Scale-Up Production Of Extracellular Vesicles From Mesenchymal Stromal Cells Isolated From Pre-Implantation Equine Embryos

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Abstract

Mesenchymal stromal cells (MSC) have recently been explored for their potential use as therapeutics in veterinary medicine applications. Alternatively, MSC-derived extracellular vesicles (EVs) may also provide therapeutic benefits but as an off-the-shelf solution, provided they can be produced in large enough quantities and without the risk of contamination from bovine EVs contained in fetal bovine serum that is a common component of cell culture media. Towards this aim, we demonstrated the successful isolation and characterisation of equine MSCs from pre-implantation embryos. We also demonstrate that many of these lines can be propagated long-term in culture and conducted a head-to-head comparison of two bioreactor systems for scalable EV production including in serum-free conditions. Based on our findings, the CELLLine™ AD 1000 flasks enabled higher cell density cultures and significantly more EV production than the Fibercell™ system or conventional culture flasks. These findings will enable future isolation of equine MSCs and the scalable culture of their EVs for a wide range of applications in this rapidly growing field.

Introduction

Mesenchymal stromal cells (MSCs) have recently been used as therapeutics in several experimental settings including veterinary applications. Typically, MSCs express CD73, CD90, and CD105 cell surface markers, adhere to plastic, and can be differentiated into three lineages, but lack hematopoietic and endothelial markers ^{1,2}. In veterinary applications it is common to conduct autologous MSC transplantation, in which MSCs are isolated from the animal to be treated, expanded by *in vitro* culture, then transferred back to the original animal ³. This process is invasive to the animals and due to the time required to expand MSCs *in vitro*, may delay treatment and potentially reduce the effectiveness of MSC therapy. Therefore, an off-the-shelf product that could be used in allotransplantation may be advantageous. There is considerable and growing evidence that the anti-inflammatory and regenerative properties of MSCs are not the result of engraftment of the MSCs themselves, but rather are affected by extracellular vesicles (EVs) extruded from the MSCs ^{3,4}.

EVs are lipid membrane bound vesicles that are released from all cells ⁵. They contain cargos of proteins, DNA and RNA, including short and long regulatory RNAs ^{6,7}. Compared to live cells, EVs have several advantages as a therapeutic agent. EVs are not live cells and therefore, present no risk of unintentional growth at sites of engraftment and appear to be less antigenic than the cells from which they are derived ^{7,8}. However, a major problem facing EV researchers is the limited yield of EVs from conventional cell culture systems, particularly from primary cells. This is a significant obstacle in the preparation of EVs, especially for the production of a large scale, off-the-shelf therapeutics. A further barrier to obtaining large quantities of MSC-derived EVs is that cultures typically utilize fetal calf serum, which itself contains many EVs that cannot be effectively separated from the MSC-derived EVs ⁹.

Bioreactors that support high-density culture may be a solution to both EV yield and contaminating exogenous serum EV problems. Some devices use semipermeable membranes to separate the bioreactors into a large volume medium chamber and a small cell chamber. The membrane allows transfer of essential nutrients to the cells while preventing the exchange of larger products such as EVs between the compartments. At the same time, EVs extruded from the cells are concentrated in the smaller volume cell compartment, reducing downstream isolation requirements. Examples of such bioreactors include the FiberCell™ system that is based on semipermeable hollow-fibers through which medium is pumped and the CELLline™ system that has static media in two chambers separated by a semipermeable membrane. While both systems were developed for hybridoma cell culture for production of monoclonal antibodies in the past, they have recently been reported for EV harvesting from cell lines^{10–16}. However, a direct comparison of these two types of bioreactors for the preparation of EVs from primary cultures of MSCs has not yet been undertaken.

In this study, we report the isolation, culture, and characterisation of cells from pre-implantation equine embryos that display characteristics of MSCs. We were then able to grow these cells in CELLline™ and FiberCell™ bioreactors in the absence of FBS and compared EV yield from these systems to conventional cultures. To the best of our knowledge, this is the first example of culturing equine MSCs in bioreactor systems for the large-scale production of EVs.

Results

Isolation and characterisation of equine MSC-like cells

We isolated putative MSC lines from six equine pre-implantation embryos based on plastic adhesion. While the cultures initially contained multiple cell types, cells with the morphology of MSCs rapidly became the predominant cell type due to their high rate of proliferation. Very few non-MSCs survived passage to fresh flasks and by passage three, the cultures were essentially pure (Fig. 1). We continued to passage the cells to monitor when they would cease proliferation. Three of the lines (PL, T, and DP) stopped rapid proliferation between passages 8–11, two lines (J and FM) continued proliferating to passage 20–21 but the proliferation rate of FM slowed from passage 16. One line (SF) continued to proliferate to passage 32.

