<table>
<thead>
<tr>
<th>Title</th>
<th>Enhanced ex vivo expansion of adult mesenchymal stem cells by fetal mesenchymal stem cell ECM (Main article)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Ng, Chee Ping; Mohamed Sharif, Abdul Rahim; Heath, Daniel E.; Chow, John W.; Zhang, Claire B. Y.; Chan-Park, Mary B.; Hammond, Paula T.; Chan, Jerry Kok Yen; Griffith, Linda G.</td>
</tr>
<tr>
<td>Date</td>
<td>2014</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10220/19361">http://hdl.handle.net/10220/19361</a></td>
</tr>
<tr>
<td>Rights</td>
<td>© 2014 The Author(s). This is the author created version of a work that has been peer reviewed and accepted for publication by Biomaterials, Elsevier Ltd. It incorporates referee’s comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [DOI:<a href="http://dx.doi.org/10.1016/j.biomaterials.2014.01.081">http://dx.doi.org/10.1016/j.biomaterials.2014.01.081</a>] .</td>
</tr>
</tbody>
</table>
Enhanced Ex Vivo Expansion of Adult Mesenchymal Stem Cells by Fetal Mesenchymal Stem Cell ECM

Chee Ping Ng a,*, Abdul Rahim Mohamed Sharif a, Daniel E Heath a, John W Chow a, Claire BY Zhang a, Mary B Chan-Park b, Paula T Hammond a,c, Jerry KY Chan d,e,f, Linda G Griffith a,g,h.

a BioSystems and Micromechanics Interdisciplinary Group, Singapore-MIT Alliance for Research and Technology, 1 CREATE Way, Singapore 138602
b School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459
c Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139
d Experimental Fetal Medicine Group, Department of Obstetrics & Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore and National University Hospital Systems, Singapore, 5 Lower Kent Ridge Rd, Singapore 119074
e Department of Reproductive Medicine, KK Women's and Children's Hospital, 100 Bukit Timah Road, Singapore 229899
f Cancer and Stem Cell Biology, Duke-NUS Graduate Medical School, 8 College Road, Singapore 169857
g Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139
h Department of Mechanical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

* Address all correspondences to
Chee Ping Ng
BioSystems and Micromechanics
Singapore-MIT Alliance for Research and Technology
1 CREATE Way
Singapore 138602
Email: ng.chee.ping@smart.mit.edu
Phone: SG(+65) 6601 1380
Fax: SG(+65) 6684 2118
Abstract
Large-scale expansion of highly functional adult human mesenchymal stem cells (aMSCs) remains technologically challenging as aMSCs lose self renewal capacity and multipotency during traditional long-term culture and their quality/quantity declines with donor age and disease. Identification of culture conditions enabling prolonged expansion and rejuvenation would have dramatic impact in regenerative medicine. aMSC-derived decellularized extracellular matrix (ECM) has been shown to provide such microenvironment which promotes MSC self-renewal and "stemness". Since previous studies have demonstrated superior proliferation and osteogenic potential of human fetal MSCs (fMSCs), we hypothesize that their ECM may promote expansion of clinically relevant aMSCs. We demonstrated that aMSCs were more proliferative (~1.6x) on fMSC-derived ECM than aMSC-derived ECMs and traditional tissue culture wares (TCPS). These aMSCs were smaller and more uniform in size (median ± interquartile range: 15.5±4.1µm versus 17.2±5.0µm and 15.5±4.1µm for aMSC ECM and TCPS respectively), exhibited the necessary biomarker signatures, and stained positive for osteogenic, adipogenic and chondrogenic expressions; indications that they maintained multipotency during culture. Furthermore, fMSC ECM improved the proliferation (~2.2x), size (19.6±11.9µm vs 30.2±14.5µm) and differentiation potential in late-passaged aMSCs compared to TCPS. In conclusion, we have established fMSC ECM as a promising cell culture platform for \textit{ex vivo} expansion of aMSCs.

Keywords: Bone Marrow, Bioactivity, Biomimetic Material, Cell Proliferation, ECM (extracellular matrix), Mesenchymal Stem Cells
1. Introduction

Human mesenchymal stem cells (MSCs) are bone marrow-derived multipotent cells which are involved in tissue regeneration throughout a person's life. Due to their self renewal capacity, differentiation and therapeutic potential these cells are also of great clinical interest. Currently researchers are exploring the utility of these cells for the treatment of many acute, chronic, and degenerative diseases including Crohn's disease, multiple sclerosis, graft-versus-host disease, type 1 diabetes, bone fractures, cartilage defects, etc [1-3]. However, a major bottleneck in clinical use of adult mesenchymal stem cells (aMSCs) is their low prevalence which requires prolonged ex vivo expansion to achieve a useful number of cells; expansion which results in the cells' loss of many of their useful stem cell properties.

The standard method of isolating and culturing aMSCs is through plastic adherence of mononuclear cells from bone marrow (BM) aspirate. When cultured under appropriate conditions the aMSCs adhere and proliferate while contaminating cell types perish resulting in a relatively pure population of progenitors after 2-3 passages. However, the percentage of progenitors collected from BM aspirate is small. Using colony forming unit assays (CFUs), the prevalence of aMSCs in BM aspirates has been measured to be between 0.001 and 0.01 % of the nucleated cells within the sample[4-6]. This means these cells must undergo prolonged expansion in order to obtain clinically relevant cell numbers. For example, a dose of 1-2 millions cells per kg of body weight or more (about 70-140 million cells for average human of weight 70kg) is required for intravenous infusion and treatment of a wide variety of conditions such as graft-versus-host disease (GVHD), bone and cartilage defects, myocardial infarction and autoimmune diseases [2, 7].

Currently large-scale expansion of highly functional aMSCs is an unaddressed challenge as MSCs undergo drastic phenotypic changes during prolonged culture on tissue culture polystyrene (TCPS). Specifically, the proliferation capacity of the cells decreases, the self renewal capacity of the cells diminishes, and the cells often begin to express behavior of cells with a committed lineage or reach senescence. Furthermore, MSC quantity/quality declines with donor age and disease, thus it is indeed challenging to expand sufficient numbers for treatments in older donors who are typically the patients in need of such therapy. These limitations clearly illustrate the need to develop cell culture conditions which enable prolonged expansion of the stem cells without loss of their stem cell characteristics. One type of cell culture substrate which has recently shows promise in maintaining aMSC phenotype during ex vivo expansion is decellularized extracellular matrix (ECM). During ex vivo culture cells produce their own ECM. Through appropriate processing the matrix can be decellularized, meaning the cells and cellular debris are removed leaving behind only the extracellular components. Previously, Lin et al. showed that decellularized aMSC ECM improved the proliferation, attachment, spread, migration, and multi-lineage differentiation capacity of aMSCs in comparison to cells grown in collagen type I [8]. Lai and coworkers demonstrated the expansion of human MSCs on their ECM strongly promoted cell proliferation, maintained stem cell properties, and resulted in low levels of reactive oxygen species being produced [9]. In contrast, bone formation capacity of cells
expanded on tissue culture plastic was dramatically diminished after 6–7 passages. Also, Sun et al. has shown that culturing freshly isolated human bone marrow mononuclear cells on stromal cell-derived extracellular matrix enhances the formation of colonies comprised of either osteoblast-like, fibroblast-like, or adipocyte-like cells [10]. In addition, they also showed that culturing late-passage MSCs on fresh ECM recovered or at least retained the desired properties in these older cells. These recent studies clearly illustrated the potent ability of decellularized ECM to maintain, and in some cases recover desirable aMSC behavior. The cellular mechanisms which result in loss of aMSC "stemness" during prolonged expansion remain unclear; however, these previous works illustrate that ECM may be an important factor. One could envision that over multiple passages compositional changes in ECM may occur which results in an extracellular environment lacking certain cues necessary for the maintenance of aMSC phenotype, and by culturing the stem cells on a more biologically complete matrix the desired cell behaviors can be maintained/recovered. Plating of cells on ECM isolates such as collagen or laminin or synthetic substrates would likely lack the appropriate cues. Thus, MSC ECM may be a more biologically complete substrate and aid in the maintenance of the desired aMSC phenotype.

