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Improved *ex vivo* expansion of mesenchymal stem cells on solubilized acellular fetal membranes

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Abstract

Coatings produced from extracellular matrixes (ECM) have emerged as promising surfaces for the improved *ex-vivo* expansion of mesenchymal stem cells (MSCs). However, identifying a readily available source of ECM to generate these coatings is currently the bottleneck of this technology. In this study, we assessed if ECM coatings derived from decellularized fetal membranes were a suitable substrate for MSC expansion. We separated and decellularized the two main components of the fetal membranes, the amnion and the chorion. Characterization of the decellularized membranes revealed that each membrane component has a distinct composition, implying that coatings produced from these materials would have unique biological properties. The membranes were processed further to produce solubilized forms of the decellularized amniotic membrane (s-dAM) and decellularized chorionic membrane (s-dCM).On s-dAM coatings *decidual* MSCs (DMSC) were more proliferative than those cultured on tissue culture plastic alone or on Matrigel coatings; were smaller in size (a measure of MSC potency); exhibited greater adipogenic differentiation capacity; and improved osteogenic capacity. Additionally, long term culture studies showed late passage DMSCs (passage 8) cultured on s-dAM showed a decrease in cell diameter over three passages. These data support the use of s-dAM as a substrate for improved MSC expansion.

Keywords

Extracellular matrix (ECM), decellularization, cell-ECM interactions, biomimetic material, stem cell transplantation

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1. Introduction

MSCs have considerable potential in the fields of cell therapies, tissue engineering, and regenerative medicine [1]. According to clinicaltrials.gov, MSCs are employed in more than 700 registered clinical trials worldwide as potential treatments for musculoskeletal diseases, immune system disease, central nervous system disease, skin wounds, etc. However, the clinical application of MSCs is limited by their low prevalence in the human body and inefficient methods for large-scale *ex vivo* production. During the expansion process, MSCs lose many of their desirable properties including their proliferative capacity, differentiation potential, and expression of homing molecules, making it difficult to obtain the large numbers of high quality MSCs needed for therapeutic applications [2]. During *ex vivo* expansion, MSCs experience a vastly different environment compared to their natural environment (i.e. the MSC niche), and these environmental differences are believed to be main drivers for the loss of key MSC properties [3-5].

Developing an expansion platform that enables large numbers of MSCs to be produced while preserving their key properties is a challenge yet to be overcome. Thus, the identification of culture conditions that maintain MSC potency during expansion is an active area of research. Previous studies have attempted to reproduce a better growth environment through optimizing cell culture conditions such as changing the source of serum and adding media supplements [6-8], changing oxygen tension [9-10], introducing three dimensional culture systems [11-12], and/or engineering more favourable substrates [13-15].

In vivo, MSCs interact physically and biochemically with the extracellular matrix (ECM), a network of proteins and proteoglycans in which the cells reside. The ECM is a complex and critical

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component of the MSC microenvironment, which varies in composition and structure among different tissue types and even between different parts of the same tissue. This complexity makes it difficult to generate cell culture substrates that recapitulate the various de novo ECM functions. MSCs interactions with ECM influence various cell behaviours including proliferation, differentiation, migration, and secretion of paracrine factors [16-17].

Outside the body MSCs are often cultured on a monolayer of purified and adsorbed proteins, most notably collagen, laminin, or fibronectin. However, these substrates do not maintain the MSC phenotype in culture for long periods [5]. An alternative method of producing biomaterials that more closely represent the MSC niche was introduced by Chen et al. (2007) [18]. The researchers isolated the ECM deposited by MSCs during *in vitro* culture through a decellularization process, and then cultured MSCs on these ECMs. We recently reviewed this technology, and the relevant studies have demonstrated that MSCs cultured on ECM substrates exhibit significantly improved *ex vivo* expansion including higher proliferation rates, improved multi-lineage differentiation capacity, increased colony forming unit potential, maintenance of cell size (an indicator of MSC potency), and sustained or increased expression of MSC-specific markers [2]. Additionally, we recently showed that stable, hTERT-transformed MSC lines are a reliable and reproducible source for the production of larger quantities of high quality ECM compared to primary MSCs [19]. However, a limitation of this technology remains. Currently, only small quantities of cell-derived ECM that can be produced [2]. In this study we investigate tissue-derived ECM as an alternative to cell-derived ECM.

