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State of the art: Stem cells in equine regenerative medicine

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Summary

According to Greek mythology, Prometheus' liver grew back nightly after it was removed each day by an eagle as punishment for giving mankind fire. Hence, contrary to popular belief, the concept of tissue and organ regeneration is not new. In the early 20th century, cell culture and *ex vivo* organ preservation studies by Alexis Carrel, some with famed aviator Charles Lindbergh, established a foundation for much of modern regenerative medicine. While early beliefs and discoveries foreshadowed significant accomplishments in regenerative medicine, advances in knowledge within numerous scientific disciplines, as well as nano- and micromolecular level imaging and detection technologies, have contributed to explosive advances over the last 20 years. Virtually limitless preparations, combinations and applications of the 3 major components of regenerative medicine, namely cells, biomaterials and bioactive molecules, have created a new paradigm of future therapeutic options for most species. It is increasingly clear, however, that despite significant parallels among and within species, there is no 'one-size-fits-all' regenerative therapy. Likewise, a panacea has yet to be discovered that completely reverses the consequences of time, trauma and disease. Nonetheless, there is no question that the promise and potential of regenerative medicine have forever altered medical practices. The horse is a relative newcomer to regenerative medicine applications, yet there is already a large body of work to incorporate novel regenerative therapies into standard care. This review focuses on the current state and potential future of stem cells in equine regenerative medicine.

Keywords: horse; stem cell; scaffold; adipose; bone marrow; tissue engineering

Introduction

The premise of regenerative medicine is to restore structure and function to tissues or organs damaged by time, disease or injury. Typically, cells, bioactive molecules and biocompatible materials, alone or together, are applied to guide tissue formation by native cells in the recipient or to create viable structures within the laboratory for implantation. Concentrated efforts in the field of regenerative medicine and advances in biotechnology contributed to robust discovery and a multitude of potential therapeutic options. Loss of normal musculoskeletal tissue function can be career- and, potentially, life-ending in horses. It is no surprise that tissue targets of equine regenerative medicine include cartilage [1–7], tendon/ligament [8–24], bone [25–32] hoof lamina [33] and meniscus [34]. Equine skin [35] and cornea [36], among others, have also been considered for regenerative therapy.

Cells and biomaterials may augment and accelerate healing rates or overcome chronic conditions that are refractory to available treatments. However, regenerative medicine components and procedures are inherently diverse, and implementation of safe and effective treatment strategies requires incremental steps. As such, there are a number of important considerations surrounding incorporation of novel regenerative medical therapies into standard practice. Cells at various stages of development harvested from different donors and tissues are not identical, and they respond differently to environmental stimuli. Although controlled laboratory conditions are designed to replicate natural tissue milieus, native environments cannot be recreated fully in the laboratory setting. Hence, preclinical trials that incorporate multiple assessments over a meaningful time period are vital to develop standardised treatments. Perhaps of utmost importance is that the effectiveness of a treatment designed to replace or regenerate tissue depends on the inherent response of the recipient and the quality of the treatment itself. Standardised approaches for specific clinical needs that are validated with established procedures will contribute to forward progress in the field of equine regenerative medicine.

Regulation

In the US, the Food and Drug Administration (FDA) oversees human stem cell treatments and associated products. Regulations surrounding the use of human cells, tissues and cellular- and tissue-based products were published in 2006 [37,38]. Products are regulated as either biologics or drug medical devices, depending on their intended use and the amount of processing required to prepare them. In either case, the FDA has enforcement authority.

Oversight of veterinary regenerative medicine products is provided by the Center for Veterinary Biologics of the US Department of Agriculture (USDA), the Center for Veterinary Medicine (CVM) branch of the FDA or both. The USDA oversees biologics, while the FDA oversees food additives, drugs and devices, and both oversee animal biologics that also qualify as new animal drugs. Minimal manipulation of human cells or nonstructural tissue is defined by the FDA as 'processing that does not alter the relevant biological characteristics of cells or tissues' (21CFR1271.3(f)(1)). To date, there are no published regulations specific to veterinary stem cell-based products and treatments. However, those that exceed minimal manipulation standards as defined and interpreted by the regulating authority may be treated as drugs that require review and approval prior to marketing and sale. Furthermore, a new animal drug is defined, in part, as

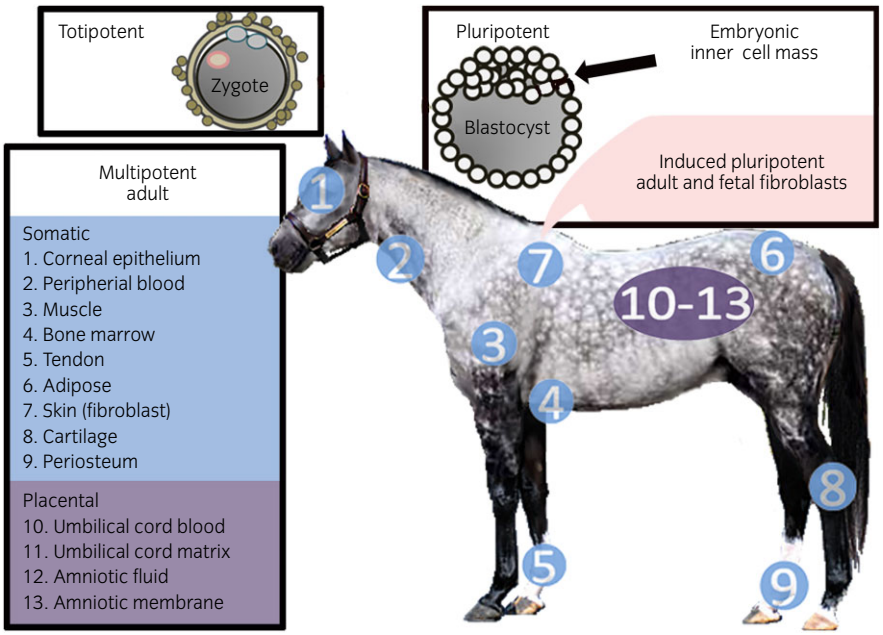

Fig 1: Schematic diagram of equine undifferentiated cell sources and plasticity.

