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Equine-Induced Pluripotent Stem Cells Retain Lineage Commitment Toward Myogenic and Chondrogenic Fates

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SUMMARY

Induced pluripotent stem cells (iPSCs) hold great potential not only for human but also for veterinary purposes. The equine industry must often deal with health issues concerning muscle and cartilage, where comprehensive regenerative strategies are still missing. In this regard, a still open question is whether equine iPSCs differentiate toward muscle and cartilage, and whether donor cell type influences their differentiation potential. We addressed these questions through an isogenic system of equine iPSCs obtained from myogenic mesoangioblasts (MAB-iPSCs) and chondrogenic mesenchymal stem cells (MSC-iPSCs). Despite similar levels of pluripotency characteristics, the myogenic differentiation appeared enhanced in MAB-iPSCs. Conversely, the chondrogenic differentiation was augmented in MSC-iPSCs through both teratoma and in vitro differentiation assays. Thus, our data suggest that equine iPSCs can differentiate toward the myogenic and chondrogenic lineages, and can present a skewed differentiation potential in favor of the source cell lineage.

INTRODUCTION

Horses are invaluable animals for companionship and sport. The equine industry creates an estimated economic impact of US$300 billion worldwide, and novel means for addressing equine health issues are constantly required (Tecirlioglu and Trounson, 2007). Musculoskeletal problems, including pathologies or injuries of muscle and cartilage, constitute a leading health threat among horses (Smith et al., 2014). As an example, equine atypical myopathy has been increasingly reported over recent years (Votion and Serteyn, 2008), and equine osteochondrosis is relatively frequent across different breeds (van Weeren and Jeffcott, 2013). Therefore, the quest for novel tools for muscle and cartilage repair is still compelling. In this regard, stem cells may support the needs of veterinary medicine (Cebrian-Serrano et al., 2013). In equine practice, mesenchymal stem cells (MSCs) are commonly used to treat tendinitis and osteoarthritis (Schnabel et al., 2013). However, a comprehensive regenerative approach tailored to both muscle and cartilage is still missing, especially for large-scale applications. Importantly, considering the obvious difficulties of in vivo trials, in vitro models constitute a useful, first-line trial platform for addressing differentiation and heterogeneity of stem cells (Goodell et al., 2015). To this end, induced pluripotent stem cells (iPSCs) hold great potential, in light of their tremendous expansion capacity and wide differentiation potential (Yamanaka, 2009). Recently, iPSCs have been generated from equine fibroblasts (Breton et al., 2013) and keratinocytes (Sharma et al., 2014); however, their differentiation potential toward myocytes or chondrocytes remains unknown. Furthermore, iPSCs tend to retain, at least partially, the intrinsic fate propensity of the cell source (Sanchez-Freire et al., 2014). In mice, iPSCs derived from resident myogenic pericytes, i.e. mesoangioblasts (MABs), show biased myogenic differentiation in both teratoma and in vitro differentiation assays (Quattrocelli et al., 2011). However, it is still unknown whether it is possible to discriminate the intrinsic equine iPSC propensity toward the myogenic and the chondrogenic lineages. To address this question, isogenic settings need to reduce the variability introduced by genetic background (Kotini et al., 2015). Relevantly for putative veterinary applications, the choice of the cell source should be confined to somatic compartments at facilitated reach, e.g. blood and superficial muscle biopsies. Equine peripheral blood has been recently used to isolate circulating progenitors, exhibiting MSC properties (Spaas et al., 2013) and differentiating in chondrocytes (Broeckx et al., 2014). With regard to the muscle, it is still unknown whether equine MABs can be isolated with similar characteristics to murine, canine, or

human MABs (Quattrocelli et al., 2014; Sampaolesi et al., 2006), and to which extent equine MABs display myogenic propensity, once cultured in vitro and after reprogramming.

Thus, the objective of this study is to compare iPSCs obtained from equine MABs and peripheral blood MSCs in isogenic conditions to evaluate the intrinsic iPSC propensity for myogenic and chondrogenic lineages.