Many types of tissues from allogenic and xenogenic sources has been decellularized [20-22], and the promise of these ECM-based biomaterials is well documented. Specifically, it has been shown that

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the ECM of different tissues can evoke a regenerative responses from their naturally residing stem/progenitor cells *ex vivo* [20]. Decellularized allogenic tissues are preferable compared to their xenogenic counterparts. For instance, allografts have a higher chances of receiving approval from regulatory organisations such as the Food and Drug Administration (FDA) due to absence of α-gal epitopes in human-derived tissues [23]. However, allogenic tissues are often only available in limited supply. Therefore, finding a readily available human tissue that produces useful ECM-based biomaterials will have a great impact in the translation of this technology into clinics.

In seeking a human tissue source to produce cell growth substrates, our attention was drawn to fetal membranes. The fetal membranes envelops the fetus and amniotic fluid during pregnancies and, along with other birth-associated tissues, are discarded postpartum. Fetal membranes possess several advantages over other tissue types: 1) they are one of the few human tissue sources that are readily available, inexpensive to harvest, and available in large quantities (~15 million m^2/year, globally); 2) the membranes and other birth-associated tissues harbour various MSC populations [24]; 3) they contain tissue from both fetal and maternal origin [25]; 4) the safety and effectiveness of fetal membranes have been demonstrated clinically for other purposes [26-28] and commercially available product such as AmnioGraft® are currently use, and 5) biological advantages including low immunogenicity and anti-microbial properties via endogenous production of antimicrobial molecules during pregnancy [29-31]. In this study, we assess the use of fetal membranes as a source of decellularized ECM, specifically through the lens of improved MSC expansion.

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In this contribution, we test the hypothesis that coatings produced from ECM obtained from fetal membranes could be useful as a substrate in MSC expansion (**Fig. 1**). Specifically, the membranes were split into their chorion and amnion components and decellularized. After decellularization, the membranes were solubilized via exposure to pepsin in order to create two distinct solutions of biomolecules that could be used to coat various surfaces of interest. The biochemical nature of the solutions was characterized by SDS-PAGE and sulphated glycosaminoglycan (sGAG) quantification. Differences in the composition of the materials were observed, and we postulated that these differences would result in coatings that would impact MSCs expansion in distinct ways, a hypothesis that was borne out in these data. Specifically, coatings produced from the solubilized amniotic membrane improved MSC proliferation and differentiation capacity.

2. Materials and Methods

2.1. Placenta collection

Term placentae, after 37 weeks gestation, were collected following caesarean section or normal vaginal delivery from healthy women with uncomplicated pregnancies. Exclusion criteria consisted of the factors known to affect the placenta and fetal membranes, including gestational age below 37 weeks, genetic abnormalities, multiple pregnancies, and body mass index (BMI) above 30. Clinical characteristics of the donors are compiled in **Table S1**. All human tissue collection was approved by the Royal Women's Hospital Research and Ethics Committee, Victoria, Australia (study number 14/35). In all the cases, patients were supplied with an information sheet and signed a separate written consent form. Upon collection of the placenta, fetal membranes (i.e. amnion and chorion) were removed from placenta, and then manually separated from each other.

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2.2. Isolation, characterization, and maintenance of primary DMSCs

Cells were isolated from the *decidua basalis* that remained attached to the maternal side of the placenta as described previously [19, 32]. Briefly, tissue from the maternal side of the placenta, was finely minced and washed 3 times with phosphate buffered saline (PBS). The minced tissues were then digested in 0.25% trypsin and 0.5% DNase I solution (Worthington) in Hank's Balance Salt Solution without calcium or magnesium (HBSS[-]) overnight at 4°C with gentle agitation. The following day, the solution was removed by centrifugation and the processed tissue was further digested in 3 mg/mL collagenase type 1 (Worthington) and 0.05 mg/mL DNase I for 10 min in a 37°C shaking water bath. The digest was strained through a 100 µm sieve and cellular material passing through the sieve was fractionated by gradient centrifugation using Histopaque®. The top fraction, which is a mixture of serum and cells, was collected and spun at 300g to collect the cell pellet. The cell pellet was plated on tissue culture plastic in α-MEM complete medium (Sigma-Aldrich) containing 10% newborn calf serum (NBCS), 1% penicillin/streptomycin and 2 mM L-glutamine. The medium was changed every 3-4 days. All the reagents were from Gibco®, Life Technologies unless otherwise noted.

