Stem Cell Research & Therapy

RESEARCH Open Access

Generation of equine induced pluripotent stem cells from cells of embryonic, perinatal and adult tissues

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Abstract

Background Regenerative therapies are quickly expanding to application in equine patients because of their importance as sporting and companion animals. Furthermore, aligning with a One Health concept, veterinary medicine offers a unique platform for preclinical studies. While mesenchymal stem/stromal cells (MSCs) therapies are already used in treating horses, strategies involving induced pluripotent stem cells (iPSCs) are poorly developed. iPSCs present great potential for therapy and disease modelling, but their consistent generation in horses requires further investigation into the source of somatic cells and the reprogramming method and conditions.

Methods The reprogramming potential of equine cells from tissues of three developmental origins was compared: prenatal (embryo-derived MSCs, eMSCs), perinatal (cord blood-derived MSCs, CB-MSCs) and adult (articular chondrocytes, ACs). Two reprogramming methods (retroviral, lentiviral) and different culture conditions (serum/serum-free, feeder cells/feeder-free, with/without small molecules) were tested. Pluripotent gene expression was analyzed at different time-points to reveal transcriptomic changes associated with reprogramming. The generated equine iPSCs (eqiPSCs) were characterized by alkaline phosphatase (AP) staining, expression of pluripotent genes and proteins, three-germ layer differentiation (embryoid body) and karyotype.

Results Using a lentiviral vector with serum-free media and feeder cells resulted in the most favorable conditions for eqiPSCs reprogramming, but adding small molecules had a negative effect. Equine CB-MSCs and ACs were only partially reprogrammed and could not be efficiently expanded in culture. Only eMSCs generated putative eqiPSCs that met the cellular, molecular and functional criteria of pluripotent cells. Equine eMSCs showed higher proliferation and basal expression of pluripotent genes compared to CB-MSCs and ACs, and showed the highest upregulation of pluripotent genes along reprogramming.

Conclusions The developmental stage of the starting cell strongly influences their reprogramming potential in equine species. This has been suggested for human and other animal species, but direct comparison of equine cells from prenatal, perinatal and adult sources has not been reported before. Novel preliminary insight into the transcriptomic changes of different equine cell types during reprogramming, and on the effect of different culture

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conditions, can contribute improving the generation of eqiPSCs. While transgene-free methods are the goal, putative eqiPSCs are critical to enlarge our knowledge on animal iPSC biology.

Keywords Horse, Equine, Pluripotency, iPSC, Reprogramming, Gene expression

Introduction

Induced pluripotent stem cells (iPSCs) are quickly reshaping the regenerative medicine field by overcoming the limitations of both embryonic stem cells (ESCs) and adult stem cells. Embryonic stem cells present unlimited capacity for self-renewal and differentiation, but access is problematic in the context of human application. On the other hand, adult stem cells are more accessible but their proliferation and differentiation are limited. The report in 2006 on a method to reprogram adult cells into pluripotent cells, known as iPSCs, using four transcription factors (Oct4, Sox2, Klf4, c-MYC; i.e. OSKM) [1] has been transformative. A wide array of patient-specific cells can be derived from iPSCs, constituting a game-changer for therapy, disease modelling or drug screening [2]. While this technology has rapidly advanced in human medicine, its unique advantages have not been significantly transferred to veterinary medicine [3].

Companion animals are now considered as family members and contribute to human well-being and positive social development. In the case of horses, this is especially true as they serve mounted forces, provide leisure and, in certain regions, play a key role in farm work and transport. Because of their global attraction as sporting animals, horses also have economic importance, with the equine industry estimated at €100 billion/year in Europe [4]. Furthermore, equine anatomy and physiology have resemblance to humans, and suffer from analogous diseases such as osteoarthritis, asthma, metabolic syndrome or ophthalmologic diseases. Most of these pathologies have no effective treatments either in humans or in veterinary patients, and thus iPSCs constitute an important promise not only for treatment but also to unravel their pathophysiology. Therefore, veterinary regenerative medicine poses a unique scenario for stem cell translational research in which animals can benefit from advanced therapies while informing safety and effectiveness for human treatments in naturally-occurring pathology models. In fact, the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) recommend the use of large animals such as horses to develop advanced cell-based therapies using a 'step-wise' approach [5]. This two-way flow of knowledge, known as One Medicine, is particularly important for iPSCs as their technical and safety challenges slow the progress to clinical trials compared to other cell therapies [6].

Jointly advancing iPSC applications in veterinary and human medicine critically requires reliable generation of animal iPSCs, which is still a work in progress. So far, generation of equine iPSCs (eqiPSCs) has been pursued by extrapolating human and mouse protocols in a 'trial-and-error' manner. By these means, some groups have generated putative eqiPSCs, but the reprogramming efficiency is very low and, more importantly, it is common that these cells do not become fully reprogrammed and rely on the expression of exogenous OSKM (transgene, Tg) [3]. The challenges of generating eqiPSCs have discouraged many researchers from working with these cells, further limiting progress in the field. To facilitate progress, a more in depth understanding of pluripotency reprogramming in animal cells is needed. This is a highly complex process that requires turning on and off pluripotent and somatic genes (transcriptomic changes). This is achieved by changes in chromatin structure (epigenomic changes), which underlying mechanisms are also influenced by other processes like cellular signaling pathways or cell proliferation [7, 8]. In-depth understanding of this process is critical to effectively improve it, but this information is currently missing in the equine species.

A few reports have compared transcriptomic/epigenomic features of putative eqiPSCs to their parental cell line [9, 10], but the stages in between have been overlooked. Furthermore, each cell type has a particular identity that needs to be erased to become pluripotent, contributing to differences in reprogramming efficiency. In human [11] and dogs [12] it has been described that less mature cells from prenatal origins may be more amenable for reprogramming, as well as cells in a less differentiated state (e.g. stem cells, progenitor cells) [13]. However, in equine, very few cell types have been directly compared for iPSC reprogramming. One study compared the potential of equine MSCs of different origins to become eqiPSCs and included a perinatal source [14], but there are no reports directly comparing embryo/fetal cells and adult cells in equine species. In addition, different studies have used varying methodologies for eqiPSC reprogramming, with very few of them directly comparing protocols to determine the actual advantages of each one [3]. For instance, certain small molecules could increase reprogramming efficiency by inhibiting specific signaling pathways or by acting on enzymes involved in epigenomic changes, but their role in eqiPSC reprogramming is unclear since their effects have not been established over control conditions [15].

To contribute addressing these gaps, in this study we directly compared the reprogramming potential of equine cells from different developmental origins by

using different reprogramming methods and culture conditions for eqiPSC generation. Furthermore, we analyzed the expression of key pluripotent genes at different moments of reprogramming to seed light into the transcriptomic changes that equine cells experience along reprogramming. We hypothesized that the less developed/differentiated the cells, the more efficient would be their reprogramming.

Specifically, we used three cell types that have never been tested before to obtain eqiPSCs: embryo-derived mesenchymal stem/stromal cells (eMSCs), cord blood-derived MSCs (CB-MSCs) and articular chondrocytes (ACs). Embryo-derived MSCs are a novel type of MSCs derived from early embryos [16], thus combining both an early origin and a low differentiated state. Perinatal tissues are a non-invasive source of stem/stromal cells with distinctive properties compared to adult cells, attributed to their earlier origin [17]. Umbilical cord tissue MSCs have been suggested as a potential source of eqiPSCs [14], but the use of CB-MSCs has not been reported to date even though they are more commonly used for clinical applications in the horse [18, 19]. Furthermore, human iPSCs obtained from cord blood cells have shown a higher chondrogeneic potential [20], a feature that would be therapeutically advantageous in the treatment of joint pathologies. Similarly, human iPSCs derived from ACs also present a higher commitment towards the chondrogeneic lineage, possibly due to epigenetic memory [21], but ACs have not been explored to generate eqiPSCs. To the best of our knowledge, this is the first work in equine comparing the reprogramming capacity of embryonic, perinatal and adult cells, and studying the intermediate stages of reprogramming.

Results

Selection of reprogramming conditions

In order to select reprogramming conditions, we started testing the reprogramming potential of equine CB-MSCs, as these represent an intermediate perinatal developmental stage. We initially used retroviral vectors containing human OSKM, similarly to that previously described [22, 23]. We compared different culture systems by combining three types of culture media: with fetal bovine serum (FBS), serum-free media (knock-out serum replacement, KOSR) or chemically defined media (E8 StemFlex, Gibco) along with two types of basement systems: feeder cells (irradiated mouse embryonic fibroblasts, iMEF) or feeder-free matrix membrane (Geltrex, Gibco).

None of the conditions tested was fully successful for reprogramming, and putative eqiPSCs were not obtained from CB-MSCs when using retroviral vectors. Nevertheless, morphological and transcriptomic changes were observed that suggested partial reprogramming of transduced CB-MSCs. The combination of KOSR media with iMEF as feeder layers led to more consistent changes and thus was selected for subsequent reprogramming experiments. Results in full and methodological details about the retroviral reprogramming can be found in Additional file 1.

Results from the retroviral reprogramming attempt also revealed a low transduction efficiency and an uneven delivery of the four transgenes. In view of this, we decided to test a lentiviral cassette to deliver the four OSKM factors as a single Tg, and after prior selection of the multiplicity of infection (MOI) based on the transduction efficiency of a green fluorescent protein (GFP)-containing lentiviral vector. Methodology and results for MOI selection can be found in Additional file 2. In addition, we investigated the effects of a cocktail of small molecules (SM), in parallel to standard conditions, to determine whether SM supplementation could improve reprogramming. For this second experiment, equine eMSCs, CB-MSCs and ACs were tested in parallel.

Equine eMSCs, but not CB-MSCs and ACs, can be reprogrammed using a lentiviral OSKM cassette vector

Morphological changes in equine eMSCs, CB-MSCs and ACs undergoing reprogramming

Lentiviral transduction of CB-MSCs and ACs resulted in morphological changes and positive AP staining, but characteristic iPSC colonies with well-defined borders were not obtained. This was also the case when the SM cocktail was used in the reprogramming of these cells. In fact, the addition of the SM cocktail resulted in lower proliferation in all cases with formation of fewer colony-like structures. Some colony-like areas from transduced CB-MSCs and ACs were picked and expanded individually in the same conditions used for reprogramming, but these cells either stopped proliferating or differentiated after 2–3 passages, even when positive AP staining was maintained.