In this report, we investigate the use of fMSC to generate ECM-based substrates for ex vivo expansion of aMSCs. There are several potential reasons why fMSC ECM may offer a superior cell growth substrate compared to aMSC ECM and other tissue-derived MSC ECMs. In previous work, Zhang and co-workers have illustrated higher proliferative and osteogenic capacity of fMSC compared to aMSCs, human umbilical cord MSCs, and human adult adipose tissue MSCs [11]. The higher proliferation capacity of the cells may indicate greater amounts of ECM production meaning more ECM is produced making these cells a more productive source of ECM. Also, since the quality and quantity of MSCs has been documented to decline with age, less ECM will be collected from cell isolated from an adult bone marrow sample and the ECM which is produced will likely be of less consistent quality. The most primitive and proliferative BM MSCs are from fetal bone marrow in the constant state of tissue development indicating that they may be the best source of ECM for the maintenance of aMSC phenotype. Furthermore, the process of decellularization destroys the cells that are used to produce them; this implies loss of a substantial amount of the expanded aMSCs to produce the matrix; cells which could instead be used for therapeutics. Here, we perform 2 studies to evaluate the utility of fMSC ECM as a cell culture substrate for the expansion of highly functional aMSCs. First, we assess the (1) proliferation, (2) cell size distribution (as indicators of “stemness” [12, 13]), (3) immunophenotypic expressions and (4) differentiation capacities of expanded aMSCs seeded on fMSC ECMs in comparison to substrates such as standard TCPS and ECMs regenerated from aMSCs and human neonatal dermal fibroblasts (nHDF). In the second study, we explore the effects of fMSC ECMs on late-passaged aMSCs versus TCPS in terms of the 4 parameters as indicated earlier for the first study. We also quantify the amount of ECM generated from the various cell types to understand their scalability potentials for large-scale therapeutic applications.
2. Materials and Methods

2.1 Cell culture and maintenance of fMSC, nHDF and aMSC

Human fetal mesenchymal stem cells (fMSC) were isolated from fetal femurs after clinically indicated termination of pregnancy as previously described [11]. All human tissue collection for research purposes was approved by the Domain Specific Review Board of National University Hospital Singapore, in compliance with international guidelines regarding the use of fetal tissue for research. In all cases, patients gave separate written consent for the use of the collected tissue. Briefly, single-cell suspensions were prepared by flushing the BM cells out of femurs using a 22-gauge needle, passing through a 70-µm cell strainer (BD Biosciences, San Diego, CA), and plating on NuclonTM Delta T75 culture flasks (Thermo Scientific Nunc, Rochester, NY) at $10^6$ cells/ml. Adherent spindle-shaped cells were recovered from the primary culture after 4–7 days. Non-adherent cells were removed with initial medium changes every 2–3 days. fMSCs were isolated and cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) for fMSC supplemented with 10% MSC qualified fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA), hereafter referred to as D10 medium.

Passage 2 human adult mesenchymal stem cells (aMSCs) were obtained from Lonza (Basel, Switzerland) and cultured according to manufacturers’ instructions. aMSC were cultured in low glucose Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% MSC qualified FBS and 1% penicillin/streptomycin (Life Technologies), hereafter referred to as DLG10 medium.

Passage 1 human neonatal dermal fibroblasts (nHDF, ATCC® PCS-201-010™) were obtained from ATCC (Manassas, VA) and expanded according to manufacturers’ instructions. Neonatal dermal fibroblasts were cultured and expanded to passage 3 in Fibroblast Basal Medium (ATCC® PCS-201-030™) supplemented with Fibroblast Growth Kit-Low serum (ATCC® PCS-201-041™) and 1% penicillin/streptomycin (Life Technologies).

Cells were plated in Nuclon™ Delta T-75 flasks at 500 cells/cm², incubated at 37°C and 5% CO2, and medium was changed every 2-3 days. Passage 2 cultures were expanded up to a maximum of 70% confluence, detached with 1X TrypLE Express (Life Technologies, Carlsbad, CA) unless otherwise stated and cryopreserved in appropriate growth medium with 10% DMSO (Sigma Aldrich, St Louis, MO) and 30% MSC qualified FBS to create a stock of cells. For cell experiments, cells from the stock were thawed and cultured according to the above protocol. Passages 4 were used in cell experiments to screen and characterize the various substrates. D10 medium as described previously was used in all experiments.

2.2 Preparation of decellularized ECM, fibronectin-coated substrates and conditioned medium
Decellularized ECM substrates were prepared with modifications from a previous protocol [14] by plating cells in 6-well plates at 1000 cells/cm² and cultured for 14 days with fresh media replacement every 2-3 days (Fig 1a). A final concentration of 50µM ascorbic acid (Sigma Aldrich, St Louis, MO) was added to the media for the last 7-8 days of culture to increase the production of ECM. Passage 3 cells were used to produce the matrices (Fig 1b) unless otherwise stated. The cells were rinsed with PBS and treated with PBS containing 0.5% Triton X-100 (Sigma Aldrich, St Louis, MO) and 20mM NH₄OH (Sigma Aldrich, St Louis, MO) for 5 min at 37 °C. ECM remaining in the wells were rinsed with PBS and treated with DNAse (Sigma, ≥100 units/mL PBS) for 1 h at 37 ºC. Following additional PBS rinsing, plates were allowed to dry within a sterile biosafety cabinet. ECM-containing plates were stored at 4°C in the dark in a closed sterile container or sterile sealable bag until use within 1 month.

Fibronectin-coated substrates were prepared according to Marcantonio et. al with slight modifications [5]. Briefly, fibronectin (Sigma, St. Louis, MO) was adsorbed to tissue culture substrates by incubating substrates with a 10 μg/mL solution in PBS for 2 h at room temperature, rinsing 3x with PBS, then blocking for 1 h at room temperature with 1% bovine serum albumin (Sigma, St. Louis, MO), and rinsing 3x again with PBS. Fibronectin coated culture wares were stored for 2-4 weeks at 2-8°C in a closed sterile container or sterile sealable bag.

To generate conditioned media, fMSCs were plated at a density of 1000 cells/cm² with D10 medium. On the following day, the medium was replaced with DLG10 medium for conditioning. The conditioned DLG10 medium was collected every 24 hours until the cells reached confluence, sterile filtered with a 0.22μm syringe filter and stored at -20°C until use.

2.3 Cell Proliferation study
aMSC were inoculated at 100 cells/cm² on the decellularized fMSC ECM and cultured for 10 days with fresh media change every 3 days. Cells were detached using TrypLE Express or Accutase (Sigma Aldrich, St Louis, MO), samples counterstained with trypan blue (0.4%) (Sigma Aldrich, St Louis, MO) and counted using a hemocytometer. Images were also acquired for cell size distribution and analyzed using ImageJ image processing software from NIH, Bethesda, MD.

