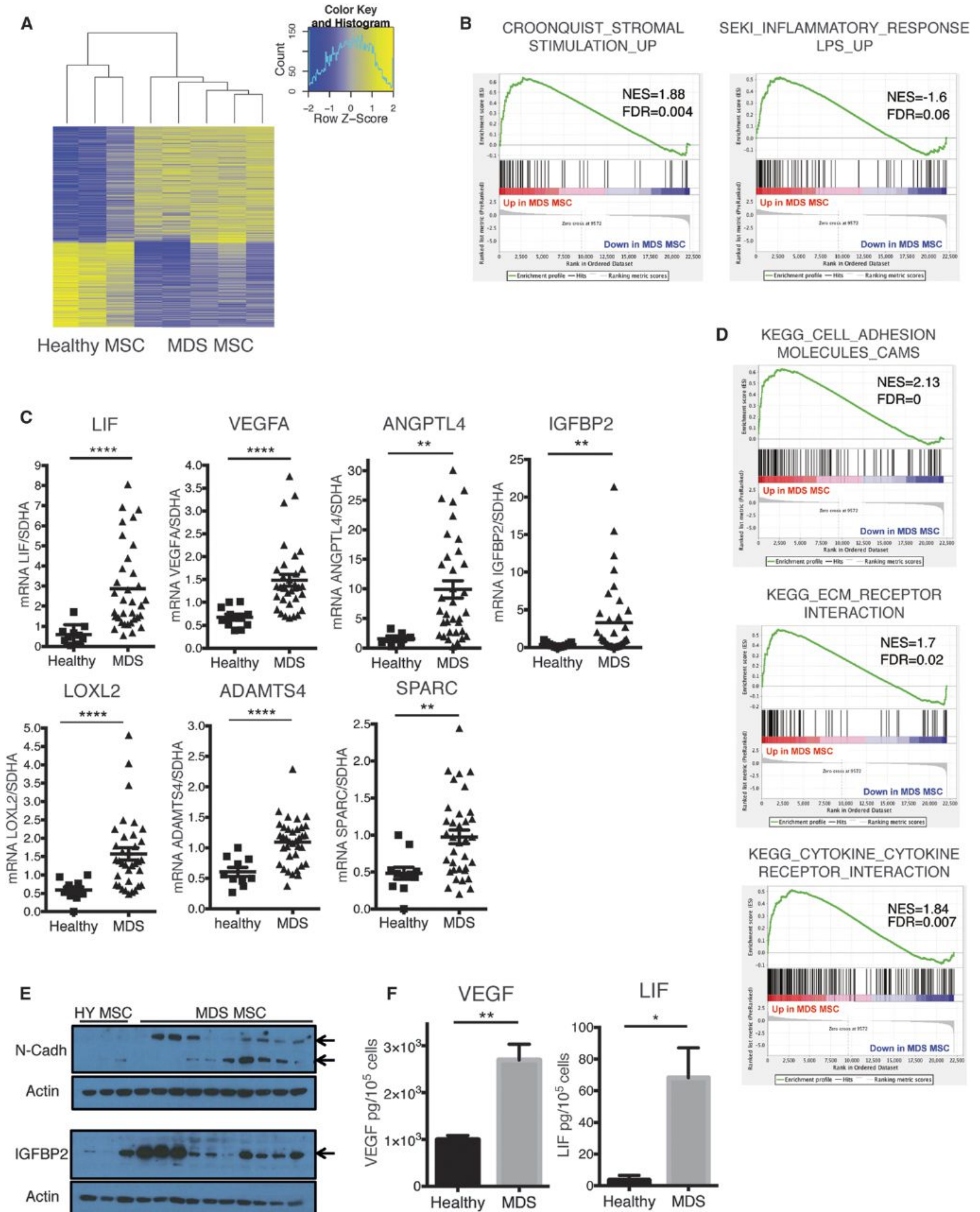
(B) Summary of paired analysis performed as described in (A) with five different primary patients (MDS14, MDS28, MDS38, MDS52, and MDS53). Data show a significant increase in the mean frequency of MDS cells engrafting when using MDS-derived MSCs (one-sided Wilcoxon test; $p = 0.03$).

See also Table S1.

MSCs (healthy MSCs, n = 3; MDS MSCs, n = 5) by RNA NGS. This analysis revealed 1,008 differentially expressed genes (q value < 0.1; 584 upregulated and 424 downregulated in MDS MSCs; Figure 6A). Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) revealed that MDS MSCs exhibit a significant depletion of gene sets associated with adipogenesis with a concomitant enrichment in gene sets reflective of a mesenchymal and osteoprogenitors cell fate (Figures S4A and S4B). In addition, MDS MSCs exhibit signs of ongoing stromal stimulation and response to an inflammatory environment (Figure 6B). This is paralleled by the increased expression of genes associated with fibrosis (*LOXL2*, *SPARC*, and *ADAMTS4*), a clinical feature often observed in MDS (Figure 6C). Most importantly, our analysis also identified gene sets including cellular adhesion, extracellular matrix remodeling, and cytokine-cytokine receptor interaction to be significantly enriched in MDS MSCs (Figure 6D). These data support the view that patient MSCs might establish a specific pattern of MSC-hematopoietic MDS cell interaction within the diseased bone marrow. To validate some of these candidates, real-time RT-PCR, western blotting, and ELISA analysis were performed (Figures 6C, 6E, 6F, and S4C). Although statistical significance was achieved in the entire cohort for several factors (*VEGF-A*, *LIF*, and *ANGPTL4* and *SPARC*, *IGFBP2*, *ADAMTS4*, and *LOXL2*), others showed robust differential expression in only few patients' samples (*CCL26* and *ANG1*), reflecting an expected interpatient heterogeneity (data not shown). CDH2 (N-Cadherin), an important adhesion molecule involved in the control of HSC niche interactions and MSC-mediated protection of CML progenitors from tyrosine kinase inhibitors (Zhang et al., 2013), is also found to be highly upregulated in MDS MSCs (Figure 6E). In addition, despite being cultured in normoxic conditions, MDS MSCs maintain a strong hypoxia signature, suggesting that this program is maintained by cell-intrinsic changes at the genetic or epigenetic level (Figure S4D). Altogether, these data show that MDS MSCs have an intrinsically altered pattern of gene expression, including a number of processes involved in intercellular crosstalk, that may all contribute to their capacity to support MDS hematopoietic cells in the secondary host.

Finally, in order to test the possibility that hematopoietic MDS cells may directly induce changes in their surrounding stromal cells, we developed an in vitro coculture system in order to evaluate the effect of these cells on a healthy age-matched stroma. Healthy old MSCs were isolated from several primary donors and cocultured with either an MDS cell line, MDSL (Matsuoka et al., 2010), or primary whole bone marrow isolated from lower-risk MDS patients (Figures 7A–7C). We FACS purified the stromal cells 24 hr postincubation and evaluated the expression of *LIF* as a read out. The data show strong *LIF* induction by the exposure of healthy MSC to the MDSL (Figure 7B) or different patient-derived primary MDS bone marrow cells (Figure 7C). Importantly, exposure of the same MSCs to healthy age-matched bone marrow only marginally affected *LIF* expression (Figure 7C). These data indicate that diseased bone marrow cells are likely to play an active role in the "reprogramming" of their bone marrow niche during disease development and/or progression by possibly converting it into a self-supportive one.

Notably, in our xenotransplant model, the injected MSCs (both MDS and healthy) remained present exclusively in the injected bones for up to 4 weeks posttransplantation (Figures S5A–S5C). However, we could demonstrate engraftment of MDS-derived cells in both injected and noninjected femurs (Figure S5D). Combined with the observation that MDS hematopoietic cells can reprogram a healthy niche, these data support a model in which MDS cells further expand and migrate after the initial engraftment of the injected bone and then install disease



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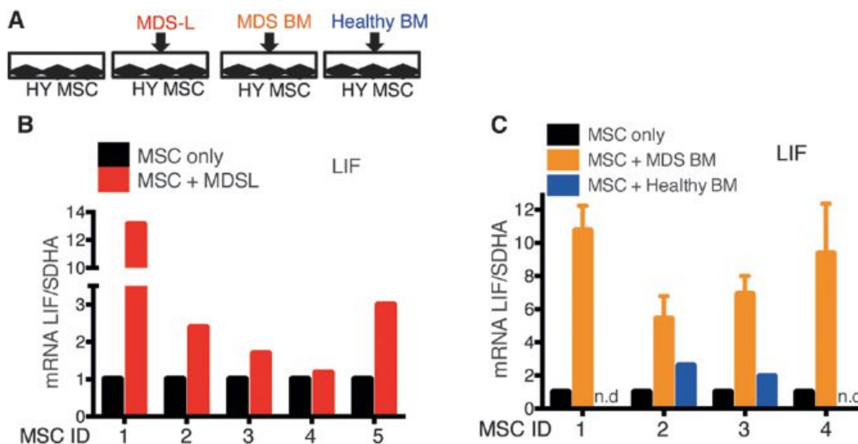

Figure 7. Exposure of Healthy MSCs to MDS Bone Marrow Leads to Altered Gene Expression

(A and B) Experimental scheme and analysis of *LIF* expression levels by qRT-PCR in five independent primary healthy old MSC cultures that were exposed to an MDS cell line (MDS-L). Data are depicted as fold change in comparison to the MSC only control culture.

(C) Analysis of *LIF* expression levels by qRT-PCR in four independent primary healthy old MSC cultures (HY MSC, black bars) that were cocultured with primary bone marrow cells derived from lower-risk MDS patients (MDS BM, orange bars). Healthy MSC culture number 1 (MSC1) was cocultured with samples MDS101 and MDS102 in two independent experiments. MSC2 was cocultured with samples MDS101, MDS102, and MDS111 in three independent experiments. MSC3 was cocultured with samples in five independent experiments (MDS101, MDS102, MDS111, MDS25, and MDS54). MSC4 was cocultured with samples MDS101 and MDS102 in two independent experiments. Statistical analysis reveals that *LIF* induction is significantly different in settings with versus without MDS cells (paired Student's t test, ****$p < 0.0001$). Healthy MSC2 and MSC3 were also exposed to healthy old (65 years old) bone marrow as a control. Data are presented as mean fold change in comparison to the corresponding MSC culture only ± SEM. All MSC cultures were depleted of any hematopoietic cells by gating out $CD45^+$ and $CD235a^+$ cells by flow cytometry before being further processed for qRT-PCR analysis (data not shown). n.d., not determined.

to the noninjected bones because of their potential to influence the mouse bone marrow stromal environment. Future studies will be necessary to explore the exact mechanism of this phenomenon.

DISCUSSION

Critically, our study identifies an intricate interplay in human MDS between mutant hematopoietic cells and their MSCs. Our data show that patient-derived hematopoietic cells instruct healthy MSCs to acquire MDS MSC-like features. In turn, MDS MSCs produce a number of cytokines and other factors to further promote the development and expansion of diseased hematopoietic MDS stem cells and their progeny. The functional relevance of this diseased ''hematopoietic niche unit'' for the development and progression of MDS in patients is demonstrated by its capacity to propagate MDS after orthotopic intrafemoral transplantation into NSG or NSGS mice. In contrast, the sole transplantation of $CD34^+$ MDS hematopoietic cells by our group and others only gave rise to inefficient and often transient engraftment (Martin et al., 2010; Nilsson et al., 2000; Thanopoulou et al., 2004). The use of NSGS mice, which produce human IL3, GM-CSF, and SCF (Wunderlich et al., 2010) as recipients, not only further enhanced the level of engraftment of human MDS but also retained dysplastic morphologic features typical of MDS pathology. Nevertheless, preliminary data indicate that patient MSCs remain essential for the efficient engraftment of lower-risk MDS cells even in the context of NSGS recipients, further highlighting their essential role in MDS pathogenesis (data not shown).

Importantly, beyond MDS, other myeloid neoplasms, such as CML and a subset of AMLs, have proven to be very challenging to propagate in xenografts. In addition, some studies reported that AML and MDS MSCs carry karyotype abnormalities that might hint toward functional significance for disease pathogenesis (Blau et al., 2007; Flores-Figueroa et al., 2005). Therefore, it is tempting to speculate that, similar to lower-risk MDS, CML and at least the fraction of ''nonengrafter'' AMLs might fail to engraft NSG mice because of a lack of a supportive environment. Niche contribution to these human myeloid neoplasms remains underappreciated, and our data point to an important area for future investigations. Consequently, this suggests that higher-risk MDS or AMLs that are transplantable by injection of $CD34^+$ cells alone have most likely acquired molecular lesions allowing them to become independent of the supporting stromal signals. Alternatively, they exhibit increased potential to rapidly reprogram the mouse stroma in order to allow disease propagation.

Figure 6. Molecular Features of MDS MSCs in Comparison to Age-Matched Healthy MSCs

(A) Heat map of 1,008 differentially regulated genes between MDS MSCs (n = 5; patients 14, 16, 17, 18, and 20; median age = 71; mean age = 68.6 ± 4) and healthy age-matched MSCs (n = 3; median age = 74; mean age = 74.4 ± 12) as determined by RNA sequencing.

(B) GSEA of RNA sequencing data showing enrichment for stromal stimulation gene sets (left) and inflammatory response (right) in MDS MSCs.

(C) Validation of differential gene expression by quantitative RT-PCR of candidate genes in a larger and independent cohort of MDS (n = 36) and age-matched healthy MSCs (n = 10; Mann-Whitney test; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

(D) GSEA showing enrichment for adhesion molecules (left), extracellular matrix and receptor genes (middle), and cytokine receptor interaction (right) in MDS MSCs.

(E) Western Blot showing differential protein expression of CDH2 (N-Cadherin) and IGFBP2 in MDS MSCs as compared to healthy MSCs.

(F) Confirmation of differential levels of VEGFA and LIF by ELISA in culture supernatants of MDS derived MSCs in comparison to age-matched healthy MSCs (Mann-Whitney test, **$p < 0.01$, *$p < 0.05$).

See also Figure S4.