In contrast, eMSCs reprogrammed under the same conditions formed indicative iPSC colonies consisting of small cells with high nucleus: cytoplasm ratio with tightly packed colonies with well-defined borders. Twenty-one colonies were picked up individually from the plates without the SM cocktail, of which 14 were successfully expanded and putative eqiPSC lines were established. Colonies that formed in plates with the SM cocktail presented less border definition. From seven SM-colonies picked, only two lines could be established. After passaging these lines twice, the SM cocktail was removed and the colony morphology changed towards that from non-SM conditions with better defined borders (Fig. 1).

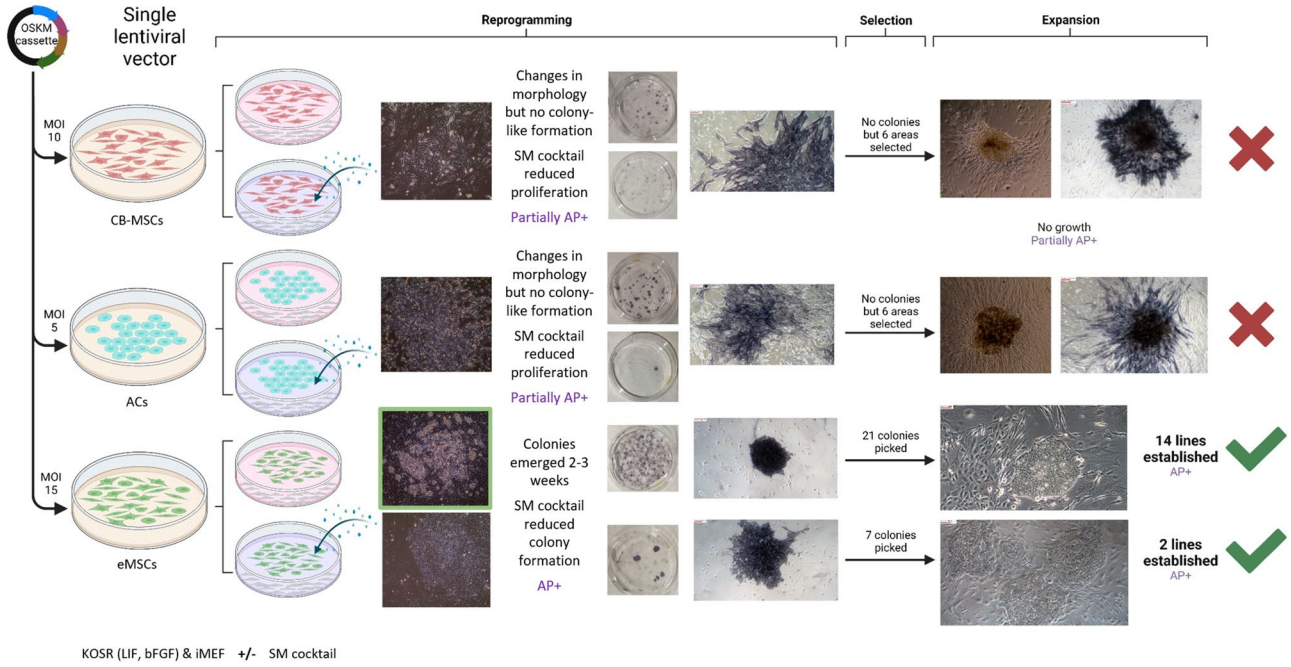

Fig. 1 Overview of the lentiviral reprogramming conditions tested and the results obtained. Equine cord blood-derived mesenchymal stem/stromal cells (CB-MSCs), articular chondrocytes (ACs) and embryo-derived MSCs (eMSCs) were transduced with a lentiviral human OSKM (Oct4, Sox2, Klf4, c-MYC) cassette using a previously selected multiplicity of infection (MOI). Transduced cells were seeded onto irradiated mouse embryonic fibroblasts (iMEF) and cultured with media containing knock-out serum replacement (KOSR) supplemented with leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF). Cultures were duplicated to test in parallel a cocktail of small molecules (SM). Representative images of morphology changes and alkaline phosphatase (AP) staining are shown

Transcriptomic changes in equine eMSCs, CB-MSCs and ACs undergoing reprogramming

Expression of pluripotency related genes and of the lentiviral Tg was assessed in the three types of equine cells at 5 days after transduction and at 3 weeks after plating transduced cells into iPSC conditions, with or without the SM cocktail (Fig. 2).

At 5 days post-transduction, eMSCs showed overexpression of SRY-box transcription factor 2 (*SOX2*), POU class 5 homeobox 1 (*POU5F1;* coding for octamer-binding transcription factor 4, OCT4) and fibroblast growth factor 5 (*FGF5*) compared to the non-transduced (basal) cells; while CB-MSCs upregulated only Nanog homeobox (*NANOG*), and ACs upregulated *SOX2*, *POU5F1 (OCT4)*, *NANOG* and zinc finger protein ZFP42 (*ZFP42*, coding for *REX1)*. At this time-point, eMSCs showed the highest expression of *SOX2* and *FGF5*, and ACs the highest expression of *ZFP4 (REX1)*, while *POU5F1 (OCT4)* and *NANOG* were similarly expressed in the three cell types.

After 3 weeks, transduced eMSCs plated in non-SM conditions showed the highest expression of all five pluripotent markers. Interestingly, the SM cocktail downregulated the expression of the pluripotent genes in eMSCs, while on the contrary, the SM cocktail generally increased the expression of pluripotent genes in CB-MSCs and ACs. However, characteristic iPSC colonies did not form from any CB-MSC or AC condition.

The lentiviral Tg expression was induced at day 5 after transduction at similar levels among the three cell types. However, Tg expression decreased at 3 weeks in all the conditions. We hypothesize that only cells expressing high levels of Tg were able to form colonies or colony-like areas, which were picked up and thus would have been depleted in the plates at 3 weeks.

Comparative analysis of equine cell types used for reprogramming: eMSCs, CB-MSCs and ACs

Directly comparing the reprogramming potential of equine somatic cells from different developmental origins revealed that only those from the earliest stage were able to form characteristic iPSC colonies. In order to explore differences among parental cells potentially related to their different reprogramming potential, we studied their proliferation and gene expression of pluripotent markers.

Embryo-derived MSCs showed the highest proliferation (cell doubling time, CDT = 1.20 days), followed by CB-MSCs (CDT = 3.05 days) and ACs (CDT = 3.49 days). Interestingly, 5 days after the lentiviral transduction, proliferation increased in CB-MSCs (CDT = 1.49 days) and ACs (CDT = 1.79 days) but decreased in eMSCs (CDT = 2.74 days).

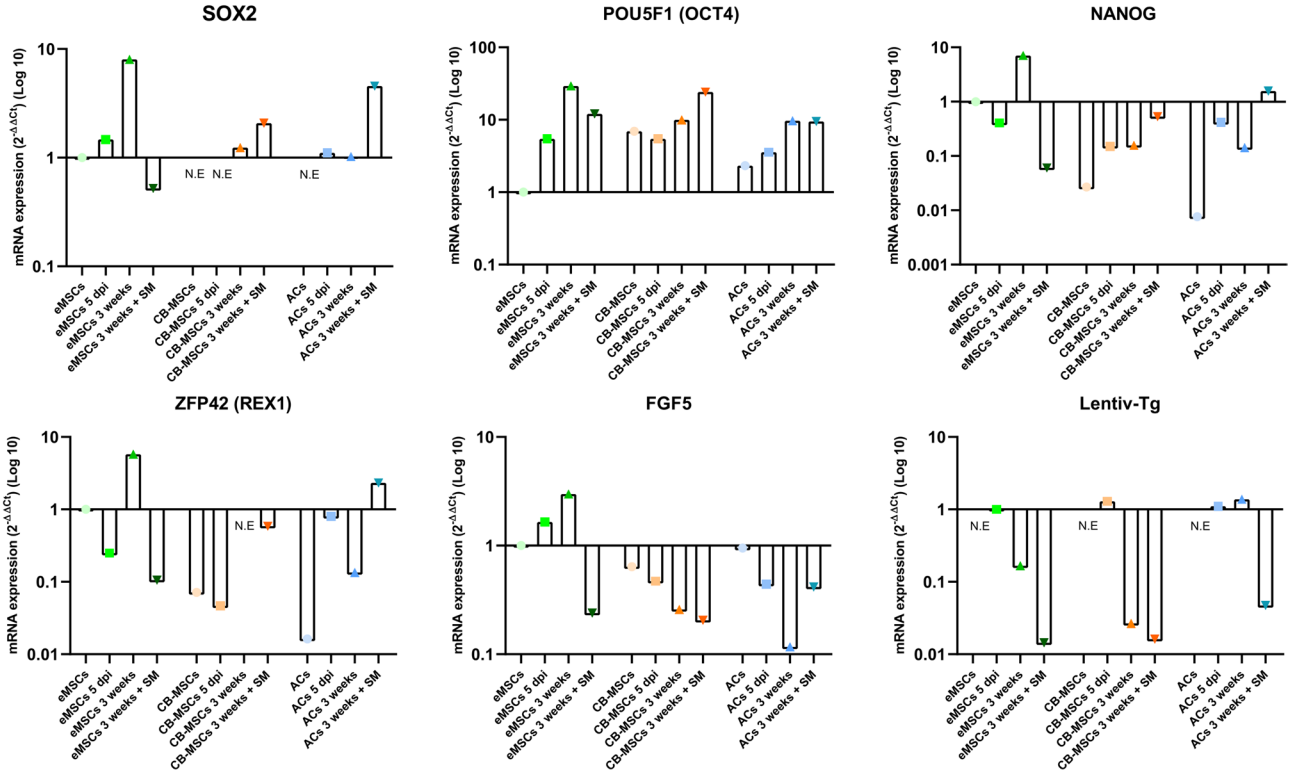

Fig. 2 Transcriptomic changes in equine eMSCs, CB-MSCs and ACs during reprogramming with lentiviral vector. Mean ± SD mRNA expression of the pluripotency-related genes *SOX2*, *POU5F1 (OCT4)*, *NANOG* (core markers), *ZFP42 (REX1)* (naïve marker) and *FGF5* (primed marker), and of the lentiviral transgene (Lentiv-Tg) in equine embryo-derived MSCs (eMSCs), cord blood-derived MSCs (CB-MSCs) and articular chondrocytes (ACs) along the reprogramming process: at 5 days post infection (dpi) and at 3 weeks under iPSC conditions (SM = small molecules cocktail added). Reference sample (value 1) = parental eMSC line, except for Lentiv-Tg (not expressed in parental cells) = eMSC 5 dpi. $n = 1$ for all conditions. N.E, no expression detected. SOX2, SRY-box transcription factor 2; POU5F1 (OCT4), POU class 5 homeobox 1 (octamer-binding transcription factor 4); NANOG, Nanog homeobox; ZFP42 (REX1) zinc finger protein ZFP42; FGF5, fibroblast growth factor 5

The comparison of pluripotent gene expression among equine cells also included bone marrow-derived (BM)-MSCs and newly established putative eqiPSCs. Equine BM-MSCs were not used for reprogramming since previous reports have already shown their very limited efficiency [14], but they were analyzed here to represent an intermediate stage between perinatal (CB-MSCs) and fully differentiated cells (ACs). This way, prenatal (eMSCs), perinatal (CB-MSCs) and adult (BM-MSCs) equine MSCs, along with adult differentiated cells (ACs) were compared in terms of pluripotent gene expression. Putative eqiPSCs were included in this assay for comparative purposes but characterization in full will be presented in the next section. Two to four biological replicates of each cell type were used.

