

Generation, Characterization, and Multilineage Potency of Mesenchymal-Like Progenitors Derived from Equine iPSC Cells

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Multipotent mesenchymal stromal cells (MSCs) are more and more frequently used to treat orthopedic injuries in horses. However, these cells are limited in their expandability and differentiation capacity. Recently, the first equine-induced pluripotent stem cell (iPSC) lines were reported by us [1]. In vitro differentiation of iPSCs into MSC-like cells is an attractive alternative to using MSCs derived from other sources, as a much larger quantity of patient-specific cells with broad differentiation potential could be generated. However, the differentiation capacity of iPSCs to MSCs and the potential for use in tissue engineering have yet to be explored. In this study, equine iPSCs were induced to differentiate into an MSC-like population. Upon induction, the iPSCs changed morphology toward spindle-shaped cells similar to MSCs. The ensuing iPSC-MSCs exhibited downregulation of pluripotency-associated genes and an upregulation of MSC-associated genes. In addition, the cells expressed the same surface markers as MSCs derived from equine umbilical cord blood. We then assessed the multilineage differentiation potential of iPSC-MSCs. Although chondrogenesis was not achieved after induction with transforming growth factor-beta 3 (TGFβ3) and/or bone morphogenic protein 4 (BMP-4) in 3D pellet culture, mineralization characteristic of osteogenesis and lipid droplet accumulation characteristic of adipogenesis were observed after chemical induction. We demonstrate a protocol for the derivation of MSC-like progenitor populations from equine iPSC cells.

Introduction

MULTIPOTENT MESENCHYMAL STROMAL CELLS (MSCs), also known as mesenchymal stem cells, have demonstrated great potential for cell-based treatment of disease and injury over recent years [2–5]. MSCs show promise in areas such as immunomodulation, enhanced wound healing, regenerative medicine, and tissue engineering, especially in tissues such as the bone and cartilage [3,4,6]. Clinical studies have shown that injection of MSCs into a site of injury speeds healing. MSCs may directly contribute to regeneration by differentiating into the appropriate tissue type *in vivo* or may more likely contribute to healing in an indirect manner through secreting factors that reduce inflammation and stimulate endogenous repair [7–10].

In horses, several reports have been published on the treatment of orthopedic defects using MSCs derived from

various sources, typically isolated from the bone marrow and adipose tissue [9]. Although these tissues provide a source of MSCs, collection is an invasive process and the capacity for proliferation and differentiation of these cells declines with the age of the horse [11,12]. In addition, MSC isolation and expansion from bone marrow and adipose tissue take weeks to reach therapeutic cell numbers. MSCs isolated from umbilical cord blood (CB-MSCs) of newborn foals can eliminate some of these issues. The cord blood is collected in a minimally invasive manner, and the cells are phenotypically young when compared to adipose- and bone marrow-derived MSCs from adult donors [13,14]. Cryopreservation allows CB-MSCs to be used as required. The cells can be banked for autologous and allogeneic use [15]. However, there are unique limitations to CB-MSC isolation, in particular failure to collect blood at the time of foaling [16]. Like MSCs from other sources, CB-MSCs are ultimately limited in their

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proliferation and differentiation potential *in vitro*. Therefore, there is a need for an MSC source that has greater expandability and high differentiation potential.

Embryonic stem cells are capable of proliferating indefinitely and are considered pluripotent. Equine embryonic-like stem cells have been reported, but no research groups have described equine embryonic stem cells that fulfill all criteria of pluripotency [17]. Therefore, focus has turned to generating equine-induced pluripotent stem cells (iPSCs) as a cell source that could be utilized for developmental studies and cell-based therapies in the horse [1,18,19]. Our equine iPSC lines [1] were generated from fetal fibroblasts using the *piggyBac* transposon transgene delivery system to transfect the reprogramming factors Oct4, Sox2, Klf4, and c-Myc, as well as green fluorescent protein (GFP) [20]. The cells display pluripotency characteristics: expression of hallmark pluripotency genes, the ability to self-renew, form embryoid bodies, and form teratomas containing all three embryonic germ layers in NOD/SCID mice [1].

Since iPSCs can theoretically be propagated indefinitely, a possible treatment would be to inject these cells directly into the affected individual (systemically or at the site of injury); similar to what has been done with MSCs. However, there is a substantial risk of teratoma formation through direct iPSC injection in wild-type and immunocompromised animals, both syngeneically and allogeneically [21]. The differentiation of iPSCs into specific tissue types before implantation is bound to decrease the risk of teratoma formation, as it reduces both the expandability and pluripotency of the iPSCs [21,22]. Nevertheless, directed differentiation of iPSCs into a homogenous population of differentiated cells is technically difficult and can result in immature and/or inferior tissue with regard to cell and extracellular matrix organization [23]. Therefore, differentiating iPSCs into an intermediate multipotent cell type, such as MSCs, may not only reduce or eliminate the risk of teratoma formation upon implantation but may also provide a more homogenous and mature cell type for tissue engineering [24]. Recently, successful differentiation of human and mouse iPSCs into MSC-like cells has been reported [25–27] and shows that a high yield of MSCs can be generated with enhanced proliferation and differentiation capabilities compared to adult MSCs [27]. These MSC-like cells appear to incorporate the benefits of both iPSCs and MSCs, making them a promising new cell type for regenerative medicine.

We hypothesized that equine iPSCs are a robust and highly expandable source of MSCs. We report on the differentiation of equine iPSCs into MSC-like progenitor cells under specific culture conditions. The resulting equine iPSC-MSCs are plastic adherent and display a fibroblastoid morphology that resembles MSCs from other sources. They show a downregulation of the pluripotency-associated endogenous genes, *Oct4*, *Nanog*, *Sox2*, and *Klf4*, an upregulation of the MSC-associated genes, *Sox5*, *Sox6*, *Sox9*, *CD29*, *CD44*, and *CD90*, and an upregulation of mesodermal lineage genes, *BMP7* and *SNAI3*, under induction conditions. In addition, these cells express MSC surface markers CD90, CD29, and CD44 and did not express the markers CD4, CD8, CD73, CD105, MHC I, CD11a/18, CD45, and MHC II. Our equine iPSC-MSCs are capable of undergoing adipogenesis and osteogenesis, but not chondrogenesis *in vitro*.