Figure 1. Phase-contrast photomicrograph demonstrating the morphology of the MSC-like cells cultured from equine pre-implantation embryos. Image taken using a 4x non-confocal objective. Scale bar, 500 µm.

Lineage markers suggest MSCs

To investigate if the cultured equine embryo-derived cells exhibited MSC-like phenotypes, the expression of MSC markers was examined for the three cell lines that grew beyond passage 11. Flow cytometry and RT-PCR confirmed the expression of CD90 and CD44 (Fig. 2 Table S3), while due to the absence of suitable antibodies the expression of CD73 and CD105 were demonstrated by RT-PCR (Fig. 2, Table S3)¹.

Cells were additionally found to weakly express class I MHC. An additional marker, CD146 was shown to be strongly expressed by the MSCs by quantitative RT-PCR but, levels of CD146 declined in very late passages of each cell line (Table S3) ¹⁷.

We continued characterisation of MSC-like cells using flow cytometry which confirmed the absence of CD14 and MHC-class II markers from all three cell lines (Fig. 2). Flow cytometry suggested very weak expression of CD45 in some passages for two of the three MSC lines but RT-PCR confirmed the absence of CD45 expression (Fig. 2, Table S3). Quantitative RT-PCR also confirmed low or no CD31 expression and low CD34 expression that declined overtime in culture (Fig. 2, Table S3).

In summary, our MSC-like cells expressed CD90, CD105, CD73, CD44, and CD146 but did not express CD14, CD34 CD45, and MHC-class II consistent with the characteristic phenotype of MSCs.

Trilineage differentiation assays confirm MSCs

Cultured MSCs have the ability to differentiate into three mesenchymal lineages: adipogenic, osteogenic and chondrogenic. To further investigate whether the equine embryo-derived cells were MSCs, cells from the three lines were incubated in media for 14 or 21 days to stimulate differentiation and representative images at day 21 are presented (Fig. 3). Adipogenic differentiation was confirmed by positive staining with Oil Red-O and no lipid droplet formation was detected in control untreated cells (Fig. 3A, Table S4). Following incubation in osteogenic differentiation conditions the cells showed a pronounced change in morphology (Figure S3A) accompanied by the presence of Alizarin Red S-stained calcium deposits; however, this staining was variable between cell lines and passages (Fig. 3B, Table S4). Chondrogenic differentiation was confirmed by altered morphology and larger cell pellet, in addition to increased Alcian Blue staining (Fig. 3C, Figure S3B).

Equine embryo-derived MSCs cultured in high-density bioreactors

The yield of EVs from MSCs cultured in traditional flat surfaces is not practical or scalable enough for use as a therapeutic. To improve the yield of EVs we tested the ability of one cell line (SF) to grow in bioreactors under serum-free conditions to avoid contamination of MSC-derived EVs with exogenous bovine serum EVs (Fig. 4A).

Two different bioreactors were tested in this study, including a 1L CELLLine™ flask for adherent cells and a FiberCell™ with two different hollow fiber cartridges, 2025D and 2025G. The 2025D was coated with fibronectin to help improve cell adhesion. The cells were seeded into the bioreactors in DMEM/F12 containing 10% FBS and cultured for 27 days to establish the high-density cultures. Direct observation of the cells was not possible in any of the bioreactors. Therefore, glucose consumption was used as an indirect measure for cell growth. An increase in daily glucose consumption was observed in the CELLLine™ flask in this establishment phase of the culture, but there was no increase in glucose consumption in either cartridge in the FiberCell™ system (Fig. 5A). At the end of the growth period, the bioreactors were

dismantled to assess cell density and surface morphology. Although we did not attempt to quantify cell numbers, cells covered most of the mesh surface within the CELLLine™ flask with very little exposed surface (Fig. 5B). DAPI staining confirmed the presence of densely packed cells which were absent in an unused flask. Little biomaterial was found on the semipermeable dialysis membrane and the gas-permeable plastic sheets underneath the fibrous growth surface (Figure S4). In contrast, SEM of both FiberCell™ systems indicated the presence of relatively sparse cell clusters with limited coverage of the fiber surface (Fig. 5C).