Passage 2-3 cells were characterized for purity and basic MSC characteristics following recommendations of the International Society for Cell Therapy (ISCT) [33] and those of Parolini et al for placenta derived MSCs [34]. Briefly, fluorescence in situ hybridization (FISH) analysis was used to determine if cells were of maternal origin and free from contamination with fetal cells. To achieve this, cells were prepared from the placenta of a male baby. A droplet of diluted cell suspension (~

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200 cells/droplet) was fixed on a glass slide and hybridized with fluorescent X/Y chromosome probes as described previously [32]. The X chromosome was detected with the fluorescent CEP X Alpha Satellite DNA Probe (Spectrum Green) and the Y chromosome with the CEP Y Alpha Satellite DNA Probe (Spectrum Orange).

Cells were also characterized for their colony forming efficiency, expression of MSC positive markers (CD44, CD73, CD90, CD105, CD146, CD166) and negative markers (CD19, CD45, and HLA-DR) using an LSRII flow cytometer (BD) with FACSDiva software (BD). All of the antibodies were purchased from BD Biosciences. Differentiation potential into adipo- and osteo-lineages was performed as described previously [19]. Cell preparations met all the above criteria and were therefore subsequently called decidual MSCs (DMSCs). DMSCs from at least three patient samples were pooled to minimise patient-to-patient variation.

2.3. Decellularization of fetal membranes

The separated amnion and chorion membranes were thoroughly washed with HBSS[-] to remove excess blood and kept at -80ºC until decellularization. Two decellularization methods were compared, as described below. Decellularization was verified through DAPI staining (Vector Laboratories) and by imaging with a Olympus Provis AX70 fluorescence microscope.

Decellularization Method 1. The membranes were incubated in hypotonic Tris (10 mM) buffer, pH 8 containing 0.1% w/v ethylenediaminetetraacetic acid (EDTA) at 4°C for 16 hours with a gentle agitation. Membranes were then treated with 0.03% sodium dodecyl sulphate (SDS) in Tris buffer (1

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M, pH 7.4) containing 0.1% w/v EDTA at room temperature for 24 hours with agitation. After washing twice with Tris buffer, residual nuclear DNA was removed by incubating with DNase (50 U/mL) in a buffer consisting of 50 mM Tris-HCL, 10 mM $MgCl_2$, 50 µg/mL bovine serum albumin (pH 7.5) for 3 hours 37°C with gentle agitation [35]. The resulting dECM was then washed thoroughly for at least 30 minutes in Tris buffer to remove the detergent.

Decellularization Method 2. The membranes were agitated for 3 days at room temperature in distilled water, and the water was changed three times per day. The membranes were then immersed in a 2% Triton X-100 and 0.1% ammonium hydroxide solution in distilled water for 5 days, followed by washing in distilled water for 3 days at 4ºC [36].

The resulting ECM was sterilized with 0.1% (v/v) peracetic acid in 4% (v/v) ethanol for 3 hours at room temperature, washed thoroughly several time with tris buffer [35], lyophilized for 24-48 hours, snap frozen with liquid nitrogen, and pulverized into a powder. The dry weight of each patient sample was recorded to determine the yield per patient. Powders were stored at -20°C.

2.4. Solubilisation of fetal membranes

The dECM powders were solubilised in 0.5M acetic acid containing a 1:10 mass ratio of pepsin to powder at a final concentration of 10 mg/mL of dECM powders for 48 hours at room temperature with gentle agitation until a milky solution without any visible particles was attained. While keeping the solution on ice, the pH was adjusted to 7.2-7.4 through dropwise addition of 10M NaOH to

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irreversibly deactivate the pepsin. The solubilized dECMs were pooled from at least four patient samples and stored at -20°C [37].

2.5. Biochemical characterization of solubilized fetal membranes

dECM powder (10 μg) was digested with pepsin and loaded onto a 4-12% gradient Bis-Tris Criterion XT Precast Gel (Bio-Rad) along with Precision Plus protein standard. Samples were electrophoresed at 120V for 60-120 min in Bio-Rad XT MOPS running buffer. The gels were then stained with BioSafe Coomassie (Bio-Rad) for at least 2 hours and the excess stain was removed by washing the gels 3 times with deionized water for 5 min each. The bands were visualized using GE Image Scanner III densitometer and analysed with ImageQuant TL software (GE Healthcare).