Materials and Methods

iPSC culture and maintenance

Equine H3-B iPSCs were grown in Dulbecco's modified Eagle medium-high glucose (DMEM-HG, No. 11960-044; Invitrogen) supplemented with 2 mM GlutaMAX™ (No. 35050; Invitrogen), 0.1 mM nonessential amino acids (No. 11140; Invitrogen), 0.1 mM betamercaptoethanol (No. M7522; Sigma-Aldrich), 1 mM sodium pyruvate (No. 11360-070; Invitrogen), 50 U/mL penicillin/streptomycin (No. 15070; Invitrogen), 15% fetal bovine serum (FBS; HyClone), 1,000 U/mL leukemia inhibitory factor (No. ESGRO; Millipore), 10 ng/mL basic fibroblast growth factor (bFGF, No. 100-18B; PeproTech), and 1.5 µg/mL doxycycline (No. D9891; Sigma-Aldrich). The cells were plated on mitomycin-inactivated mouse embryonic feeder cells and cultured in a humidified incubator at 37°C and 5% CO₂. They were passaged every 2–3 days by enzymatic digestion with TrypLE Select (No. 12563; Invitrogen) at a 1:4 ratio.

Derivation of an MSC-like population from equine iPSCs

A serial plating strategy was used to differentiate the iPSC line H3-B [1] into MSC-like cells (Fig. 1). To separate the iPSCs from their mouse embryonic fibroblast feeder layer, the cells were enzymatically digested as described above, centrifuged, and resuspended in the sorting medium containing phosphate-buffered saline (PBS), 25 mM HEPES, and 0.2% FBS. Fluorescent-activated cell sorting of GFP-positive iPSCs was performed using the MoFlo Astrios Cell Sorter (Beckman Coulter). GFP-positive cells were seeded onto gelatin-coated plates at 1×10^4 cells/cm² in MSC induction media consisting of DMEM-HG, 10% FBS, 0.1 mM nonessential amino acids, 50 U/mL penicillin/streptomycin, 10 µM anaplastic lymphoma kinase (ALK) inhibitor SB431542 (Stemgent No. 04-0010), and 5 ng/mL bFGF. Cells were expanded to 80% confluency and then passaged onto a second gelatin-coated plate for one passage. Hereafter, the cells were passaged onto uncoated tissue culture plates and propagated for 3–11 passages. The cells gradually obtained a fibroblast-like spindle-shaped morphology. ALK inhibitor was removed from the MSC induction media after 10 days. Cells were routinely passaged every 3–4 days, at a 1:3 split ratio, until reaching the eighth passage. This process was reproduced thrice with H3-B iPSCs ($n=3$). The resulting iPSC-MSCs were evaluated at an early passage (passage 3–4) and late passage (passage 6–8) by gene expression, flow cytometry, and functional differentiation.

Quantitative real-time PCR analyses

Total RNA was harvested from iPSC-MSCs using the Qiagen RNeasy Kit (Qiagen), and 1 µg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). The reaction was performed at 42°C for 15 min. The cDNA was subsequently analyzed by qPCR using LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich) using the CFX384 Touch™ Real-Time polymerase chain reaction detection system (Bio-Rad). For all samples, a negative control was included that did not

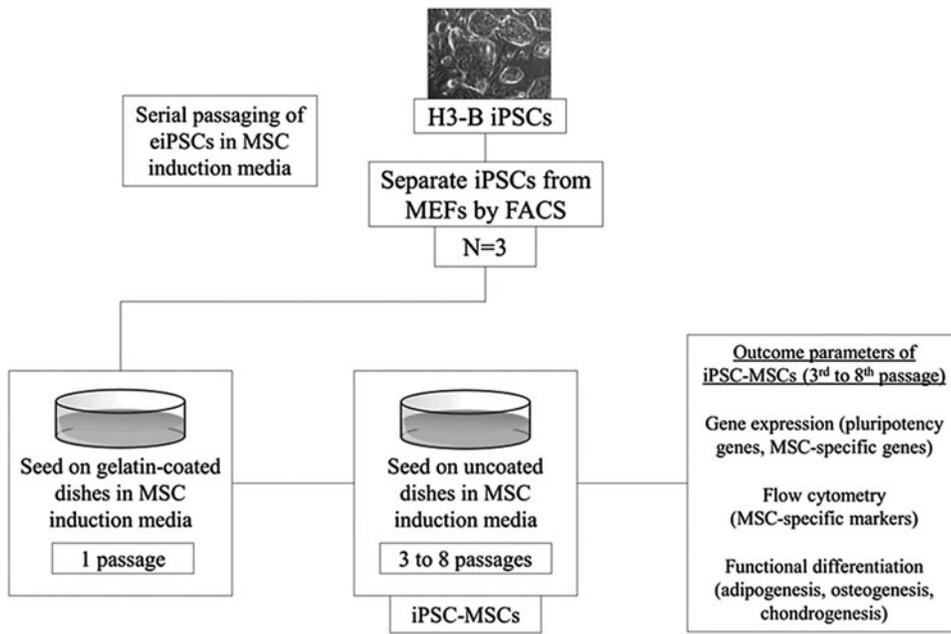


FIG. 1. Study design on the derivation and subsequent evaluation of iPSC-MSCs from equine iPSCs. iPSC, induced pluripotent stem cell; MSCs, mesenchymal stromal cell.

contain cDNA. All reactions were completed in triplicate. Values are expressed as $2^{\Delta\Delta Ct}$, with $\Delta\Delta Ct$ defined as the difference in crossing threshold (Ct) values between experimental and control samples, as described using β -actin and *Hprt1* as internal standards. Fold difference was compared to the H3-B iPSC parent line. Oligonucleotide primers are listed in Table 1. *Oct4*, *Sox2*, and *Klf4* primer pairs do not amplify mouse transcripts from the inserted transgenes [1].