CELLLine™ AD 1000 bioreactor supports high EV production

After 27 days in FBS containing medium we changed to media with CDM-HD serum replacement and began harvesting EVs. EV were harvested from the CELLLine™ flask at a stable rate ranging from 4–10 $\times 10^{10}$ particles/day. On average, this is approximately equal to the number of EVs produced by MSCs grown in 20 T175 flasks. In contrast, both cartridges in the FiberCell™ system produced only 0.1–0.6 $\times 10^{10}$ EVs/day, which is approximately the same number of EVs produced by MSCs grown in one T175 flask. Nanoparticle tracking analysis (NTA) indicated that the average EV size (~ 100 nm) was similar regardless of the culture system used (Fig. 6B,C), and TEM images of EVs from the CELLLine™ flask show the expected rounded and cup-like morphologies (Fig. 6D). Lower magnification TEM images, as required by the MISEV guidelines, are presented in Figure S6.

Due to the low EV production, the FiberCell™ system was terminated after 46 days of culture while the CELLLine™ system was maintained to examine if cells could survive and continue to produce EVs with different medium combinations. To do this, the medium in the large chamber was changed to advanced DMEM/F12 with 2% FBS and the medium in cell chamber was changed to basal DMEM/F12 without FBS or CDM-HD. Cells were cultured for additional 4 weeks and after an initial rise, EV production was maintained at an average 6 $\times 10^{10}$ /day, similar to that observed when the cell chamber medium contained the CDM-HD serum replacement (Figure S5).

Extracellular vesicles cannot pass between chambers of the CELLLine™ flask

The CELLLine™ flask system has a large (~ 1 L) chamber that contains growth medium and a much smaller 15 mL cell chamber into which EVs are secreted (Fig. 4). In theory, the semipermeable membrane that separates the chambers should prevent any bovine serum EVs from the growth medium chamber passing into the lower cell chamber from which EVs are harvested. To test this, we added 500 mL Advanced DMEM/F12 containing 2% FBS to the growth medium chamber and 15 mL of PBS to the lower chamber of three fresh CELLLine™ flasks without cells. Following three days of incubation at 37°C, NTA analysis indicated there were 1.14×10^9 ($\pm 1.36 \times 10^7$) particles/13 mLs of medium in the large chamber and 3.44×10^7 ($\pm 4.13 \times 10^7$) particles/13 mLs in the lower chamber indicating that despite a substantial concentration gradient, EVs were unable to pass through the semipermeable membrane (Table S5).

The economics of using the CELLLine™ bioreactor for EV production

There is an initial cost of approximately \$(US) 400 to purchase a single CELLLine™ flask or Fibercell™ cartridge. The FiberCell™ system requires an additional one-off investment in the pump required to circulate the medium. The daily yield of EVs from our cells grown in the CELLLine™ system was approximately 20 time the yield from traditional T175 flasks. Assuming one medium change and one passage per week, between 1–2 days of technician time would be required to maintain the 20 T175 flasks. In contrast, only 1–2 hrs/week was required to maintain the CELLLine™ system. There is also a considerably reduced environmental impact from the use of a single bioreactor which we maintained for approximately three months compared to the use of 20 T175 flasks which are traditionally replaced after each passage. Furthermore, the CELLLine™ flask provides EVs in much lower volume, potentially reducing the total isolation and purification time and cost depending on the specific downstream application. A comparison of the costs of generating equivalent numbers of EVs bioreactors and conventional flasks is presented in Table 1.

Table 1
Cost and efficiency comparison of CELLLine™, FiberCell™ and traditional culturing system.

	CELLLine™	Fibercell™	T175
Length of culture	82 days	46 days	Passage every 3–4 days
Price (USD)	345.8	321 (small cartridge) ¹	70.6 (20 T175 for 1 passage)
Medium/Week	500 ml	500 ml	At least 1L
TC work cost/week (USD 2 harvests) ²	17.6 (1 hr)	17.6–35.2 (1–2 hrs)	140.85–281.7 (1–2 days)
¹ need additional cost for pump			
² value was calculated based on a technician’s salary of (\$NZ) 60,000/year			

Discussion

Mesenchymal stromal cells are typically isolated from adipose tissue or bone marrow, but numerous other sources of MSCs have been described, including the placenta, endometrium, and cord blood ^{18–20}. It has been suggested that MSCs obtained from younger tissues, such as placentae, have greater differentiation potential, are more anti-inflammatory, and may proliferate longer in culture than cells derived from older tissues ^{20,21}. Here, we isolated multiple MSC-like lines from several preimplantation equine embryos. These lines all proliferated at a high rate when initially isolated, but several lines continued to rapidly proliferate beyond passage 11 which is much higher than previously isolated equine

MSCs²². This continued proliferation of embryo-derived cells in culture is a significant advantage if these cells are used as a source of MSCs or their EVs for regenerative medicine.