any article intended for use in the diagnosis, cure, mitigation, treatment, prevention of disease or to affect the structure or function of the body. Although it is possible that homologous stem cell therapy may fall under regulation of the practice of veterinary medicine, many regenerative medicine products are subject to laws and regulations that apply to new animal drugs, including those for investigational use (21CFR511.3).

In the UK, the collection, storage, processing, production and administration of equine stem cells for use as an autologous treatment for nonfood-producing horses is regulated by the Veterinary Medicines Directorate (VMD) through Equine Stem Cell Centre Authorisations (PCDOCS #431064-v3-VMGN 15 Manufacturing Authorisations). This authorisation is granted by the VMD when they are satisfied that: 1) the welfare of the animals used in the collection of equine stem cells is respected; 2) the production process will produce a consistent, safe product; and 3) the centre is under the supervision of a veterinary surgeon, or a person who is suitably qualified to operate the centre.

Authorisation is given after submission of an application and an inspection of the facilities. Periodic re-inspection of the facilities will occur subsequent to granting of the authorisation. It is likely that the regulatory environment relating to the processing and use of equine stem cells will evolve over time. In other jurisdictions, similar regulations may exist or may develop over time.

Regardless of jurisdiction, products for patient administration must meet safety and efficacy criteria and minimal standards of sterility, purity and potency, among others. Additional cell-based product considerations include, in part, product characterisation, potential for disease transmission, demonstrated control of the manufacturing process, immunogenicity and freedom from tumour or other unintended tissue formation. Only products approved by one or both agencies can be legally marketed in the USA. Manufacturers and sponsors are permitted to self-determine whether their products require agency review. However, if either agency objects after a product is made available without their review, the manufacturer can be faced with enforcement sanctions and be required to withdraw the product. Although specific guidelines are not currently available for veterinary regenerative medicinal products, including stem cells, it is possible that future regulations may be similar to current FDA standards for human products as well as FDA and USDA rules surrounding veterinary biologics.

Cells

The International Society for Cellular Therapy (ISCT) defines a stem cell as 'one attributed to producing all necessary components in a given tissue'. This may contrast with the more colloquial understanding that a stem cell is capable of producing every component of all tissues. Nomenclature for cell potential to develop into diverse tissue lineages, known as differentiation capacity or plasticity, is described in detail elsewhere [32,39]. Briefly, plasticity decreases in order from totipotent (all cell types) to pluripotent (most cell types), multipotent (some cell types) and unipotent (one cell type). The plasticity of undifferentiated cells within an organism decreases with increasing stage of development [40,41]. Hence totipotent cells occur only in early stages of embryonic development, pluripotent in later embryonic and early fetal stages, multipotent in late fetal and fully developed (adult) tissue stages, and unipotent, sometimes called progenitor, cells only in adult stages. Tissue harvest sites for undifferentiated equine cells from fully developed tissue include adipose tissue [28,42–48], bone marrow [1–5,7,11,22,34,49–56], peripheral blood [14,35,57], amnion [13,53], amniotic fluid [58], umbilical cord tissue [40,59], umbilical cord blood [29,48,60–62], tendon [63], muscle [28,63], periosteum [28] and cornea [36] (Fig 1, Table S1). Cells have also been isolated from equine embryonic [64–68] and fetal tissue [24]. Umbilical tissues may contain more primitive cells with greater plasticity and expansion capacity than other adult tissues [40,43,60]. As indicated above, cell plasticity within a tissue ultimately depends on the individual donor [60].

Regardless of maturity and tissue source, undifferentiated cells isolated from a given tissue are not identical. Even those isolated from the blastocyst inner cell mass may vary in developmental potential [69]. Heterogeneity among cells within a tissue increases with donor age owing to commitment towards specialisation [70]. The variety of cell phenotypes among and within cell isolates is well recognised in equine regenerative medicine [41,60]. Specific cell phenotypes are selected at many stages during the process of cell isolation and culture, including the process of passaging (detaching cells from one culture vessel and adding them to another) [29]. The information below provides an overview of the inherent diversity of the undifferentiated cell component of equine regenerative medicine.

Cell isolates

Distinctions among cell isolates from the same tissue are illustrated by adult multipotent stromal cells (MSCs). Bone marrow-derived multipotent stromal cells (BMSCs) in sequential 5 and 50 ml bone marrow aspirates from ileum and sternebrae in 2- to 5-year-old horses were compared [52]. In aspirates from the same harvest site, the first 5 ml fraction had the highest BMSC density, but *in vitro* osteogenic

and chondrogenic differentiation were similar among fractions. Comparing harvest sites, the ileum had the highest overall cell yield and better chondrogenic differentiation than the sternum. In contrast, comparisons of sequential 12 ml bone marrow aspirates from sternebra and tuber coxae in middle-aged horses showed higher BMSC yield from the sternum than the ilium [71]. There was no difference between the 2 sites in sequential 60 ml aspirates in a separate investigation [29]. In all of the studies, cell yield was always lower from second aspirates. An evaluation of the number of adipose-derived multipotent stromal cells (ASCs) from subcutaneous adipose tissue revealed a positive correlation between vessel number and the number of colony-forming cells per gram of tissue [72]. There are doubtless innumerable donor site characteristics that contribute to dissimilar findings within tissues.