Flow cytometry

iPSC-MSCs and CB-MSCs (1 cell line at passage 3) were expanded and subsequently harvested using ACCUMAX (Stem Cell Technologies) and washed with flow buffer (PBS, 5 mM EDTA, 1% horse serum, and 0.1% sodium azide). The following primary antibodies were used to stain

the cell suspensions (150,000–200,000 cells): mouse anti-human CD73 (Abcam), mouse anti-human CD90 (VMRD), mouse anti-human CD105 (ABD Serotec), mouse anti-horse CD44 (ABD Serotec), fluorescein isothiocyanate (FITC) mouse anti-human CD29 (Beckman Coulter), mouse anti-human CD45 (VMRD), mouse anti-horse CD4 (ABD Serotec), mouse anti-horse CD8 (ABD Serotec), mouse anti-horse CD11a/18 (ABD Serotec), mouse anti-horse MHC I (ABD Serotec), and mouse anti-horse MHC II (ABD Serotec). Rat anti-mouse IgM-FITC and goat anti-mouse IgG1-FITC were used as secondary antibodies. Specificity of all antibodies has been validated in our laboratory using positive controls for the target species of the antibody (horse or human) and with three negative controls: isotype-negative control, secondary antibody, and unstained control [28]. Ten thousand events were acquired for each experiment on BD FACScan (BD Biosciences) using CellQuest (Becton

TABLE 1. PRIMER PAIRS USED FOR qRT-PCR

Gene	Forward primer	Reverse primer	Source
<i>B-actin</i>	ATGGTGGCAATGGGTCAGAAGGAC	CTCTTTGATGTACGCACGATTTC	[1]
<i>Klf4</i>	AGGCATCTCCATTTATATTTA	CATGGGGAGAGCTCCTC	[1]
<i>Nanog</i>	TCCTCAATGACAGATTTTCAGAGA	TGGCAAATTGCTCGAGGTCT	[1]
<i>Sox2</i>	ACGCTTTCATGGTATGGCCCCA	TTCAACTCGGCCGCCAAGC	[1]
<i>Hprt1</i>	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT	[1]
<i>Sox9</i>	ATCTGAAGAAGGAGAGCGAG	TCAGAAGTCTCCAGAGCTTG	Unpublished
<i>Sox5</i>	ATGGAGAGGTAGCCATGGTG	TGCTGAGAAGTGGGAGTCCT	[30]
<i>Sox6</i>	CTTACTCGGCCAGAAGATGC	GAAGGCCTGAAGGATTTTCC	Unpublished
<i>CD29</i>	CCATTGTTACGTTGTGGAG	TTGGCAAATTCCCTTCTGTC	Unpublished
<i>CD44</i>	ATCCTCACGTCCAACACCCTC	CTCGCCTTCTTGGGTGTAGC	Unpublished
<i>CD90</i>	GACTGCCGCCATGAGAATAC	GTGTAGAGCCCCCTCGTCCTT	Unpublished
<i>BMP7</i>	GCCATCGTGCAGACACTGGT	GGCCCCAGAGGTCTGACTT	Unpublished
<i>SNAI3</i>	CCGCATCAGCGTGTACCAAG	CTCTGCCCGTCCAAGGTCTC	Unpublished
<i>LPL</i>	ATCAACTGGATGGCGGAGGA	ATCCGCATCATCAGGGGAAA	Unpublished
<i>PPARγ</i>	ATGCCTTGCTGTGGGGATGT	TCAAATTGCCCTCGCCTTC	Unpublished

qRT-PCR, quantitative real time-PCR.

Dickinson) software, and analysis was conducted using FlowJo (Tree Star).

Osteogenesis assay

Osteogenic differentiation was induced as previously described [2]. In brief, passage 4 iPSC-MSCs were seeded in six-well plates at a density of $\sim 3,000$ cells/cm² and grown until 90%–100% confluency. To induce osteogenesis, cells were cultured for 10 days in osteogenic media containing Dulbecco's modified Eagle medium-low glucose (DMEM-LG), 10% FBS, 1% L-glutamine, 1% antibiotic antimycotic (ABAM), 100 nM dexamethasone, 10 mM β -glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. An equal number of control cells were maintained in the MSC expansion medium. Media were changed every 3 days. Osteogenesis was evaluated using Alizarin Red S staining for calcium deposition and von Kossa staining for phosphate accumulation.

Adipogenesis assay

Induction of adipogenesis was performed as previously described [29]. Passage 4 iPSC-MSCs were seeded at a density of $\sim 2.1 \times 10^4$ cells/mL in six-well plates. Adipogenesis was carried out in adipogenic induction media, containing DMEM-LG, 15% rabbit serum, 500 μ M IBMX, 1% L-glutamine, 1% ABAM, 1 μ M dexamethasone, 10 μ g/mL insulin, and 200 μ M indomethacin. Control cells were maintained in expansion media. Evaluation of positive adipogenesis was done by Oil Red O staining to observe lipid droplets.