There are clear criteria, issued by the International Society for Cell Therapy that human cells need to meet before they are considered MSCs²³. However, it is less clear whether equine MSCs fit all of these criteria and this is further complicated by the limited availability of characterised reagents to interrogate these markers²⁴. The isolated embryo-derived MSC-like cells in the current study expressed markers typical of equine MSCs and lacked hematopoietic markers, comparable to previous reports of the characterisation of MSCs derived from equine adipose, bone marrow, placenta, and umbilical cord blood^{25–27}. The low or variable expression of CD34 and CD73 observed in the current study was not unexpected and indicates potential differences between MSCs from human and equine origins^{25,26}. Although it was confirmed that embryo-derived MSCs do not express the CD45 hematopoietic marker, the variable low expression observed by flow cytometry confirms the problematic nature of some antibodies for some less commonly researched species such as equids. In addition to the classical MSC panel of markers, prior studies have also reported the expression of CD146, a marker suggested to identify MSCs with higher multipotency, which the embryo-derived MSC-like cells in the current study strongly expressed^{17,28}. Expression of this marker indicates that the cells within this study may have the potential to treat a wider range of conditions than classical MSCs.

An important secondary characteristic of MSCs is their ability to differentiate into multiple lineages. Comparable to prior studies, the equine embryo-derived cells readily underwent differentiation to adipocytes. However, other studies have suggested that this requires modification to typical media, especially the addition of rabbit serum^{22,24,26}. Like placental MSCs, these embryo-derived cells were less inclined to commit to the osteogenic lineage than bone marrow-derived MSCs²⁹. Similarly, cells in chondrogenic differentiation medium underwent clear morphologic changes but showed variable staining with Alcian blue. Overall, the expression of MSC markers, lack of lineage-specific markers and ability to undergo differentiation suggests the equine embryo-derived cells are MSCs.

In many cases, MSCs used in equine regenerative medicine are derived from either bone marrow or adipose tissue of the animal that is being treated^{30,31}. This requires invasive extraction of tissue or cells from the animal and due to the low numbers of MSCs, these cells are then expanded *in vitro* in a lengthy process that takes approximately 1–2 weeks. The enriched MSCs are then used to treat the animal. There is potentially considerable therapeutic and economic value in being able to treat these animals at a time more proximal to the time of injury. Having a ready source of allogeneic MSCs with high regenerative potential available that could be rapidly accessed would achieve more timely treatment of injured animals, as previously described for the use of human MSCs³². Since our MSCs are derived from young tissues it is likely that they will also possess more anti-inflammatory and regenerative properties than the adult MSCs that are used in current practice and they could potentially a valuable allogeneic therapeutic.

The utility of MSCs in regenerative medicine has been recognised for many years. However, in more recent times it was noted that there is a failure of large-scale engraftment of the MSCs and attention has focused more on the beneficial effects of the factors that these cells release^{33–37}. In this light, EVs are of particular interest, since they are thought to convey many of the anti-inflammatory and regenerative properties of MSCs and appear to be amenable to long-term storage^{3,4}. Reflecting this, there are more than 1300 registered trials of MSCs for various conditions and a growing number of clinical trials that investigate the use of EVs from MSCs as a therapeutic treatment (<https://clinicaltrials.gov/ct2/home> assessed 15th November 2021). MSC-derived EVs have shown value in veterinary medicine as therapies for persistent mating-induced endometritis, increasing oocyte yield, as well as in many non-reproductive conditions such as osteoarthritis and tendonitis^{38–40} and there is potential that EVs from our equine embryo-derived MSCs would also show similar benefits^{41–43}.

A major limitation to the use of EVs as a therapeutic is the limited capacity of MSCs to produce EVs in traditional two-dimensional culture flasks. Small bioreactors have been used for purposes such as monoclonal antibody production for many years and more recently investigators have started to explore their use for the production of EVs^{10,44}. Here we conducted a head-to-head comparison of two common, commercially available small bioreactor systems, the CELLLine™ flask system and the FiberCell™ system. These systems use two different growth surfaces and the CELLLine™ flask is static while the FiberCell™ uses a continuous flow of media. In this comparison, the CELLLine™ flask system clearly outperformed the FiberCell™ in terms of cell density and quantity of EVs produced. Guerreiro *et al.* reported the use of CELLLine™ flask with PE/CA-PJ49/E10; BxPC3 and H3 cell lines¹⁵. Similar to our observation, in that study and others, adherent cells grew well in CELLLine™ and produce EVs in range of 10¹⁰ particles/harvest^{11,15}. The MSC line that we isolated and trailed adapted well to culture in the CELLLine™ flask and showed the potential to allow long term, stable, EV production. Moreover, this production was sustained in the absence of FBS in the cell chamber. In contrast to the report of Guerreiro *et al*¹⁵, our MSCs mainly attached to and grew on the mesh net rather than spread to the semipermeable membrane or plastic sheets in the CELLLine™ flask. This suggested the behaviour of cells in the bioreactors is likely to be cell type/line dependent. While there was little attachment of our MSCs to either of the growth surfaces in the FiberCell™ systems we tested, it is possible that other surfaces or hollow fibre cartridges might be more suited to adherent cell growth. We have for many years used, and continue to use, the FiberCell™ system to produce monoclonal antibodies in serum-free conditions from hybridoma cells. It may be that the FiberCell™ system might be more successful in producing EVs from non-adherent cells such as lymphocytes.