Sulphated GAG quantification was performed using an absorbance assay. A working solution of dye was prepared by dissolving 16 mg of 1,9-dimethyl-methylene blue (DMMB) in 1 L water containing 3.04 g glycine, 1.6 g NaCl and 95 mL of 0.1 M acetic acid and then filtering through 0.45μm filter. 40μL of each solubilized dECM sample and 250 μL of DMMB solution were pipetted into a plate reader and the absorbance was measured at 525nm using a SpectraMax Plus microplate reader.

2.6. Cell proliferation assay

Various concentrations of solubilized fetal membranes were added to the wells of 96-well plates (150 μL/cm^2 per well) and incubated for least three hours at room temperature to allow biomolecules from the solution phase to absorb onto the surface of the tissue culture plastic (TCP). The wells were rinsed to remove non-adsorbed biomolecules, and DMSCs were then seeded at 6000 cell/cm^2 and

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cultured for 7 days with fresh medium added at day 3. The cell counting kit (CCK-8) proliferation assay was performed according to the manufacturer's protocol. Briefly, 10% CCK-8 reagent in media was added to each well and incubated for 4 hours. The absorbance at 450 nm was measured on a SpectraMax Plus microplate reader. DMSCs were also cultured on TCP or on an absorbed layer of Matrigel growth factor reduced basement membrane matrix (Corning®, Life Technologies) as controls.

2.7. Cell size distribution

DMSCs were cultured on different substrates for 7 days in 6-well plates and trypsinized to prepare a single cell suspension. DMSCs (10^5 per sample in duplicate) were collected and washed twice with HBSS[-] containing 2% new born calf serum. DMSCs were then stained with DAPI and analysed immediately with a BD X-20 Fortessa flow cytometer, which recorded 10^5 events for each sample. As previously described, the light-scattering properties of DMSCs were measured as forward light scattering (FCS), as an indicator of cell size [38].

2.8. Differentiation Assays

2.8.1. Adipogenesis

DMSCs were seeded onto the desired surfaces at the density of 2.1 x 10^4 cells/cm^2 in maintenance medium for 1-3 days until the plates were 100% confluent. The maintenance medium was then replaced by medium with adipogenic supplement (human mesenchymal stem cell functional identification kit, R&D systems) and the medium was changed every 3-4 days. After 14 days, samples were fixed with 4% (wt/v) paraformaldehyde (PFA) for 20 min. Samples were washed with

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PBS twice, followed by adding 60% (v/v) isopropanol for 5 min at room temperature. Finally, DMSCs were stained with 0.18% (wt/v) Oil Red O dye (Sigma) in 60% isopropanol.

2.8.2. Osteogenesis

DMSCs were seeded onto the desired surfaces at a density of 4.2 x 10^3 cells/cm^2 in maintenance media for 1-3 days until the plates were 50-70% confluent. Maintenance media was then replaced by media with osteogenic supplement (human mesenchymal stem cell functional identification kit, R&D systems) and the medium was changed every 2-3 days. After 3 weeks, samples were washed and fixed 4% (wt/v) paraformaldehyde (PFA) for 20 min. Samples were washed with milliQ water twice, and stained with 0.02 mg/mL Alizarin Red (Sigma) solution in milliQ with a pH of 4.1-4.3 using 0.5% ammonium hydroxide.

2.8.3. Quantification

An Olympus DP80 microscope was used to take images of the stained cultures. The area covered by either Oil Red O or Alizarin Red in each image was measured using ImageJ (NIH). The quantification method was adopted from the protocol available online (http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html). Briefly, the images were converted to grayscale and the channel that had the best separation of stains from background was chosen. Finally, the stained area was isolated and measured from non-staining area by adjusting the threshold [5].

2.9. Long-term culture study

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To observe the long-term effect of ECM coatings on DMSCs, we cultured 6000 DMSCs/cm^2 on various substrates from passage 8 to 11 in 6 well plates. Once the cells reach 70% confluence, DMSCs were passaged and re-seeded on a freshly coated plate up to passage 11. The cell diameter was measured by a Countess^{TM} Automated Cell Counter (Thermo Fisher Scientific).

2.11. Statistical analysis

GraphPad Prism software was used for statistical analyse. Statistical difference between two means was determined using a student t-test. For tests where more than two means were compared, an Analysis of Variances (ANOVA) was performed followed by Tukey's post-hoc test. All data are presented as mean ± standard error.