Chondrogenesis assay

Passage 6 and 11 iPSC-MSCs were induced to undergo chondrogenesis in pellet culture as previously described [30]. Approximately 2.5×10^5 iPSC-MSCs were resuspended in 200 μ L of chondrogenic induction media, containing high-glucose DMEM (DMEM-HG), 200 mM GlutaMAX, 100 mM sodium pyruvate, 1% ABAM, 0.1 mM dexamethasone, 100 mg/mL ascorbic acid-2 phosphate, 40 mg/mL proline, 1 \times insulin,

transferrin, and selenious acid (ITS) premix (No. 515 00-056; Life Technologies), and 10 ng/mL transforming growth factor-beta 3 (TGF β 3, No. 243-B3; R&D Systems). The resuspended cells were plated in V-bottom polypropylene 96-well plates (No. MPG-651201; Phenix) and centrifuged to pellet the cells at the bottom of the wells. Bone morphogenic protein 4 (BMP-4, No. E12512; Roche) was added alone or in conjunction with TGF β 3 in various final concentrations (10, 50, and 100 ng/mL) to the chondrogenic media in a subset of pellets. BMP-4 was removed from the media after 1 week of pellet culture, as previously described [11]. Pellets were incubated at 37°C in 5% CO₂ and 21% O₂ for 2–4 weeks. Media were changed every 2–3 days.

Statistical analysis

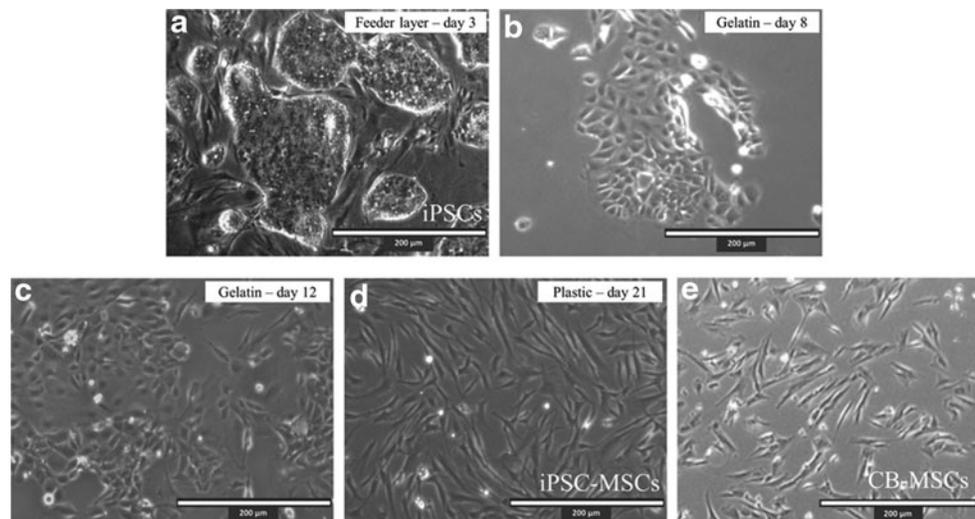
GraphPad Prism 6 Software was used to perform statistical analysis on qRT-PCR data. One-way ANOVA, followed by Tukey's post hoc test, was used to determine expression differences between groups. *P*-values <0.05 were considered to be statistically significant.

Results

Derivation of iPSC-MSCs from equine iPSCs

Derivation of MSCs from iPSCs has been done indirectly through the formation of embryoid bodies and subsequent selection of adherent outgrowths, or directly through the addition of specific differentiation media [26,31,32]. Since attempts at propagating adherent cells from embryoid bodies were unsuccessful (data not shown), we opted to explore the latter method and include serial passaging onto uncoated Petri dishes to select for a more homogenous population of MSC-like cells. After 8 days on gelatin in MSC induction media, the iPSCs lost their colony forming phenotype and acquired a mixed cuboidal and spindle-like morphology (Fig. 2a, b). Upon subsequent passaging onto uncoated Petri dishes, the spindle-like morphology predominated (Fig. 2c) and eventually the majority of the differentiated population maintained this MSC-like phenotype (Fig. 2d).

FIG. 2. Derivation of an MSC-like population from equine iPSCs. iPSCs maintained in iPSC media form ES-like colonies (a). iPSCs cultured in MSC media on gelatin acquired a heterogeneous cuboidal-like morphology with time (b). Upon passaging onto uncoated tissue culture plastic, cells adherent to plastic acquired a more homogenous spindle-like morphology (c, d) similar to MSC (e).



Gene expression of iPSC-MSCs

To further characterize and classify iPSC-MSCs, gene expression analyses were conducted. Gene expression of equine pluripotency markers (*Oct4*, *Sox2*, *Nanog*, and *Klf4*) in three separately derived iPSC-MSC lines was compared to their parent H3-B iPSC line. In both early passage (passage 3–4) and late passage (passage 6–8) cells, iPSC-MSCs showed consistent downregulation of expression in each gene relative to H3-B iPSC (Fig. 3a). In addition, iPSC-MSCs displayed significant upregulation of MSC-associated genes *Sox5*, *Sox6*, *Sox9*, and *CD90* as well as an upregulation of mesodermal lineage genes *BMP7* and *SNAI3* relative to H3-B iPSC (Fig. 3b). In addition, *Sox5*, *Sox6*, *Sox9*, and *CD44* and *CD90* transcription levels approached that of CB-MSCs.

Flow cytometry analyses and derivation efficiency of iPSC-MSCs

Another criterion for the classification of MSCs is expression of a certain panel of surface markers [33]. As equine MSCs lack a standardized set of surface markers associated with MSCs of various sources, our laboratory has established surface antigen expression for MSCs derived from cord blood [28]. Upon flow cytometry analysis, we observed that iPSC-MSCs display a similar immunophenotype as CB-MSCs, namely, positive expression for CD29, CD44, and CD90 and low or absent expression of CD4, CD8, CD105, CD45, CD73, CD11a/18, MHC I, and MHC II (Fig. 4a, b). Of cells that remained plastic adherent throughout the MSC induction process, 74.4% of iPSC-MSCs expressed CD90, 97.5% expressed CD29, and 97.7% expressed CD44 (Fig. 4). As these values are nearly identical to the percent-positive cells in CB-MSCs (CD90: 76.6%; CD29: 97.0%; and CD44: 90.9%; Fig. 5), MSC derivation efficiency of adherent iPS cells using our serial plating method is close to 100%.