One real highlight of using bioreactors is the potential to harvest large numbers of EVs that are free from contaminating bovine EVs derived from FBS. In the CELLLine™ flask system this can be achieved using either a serum replacement or a combination of serum-containing medium in the large medium compartment and serum-free medium in the cell growth compartment from which EVs are harvested. Here we confirmed using flasks without cells, that the semipermeable membrane does indeed prevent the passage of EVs from the large medium chamber to the small cell chamber of the CELLLine™ flask system.

Other major advantages of using the CELLline™ bioreactor that we have characterised are; 1) substantially reducing the person time required to maintain the culture and harvest EVs, 2) a substantial reduction in environmental impact by reducing the number of flasks and other disposable culture consumables required, and 3) a major reduction in the cost of cell culture and EV processing consumables. Although culturing of MSCs using bioreactors is beneficial, there are also drawbacks to their current designs. Neither of the systems used in the current study provided any visual access point to check the health and/or growth of adherent cells in the bioreactors. Although, as used here, glucose consumption may provide some insight to the cell's metabolic activity, it may not always represent the actual growth of the cells, particularly when using a commercial serum replacement, which may contain an alternative energy source. Finally, it has recently been shown that changing the growth conditions of MSCs or other cells following adaptation to culture in bioreactors altered the contents and therefore possibility the biological function of EVs ⁴⁵.

In this study, we demonstrate that a novel source of MSCs can be isolated from pre-implantation equine embryos, that the isolated cells express characteristic MSC markers and can be differentiated into the expected three lineages. In addition, given the recent interest in using MSC-derived EVs in therapeutic applications, we tested the performance of the MSCs in two commonly reported small bioreactor systems for EV production. Based on our findings, the CELLline™ AD 100 bioreactor flask outperformed both conventional culture systems and the two Fibercell cartridges tested, in supporting high cell density and total EV production. We hope these findings will encourage further exploration of these novel MSCs and their EVs in veterinary regenerative medicine research. Furthermore, we hope our findings on different bioreactor culture systems will enable researchers to produce EVs in quantities that will ultimately facilitate clinical therapeutic application in veterinary medicine at a commercial level.

Methods

Materials

Culture medium and additives were purchased from Gibco™ (Thermo Fisher Scientific, NZ) unless indicated otherwise. PBS (CAT.D8537) was supplied from Sigma-Aldrich (USA). Primary and secondary antibodies for flow cytometry are as described in Table S1. FiberCell cartridges and CDM-HD were purchased from the FiberCell System (USA) and the CELLline AD 1000 flasks from Sigma-Aldrich (MO, USA).

Isolation and culture of pre-implantation equine embryo MSC

The mesenchymal stromal cells used in this study were isolated from 33–34 day old equine embryos produced in our laboratory (ethics approval AEC1431). The embryos were harvested following the method of de Mestre, et al. ⁴⁶. Equine embryos were transported in PBS with 2% anti-anti until processed. Equine embryos were examined under a dissecting microscope and any fetal membrane removed. The embryos

were minced with a scalpel then passed through a 2 mm stainless steel mesh and 1/10 of the cells used for subsequent culture. Cells were initially cultured and characterised in DMEM/F12 containing 150 mM HEPES, 10% FBS (Life Technologies, NZ), 0.4 µg/ml insulin (Sigma-Aldrich), 1% anti-anti, 1% GlutaMAX, and 0.5 µg/ml Vitamin C (Sigma-Aldrich), and grown in a humidified incubator at 37°C with 5% CO₂. Cells were subsequently cultured in Advanced DMEM/F12 supplemented with 2% FBS, 1% anti-anti and 1% GlutaMAX in T75 filter-cap flasks. Cells were passaged with 0.05% Trypsin for RNA extraction and differentiation assays or detached using TrypLE or accutase for flow cytometry experiments.

cDNA synthesis, RT-PCR and quantitative RT-PCR

RNA was extracted from 1 million equine MSC-like cells or PBMCs using TriZol and the PureLink RNA mini kit (Invitrogen, MA, USA). RNA was reverse-transcribed to cDNA using the the qScript cDNA Supermix (Quanta Biosciences, MD, USA) or the High-capacity RNA-to-cDNA kit (Applied Biosystems, MA, USA) as per the manufacturer's instructions.