To date, there are no surface markers that allow us to distinguish MDS stem cells from normal HSCs. Thus, when establishing xenograft models, it is essential to prove that the engrafted cells are indeed disease-derived as opposed to reflecting the engraftment of residual healthy HSCs present in the patients' bone marrow. The mutational tracking effort in this study demonstrates that our niche-mediated xenograft model supports the engraftment of bona fide MDS clones. In total, our approach revealed significant engraftment in 70% (14 of 20) of the patient samples transplanted with MDS MSCs. The engraftment behavior did not correlate with any clinical feature or comorbidities. Importantly, among the 14 patient samples that showed significant engraftment activity with MDS MSCs, 72% (10 of 14) showed engraftment of an MDS clone, as characterized by molecular lesion and/or a strong myeloid-biased output, whereas the remaining 28% (4 of 14) showed coengraftment of a healthy and an MDS-derived stem cell. Furthermore, the presence of stem cell ($CD34^+CD38^-$) and progenitor phenotypes ($CD34^+CD38^+$) in all xenografts 16–28 weeks posttransplantation, combined with the detection of typical MDS molecular lesions in both lymphoid and myeloid lineages in xenografts, is consistent with the engraftment of a patient derived "diseased" stem cell capable of long-term multilineage hematopoiesis. This finding argues in favor of a multipotent stem cell origin of the disease, supporting the hypotheses of others (Lawrence et al., 1987; Tehranchi et al., 2010; Thanopoulou et al., 2004; White et al., 1994). The demonstration that only $Lin^-CD34^+CD38^-$ MDS cells are able to transplant the disease further supports this conclusion and is in line with other myeloid disorders, such as CML and AML, which are believed to be initiated by mutations in normal HSCs (Corces-Zimmerman et al., 2014; Jan et al., 2012; Shlush et al., 2014; Sloma et al., 2010). This is corroborated by the results showing serial engraftment capacity, even if the MDS chimerism was relatively low in this setting. However, similar low levels of engraftment in a serial transplant setting have also been reported for aged normal HSCs (Dykstra et al., 2011), reflecting the likely reduced self-renewal capacity of aged stem cells in mouse and human.

The presence of human preleukemic HSCs, which can survive chemotherapy and provide a potential source for relapse, has recently been demonstrated by sequencing human cells in AML xenografts (Shlush et al., 2014; Corces-Zimmerman et al., 2014). In addition, whole-exome sequencing revealed that MDS bone marrow consists of distinct subclones, which are in continuous evolution during disease progression (Walter et al., 2012). The combination of our xenotransplantation workflow with quantitative mutational analyses now allows the investigation of such clonal composition and hierarchy in MDS in vivo. For example, our data imply that lower-risk MDS is driven by founder mutations such as *TET2* that initially occur in normal HSCs but then form the basis for further clonal evolution. Importantly, identification of mutations present at the stem cell level in the founder clone, as well as their specific targeting, is of relevance for the development of new treatment strategies and disease monitoring.

The identification of patient-derived MSCs as a critical functional component of lower-risk human MDS may well be relevant for other less aggressive hematological malignancies. Although it is difficult to exclude the formal possibility that a large excess of human cells might abrogate "nonspecific" causes of decreased engraftment, our data show that, in comparison to age-matched healthy MSCs, MDS MSCs significantly enhance MDS engraftment. This strongly argues in favor of the existence of a specific mechanism by which MDS MSCs support MDS $CD34^+$ engraftment in vivo. Our data are in line with recent evidence from mouse genetic studies suggesting that alterations in niche cells alone are sufficient to drive the development of myeloid malignancies in mice (Kode et al., 2014; Raaijmakers et al., 2010; Walkley et al., 2007). All of the factors differentially expressed between MDS and healthy MSCs, such as *LIF* (da Silva et al., 2005; Escary et al., 1993), *VEGFA* (Rehn et al., 2011), IGFBP2 (Garcia-Manero, 2012; Huynh et al., 2011), and *ANGPTL4* (Drake et al., 2011; Zheng et al., 2012), are known to promote survival and proliferation of both mouse and human HSPCs. Our finding that some of these factors can be induced by exposure of healthy MSCs to diseased primary MDS bone marrow cells, but not a healthy age-matched counterpart, is consistent with recent reports suggesting that leukemic cells can alter their niche counterpart in genetic mouse models of CML and AML (Schepers et al., 2013; M. Hanoun, 2013, Am. Soc. Hematol., abstract). These data support the view that a specific pattern of MSC-hematopoietic cell interaction exists within the diseased bone marrow and most likely contributes to the progressive bone marrow clonality and fibrosis frequently observed in MDS patients. Thanks to this approach, it is now possible to dissect the cellular and molecular components of this MDS niche unit in vivo, which may lead to the design of targeted strategies aimed at disrupting essential MDS MSC niche interactions. Moreover, the possibility to efficiently establish MDS xenografts from lower-risk MDS patients generates a platform for personalized oncology. Patients are still alive at the time the models are established, allowing assessment and possibly targeting of MDS pathology at the level of individual patients.

EXPERIMENTAL PROCEDURES

Detailed procedures can be found in the Supplemental Experimental Procedures.

Patient and Healthy Donor Bone Marrow Samples

MDS samples were collected from diagnostic bone marrow aspirations of MDS patients treated in the Department of Hematology and Oncology of the University Hospital Mannheim, Germany, after written informed consent. Bone marrow samples of healthy age-matched donors were obtained from residual femur specimen accrued from hip replacement surgery after written informed consent. The use of human samples was approved by the Institutional Review Board of the Medical Faculty Mannheim, University of Heidelberg, Germany, in accordance with the Declaration of Helsinki. Patient characteristics are summarized in Table S1.

Molecular Analyses

High-Density SNP Array Analyses

High-density SNP array analysis was carried out as previously reported (Nowak et al., 2012).

Screening for Commonly Mutated Genes in MDS

NGS with a screening panel for commonly mutated genes in MDS was performed as previously described (Grossmann et al., 2013; Kohlmann et al., 2011). In addition, exome sequencing was performed on an Illumina HiSeq2000 platform.

Pyrosequencing

For all mutations detected in MDS samples (single-nucleotide variations and InDels up to 4 bp), custom primer sets for validation and molecular tracking

were designed. Pyrosequencing and data analysis were performed on a Pyromark ID system (QIAGEN).

Fluorescence In Situ Hybridization

Interphase FISH was performed with the following probes: XL 5q33, D-5057-100-OG, XL AML1, and D-5027-100-OG (Metasystems), and quantitative analyses were carried out in the Munich Leukemia Laboratory.

RNA Sequencing Analysis and GSEA

RNA transcriptome sequencing was performed on a HiSeq2000 platform (Illumina). Aligned reads were converted into count tables with the htseq-count program version 0.5.4 with the gene annotation file used for read mapping. Differentially expressed genes were called with the DESeq2 package in R/Bioconductor according to the procedure outlined in the vignette. Correction for multiple testing was performed with the Benjamini-Hochberg procedure. All genes were ranked according to their log fold change and submitted to the Pre-Ranked GSEA tool and compared with 3,786 gene sets from the C2 collection of MSigDB.

Quantitative RT-PCR

RNA samples were transcribed with the SuperScript VILO cDNA synthesis kit according to the manufacturer's instructions with additional oligo-dT primers (Invitrogen). Quantitative RT-PCR was performed with the ABI Power SYBR Green Master Mix (Life Technologies). PCR reactions were performed on a Viia7 (Life Technologies) with the primers listed in Supplemental Experimental Procedures.

Flow Cytometry Analysis and Cell Sorting

FACS analysis was performed on a BD LSR Fortessa, and sorting was performed on FACSAria II and FACSAria III systems (BD Biosciences). Antibodies used are described in the Supplemental Experimental Procedures. The FACS-sorted populations used in the experiments described in Figures 3D and 3E were reanalyzed and showed over 98% purity (data not shown).

Mouse Experiments

NSG and NSGS mice were purchased from the Jackson Laboratory. Females 6–8 weeks of age were sublethally irradiated (200 cGy) before the cells were injected in the femoral bone marrow cavity. All xenotransplants were performed with 10^5 $CD34^+$ cells along with 5×10^5 MSCs unless otherwise indicated. Sample preparation is described in the Supplemental Experimental Procedures. Where indicated, fine needle aspirates from the noninjected femur were performed in order to estimate engraftment. Primary mice were analyzed 16–28 weeks posttransplanation unless indicated otherwise. For secondary transplants, FACS-sorted cells were mixed with MDS MSCs and injected according to the same procedure used for primary mice. Animals were housed under specific pathogen-free conditions at the central animal facility of the German Cancer Research Centre. All animal experiments were approved by the Animal Care Committee under Tierversuchsantrag numbers G74/12 and G210/12.

ACCESSION NUMBERS

The RNA sequencing data has been uploaded to the European Genotype Archive database for European Bioinformatics Institute and can be accessed under accession number EGAS00001000716.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, five figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.02.014.

AUTHOR CONTRIBUTIONS

H.M., D.N., A.T., and W.-K.H. designed the study and wrote the manuscript. H.M. and D.N. executed most of the experiments and supervised collaborators. A.T. and W.-K.H. supervised the entire study. M.M. and J.-C.J. performed the molecular analysis and assisted with data interpretation and manuscript preparation. A.K. assisted in the establishment of the NGS analysis for molecular tracking. M.S. and C.H. performed FISH analysis. A.F. performed cytogenetic analysis. V.N., B.Z., J.O., C.K., K.M., S.F., and J.V. provided technical assistance for molecular analysis, primary MSC expansion, and mouse procedures. C.H., A.L., and C.E. assisted with RNA sequencing analysis. F.N., U.P., and N.M. contributed primary MDS patient samples. S.R., T.J., and H.R. provided healthy old bone marrow samples. E.R. and G.M. assisted with cytomorphological analysis.

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REFERENCES

Abdel-Wahab, O., Gao, J., Adli, M., Dey, A., Trimarchi, T., Chung, Y.R., Kuscu, C., Hricik, T., Ndiaye-Lobry, D., Lafave, L.M., et al. (2013). Deletion of Asxl1 results in myelodysplasia and severe developmental defects in vivo. J. Exp. Med. *210*, 2641–2659.

Bejar, R., Stevenson, K., Abdel-Wahab, O., Galili, N., Nilsson, B., Garcia-Manero, G., Kantarjian, H., Raza, A., Levine, R.L., Neuberg, D., and Ebert, B.L. (2011). Clinical effect of point mutations in myelodysplastic syndromes. N. Engl. J. Med. *364*, 2496–2506.

Bejar, R., Stevenson, K.E., Caughey, B.A., Abdel-Wahab, O., Steensma, D.P., Galili, N., Raza, A., Kantarjian, H., Levine, R.L., Neuberg, D., et al. (2012). Validation of a prognostic model and the impact of mutations in patients with lower-risk myelodysplastic syndromes. J. Clin. Oncol. *30*, 3376–3382.

Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., and Wang, C.Y. (2013). The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat. Med. *19*, 35–42.

Blau, O., Hofmann, W.K., Baldus, C.D., Thiel, G., Serbent, V., Schümann, E., Thiel, E., and Blau, I.W. (2007). Chromosomal aberrations in bone marrow mesenchymal stroma cells from patients with myelodysplastic syndrome and acute myeloblastic leukemia. Exp. Hematol. *35*, 221–229.

Corces-Zimmerman, M.R., Hong, W.J., Weissman, I.L., Medeiros, B.C., and Majeti, R. (2014). Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc. Natl. Acad. Sci. USA *111*, 2548–2553.

da Silva, C.L., Gonçalves, R., Crapnell, K.B., Cabral, J.M., Zanjani, E.D., and Almeida-Porada, G. (2005). A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Exp. Hematol. *33*, 828–835.

Drake, A.C., Khoury, M., Leskov, I., Iliopoulou, B.P., Fragoso, M., Lodish, H., and Chen, J. (2011). Human CD34+ CD133+ hematopoietic stem cells cultured with growth factors including Angptl5 efficiently engraft adult NOD-SCID Il2rγ-/- (NSG) mice. PLoS ONE *6*, e18382.

Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M., and de Haan, G. (2011). Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. J. Exp. Med. *208*, 2691–2703.

Escary, J.L., Perreau, J., Duménil, D., Ezine, S., and Brûlet, P. (1993). Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. Nature *363*, 361–364.

Flores-Figueroa, E., Arana-Trejo, R.M., Gutiérrez-Espíndola, G., Pérez-Cabrera, A., and Mayani, H. (2005). Mesenchymal stem cells in myelodysplastic syndromes: phenotypic and cytogenetic characterization. Leuk. Res. *29*, 215–224.

Garcia-Manero, G. (2012). Myelodysplastic syndromes: 2012 update on diagnosis, risk-stratification, and management. Am. J. Hematol. *87*, 692–701.

Grossmann, V., Roller, A., Klein, H.U., Weissmann, S., Kern, W., Haferlach, C., Dugas, M., Haferlach, T., Schnittger, S., and Kohlmann, A. (2013). Robustness of amplicon deep sequencing underlines its utility in clinical applications. J. Mol. Diagn. *15*, 473–484.

Haferlach, T., Nagata, Y., Grossmann, V., Okuno, Y., Bacher, U., Nagae, G., Schnittger, S., Sanada, M., Kon, A., Alpermann, T., et al. (2014). Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. Leukemia *28*, 241–247.

Huynh, H., Zheng, J., Umikawa, M., Zhang, C., Silvany, R., Iizuka, S., Holzenberger, M., Zhang, W., and Zhang, C.C. (2011). IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells. Blood *118*, 3236–3243.

Jan, M., Snyder, T.M., Corces-Zimmerman, M.R., Vyas, P., Weissman, I.L., Quake, S.R., and Majeti, R. (2012). Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. Sci. Transl. Med. *4*, ra118.

Kode, A., Manavalan, J.S., Mosialou, I., Bhagat, G., Rathinam, C.V., Luo, N., Khiabanian, H., Lee, A., Murty, V.V., Friedman, R., et al. (2014). Leukaemogenesis induced by an activating β-catenin mutation in osteoblasts. Nature *506*, 240–244.

Kohlmann, A., Klein, H.U., Weissmann, S., Bresolin, S., Chaplin, T., Cuppens, H., Haschke-Becher, E., Garicochea, B., Grossmann, V., Hanczaruk, B., et al. (2011). The Interlaboratory RObustness of Next-generation sequencing (IRON) study: a deep sequencing investigation of TET2, CBL and KRAS mutations by an international consortium involving 10 laboratories. Leukemia *25*, 1840–1848.

Lawrence, H.J., Broudy, V.C., Magenis, R.E., Olson, S., Tomar, D., Barton, S., Fitchen, J.H., and Bagby, G.C., Jr. (1987). Cytogenetic evidence for involvement of B lymphocytes in acquired idiopathic sideroblastic anemias. Blood *70*, 1003–1005.

Martin, M.G., Welch, J.S., Uy, G.L., Fehniger, T.A., Kulkarni, S., Duncavage, E.J., and Walter, M.J. (2010). Limited engraftment of low-risk myelodysplastic syndrome cells in NOD/SCID gamma-C chain knockout mice. Leukemia *24*, 1662–1664.

Matsuoka, A., Tochigi, A., Kishimoto, M., Nakahara, T., Kondo, T., Tsujioka, T., Tasaka, T., Tohyama, Y., and Tohyama, K. (2010). Lenalidomide induces cell death in an MDS-derived cell line with deletion of chromosome 5q by inhibition of cytokinesis. Leukemia *24*, 748–755.