Characterization of functional differentiation of iPSC-MSCs

To elucidate the multilineage differentiation potency of iPSC-MSCs, we performed adipogenic, osteogenic, and chondrogenic induction assays in vitro. After 10 days in osteogenic induction media, von Kossa staining and Alizarin Red staining revealed that the iPSC-MSCs acquired mineralization after induction, indicative of early osteogenesis (Fig. 6). Furthermore, after 14 days in adipogenic induction media, morphology changes and lipid droplets were observed in the iPSC-MSCs following Oil Red O staining (Fig. 6). The results obtained were consistent with what was observed in CB-MSCs induced for osteogenesis and adipogenesis for the same time period (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Analysis of adipogenic-associated genes LPL and PPAR γ showed no significant difference in gene expression in induced iPSC-MSCs (for LPL: $P=0.2156$; for PPAR γ : $P=0.0633$) and CB-MSCs (for LPL: $P=0.3504$; for PPAR γ : $P=0.1081$) compared to noninduced cells (Supplementary Fig. S2). Chondrogenesis was not successful despite several attempts to induce chondrogenesis in 3D pellet culture for 2 and 4 weeks using TGF β 3 at 21% oxygen. The pellets were either too small for analysis and/or contained mostly necrotic tissue (Supplementary Fig. S3a, b). In contrast, CB-MSCs induced for 4 weeks showed proteoglycan accumulation and lacunae formation typical of hyaline-like cartilage (Supplementary Fig. S3c). iPSC-MSCs also did not undergo chondrogenesis when treated with BMP-4 alone or with TGF β 3 in 21% or 5% oxygen conditions (data not shown).

Discussion

Our aim in this study was to determine whether equine iPSCs could be successfully differentiated into MSC-like cells that exhibit properties similar to MSCs from other

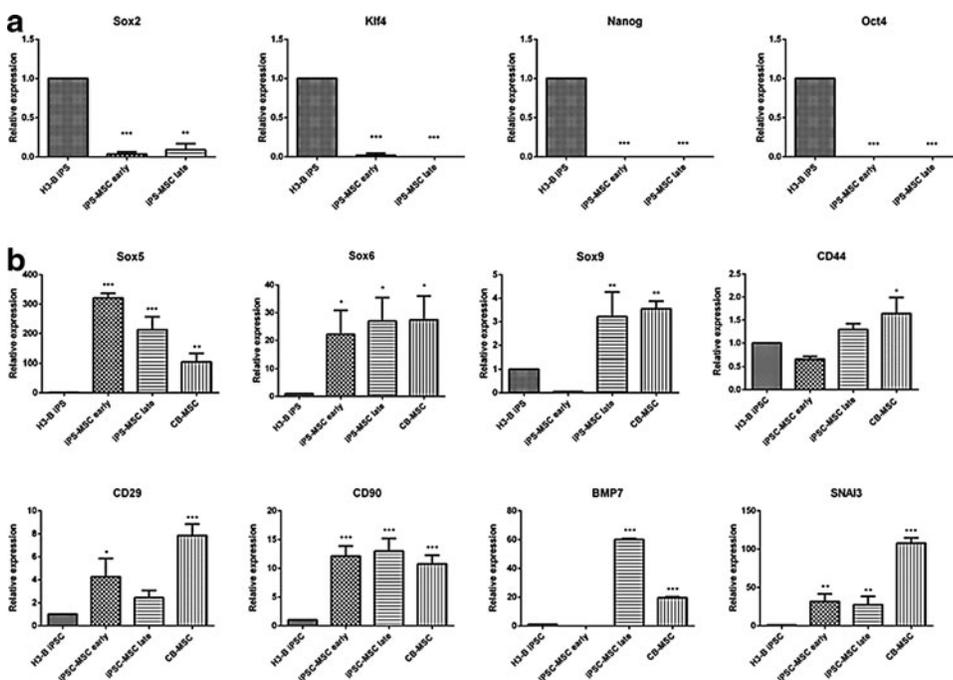


FIG. 3. Gene expression analysis of pluripotency and mesenchymal/mesodermal-related genes by qRT-PCR. (a) Expression data of pluripotency genes *Oct4*, *Sox2*, *Klf4*, and *Nanog*. (b) Expression data of MSC-associated genes *Sox5*, *Sox6*, *Sox9*, *CD44*, *CD29*, *CD90* and mesodermal-associated genes *BMP7* and *SNAI3*. H3-B iPSC: parent iPSC line. iPSC-MSC early: passage 3–4. iPSC-MSC late: passage 6–8. CB-MSC: MSCs derived from equine cord blood. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ compared to parent iPSC line. CB-MSC, cord blood mesenchymal stromal cell.

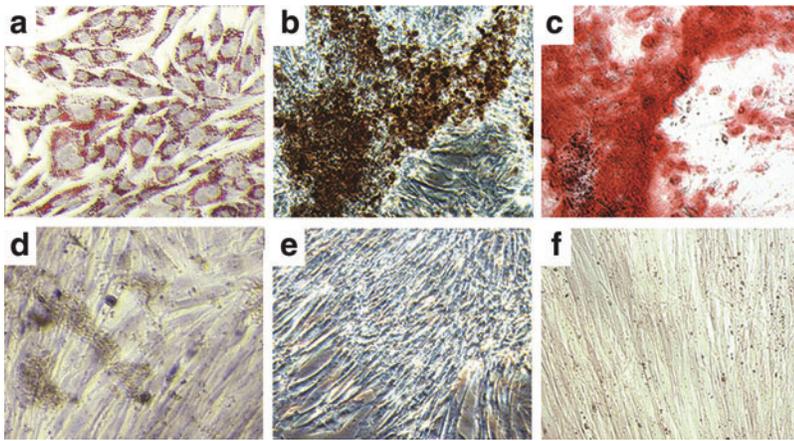


FIG. 6. In vitro adipogenesis and osteogenesis assays of iPSC-MSCs (200 \times magnification). (a) iPSC-MSCs cultured in the adipogenic induction medium showed marked changes in morphology and lipid droplet accumulation as shown by positive Oil Red O staining. (d) Adipogenic control stained negative for Oil Red O after 14 days in the MSC expansion medium. iPSC-MSCs cultured in the osteogenic induction medium acquired mineralization by positive von Kossa staining for phosphate accumulation (b) and Alizarin Red staining for calcium accumulation (c). Osteogenic controls stained negative for von Kossa and Alizarin Red after 14 days in the MSC expansion medium (e, f).

fibroblastic morphology typical of MSCs. A homogenous population of cells resulted after only 2–3 passages on uncoated Petri dishes in MSC induction media; this technique selected for cells that were plastic adherent, as any floating cells were lost when changing media. MSC induction media were found to be required for the differentiation of plastic-adherent cells through the serial passaging method; simply removing doxycycline from the iPSC culture media was not sufficient in inducing the cells to differentiate into MSC-like cells (data not shown).