To perform cell size distribution analysis, cells monodispersed by trypsinization was examined by cellular image analysis using ImageJ. The major and minor axes of the cells were measured, averaged and presented as the cell sizes. Their normalized (relative) frequency distributions, normalized against the total number of cells counted per condition, were plotted against the average cell size (determined by averaging the major and minor axes) in 3 μm bins. For quantification of the cell size distributions, the median, 25% percentile, 75% percentile and the interquartile range (IQR, a measure of statistical dispersion equal to the difference between the upper 75% and lower 25% quartiles) of the normalized frequency distributions were determined using GraphPad Prism 5 software (La Jolla, CA).
Cumulative population doubling level at each subcultivation was calculated from the cell count by using the equation [15]:

\[
\frac{N_{H}}{N_{I}} = 2^{X},
\]

or

\[
X = \frac{\log_{10}(N_{H}) - \log_{10}(N_{I})}{\log_{10}(2)},
\]

where \(N_{I}\) = inoculum number, \(N_{H}\) = cell harvest number, and \(X\) = population doublings. The population doubling increase that was calculated was then added to the previous population doubling level (PDL), to yield the cumulative population doubling level.

2.4 Plating Efficiency Study

MSCs were harvested by trypsinization and washed twice with a serum-free medium, and \(5 \times 10^{4}\) aMSCs were inoculated per well of 2-well LabTek chambered glass well slides (Thermo Scientific Nunc, Rochester, NY) prepared with the ECM substrates. For TCPS comparison, cells were seeded on 12 wells Nunc Nuclon™ Delta plates, which had an equivalent surface area (4cm²) to the well slides. After incubation for 2 hours, the wells were rinsed twice to remove unattached cells. The attached cells were fixed using 4% paraformaldehyde (PFA) (Sigma Aldrich, St Louis, MO) for 15 min and stained with DAPI (5µg/ml, Life Technologies, Carlsbad, CA) for 20min. Mosaic images of the entire wells were acquired by epi-fluorescence microscopy using a customized fast-rastering Leica DMI 6000B microscope (ImageIQ, Cleveland, OH). Cells were counted using the "analyze particles" plugin in ImageJ image processing software (NIH, Bethesda, MD). Plating efficiencies (PE) measured how efficient the cells adhered to the substrates and were calculated as a fraction:

\[
PE = \frac{N_{A}}{N_{P}}
\]

where \(N_{A}\) = adhered cell number and \(N_{P}\) = plated cell number. A PE of 1 implied that all the plated cells adhered while on the other end of the spectrum, a PE of 0 indicated no adhesion.

2.5 Immunophenotyping

To minimize the variations, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed minimal criteria to define hMSC populations. These include: (i) hMSCs must be plastic-adherent when maintained in classical culture conditions; (ii) hMSCs must express high levels (≥95% positive) of CD105, CD73, and CD90 and lack expression. (≤2% positive) of CD45, CD34, CD14, or CD11b, CD79α or CD19, and HLA-DR (unless stimulated by interferon-γ) surface molecules; (iii) hMSCs must differentiate into osteoblasts, adipocytes, and chondroblasts under specific in vitro differentiation conditions [2, 16].

The various biomarkers and negative control isotypes used for screening were purchased from BD Biosciences (San Jose, CA) and listed in Supplementary Table S1. Some of the markers were not
tested on the later passaged cells due to their limited yield. CD14 or 11b expression was not also
tested. Briefly, cells were rinsed with PBS, detached with accutase and incubated at 37°C in
incubator for 15min to detach cells. Cells were washed, samples counterstained with trypan blue
(0.4%) (Sigma Aldrich, St Louis, MO) and counted using a hemocytometer. The rest of the cells were
divided appropriately into tubes containing the antibodies, mixed well and incubated for 1h in the dark.
The labeled cells were washed with PBS before fixation in 1% PFA, and screened using an Accuri C6
flow cytometer (BD Biosciences, San Jose, CA) within 1 week.

2.6 Differentiation Assays

Osteogenesis induction was performed with StemPro(R) Osteogenesis Differentiation Kit (Gibco, Life
Technologies, Carlsbad, CA) according to manufacturer’s instructions. Briefly, aMSCs were plated on
various substrates at 5000 cells/cm² and cultured in DLG10 medium for 48h before changing to
differentiation media. The cells were subsequently cultured for 13-14 more days with media change
every 3 days before detection for differentiation. Osteoblast differentiation was detected by alkaline
phosphatase (AP) expression using SIGMAFAST™ BCIP/NBT substrate (Sigma Aldrich, St Louis,
MO). AP activity is an indication of successful differentiation of MSC into osteoblasts. Undifferentiated
MSCs show weak alkaline phosphatase (AP) activity, whereas differentiated osteoblasts feature very high AP and stain blue violet using BCIP/NBT as a substrate. Cell were
washed with PBS and fixed with pre-cooled methanol for 5min at -20°C. This was followed by rinsing
with mQ-H₂O before incubation with dissolved SIGMAFAST™ BCIP®/NBT substrate for 10min under
slow agitation on a plate shaker at room temperature. The stain solution was removed and the cells
were washed with mQ-H₂O and imaged immediately while kept moistened.

Adipogenesis induction was performed with StemPro(R) Adipogenesis Differentiation Kit (Gibco, Life
Technologies, Carlsbad, CA) according to manufacturer’s instructions. Briefly, aMSCs were plated on
various substrates at 5000 cells/cm² and cultured in DLG10 medium for 48h before changing to
differentiation media. The cells were subsequently cultured for 13-14 more days with media change
every 3 days before detection for differentiation. Adipocytes were detected by their large numbers of
intracellular lipid vesicles using Oil Red O staining (Sigma Aldrich, St Louis, MO) and identified by
their bright red color. Cells were washed with PBS and fixed with neutral buffered formalin (10%) for
30min at room temperature. This was followed by rinsing with mQ-H₂O before incubation with 60%
isopropanol to cover the cell monolayer for 5 min at room temperature. The isopropanol was replaced
with 0.3% Oil Red O in isopropanol and incubated for 20min under slow agitation on a plate shaker at
room temperature. The cells were washed with mQ-H₂O and imaged immediately while kept
moistened.

Chondrogenic differentiation was done using StemPro(R) Chondrogenesis Differentiation Kit (Gibco,
Life Technologies, Carlsbad, CA) according to manufacturer’s instructions. Briefly, aMSCs were
plated in non-adherent u-bottom 96-well plates at 100000 cells/well to generate spheroids and
cultured in DLG10 medium for 48h before a change to differentiation media. The cells were
subsequently cultured for 13-14 more days with media change every 3 days before detection for differentiation. Chondrogenic differentiation of MSC in 3D spheroid culture results in formation of cartilage with proteoglycan aggrecan. Aggrecan was detected with Alcian Blue (Sigma Aldrich, St Louis, MO), a dark-blue copper-containing dye. Spheroids were rinsed with PBS before fixation with 10% neutral buffered formalin for 60 min at room temperature. Following thorough rinsing with PBS to remove the fixatives, the spheroids were stained in 1% Alcian Blue solution in 0.1N HCl (prepared from Alcian Blue-8GX, Sigma Aldrich, St Louis, MO) and agitated slowly on a plate shaker for 30 min at room temperature. The spheroids were washed thoroughly with 0.1N HCl to remove the Alcian Blue stain and imaged immediately while kept moistened.

2.7 Image Quantification of Differentiation Expression Levels
The expression level in each image was defined by the intensity density of the stain in the image, which is the product of area and mean intensity of the stains in the images. This was determined using ImageJ by adopting the methods from their online example provided to quantify stained liver tissues (http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html). Briefly, there were five steps to obtaining the quantification:

1. Converting the color images to grayscale images of individual red, green and blue channels,
2. Determining the channel with the best separation (BCIP NBT and Oil Red O - green channel; Alcian Blue - red channel)
3. Isolating the stained areas using thresholding and converting the thresholded portion of the image into a selection mask using the 'create selection' function
4. Transferring the selection mask back to the original color image
5. Measuring the intensity density of the selection in the image using the 'measure' function

The ImageJ macro for the BCIP NBT expression quantification is provided in the supplementary materials as an example (Supplementary Fig S1).