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3. Results

3.1. Isolation and characterisation of DMSCs

Cells were isolated from the maternal side of the placenta. The characteristics of patients from whom the placentae were collected are summarised in **Table S1**. FISH analysis confirmed that the cells collected from pregnancies with male babies possessed two X chromosomes and therefore were of maternal origin and without fetal cell contamination, which attests to their purity. Cells adhered to tissue culture plastic and displayed the characteristic fibroblast morphology, produced colonies when plated at low density, and possessed multilineage differentiation capacity. Cells were positive for the standard MSC markers and negative for the standard non-MSC markers via flow cytometry. These various properties were consistent with cells displaying the MSC phenotype according to the ISCT criteria (**Fig. S1**). Since the cells were also of maternal origin and obtained from the *decidua basalis* that remains attached to the maternal side of the placenta we subsequently referred to them as DMSCs.

3.2. Optimizing the decellularization method

Two fetal membrane decellularization methods were assessed. In Method 1, the fetal membranes were soaked in a hypotonic buffer to rupture the cell membranes through an osmotic pressure difference, the cells were further lysed through exposure to the anionic detergent SDS, and residual DNA was degraded through exposure to DNase [29]. In Method 2, the membranes were soaked in distilled water to rupture the cell membranes through an osmotic pressure difference, the cells were further lysed through exposure to the non-ionic detergent Triton X-100, and the chromosomal DNA was denatured through exposure to ammonium hydroxide [29]. Method 1 resulted in robust DNA

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removal as assessed through DAPI staining, while Method 2 showed considerable DNA contamination compared to the control membrane (**Fig. 2**). Based on these results, the first decellularization method was chosen for the rest of the work.

3.3. Solubilized amnion and chorion membranes have different biochemical characteristics

The yield and biochemical characteristics of the decellularized membranes were assessed for five patient samples (**Fig. 3**). The chorion produced larger quantities of decellularized ECM per patient in comparison to the amnion (~1.1g and 0.4g, respectively; **Fig. 3A**). The SDS-PAGE banding patterns for s-dAM and s-dCM were similar. However, the s-dAM bands had a higher pixel density (**Fig. 3B**). The gels were run with the same starting protein mass (1 μL of 10 μg/mL) of s-dAM and s-dCM. The pixel densities of two of the main bands present in the SDS-PAGE gel, identified by the blue boxes, were quantified and the intensity of the bands was statistically greater for s-dAM (**Fig. 3C**). This indicates that s-dAM produces a more protein rich ECM. As the ECM is largely composed of proteins and proteoglycans, we expected that the chorion-derived ECM would be richer in GAGs. Quantification confirmed that s-dCM was a more sGAG-rich material (**Fig. 3D**).

3.4. s-dAM promotes DMSC proliferation in a dose dependent manner

The potential for dECM coatings to support DMSC growth *in vitro* was assessed in comparison to the traditional growth surfaces of TCP and Matrigel. ECM coatings were prepared through the adsorption of biomolecules from three different concentrations of s-dECMs: 0.01, 0.1, and 0.5 mg/mL [0.5 mg/mL is the highest achievable concentration before the dECMs form gels (data not shown)]. Phase contrast microscopy reveals that DMSCs on all surfaces display an elongated,

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spindle-like morphology (**Fig. 4A**). All of the samples resulted in cell numbers that is at least as high as TCP. Additionally, the number of cells qualitatively appear to be greater on the ECM substrates compared to the TCP. These data were corroborated through the metabolic assay and illustrate that the adherent cell density at day 7 was higher on both s-dCM (~1.4x and ~1.7x, at 0.1mg/mL and 0.5 mg/mL, respectively) and s-dAM (~1.7x and ~2x, at 0.1mg/mL and 0.5 mg/mL, respectively) compared to TCP. Interestingly, the highest concentration of s-dAM also resulted in a higher number of cells compared to Matrigel (~1.3x) (**Fig. 4B**).