Miller, P.H., Cheung, A.M., Beer, P.A., Knapp, D.J., Dhillon, K., Rabu, G., Rostamirad, S., Humphries, R.K., and Eaves, C.J. (2013). Enhanced normal short-term human myelopoiesis in mice engineered to express human-specific myeloid growth factors. Blood *121*, e1–e4.

Muguruma, Y., Matsushita, H., Yahata, T., Yumino, S., Tanaka, Y., Miyachi, H., Ogawa, Y., Kawada, H., Ito, M., and Ando, K. (2011). Establishment of a xenograft model of human myelodysplastic syndromes. Haematologica *96*, 543–551.

Muto, T., Sashida, G., Oshima, M., Wendt, G.R., Mochizuki-Kashio, M., Nagata, Y., Sanada, M., Miyagi, S., Saraya, A., Kamio, A., et al. (2013). Concurrent loss of Ezh2 and Tet2 cooperates in the pathogenesis of myelodysplastic disorders. J. Exp. Med. *210*, 2627–2639.

Nilsson, L., Astrand-Grundström, I., Arvidsson, I., Jacobsson, B., Hellström-Lindberg, E., Hast, R., and Jacobsen, S.E. (2000). Isolation and characterization of hematopoietic progenitor/stem cells in 5q-deleted myelodysplastic syndromes: evidence for involvement at the hematopoietic stem cell level. Blood *96*, 2012–2021.

Nowak, D., Klaumuenzer, M., Hanfstein, B., Mossner, M., Nolte, F., Nowak, V., Oblaender, J., Hecht, A., Hütter, G., Ogawa, S., et al. (2012). SNP array analysis of acute promyelocytic leukemia may be of prognostic relevance and identifies a potential high risk group with recurrent deletions on chromosomal subband 1q31.3. Genes Chromosomes Cancer *51*, 756–767.

Pang, W.W., Pluvinage, J.V., Price, E.A., Sridhar, K., Arber, D.A., Greenberg, P.L., Schrier, S.L., Park, C.Y., and Weissman, I.L. (2013). Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes. Proc. Natl. Acad. Sci. USA *110*, 3011–3016.

Papaemmanuil, E., Gerstung, M., Malcovati, L., Tauro, S., Gundem, G., Van Loo, P., Yoon, C.J., Ellis, P., Wedge, D.C., Pellagatti, A., et al.; Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium (2013). Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood *122*, 3616–3627, quiz 3699.

Raaijmakers, M.H. (2012). Myelodysplastic syndromes: revisiting the role of the bone marrow microenvironment in disease pathogenesis. Int. J. Hematol. *95*, 17–25.

Raaijmakers, M.H., Mukherjee, S., Guo, S., Zhang, S., Kobayashi, T., Schoonmaker, J.A., Ebert, B.L., Al-Shahrour, F., Hasserjian, R.P., Scadden, E.O., et al. (2010). Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature *464*, 852–857.

Rehn, M., Olsson, A., Reckzeh, K., Diffner, E., Carmeliet, P., Landberg, G., and Cammenga, J. (2011). Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. Blood *118*, 1534–1543.

Schepers, K., Pietras, E.M., Reynaud, D., Flach, J., Binnewies, M., Garg, T., Wagers, A.J., Hsiao, E.C., and Passegué, E. (2013). Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. Cell Stem Cell *13*, 285–299.

Shlush, L.I., Zandi, S., Mitchell, A., Chen, W.C., Brandwein, J.M., Gupta, V., Kennedy, J.A., Schimmer, A.D., Schuh, A.C., Yee, K.W., et al.; HALT Pan-Leukemia Gene Panel Consortium (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature *506*, 328–333.

Sloma, I., Jiang, X., Eaves, A.C., and Eaves, C.J. (2010). Insights into the stem cells of chronic myeloid leukemia. Leukemia *24*, 1823–1833.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA *102*, 15545–15550.

Tehranchi, R., Woll, P.S., Anderson, K., Buza-Vidas, N., Mizukami, T., Mead, A.J., Astrand-Grundström, I., Strömbeck, B., Horvat, A., Ferry, H., et al. (2010). Persistent malignant stem cells in del(5q) myelodysplasia in remission. N. Engl. J. Med. *363*, 1025–1037.

Thanopoulou, E., Cashman, J., Kakagianne, T., Eaves, A., Zoumbos, N., and Eaves, C. (2004). Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome. Blood *103*, 4285–4293.

Walenda, T., Bokermann, G., Ventura Ferreira, M.S., Piroth, D.M., Hieronymus, T., Neuss, S., Zenke, M., Ho, A.D., Müller, A.M., and Wagner, W. (2011). Synergistic effects of growth factors and mesenchymal stromal cells for expansion of hematopoietic stem and progenitor cells. Exp. Hematol. *39*, 617–628.

Walkley, C.R., Olsen, G.H., Dworkin, S., Fabb, S.A., Swann, J., McArthur, G.A., Westmoreland, S.V., Chambon, P., Scadden, D.T., and Purton, L.E. (2007). A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell *129*, 1097–1110.

Walter, M.J., Shen, D., Ding, L., Shao, J., Koboldt, D.C., Chen, K., Larson, D.E., McLellan, M.D., Dooling, D., Abbott, R., et al. (2012). Clonal architecture of secondary acute myeloid leukemia. N. Engl. J. Med. *366*, 1090–1098.

White, N.J., Nacheva, E., Asimakopoulos, F.A., Bloxham, D., Paul, B., and Green, A.R. (1994). Deletion of chromosome 20q in myelodysplasia can occur in a multipotent precursor of both myeloid cells and B cells. Blood *83*, 2809–2816.

Wunderlich, M., Chou, F.S., Link, K.A., Mizukawa, B., Perry, R.L., Carroll, M., and Mulloy, J.C. (2010). AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia *24*, 1785–1788.

Zhang, B., Li, M., McDonald, T., Holyoake, T.L., Moon, R.T., Campana, D., Shultz, L., and Bhatia, R. (2013). Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-β-catenin signaling. Blood *121*, 1824–1838.

Zheng, J., Umikawa, M., Cui, C., Li, J., Chen, X., Zhang, C., Huynh, H., Kang, X., Silvany, R., Wan, X., et al. (2012). Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development. Nature *485*, 656–660.

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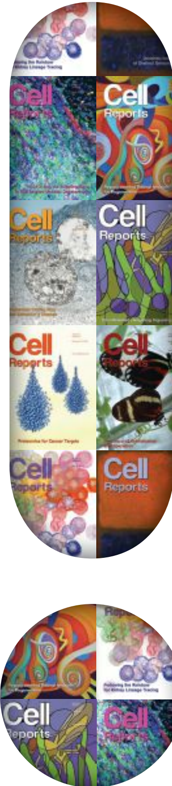

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Glioblastoma Stem Cells Generate Vascular Pericytes to Support Vessel Function and Tumor Growth

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SUMMARY

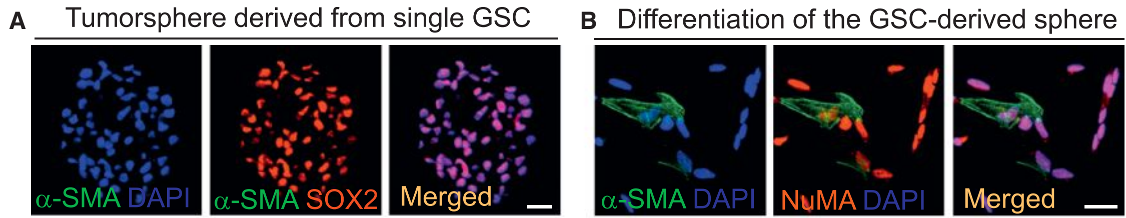
Glioblastomas (GBMs) are highly vascular and lethal brain tumors that display cellular hierarchies containing self-renewing tumorigenic glioma stem cells (GSCs). Because GSCs often reside in perivascular niches and may undergo mesenchymal differentiation, we interrogated GSC potential to generate vascular pericytes. Here, we show that GSCs give rise to pericytes to support vessel function and tumor growth. In vivo cell lineage tracing with constitutive and lineage-specific fluorescent reporters demonstrated that GSCs generate the majority of vascular pericytes. Selective elimination of GSC-derived pericytes disrupts the neovasculature and potently inhibits tumor growth. Analysis of human GBM specimens showed that most pericytes are derived from neoplastic cells. GSCs are recruited toward endothelial cells via the SDF-1/CXCR4 axis and are induced to become pericytes predominantly by transforming growth factor β. Thus, GSCs contribute to vascular pericytes that may actively remodel perivascular niches. Therapeutic targeting of GSC-derived pericytes may effectively block tumor progression and improve antiangiogenic therapy.

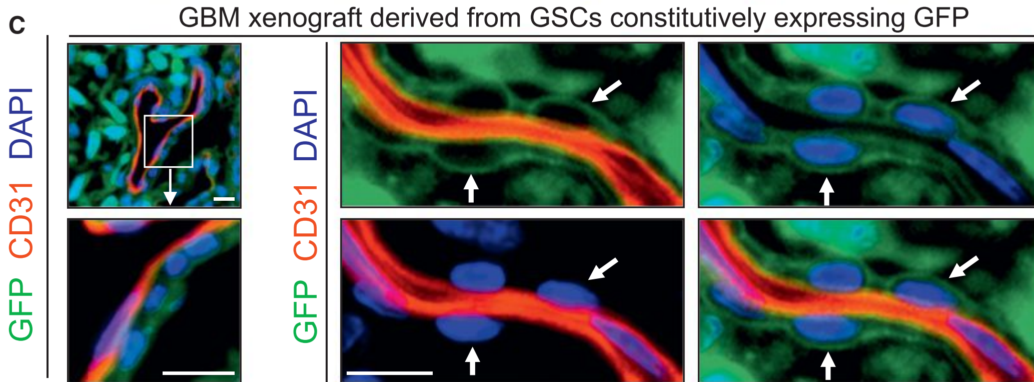
INTRODUCTION

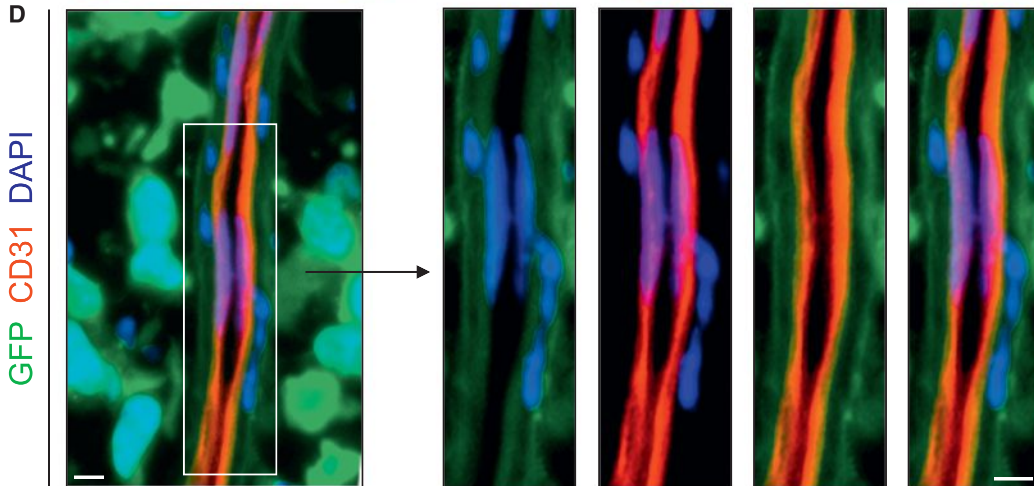
Glioblastomas (GBMs) are fatal tumors with florid vascularization that correlates with tumor malignancy and clinical prognosis (Norden et al., 2009). Targeting endothelial cells (ECs) has been a major focus of antiangiogenic therapeutics, although tumor vessels consist of two distinct but interdependent cellular compartments: ECs and pericytes (Bergers and Song, 2005; Carmeliet and Jain, 2011). However, most current therapies targeting ECs are not curative and may transform tumor growth pattern toward a more invasive phenotype in GBMs (Pàez-Ribes et al., 2009), suggesting that targeting ECs alone is not sufficient for effective tumor control. Therefore, further insights into tumor vascular development and maintenance have direct translational implications.

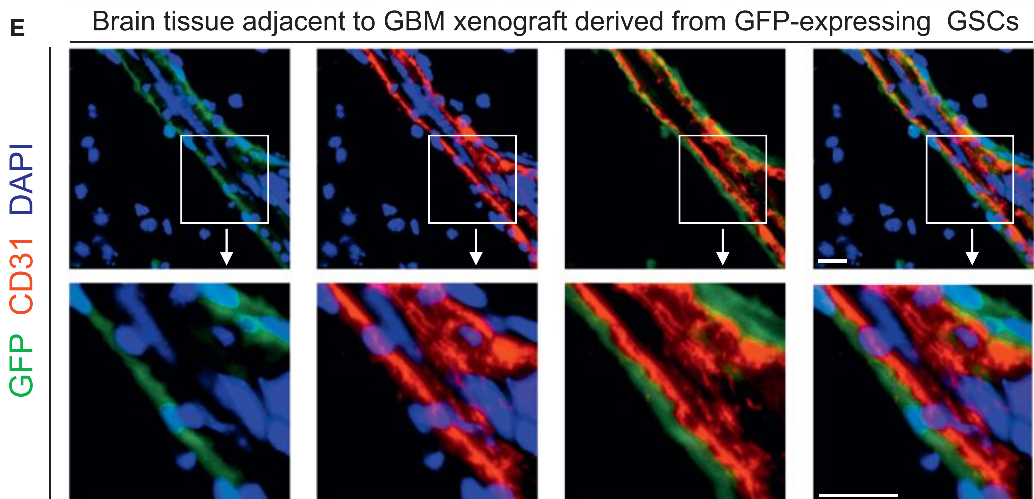
Vascular pericytes play critical roles in various physiological contexts, including support of vascular structure and function, maintenance of blood-brain barrier, facilitation of vessel maturation, and initiation of vessel sprouting (Armulik et al., 2010; Bell et al., 2010; Bergers and Song, 2005; Winkler et al., 2011). Pericytes and ECs communicate with each other by direct physical contact and reciprocal paracrine signaling to maintain vessel integrity and function (Franco et al., 2011; Carmeliet and Jain, 2011; Song et al., 2005). Altered association between pericytes and ECs has been shown in tumor vessels (Carmeliet and Jain, 2011; Winkler et al., 2011). Tumor vessels with less pericyte coverage appear more vulnerable to radiation and chemotherapy, suggesting that pericytes are critical to protect ECs and may promote therapeutic resistance (Bergers et al., 2003; Franco et al., 2011). When therapies target ECs in tumors, the pericyte network often maintains a functional core of pre-existing blood vessels (Carmeliet and Jain, 2011). The tumor vasculature frequently exhibits structural and functional abnormality with irregular pericytes on endothelial tubules. The pericyte-EC interaction also differs substantially between tumors and normal tissues (Morikawa et al., 2002; Winkler et al., 2011). However, the mechanisms underlying the abnormality and difference are poorly understood. To better understand the vascular development and maintenance in tumors and lay the foundation for improved targeting therapy, it is essential to determine the interplay between cancer cells and vascular compartments.