Harvest sites

A goal to identify harvest sites with concentrations of MSCs that divide (expand) rapidly *in vitro* and also possess potential for specific tissue formation drives continuous investigative efforts. Epigenetic factors are thought to contribute to the ability of cells to differentiate into the tissue from which they were derived [73], a concept that is supported by equine studies [3,29]. However, results are not conclusive. Using cryopreserved tissues, equine periosteum yielded more MSCs than bone marrow, muscle and adipose tissue; BMSCs had the slowest proliferation rate, and all 3 had comparable osteogenesis [28]. Cell yield from fresh adipose, tendon and umbilical cord tissue was over 200 times higher than from bone marrow or cord blood [43]. Furthermore, cells from tendon and adipose tissue showed the most rapid proliferation, and osteogenesis was strongest in cells from bone marrow, while chondrogenesis was weakest. Among cryopreserved MSCs from bone marrow, adipose tissue and umbilical cord blood and tissue, BMSCs had the highest osteogenic capacity [29]. In another study, fresh BMSCs and ASCs appeared to have the best osteogenic and adipogenic potential, respectively [74], and cryopreserved BMSCs had superior chondrogenic potential to ASCs [6]. Diversity among studies such as these is attributable in part to variable cell isolation, culture and characterisation procedures.

Isolation procedures

Density gradients and proteolytic enzyme digestion are the most common mechanisms to isolate the mononuclear cell fraction from adult bone marrow and the stromal vascular fraction from solid tissue, respectively. Techniques to isolate equine ASCs were originally patterned after those for humans [74]. However, to date, there is no standard isolation method from any tissue in the horse. Isolation steps are often followed by maintaining cells in plastic cultureware in ideal cell growth conditions. Use of plastic adherence alone to isolate and enrich for MSCs, owing to their strong affinity for plastic, is also reported [50].

Small differences among isolation methods can significantly affect cell isolates. A Percoll colloidal silica density gradient resulted in the highest initial BMSC yield and BMSC number after 14 days of culture compared to plastic adherence alone or a Ficoll polysaccharide gradient [50]. The authors proposed that disparate results from the 2 gradients may have been due to MSCs settling in a band that was not harvested from the Ficoll gradient. Enzymatic activity of collagenase solutions is inconsistent, and digestion protocols are not standardised for cell isolation from solid tissues. Tissue digestion duration, sample size and harvest location influence MSC yield [74]. Explant isolation, the process of MSC migration from minced tissue in ideal culture conditions, was proposed to reduce the potential effects of tissue digestion on the cells. Collagenase digestion of equine adipose tissue, tendon and umbilical cord matrix resulted in higher MSC yield from all tissues than from explant isolation, and there were no differences in cell proliferation or differentiation characteristics between the techniques [75]. Future work to optimise cell isolation that includes determination of cell phenotype will be important to elucidate the effects of gradients, digestion and associated protocols on cells.

Cell culture

The culture environment of undifferentiated cells is designed to facilitate survival and induce specific behaviours for evaluation and comparison. Two of the 3 requirements for determination of adult MSC identity according to the ISCT are based on *in vitro* behaviour, adherence to plastic and trilineage differentiation into adipocytes, osteoblasts and chondroblasts [76]. Culture conditions for equine cells were patterned after other species, and much of the original information was specific to bone marrow-derived cells [41]. However, species- and cell-specific culture conditions are required for optimal cell growth and differentiation [60]. Numerous basic culture media for cells harvested from assorted tissues and induction media for diverse lineages have been customised for equine cells, largely over the last decade (Table S1).

Fetal bovine serum

Fetal bovine serum (FBS), one of the most common components of cell culture media, provides hormones, nutrients, plasma proteins for cell adhesion and endogenous growth factors for proliferation [55]. Varying concentrations are also frequently used in cryopreservation medium [6,29]. Some limitations of FBS include variability among preparations, possible disease transmission, and the potential for xenogeneic proteins to trigger cell behaviour that would not be caused by autologous or allogeneic serum [77]. Clinical translation of cell therapies may be also be hampered by immunological concerns [78], especially as FBS may not be removed from cells even with extensive washing [29]. Some alternatives to FBS for culture of equine cells include allogeneic platelet lysate [55], autologous serum [29,79] and commercially available serum substitute [79]. Changes in cell adhesion, morphology and growth patterns [55] as well as slower proliferation have been associated with FBS alternatives [25,29,79]. There are also concerns surrounding quality between preparations and potential for immunological stimulation. Efforts to find replacements for FBS with comparable properties may be key to clinical translation of cell therapies.