2.8 Late Passage Study
In the late passage studies to assess if fMSC ECM substrates could rejuvenate aged aMSCs (Schematic in Fig 5a), Passage 6 cells previously cultured on TCPS were seeded on fMSC ECM substrates at 100/cm² and cultured in similar conditions for 3 passages as stated above. They are then assessed for cell proliferation, cell size, differentiation capabilities and MSC biomarker expression as described in the earlier sub-sections.

2.9 Quantification of ECM yield per cell plated
Decellularized ECM substrates were prepared as described in Section 2.2. The substrates were mechanically detached in deionized water using cell scrapers (SPL Life Sciences, South Korea) and weighing them in 1.5ml eppendorf tubes (pre-weighed and post-dried after drying in a 70°C oven overnight). The weight of the ECM was normalized to the number of cells plated instead of the number of cells at the time of decellularization since it was not possible to determine the latter number.
due to the destructive nature of the decellularization protocol. In addition, from a scalability perspective, it might be more of interest to determine how much ECM could be generated from the number of cells plated instead.

2.10 Statistical Analysis
All data are presented as mean ± standard deviation calculated, with n = 3 replicates or otherwise stated, depending on the experiments. Using GraphPad Prism 5 software, multiple means were compared with either an unpaired t-test or an analysis of variance (ANOVA) test to determine if the means were statistically different. If ANOVA indicated statistical difference, then the Tukey-Kramer Honestly Significant Difference Test was used to determine which of the pairs of means were statistically different. Pairs of means are marked with either (ns) meaning the means or not statistically different or asterisks (*) if the means are significantly different. The number of asterisks indicates the P-value: p ≤ 0.5 for *, p ≤ 0.01 for **, and p ≤ 0.001 for ***.

3. Results
3.1 Effect of proliferation of aMSCs on various substrates:
We first started by screening various substrates and conditions, specifically, conditioned medium using expanded passage 4 aMSCs (Fig 1a). Passage 3 cells were used to prepare the decellularized matrices unless otherwise stated. They were successfully decellularized as visualized by light microscopy (Fig 1b). The reason for the choice of passage 3 was to maximize the number of cells while ensuring the cells still maintain their undifferentiated phenotypes. Furthermore, early passage BM cultures contained contaminated non-MSCs of mostly haematopoietic nature that usually cleared up within the 2 passages. It had also been shown by Binato and co-workers that MSCs retain their chromosome stability up to passage 4 [7]. Morphologically, aMSCs cultured on fMSC ECM retained spindle-shaped morphology (Fig 1c) but qualitatively more in numbers. They also appeared more aligned, probably due to the orientation of the fMSC ECM. aMSCs cultured on fMSC ECM resulted in significantly higher growth than on TCPS as indicated by the cell yield at day 10 (Fig 1d). On average, the cell number at the end of day 10 culture on fMSC ECM is about 1.6x more than their TCPS counterpart (n=3, p≤0.01). In addition, the passage/age of the cells used for generating the matrices appear to affect the quality of the ECM as P4 fMSC ECM were less effective compared to P3 fMSC ECM. Growth on P4 ECM was also higher (about 1.3x) than that of TCPS (n=3, p>0.05) but lower than culture on P3 ECMs. aMSCs cultured on fMSC ECMs were also more proliferative (about 1.8x) than their counterparts on aMSC ECMs. Interestingly here, we did not observed any significant difference (p > 0.05) between aMSC yield on aMSC ECMs in comparison to TCPS.

From the cell size distribution data (Fig 1e and Table 1) in terms of median and interquartile range (IQR), we observed that aMSCs cultured on P3 fMSC ECM (median ± IQR: 15.5±4.1µm) have slightly smaller and more uniform sizes than TCPS (17.5±4.1µm) and others (e.g. aMSC ECM: 17.2±5.0µm; P4 fMSC ECM: 18.5±5.3µm; Fibronectin: 22.4±11.5µm). It has been demonstrated that smaller MSCs share similar characteristics as early passage multi-potent MSCs and may be more "stemness"
Thus the result here may suggest that culturing aMSCs on the fMSC ECMs may help the adult cells to retain their "stemness" characteristics.

In addition in a separate screening, we investigated the ECM of neonatal fibroblasts (nHDF) for the expansion of aMSCs in comparison to TCPS (Supplementary Fig S2). Fibroblast ECMs are of interest since they are one of the substrates used for expanding stem cells. While aMSCs proliferated more (about 1.7x fold) on nHDF ECM compared to TCPS (Supplementary Fig S2a), the cell size distribution for the aMSCs cultured on both substrates are similar however (Supplementary Fig S2b). Furthermore, we tested some of the commercially available xenogenic-free substrates (Supplementary Fig S3). However, the proliferation and size of the aMSCs on these substrates were not significantly improved and in some conditions, performed worse than our control TCPS substrates. Since the fibronectin coating, conditioned media conditions and xenogenic-free substrates did not improve or fared significantly worse than TCPS in terms of cell yield (Fig 1d, n=3, p<0.001 and p<0.05 for fibronectin and conditioned media respectively and Supplementary Fig S2 for xenogenic-free substrates) and size (Table 1 and Supplementary Fig S2), we did not include them in subsequent evaluations, which we focused on the ECM substrates.

3.2 Plating Efficiency
All the ECM substrates (aMSC, fMSC and nHDF) were very effective in capturing the aMSCs within 2 hours (Fig 2), with plating efficiencies around 1 (Mean±S.D: 0.94±0.09, 1.07±0.08, 1.03±0.06 for aMSC, fMSC and nHDF respectively, n=4 for all). In contrast, the performance of glass substrates were significantly poor (0.27 ± 0.06, n=6) while TCPS were efficient (0.60 ± 0.06, n=4) but still significantly less than the ECM substrates (p<0.001).

3.3 Flow Cytometry Analysis
hMSCs must express high levels of CD73, CD90, CD105 and lack expression of CD45, CD34, CD79a and HLA-DR, as defined by the Mesenchymal and Tissue Stem Cell Committee of the ISCT [16]. Our results in Fig 3 shows that similar to their counterparts cultured on TCPS, aMSCs retain their immunophenotype when cultured on fMSC ECM.

3.3 Differentiation Assay
We have also demonstrated through in vitro assays that aMSCs plated on fMSC ECM are multipotent as they are able to differentiate into osteogenic, adipogenic and chondrogenic lineages based on positive alkaline phosphatase (AP) (purple stain in Fig 4a), oil red o (red stain in Fig 4c) and alcian blue (blue stain in Fig 4e). The expression levels of the various stains in the images were quantified and presented as intensity densities (Fig 4b, d and f). For osteogenic differentiation, the AP expression levels of aMSCs differentiated on aMSC ECM and fMSC ECMs were significantly higher (p ≤ 0.01, ~1.6x and ~1.7x respectively, n=19 and 15 respectively) than that of their controls on TCPS (n=15) (Fig 4b). Interestingly, the AP levels for aMSC on nHDF ECMs were significantly poor compared to the other conditions (p ≤ 0.001, ~9.3x, 9.5x and 5.7x lower to aMSC ECM, fMSC ECM
and TCPS respectively, n=15). For adipogenic differentiation (Fig 4d), only the Oil Red O expression levels of the cells differentiated on fMSC ECM were significantly higher to their TCPS counterparts (p ≤ 0.05, ~9.3x, n=15 for both). The average expression levels of aMSCs on the other ECMs were higher (~3.0x and ~2.4x for aMSC ECM and nHDF ECM respectively, n=15 for all) but not significantly different to that of TCPS (p > 0.05), suggesting that adipogenic differentiation potential of aMSCs were better retained when cultured on ECM substrates. Furthermore, the average Alcian blue expression levels for all ECM conditions were similar and higher than the TCPS (~1.4x, ~1.4x and ~1.3x for aMSC ECM, fMSC ECM and nHDF ECM respectively, n=15, 21, 20 and 20 for aMSC ECM, fMSC ECM, nHDF ECM and TCPS respectively) (Fig. 4f). The quantitative results here agreed with our qualitative observations that multi-potency of the aMSCs are better retained on fMSC ECM in contrast to their counterparts on TCPS and aMSC ECM. They also suggested that the fMSC ECM might be a superior, if not comparable, platform to TCPS for the culture of aMSCs.