GBMs display remarkable cellular hierarchies with tumorigenic glioma stem cells (GSCs) at the apex (Bao et al., 2006a; Calabrese et al., 2007; Zhou et al., 2009), although the cancer









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stem cell (CSC) model remains controversial for some tumor types (Magee et al., 2012). We previously demonstrated that GSCs promote tumor angiogenesis through elevated expression of vascular endothelial growth factor (VEGF) (Bao et al., 2006b). This study has been extended by others (Ehtesham et al., 2009; Folkins et al., 2009). GSCs are often located in perivascular niches and interact with ECs in a bidirectional manner (Bao et al., 2006b; Calabrese et al., 2007). Within this context, there was an excitement generated by reports suggesting that GSCs may transdifferentiate into ECs (Ricci-Vitiani et al., 2010; Soda et al., 2011; Wang et al., 2010). These reports have been controversial because the frequency of GSC-EC conversion was not defined, and ECs do not contain cancer genetic alterations in human GBMs (Kulla et al., 2003; Rodriguez et al., 2012). Because pericytes are physically proximal to ECs on vessels, distinguishing ECs and pericytes by location alone poses a challenge. A complementary or competing hypothesis would be a lineage commitment of GSCs to vascular pericytes. There are important reasons to consider GSCs as potential pericyte progenitors. GSCs have the ability to undergo mesenchymal differentiation (deCarvalho et al., 2010; Ricci-Vitiani et al., 2008). GSCs share properties with neural stem cells (NSCs) that display the potential to transdifferentiate into pericytes (li et al., 2009; Morishita et al., 2007). Further, pericytes are similar to mesenchymal stem cells (MSCs) (Crisan et al., 2008). Thus, we investigated the potential of GSCs to generate vascular pericytes and contribute to the remodeling of perivascular niches and determined the significance of GSC-derived pericytes (G-pericytes) in maintaining functional vessels to support GBM tumor growth.

RESULTS

GSCs Are Able to Assume a Pericyte Lineage In Vitro

To investigate a potential lineage link between GSCs and pericytes, we initially examined the capacity of GSCs to differentiate into pericytes in vitro. GSCs were isolated from GBM tumors and validated through functional assays (self-renewal, multipotency, and tumor formation) as previously described (Bao et al., 2006a; Guryanova et al., 2011). Immunofluorescent (IF) staining of freshly sorted GSCs from primary GBMs and the GSC-generated tumorspheres demonstrated SOX2 expression but complete absence of the pericyte markers α smooth muscle actin (α-SMA) and NG2 (Figures S1A and S1B available online), supporting a lack of contamination of GSC populations by pericytes. After GSCs or tumorspheres were induced for differentiation, the differentiated cells contained a fraction (4%–11%) of cells expressing multiple pericyte markers (α-SMA, NG2, CD248, and CD146) (Figures S1C–S1E). To further determine GSC ability to assume a pericyte lineage, we examined the cellular fate of single GSC-derived tumorsphere that did not contain any cell expressing pericyte markers (Figure 1A). Upon differentiation, cells derived from the single GSC-derived tumorsphere contained a fraction of cells expressing pericyte markers (Figure 1B). To rule out potential contamination of host-derived pericyte progenitors in xenograft-derived GSCs, we performed secondary sorting of enriched GSCs with positive selection for the human cell-specific surface antigen TRA-1-85 and negative selection for the pericyte marker CD146. We confirmed that the single GSC-generated spheres derived from the resorted GSCs ($SOX2^+$) did not contain any cell expressing pericyte markers (Figure S1F), whereas differentiated cells derived from the single GSC-derived sphere contained pericyte-marker-expressing cells (Figure S1G). These pericyte-marker-positive cells also expressed the human-cell-specific nuclear antigen NuMA (Figure S1G), confirming that these pericytes were derived from human GSCs, but not from murine pericytes or their progenitors. Collectively, these data demonstrate that GSCs have the capacity to assume a pericyte lineage in vitro.

GSCs Give Rise to Vascular Pericytes in GBM Xenografts In Vivo

To extend the lineage analysis of GSCs in vivo, we examined the origin of pericytes in GBM xenografts and found that pericytes ($CD146^+CD248^+$; 2.63%–6.14% of total cells) sorted from the xenografts were largely positive for human NuMA and TRA-1-85 (Figures S1H, S1I, and S1L). In contrast, purified ECs ($CD31^+CD105^+$) from GBM xenografts were completely negative for human NuMA and TRA-1-85 (Figures S1J–S1L). We then performed a lineage tracing study by transducing GSCs with GFP constitutive expression and implanted the GSCs orthotopically to establish xenografts. Tumor sections of the xenografts derived from the green fluorescent protein (GFP)-labeled GSCs were immunostained for an EC marker (CD31) and several pericyte markers (α-SMA, Desmin, NG2, CD146, CD248, Ang1, CD13, and platelet-derived growth factor receptor β [PDGFRβ]) because these pericyte markers are expressed in normal brain and primary GBMs (Figures S2A–S2C). No tumor showed GFP-positive ECs, but most tumor vessels were adorned with GFP-positive cells with typical pericytic location and morphology on the vascular external surface (Figures 1C and 1D). IF analyses of pericyte markers further confirmed that expression of pericyte markers (Desmin, α-SMA, NG2, PDGFRβ, CD248, and CD146) overlapped with GFP in the majority (mean 78%, range 57%–89%) of pericytes (Figures 2A and 2B; Figures S2D and S2E), indicating that the majority of vascular pericytes were derived from GSCs. We validated this result in 21 GBM xenografts using GFP-labeled GSCs isolated from 12 primary GBMs and 9 GBM xenografts, suggesting that the contribution of GSCs to pericytes is a common event during

Figure 1. GSCs Have the Potential to Assume a Pericyte Lineage

(A) IF staining of SOX2 (a GSC marker) and α-SMA (a pericyte marker) in the single GSC-derived tumorsphere. Nuclei were stained with DAPI.

(B) IF staining of α-SMA and NuMA (a human cell-specific nuclear antigen) in differentiated cells derived from the single GSC-derived tumorsphere.

(C and D) In vivo lineage tracing of GSCs with GFP constitutive expression. Sections of GBM tumors derived from the GFP-labeled GSCs (D456 or CCF2170) were immunostained for CD31 to mark ECs and counterstained with DAPI. Arrows indicate GFP^+ cells with pericytic location.

(E) IF staining of CD31 in peritumoral brain adjacent to the GBM tumor derived from GFP-labeled GSCs (CCF2170).

All scale bars represent 25 μm. See also Figure S1.

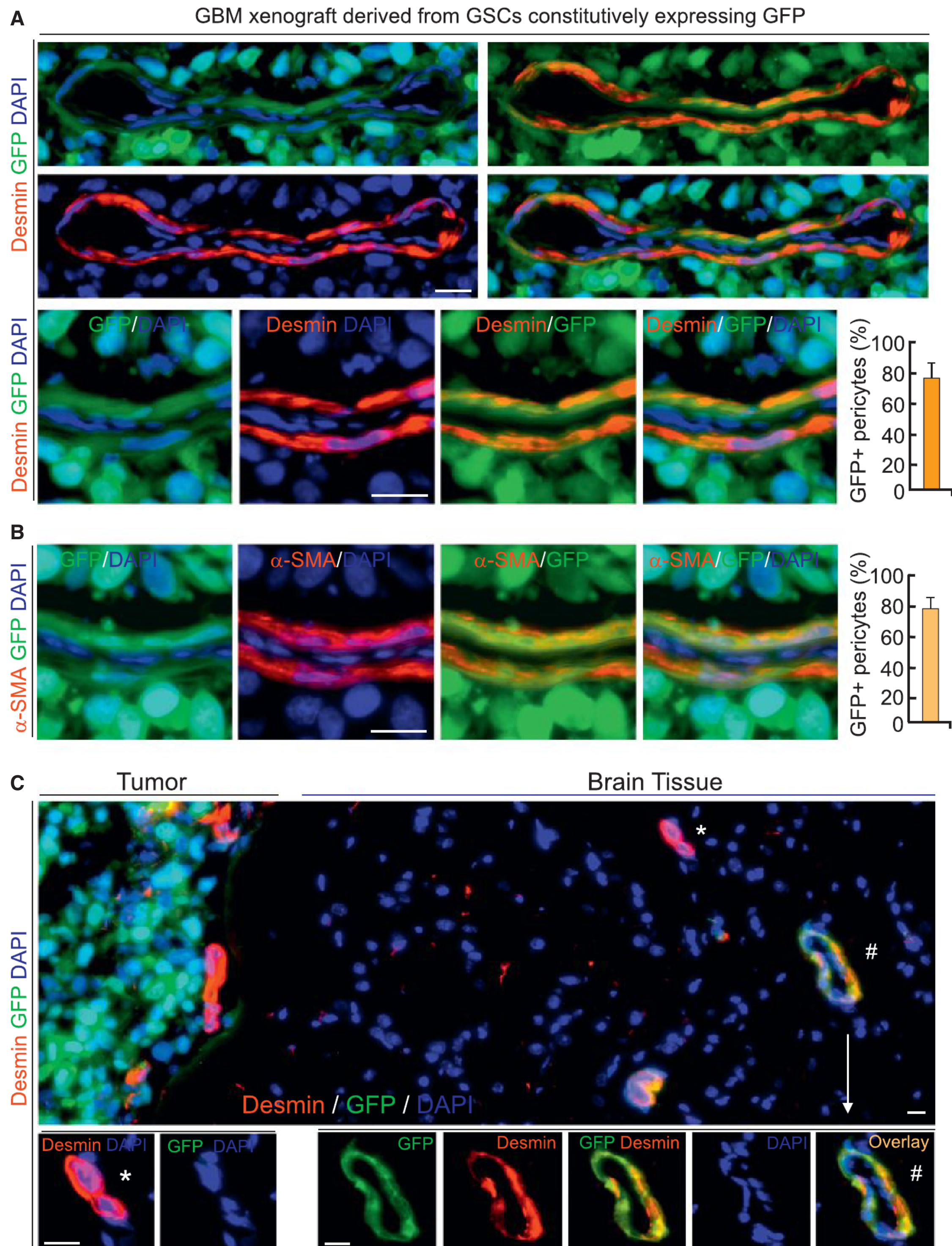


Figure 2. GSCs Generate Pericytes Expressing Specific Markers In Vivo

(A and B) In vivo lineage tracing of GSCs and IF staining of pericyte marker Desmin (A) or α-SMA (B) in GBM tumors derived from GFP-labeled GSCs (D456). Quantifications show fractions of G-pericytes (GFP^+ and $Desmin^+/\alpha\text{-}SMA^+$).

(C) IF staining of Desmin in peritumoral brain adjacent to GBM tumor derived from GFP-labeled GSCs (CCF1468). A vessel containing G-pericytes ($Desmin^+$ and GFP^+) in peritumoral brain was marked (#) and enlarged.

All scale bars represent 20 μm. The error bars represent SD. See also Figure S2.

GBM growth. Notably, a minor fraction (mean 22%, range 11%–43%) of vascular pericytes in the GBM xenografts did not overlap with GFP expression, indicating that these pericytes were host derived. Most tumor vessels had a mixture of GSC- and host-derived pericytes (Figure 2A). Taken together, these data demonstrate that GSCs have the capacity to generate the majority of vascular pericytes in GBM xenografts.

Peritumoral Brain Vessels Contain G-Pericytes

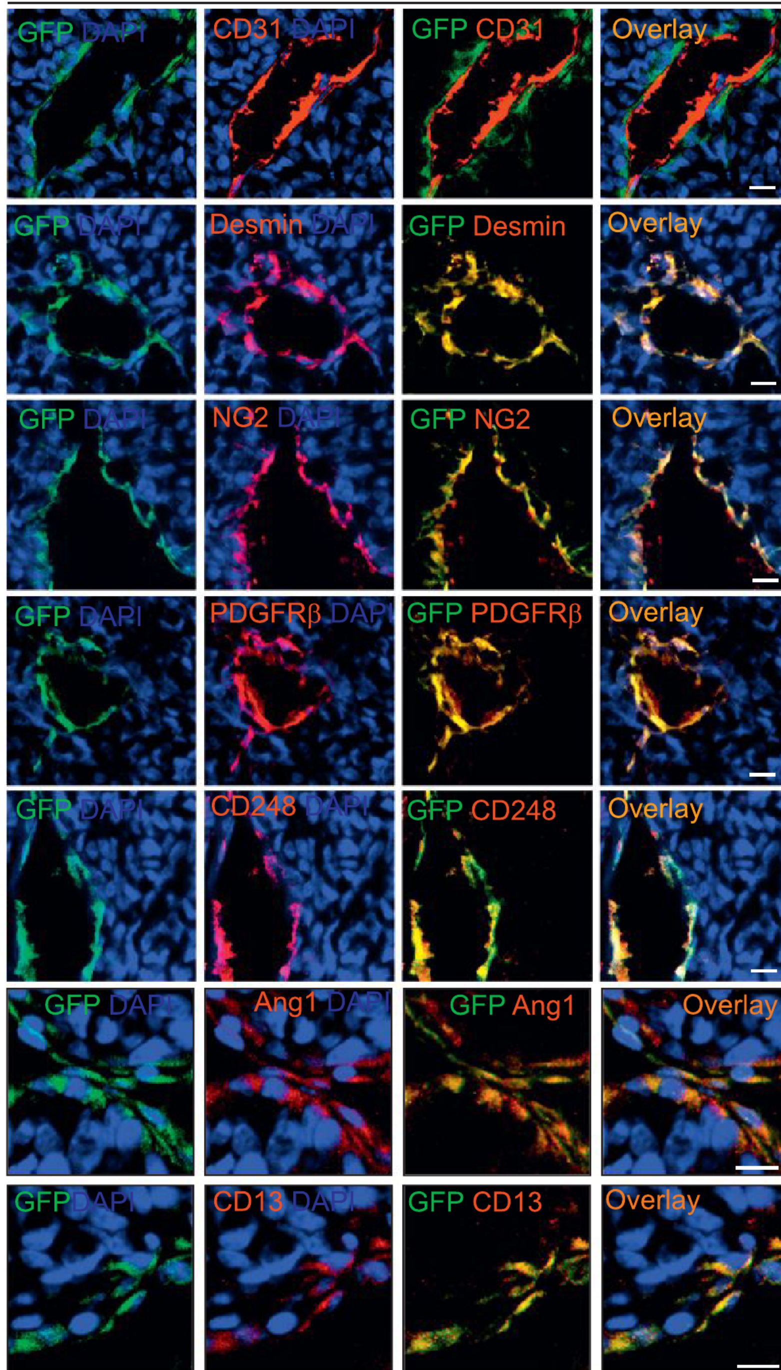
Because GBMs commonly invade into normal brain, we examined whether GSCs contribute to vascular pericytes in peritumoral brain. We found that a subset of vessels in peritumoral brain adjacent to the GFP-labeled GSC xenograft also contain GFP-positive pericytes (Figure 1E). IF analyses validated a fraction of vessels coexpressing pericyte markers and GFP and in brain tissue near the GFP tumor (Figure 2C). These data indicate that GSCs can also give rise to pericytes in the peritumoral brain. Notably, G-pericytes (GFP^+) were detectable not only in peritumoral brain but also in tumor-free brain up to 0.86 mm distant from the tumor edge, suggesting that GSCs were recruited by ECs in the peritumoral brain to generate pericytes. Thus, GSCs also generate vascular pericytes in the peritumoral brain.