3.5. DMSCs grown on s-dAM coatings retained a smaller cell size

Cell size has emerged as a potent biophysical marker of MSC potency. MSCs that retain smaller sizes during culture are reported to have greater proliferative capacity and rate, increased colony forming unit potential, and improved differentiation capacity [39-40]. We assessed the cell size distributions of DMSCs cultured on the various surfaces via flow cytometry, by measuring the forward light scattering area (FSC-A, **Fig. 5**) [38]. Forward scattering is a result of light interacting with particles larger than the wavelength of the incident light, and increases with the particle size [41]. Thus, the extent of forward scattering is an indirect measure of the cell size and was reported as the median value for each cell population. DMSCs cultured on all surfaces had approximately the same forward scattering area (~$1.5x10^5$ FSC-A) except those cultured on coatings produced from the higher concentrations of s-dAM. These cells exhibited a significantly lower forward scattering area (~$1.3x10^5$ FSC-A) compared to all other treatments. Thus, coatings produced from 0.5mg/mL solutions performed the best at improving cell proliferation and maintaining a small cell size. Therefore, only coatings produced from this concentration of coating solutions were used in the remainder of the studies.

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3.6. s-dAM coatings improved adipogenic and osteogenic differentiation

The adipogenic and osteogenic differentiation of DMSCs expanded on varied substrates was assessed. DMSCs cultured on s-dAM coatings showed significantly improved adipogenesis compared to all other surfaces after 14 days in adipogenic medium (**Fig. 6**). Brightfield images show that DMSCs on s-dAM coatings contained a larger number of perinuclear lipid droplets (**Fig. 6A**). Image analysis confirmed this observation. DMSCs cultured on s-dAM coatings exhibited ~3.5, 2.5, and 1.8 fold increases in lipid accumulation compared to TCP, Matrigel, and s-dCM coatings, respectively (**Fig. 6B**).

DMSCs cultured on s-dAM coatings showed significantly improved osteogenesis compared to all other surfaces after 21 days in osteogenic medium (**Fig. 7**). The area covered by calcium deposits as stained by Alizarin Red S were ~ 12% of the total area covered by cells on s-dAM, and ~ 7% on TCP and ~ 5% on s-dCM (**Fig. 7B**).

3.7. s-dAM decreases the cell size at late passages

DMSCs were passaged on TCP for 8 passages and then cultured on ECM coatings for an additional 3 passages to determine if the ECM coatings could improve the potency of late passage DMSCs, as assessed by measurement of cell diameter. After 8 passages on TCP, the DMSCs had an average diameter of 16μm. We observed significantly smaller cell diameter for DMSCs that were subsequently expanded on s-dAM (~11.5 μm) in comparison to DMSCs cultured on TCP (~17μm), s-dCM (~16.5μm), and Matrigel (~14.5 μm) (**Fig. 8**).

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4. Discussion

Mesenchymal stem cell fate is regulated through a combination of intrinsic mechanisms that are governed by the cell's own transcriptional network and extrinsic factors present in the cell's microenvironment such as soluble growth factors, other resident cells, and the surrounding ECM [42]. Both intrinsic and extrinsic factors are critical for the preservation of MSC potency. However, the microenvironmental factors experienced by cells during *ex vivo* expansion are significantly different to those found in their natural microenvironment [42]. These environmental changes are thought to be key contributors to the loss of MSC potency. Therefore, attempting to reproduce this environment *ex vivo* is a rational strategy to preserve MSC potency.

We previously reviewed the long-standing use of fetal membranes as biomaterials, in partially or fully decellularized form, in applications such as skin replacement, ocular pathologies, and many other tissue repair applications [29]. Despite their successful history of clinical use, handling decellularized membranes and preparing a reproducible surface for cell culture is challenging. Therefore, our aim was to generate solubilised forms of the decellularized fetal membranes, which could be used to create readily available, reproducible, and uniform surfaces for MSC growth through a simple adsorption step (**Fig. 1**). We selected a rapid decellularization method that resulted in robust removal of DNA (**Fig. 2**). Furthermore, we solubilised the membranes through enzymatic digestion via pepsin because this enzyme is present naturally in most mammals, and other tissues

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decellularized with pepsin retain ECM bioactivity. Finally, pepsin is irreversibly deactivated through a simple pH adjustment [43].