RESULTS

To obtain source cells for reprogramming in isogenic settings (Figure 1A), we isolated equine MABs in isogenic settings with MSC lines, which were already established from peripheral blood (Spaas et al., 2013), and demonstrated chondrogenic commitment (Broeckx et al., 2014). We adapted the procedures that were previously reported for murine and human MABs (Quattrocelli et al., 2012) to biopsies obtained from the splenius muscles of three syngeneic horses, and accordingly sorted the heterogeneous cell population for alkaline phosphatase (AP) (1.5% ± 0.6% [mean ± SD; n = 3]). AP activity was confirmed in the AP^+ but not the AP^- fraction (Figure 1B). At early passage (<3), AP^+ cells proliferated with a doubling time of approximately 27 hr and stained positively for NG2, CD140a, CD140, and CD44, pericytic surface markers reported for murine and human MABs (Quattrocelli et al., 2012) (Figure 1C). Furthermore, after 7 days in serum starvation, the AP^+ cells robustly differentiated into multinucleated, $MyHC^+$ myotubes (fusion index, 27.5% ± 7.01% [mean ± SD; n = 3]), unlike the isogenic MSC controls (Figure 1D). We thus identified the AP^+ cells as bona fide equine MABs, which show robust myogenic propensity compared with the isogenic MSCs. To obtain iPSCs, we transduced equine isogenic MABs and MSCs with retroviral vectors carrying human reprogramming factors (*OCT4*, *SOX2*, *KLF4*, *cMYC*), then picked and expanded single-cell clones (n = 3/cell type) on a feeder layer. Reprogramming efficiency was approximately 0.0001% for both cell types. Colonies of both MAB- and MSC-iPSCs appeared round-shaped and with compact borders, and were positive for AP, OCT4, SOX2, NANOG, and LIN28 staining (Figure 1E). The expression of the pluripotency markers *OCT4*, *SOX2*, *NANOG*, and *LIN28* was confirmed with specifically cross-reacting primers, using human H9 cells as positive control. Moreover, expression of retroviral transgenes was not detected in both equine iPSC types (Figure 1F). Also, equine iPSCs showed a euploid, donor-matching karyotype (n = 62, XX; Figure 1G). Furthermore, after 20 days of spontaneous in vitro differentiation, both MAB- and MSC-iPSCs differentiated in ectodermal ($TUJ1^+$), endodermal (αFP^+), and mesodermal (αSMA^+) derivative cells (Figure 1H). Thus, equine isogenic MAB- and MSC-iPSCs shared common markers of pluripotency. To gain insight into iPSC intrinsic propensity, we subcutaneously injected equine iPSCs in immunodeficient mice and analyzed the teratomas at 4–6 weeks after injection. Both iPSC types generated teratomas containing immature derivatives of ectoderm, endoderm, and mesoderm, confirming their pluripotency (Figure 2A). However, MAB-iPSC teratomas showed a significantly higher quantity of immature muscle patches in comparison with MSC-iPSCs (Figure 2B). Conversely, MSC-iPSC teratomas showed significantly larger chondrogenic patches (Figure 2C). To exclude the contribution of host cells to teratoma derivatives, we stained equine iPSC teratoma sections for lamin A/C, using murine iPSC- and human iPSC-derived teratomas as negative and positive controls, respectively. Both MAB- and MSC-iPSC-derived teratomas stained positively to lamin A/C (Figures 2D and 2E), indicating that the teratoma tissues derived from equine iPSCs. Intrigued by the propensities shown in the teratoma assays, we asked whether the source-related propensity was significantly skewing the iPSC fate in dedicated differentiation assays. We tested the myogenic differentiation of iPSCs and related source cells under conditions of bone morphogenetic protein (BMP)/transforming growth factor β (TGF-β) blockade and assayed for $MyHC^+$ myocytes and myotubes. After 30 days, MABs and MAB-iPSCs showed higher differentiation rates compared with isogenic MSCs and MSC-iPSCs (Figures 3A and 3B). Moreover, equine *MYH2* expression levels were significantly higher in differentiated MABs and MAB-iPSCs (Figure 3C). We then tested the chondrogenic differentiation in compacted spheres (Spaas et al., 2013) and assayed for Alcian blue-positive structures. Only MSC- and MSC-iPSC-derived spheres showed consistent chondrogenic differentiation, assayed as larger chondrogenic patches and lower cell density when compared with undifferentiated spheres (Figures 4A and 4B). Accordingly, equine *COMP* expression levels were significantly induced only in differentiated MSC and MSC-iPSC spheres, and appeared non-detectable in MABs and MAB-iPSCs (Figure 4C). Furthermore, in light of the adipogenic potential of equine MSCs (Spaas et al., 2013), we tested the differentiation efficiency of equine iPSCs toward the adipogenic lineage. Intriguingly, albeit generally limited and variable, the differentiation efficiency into Oil Red^+ adipocytes appeared higher in MSC-iPSCs than in MAB-iPSCs (Figure 4D), indicating a possible retention of MSC propensity toward other lineages as well. Thus, in both teratoma and in vitro assays, equine isogenic iPSCs showed intrinsic, discriminable propensities toward the lineages of source stem cells, e.g. myogenic in MAB-iPSCs and chondrogenic in MSC-iPSCs.