Only eMSCs expressed *SOX2* in all the three populations analyzed, while none of the three CB-MSCs populations expressed this gene, and only one BM-MSC population and one AC population expressed it. All the cell populations of each type expressed *POU5F1 (OCT4)*, while there were some populations that did not express a particular gene: two eMSC populations did not express *ZFP42 (REX1)* (only the population used for reprogramming expressed this gene), one CB-MSC population did not express *NANOG*, and one BM-MSC population did not express *FGF5*. Overall, eMSCs expressed higher levels of *SOX2*, *NANOG*, *ZFP42 (REX1)* and *FGF5* compared to the other primary cells. However, there were no statistically significant differences among primary cells, likely due to large inter-donor variability within each cell type. Putative eqiPSCs significantly increased the expression of pluripotency genes over the parental eMSCs ($p < 0.05$), except for *FGF5*, whose expression decreased (Fig. 3).

Characterization of the putative EqiPSCs obtained from eMSCs

A total of 16 putative lines of eqiPSCs were obtained from eMSCs: 14 came from regular (non-SM) eqiPSCs culture conditions, and two lines came from the plates in which the SM cocktail was added. All the 16 eqiPSC lines were expanded from single colonies so they are considered as monoclonal. Characterization was conducted at passage 10 at the cellular (morphology), molecular (pluripotency

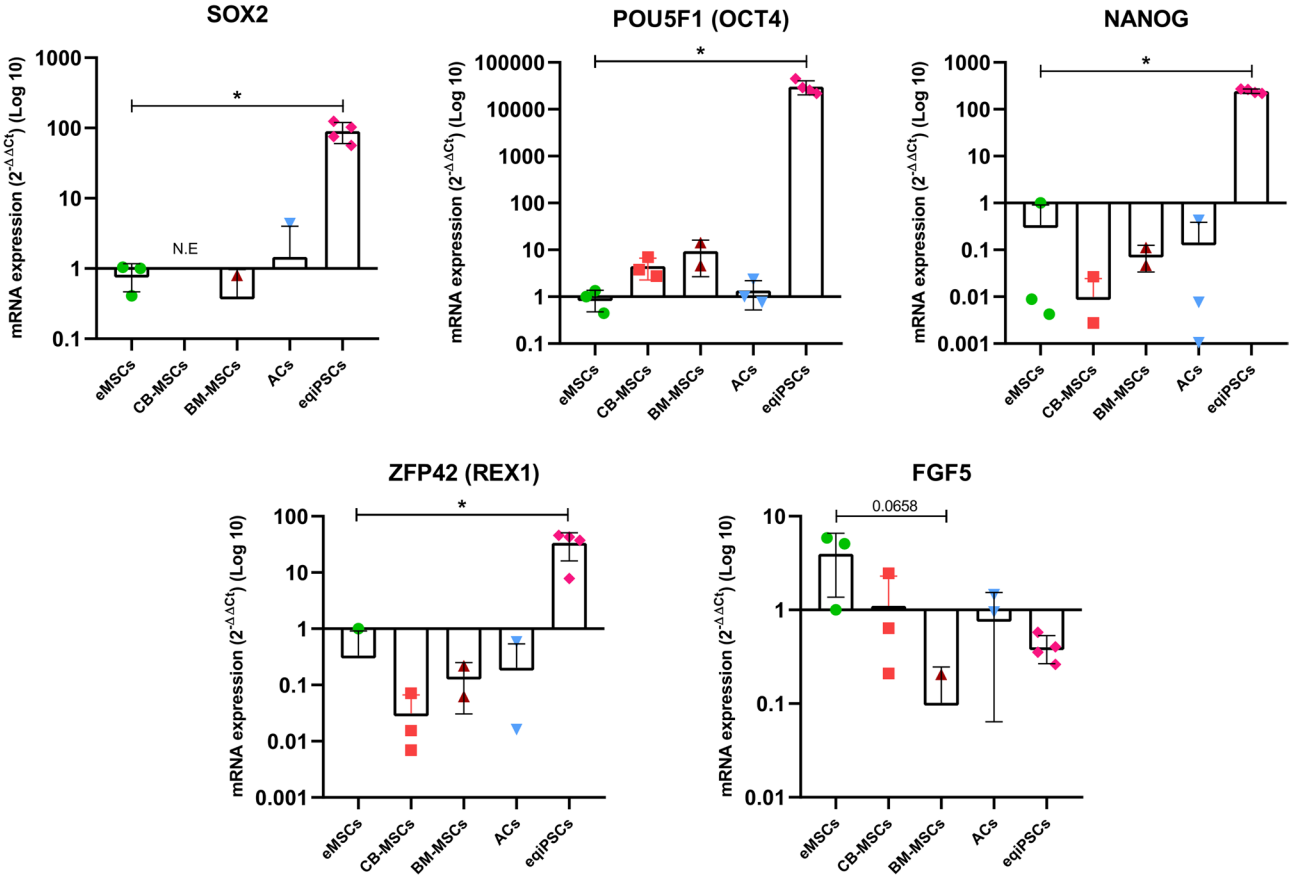

Fig. 3 Expression of pluripotency-related genes in different equine primary cells. Mean ± SD mRNA expression of the pluripotency-related genes *SOX2*, *POU5F1 (OCT4)*, *NANOG* (core markers), *ZFP42 (REX1)* (naïve marker) and *FGF5* (primed marker) in equine embryo-derived MSCs (eMSCs, $n = 3$), cord blood-derived MSCs (CB-MSCs, $n = 3$), bone marrow-derived MSCs (BM-MSCs, $n = 2$), articular chondrocytes (ACs, $n = 3$) and equine induced pluripotent stem cells (eqiPSCs, $n = 4$). Each dot represents one cell line. Reference sample (value 1) = parental eMSC line. N.E, no expression detected; SOX2, SRY-box transcription factor 2; POU5F1 (OCT4), POU class 5 homeobox 1 (octamer-binding transcription factor 4); NANOG, Nanog homeobox; ZFP42 (REX1) zinc finger protein ZFP42; FGF5, fibroblast growth factor 5. * = $p < 0.05$

markers) and functional (differentiation) levels. All the lines stained positive for AP (Fig. 1) and showed the typical iPSC morphology, growing in well-defined colonies of small cells with high nuclei, with dome-shaped and multilayered morphology (Fig. 4A). All the 16 eqiPSC lines expressed high levels of the core pluripotent genes *SOX2*, *POU5F1* and *NANOG* compared to the parental eMSCs. Interestingly, the eqiPSCs lines obtained under SM conditions ($n = 2$) expressed significantly higher levels of *SOX2* and *NANOG* ($p < 0.05$), even though these colonies initially had a less defined morphology. All the lines also expressed the marker of naïve pluripotency *ZFP42 (REX1)* to similar levels. However, the marker of primed pluripotency *FGF5* was expressed at lower levels and two of the non-SM lines did not show any expression. Importantly, all the lines also expressed high levels of the Tg, and it was significantly higher in the SM-eqiPSC lines ($p < 0.05$) (Fig. 4B). This suggests that reprogramming was not completed to the stabilization stage, where pluripotency would not rely on Tg expression, thus these eqiPSC lines are considered as putative.

Five eqiPSC lines (all from non-SM conditions) were selected for immunofluorescence staining and in vitro differentiation. All the five lines expressed the core pluripotent factors SOX2, OCT4 and NANOG at the protein level (Fig. 4C), and all of them were able to differentiate into cells of the three germ layers in the in vitro EB formation assay (Fig. 5). After 3 weeks of differentiation, we observed outgrowing of heterogeneous cells from the EB, including fibroblastic-like, epithelial-like and neuron-like morphologies (Fig. 5A). We confirmed positive expression of alpha-fetoprotein (AFP, endoderm), beta-3-tubulin (βIII-tubulin, ectoderm) and alpha smooth muscle actin (α-SMA, mesoderm) by immunofluorescence in all the five lines (Fig. 5B). Marked differences were not noted among eqiPSC lines in terms of differentiation capacity.

Finally, three of these eqiPSC lines (named FD7, FD8.1 and FD8.6) and the parental eMSC line were subjected to karyotyping to assess chromosomic stability (Fig. 6).