Induction media

Induction media (Table S1) for lineage differentiation in cultured cells create environmental conditions that do not necessarily replicate the natural milieu. Most contain bioactive molecules to upregulate lineage-specific gene expression and also provide necessary components for subsequent cell generation of intra- (adipogenesis) or extracellular products (chondrogenesis and osteogenesis). While these basic elements are generally included, the components vary, as do concentrations and culture times. A specific example is rabbit serum, which contains high concentrations of free fatty acids [41] and is frequently added to equine adipogenic medium to increase lipid production. Dexamethasone upregulates osteogenic target gene expression, including runt-related transcription factor 2 (Runx2), a key transcription factor for osteoblastic differentiation [3]. Increased concentrations were reportedly required to stimulate equine peripheral blood-derived fibroblast-like cell (PB-FLC) osteogenesis [41]. Growth factor additives are often customised for specific purposes. Transforming growth factor (TGF) β1 is standard for equine chondrogenesis medium, but TGFβ3 is reported to stimulate more robust PB-FLC and BMSC chondrogenesis [41]. Fibroblastic growth factor (FGF) 2 enhanced ASC proliferation but reduced that of umbilical cord blood stem cells, while FGF5 had the opposite effect [48]. The variability in response to induction medium by cells harvested from the same tissues is highlighted by chondrogenic differentiation. In one study, chondrogenic differentiation was prominent in MSCs from umbilical cord blood and weak in BMSCs [43]; in another, there was no umbilical cord blood MSC chondrogenesis and moderate BMSC chondrogenesis [80], and still others showed intense BMSC chondrogenic differentiation [6,41]. Progessive improvements and standardisation of induction protocols will increase consistency among results.

Surface conditions

Culture surface modifications are applied to optimise conditions for specific undifferentiated cell responses, often specific to lineage. Standard

options include 2-dimensional monolayer culture, 3-dimensional cell pellet cultures, co-culture with other cells and matrix surface protein coatings [3]. Identical conditions can elicit different responses based on cell origin, as in the effect of culture surface on tenogenic differentiation by ASCs and umbilical cord blood MSCs. Cells were cultured on 1% gelatin-coated tissue culture plates, dextran beads coated with porcine skin collagen and in 30% Matrigel, a 3-dimensional culture matrix composed of laminin, collagen IV and entactin containing growth factors (insulin growth factor-1, platelet-derived growth factor, TGFβ and epidermal growth factor) [48]. Gene expression of scleraxis, a tenogenic transcription factor, was increased in both cell types cultured in Matrigel, but only in ASCs cultured on beads. Expression of the gene for tenascin C, an extracellular matrix protein in tendons, was increased only in ASCs cultured in Matrigel.

In vitro limitations

Unavoidable differences between *in vitro* and *in vivo* conditions limit extrapolation of data between the two. Oxygen concentrations vary between culture and physiological conditions, with much higher, ~20%, oxygen in the incubator environment vs. ~1–8% in bone marrow and adipose tissues. Proliferation of both ASCs and BMSCs was higher at 20% oxygen, and there were higher numbers of nonviable ASCs, increased expression of pluripotency-associated genes in cells from both tissues and differences in surface antigen gene expression between ASCs and BMSCs at lower (5%) oxygen levels [47]. In a separate report, BMSC chondrogenesis target gene expression and sulfated glycosaminoglycan deposition were enhanced by lower oxygen tension, whereas adipogenesis, osteogenesis and proliferation were not affected [5]. Low-dose antimicrobials are standard in undifferentiated cell culture media. However, at therapeutic concentrations, gentamicin and amikacin are toxic to BMSCs [49]. Cells can also vary in response to normal and pathological conditions. The influence of synovial fluid from normal and osteoarthritic joints on a BMSC secretory profile showed that osteoarthritic but not normal joint synovial fluid induced gene expression of anabolic cytokines, and neither induced a proinflammatory cytokine response [56]. The authors cautioned that the synovial fluid from osteoarthritic joints may have varied in composition and synovial fluid dilution may have diminished the effects. Efforts such as these to relate *in vitro* culture findings to clinical applications continue to strengthen progress towards practical applications.

Cell characterisation

A challenging aspect of equine regenerative medicine research is cell phenotype identification. As mentioned previously, heterogeneous cell phenotypes at different stages of maturity and lineage commitment compose initial cell isolates. Cell morphology, plastic adherence, trilineage differentiation and cell surface marker expression are used to characterise adult equine MSCs both before and after induction to tissue lineages [30,41,81]. Minimal ISCT criteria for human multipotent mesenchymal stromal cells published in 2006 are adherence to plastic, specific surface antigen expression and trilineage differentiation based on histochemical staining [76]. More recent ISCT and International Federation for Adipose Therapeutics and Science (IFATS) criteria for human stromal vascular fraction cells and ASCs include additional confirmation of cell differentiation by target gene and protein expression [82]. Many recent equine studies with undifferentiated cells use several of these criteria, including expression of CD90 and CD44 without CD34 and CD45, but there are no minimal standards [28,81] (Table S2).

Rigorous and consistent phenotyping of undifferentiated cells is critical to production of robust, repeatable results with the greatest potential for translational value. A shortage of monoclonal antibodies directed against equine cell surface markers contributes to the lack of an established set of markers for equine undifferentiated cells that parallels that of humans [43]. As such, antibodies that are not specific for the horse are frequently employed [31,81]. Many (>95% in some cases) antibodies for xenogeneic proteins do not react with equine surface antigens [83]. This highlights the need for confirmation of reactivity with the target species protein [31,83].