3.4 Effect of plating late passaged TCPS-cultured cells on fMSC ECM
To elucidate if late-passaged cells can be "rejuvenated" by fMSC-derived ECM, we took aMSCs that had been cultured on TCPS for 6 passages and plated them on fMSC ECM. After 3 passages, we assessed their cell yield, cell size and osteogenic potential in comparison to their TCPS counterpart (Fig 5a). We observed significantly higher cell numbers (about 2.2-fold, p ≤ 0.01, n=3) compared to TCPS controls (Fig 5b). In addition, culturing late passage aMSCs on fMSC ECM (labeled TCPS-FMSC ECM in the figure) retarded but did not prevent their gradual increase in cell size and reduction in uniformity (Fig 5c). The cell size distribution for TCPS-FMSC ECM-cultured cells (P9) increased to 19.6±11.9µm (Median±IQR) in contrast to the size distribution of the starting passage (P6, 16.8±5.9µm) (Table 2). In comparison, the TCPS-cultured cells (P9) had a higher median and wider distribution of 30.2±14.5µm. Furthermore, higher frequency of TCPS-FMSC ECM cultured cells (about 20% compared to about 10% for TCPS cultured cells) was observed to possess similar cell size distribution in the 5-20µm range compared to the starting passage (P6, green, about 40% in Fig 5c). Main positive and negative biomarkers were screened for the 2 culture conditions (Fig 5d) and their expressions were comparably similar. One exception was that CD105 expression of late passaged TCPS-FMSC cultured aMSCs were lower than that of TCPS cultured aMSCs (74.4% vs 86.0%).

Interestingly, TCPS-FMSC ECM cultured cells improved their differentiation potential in contrast to TCPS cultured cells as evidenced by the positive stain expressions after application of differentiation media (Fig 6a). In addition, their average expression levels were higher compared to the TCPS cultured counterparts. Quantification of the AP levels using SIGMAFAST™ BCIP NBT for TCPS-FMSC ECM cultured cells showed higher but not significantly different expression (~1.6x, p > 0.05, n=9) to the TCPS cultured controls (Fig 6b). Oil Red O intensity densities were significantly higher for adipogenic differentiated TCPS-FMSC ECM cultured cells compared to TCPS-cultured cells (~2.1x, p ≤ 0.05, n=7 and 11 respectively). Furthermore, we found that there was a trend towards higher but
not significantly different intensity densities of chondrogenic (Alcian Blue) expression for TCPS-FMSC ECM cultured cells in comparison to the TCPS-cultured cells (~18.7x, > 0.05, n=5 and 6 respectively).

In addition, virtually all TCPS cultured cells lost their chondrogenic potential as indicated by their Alcian Blue expression (Fig 6a and b). Taken together, the study with late passaged TCPS-cultured cells suggested the ability of the fMSC ECM to retard the senescence and rejuvenate the "stemness" of expanded aMSCs.

3.5 Quantification of ECM yield

Lastly to understand the scalability potential of the substrate for large-scale expansion of aMSCs for therapeutic applications, we quantified the amount of ECM produced per cell plated. We obtained (0.5 ± 0.2) µg, (1.4 ± 0.6) µg and (0.6 ± 0.5) µg per cell plated for aMSC (n=6), fMSC (n=4) and nHDF (n=6) respectively (Fig 7). The ECM yield for fMSC is significantly higher than aMSC (p ≤ 0.05). In addition, the yield for fMSC was also higher to that of nHDF but not significantly different (p > 0.05).

4. Discussion

Traditionally, MSC cells are expanded on tissue culture polystyrene (TCPS). However, they lose their regenerative capabilities after a few passages. Furthermore, results vary depending on the quality and brand of tissue culture polystyrene used [17]. To overcome these shortfalls, others have deposited individual ECM proteins such as fibronectin, collagen or laminin on the plastic substrates to culture and expand aMSCs. Recent alternatives are the development of xenogenic-free substrates, which includes synthetic polymers or recombinant human matrices or matrix isoforms [2, 3, 18, 19]. Many have become commercially available in recent years or are in the process of development. One of the drawbacks in growing cells in vitro using conventional tissue culture techniques and other above mentioned substrates is that these culture environments do not mimic their natural biological support enough or adequately in the physiological relevant manner. This natural support is a complex network of numerous macromolecules known as the extracellular matrix or ECM. The ECM holds cells and tissues together and provides a highly organized lattice within which cells can migrate and interact with each other. The matrix also plays an active and complex role in regulating the behavior of cells that are in contact with it, influencing their shape, migration, proliferation and metabolic functions [20, 21]. Some of the ECM proteins found in the MSC ECMs are Collagen Type I, Collagen Type III, Fibronectin, Biglycan, Decorin, Perlecan and Laminin [9]. In contrast, cells grown on plastic and isolated proteins lose many of their natural multipotent properties since this approach fails to accurately mimic the complex protein interaction with the endogenous ECM, an environment that can be efficiently generated by a number of cellular populations.