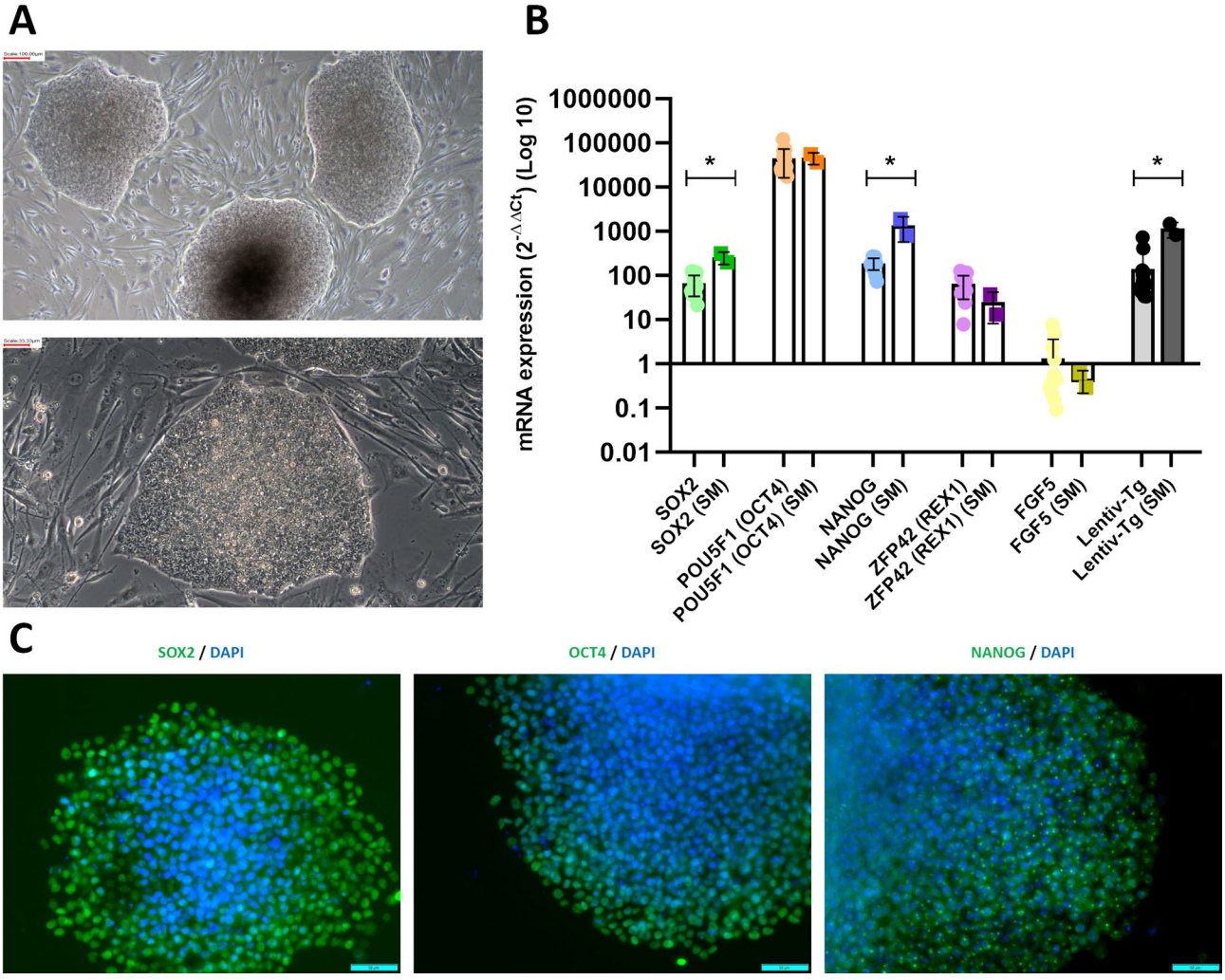

Fig. 4 Cellular and molecular characterization of eqiPSCs. **A** Characteristic morphology of equine induced pluripotent stem cells (iPSCs) colonies. Scale bar 100 µm above, 33.3 µm below. **B** Mean ± SD mRNA expression of the pluripotency-related genes *SOX2*, *POU5F1 (OCT4)*, *NANOG* (core markers), *ZFP42 (REX1)* (naïve marker), *FGF5* (primed marker) and of the lentiviral transgene (Lentiv-Tg) in eqiPSCs obtained either under standard conditions ($n = 14$, except for *FGF5* where two lines did not express this gene) or adding a small molecule (SM) cocktail ($n = 2$). Each dot represents one cell line. Reference sample (value 1 not shown) = parental eMSC line, except for Lentiv-Tg (not expressed in parental cells) = eMSC 5 days post infection. N.E = no expression detected. **C** Positive immunofluorescence staining for the core pluripotency markers SOX2, OCT4 and NANOG (green). Nuclei are counterstained with DAPI (blue). Scale bar 50 µM. SOX2, SRY-box transcription factor 2; POU5F1 (OCT4), POU class 5 homeobox 1 (octamer-binding transcription factor 4); NANOG, Nanog homeobox; ZFP42 (REX1) zinc finger protein ZFP42; FGF5, fibroblast growth factor 5. * = $p < 0.05$

Equine eMSCs presented a normal karyotype of 64, XX with no abnormalities detected (Fig. 6A). Equine iPSCs also had a normal 64, XX karyotype in most of the metaphases analyzed (Fig. 6B). The eqiPSC line FD7 had a normal karyotype in 88% of the metaphases analyzed. The remaining cells had 63 to 65 chromosomes but there was no consistency in the missing or extra chromosome. Equine iPSC line FD8.1 presented a normal karyotype in 74% of the metaphases analyzed, with 10% of cells with 63 chromosomes and 16% with 65 chromosomes. The eqiPSCs line FD8.6 presented 78% of metaphases with normal karyotype, and there were 16% of cells with 63 chromosomes and 6% with 65 chromosomes. In FD8.1 and FD8.6 lines, half of the cells with 63 chromosomes were missing one X chromosome, while the other half was missing a random chromosome. In the cells with an extra chromosome, it was a small acrocentric one that could not be identified. While actual chromosome number abnormalities cannot be fully discarded, the inconsistency in the type of missing or extra chromosome suggest that these observations could be artifacts.

Methods

Isolation and characterization of equine primary cells

Tissues for isolating all the primary cell populations were harvested under corresponding ethics approval and/or owner's consent under the applicable regulations (see

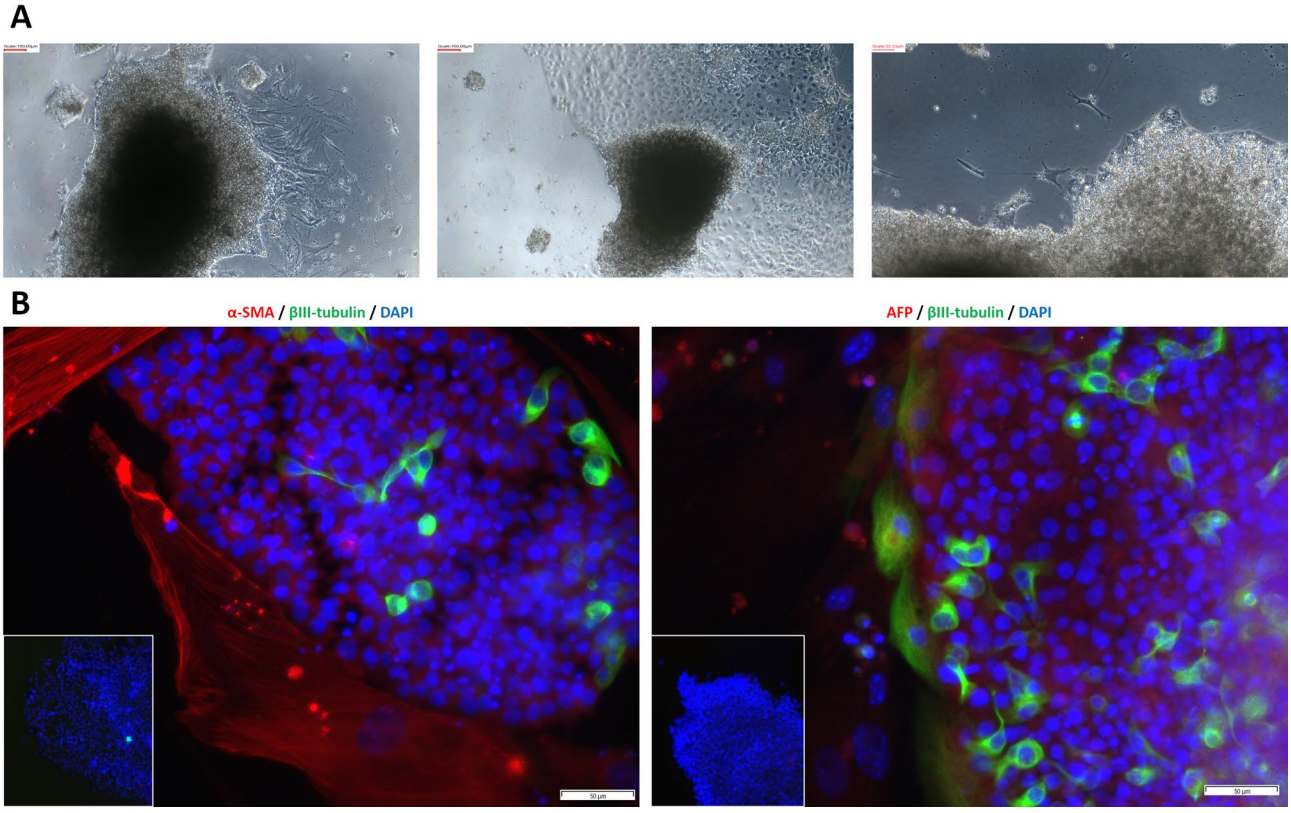

Fig. 5 Functional characterization of eqiPSCs: spontaneous differentiation into cells of the three-germ layers (embryoid body assay). **A** Heterogeneous morphology of cells outgrowing from embryoid bodies derived from equine induced pluripotent stem cells (eqiPSCs), including fibroblast-like (left), epithelial-like (center) and neuron-like (right). Scale bar 100 µm left and center; 33.3 µm right. **B** Positive immunofluorescence staining for mesoderm marker alpha smooth muscle actin (α-SMA, red in left image), endoderm marker alpha-fetoprotein (AFP, red in right image), and ectoderm marker beta-3-tubulin (βIII-tubulin, green in both images). Images of cells incubated only with secondary antibodies are provided in the bottom left corner of each image to show lack of non-specific staining. Nuclei are counterstained with DAPI (blue). Scale bar 50 µM. All images were selected to be representative of all lines