Identification of surface marker expression is further complicated by effects of isolation, culture time and cell passaging [28]. Quantification of mRNA levels to confirm flow cytometry or immunocytochemistry results is appealing owing to increasing knowledge of equine genetic sequences (Table S3) [80]. However, some equine genes are not fully sequenced, and gene transcription determined by quantification of mRNA message is not equal to protein expression, because not all mRNA is translated [83]. Multicolour flow cytometry is a technique for discrimination of distinct cell phenotypes by the presence or absence of multiple surface antigens and intracytoplasmic proteins simultaneously. Using this technology, the phenotype of umbilical cord blood MSCs using validated antibodies was CD29, CD44 and CD90 positive and CD45, CD79α and major histocompatibility complex (MHC) II negative [83]. It is possible to 'sort' or isolate cell phenotypes based on the presence and absence of proteins using similar strategies. This mechanism may be one of the best options for standardisation of undifferentiated cell phenotypes.

Detection and quantification of target gene expression based on mRNA levels is routine among equine undifferentiated cell assessments (Table S3). Gene expression is part of a standard toolkit to confirm cell identity and response to environmental factors and to assess the relative potential of the cell to produce proteins related to specific functions [29]. However, protein expression vs. the presence of mRNA is recommended to measure and confirm undifferentiated cell markers, given differences among mRNA transcription vs. protein translation [65]. Inconsistencies between studies are no doubt related to the many points identified above, among others. Mechanistic studies aimed at cell signalling pathways in undifferentiated equine cells, such as the one which showed differences in specific pathway activation among fibroblastic growth factors [48], will enhance knowledge of pathological processes, therapeutic strategies and undifferentiated cell potential.

Embryonic stem-like cells

Embryonic stem cells derived from the preimplantation or peri-implantation embryo are capable of unlimited proliferation *in vitro* without differentiation, and maintain the potential to form all 3 embryonic germ layers after prolonged culture. Undifferentiated cells from the inner cell mass of the blastocyst embryo could provide fully characterised, pluripotent cell lines with nearly unlimited proliferation potential and longevity that may be an ideal cell source for *de novo* tissue generation intended for implantation. However, there are ethical concerns: the potential for immunological reactions, the fact that cell isolates vary in developmental potential [69] and the fact that undifferentiated embryonic stem cells can form teratomas may limit direct implantation. In fact, teratomas did not occur following injection of cell lines derived from equine blastocysts into immunocompromised mice [66] or equine superficial digital flexor tendons [64]. However, this raises concerns that they may not be true embryonic stem cells [67]. As such, the cells are often designated embryonic stem-like cells (ESCs).

Isolation and culture of equine ESCs is relatively new [64–68]. Consistent with other species, a report indicates that equine ESCs require a feeder cell layer and leukaemia inhibitory factor to maintain an undifferentiated state during cell passage [68], whereas others report that leukaemia inhibitory factor is not necessary [65,66]. One report indicates that cell lines developed from fresh blastocysts are capable of expressing proteins specific for the 3 germ layers after prolonged culture [66]. Another report confirmed that cell lines developed from frozen blastocysts maintained a normal karyotype following prolonged *in vitro* culture and were capable of expressing neural and haematopoietic precursor cell proteins [68]. However, to date, chimerism and germline transmission required to confirm embryonic stem cell status have not been established in the horse. Problems with equine ESC characterisation are comparable to those of MSCs [67], and gene and protein expression of factors associated with undifferentiated pluripotency vary from other species and among equine studies [65,66,68]. This may be due to differences between isolation methods, variability in early embryogenesis among species and limited knowledge of the function of the various markers. Efforts are continuing to realise the full opportunities offered by equine ESCs.

Induced pluripotent stem cells

Contemporary science allows adult and fetal cells to be returned to an embryo-like state by artificial induction of pluripotency to recapture some of the differentiation and expansion capacity lost during maturation [84,85]. The cells, known as induced pluripotent stem cells (iPSCs), are differentiated cells that are reprogrammed by inducing overexpression of endogenous pluripotency transcription factors such as octamer-binding transcription factor 4 (Oct4), v-myc myelocytomatosis viral oncogene homologue (c-Myc), sex-determining region Y-box 2 (Sox2) and kruppel-like factor 4 (Klf4). They do not have the ethical concerns of ESCs and overcome the problem of immunological incompatibility because they can be derived from the patient. Since the original work in mouse fibroblasts, iPSCs have been produced from somatic cells in many species, including the horse [84–86]. It is possible that custom iPSCs may be used to 'create' cells for study in the laboratory by inserting genes with defects known to cause pathological conditions into the cells [87]. By combining iPSCs with gene therapy, autologous iPSCs may someday be created for treatment of specific genetic conditions by placing corrected copies of genes into cells harvested from a patient. The cells may have long-term expression of the normal protein following administration. A landmark proof-of-principle study demonstrated effective iPSC treatment of haemophilia for up to 10 months in a transgenic mouse model [88]. There are many equine conditions that could potentially benefit from iPSC technology, such as hyperkalaemic periodic paralysis, glycogen branching enzyme deficiency and *type 1* polysaccharide storage myopathy [89].