RT-PCR experiments were performed using the EmeraldAmp Max HS PCR master mix (Takara, Japan) as per the manufacturer's instructions. Briefly, 1 µL cDNA (< 50 ng/µL) was added to 10 µL Emerald Master Mix, 0.5 µL each of forward and reverse primers (Table S2, 10 µM), and 8 µL MilliQ water. Reactions were run using a PCR thermal cycler (Applied Biosystem, Thermo Fisher Scientific) as follows: 30 cycles of 98°C for 10 s, 58–62°C for 30 s followed by 72°C for 1 min/kb. PCR products were then run on a SYBR safe stained 1–2% agarose gel at 100 V.

qPCR experiments were performed using SYBR Green qPCR Supermix-UDG with ROX (Invitrogen) as per the manufacturer's instructions. Briefly, 2 µL cDNA (100–150 ng/µL) was added to 5 µL SYBR Green qPCR Supermix, 0.2 µL each of the forward and reverse primers (Table S2, 10 µM) and 2.6 µL MilliQ water. Samples were added to a 384-well plate and reactions run using the following conditions: 50°C for 2 min, 95°C for 10 min then 40 cycles of 95°C for 15 s and 60°C for 1 min followed by a melt curve analysis. Each condition was performed in triplicate and β-actin was used as the housekeeping gene. The expression level of the target gene was plotted as the averaged mean CT value ± SEM of the three technical replicates.

Flow cytometry

Cells were detached with TrypLE or accutase to avoid cleaving cell surface markers. Equine PBMCs were used as the positive control for negatively expressed MSC cell surface marker proteins. For each experimental condition, ~ 500,000 cells were washed in FACS buffer (1% FBS in PBS), resuspended in 100 µL fresh FACS buffer containing 5 µL primary antibody and incubated in the dark for 30 min on ice. Cells were washed in FACS buffer, and for unconjugated primary antibodies, cells were incubated with 1 µL secondary antibody in 1 mL FACS buffer for a further 30 min on ice. Cells were washed a final time, resuspended in 1 mL buffer, and expression of cell surface markers immediately examined using a fixed voltage CyFlow Cube8 flow cytometer (Sysmex, Japan). The forward and side scatter parameters were used to determine and gate cell populations. At least 10,000 events were acquired, with the no primary antibody control used to discriminate positive from negative staining.

Tri-lineage differentiation assays

Tri-lineage differentiation was examined for the SF, J and FM cell lines. Where possible, differentiation into adipogenic, osteogenic, and chondrogenic lineages were examined at an early, middle, and late passage of the cell line growth potential. It was attempted to match experimental passages across differentiation assays and is noted where this was not possible. For all differentiation assays, two wells or cell pellets were subjected to differentiation medium while a third was used as an undifferentiated control. Differentiation was examined using an It-UEclipse inverted microscope (Nikon) with 10x, 20x or 40x non-confocal objective lens.

Adipogenic differentiation

Cells were plated at a density of 500,000 cells per well in a 6-well plate. The following day, growth media was removed and replaced with 2 mL complete MesenCult Adipogenic differentiation medium (Stemcell Technologies, Canada). Cells were incubated at 37°C with 5% CO₂ for 14 or 21 days with the media changed every 3 days. On days 14 and 21, cells were washed in PBS and fixed with 4% PFA for 30 min. Cells were washed and incubated in 60% isopropanol for 5 min before replacement with Oil Red O working solution for 5 min. The cells were then washed with water, counterstained with Hematoxylin and imaged for lipid formation.

Osteogenic differentiation

Cells were plated at a density of 1,000 cells per well in a 6-well plate. The day after plating, growth media was replaced with Osteogenic Stimulatory medium containing 10% FBS, 0.01 µM dexamethasone, 10 mM β-Glycerophosphate, 50 µM ascorbic acid, and 1% anti-anti. Plates were incubated at 37°C with 5% CO₂ for 14, 21, or 24 days with the media being changed every 3 days. On days 14, 21, and 24, cells were washed in PBS and fixed with 4% PFA for 30 minutes. Following fixation, cells were washed and incubated in the dark with Alizarin Red S staining solution for 45 minutes. The cells were then washed with water and imaged for calcium deposits.