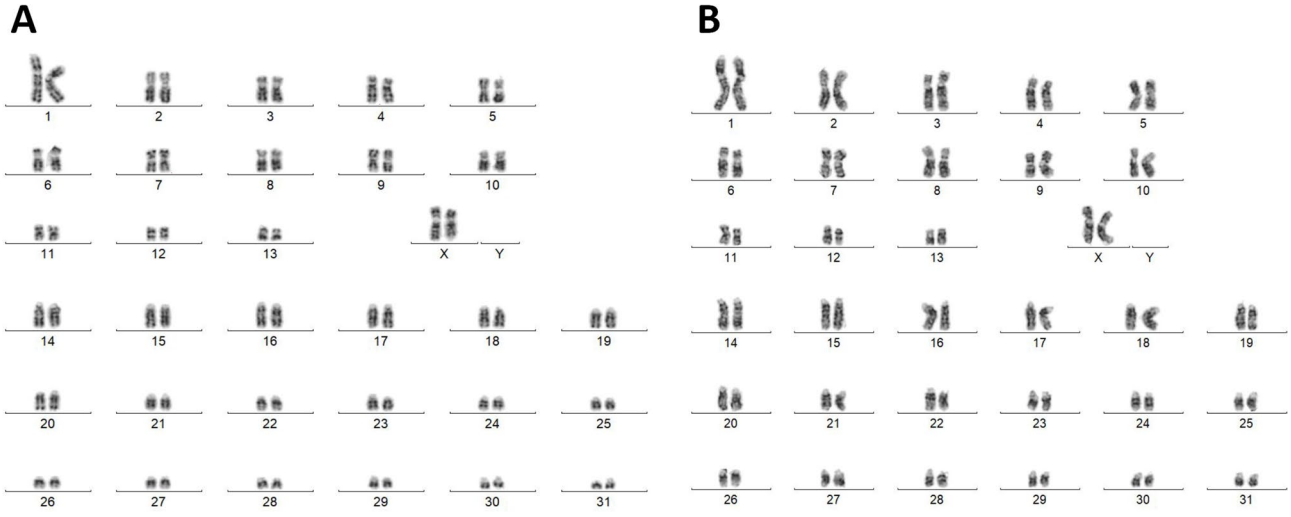

Fig. 6 Karyotype study of equine eqiPSCs. Representative images of normal 64, XX equine karyotype in (**A**) the parental cell line of embryo-derived mesenchymal stem/stromal cells, and in (**B**) one putative line of equine induced pluripotent stem cells (eqiPCs, FD8.6)

Ethics Statement). The work has been reported in line with the ARRIVE guidelines 2.0.

Equine eMSCs were obtained as previously described [16]. Briefly, eMSCs were isolated from 33- to 34-day-old pre-implantation equine embryos harvested by uterine flushing. Equine embryos were examined under a dissecting microscope and any fetal membrane was removed. The embryos were then minced with a scalpel and then passed through a 2 mm stainless steel mesh. Cells were cultured in Dulbecco's modified eagle's medium (DMEM)/F12 containing 150 mM HEPES, 10% FBS (all from Gibco), 0.4 µg/mL bovine insulin, 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 U/mL penicillin, and 0.5 µg/mL Vitamin C (all from Sigma-Aldrich) at 37 °C with 5% CO_2. Cells were passaged with 0.05% Trypsin-EDTA (Gibco) when 80–90% confluent and cryopreserved in 90% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). A total of three populations of equine eMSCs (from 1 male and 2 female embryos, all Thoroughbred) were used between passages 1–2 for all the experiments.

Equine CB-MSCs were obtained from umbilical cord blood collected immediately after foal delivery, following methodology previously described [24]. Briefly, 60 to 300mL of cord blood were collected in blood bags and transported refrigerated to the lab. The mononuclear cell fraction was isolated by density gradient centrifugation of cord blood and cells were seeded in isolation media consisting of low glucose DMEM with 10% FBS, 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 U/mL penicillin and 100 mM dexamethasone (Sigma-Aldrich). Cells were expanded at 37℃ and 5% CO_2 with the same culture media but omitting dexamethasone. Cells were passaged and frozen as described for eMSCs. A total of three populations of CB-MSCs (from 1 male and 2 female newborn foals, all Thoroughbred) were used between passages 1–3 for all experiments.

Equine BM-MSCs were obtained as previously described [25]. Briefly, 30–40 mL of bone marrow were harvested from the sternum under sedation and local analgesia. Mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Fisher Scientific) and seeded in medium consisting of low glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin at 37 ℃ and 5% CO_2. BM-MSCs were passaged and cryopreserved as described for eMSCs and CB-MSCs. A total of two populations of BM-MSCs (from 1 male and 1 female 2–3 y.o. donors; mixed breed) were used between passages 3–4 for all the experiments.

Equine ACs were obtained as previously described [26]. Briefly, articular cartilage was excised from the subchondral bone of metacarpophalangeal joints, finely diced and subjected to enzymatic digestion with collagenase type II (Sigma-Aldrich) overnight. The resulting cell suspension was passed through a 100µM cell strainer, centrifuged and cells seeded in media consisting of low glucose DMEM with 10% FBS, 0.1 mg/mL streptomycin, 100 U/mL penicillin and 500 ng/mL amphotericin B (Gibco). Cells were passaged and cryopreserved as described for the other cell types above. A total of three populations of ACs (from 1 female and 2 male 2–7 y.o. donors; all Thoroughbred) in passages 1–2 were used for all the experiments.

Equine eMSCs, CB-MSCs and BM-MSCs were characterized by their immunophenotype and tri-lineage differentiation capacity as previously described [25]. Methodology and results for characterization of eMSCs and CB-MSCs can be found in Additional file 3. Characterization of the two populations of BM-MSCs has already been published and the original article is openly accessible [25]. Characterization of ACs is not customary since these are the only cell type present in the cartilage, and this tissue is harvested separately from other joint tissues.

One population of eMSCs (EV06, Thoroughbred, female), one of CB-MSCs (Eq. 1, Thoroughbred, female) and one of ACs (BB182, Thoroughbred, male) were used for all the transduction experiments. For MOI selection using a GFP-containing lentiviral vector, BM-MSCs from one donor (BMQ-D1, mixed-breed, male) were also used. Biological replicates were added for the transcriptomic study of primary cells: two additional populations of eMSCs, CB-MSCs and ACs (total $n = 3$), and one additional population of BM-MSCs (total $n = 2$) were used.

Lentiviral transduction of equine cells

Equine eMSCs, CB-MSCs and ACs were subjected to two rounds of transduction using a commercially available lentiviral vector containing human Oct4, Sox2, Klf4 and c-MYC in a single cassette (CRE-excisable OSKM lentifect purified particles, Cat No. LP801-100, GeneCopoieia, Labomics). Equine eMSCs ($n = 1$, passage 2), CB-MSCs ($n = 1$, passage 2) and ACs ($n = 1$, passage 2) were seeded at 2,500 cells/cm^2 (eMSCs) or 5,000 cells/cm^2 (CB-MSCs and ACs) in 6-well plates using their corresponding media for 24 h prior to the first transduction.

On day 0, virus suspension was added for a MOI of 5 (ACs), 10 (CB-MSCs) or 15 (eMSCs), selected as described in Additional file 2, and for a total of 100,000 cells of each type. For this, the corresponding volume of viral suspension was added to the culture media along with 5 µg/mL of polybrene and plates were centrifuged (spinfection). The procedure was repeated the next day (day 1 pi) for a total of two rounds of transduction. On day 2 pi, culture supernatant containing viral particles was removed, cells washed with phosphate-buffered

saline (PBS), and corresponding fresh media added. Media was changed again on day 4 pi.

On day 5 pi, all the cells were passaged and seeded at three different densities (100, 500 or 1,000 cells per cm^2) in 10-cm dishes prepared the day before with feeder (iMEF) layers and KOSR media. To prepare feeder layers, CF1 Mouse embryonic fibroblasts irradiated (Gibco) were seeded 24 h in advance onto 0.1% gelatin (Attachment Factor, Gibco) coated wells. 150,000 iMEF/well were cultured in 6-well plates with high glucose DMEM supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin. KOSR media consisted of Knock-out DMEM (Gibco), 20% KOSR (Gibco), 2mM L-glutamine, 1% non-essential amino acids solution (Gibco), 0.1 mM 2-Mercaptoethanol (Gibco), 1,000 U/mL of human leukaemia inhibitory factor (LIF) (Reprocell) and 10 ng/mL of human basic fibroblast growth factor (bFGF) (Peprotech).

All cultures were duplicated to test in parallel the supplementation with a SM cocktail that was added to the KOSR media for a final concentration of 0.5 µM PD0325901 (mitogen-activated protein kinase kinase inhibitor, Cat No. HY-10254, MedChemExpress), 0.25 µM A83-01 (transforming growth factor beta [TGF-β] antagonist, Cat No. HY-10432, MedChemExpress), 3 µM CHIR99021 (glycogen synthase kinase-3 beta [GSK3b] inhibitor, Cat No. HY-10182, MedChemExpress) and 1 mM valproic acid (histone deacetylase [HDAC] inhibitor; Cat No. HY-10585, MedChemExpress). This cocktail was selected based on previous reports on equine [15, 27] and canine [28] iPSC reprogramming and according to the roles of these molecules in inhibiting certain signaling pathways or favoring chromatin remodeling to facilitate reprogramming. Transduced and non-transduced (negative control) eMSCs, CB-MSCs and ACs were snap-frozen on day 5 pi for gene expression analysis.

Media with or without the SM cocktail was changed every other day until colony-like formations emerged, and then switched to daily change. Changes in morphology and cell clustering started to be observed at day 12 pi. Colony-like formations were manually picked between weeks 2 to 3 pi and individually cultured under the same conditions as used during reprogramming with change of media every other day. The remaining cells were snap-frozen for RNA isolation and gene expression analysis at week 3 pi.

The colony-like clusters picked from transduced CB-MSCs and ACs were discarded after only 1 passage as they were not viable. The putative colonies picked from eMSCs were individually expanded in the same conditions (KOSR media + iMEF), and for the lines reprogrammed in the presence of SM cocktail, it was maintained until passage 2. All the lines were passaged mechanically every 4–6 days up to passage 6, after which enzymatic passaging with short Accutase (Gibco) treatment was used. At passage 8, tolerance to cryopreservation was tested using 90% KOSR and 10% DMSO, and adding 10 µM Y-27,632 (ROCK pathway inhibitor, Cat No. 688000, Sigma-Aldrich) to the culture media for 18 h after thawing. At passage 10, a sample from all eqiPSCs putative lines was snap-frozen for gene expression studies.