As reported in 2011 and 2012, iPSCs were generated from skin fibroblasts of equine fetuses [85], a newborn male foal and a 2-year-old gelding [84]. There are distinct differences among the 2 studies that include cell donor ages as well as reprogramming and culture techniques. In the former study, fetal fibroblasts were reprogrammed using a transposon method to deliver transgenes containing Oct4, Sox2, Klf4 and c-Myc that were induced by exposure to doxycycline [85]. Following transfection, cells were cultured on a feeder layer composed of equine and murine fetal fibroblasts. Resulting cell lines maintained a stable karyotype during long-term culture, formed embryoid-like bodies, expressed pluripotency marker mRNA and proteins, differentiated into mesodermal, endodermal and ectodermal lineages *in vitro*, and formed teratomas when injected subcutaneously into immunocompromised mice. In the latter study, retroviral transduction was used to reprogramme the cells with the same set of genes as the first [84]. Selected colonies were cultured on a feeder layer of inactivated SNL cells, an immortal murine cell line that expresses neomycin resistance and leukaemia inhibitory factor genes. In 4 cell lines generated from the cells of the newborn foal, reverse transcriptase-PCR analysis and immunofluorescence revealed expression of endogenous pluripotency markers that were not expressed in the parental equine fibroblasts. Immunostaining for lineage-specific proteins verified endodermal, ectodermal and mesodermal differentiation capabilities *in vitro*. *In vivo* pluripotency of one cell line was demonstrated by generation of teratomas in the kidney capsule of immunodeficient mice. Similar outcomes minus teratoma validation were achieved with a cell line derived from the 2-year-old. These early accomplishments confirm the potential for iPSC technology in the horse. Prior to therapeutic implementation, however, there are several points to be addressed, including activation of oncogenes during the reprogramming process and insertional mutagenesis or incomplete transgene silencing associated with retroviral vectors. Mechanisms to generate iPSC lines using integration-free or nongenetic approaches may be crucial to advance iPSC technology.

Biocompatible scaffolds

One of the most relevant aspects of regenerative therapeutics is restoration of damaged or nonfunctional tissue. The microenvironment of undifferentiated cells controls proliferation and commitment through complex interactions among cells and matrix components. This microenvironment or 'niche' is often highly conserved within tissues among species [90]. There is tremendous effort to recreate the microenvironment in the form of biocompatible scaffolds with a high affinity for undifferentiated cells that will promote implant integration and 'instruct' or guide native and implanted cells to assume target tissue lineages. Scaffold composition varies with target tissue types and among clinical targets, and can range from undifferentiated cell carriers [91] to more mature tissue implants grown *in vitro* [31]. Accordingly, scaffolds range from highly specialised to single proteins and biopolymers.

Current work showcases the effects of cell interactions with extracellular matrix proteins. Based on histochemical staining, BMSCs were successfully induced to osteogenic and chondrogenic lineages on bacterial cellulose hydrogel scaffolds [91]. A study of MSCs from bone marrow, periosteum, muscle and adipose tissue showed that BMSCs on electrochemically aggregated bovine type I collagen had superior histochemical staining for osteogenesis after 168 h of culture in osteogenic medium compared to the other cell sources and BMSCs cultured on monomeric collagen, on rat tail collagen or in fibrin glue [27]. Contrasting results are provided by a study showing that bovine collagen type I supports similar osteogenic, adipogenic and chondrogenic extracellular matrix production by both ASCs and BMSCs following cell seeding with a perfusion bioreactor [31]. Although tissue-specific gene expression was initially different among cell harvest sites, the differences resolved after 21 days of culture on the scaffolds in differentiation medium. Distinctions between the latter studies include use of cryopreserved vs. fresh cells, duration of evaluation and outcome measures. *In vitro* knowledge of interactions between stem cells and implant materials is required to anticipate the *in vivo* response of exogenous and native stem cells.

In addition to extracellular matrix components, tissue architecture and mechanical properties are central to undifferentiated cell commitment [92]. Native tissue can be decellularised to remove potentially immunogenic cells while conserving extracellular matrix components in a natural 3-dimensional structure. A method to decellularise superficial digital flexor tendon reportedly removes cellular debris, does not alter tendon composition, ultrastructure or mechanical properties and is biocompatible with BMSCs [92]. Conditions that closely mimic the natural environment of the clinical target site provide valuable insight for target tissue regeneration.

Cell migration

Investigative results confirm undifferentiated cell migration *in vitro* and *in vivo* [43,75,93]. Cell migration from spheroids generated with passage 3 MSCs from adipose tissue, superficial digital flexor tendon and umbilical cord matrix was measured after 24 h of spheroid culture in standard culture medium on adherent cultureware [75]. There were no differences in migration among cell harvest sites, though cells isolated by enzymatic digestion vs. explant tended to show higher migration potential. In another study using the same cell passage and similar techniques, MSCs from adipose tissue and tendon migrated faster than those from umbilical cord tissue and bone marrow [43]. The authors suggested that MSC migration may be fundamental to systemic application [43] and graft integration into recipient tissue [43,75].

Equine ESCs have higher survival and greater migration than BMSCs following injection into mechanically induced superficial digital flexor tendon lesions [64]. For the study, 4 lesions were mechanically induced in one tendon of each horse and subsequently injected with autologous or allogeneic BMSCs expressing green fluorescent protein, allogeneic ESCs expressing β-galactosidase or autologous serum. Labelled cells were quantified in tissues harvested 10, 30, 60 and 90 days after injection. At all time points, labelled BMSCs were found in the injected lesion as well as in healthy tissue and epitenon around it, but <5% of cells were detectable at 10 days and <0.2% at 90 days after injection. The ESCs were abundant, and their migration was too great to allow calculation of survival. While ESCs were found in the same tissues as the BMSCs surrounding the lesion, they were also found in the other tendon lesions as early as 10 days after injection. Additionally, ESCs were located within vessels in tissue between lesions and in the epitenon above and below the upper and lowermost lesion sites. Migration to other tissues and organs could not be ruled out, and the authors recommended that functional effects of undifferentiated cells on tendon regeneration be investigated with a single lesion in individual animals that receive only one stem cell treatment.