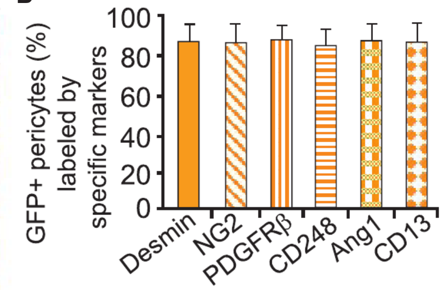
Validation of G-Pericytes by Lineage-Specific Fluorescent Reporters

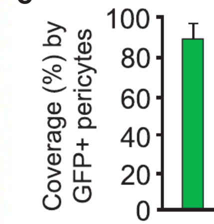
To provide direct evidence validating GSC capacity to generate pericytes in vivo, we performed in vivo cell lineage tracing of GSCs with a pericyte marker (Desmin or α-SMA) promoter-driven expression of GFP or mCherry, which served as fluorescent reporters of pericyte lineage. We cloned the human Desmin promoter (Li and Paulin, 1991) and α-SMA core promoter (Keogh et al., 1999; Nakano et al., 1991) and then generated lentiviral constructs for the Desmin promoter-driven GFP expression (DesPro-GFP) or α-SMA promoter-driven mCherry expression (αSMAPro-mCherry). We confirmed that the cloned Desmin and α-SMA promoters were functional and pericyte specific because GFP or mCherry expression occurred specifically in human brain vascular pericytes (HBVPs) (Figure S3A, left). We then implanted DesPro-GFP-transduced GSCs into mouse brains and examined tumor vessels by IF analysis. DesPro-driven GFP expression specifically marked perivascular cells that expressed pericyte markers, including Desmin, NG2, PDGFRβ, CD248, Ang1, and CD13 (Figures 3A–3C), validating that GSCs generated vascular pericytes in the GBM xenografts. The G-pericytes also expressed the gap junction protein connexin45 (Cx45) that is often localized at pericyte-EC contacts (Figures 3D and 3E). Notably, GFP-positive cells were mainly located in perivascular regions close to vessels but rarely detected in regions distant from vessels in tumors (Figure 3F). We further performed an additional pericyte lineage tracing of GSCs cotransduced with DesPro-GFP and αSMAPro-mCherry and detected coexpression of mCherry and GFP in perivascular cells (Figures 3G and 3H). GFP^+ perivascular cells were abundant around vessels and the majority of pericyte-marker-positive cells (>83%) expressed GFP (Figures 3A–3F), confirming that GSCs generated the majority of pericytes in these tumors. Tumor pericytes often exhibit abnormal morphologies, sometimes extending their processes away from the endothelium (Morikawa et al., 2002). The G-pericytes often displayed such irregular morphology (Figures 3A and 3F). Recent appreciation of intertumoral heterogeneity of GBMs has informed a mesenchymal subtype in contrast to proneural and classical subtypes (Verhaak et al., 2010). Interestingly, in vivo lineage tracing showed that mesenchymal GSCs have significantly greater ability to generate pericytes than classic and proneural GSCs in xenografts (Figures S3B and S3C; Table S1). Collectively, these data provide direct evidence demonstrating that GSCs have the capacity to generate pericytes in vivo.

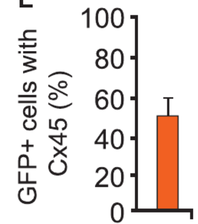
Because our in vivo cell fate tracing of GSCs with GFP constitutive expression failed to detect GSC-derived ECs (Figures 1C and 1D), we performed the cell lineage tracing of GSCs with an EC marker (CD31 or CD105) promoter-driven GFP expression to directly address whether GSCs generate ECs. We cloned the human CD105 (endoglin) promoter (Ríus et al., 1998) and the CD31 (PECAM-1) promoter restricted to ECs (Almendro et al., 1996; Gumina et al., 1997) and then generated lentiviral constructs for conditional GFP expression driven by CD31 or CD105 promoter (CD31Pro-GFP or CD105Pro-GFP). We validated that the cloned CD31 and CD105 promoters were functional and EC specific because CD31Pro- or CD105Pro-driven GFP expression specifically occurred in ECs (human brain microvessel endothelial cells [HBMECs]) (Figure S3A, right). To perform EC lineage tracing of GSCs, GSCs with CD31Pro-GFP or CD105Pro-GFP were orthotopically implanted into mouse brains. In confirmation with our earlier studies, no GFP expression was detectable in tumor ECs marked by CD31 and Glut1 staining (Figures S3D and S3E), further ruling out the possibility of GSC-derived ECs in GBM xenografts.

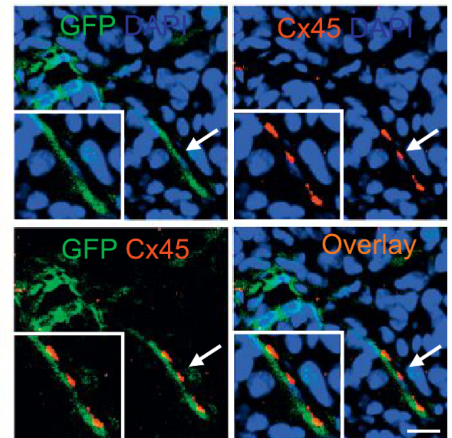
To further characterize the G-pericytes, we examined pericyte marker expression in G-pericytes and HBVP pericytes. We isolated G-pericytes by sorting GFP^+CD146^+ cells from GBM xenografts derived from the DesPro-GFP-GSCs. Comparative RT-PCR analyses of key pericyte markers (α-SMA, Desmin, CD248, NG2, CD146, and PDGFRβ) in the sorted G-pericytes and HBVPs confirmed similar marker expression in the GBM xenografts (Figure S4A). To address whether G-pericytes still express GSC markers after lineage switching, we examined expression of several putative GSC markers (SOX2, OLIG2, CD133, and Nestin) and pericyte markers in sorted GSCs ($CD15^+L1CAM^+$) and G-pericytes (GFP^+CD146^+). RT-PCR analyses showed that G-pericytes no longer express the GSC markers (Figure S4B). This result was confirmed by IF staining of SOX2, OLIG2, or Nestin on frozen sections of the DesPro-GFP-GSC xenografts. Consistently, GFP expression was turned on specifically in perivascular cells that rarely (<0.8%) expressed SOX2, OLIG2, or Nestin (Figure S4C, S4D, and S4F). In contrast, the SOX2, OLIG2, or Nestin-expressing cells (GSCs) are localized near perivascular niches (Figure S4C and S4D). The mutually exclusive expression of GSC and pericyte markers suggests that GSCs undergo differentiation to generate G-pericytes rather than being a GSC subpopulation adjacent to ECs in GBM tumors. In addition, G-pericytes do not express astrocyte markers such as glial fibrillary acidic protein (GFAP) and S100β (Figures S4A, S4E, and S4F), indicating that G-pericytes are

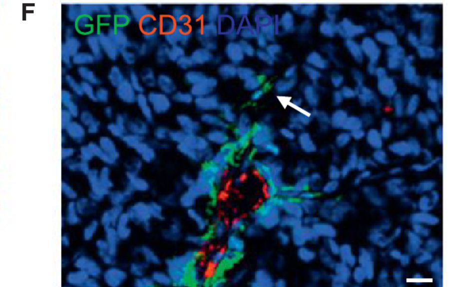


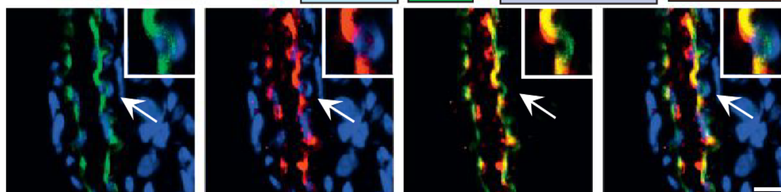


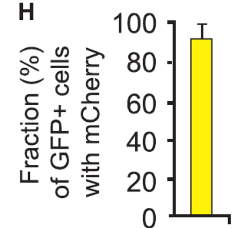












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not a subpopulation of astrocytes. Consistently, pericytes and astrocytes are distinct cell populations without overlapping expression of specific markers in primary GBMs (Figure S4G). These data demonstrate that G-pericytes are unique cells expressing specific pericyte markers.

Pericytes in Primary GBMs Are Commonly Derived from Neoplastic Cells

To examine whether pericytes are lineage related to cancer cells in human primary GBMs, we performed fluorescence in situ hybridization (FISH) analyses of common GBM genetic changes (Cancer Genome Atlas Research Network, 2008) in combination with IF staining of a pericyte marker (α-SMA) to determine if pericytes carry cancer genetic alterations in GBMs. Because gains of chromosome 7 (*EGFR* amplification) or losses of chromosome 10 (*PTEN* loss) are frequent in GBM cells, permitting a lineage tracing to the neoplastic cells, we employed DNA probes for centromeres of chromosome 7 (CEP-7) and 10 (CEP-10), *EGFR*, and *PTEN* to detect cancer genetic alterations in pericytes, ECs, and tumor cells in GBM tissue microarrays. FISH analyses showed the majority of tumor pericytes (mean 76%, range 58%–83%) carried the same genetic alterations (CEP-7 polysomy, *EGFR* trisomy or amplification, CEP-10 loss, or *PTEN* loss) as cancer cells in 49 GBMs (Figures 4A and 4B), indicating that tumor pericytes are commonly derived from cancer cells. In contrast, we rarely detected relevant genetic changes in ECs in these GBMs (Figures S5A and S5B). To further confirm these results, we isolated pericytes ($CD146^+CD248^+$; 2.18%–5.26% of total cells) and ECs ($CD31^+CD105^+$) from primary GBMs and performed similar FISH analyses. The majority (>72%) of sorted tumor pericytes (α-SMA$^+$) carried the same genetic alteration (CEP-7 polysomy) as matched GSCs (Figures 4C and 4D). In contrast, sorted ECs ($CD31^+CD105^+$) expressed Glut1 but did not share the GSC genetic alterations (Figure S5C and S5D). These results support a tumor source for pericytes, but not for ECs in human primary GBMs.

To further address whether pericytes in endogenous GBMs are derived from cancer cells, we examined pericytes in the genetically engineered mouse GBMs (Nestin-tva/Ink4a/Arf$^{-/-}$/HA-PDGFB models; Hambardzumyan et al., 2009). IF staining of hemagglutinin-tagged platelet-derived growth factor B (HA-PDGFB) and pericyte markers (Desmin, NG2, CD248, or α-SMA) showed that a significant fraction (mean 63%) of tumor pericytes expressed HA-PDGFB, supporting a tumor origin (Figure 4E, 4F; data not shown). In contrast, staining of EC markers (CD31 or Glut1) and HA-PDGFB showed no tumor-cell-derived ECs in these mouse GBMs (Figure S5E). These data demonstrate that pericytes in the genetically engineered mouse GBMs are also largely derived from neoplastic cells.

Selective Elimination of G-Pericytes Disrupts Tumor Vessels and Inhibits Tumor Growth

To determine the functional significance of G-pericytes, we examined effects of selective elimination of G-pericytes on vessels and tumor growth. GSCs were transduced with Desmin-promoter-driven expression of herpes simplex virus thymidine kinase (HsvTK) (Figure 5A) to achieve conditional HsvTK expression in G-pericytes. Because HsvTK metabolizes ganciclovir (GCV) into a toxic agent specifically in cells expressing HsvTK (Culver et al., 1992), G-pericytes expressing HsvTK should be sensitive to GCV and thus eliminated by GCV treatment. To confirm selective killing of G-pericytes expressing Desmin-promoter-driven HsvTK by GCV treatment, we generated a construct for coexpression of HsvTK and GFP under the same promoter (DesPro-TK-GFP) (Figures 5A and S6A). As expected, after the DesPro-TK-GFP-transduced GSCs were induced to differentiate, GFP was expressed in a fraction of differentiated cells (G-pericytes) (Figure S6B). Apoptotic detection showed that GCV treatment selectively induced apoptosis in cells coexpressing GFP and HsvTK (Figure S6C). These data indicate that selective elimination of G-pericytes is achievable by using Desmin-promoter-driven HsvTK conditional expression with GCV treatment.

To examine the impact of selective targeting of G-pericytes on tumor vessels, we implanted DesPro-TK-GFP-GSCs into mouse brains. Mice bearing the tumors were treated with vehicle control or GCV daily to induce HsvTK-mediated toxicity to G-pericytes. Apoptotic detection by TUNEL staining demonstrated that GCV treatment for 3 days selectively induced cell death in G-pericytes (GFP^+) in vivo (Figure 5B). Further, GCV treatment for 1 week caused almost a complete depletion of G-pericytes, collapse of vessel lumens, and disruption of endothelial walls in GBM tumors (Figures 5C, 5D, S6D and S6E). Moreover, measurement of vascular function by fluorescein isothiocyanate (FITC)-conjugated mega-dextran showed that GCV treatment for 1 week to deplete G-pericytes severely attenuated vascular function in the DesPro-TK-GSC xenografts, because perfusion of FITC-mega-dextran into the tumors was dramatically reduced (Figures 5E, 5F, S6F and S6G). Collectively, these data demonstrate that selective elimination of G-pericytes potently disrupts vascular structure and function in GBM tumors.