Table 1 Summary of samples used in gene expression assays in this study

Cell type	Non-transduced	5 days pi	3 weeks pi	Putative eqiPSC
CB-MSCs	Standard culture conditions	KOSR + iMEF +/- SM	KOSR + iMEF +/- SM	–
ACs	Standard culture conditions	KOSR + iMEF +/- SM	KOSR + iMEF +/- SM	–
eMSCs	Standard culture conditions	KOSR + iMEF +/- SM	KOSR + iMEF +/- SM	*n* = 14 non-SM *n* = 2 SM
BM-MSCs	Standard culture conditions	–	–	–
Human iPSCs	Standard culture conditions	–	–	–
iMEF	Standard culture conditions	–	–	–

MSCs mesenchymal stem/stromal cells, *CB-MSCs* cord blood-derived MSCs, *ACs* articular chondrocytes, *eMSCs* embryo-derived MSCs, *BM-MSCs* bone marrow-derived MSCs, *iPSCs* induced pluripotent stem cells, *iMEF* irradiated mouse embryonic fibroblasts, *pi* post infection, *KOSR* knock out serum replacement, *SM* small molecules cocktail

Gene expression analysis by real time quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen), genomic DNA was removed using the PureLink™ DNase kit (Invitrogen) and complementary DNA (cDNA) was synthetized with the qScript™ cDNA SuperMix (Quanta Biosciences). All the procedures were performed according to manufacturer's instructions.

RNA was isolated from equine CB-MSCs, ACs and eMSCs non-transduced, at 5 days pi, at 3 weeks pi and from putative eqiPSC lines (only from eMSCs). In addition, RNA was extracted from equine BM-MSCs as reference of adult MSCs, as well as from one human iPSC line established in our lab [29] and from iMEF to serve as controls for equine-specific amplification during primer optimization. A summary of the samples used in gene expression assays is provided in Table 1.

Primers were designed using the Primer-BLAST online tool of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on equine sequences, or on plasmid sequence for the transgene. Some equine primers were selected from bibliography. Because equine and human sequences for *POU5F1 (OCT4)*, *SOX2*, *KLF4* and *c-MYC* are highly homologous, primers for the lentiviral Tg were designed

to span the Klf4/c-MYC junction of the plasmid, and equine primers were designed with one primer in the pair outside of the plasmid ORF (open reading frame), in order to differentiate the exogenous expression of the human Tg from the endogenous equine expression [30]. All the primers used were tested for efficiency using a standard curve and for specific amplification using human iPSCs and iMEFs as controls. Details on primers are provided in Table 2.

RT-qPCRs were performed and monitored with a Step One Plus Real-Time PCR System (Applied Biosystems). All reactions were carried out in a total volume of 10 µL with 2 µL of cDNA as the template and 5 µL Fast SYBR Green Master Mix (Applied Biosystems). Amplification was performed in duplicate for each sample as follows: 20" at 95℃ for initial activation, followed by 40 cycles consisting of 3" at 95℃ and 30" at the annealing temperature (Ta) indicated in Table 2, and a dissociation curve protocol run after every PCR. The levels of gene expression were determined using the comparative $2^{-\Delta\Delta Ct}$ method. The normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-2-microglobulin (B2M). The reference sample used in each analysis is stated within the corresponding figure.

Alkaline phosphatase staining

Cells were fixed with 4% paraformaldehyde (PFA) in PBS (Fisher Scientific) 1' room temperature (RT), washed twice with PBS and incubated at 64℃ for 20' to inactivate the AP activity of iMEF. AP staining solution was prepared by diluting 1 BCT/NBT reagent tablets ready to use (Sigma-Aldrich) into 10mL of ultrapure milliQ H_20, and 500 µL per well (6-well plates) were added to the fixed cells. After incubating 30' RT in the dark, staining solution was removed and cells washed twice with PBS. Negative AP staining of primary equine cells (eMSCs, CB-MSCs and ACs) was confirmed.

Embryoid body assay

Five putative eqiPSC lines were selected for characterization at passage 10 by their ability to differentiate into cells of the three germ layers. To do this, an in vitro embryoid body (EB) assay was conducted similarly to that previously described [33]. For each eqiPSC line, one well (6-well plate) with confluent eqiPSCs cultured under standard conditions was passaged with Accutase and cells were transferred into non-adherent V-bottom 96-well plates (no feeders or basement membrane) using KOSR media (200 µL/well). The plates were centrifuged 1000 g 3' and incubated for 24 h. The next day, the formation of EBs was checked and media was replaced by EB media (KOSR media without LIF and bFGF). Cells were maintained for 5 days with change of media every other day. Subsequently, the EBs were transferred to 8-well chambered coverslips (Ibidi) coated with Geltrex and cultured for additional 3 weeks with change of media three times a week. The outgrowth of cells with different morphologies was regularly monitored. On week 3, cells were stained to assess specific markers by immunofluorescence.

Table 2 Details of primers used for gene expression in this study

Gene	Accession number	Primer sequence (5′ – 3′)	Amplicon (bp)	Source	Ta	[F′/R′] (nM)
GAPDH	NM_001163856	F: GCAAGTTCCATGGCACAGT R: CACAACATATTCAGCACCAGCAT	128	[31]	60	300/300
B2M	NM_001082502	F: TCGTCCTGCTCGGGCTACT R: ATTCTCTGCTGGGTGACGTGA	102	[31]	60	300/300
NANOG	XM_023643093.1	F: CTCGATTTGGGCAGTGGCTA R: CGAGCCCTCTAGAATCCGTC	117	[32]	60	300/300
POU5F1 (OCT4)	XM_001490108.6	F: AGAAGGACGTGGTACGAGTG R: GTGCCAGGGGAAAGGATACC	138	[32]	60	300/300
SOX2	XM_023623361.1	F: CCATTAACGGCACACTGCCC R: AGAATTTCTCCCCCACCTCCAG	72	Own design	61	300/300
ZFP42 (REX1)	XM_001489519.4	F: TGGAGGAATATCCAGCGTTGA R: GCTTTCCCACATTCTGCACATA	213	[32]	60	300/300
FGF5	XM_014738875.2	F: GACCCGTTGCCACTGATAGG R: TCGTGGGAGCCATTGACTTT	250	Own design	60	300/300
EIF3L	XM_001501206.6	F: ATGCCAAAGTCAGTGGAGGA R: CCTCTGACTTCTTGGCGGTC	207	Own design	61	300/300
Lentiv-Tg	Sequence from supplier	F: CCACCTCGCCTTACACATGA R: TGCTGGTTTTCCACTACCCG	141	Own design	60	300/300

GenBank accession numbers or plasmid references of the sequences used for primers design, primers sequence (F: forward, R: reverse), length of the amplicon in base pair (bp), source of the primers, annealing temperature (Ta) and concentration (nM) of each primer used per reaction are presented. *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *B2M* beta-2-microglobulin, *NANOG* Nanog Homeobox, *POU5F1* POU class 5 homeobox 1, *OCT4* octamer-binding transcription factor 4, *SOX2* sex determining region Y-box 2, *ZFP42 (REX1)* zinc finger protein 42, *FGF5* fibroblast growth factor 5, *EIF3L* eukaryotic translation initiation factor 3 subunit L, *Lentiv-Tg* lentiviral transgene

Table 3 Details for antibodies used for Immunofluorescence staining in this study

Antibody	Reference/supplier	Dilution	Previously reported for equine
Rabbit anti-human SOX2	34516 Cell Signaling Technology	1:200	Yes [34]
Rabbit anti-human OCT4	94310 Cell Signaling Technology	1:200	Yes [34]
Rabbit anti-human NANOG	99399 Cell Signaling Technology	1:200	No
Mouse anti-human AFP	A8452 Sigma-Aldrich	1:200	No
Mouse anti-human α-SMA	48938 Cell Signaling Technology	1:400	No
Rabbit anti-human βIII-tubulin	5568 Cell Signaling Technology	1:400	No
Anti-rabbit IgG secondary antibody (Alexa Fluor 488)	4412 Cell Signaling Technology	1:1000	–
Anti-mouse IgG secondary antibody (Alexa Fluor 555)	4409 Cell Signaling Technology	1:1000	–

All the primary antibodies were used for intra-cellular staining. *SOX2* SRY-box transcription factor 2, *OCT4* octamer-binding transcription factor 4, *NANOG* Nanog homeobox, *α-SMA* alpha smooth muscle actin, *AFP* alpha-fetoprotein; βIII-tubulin, beta-3-tubulin

Immunofluorescence

Immunofluorescence was used to assess EB differentiation and pluripotent markers expression in undifferentiated eqiPSCs. For the latter, the same five lines used in the EB assay (passage 10) were cultured in their standard conditions in 8-well chambered coverslips for 3 days prior to the staining. Primary and secondary antibodies used are listed in Table 3.

Cells were fixed with 4% PFA for 15' RT, washed three times with PBS, and permeabilized with 0.3% Triton X (Thermo-Fisher) solution in PBS for 15' RT. Subsequently, cells were washed and blocked with blocking buffer consisting of 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.3% Triton X in PBS. Corresponding primary antibodies were diluted in blocking buffer as shown in Tables 3 and 150 µL of the dilution were added to the cells. Primary antibodies for SOX2, OCT4 and NANOG were added individually (undifferentiated iPSCs), while antibody for βIII-tubulin was used together with either AFP or α-SMA antibodies (EB-differentiated cells). Cells were incubated with primary antibodies at 4 ℃ overnight.

The next day, cells were washed three times with PBS and corresponding secondary antibodies were added at the dilutions shown in Table 3 (150 µL/chamber). For each eqiPSCs line, a chamber was stained with only the secondary antibody as control for non-specific staining. Secondary antibodies were incubated for 60' RT in the dark. After washing the cells (PBS x 3), nuclei were counterstained with DAPI (dilution 1:2000 of 1 mg/mL of stock solution) (Thermo-Fisher) for 5' RT (200 µL/chamber). Chambers were washed again and cells were left on PBS for fluorescence imaging using an Olympus CKX53 inverted microscope. Images from different fluorescent channels were merged with Image J.