Similar results were found in an investigation comparing fourth metacarpal ostectomy healing in contralateral limbs treated with fibrin

glue alone or in combination with autologous, periosteal MSCs labelled with a lipophilic dye that intercalates into the cytoplasm and plasma membrane. Cells were cultured in osteogenic medium prior to application via percutaneous injection 2, 4 and 6 weeks following the ostectomy procedures. Subsequently, labelled cells were found in both the MSC plus fibrin and the fibrin-only ostectomy sites as well as pelvic flexure tissues 12 weeks later [94]. The authors recommended single lesions in individual horses for future studies to evaluate undifferentiated cell effects on equine bone healing, similar to the authors of the tendon injury study. The results from these independent studies with cells harvested from distinct tissues and targeting different organs confirm that meaningful results about tissue regeneration are derived from evaluation of one treatment per animal at a single anatomical site.

Immunogenicity

The current undifferentiated cell paradigm is that they function primarily through paracrine effects rather than proliferation and differentiation to specific tissue lineages [56]. Undifferentiated cells are thought to improve regeneration by production of growth and immunomodulatory factors to recruit native cells with varying degrees of plasticity and direct differentiation, maturation and extracellular matrix production [43,95]. There are many questions surrounding cell activity and persistence following implantation. Likewise, the potential to stimulate cellular and humoral immune reactions can limit multiple treatments. Current thought is that undifferentiated cells have some level of immune privilege and produce trophic factors to modulate the inflammatory process [29]. This makes it plausible that allogeneic cells may be as effective as autologous cells for therapeutic application [93]. The potential that preserved cells may have 'off-the-shelf' accessibility drives work to confirm their safety and efficacy.

Current *in vitro* evidence provides valuable information surrounding potential immunomodulatory capabilities of undifferentiated equine cells. Five cell lines derived from bone marrow, adipose tissue, umbilical cord blood and tissue were evaluated for inhibition of lymphocyte proliferation and mediator secretion [44]. None of the MSCs affected lymphocyte proliferation or produced measurable levels of mediators in a quiescent environment, but activated MSCs secreted interleukin 6 and prostaglandin E_2 and inhibited lymphocyte proliferation and production of tumour necrosis factor α and interferon γ. The cytokine TGFβ1 was constitutively produced by all cell lines. These results offer some explanation of the mechanism by which MSCs may modulate the immune response, because interleukin 6, prostaglandin E_2 and TGFβ1 are factors that may inhibit lymphocyte production. Adding to information from this study, the immunomodulatory mRNA profile of umbilical cord blood and matrix as well as peripheral blood MSCs was determined [93]. Co-stimulatory molecules, those necessary to trigger and amplify T-helper cell responses following T cell activation, CD40 and CD80 were moderately to strongly expressed by all MSCs. Transforming growth factor β and hepatocyte growth factor, which synergistically suppress T cell proliferation, were strongly and moderately to weakly expressed, respectively. The genetic expression of co-stimulatory molecules found in this study is an important consideration for potential MSC immunogenicity.

As described above, allogeneic ESCs and BMSCs appear to persist in and around tendon tissues for up to 90 days after injection [64], and autologous periosteal MSCs are present in musculoskeletal and gastrointestinal tissues up to 12 weeks [94]. In an earlier study, there were leucocytes in and around mechanically induced superficial flexor tendon lesions injected with autologous and allogeneic BMSCs 10 and 34 days after administration [96]. In the 2 tendon studies, determination of leucocyte infiltration in response to cell implantation was hampered by cell migration. However, persistence of the cells in all studies potentially supports lack of active cell removal.

Lack of response to repeated injections of allogeneic cells was determined by similar local and lymphocytic responses to sequential sets of intradermal injections of autologous and allogeneic umbilical cord tissue MSCs 3–4 weeks apart [97]. Dermal induration and wheal area were compared with saline and phytohaemogglutinin injections administered at the same time, and mixed leucocyte reactions were used to quantify lymphocytic responses. Potential MSC migration among skin injection sites was not evaluated. Local dermal reactions were similar between cell sources, and *ex vivo* T cell proliferation was not affected by either, indicating no detectable cellular immune response to multiple intradermal MSC injections.

Two sequential studies to quantify responses to intra-articular injection of autologous, autologous transduced with human bone morphogenetic protein 2, allogeneic and xenogeneic (human) BMSCs provide additional information [98,99]. Bone marrow-derived multipotent stromal cells from each group were simultaneously injected into normal fetlocks of 6 horses (one fetlock per BMSC group). Sixty and 120 days after injection, synovial joint biopsies and peripheral blood mononuclear cells were isolated, respectively. Joint parameters and synovial fluid were evaluated 1, 2, 7, 14, 21 and 28 days after injection. Joint inflammation was evident in all treated fetlocks, but most severe between 24 and 48 h in joints that received allogeneic and xenogeneic BMSCs. Synovial joint fluid parameters returned to normal ranges between 14 and 28 days. There was a persistent mononuclear cell infiltrate in the synovium of all joints that received MSCs. In co-culture experiments, xenogeneic MSCs induced the largest increases in CD4+ lymphocytes, interleukin 6 and interferon γ, though interleukin 6 and interleukin 10 were elevated in all groups compared with unstimulated MSCs. Cell presence in the joints could not be demonstrated at either of the latter 2 time points, in contrast to previous studies, and cell migration among joints and tissues was not evaluated.