To evaluate the impact of selective targeting of G-pericytes on tumor growth, we initially used subcutaneous tumor experiments to track sequential tumor volumes. The established subcutaneous tumors derived from the DesPro-TK-GFP-GSCs were treated with GCV or vehicle control for 3 weeks. GCV treatment caused significant regression of the tumors (Figures 5G and 5H), indicating that selective elimination of G-pericytes by HsvTK-induced GCV toxicity inhibited tumor growth. To further validate

Figure 3. In Vivo Lineage Tracing of GSCs with Pericyte-Specific Promoter-Driven Fluorescent Reporters

(A–F) In vivo lineage tracing of GSCs with Desmin promoter-driven GFP (DesPro-GFP). Sections of GBM tumors derived from DesPro-GFP-GSCs were immunostained for an EC marker (CD31), a pericyte marker (Desmin, NG2, PDGFRβ, CD248, Ang1, or CD13) (A), or the pericyte-EC junction marker Cx45 (D). Quantifications show fractions of GFP^+ pericytes (B), coverage by GFP^+ pericytes (C), or the fraction of GFP^+ pericytes expressing Cx45 (E). An arrow indicates rare GFP^+ pericytes away from vessels (F).

(G and H) In vivo lineage tracing of GSCs with coexpression of Desmin promoter-driven GFP and α-SMA promoter-driven mCherry in GBMs. Quantification shows the fraction of GFP^+ cells with mCherry.

All scale bars represent 25 μm. The error bars represent SD. See also Figures S3, S4, and Table S1.

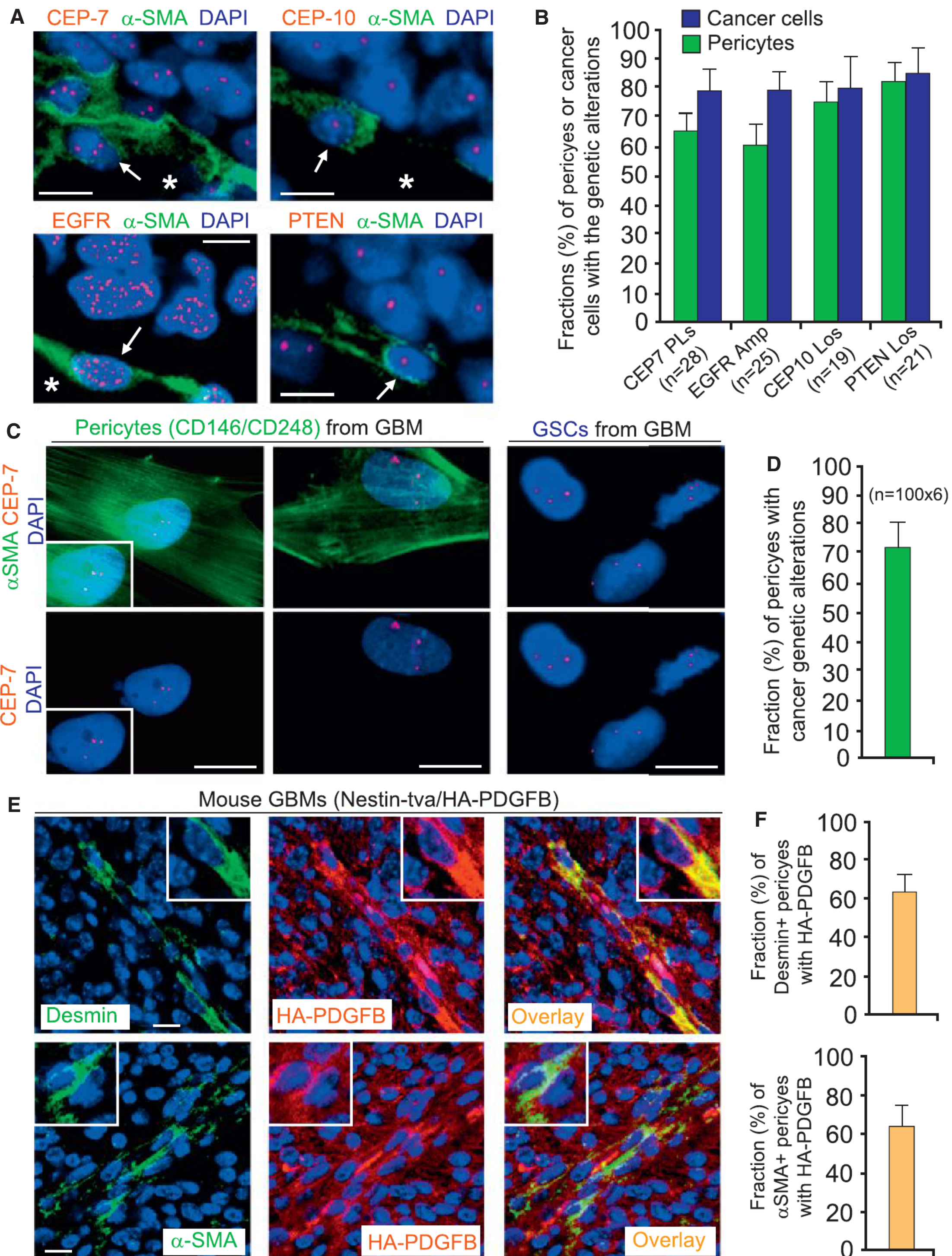


Figure 4. Pericytes Are Commonly Derived from Neoplastic Cells in Primary GBMs

(A and B) FISH analyses of genetic alterations with the CEP-7, CEP-10, EGFR, or PTEN probe (in red) in pericytes (α-SMA^{+}) in primary GBMs. Quantification shows average fractions of pericytes carrying the cancer genetic alterations (CEP-7 polysomy, EGFR amplification or trisomy, CEP-10 loss, or PTEN loss) in GBM tissue arrays (B).

(C and D) FISH analyses with CEP-7 probe in sorted pericytes (α-SMA^{+}) and GSCs from primary GBMs. Quantification shows the fraction (mean 72%) of pericytes carrying the GSC genetic alterations (D).

(E and F) IF staining of a pericyte marker (Desmin or α-SMA) and HA-PDGFB in the genetically engineered mouse GBMs (Nestin-tva/Ink4a/Arf$^{-/-}$/HA-PDGFB model). Quantifications show fractions (mean 63%) of HA-PDGFB^{+} pericytes (F)

The scale bars represent 10 μm (A and C) and 25 μm (E). The error bars represent SD. See also Figure S5.

this result in orthotopic tumors, we transduced GSCs either with DesPro-GFP (control) or DesPro-TK and implanted these GSCs into mouse brains to establish GBM xenografts. Both groups of mice bearing the tumors were administered with GCV to eliminate the G-pericytes expressing HsvTK. GCV treatment for 2 weeks caused extensive vessel regression in GBM tumors derived from DesPro-TK-GSCs, but not from DesPro-GFP-GSCs (Figures S6H and S6I). Moreover, GCV treatment for 3 weeks markedly inhibited intracranial tumor growth in GBM xenografts derived from DesPro-TK-GSCs, but not in control tumors from DesPro-GFP-GSCs (Figures 5I and 5J). Alternatively, treatment by GCV, but not vehicle control suppressed intracranial tumor growth in the GBM xenografts derived from DesPro-TK-GSCs (Figure S6J). As a consequence, GCV treatment significantly increased survival of animals implanted with the DesPro-TK-GSCs (Figure 5K). These data demonstrate that selective elimination of G-pericytes suppressed GBM tumor growth and malignant progression.

GSCs Are Recruited toward ECs via the SDF-1/CXCR4 Axis

To understand the mechanisms underlying GSC recruitment toward ECs, we examined whether GSCs can be recruited by HBMECs to support the maintenance of EC complexes in vitro. GSCs labeled with the green fluorescent tracer CFSE were mixed with HBMEC complexes labeled with the red fluorescent tracer CMTRX. Integration of GSCs-derived cells into EC complexes was detected on day 2 after cell mixing, and the integration stabilized the EC complexes for extended periods (2.6-fold) relative to EC complex alone (Figure 6A). To address whether pericyte lineage specification of GSCs can be induced by EC complexes, we cocultured GSCs with HBMECs and detected integration of G-pericytes by α-SMA staining (Figure 6B). Because the attachment of pericytes to ECs can be mediated through adherens junctions containing N-cadherin (Gerhardt et al., 2000), we examined N-cadherin expression and found that N-cadherin was localized to the contact sites between G-pericytes (α-SMA$^+$) and EC complexes (Figure 6B).

To define the molecular mechanisms underlying GSC recruitment by ECs, we analyzed the effect of several chemotactic factors (SDF-1α, PDGFB, and transforming growth factor β [TGF-β]) secreted by HBMECs on GSC migration. We found that SDF-1 potently stimulated GSC migration (Figures S7A and S7B), whereas PDGFB only modestly attracted GSCs. To further address whether HBMECs attract GSCs via SDF-1, we cocultured GSCs and HBMECs in separate chambers of transwells and detected that HBMECs potently attracted GSCs, an effect dependent on SDF-1 because an anti-SDF-1 antibody attenuated the effects (Figures S7C and S7D). Because ECs in brain and GBMs constitutively express SDF-1 (Kokovay et al., 2010; Komatani et al., 2009), we confirmed that abundant SDF-1 formed a gradient around vessels with greater SDF-1 proximal to vessels in brain and GBMs (Figure S7E).

Because SDF-1 is secreted by ECs and GSCs express the SDF-1 receptor CXCR4 (Ehtesham et al., 2009; Folkins et al., 2009), we hypothesized that brain ECs may recruit GSCs at least in part through the SDF-1/CXCR4 axis. CXCR4 knockdown in GSCs reduced the recruitment of GFP-labeled GSCs to EC complexes (Figures 6C and 6D). In addition, an SDF-1 blocking antibody significantly reduced the integration of GFP-labeled GSCs into HBMEC complexes (Figures S7F and S7G). As a further confirmation, we examined the effect of a CXCR4 inhibitor (AMD3100) on GSC recruitment to EC complexes. AMD3100 treatment significantly reduced the integration of GSCs (CMTRX labeled, in red) into CFSE-labeled HBMECs (in green) (Figures S7H and S7I).

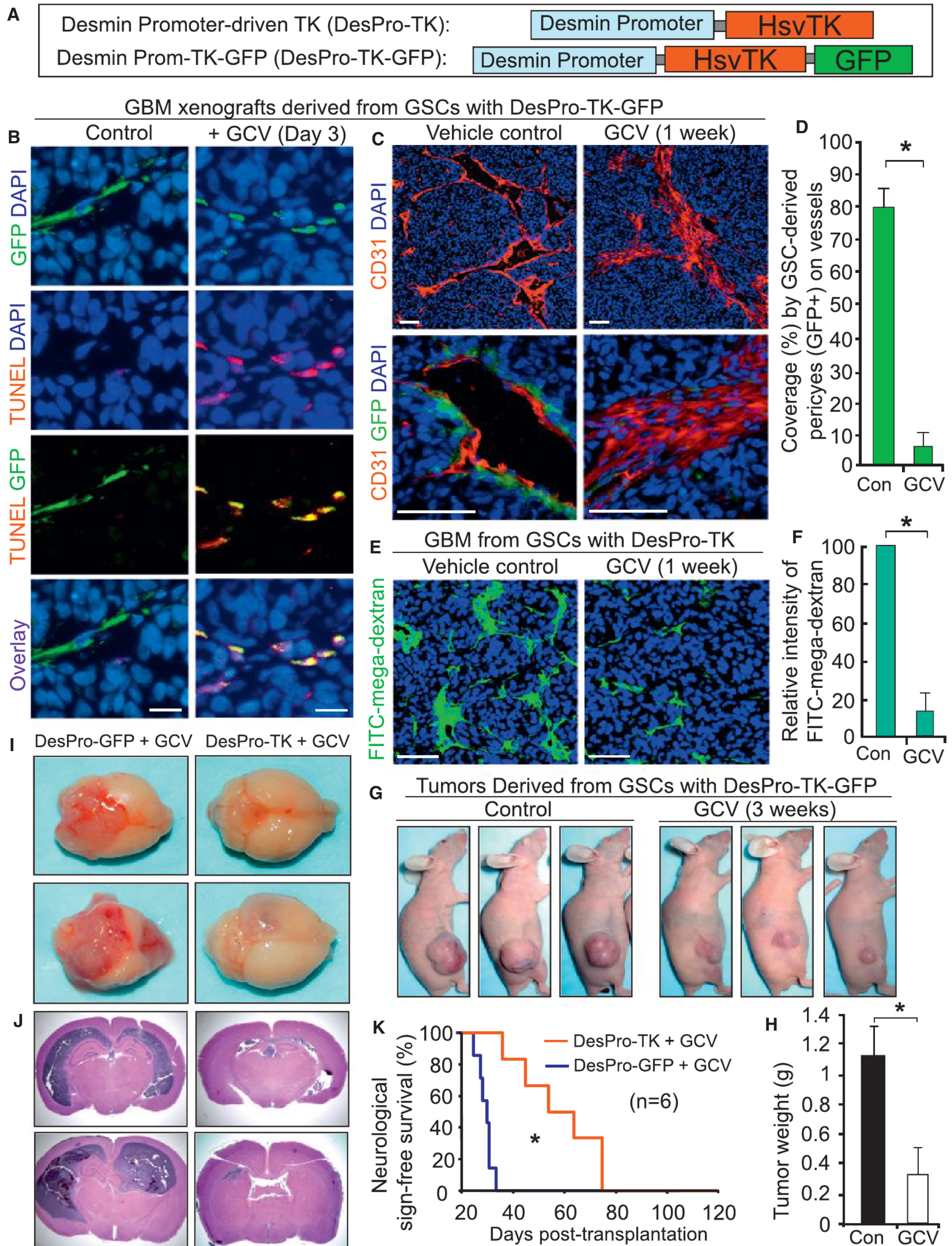
To further determine whether GSC recruitment to ECs depends on the SDF-1/CXCR4 axis during tumor vascularization, GFP-labeled GSCs were transduced with shCXCR4 or nontargeting small hairpin RNA (shNT) and implanted into mouse brains. In shNT xenografts, tumor vessels were covered with abundant G-pericytes (GFP$^+$ and Desmin$^+$), whereas G-pericytes and total pericyte coverage on vessels was significantly reduced in shCXCR4 xenografts (Figures 6E–6G). Immunohistochemical (IHC) staining confirmed that CXCR4 knockdown significantly decreased vessel density in the tumors (Figures S7J and S7K). Collectively, these data suggest that ECs recruit GSCs via the SDF-1/CXCR4 axis and that targeting this pathway reduces G-pericytes in GBMs.