Karyotyping

Three putative eqiPSCs lines characterized as described above (passage 14), and their parental eMSC primary line (passage 3), were subjected to karyotyping to assess chromosomic stability. Cells were grown in their regular conditions in 6-well plates and harvested when confluent. Colcemide solution in Hanks's Balanced Salt Solution (HBSS) (KaryoMAX Colcemid, Gibco) and ethidium bromide solution (1 mg/mL in distilled water, Sigma-Aldrich) were added to the cultures (20 µL of each per well) and incubated for 2 h at 37 ℃. Subsequently, plates were manually shacked to collect loose cells, and adherent cells were detached by Accutase treatment to obtain a single cell suspension. Harvested cells were treated with hypotonic Potassium Chloride solution (KaryoMAX Potassium Chloride Solution, Gibco) for 15' at 37 ℃ and fixed using 3:1 solution of methanol and glacial acetic acid (both from Sigma-Aldrich). Cells were preserved at -20℃ and submitted to the Molecular Cytogenetics Laboratory at the Texas A&M University (Texas, US) for analysis. Chromosome preparations were made on wet glass slides by dropping the cell solution and G-banding staining was performed. Up to 50 cell metaphases were analyzed per cell line to assess number and structure of chromosomes.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0.1 software (San Diego, CA, USA). Data sets were checked for normality using the Shapiro–Wilk test, and parametric or non-parametric tests were chosen accordingly. Differences in the mRNA relative expression between cell types (eMSCs, CB-MSCs, ACs, BM-MSCs, eqiPSCs +/- SM) were analyzed by unpaired t-test (parametric) or Mann-Whitney test (non-parametric) when two groups were compared, and by either One-way ANOVA (parametric) or the Kruskall–Wallis test (non-parametric) followed by Dunn's *post-hoc* test for multiple (> 2 groups) comparisons. Significance was set as $p < 0.05$ in all cases.

Discussion

This study demonstrated for the first time that the developmental origin of equine cells has a dramatic influence on the efficiency of their reprogramming into eqiPSCs. We studied three types of equine cells that had not previously been assessed for the generation of eqiPSCs, and found that only those from an early developmental stage could be reprogrammed into eqiPSCS. Interestingly, such prenatal cells presented higher proliferation and higher expression of pluripotent markers. Moreover, different gene expression profiles were found across equine cell types along the reprogramming process, providing a first insight into the transcriptomic changes that equine cells undergo to become eqiPSCs. In addition, the role of pathway inhibitors was explored, highlighting that cell type and species specific requirements need to be investigated. The generated eqiPSCs were termed as putative lines because they maintained transgene expression, but met the standard pluripotent criteria.

It should be noted that only one cell line representing each developmental stage was used for reprogramming in this study because of the multiplicity of conditions tested in parallel to enable direct comparison. Similarly, previous eqiPSC studies have also used a single donor/cell line [15, 34, 35]. For similar reasons, we only tested a single MOI for each cell type. This MOI was selected based on the transduction efficiency using the same lentiviral vector but containing GFP, which is a common approach to estimate the percentage of cells infected by a given vector [22, 36–38]. However, the retention and expression of the transcripts of interest cannot be predicted by these means, so we cannot discard that the use of different MOIs in the reprogramming experiments might have yielded different results. Moreover, provided the significantly lower transduction of eMSCs compared to other cell types, a higher MOI was set for reprogramming experiments, even though it had not been tested with the GFP-containing vector. Thus, the actual transduction efficiency of reprogrammed eMSCs is unknown, constituting a limitation of the study and an area for further refinement. In the current study, we prioritized demonstrating that eMSCs can be reprogrammed into eqiPSCs, while determining the optimal conditions to achieve such reprogramming, including confirmation of the ideal MOI, remain for subsequent efforts.

We compared different reprogramming methods and different culture conditions to shed light on the requirements for pluripotency induction in equine cells. In our hands, the use of a serum-free media (KOSR, supplemented with both LIF and bFGF) and iMEF layers as feeder cells, resulted in the most adequate conditions among those tested, in agreement with other works [35, 37, 41]. In spite of some eqiPSCs studies using FBS [42–44], this was the least appropriate condition in our study. It has been reported that FBS can lead to reprogramming arrest at intermediate stages by maintaining cell program and inhibiting the activation of pluripotent genes [45]. Previous works have used either only LIF or only bFGF for eqiPSCs culture, but most of the evidence points at the co-dependency of both factors [15, 37]. Only a few works have reported expansion of eqiPSCs in feeder-free systems [34, 43], while most of the studies have used iMEF layers to generate and expand eqiPSCs [14, 22, 23, 35].

Interestingly, eMSCs showed the lowest transduction efficiency by lentiviral vectors but yet were the only cell type that could be reprogrammed. It has been suggested that stem cells possess intrinsic immunity to viral infection [46] that would be mediated by interferon-stimulated genes (ISGs) [47]. Thus, the expression of *EIF3L* (eukaryotic translation initiation factor 3 subunit L), a gene of the ISG family, was assessed in four types of equine primary cells and in eqiPSCs (Additional File 2), but the expression of this particular gene could not be related either to transduction or to reprogramming efficiencies. Future studies including more ISG targets might reveal mechanisms behind differences in transduction efficiency of equine cells. Nevertheless, lentiviral transduction of equine CB-MSCs and ACs was highly efficient but these cells could only be partially reprogrammed. Therefore, the origin of the equine cells would have a more significant effect than the reprogramming methodology.

Equine eMSCs presented distinct features over perinatal and adult cells that may explain their higher reprogramming potential. Equine eMSCs showed higher proliferation than CB-MSCs and ACs, which has been related to higher reprogramming efficiency in human [48] and animal cells. Canine adult fibroblasts are often refractory to reprogramming, while fetal canine fibroblasts can be reprogrammed more efficiently, and indeed show faster proliferation [49]. Zhang et al. [10] generated iPSCs from adult fibroblasts obtained from horses, donkeys and mules as interspecies hybrid. Higher proliferation rate of mule fibroblasts was associated with higher generation efficiency and higher pluripotency expression of mule iPSCs. Furthermore, Pessoa et al. [14] reported the obtainment of higher number of colonies from the equine cell types showing lower cell doubling time. It has been suggested that stimulating cell proliferation could increase reprogramming efficiency. Actually, canine BM-MSCs treated with simvastatin not only enhanced their proliferation but also increased the expression of the pluripotent markers REX1 and OCT4 [50]; however, reprogramming was not attempted in that study. Interestingly, the proliferation rate of CB-MSCs and ACs increased after OSKM transduction, which has been proposed as a feature of early reprogramming (initiation stage) [8]. This

observation aligns to changes in morphology, AP staining and upregulation of pluripotent genes, suggesting all together that reprogramming was induced in CB-MSCs and ACs but could not be completed.

To further investigate differences among equine cell types, transcriptomic analysis revealed that eMSCs basally expressed higher levels of pluripotent markers, which were further increased during reprogramming. Reprogramming of somatic cells into iPSCs requires activation of pluripotency networks and silencing of the somatic program (cell identity) by turning on and off specific genes. Recently, two studies [9, 10] analyzed the bulk transcriptome of putative eqiPSCs and compared it over the transcriptome of their parental cells. While these studies provide highly valuable information, they exclusively focus on the start (parental cells) and the end (putative eqiPSCs) points of the process, but omit the intermediate stages of reprogramming. Revealing the changes that cells undergo during reprogramming is key to understand the process and to unveil its barriers and enhancers [39, 40]. To date, only one study has analyzed targeted transcriptomic changes at different moments of the reprogramming of canine cells [49], while no reports exist for equine cells.

Tobias et al. [49] found that adult canine fibroblasts were refractory to reprogramming, with partially reprogrammed clones upregulating *POU5F1 (OCT4)* but not *SOX2* and *NANOG*. In line with these results, our study showed that *POU5F1 (OCT4)* was upregulated by all the three types of equine cells even when reprogramming was not complete, but the highest expression of *SOX2* and *NANOG* was found for eMSCs, coinciding with the emergence of colonies. Thus, the expression dynamics of these genes may also be critical during the initiation stage of equine cell reprogramming. Moreover, eMSC was the only cell type that consistently expressed *SOX2* prior to reprogramming, which in human cells has been related to higher reprogramming efficiency [30]. While the data is still limited to make any claim, these observations suggest that parameters like proliferation and pluripotency expression might be used in equine cells as indicators of reprogramming efficiency.

Another strategy to enhance the reprogramming efficiency is to add certain chemical inhibitors to regulate specific signaling pathways. The use of these small molecules is reported in a few equine studies [15, 27], but the extent of their effects is unclear. The GSK3 signaling pathway can block other pathways required for reprogramming, while MEK and TGFβ pathways are involved in the maintenance of cell identity, which needs to be erased to induce pluripotency. Finally, valproic acid can increase chromatin accessibility by inhibiting histone deacetylase (reviewed in [51]). Contrary to that expected, the combination of these inhibitors did not improve reprogramming efficiency but actually decreased cell proliferation and colony formation in all cases. However, SM induced the upregulation of pluripotent genes in CB-MSCs and ACs, but not in eMSCs.

These counterintuitive effects might be explained by the SM dose, combination and exposure time. For example, inhibiting MEK and TGFβ pathways can silence somatic programs and upregulate pluripotency markers, but can also induce a cytostatic effect [52]. In addition, while valproic acid can facilitate OSKM target access and thus induce expression of pluripotent genes, it can also trigger cell-cycle arrest [53]. Moreover, the prolonged exposure to these SMs might accentuate these effects. In addition, the epigenetic profile of each cell type may add further variation to the effects displayed by SMs. For instance, prenatal cells often present a higher baseline pluripotency profile, as also suggested in this study for equine eMSCs. These cells might rely on TGF-β signaling for proliferation/survival [54], and thus its blocking may limit expression of pluripotent genes. Therefore, while certain SM have potentially beneficial effects for reprogramming, our results stress the need of adjusting their dose, combination and time of exposure depending on the species and the cell type. Future studies should explore the individual and combined use of different SMs to determine species specific and cell type specific needs for iPSC reprogramming.