Gene expression analysis confirmed that pluripotency-associated genes *Oct4*, *Sox2*, *Nanog*, and *Klf4* were down-regulated in iPSC-MSCs upon differentiation. This suggests a loss of pluripotency, which was correlated with an up-regulation of MSC-associated genes *Sox5*, *Sox6*, *Sox9*, *CD29*, and *CD90* and an up-regulation of mesodermal lineage markers *BMP7* and *SNAI3*. *Sox5* and *Sox6* are transcription factors involved in the osteochondroprogenitor lineage program and act upstream of *Sox9* [41]. The *Sox9* transcription factor is the key regulator in chondrogenesis, initiating the expression of cartilage-specific extracellular matrix genes such as *Col2a1* [42]. *Sox9* is required for the commitment of chondroprogenitors, mesenchymal condensation, and proper chondrocyte proliferation, differentiation, and maturation [43]. Our data revealed that although *Sox5* and *Sox6* were up-regulated in iPSC-MSCs in both early and late passages, *Sox9* expression was only up-regulated in late passage cells (Fig. 3b). This variable expression of the *Sox* trio of transcription factors has been observed before in iPSC-MSCs, although chondrogenesis was observed in these cell lines [26].

BMP7 is a member of the TGF β superfamily and is an early mesodermal lineage marker [44,45]. It has been found to be expressed at numerous sites throughout development. Late passage iPSC-MSCs show a distinct up-regulation of *BMP7* over H3-B iPSC, although no expression was detected in early passage iPSC-MSCs (Fig. 3b). *SNAI3* is a zinc finger protein, a member of the Snail protein family, and is also an early mesodermal marker. Snail proteins function primarily as transcriptional repressors and are capable of inducing epithelial–mesenchymal transition [46]. *SNAI3* was up-regulated in both early and late passage iPSC-MSCs (Fig. 3b).

Flow cytometry of MSC markers (*CD90*, *CD29*, and *CD44*) predicted a homogenous population of differentiated MSC-like cells successfully derived from iPSCs. This indicates that the efficiency of derivation among plastic-adherent cells is close to 100% (Fig. 4). We chose this efficiency assessment method as it provides the most accurate evaluation of the percentage of cells with an MSC phenotype. However, it must be recognized that this is not a true measure of derivation efficiency from undifferentiated iPSCs to MSCs. This is because of the nature of the serial plating method, where some iPSCs were lost during media replacement due to lack of adherent properties. Efficiency of MSC generation is difficult to establish, and reports on iPSC-MSC or embryonic cell conversion often do not include efficiency data [22,26,47–49]. However, the flow cytometry results show that the cells that remained attached throughout iPSC-MSC conversion are homogenous and phenotypically similar to CB-MSCs.

Our iPSC-MSCs successfully underwent adipogenesis and osteogenesis after chemical induction, as determined by morphology and staining assays (Fig. 6). Analysis of the expression of adipogenesis-associated genes *LPL* and *PPAR γ* did not yield significant results in induced iPSC-MSCs or CB-MSCs (Supplementary Fig. S2). We believe this to be a power issue rather than an indication of minimal adipogenic differentiation, as clear morphological changes and lipid vacuole formation are seen in induced iPSC-MSCs and CB-MSCs (Fig. 6 and Supplementary Fig. S1).

Intriguingly, iPSC-MSC response to chemical chondrogenic induction in 3D pellet culture was lower than expected. This may be associated with the low expression of *Sox9* in early passage cells; although *Sox9* expression increases in late passage cells, the increased passage number may correlate with decreased chondrogenic potential as is seen in MSCs [50]. Other studies have demonstrated the benefit of overexpressing *Sox9* to enhance chondrogenesis [43,51,52]. It may be that the use of only one equine iPSC line reflects donor-to-donor variation in the chondrogenic potency of MSCs from adult tissues, a known phenomenon [11,53,54]. iPSC-MSCs generated from other equine iPSC lines may possess chondrogenic potency under the described conditions. Several unsuccessful attempts at direct differentiation of iPSCs to chondrocytes were attempted as well;

these included embryoid body formation, pellet culture of iPSCs, membrane culture of iPSCs alone, and membrane culture of iPSCs cocultured with bovine chondrocytes (data not shown). This work exemplifies the caveat that established mouse and human protocols for differentiation cannot simply be recapitulated in the horse. This report is a first step toward development of equine specific protocols for iPSC cell fate conversion toward MSCs.

In conclusion, the present study provides an important look at the capacity of horse iPSCs to differentiate into an MSC-like progenitor population. Using a simplified serial plating technique, we demonstrate that MSC-like cells with properties similar to CB-MSCs can be derived from equine iPSCs. Although this technique is reproducible in one equine iPSC line, the general robustness of the protocol should be tested using a larger number of unrelated iPSC lines. Testing several iPSC lines would also help answer whether the lack of chondrogenic potential is a general feature of equine iPSC-MSCs or the iPSC line used in this study.