TGF-β Induces Differentiation of GSCs into Pericytes

We next sought to understand the molecular mechanisms underlying the pericyte lineage specification of GSCs. To identify the potential factors inducing GSC differentiation into pericytes, we examined the effect of several EC-secreted cytokines (SDF-1, PDGFB, and TGF-β) on GSC differentiation into pericytes. Immunoblot analysis showed that TGF-β dominantly induced expression of α-SMA when GSCs were cultured in differentiation media (Figures 7A and 7B). IF staining of multiple pericyte markers (NG2, α-SMA, CD146 and CD248) confirmed that TGF-β treatment increased the fraction of cells expressing pericyte markers in the differentiated cells (Figures 7C and 7D; data not shown). Further, TGF-β treatment induced GFP-expressing cells in differentiated cells derived from DesPro-GFP-GSCs (Figures 7E and 7F). To address whether ECs induce GSC differentiation into pericytes through TGF-β, we cocultured DesPro-GFP-GSCs and HBMEC complexes and monitored GFP-expressing cells (G-pericytes) over time. GFP$^+$ cells were induced and integrated into EC complexes, an effect that was attenuated by incubation of the EC complexes with an anti-TGF-β antibody (Figure 7G). Immunoblot analysis validated that coculture of GSCs with HBMECs or their conditioned media induced expression of pericyte marker α-SMA in differentiated cells, an effect that was reduced by a TGF-β neutralizing antibody (Figure 7H). Collectively, these data demonstrate that HBMECs induce pericyte lineage specification of GSCs at least in part through TGF-β. Thus, the recruitment of GSCs toward ECs via the SDF-1/CXCR4 axis and the induction of GSC differentiation into pericytes by TGF-β are two events controlled by different molecular mechanisms (Figure 7I).

DISCUSSION

Pericytes play essential roles to maintain functional vessels to support tumor growth. Tumor pericytes are thought to be derived from their progenitors from the surrounding normal



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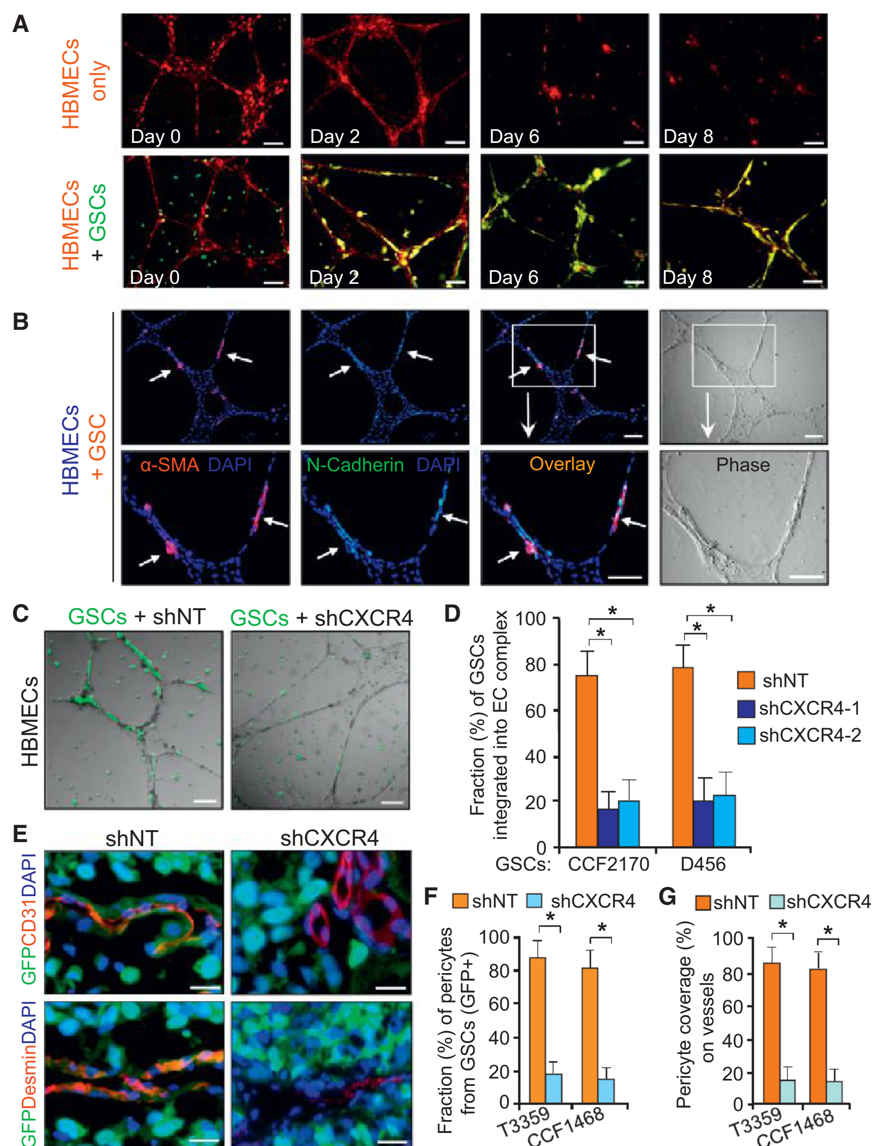

Figure 6. GSCs Are Recruited toward ECs through the SDF-1/CXCR4 Axis to Support Endothelial Complex

(A) In vitro endothelial complex formation of HBMECs (labeled with red fluorescent tracer CMTRX) with or without GSCs (labeled with CFSE, in green).

(B) IF staining of α-SMA and N-cadherin in complexes of HBMECs and GSC-derived cells. Nuclei were stained with DAPI.

(C and D) Endothelial complexes of HBMECs with GFP-labeled GSCs expressing shCXCR4 or shNT. Quantification shows fractions of GSC-derived cells (GFP^+) on HBMEC complexes (D). *$p < 0.001$.

(E–G) In vivo lineage tracing of GSCs with GFP constitutive expression and IF staining of CD31 or Desmin in tumors derived from GSCs expressing shCXCR4 or shNT. Quantifications show fractions of G-pericytes (GFP^+) (F) and total pericyte coverage (G) on vessels. *$p < 0.001$.

The scale bars represent 100 μm (A–C) and 25 μm (E). The error bars represent SD. See also Figure S7.

in GBMs are derived from GSCs. Because G-pericytes express similar pericyte markers as normal brain vascular pericytes, GSCs function as pericyte progenitors and contribute to vasculature formation in GBMs. The ability of GSCs to generate vascular pericytes in vivo suggests that GSCs may actively remodel their microenvironment and create a supportive niche, permitting functional vessels to augment tumor growth without depending on the limited source of normal pericyte progenitors from surrounding tissues.

Because NSCs can transdifferentiate into pericytes (Li et al., 2009; Morishita et al., 2007), a lineage link between NSCs and pericytes is present in normal tissues. Because GSCs share regulatory programs with NSCs, the plasticity of GSCs toward a pericyte lineage may be a product of aberrant

tissue or from the bone-marrow-derived cells homing in tumors after treatments (De Palma et al., 2005; Du et al., 2008). In this study, we demonstrate that the majority of vascular pericytes

Figure 5. Selective Elimination of G-Pericytes Disrupts Tumor Vessels and Inhibits Tumor Growth

(A) Schematic illustrations of Desmin-promoter-driven expression of HsvTK and coexpression of HsvTK and GFP.

(B) TUNEL assay detecting selective apoptosis (in red) of G-pericytes (GFP^+) induced by ganciclovir (GCV) in GBM tumors derived from DesPro-TK-GFP-GSCs (CCF2170).

(C and D) IF staining of CD31 (in red) shows effects of selective elimination of G-pericytes (GFP^+) by GCV on vessels in GBM tumors derived from DesPro-TK-GFP-GSCs. Quantification shows the reduced G-pericyte coverage by GCV treatment (D). *$p < 0.001$.

(E and F) Assessment of vascular function using the FITC-conjugated mega-dextran after selective elimination of G-pericytes in GBM tumors derived from DesPro-TK-GSCs. Quantification shows intensity of perfused FITC-mega-dextran into the control or GCV-treated tumors (F). *$p < 0.001$.

(G and H) The effect of targeting G-pericytes by GCV treatment on growth of subcutaneous tumors derived from DesPro-TK-GFP-GSCs. Quantification shows mean tumor weights in the control and GCV-treated mice (H). *$p < 0.001$ ($n = 12$).

(I and J) The effect of selective elimination of G-pericytes on GBM growth in mouse brains. Mice bearing tumors derived from DesPro-TK-GSCs or DesPro-GFP-GSCs (control) were treated with GCV for 3 weeks. Images of whole brains (I) and histological analysis (hematoxylin and eosin [H&E] staining) on brain sections (J) are shown.

(K) Kaplan-Meier survival curves of mice bearing GBM tumors derived from DesPro-TK-GSCs or DesPro-GFP-GSCs (control) after GCV treatment. *$p < 0.001$ ($n = 6$).

The scale bars represent 25 μm (B) and 100 μm (C and E). The error bars represent SD. See also Figure S6.

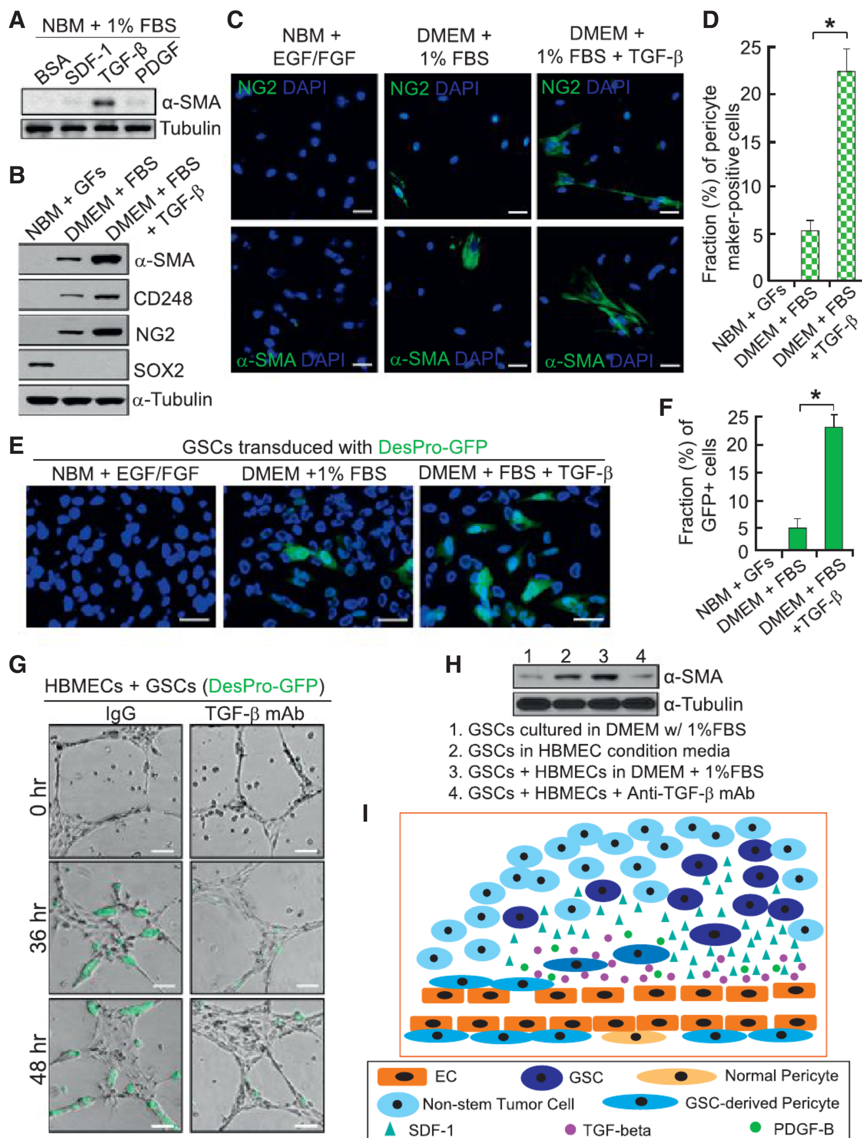

Figure 7. TGF-β Induces Differentiation of GSCs into Pericytes

(A) Immunoblot (IB) analysis of pericyte marker (α-SMA) expression in differentiated cells from GSCs (CCF1992) in the presence of indicated cytokines (1 ng/ml) in culture media.

(B) IB analysis of pericyte markers (α-SMA, CD248, and NG2) and a GSC marker (SOX2) in GSCs and differentiated cells with or without treatment of TGF-β (2 ng/ml).

(C and D) IF staining of pericyte markers (NG2 and α-SMA) in GSCs (CW1217) and differentiated cells induced by serum or TGF-β. Quantification shows pericyte fractions (D). *p < 0.001.

(E and F) In vitro pericyte lineage tracing of GSCs with Desmin promoter-driven GFP induced by serum or TGF-β (2 ng/ml). Quantification shows fractions of GFP$^+$ cells in the differentiated cells (F). *p < 0.001.

(G) In vitro HBMEC complex formation with DesPro-GFP-GSCs in the presence of anti-TGF-β antibody (monoclonal antibody [mAb]) or immunoglobulin G (IgG).

(H) IB analysis of α-SMA expression after coculture of GSCs with HBMECs or their conditioned media in the presence of anti-TGF-β antibody or IgG.

(I) A schematic illustration showing the recruitment of GSCs toward ECs and the differentiation of GSCs into pericytes in GBMs. GSCs expressing CXCR4 are recruited toward ECs by SDF-1 and induced predominantly by TGF-β to become pericytes to support vessel function and tumor growth. The scale bars represent 25 μm (C and E) and 100 μm (G). The error bars represent SD.

developmental biology. Although previous reports suggest that GSCs may give rise to ECs in GBMs (Ricci-Vitiani et al., 2010; Soda et al., 2011; Wang et al., 2010), such an event may be very rare because ECs in GBMs rarely carry the cancer genetic mutations as demonstrated in our study and others (Kulla et al., 2003; Rodriguez et al., 2012). Moreover, our complementary lineage tracing studies failed to demonstrate GSC-derived ECs in vivo, although in the culture condition we occasionally observed rare EC-marker-expressing cells (<0.6%) in differentiated cells from GSCs. Because vascular pericytes closely attach to ECs and both cells appear very thin, prior studies may have missed the true identity of tumor-derived cells on vessels. Because both ECs and pericytes express Tie2 (De Palma et al., 2005), the use of Tie2 promoter-driven HsvTK expression for targeting “GSC-derived ECs” (Ricci-Vitiani et al., 2010) might actually eliminate the G-pericytes. Our in vivo lineage tracing with pericyte- or EC-specific promoter-driven fluorescent reporters directly demonstrated that GSCs give rise to pericytes rather than ECs in vivo.