The design of niche-like cell culture interfaces that maintain cells in their native behavioral state or instruct their phenotypic transformation towards that of a desired tissue may be the key to expanding MSC effectively. It has been previously demonstrated that cells cultured on ECM adopt growth
characteristics, morphological appearance and biological responses which were not expressed when maintained on artificial plastic or glass substrata. This was observed even if the same substrates were coated with isolated constituents of ECM such as purified collagen or glycoproteins [22-26]. In recent years, there were studies that employed decellularized matrices from epithelial cells, endothelial cells, fibroblasts and adult bone marrow mesenchymal stem cells to expand the MSCs [8, 9, 14, 27, 28]. These studies concluded that decellularized matrices offer superior platforms to proliferate the cells in contrast to traditional methods. The use of naturally produced ECM, may also assure that the various matrix components will be found in their natural configuration and proportion. In this report, we investigated employing fMSCs, the most primitive and generative versions of MSCs, to produce the matrices for culturing the adult mesenchymal stem cells. Being similar cell types (except this is the fetal version), we hypothesized that their microenvironment would be the most similar, and therefore the most suitable for culturing these cell types. Furthermore, the process of decellularization destroyed the cells used to produce the matrices. Thus if autologous adult mesenchymal stem cells were used to generate the ECM, a large portion of the cells would be destroyed during the process resulting in the sacrifice of the already limited aMSCs. An alternative would be to rely on allogeneic sources for preparing the substrates. However, aMSCs suffered from more inconsistency since proliferation capacity and multipotency depended on the age/health of the donor. Furthermore, fMSC had been demonstrated to possess higher proliferative and osteogenic capacity compared to aMSCs and MSCs from other sources [11]. This implied that their ECM might be a superior substrate to expand and perhaps regenerate the stem cell behavior of adult cells. In addition, we might be able to generate larger quantities of ECM due to their superior growth. We observed here that only the decellularized fMSC matrices had shown significant increase in numbers of aMSC compared to TCPS during expansion. In contrast to previous literature [8, 9], we did not observed increased proliferation on aMSC ECM in comparison to TCPS. It was possible that the TCPS substrates employed in our studies were more conducive for the expansion of aMSCs compared to those used by others in previous literature; studies have shown that aMSCs plated on TCPS of various brands could vastly differ in their isolation, expansion and characteristics [17, 29]. Alternatively, the aMSCs used in our studies might not be as potent (which according to the manufacturer, our lot was isolated from a 43-year old male donor and estimated to had undergone 7-8 population doublings by passage 2; a further 7 doublings before they were used to generate the substrates) as their equivalents used in previous studies. Interestingly as well, we obtained higher ECM yield from fMSC compared to the other cell types (aMSCs and nHDFs) (Fig 7); this finding might explain why we experienced higher cell yield when aMSCs were expanded on ECM generated from the fMSCs as the increased ECM yield might contain higher amount of sequestered growth factors and provide a more conducive environment for proliferation. Our results thus suggested that fMSC ECMs as promising substrates to improve the ex vivo expansion of aMSCs for potential autologous applications such as clinical transplantation. However, this needs to be further validated through several different fMSC lines in order to reaffirm and improve the power of our observations. In addition, further studies as described below will be necessary to optimize the ECM substrates for translational applications.
Since the studies here were performed on mid-passages cells, one of the future studies is to culture the cells from the point of isolation where mononuclear cells from fresh bone marrow aspirates to their senescence to elucidate the long term effectiveness of the ECM substrates on expansion and maintenance of their progeny. We had performed a long-term study where we cultured and tracked the cell yield over 5 passages until the cultured aMSCs reached senescence. Commercially available cryopreserved purified aMSCs were plated directly on the various substrates and replated on the same kind of substrates after 10 days of expansion. We observed the loss of aMSC proliferation capacity and "stemness" in the later passages for all conditions and surprisingly, aMSCs reached senescence faster when cultured on fMSC ECM compared to TCPS and other substrates (data not shown). In addition in this study, long term culturing on fMSC ECM also did not maintain the multipotency of the aMSCs as we observed an increase in the cell size distribution. However, it was possible that our long term study was not conducted optimally. For example, we cultured them for 10 days before passaging; this usually resulted in almost confluent conditions (beyond the recommended 70%) for the aMSCs cultured on fMSC ECM in contrast to the other substrates due to its higher proliferative capacities in the early passages. As a result, this might have caused the cells to lose their proliferative potential and reach senescence faster in the later passages. To ascertain if this is the case, it will be interesting to repeat the long term studies but passage the cells at 70% confluency instead of every 10 days. Alternatively, the results may suggest that there are limitations to the life span of the aMSC population and culturing them under any of the tested ECM substrates will not extend their life spans. In addition, commercial available expanded aMSCs were used in the studies. Their preparation, expansion and cryopreservation methods may already committed some of the progenitors down certain lineages and may affect their subsequent fates on these substrates [30, 31]. Indeed, we failed to detect one of the early positive biomarkers, particularly CD271 [32-35], in the early passages (Fig 3).

Furthermore, our results (Fig 1) indicated a passage-dependence on the quality of the ECM with the passage 3 ECM substrates performing better than ECM generated from Passage 4 in terms of cell yield and size. Therefore, we may to elucidate the effects of ECM produced by earlier passage (1 and 2) fMSCs. In addition, earlier passage cell populations contained contaminating cells from their isolation such as hematopoietic cells. The ECM of these contaminating cells might synergistically provide a more supportive environment on maintaining the multipotency of the MSCs. However, these series of studies may face difficulties to implement and may also not be feasible in terms of scaling up for applications due to limitations in their cell numbers.

Lastly, the ECM substrates were generated by culturing the cells to confluence and maintaining them under confluence conditions for one more week. This method from previous decellularization protocols for aMSC ECMs [8, 9, 14] were based on protocols from studies using epithelial and endothelial cells [36]. Culturing to confluence and beyond might not be ideal for MSCs since it was generally recommended to passage these cells at 70-80% confluence for optimal growth and prevent
them from becoming senescent and lose pluripotency. The confluency of the MSCs might alter their phenotypes, and thereby changing the nature of the ECM they produced to one that is more committed towards certain lineages or provide cues for senescence. Therefore for it would be interesting to investigate the ECM generated during the growth phase. In addition, we noticed that the ECM may delaminate easily during the rinsing and treatment steps in our decellularization protocol; this was previously by others using similar decellularization protocols [37]. While we minimized the delamination through delicate handling of our ECM culture wares, it may be necessary to improve the adhesion of the ECM to substrate through immobilization [37]. Alternatively, since the quantity produced may be limited, it might be necessary to develop transferable versions by retrieving and pooling the decellularized matrices to obtain sufficient amounts for testing. Nevertheless, our results overall represented the first steps towards the potential use of fMSC ECMs to improve the expansion and maintenance of aMSCs. To our knowledge, this is the first study in which matrices derived from BM fMSCs were generated and characterized for their expansion and differentiation potential. More studies, such as genetic stability characterizations, will be required to better understand the biological effects of aMSCs cultures under fMSC ECMs and translate the fMSC ECM substrate to expand aMSCs ex vivo for clinical applications.

5. Conclusions
In summary, fMSC ECM promotes aMSC expansion better than the other conditions including TCPS, aMSC ECM and nHDF ECMs in terms of superior growth, smaller and more uniformed-sized cells. This phenomenon may be dependent on the age (passage number) of the cells producing the substrate. Late-passage aMSCs (previously cultured on TCPS for 5 passages) were significantly improved on the fMSC ECM platform in terms of proliferation, stemness and differentiation potential compared to continuous culture in standard TCPS conditions. We also quantified amount of ECM produced by each plated cell, which suggested feasible scalability for clinical applications. The results established fMSC matrices as promising platforms for ex-vivo expansion of human aMSCs.

Acknowledgements
This research was supported by the National Research Foundation Singapore through the Singapore MIT Alliance for Research and Technology’s BioSystems and Micromechanics (BioSyM) Inter-Disciplinary Research program. We are also grateful to Mr. Kwok Chee Keong and Mr. Nyan Lin Minyt for their technical and logistical supports.
References


[34] Jarocha D, Lukasiewicz E, Majka M. Advantages of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105(+) and CD271(+) cells. Folia Histochem Cytobiol. 2008;46:307-14.


Captions (Tables and Figures)

Tables

Table 1. Size distribution characteristics of aMSCs cultured on the various substrates

Table 2. Size distribution characteristics of late passage TCPS-cultured aMSCs cultured on FMSC ECM in comparison to TCPS

Figures

Figure 1. (a) Schematic of decellularized ECM study. MSCs are plated on TCPS and cultured to generate extracellular matrices. The MSCs are decellularized to expose the layer of ECM for proliferation studies with aMSCs. In addition, media is collected from the fMSC cultures for the conditioned media condition. To assess proliferation, aMSCs of passage 4 were seeded on TCPS controls and decellularized matrices, cultured for 10 days before detachment to evaluate cell number and cell size. (b) Micrograph showing cell monolayer before and after the decellularization process. The decellularization protocol successfully remove the cells leaving a layer of extracellular matrices. Scale Bar = 200µm. (c) Micrographs showing adult mesenchymal stem cells cultured on the various conditions. Qualitatively, aMSCs cultured on decellularized matrices are morphologically similar to cells cultured on TCPS. Scale Bar = 200µm. (d) Cell yield (n=3) and (e) size distribution of aMSCs cultured under various conditions. Statistical analysis with 1-way ANOVA with Tukey’s Multiple Comparison Test; * p≤0.05, ** p≤0.01, *** p≤0.001

Figure 2. Plating Efficiency of expanded adult mesenchymal stem cells. Cells were plated at 5.0x10⁴ per well and incubated for 2hrs before rinsing to fixation. Fixed cells were stained with DAPI(5µg/ml) for 15minutes, imaged and counted using ImageJ. Statistical analysis using 1-way ANOVA with Tukey's Multiple Comparison Test; *** p≤0.001

Figure 3. Flow cytometry analysis of MSC markers for aMSCs cultured on TCPS (a-i and a-ii for MSC positive and negative markers respectively) and fMSC ECM (a-iii and a-iv for positive and negative markers respectively) and (b) surface biomarker expression of aMSC cultured on various substrates.