A recent publication examined the *in vitro* immunogenicity of BMSCs from horses with different major MHC II haplotypes using mixed leucocyte reactions [100]. In general, MHC II expression varied among individual cell isolates and, in some cases, changed with culture, but the majority of cell passage 2–4 cells were MHC II positive. The responder T cell proliferation response to MHC-mismatched MHC II was similar to that caused by the positive control, MHC-mismatched peripheral blood leucocytes, and significantly greater than to MHC-mismatched MHC II-negative and MHC-matched cells. Furthermore, stimulation of MHC class II-negative BMSCs with interferon γ caused a marked increase in MHC II expression. The results of this study highlight additional considerations surrounding allogeneic administration of MSCs, especially in cases of repeated application or at sites of active inflammation.

Conclusions

The information in this review is a small representation of the current state of stem cells in equine regenerative medicine. Current comprehensive summaries of *in vivo* equine studies to assess regenerative medical therapies are available elsewhere [17,19,23,26,95,101–106]. Points such as appropriate control groups, study designs that meet evidence-based medicine standards, limitations of induced injury models, sufficient sample sizes and consistency among treatment preparations, among others, deserve continued investigative diligence for implementation of regenerative medical therapies into standard treatment regimens.

Inconsistencies among cell isolation techniques can result in undifferentiated cells that may appear similar, but, as highlighted in this review, have very different capacities to direct or contribute to tissue regeneration. Unified isolation techniques will help to derive consistent cell isolates. Embryonic and induced pluripotent stem cell lines that maintain pluripotency and karyotype following cryopreservation and extended culture may augment current cell therapy options. The importance of multilevel, standardised cell characterisation cannot be overstated. It is reasonable to assume that distinct cell phenotypes have greater efficacy for specific clinical targets and that there is unlikely to be a single cell panacea that meets all equine health needs. Cell phenotypes with reproducible behaviours in standardised conditions are crucial.

Recreation of natural environments in the laboratory is a continuous endeavour. No single set of conditions represents every possible scenario. However, complex environments that closely recreate the natural state will make it possible to test cell survival and potential contributions to tissue formation reliably. Dynamic culture systems with multiple cell types, tissue-specific atmospheric conditions and species-specific extracellular matrix, among other conditions, will continue to enhance the impact of *in vitro* culture systems.

Demonstration of both safety and efficacy is paramount to translation of *in vitro* findings to clinical problems. In this regard, regulation lags behind discovery in the field of veterinary regenerative medicine. Assuming the same criteria as those for human drugs and biologics is not ideal, but it is a potential reality. Unanticipated outcomes from undifferentiated cells are a real possibility. It is clear that cells may persist for extended periods of time and that they are capable of migrating among limbs and from the distal limb to the gastrointestinal tract. Variability in immunogenicity among cell isolations and preimplantation culture conditions is a safe assumption. What is not clear is how long exogenous cells remain viable and functional, whether they change significantly with time and/or stimulation *in vivo*, and how dynamic changes may influence biologically active protein production and immunogenicity. The potential extent and impact of cell migration and delineation of local and systemic immune responses continue to be vital areas of work.

A natural step between product development and implementation is evaluation in animal species that are not the final target. The limitations of immunocompromised and nonspecies-specific models are well recognised and beyond the scope of this review. However, determination of relative efficacy in both ectopic and orthotopic locations allows comparisons within complex tissue that cannot be recapitulated artificially. Genetically manipulated species also offer advantages for mechanistic studies that are more difficult in immunocompetent species. Although not directly applicable, results contribute to outcome assessments that elucidate complex biological interactions to refine regenerative medical therapies.

The capacity to form tissues varies widely among patients. Comorbidities add another layer of complexity. It is unreasonable to assume that there is a 'one-size-fits-all' regenerative medical therapy for all patients and problems. Therapeutic dosages, regimens and potential are likely to vary widely among patients. The creation of viable tissue grafts in the laboratory may be on the horizon for equine regenerative medicine. Given that cells at many stages of differentiation retain their properties after cryopreservation, it is logical to assume that tissues at various stages of development can be preserved in a similar manner. As in all fields of science, increasing knowledge tends to discern the limits of current understanding to guide incremental progress towards standardised therapies for defined clinical targets.

Recognition and appreciation of the nearly limitless potential of regenerative medicine has resulted from the rapid evolution of cell-based therapies. However, mechanisms fully to harness and subsequently target that potential towards clinical applications are slower to develop. This contributes to disparities between expectations and realities of regenerative medicine. In a rapidly evolving field, new findings continuously deliver abundant new sets of unknowns. Such a dichotomy of rapid discovery paired with increasing awareness of knowledge gaps challenges efforts to design safe and effective treatment strategies for specific clinical needs based on predictable outcomes. Fortunately, the promise of restoring tissues and organs to a new and functional state maintains the momentum of this emerging field in contemporary medicine. Stepwise advances based on focused, evidence-based studies will continue to build the new reality of equine regenerative medicine.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1: Equine trilineage differentiation media components, culture conditions and stains.
Table S2: Markers reported for characterisation of undifferentiated equine cells.
Table S3: Genes reported for undifferentiated equine cell characterisation with polymerase chain reaction technology.