Tissues vary in the composition of their extracellular matrix, which impacts on the bioactivity of the resulting dECM [42, 44]. Therefore, we investigated coatings produced by simple mechanical separation of the two parts of the fetal membranes, the amnion and the chorion. Since each membrane part has a different composition, we postulated that the resulting coatings would impact on DMSC growth and functions in distinctive ways (**Fig. 3**). The decellularized and solubilized amnion (s-dAM) and chorion (s-dCM) were characterized by the amount of decellularized ECM that each produced, as well as the composition of the solubilized fetal membranes via SDS-PAGE and sGAG quantification. The chorion yielded significantly more dECM in comparison to the amnion. SDS-PAGE gel electrophoresis showed similar banding patterns of proteins in the amnion and the chorion. Two of the main bands present in the gels were at 100-150 kDA and ~250 kDa. Quantification of these bands revealed that s-dAM was a more protein rich material, while sGAG quantification showed s-dCM contained significantly more sGAGs.

Due to the unique compositional properties of each membrane, differences in cell behaviours on these materials were postulated, and subsequently observed. ECM-coated surfaces impacted DMSC proliferation in a dose dependent manner (**Fig. 4**). Specifically, coatings adsorbed from higher concentrations of solubilized ECMs resulted in increased proliferation, with the greatest proliferation occurring on coatings produced from a 0.5 mg/mL solution of s-dAM. These data suggest that at least some of the naive matrix's bioactivity can be retained through the decellularization and

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digestion steps. Additionally, the coating produced from the 0.5 mg/mL solution of s-dAM resulted in increased proliferation compared to Matrigel, the commercially available basement membrane-derived material that is one of the closest mimics of the stem cell niche.

Apart from proliferation, s-dAM coatings were the only substrates able to maintain a smaller DMSC cell size (**Fig. 5** and **8**) and improve both adipogenic and osteogenic differentiation (**Fig. 6** and **7**), which suggests that s-dAM is the most suitable substrate tested for the maintenance of MSC potency across the four assays used.

MSC size has emerged as a powerful biomarker for assessing MSC phenotype, with smaller cells being more potent [5, 45-46], but there is disagreement in the literature. Ng et al (2014) and Prewitz et al (2013) showed that cells expanded on dECM are smaller in size and more potent [5, 45], while Lin et al. (2012) showed that cells growing on dECM coatings are larger than cells expanding on TCP [43]. All the above studies measured cell size by image analysis of micrographs taken of adherent MSCs during culture. It is well established that variables such as substrate stiffness, ligand presentation, ligand density, and cell motility all impact on the apparent size and shape of adherent cells [43, 47-48], but these confounding factors were not taken into account in these previous studies. To minimise these confounding factors, we used two methods of assessing comparative MSC size for suspended cells. We posit that while in suspension, cells are more uniformly spherical in shape, thus this is a more accurate measure of relative cell size. First, the forward light scattering signal during flow cytometry was used (**Fig. 5**). This is an indirect measurement of cell size that is straightforward to collect alongside routine assessment of cell surface marker expression. Second, cell size was

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assessed using a Countess Automated Cell Counter (Thermo Fisher Scientific). Both measurement modalities found that cells expanded on the s-dAM coatings had a smaller size. Additionally these were the cells that also showed the most robust proliferation and differentiation capacity. These results support the idea that cell size can be used as a predictor of MSC potency. However, it must be acknowledged that these smaller cells were also growing on a different substrate from all other cells. Therefore, from these data, it cannot be determined if the cells were inherently more potent or if the underlying substrate was promoting their proliferation and differentiation. However, the authors do believe that suspension-based methods are superior for assessing cell size compared to traditional image analysis of adherent cells.

Most studies regarding the expansion of MSCs on decellularized materials are performed on relatively low passage cells and are only completed over a single passage. However, to illustrate usefulness in large scale expansion of MSCs, these substrates must be able to maintain MSC potency and phenotype over multiple passages to enable the production of the large quantities of high quality cells required for tissue engineering, regenerative medicine, and cell therapy applications [2]. Long term assays are particularly crucial as heterogeneity in MSC culture and loss of potency become increasingly apparent after approximately 5 passages [3]. To assess the impact of these coatings on late passage DMSCs, the stem cells were cultured on TCP until passage 8. At this stage the cells had an average cell diameter of ~16μm. When the stem cells were cultured on s-dAM or Matrigel for an additional three passages the average cell diameter decreased to 12 μm and 14 μm, respectively. Cells at passage 11 that were cultured on the s-dAM had a significantly smaller size compared to all other treatments, including Matrigel, illustrating that these surfaces are the best of those tested at

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reducing the size of the adherent cells. Further studies are required to assess the potency of these MSCs.