Figure 4. Differentiation assays of aMSCs (P3 or P4) cultured on TCPS and fMSC ECM (a for osteogenesis, b for adipogenesis and c for chondrogenesis). For negative controls, aMSCs are cultured in growth media. The purple positive stain in the micrographs are of alkaline phosphate expression after 14 days of selection pressure and evaluated using SIGMAFAST BCIP/NBT. Adipocytes are stained positively with Oil Red O and can be identified by their bright red color. Cartilage formation was detected with Alcian Blue, which stains aggrecan, an ECM component produced, dark blue. Quantification of differentiation expression levels of aMSC cultured on various substrates. The expression level is defined by the intensity density, as calculated by taking the product of the area and the mean of the stains in the images. Scale Bar = 200µm

Figure 5. (a) Schematic of late passage TCPS-cultured aMSC study, (b) Cell numbers (n=3), (c) size distribution, and (d) surface biomarker expressions of late passage (P6) TCPS-cultured aMSCs that have been
cultured under TCPS and FMSC ECM for 3 passages. Significant increase in cell proliferation observed as ascertained using 2-tailed unpaired t-test, ** p≤0.01).

**Figure 6.** (a) Representative differentiation micrographs and (b) expression levels of late passage (P6) TCPS-cultured aMSCs that have been cultured under TCPS and FMSC ECM for 3 passages. The purple positive stain in the micrographs are of alkaline phosphate expression after 14 days of selection pressure and evaluated using SIGMAFAST BCIP/NBT. Adipocytes are stained positively with Oil Red O and can be identified by their bright red color. Cartilage formation was detected with Alcian Blue, which stains aggrecan, an ECM component produced, dark blue. The expression level is defined by the intensity density, as calculated by taking the product of the area and the mean of the stains in the images. Scale bar = 200µm.

**Figure 7.** ECM yield per cell plated. Statistical analysis using 1-way ANOVA with Tukey's Multiple Comparison Test; * p≤0.05
<table>
<thead>
<tr>
<th></th>
<th>AMSC P3 ECM</th>
<th>TCPS ECM</th>
<th>Fibronectin CM</th>
<th>FMSC P2 ECM</th>
<th>FMSC P3 ECM</th>
<th>FMSC P4 ECM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (µm)</td>
<td>17.2</td>
<td>17.5</td>
<td>22.4</td>
<td>22.5</td>
<td>15.5</td>
<td>18.5</td>
</tr>
<tr>
<td>25-75% Percentile (µm)</td>
<td>15.7-20.7</td>
<td>15.8-20.2</td>
<td>17.9-29.4</td>
<td>20.0-26.5</td>
<td>13.9-18.0</td>
<td>15.3-18.5</td>
</tr>
<tr>
<td>Interquartile Range (IQR) (µm)</td>
<td>5.0</td>
<td>4.4</td>
<td>11.5</td>
<td>6.5</td>
<td>4.1</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>TCPS (P6)</td>
<td>TCPS (P9)</td>
<td>TCPS-FMSC ECM (P9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Starting Passage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (µm)</td>
<td>16.8</td>
<td>30.2</td>
<td>19.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-75% Percentile (µm)</td>
<td>14.5-20.4</td>
<td>21.3-35.7</td>
<td>14.5-26.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interquartile Range (IQR) (µm)</td>
<td>5.9</td>
<td>14.5</td>
<td>11.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figures

Figure 1

a

Figure 1

b

before decellularization

after decellularization

C

TCPS

AMSC

FMSC P3

Fibronectin

Conditioned Medium

FMSC P4

d
e

Normalized Measure of 

Average Cell 

Conductivity
Figure 2.

![Bar chart showing plating efficiency for different substrates: AMSC, FMSC, nHDF, Glass, TCPS. The chart includes error bars and statistical significance markers (***) indicating differences among the substrates.](image-url)

- AMSC (n=4)
- FMSC (n=4)
- nHDF (n=4)
- Glass (n=6)
- TCPS (n=4)
**Figure 3**

(a)  

**TCPS**  

i) Positive markers  

ii) Negative markers  

FMSC  

i) Positive markers  

ii) Negative markers  

Red = negative k uniform control  
Blue = CD marker expression

(b)  

<table>
<thead>
<tr>
<th></th>
<th>AMSC ECM</th>
<th>FMSC ECM</th>
<th>nHDF ECM</th>
<th>TCPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive Markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>100</td>
<td>100</td>
<td>99.9</td>
<td>100</td>
</tr>
<tr>
<td>CD73</td>
<td>100</td>
<td></td>
<td>100</td>
<td>99.9</td>
</tr>
<tr>
<td>CD90</td>
<td>91.9</td>
<td>95.0</td>
<td>92.4</td>
<td>96.3</td>
</tr>
<tr>
<td>CD105</td>
<td>81.6</td>
<td>81.9</td>
<td>55.8</td>
<td>84.7</td>
</tr>
<tr>
<td>CD146</td>
<td>43.1</td>
<td>43.1</td>
<td>33.9</td>
<td>50.2</td>
</tr>
<tr>
<td>CD166</td>
<td>99.6</td>
<td></td>
<td>100</td>
<td>99.8</td>
</tr>
<tr>
<td>CD271</td>
<td>0.6</td>
<td>1.2</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Negative Markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>1.1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>CD45</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CD79a</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0.4</td>
<td>0.8</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 4

Osteogenesis

(a) BCP/NBT (Alkaline Phosphatase) Neg control

TCPS

FMSC P3 ECM

(b) Intensity Density

Substrate

One-way ANOVA with Tukey's Multiple Comparison Test

** p < 0.01

*** p < 0.001

Adipogenesis

(c) Oil Red O Neg control

TCPS

FMSC P3 ECM

(d) Intensity Density

Substrate

One-way ANOVA with Tukey's Multiple Comparison Test

** p < 0.01

*** p < 0.001

Chondrogenesis

(e) Alcian Blue Neg control

TCPS

FMSC P3 ECM

(f) Intensity Density

Substrate

One-way ANOVA with Tukey's Multiple Comparison Test

not significant
Figure 6.

(a) TCPS vs TCPS-FMSC P3 ECM for 8CP/NBT (Alkaline Phosphatase), Oil Red O, and Alcian Blue staining.

(b) Graph showing intensity density comparison for BCIP NBT, Oil Red O, and Alcian Blue with unpaired t-test significance noted.
Figure 7.

![Bar chart showing ECM yield (µg/cell plated) for AMSC (n=6), FMSC (n=4), and nHDF (n=6).](image)
Figure 2.