The resulting putative eqiPSCs lines met the general cellular, molecular and functional criteria established for pluripotent cells [55], and presented a stable karyotype. However, specific pluripotent criteria for animal cells are not fully defined, arising from our limited understanding of pluripotency in veterinary species [56]. In addition, the variability of protocols for generating animal iPSCs, and the use of different pluripotent markers and of different antibodies across veterinary studies (reviewed in [57]), further limits establishing definitive criteria. While human iPSCs are considered to be in a primed pluripotent state, and murine iPSCs to represent naïve pluripotency, mixed reports exist about the pluripotent state of animal ESCs/iPSCs (reviewed in [58]). Previous reports on equine iPSCs and ESCs have described both naïve and primed morphology (reviewed in [57]).

The eqiPSCs colonies obtained in our study resembled naïve morphology and overexpressed the naïve pluripotency marker *ZFP42 (REX1)*, while the primed pluripotency marker *FGF5* was expressed at substantially lower levels. Previous studies also reported the expression of *REX1* in equine iPSCs [15, 34, 37, 59], with some of them simultaneously describing the expression of the primed marker stage-specific embryonic antigen 4 (SSEA-4) [15, 37]. It has been suggested that canine iPSCs may present an intermediate pluripotent state between those represented by human and murine cells, and that this state

would be plastic and could change upon the use of bFGF or LIF [60]. As aforementioned, several reports, including our study, point out at bFGF and LIF co-dependency of eqiPSCs, thus indirectly suggesting an intermediate pluripotent state as in canine iPSCs. Such pluripotent plasticity could also contribute to explain discrepancies in the characterization of eqiPSCs across studies. While the eqiPSCs generated in the current study would be closer to a naïve rather than to a primed state, studies specifically addressing the positioning of eqiPSCs in the pluripotency landscape are needed to define standard characterization criteria.

Another barrier yet to overcome in veterinary iPSCs is the achievement of fully reprogrammed iPSCs. The last stage of iPSC reprogramming is the stabilization phase, characterized by Tg silencing and capacity of the cells to maintain pluripotency without relying on Tg expression. Continuous Tg expression reflects incomplete reprogramming, hampers differentiation and carry safety concerns for therapeutic use [8, 61]. Stabilization usually happens spontaneously in human iPSCs [62], but it is an unlikely event in eqiPSCs. To the best of our knowledge and to date, only one equine report has directly shown spontaneous Tg silencing [23]. Thus, full reprogramming of equine cells could be achieved but it is unlikely to happen spontaneously, making the generation of stable eqiPSCs unpredictable. In spite of its high relevance, this aspect has not been explored in detail, with only one recent study showing that removing the Tg promoted instability of eqiPSCs [10]. The predominant use of Tg integrative methods in eqiPSC publications and the limited success using non-integrative methods (reviewed in [3]) further reflect the inefficient stabilization of iPSCs in this species. According to previous evidence (reviewed in [3]), the eqiPSCs generated in this study maintained Tg expression and thus were termed as putative. Nevertheless, these eqiPSCs were able to spontaneously differentiate, as shown in the EB assay, in agreement with previous eqiPSCs reports (reviewed in [57]). In this regard, it should be noted that only one marker per lineage was studied in cells obtained from the EB assay. Previous works have also based the study of eqiPSC differentiation in one marker per germ layer [14, 32, 63], owed to the difficulty in finding suitable cross-reactive antibodies for the equine species [57].

The goal of this study was to establish putative eqiPSCs from new sources along with essential characterization to ground the basis for future research, rather than thoroughly optimizing the generation and characterization of these cells. Future studies will fine-tune these aspects and will explore the capacity of these putative lines to undergo directed differentiation into intended target cells, such as chondrocytes or MSCs.

Conclusions

Our study provides first direct evidence in the equine species about the effect of the cell developmental stage on their potential to become iPSCs, and on which cell features might be related to such reprogramming potential. Furthermore, we provided novel preliminary insight into the transcriptomic changes experienced by different equine cell types during reprogramming, and on the varying effects that small molecules can induce in each cell type. This information is key to improve the generation of eqiPSCs. Embryo-derived MSCs emerge as a promising source to generate iPSCs in the equine species, which may increase access to eqiPSCs for research. While transgene-free methods and fully reprogrammed eqiPSCs are the goal, putative eqiPSCs lines are critical to advance in that direction, as they can enlarge our knowledge on animal iPSC biology, and contribute developing more standardized reprogramming protocols, culture conditions and characterization criteria. Further studies are needed to understand the reprogramming barriers in the equine species, which will importantly contribute to make the iPSC technology widely available for veterinary and translational applications.

Abbreviations

AC	Articular chondrocyte
ACAN	Aggrecan
AFP	Alpha-fetoprotein
ALPL	Alkaline phosphatase (gene)
AP	Alkaline phosphatase (activity)
α-SMA	Alpha smooth muscle actin
B2M	Beta-2-microglobulin
bFGF	Basic fibroblast growth factor
βIII-tubulin	Beta-3-tubulin
BM-MSC	Bone marrow-derived MSC
CB-MSC	Cord blood-derived MSC
cDNA	Complementary DNA
CDT	Cell doubling time
c-MYC	MYC proto-oncogene
COL2A1	Collagen type II alpha 1 chain
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
dpi	Days post infection (after transduction)
EB	Embryoid body
EIF3L	Eukaryotic translation initiation factor 3 subunit L
EMA	European Medicines Agency
eMSC	Embryo-derived MSC
eqiPSCs	equine iPSC
ESC	Embryonic stem cell
E8	Chemically defined media E8 StemFlex
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FGF5	Fibroblast growth factor 5
G	Geltrex
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3b	Glycogen synthase kinase-3 beta
HBSS	Hanks's Balanced Salt Solution
HDAC	Histone deacetylase
iMEF	Irradiated mouse embryonic fibroblasts
iPSC	Induced pluripotent stem cell
ISG	Interferon stimulated gene
KLF4	Kruppel-like factor 4
KOSR	Knock-out serum replacement

Lentiv-Tg	Lentiviral transgene
LIF	Leukemia inhibitory factor
LPL	Lipoprotein lipase
NANOG	Nanog homeobox
N.E	No expression
MEK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMLV	Moloney Murine Leukemia Virus
MOI	Multiplicity of infection
MSC	Mesenchymal stem/stromal cell
OCT4	Octamer-binding transcription factor 4
ORF	Open reading frame
OSKM	Oct4, Sox2, Klf4, cMYC (Yamanaka factors)
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
pi	Post infection (after transduction)
POU5F1	POU class 5 homeobox 1
PPARG	Perosixome proliferator activated receptor gamma
R.S	Reference sample
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
RUNX2	RUNX family transcription factor 2
SSEA-4	Stage-specific embryonic antigen 4
SM	Small molecule
SOX2	SRY-box transcription factor 2
Ta	Annealing temperature
Tg	Transgene
TGFβ	Transforming growth factor beta
TU	Transduction units
Y-27632	ROCK pathway inhibitor
ZFP42 (REX1)	Zinc finger protein ZFP42

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04671-1.

Supplementary material 1. Retroviral reprogramming of equine cells

Supplementary material 2. Selection of multiplicity of infection (MOI) for lentiviral transduction of equine cells

Supplementary material 3. Characterization of equine cord blood-derived and embryo-derived mesenchymal stem/stromal cells.

Acknowledgements

Authors wish to thank Dr Terje Raudsepp and the Molecular and Cytogenetics Laboratory at the Texas A&M University (Texas, US) for conducting the karyotype analysis of the equine cells, which was outsourced to this service. Authors would also like to thank Prof Mandy Peffers from Univesity of Liverpool (UK) for kindly providing primary cell lines of equine articular chondrocytes for this study, and Prof Angel Raya from the Centro de Medicina Regenerativa de Barcelona (CMRB, Spain) for generously providing the Moloney Murine Leukemia Virus (MMLV)-based retrovirus containing green fluorescent protein (GFP) expression vector used to study retroviral transduction efficiency in equine cells. Authors also acknowledge the use of the flow cytometry core facility at the Biomedical Sciences Building (University of Galway, Ireland) and of the Servicios Científico Técnicos del CIBA (IACS-Universidad de Zaragoza, Spain). The authors declare that they have not use AI-generated work in this manuscript.

Author contributions

LB and FB conceived and designed the work. LB, AI, TEA, AOB and AC acquired and analyzed the data. LB, MM, FH, CR and FB interpreted the data. LB drafted the work, and AI, TEA, AOB, AC, MM, FH, CR and FB substantially revised the work. LB, MM, CR and FB were responsible for funding acquisition. All authors approve the submitted version of this work and agree to be accountable for the author's own contribution and for the accuracy and integrity of any part of the work.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement no. 101026825, and from the Ministerio de Ciencia e Innovación (Spain) under grant number PID2020-116352GB-I00.

Data availability

The gene expression datasets generated and analyzed during the current study are available in the Zenodo repository, https://zenodo.org/records/13820354 , and with digital object identifier (doi) https://doi.org/10.5281/zenodo.13820354. Any other data are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Equine eMSCs were isolated at Colorado State University (Colorado, US) under ethics approval of the Institutional Animal Care and Use Committee (title: "Production of equine fetally-derived mesenchymal stem cells and isolation and characterization of their extracellular vesicles"; approval number: 4443; approval date: 17 April 2023). Equine cord blood was obtained under informed owner consent at Irish stud farms, and CB-MSCs were isolated at University of Galway (Galway, Ireland). Ethical approval was not required because the blood was collected from *peripartum* waste material by non-invasive means for the mare or the foal. Equine BM-MSCs were obtained at the University of Zaragoza (Zaragoza, Spain) under ethics approval of the in-house Advisory Ethics Committee for Animal Research (title: "Optimización del uso de MSCs alogénicas en el tratamiento de patologías articulares equinas: equilibrio inmunomodulación-inmunogenicidad" ["Optimizing the use of allogenic MSCs for treating equine joint pathologies: immunomodulation-immunogenicity balance"]; approval number: PI 15/16; approval date: 22 June 2021). Equine ACs were obtained *post mortem* at the University of Liverpool (UK) from healthy joints of animals euthanized for reasons unrelated to this study, thus ethics approval was not required but informed owner's consent was obtained.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 26 June 2025 / Accepted: 13 September 2025
Published online: 08 October 2025

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