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Author Disclosure Statement

T.G.K. acts in a volunteer capacity as a nonexecutive Director, Scientific Affairs (ex officio) of eQcell therapies Inc., Aurora, Ontario, Canada, a company for which T.G.K.'s research laboratory provides equine stem cell isolation and storage services. T.G.K. holds a minor non-controlling share in eQcell therapies Inc., S.I.M.L., K.N., H.-K.S., R.A.K., and A.N. declare no competing interests.

References

- Nagy K, HK Sung, P Zhang, S Laflamme, P Vincent, S Agha-Mohammadi, K Woltjen, C Monetti, IP Michael, LC Smith and A Nagy. (2011). Induced pluripotent stem cell lines derived from equine fibroblasts. *Stem Cell Rev* 7:693–702.
- Bieback K, P Wuchter, D Besser, W Franke, M Becker, M Ott, M Pacher, N Ma, C Stamm, et al. (2012). Mesenchymal stromal cells (MSCs): science and f(r)iction. *J Mol Med (Berl)* 90:773–782.
- Knight MN and KD Hankenson. (2013). Mesenchymal stem cells in bone regeneration. *Adv Wound Care (New Rochelle)* 2:306–316.
- Magne D, C Vinatier, M Julien, P Weiss and J Guicheux. (2005). Mesenchymal stem cell therapy to rebuild cartilage. *Trends Mol Med* 11:519–526.
- Steinert AF, L Rackwitz, F Gilbert, U Noth and RS Tuan. (2012). Concise review: the clinical application of mesenchymal stem cells for musculoskeletal regeneration: current status and perspectives. *Stem Cells Transl Med* 1:237–247.
- Patel DM, J Shah and AS Srivastava. (2013). Therapeutic potential of mesenchymal stem cells in regenerative medicine. *Stem Cells Int* 2013:496218.
- Kuroda R, K Ishida, T Matsumoto, T Akisue, H Fujioka, K Mizuno, H Ohgushi, S Wakitani and M Kurosaka. (2007). Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. *Osteoarthritis Cartilage* 15:226–231.
- Miyagi S, K Tensho, S Wakitani and M Takagi. (2013). Construction of an osteochondral-like tissue graft combining beta-tricalcium phosphate block and scaffold-free mesenchymal stem cell sheet. *J Orthop Sci* 18:471–477.
- Murphy JM, DJ Fink, EB Hunziker and FP Barry. (2003). Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* 48:3464–3474.
- Nejadnik H, JH Hui, EP Feng Choong, BC Tai and EH Lee. (2010). Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. *Am J Sports Med* 38:1110–1116.
- Choudhery MS, M Badowski, A Muise, J Pierce and DT Harris. (2014). Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *J Transl Med* 12:1–14.
- Stolz A, E Jones, D McGonagle and A Scutt. (2008). Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev* 129:163–173.
- Koch TG, T Heerkens, PD Thomsen and DH Betts. (2007). Isolation of mesenchymal stem cells from equine umbilical cord blood. *BMC Biotechnol* 7:26.
- Koch TG, PD Thomsen and DH Betts. (2009). Improved isolation protocol for equine cord blood-derived mesenchymal stromal cells. *Cytotherapy* 11:443–447.
- Kang J-G, S-B Park, M-S Seo, H-S Kim, J-S Chae and K-S Kang. (2013). Characterization and clinical application of mesenchymal stem cells from equine umbilical cord blood. *J Vet Sci* 14:367.
- Kern S, H Eichler, J Stoeve, H Kluter and K Bieback. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 24:1294–1301.
- Brivanlou AH, FH Gage, R Jaenisch, T Jessell, D Melton and J Rossant. (2003). Stem cells. Setting standards for human embryonic stem cells. *Science* 300:913–916.
- Breton A, R Sharma, AC Diaz, AG Parham, A Graham, C Neil, CB Whitelaw, E Milne and FX Donadeu. (2013). Derivation and characterization of induced pluripotent stem cells from equine fibroblasts. *Stem Cells Dev* 22:611–621.
- Khodadadi K, H Sumer, M Pashaiasl, S Lim, M Williamson and PJ Verma. (2012). Induction of pluripotency in adult