The contribution of GSCs to vascular pericytes requires GSC recruitment toward ECs. Because ECs in brain and GBMs express abundant SDF-1 forming chemoattractant gradient, the expression of CXCR4 (the receptor for SDF-1) in GSCs (Ehtesham et al., 2009; Folkins et al., 2009) may provide a paracrine loop for recruitment of GSCs toward ECs. A recent study showed that NSCs can be recruited to perivascular niches in normal brain through the CXCR4/SDF-1 axis (Kokovay et al., 2010). The recruitment of pericyte progenitors to ECs in normal tissues also depends on SDF-1/CXCR4 signaling (Song et al., 2009). SDF-1 expression has been proposed as one of the mechanisms underlying the resistance to antiangiogenic therapy in GBM trials (Batchelor et al., 2007). Elevated SDF-1 signaling may enhance GSC recruitment toward ECs and increase G-pericyte coverage to protect tumor vessels, leading to resistance to antiangiogenic therapy.

The potent capacity of GSCs to generate vascular pericytes allows active vascularization in GBMs to support tumor growth. Because GSCs contribute to the majority of vascular pericytes in GBMs, G-pericytes may have a crucial role in mediating therapeutic resistance in GBMs. Because pericytes juxtacrine to

ECs express significant levels of VEGF and other factors to support EC survival (Franco et al., 2011; Song et al., 2005; Winkler et al., 2011), G-pericytes may protect ECs and render ECs less responsive to antiangiogenic agents in GBMs. Thus, targeting G-pericytes may synergize with current therapies targeting ECs to achieve more effective outcome. Because CSCs are present in other solid cancers (Magee et al., 2012), it is important to determine whether CSCs can generate vascular pericytes in other malignant tumors with florid angiogenesis. Our studies demonstrate that GSCs not only interact with perivascular niches but also have the capacity to remodulate their microenvironment by contributing pericyte compartments of the neovasculature. Because selective elimination of G-pericytes potently disrupted vessels and inhibited tumor growth, therapeutic targeting of G-pericytes may have a significant impact on improving GBM treatment efficacy.

EXPERIMENTAL PROCEDURES

Isolation of GSCs and Non-Stem Tumor Cells from GBMs

GBM surgical specimens were collected in accordance with a Cleveland Clinic Institutional Review Board-approved protocol. GSCs and non-stem tumor cells were derived from GBM tumors and functionally validated as described previously (Bao et al., 2006a; Guryanova et al., 2011). For the detailed procedure, please see Extended Experimental Procedures.

Pericyte or EC-Specific Promoter-Driven Expression of GFP or mCherry

Human Desmin promoter (312 bp) with an enhancer (284 bp) (Li and Paulin, 1991), α-SMA promoter (262 bp) with an enhancer (123 bp) (Keogh et al., 1999; Nakano et al., 1991), CD105 promoter plus enhancer (955 bp) (Ríus et al., 1998), and CD31 promoter plus enhancer (887 bp) restricted to ECs (Almendro et al., 1996; Gumina et al., 1997) were cloned by PCR and confirmed by sequencing. The specific promoter with enhancer was inserted into pCDH-CMV-EF1-Puro lentiviral vector (System Biosciences) to replace the original CMV promoter. The ORF of GFP or mCherry was then inserted into the vector to generate lentiviral constructs. Lentiviruses were produced and tittered as described elsewhere (Guryanova et al., 2011).

Cell Lineage Tracing of GSCs

To perform cell lineage tracing, GSCs were transduced with GFP or mCherry constitutive expression or conditional expression driven by the pericyte or EC-specific promoter through lentiviral infection and then transplanted into brains of athymic BALB/c nu/nu mice to establish xenografts as described elsewhere (Guryanova et al., 2011). To trace cell lineage of GSCs in vivo, sections of mouse brains bearing the xenografts were immunostained for pericyte or EC markers and analyzed for GFP or mCherry expression. IF and IHC stainings were performed as described (Guryanova et al., 2011). Tumor sections of the genetically engineered mouse GBMs were provided by Dr. Dolores Hambardzumyan. For detailed methods and the antibody information, please see Extended Experimental Procedures.

Selective Targeting of G-Pericytes in GBM Xenografts

GSCs were transduced with Desmin or CD31-promoter-driven expression of HsvTK, GFP, or HsvTK plus GFP through lentiviral infection and then transplanted into brains of athymic mice. Mice bearing the xenografts received GCV (Sigma-Aldrich) at 75 mg/kg/day or vehicle control daily through intraperitoneal injection. The xenografts were collected for IF and IHC staining and fluorescent analysis. To evaluate the targeting effect on animal survival, mice were maintained until the development of neurological signs.

HBVPs, HBMECs, and EC Complex Formation

HBVPs and HBMECs were obtained from ScienCell. HBMECs with low passage were used for coculture and endothelial complex formation assays as described (Bao et al., 2006b). For the detailed procedure and the labeling of GSCs and HBMECs, please see Extended Experimental Procedures.

Statistical Analysis

All quantified data were statistically analyzed. Grouped data are presented as mean ± SD. The difference between experimental groups was assessed by one-way ANOVA or one-way ANOVA on ranks testing. For the animal survival experiments, log-rank survival analysis was performed.

For further details, please see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.02.021.

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REFERENCES

Almendro, N., Bellón, T., Rius, C., Lastres, P., Langa, C., Corbí, A., and Bernabéu, C. (1996). Cloning of the human platelet endothelial cell adhesion molecule-1 promoter and its tissue-specific expression. Structural and functional characterization. J. Immunol. *157*, 5411–5421.

Armulik, A., Genové, G., Mäe, M., Nisancioglu, M.H., Wallgard, E., Niaudet, C., He, L., Norlin, J., Lindblom, P., Strittmatter, K., et al. (2010). Pericytes regulate the blood-brain barrier. Nature *468*, 557–561.

Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D., and Rich, J.N. (2006a). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*, 756–760.

Bao, S., Wu, Q., Sathornsumetee, S., Hao, Y., Li, Z., Hjelmeland, A.B., Shi, Q., McLendon, R.E., Bigner, D.D., and Rich, J.N. (2006b). Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res. *66*, 7843–7848.

Batchelor, T.T., Sorensen, A.G., di Tomaso, E., Zhang, W.T., Duda, D.G., Cohen, K.S., Kozak, K.R., Cahill, D.P., Chen, P.J., Zhu, M., et al. (2007). AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell *11*, 83–95.

Bell, R.D., Winkler, E.A., Sagare, A.P., Singh, I., LaRue, B., Deane, R., and Zlokovic, B.V. (2010). Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. Neuron *68*, 409–427.

Bergers, G., and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. Neuro-oncol. *7*, 452–464.

Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D. (2003). Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J. Clin. Invest. *111*, 1287–1295.

Calabrese, C., Poppleton, H., Kocak, M., Hogg, T.L., Fuller, C., Hamner, B., Oh, E.Y., Gaber, M.W., Finklestein, D., Allen, M., et al. (2007). A perivascular niche for brain tumor stem cells. Cancer Cell *11*, 69–82.

Cancer Genome Atlas Research Network. (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature *455*, 1061–1068.

Carmeliet, P., and Jain, R.K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat. Rev. Drug Discov. *10*, 417–427.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell *3*, 301–313.

Culver, K.W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H., and Blaese, R.M. (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science *256*, 1550–1552.

deCarvalho, A.C., Nelson, K., Lemke, N., Lehman, N.L., Arbab, A.S., Kalkanis, S., and Mikkelsen, T. (2010). Gliosarcoma stem cells undergo glial and mesenchymal differentiation in vivo. Stem Cells *28*, 181–190.

De Palma, M., Venneri, M.A., Galli, R., Sergi Sergi, L., Politi, L.S., Sampaolesi, M., and Naldini, L. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell *8*, 211–226.

Du, R., Lu, K.V., Petritsch, C., Liu, P., Ganss, R., Passegué, E., Song, H., Vandenberg, S., Johnson, R.S., Werb, Z., and Bergers, G. (2008). HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell *13*, 206–220.

Ehtesham, M., Mapara, K.Y., Stevenson, C.B., and Thompson, R.C. (2009). CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett. *274*, 305–312.

Folkins, C., Shaked, Y., Man, S., Tang, T., Lee, C.R., Zhu, Z., Hoffman, R.M., and Kerbel, R.S. (2009). Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1. Cancer Res. *69*, 7243–7251.

Franco, M., Roswall, P., Cortez, E., Hanahan, D., and Pietras, K. (2011). Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. Blood *118*, 2906–2917.

Gerhardt, H., Wolburg, H., and Redies, C. (2000). N-cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. Dev. Dyn. *218*, 472–479.

Gumina, R.J., Kirschbaum, N.E., Piotrowski, K., and Newman, P.J. (1997). Characterization of the human platelet/endothelial cell adhesion molecule-1 promoter: identification of a GATA-2 binding element required for optimal transcriptional activity. Blood *89*, 1260–1269.

Guryanova, O.A., Wu, Q., Cheng, L., Lathia, J.D., Huang, Z., Yang, J., MacSwords, J., Eyler, C.E., McLendon, R.E., Heddleston, J.M., et al. (2011). Nonreceptor tyrosine kinase BMX maintains self-renewal and tumorigenic potential of glioblastoma stem cells by activating STAT3. Cancer Cell *19*, 498–511.

Hambardzumyan, D., Amankulor, N.M., Helmy, K.Y., Becher, O.J., and Holland, E.C. (2009). Modeling adult gliomas using RCAS/t-va technology. Transl. Oncol. *2*, 89–95.

Ii, M., Nishimura, H., Sekiguchi, H., Kamei, N., Yokoyama, A., Horii, M., and Asahara, T. (2009). Concurrent vasculogenesis and neurogenesis from adult neural stem cells. Circ. Res. *105*, 860–868.

Keogh, M.C., Chen, D., Schmitt, J.F., Dennehy, U., Kakkar, V.V., and Lemoine, N.R. (1999). Design of a muscle cell-specific expression vector utilising human vascular smooth muscle alpha-actin regulatory elements. Gene Ther. *6*, 616–628.

Kokovay, E., Goderie, S., Wang, Y., Lotz, S., Lin, G., Sun, Y., Roysam, B., Shen, Q., and Temple, S. (2010). Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. Cell Stem Cell *7*, 163–173.

Komatani, H., Sugita, Y., Arakawa, F., Ohshima, K., and Shigemori, M. (2009). Expression of CXCL12 on pseudopalisading cells and proliferating microvessels in glioblastomas: an accelerated growth factor in glioblastomas. Int. J. Oncol. *34*, 665–672.

Kulla, A., Burkhardt, K., Meyer-Puttlitz, B., Teesalu, T., Asser, T., Wiestler, O.D., and Becker, A.J. (2003). Analysis of the TP53 gene in laser-microdissected glioblastoma vasculature. Acta Neuropathol. *105*, 328–332.

Li, Z.L., and Paulin, D. (1991). High level desmin expression depends on a muscle-specific enhancer. J. Biol. Chem. *266*, 6562–6570.

Magee, J.A., Piskounova, E., and Morrison, S.J. (2012). Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer Cell *21*, 283–296.

Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R.K., and McDonald, D.M. (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. *160*, 985–1000.

Morishita, R., Nagata, K., Ito, H., Ueda, H., Asano, M., Shinohara, H., Kato, K., and Asano, T. (2007). Expression of smooth muscle cell-specific proteins in neural progenitor cells induced by agonists of G protein-coupled receptors and transforming growth factor-beta. J. Neurochem. *101*, 1031–1040.

Nakano, Y., Nishihara, T., Sasayama, S., Miwa, T., Kamada, S., and Kakunaga, T. (1991). Transcriptional regulatory elements in the 5′ upstream and first intron regions of the human smooth muscle (aortic type) alpha-actin-encoding gene. Gene *99*, 285–289.

Norden, A.D., Drappatz, J., and Wen, P.Y. (2009). Antiangiogenic therapies for high-grade glioma. Nat. Rev. Neurol. *5*, 610–620.

Pàez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Viñals, F., Inoue, M., Bergers, G., Hanahan, D., and Casanovas, O. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell *15*, 220–231.

Ricci-Vitiani, L., Pallini, R., Larocca, L.M., Lombardi, D.G., Signore, M., Pierconti, F., Petrucci, G., Montano, N., Maira, G., and De Maria, R. (2008). Mesenchymal differentiation of glioblastoma stem cells. Cell Death Differ. *15*, 1491–1498.

Ricci-Vitiani, L., Pallini, R., Biffoni, M., Todaro, M., Invernici, G., Cenci, T., Maira, G., Parati, E.A., Stassi, G., Larocca, L.M., and De Maria, R. (2010). Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. Nature *468*, 824–828.

Ríus, C., Smith, J.D., Almendro, N., Langa, C., Botella, L.M., Marchuk, D.A., Vary, C.P., and Bernabéu, C. (1998). Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. Blood *92*, 4677–4690.

Rodriguez, F.J., Orr, B.A., Ligon, K.L., and Eberhart, C.G. (2012). Neoplastic cells are a rare component in human glioblastoma microvasculature. Oncotarget *3*, 98–106.

Soda, Y., Marumoto, T., Friedmann-Morvinski, D., Soda, M., Liu, F., Michiue, H., Pastorino, S., Yang, M., Hoffman, R.M., Kesari, S., and Verma, I.M. (2011). Transdifferentiation of glioblastoma cells into vascular endothelial cells. Proc. Natl. Acad. Sci. USA *108*, 4274–4280.

Song, S., Ewald, A.J., Stallcup, W., Werb, Z., and Bergers, G. (2005). PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat. Cell Biol. *7*, 870–879.

Song, N., Huang, Y., Shi, H., Yuan, S., Ding, Y., Song, X., Fu, Y., and Luo, Y. (2009). Overexpression of platelet-derived growth factor-BB increases tumor pericyte content via stromal-derived factor-1alpha/CXCR4 axis. Cancer Res. *69*, 6057–6064.

Verhaak, R.G., Hoadley, K.A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M.D., Miller, C.R., Ding, L., Golub, T., Mesirov, J.P., et al.; Cancer Genome Atlas Research Network. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell *17*, 98–110.

Wang, R., Chadalavada, K., Wilshire, J., Kowalik, U., Hovinga, K.E., Geber, A., Fligelman, B., Leversha, M., Brennan, C., and Tabar, V. (2010). Glioblastoma stem-like cells give rise to tumour endothelium. Nature *468*, 829–833.

Winkler, E.A., Bell, R.D., and Zlokovic, B.V. (2011). Central nervous system pericytes in health and disease. Nat. Neurosci. *14*, 1398–1405.

Zhou, B.B., Zhang, H., Damelin, M., Geles, K.G., Grindley, J.C., and Dirks, P.B. (2009). Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat. Rev. Drug Discov. *8*, 806–823.

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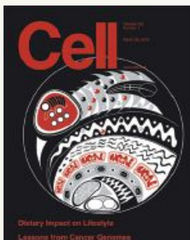

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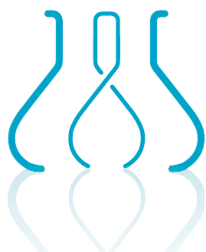

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