Overall, s-dAM coatings were the only substrate of those tested that were able to improve all of the MSC potency tests used in this work (cell size, proliferation, osteogenesis, and adipogenesis). Additionally, these ECM coatings have an additional advantage compared to cell-secreted matrixes in terms of yield, cost, and speed of production. In terms of yield, we have recently illustrated that DMSC23 stem cell lines are capable of producing 738 μg of ECM/cm^2 of cell culture surface area, the production of these matrixes required the use of costly cell culture medium, and required 14 days of culture in order to produce the matrices [19]. In comparison, a single amnion produces an equivalent amount of ECM to that which would be isolated from three 150cm^2 tissue culture flasks [19], the ECM is produced in 2-3 days, and requires less expensive reagents.

In conclusion, solubilized decellularized amniotic membranes (s-dAMs) can be used to create coatings that can maintain or reduce the cell size and promote proliferation, osteogenic, and adipogenic differentiation of DMSCs. Additionally, these ECM materials are produced from readily available human tissues, are relatively unencumbered by ethical constraints, and can be produced through rapid and inexpensive processing. These results support the use of these ECM materials for large-scale MSC expansion.

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Figure 1. Schematic illustrating the production of ECM coatings from the fetal membranes: the membranes are (A) first collected and separated into their maternal (chorion) and fetal (amnion) components, (B) decellularized, dried and pulverised to produce a powder, (C) solubilized through exposure to pepsin, and (D) used to coat culture surfaces. The wells are then rinsed to remove non-adsorbed biomolecules, and MSCs are then seeded onto these surfaces.

Figure 2. Representative image showing the results of the two decellularization methods. (A) Method 1 results in membranes where no DAPI staining is apparent. (B) Method 2 results in membranes with a large amount of residual DNA contamination as seen through comparison to (C) the control surface that did not undergo decellularization. (D) The time line shows that Method 1 is complete within 47 hours while the prolonged washing steps in Method 2 require nearly 300 hours.

Figure 3. Characterization of amnion- and chorion-derived ECMs: (A) The average yield of decellularized ECM from the amnion (s-dAM) and chorion (s-dCM) from each patient sample, (B) SDS-PAGE analysis of s-dAM and s-dCM, (C) Quantification of the two main bands present in the SDS-PAGE gel (Student's t-test, one-tailed, $^{**}p<0.01$), and (D) sGAG content present in s-dAM and s-dCM (Student's t-test, one-tailed, $^{*}p<0.05$).

Figure 4. Morphology and proliferation of primary DMSCs on ECM coatings after 7 days of proliferation: (A) Representative phase contrast images of DMSCs on ECM coatings produced from a 0.5mg/mL solution of s-dECM, and (B) quantification of cell numbers on the various surfaces. Statistical differences were determined through a one-way-ANOVA with Tukey's post-test (* $P < 0.05$, ** $P < 0.01$, $^{***}P < 0.001$ compared to TCP; $^{\#\#\#}$ $P < 0.001$ compared to Matrigel. Scale bar is 200 μm).

Figure 5. Cell size of DMSCs on various substrates: Cell size as indirectly assessed through FSC-Area via flow cytometry after a week of expansion on different substrates (One way ANOVA with Tukey's post-test (* $P < 0.05$, compared to TCP; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, compared to Matrigel).

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Figure 6. Adipogenic capacity of DMCS: (A) Representative images of Oil Red O stained DMSCs after 14 days of adipogenic induction (scale bar is 50μm), (B) quantification of staining through image analysis (One-way ANOVA with Tukey's post-test, * $P < 0.05$, ** $P < 0.01$).

Figure 7. Osteogenic capacity of DMSCs: (A) Representative images of Alizarin Red S stained DMSCs on various substrates (scale bar is 500 μm), and (B) quantification of staining through image analysis (One-way ANOVA with Tukey's post-test, * $P < 0.05$, ** $P < 0.01$).

Figure 8. Long-term study up to passage 11: Diameter of DMSCs after 8 passages on TCP (black column and red dashed line) and then cultured on ECM coatings until passage 11. No significant change in the cell size was observed at passage 9. However, at passage 11, cells cultured on s-dAM and Matrigel had significantly smaller sizes compared to passage 8 cells (One-way ANOVA with Tukey's post-test, $^{\#} P < 0.5$, $^{\#\#\#} P < 0.001$). Statistical differences in size between cells at the same passage number on different substrates are denoted with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

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