- equine fibroblasts without c-MYC. *Stem Cells Int* 2012: 429160.
20. Woltjen K, R Hamalainen, M Kibschull, M Mileikovsky and A Nagy. (2011). Transgene-free production of pluripotent stem cells using piggyBac transposons. *Methods Mol Biol* 767:87–103.
 21. Zhao T, ZN Zhang, Z Rong and Y Xu. (2011). Immunogenicity of induced pluripotent stem cells. *Nature* 474:212–215.
 22. Yen ML, CH Hou, KY Peng, PC Tseng, SS Jiang, CT Shun, YC Chen and ML Kuo. (2011). Efficient derivation and concise gene expression profiling of human embryonic stem cell-derived mesenchymal progenitors (EMPs). *Cell Transplant* 20:1529–1545.
 23. Wu SM and K Hochedlinger. (2011). Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat Cell Biol* 13:497–505.
 24. Diederichs S and RS Tuan. (2014). Functional comparison of human-induced pluripotent stem cell-derived mesenchymal cells and bone marrow-derived mesenchymal stromal cells from the same donor. *Stem Cells Dev* 23:1594–1610.
 25. Chen GY, DW Pang, SM Hwang, HY Tuan and YC Hu. (2012). A graphene-based platform for induced pluripotent stem cells culture and differentiation. *Biomaterials* 33:418–427.
 26. Guzzo RM, J Gibson, RH Xu, FY Lee and H Drissi. (2013). Efficient differentiation of human iPSC-derived mesenchymal stem cells to chondroprogenitor cells. *J Cell Biochem* 114:480–490.
 27. Lian Q, Y Zhang, J Zhang, HK Zhang, X Wu, Y Zhang, FF Lam, S Kang, JC Xia, et al. (2010). Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation* 121:1113–1123.
 28. Tessier L, D Bienzle, LB Williams and TG Koch. (2015). Phenotypic and immunomodulatory properties of equine cord blood-derived mesenchymal stromal cells. *PLoS One* 10:e0122954.
 29. Berg L, T Koch, T Heerkens, K Bessonov, P Thomsen and D Betts. (2009). Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord blood. *Vet Comp Orthop Traumatol* 22:363–370.
 30. Co C, MK Vickaryous and TG Koch. (2014). Membrane culture and reduced oxygen tension enhances cartilage matrix formation from equine cord blood mesenchymal stromal cells in vitro. *Osteoarthritis Cartilage* 22:472–480.
 31. Chen YS, RA Pelekanos, RL Ellis, R Horne, EJ Wolvetang and NM Fisk. (2012). Small molecule mesengenic induction of human induced pluripotent stem cells to generate mesenchymal stem/stromal cells. *Stem Cells Transl Med* 1:83–95.
 32. Wei H, G Tan, Manasi, S Qiu, G Kong, P Yong, C Koh, TH Ooi, SY Lim, et al. (2012). One-step derivation of cardiomyocytes and mesenchymal stem cells from human pluripotent stem cells. *Stem Cell Res* 9:87–100.
 33. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, D Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315–317.
 34. Koyama N, M Miura, K Nakao, E Kondo, T Fujii, D Taura, N Kanamoto, M Sone, A Yasoda, et al. (2013). Human induced pluripotent stem cells differentiated into chondrogenic lineage via generation of mesenchymal progenitor cells. *Stem Cells Dev* 22:102–113.
 35. Li F, S Bronson and C Niyibizi. (2010). Derivation of murine induced pluripotent stem cells (iPS) and assessment of their differentiation toward osteogenic lineage. *J Cell Biochem* 109:643–652.
 36. Jin GZ, TH Kim, JH Kim, JE Won, SY Yoo, SJ Choi, JK Hyun and HW Kim. (2013). Bone tissue engineering of induced pluripotent stem cells cultured with macrochanneled polymer scaffold. *J Biomed Mater Res A* 101:1283–1291.
 37. Liu Y, AJ Goldberg, JE Dennis, GA Gronowicz and LT Kuhn. (2012). One-step derivation of mesenchymal stem cell (MSC)-like cells from human pluripotent stem cells on a fibrillar collagen coating. *PLoS One* 7:e33225.
 38. Villa-Diaz LG, SE Brown, Y Liu, AM Ross, J Lahann, JM Parent and PH Krebsbach. (2012). Derivation of mesenchymal stem cells from human induced pluripotent stem cells cultured on synthetic substrates. *Stem Cells* 30:1174–1181.
 39. Hynes K, D Menicanin, J Han, V Marino, K Mrozik, S Gronthos and PM Bartold. (2013). Mesenchymal stem cells from iPSC cells facilitate periodontal regeneration. *J Dent Res* 92:833–839.
 40. Zou L, Y Luo, M Chen, G Wang, M Ding, CC Petersen, R Kang, F Dagnaes-Hansen, Y Zeng, et al. (2013). A simple method for deriving functional MSCs and applied for osteogenesis in 3D scaffolds. *Sci Rep* 3:2243.
 41. Ikeda T, S Kamekura, A Mabuchi, I Kou, S Seki, T Takato, K Nakamura, H Kawaguchi, S Ikegawa and UI Chung. (2004). The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum* 50:3561–3573.
 42. Ikeda T, H Kawaguchi, S Kamekura, N Ogata, Y Mori, K Nakamura, S Ikegawa and UI Chung. (2005). Distinct roles of Sox5, Sox6, and Sox9 in different stages of chondrogenic differentiation. *J Bone Miner Metab* 23: 337–340.
 43. Akiyama H. (2008). Control of chondrogenesis by the transcription factor Sox9. *Mod Rheumatol* 18:213–219.
 44. Hahn GV, RB Cohen, JM Wozney, CL Levitz, EM Shore, MA Zasloff and FS Kaplan. (1992). A bone morphogenic protein subfamily: chromosomal localization of human genes for BMP5, BMP6, and BMP7. *Genomics* 14:759–762.
 45. Dale JK, C Vesque, TJ Lints, TK Sampath, A Furley, J Dodd and M Placzek. (1997). Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* 90:257–269.
 46. Yang J and RA Weinberg. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* 14:818–829.
 47. Lian X, B Xiaoping, A Al-Ahmad, J Liu, Y Wu, W Dong, KK Dunn, EV Shusta and SP Palecek. (2014). Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. *Stem Cell Rep* 3:804–816.
 48. Takayama K, M Inamura, K Kawabata, K Katayama, M Higuchi, K Tashiro, A Nonaka, F Sakurai, T Hayakawa, M Kusuda Furue and H Mizuguchi. (2012). Efficient generation

- of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4 α transduction. *Mol Ther* 20:127–137.
49. Hynes K, D Menicanin, S Gronthos and MP Bartold. (2014). Differentiation of iPSC to mesenchymal stem-like cells and their characterization. *Methods Mol Biol* [Epub ahead of print]; DOI: 10.1007/7651_2014_142.
 50. Vacanti V, E Kong, G Suzuki, K Sato, JM Canty and T Lee. (2005). Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. *J Cell Physiol* 205:194–201.
 51. Bosnakovski D, M Mizuno, G Kim, S Takagi, M Okumura and T Fujinaga. (2005). Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res* 319:243–253.
 52. Kimura A, H Inose, F Yano, K Fujita, T Ikeda, S Sato, M Iwasaki, T Jinno, K Ae, et al. (2010). Runx1 and Runx2 cooperate during sternal morphogenesis. *Development* 137:1159–1167.
 53. Keating A. (2006). Mesenchymal stromal cells. *Curr Opin Hematol* 13:419–425.
 54. Huang S, C Feng, Y Wu, S Yang, K Ma, X Wu and X Fu. (2013). Dissimilar characteristics of umbilical cord mesenchymal stem cells from donors of different ages. *Cell Tissue Bank* 14:707–713.

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