Chondrogenic differentiation

Cell pellets were formed by resuspending 500,000 cells in 0.5 mL growth media and transferring the suspension to a 15 mL centrifuge tube. The tubes were spun at 300 x *g* for 5–10 min at RT. The caps were loosened, and pellets grown at 37°C with 5% CO₂ for 3 days. On day 3, 0.5 mL complete MesenCult-ACF differentiation medium (Stemcell Technologies, Canada) was added to give a final volume of 1 mL. The media was replaced with 0.5 mL fresh differentiation medium every 3 days until day 21. On day 21, cell pellets were washed in PBS, fixed in 4% PFA for 30 min, and stored in 70% ethanol for paraffin embedding and sectioning. Sections (6 µm) were deparafinised, rehydrated then incubated for 3 min in 3% acetic acid and washed in water. Alcian Blue solution was added to each section and incubated for 30 min in a humidified chamber. Sections were washed and cover slipped before being imaged.

Culturing cells in bioreactors

Three bioreactors were chosen to test high-density culturing of cells; one CELLLine™ AD 1000 flask for adherent cells and two FiberCell™ hollow fiber bioreactors using cartridges manufactured with different fibres referred as 2025D and 2025G. The 2025D fibres were modified by coating them with fibronectin to improve adhesion. The bioreactors were operated following the manufacturers' instructions. Both FiberCell™ bioreactors went through a pre-culturing wash step that included circulating PBS for 24 hrs, followed by DMEM/F12 and then DMEM/F12 with 10% FBS for 24 hrs each. The bioreactor 2025D was coated with fibronectin at 50 µg/mL at 37°C for 1 hr prior to circulating DMEM/F12 with 10% FBS. Each bioreactor was seeded with 30×10^6 viable MSCs in DMEM/F12 containing 10% FBS and grown for three weeks before gradually removing the FBS and replacing it with the serum replacement CDM-HD (Fig. 6A). The culturing scheme and medium composition are indicated in Figure S1.

Hoechst staining

At the end of the culture period, cells in the CELLLine™ flask were stained with hoechst (1:1000 dilution) for 10 min at 37°C. Excess stain was washed off and the cells imaged using a fluorescent microscope.

Scanning electron microscopy

All bioreactors were washed with PBS briefly and fixed with McDowell and Trump's Fixative (4% formaldehyde, 1% glutaraldehyde, 0.1M phosphate buffer, pH 7.2) for 30 min. Fixed samples were washed with PBS twice, then dehydrated with 35%, 50%, 70%, 90% (2x 10 min each) and 100% ethanol (3x 15 min). The FiberCell™ bioreactors were fixed and dehydrated while the hollow fiber bundles were still intact inside the plastic shell. The cartridge units were disconnected from the medium reservoir and the fixation and dehydration reagents were injected both inside the fibers and through the side port to the cell chamber using a syringe. The CELLLine™ AD 1000 was disassembled and cut into pieces suitable for fixation and dehydration. After the dehydration step, specimens from all bioreactors were air dried and sputter coated with 10 nm gold (Q150R S, Quorum) then imaged using a JCM-6000 benchtop scanning electron microscope (SEM) (JEOL) at 15 kV.

Collection and characterisation of EVs

Initially, to collect EVs, the MSCs were transferred to T175 flasks. The MSCs were grown to 60%-70% confluence before changing the medium to DMEM/F12 without serum, supplemented with 1x non-essential amino acids (NEAA, Thermo Fisher Scientific) and the culture continued for 3 days. The medium was then collected and centrifuged at $2000 \times g$ at 4°C for 20 min to remove any cells and large debris. EVs were harvested from the supernatant by centrifuging at $100,000 \times g$ for 1 hr at 4°C using a Sorvall WX100 + ultracentrifuge (Thermo Fisher Scientific) and resuspended in PBS.

Medium harvested from FiberCell™ bioreactors were processed in the same fashion. Collected EVs were diluted in PBS to allow detection of 1×10^7 to 1×10^9 particles/mL using a Nanosight NS300. Samples were measured using a flow rate of 50 and five 30 s videos were taken and averaged for each reading. Duplicate readings were made for each sample and the same settings were used for all samples.

Negative staining transmission electron microscopy (TEM) of equine MSC EVs was conducted by adsorption onto Formvar-coated copper grids (Electron Microscopy Sciences) for 10 minutes. Excess liquid was removed with filter paper (Whatman) and the copper grid was transferred to 20 µL of 2% filtered uranyl acetate for 2 min. Excess liquid was again removed with filter paper and the grid left to dry under a lamp for 10 min. Grids were visualized on Tecnai G2 Spirit TWIN (FEI, Hillsboro, OR, USA) TEM at 120 kV accelerating voltage. Images were captured using a Morada digital camera (SIS GmbH, Munster, Germany).

Declarations

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Author Contributions

Z.T., W.H., T.D., K.S., M.K., Y.G., and C.H. performed experiments and analyses; Z.T., W.H., C.H., and L.C. wrote the original draft; all authors contributed to the experimental design and contributed to the interpretation and discussion of results. The authors have read and approved the final manuscript.

Competing Interests

The authors have no competing interests to disclose.

Data Availability

The datasets used during this study are available from the corresponding author upon reasonable request.

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Figures

Figure 1

Phase-contrast photomicrograph demonstrating the morphology of the MSC-like cells cultured from equine pre-implantation embryos. Image taken using a 4x non-confocal objective. Scale bar, 500 μm .

B)

		FM p9/p10 [^]	FM p16	SF p8	SF p14/p13 [^]	SF p26	J p7/p8 [^]	J p13	J p18
Positive markers	CD90 (PCR/Flow)	+/+	+/+	+/+	+/+	+/X	+/+	+/+	+/+
	CD105 (PCR)	+	+	+	+	+	+	+	-
	CD73 (PCR)	+	+	+	+	+	+	+	+
	CD44 (PCR/Flow)	+/+	+/+	+/+	+/+	+/X	+/+	+/+	+/+
	MHC-I (Flow)	+	+	+	+	X	+	+	+
Negative markers	CD45 (PCR/Flow)	-/+	-/+	-/-	-/+	-/X	-/+	-/+	-/+
	CD34 (qPCR)	+	-	++	+	-	++	++	+
	CD31 (qPCR)	-	-	+	+	-	-	-	-
	CD14 (Flow)	X	-	+	-	X	-	X	-
	MHC-II (Flow)	-	-	-	-	X	-	-	-

Figure 2

Characterisation of equine MSC-like cell surface marker protein expression. A) RT-PCR detected expression of CD90, CD44, CD73, CD105 and lack of CD45 expression in SF p14. Gel lanes are cropped from original gels for presentation and are representative of the original gel (Figure S3). All markers were run on a 1% agarose gel, except CD45 which was run on a 2% gel. B) Summary table of cell surface marker expression for SF, FM and J cell lines at multiple passages by RT-PCR, flow cytometry and qPCR.

Where multiple methods were used, the expression by RT-PCR is indicated first followed by flow cytometry (Flow). For RT-PCR and flow cytometry experiments: + = marker expressed, - = marked not expressed. For qPCR experiments: + = low gene expression, ++ = moderate gene expression and +++ = high gene expression. ^ = passage used in flow cytometry experiments. *PBMCs were used as positive control. X = not tested.

Figure 3

Tri-lineage differentiation of SF equine MSC-like cells. Representative images of control and differentiated cells following 21 days in differentiation conditions A) Adipogenic differentiation, B) Osteogenic differentiation, and C) Chondrogenic differentiation. Inset images are regions shown at higher magnification. Images were taken using a 20x (A, B) or 10 x (C) objective. Scale bar, 100 μ m.

Figure 4

The A) FiberCell™ and B) CELLline™ AD 1000 flask bioreactor systems. A) The FiberCell™ bioreactor includes multiple hollow fibers enclosed in a plastic shell. Cells grow on the outside of the fibers while medium is pumped through the fibers. The hollow fiber are porous with a molecular weight cut-off of 20

kDa that allows nutrient/waste exchange. B) The CELLLine™ system employs a semipermeable membrane with a molecular weight cut-off of 10 KDa that separates the flask into an upper medium chamber with a capacity of 1 L and a small 15 mL chamber with a fibrous mesh growth surface to facilitate cell adhesion.

Figure 5

Equine MSC proliferation and growth pattern in bioreactors. A) Daily glucose consumption rate of MSC cells. SEM images showing the different morphology and growth pattern of MSCs in the B) CELLLine and C) Fibercell bioreactors. CELLLine™ flask was also stained with Hoechst to reveal the nuclei. The scale bars for B) and C) are indicated on each image.

Figure 6

EVs number and size harvested from cultured equine MSCs. A) Daily EV count from cells grown in a T175 flask or bioreactors. B) NTA histogram showing the size of EVs harvested from MSCs grown in a T175 flask. C) Size of EVs harvested from MSCs grown in bioreactors. D) Negative staining TEM of EVs harvested from the equine MSCs. Scale bar, 500 nm.

Supplementary Files

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