Plating Efficiency

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AMSC (n=4)</th>
<th>FMSC (n=4)</th>
<th>nHDF (n=4)</th>
<th>Glass (n=6)</th>
<th>TCPS (n=4)</th>
</tr>
</thead>
</table>

*** *** *** *** ***
Figure 3

(a)

(b)

<table>
<thead>
<tr>
<th></th>
<th>AMSC ECM</th>
<th>FMSC ECM</th>
<th>nHDF ECM</th>
<th>TCPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>100</td>
<td>100</td>
<td>99.9</td>
<td>100</td>
</tr>
<tr>
<td>CD73</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99.9</td>
</tr>
<tr>
<td>CD90</td>
<td>91.9</td>
<td>95.0</td>
<td>92.4</td>
<td>96.3</td>
</tr>
<tr>
<td>CD105</td>
<td>81.6</td>
<td>81.9</td>
<td>55.8</td>
<td>84.7</td>
</tr>
<tr>
<td>CD146</td>
<td>43.1</td>
<td>43.1</td>
<td>33.9</td>
<td>50.2</td>
</tr>
<tr>
<td>CD166</td>
<td>99.6</td>
<td>99.9</td>
<td>100</td>
<td>99.8</td>
</tr>
<tr>
<td>CD271</td>
<td>0.6</td>
<td>1.2</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Negative Markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>1.1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>CD45</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CD79a</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0.4</td>
<td>0.8</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 4

Osteogenesis

(a) BCP/NBT (Alkaline Phosphatase) Neg control

TCPS

FMSC P3 ECM

(b)

Intensity Density

0 1.0 x10^2 2.0 x10^2 3.0 x10^2 4.0 x10^2 5.0 x10^2

AMSC FMSC nHDF TCPS

One-way ANOVA with Tukey’s Multiple Comparison Test
** p < 0.01
*** p < 0.001

Adipogenesis

(c) Oil Red O Neg control

TCPS

FMSC P3 ECM

(d)

Intensity Density

0 2.0 x10^3 4.0 x10^3 6.0 x10^3

AMSC FMSC nHDF TCPS

One-way ANOVA with Tukey’s Multiple Comparison Test
** p < 0.01
*** p < 0.001

Chondrogenesis

(e) Alcian Blue Neg control

TCPS

FMSC P3 ECM

(f)

Intensity Density

0 5.0 x10^2 1.0 x10^3 1.5 x10^3

AMSC FMSC nHDF TCPS

One-way ANOVA with Tukey’s Multiple Comparison Test
not significant
Figure 5.

**Figure 5a:** Schematic representation of the experimental setup. The process involves the use of MSCs, PS-MSC, and decondensed ECM. The text box explains the following:
- Proliferation/doubling time
- Elongation assay (non-dilution)
- Differentiation assay (osteogenic, chondrogenic, and adipogenic)
- Pro-inflammatory (COX2, TNF, IL1B, CXCL1, CCL2, IL10)

**Figure 5b:** Graph showing cell number at Day 10. The graph compares TCPS and TCPS-FMSC ECM. The legend indicates an unpaired t-test with **p < 0.01.**

**Figure 5c:** Graph illustrating normalized frequency (%). The x-axis represents cell size (μm), and the y-axis represents normalized frequency. The graph shows three different conditions:
- TCPS (n=72)
- TCPS (n=100)
- TCPS-FMSC ECM (n=134)

**Figure 5d:** Table comparing TCPS and TCPS-FMSC ECM for different markers:

<table>
<thead>
<tr>
<th>Marker</th>
<th>TCPS</th>
<th>TCPS-FMSC ECM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD73</td>
<td>98.4</td>
<td>100</td>
</tr>
<tr>
<td>CD90</td>
<td>93.2</td>
<td>92.4</td>
</tr>
<tr>
<td>CD105</td>
<td>86.0</td>
<td>74.4</td>
</tr>
<tr>
<td>CD34</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>CD45</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CD79a</td>
<td>0.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Figure 6.

(a) TCPS vs. TCPS FMSC P3 ECM

- **BCIP/NBT (Alkaline Phosphatase)**: TCPS vs. TCPS FMSC P3 ECM
- **Oil Red O**: TCPS vs. TCPS FMSC P3 ECM
- **Alcian Blue**: TCPS vs. TCPS FMSC P3 ECM

(b) Bar charts showing intensity density for:

- **BCIP NBT**: TCPS vs. TCPS FMSC P3 ECM (n=9 vs. n=9)
- **Oil Red O**: TCPS vs. TCPS FMSC P3 ECM (n=7 vs. n=11)
- **Alcian Blue**: TCPS vs. TCPS FMSC P3 ECM (n=5 vs. n=6)

Unpaired t-test:
- Not significant (n.s.)
Figure 7.
## Supplementary Data

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC mouse anti-human CD73</td>
<td>BD Pharmingen</td>
<td>560847</td>
</tr>
<tr>
<td>FITC mouse anti-human CD90</td>
<td>BD Pharmingen</td>
<td>555595</td>
</tr>
<tr>
<td>PE mouse anti-human CD44</td>
<td>BD Pharmingen</td>
<td>550989</td>
</tr>
<tr>
<td>APC mouse anti-human CD105</td>
<td>BD Pharmingen</td>
<td>561443</td>
</tr>
<tr>
<td>FITC mouse anti-human CD146</td>
<td>BD Pharmingen</td>
<td>560846</td>
</tr>
<tr>
<td>PE mouse anti-human CD166</td>
<td>BD Pharmingen</td>
<td>559263</td>
</tr>
<tr>
<td>PE mouse anti-human CD271</td>
<td>BD Pharmingen</td>
<td>557196</td>
</tr>
<tr>
<td><strong>Negative markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE mouse anti-human HLA-DR</td>
<td>BD Pharmingen</td>
<td>555812</td>
</tr>
<tr>
<td>FITC mouse anti-human CD34</td>
<td>BD Pharmingen</td>
<td>555821</td>
</tr>
<tr>
<td>FITC mouse anti-human CD45</td>
<td>BD Pharmingen</td>
<td>555482</td>
</tr>
<tr>
<td>APC mouse anti-human CD79a</td>
<td>BD Pharmingen</td>
<td>551134</td>
</tr>
<tr>
<td><strong>Negative isoform controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC mouse IgG1, κ</td>
<td>BD Pharmingen</td>
<td>555751</td>
</tr>
<tr>
<td>FITC negative isoform control</td>
<td>BD Pharmingen</td>
<td>555748</td>
</tr>
<tr>
<td>PE mouse IgG1, κ</td>
<td>BD Pharmingen</td>
<td>555749</td>
</tr>
</tbody>
</table>

**Table S1.** Biomarkers and their catalog numbers used for immunophenotyping the MSC samples
<table>
<thead>
<tr>
<th></th>
<th>nHDF P3 ECM</th>
<th>TCPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (µm)</td>
<td>16.1</td>
<td>16.0</td>
</tr>
<tr>
<td>25-75% Percentile (µm)</td>
<td>13.8-18.9</td>
<td>13.8-18.9</td>
</tr>
<tr>
<td>Interquartile Range (IQR) (µm)</td>
<td>5.1</td>
<td>5.2</td>
</tr>
</tbody>
</table>

**Table S2.** Size distribution characteristics of aMSCs cultured on nHDF ECM substrates compared to TCPS.
// create a stack in the respective RGB channels from the original RGB images
run("RGB Stack");

// select the green channel, which has the best contrast (1-red, 2-green, and 3-blue)
setSlice(2);

// set threshold
setAutoThreshold("Default");
setThreshold(0, 18);

// create selection mask of the regions to be measured
run("Create Selection");
run("RGB Color");

// measure the density intensity
run("Set Measurements...", "area mean modal min centroid perimeter integrated area_fraction limit
display redirect=None decimal=3");
run("Measure");
selectWindow("Results");

**Fig S1.** ImageJ macro for quantification of osteogenic differentiation expression (via intensity density).
Fig S2. (a) Cell yield and (b) cell size distribution of adult mesenchymal stem cells cultured on neonatal HDF ECM. Statistical analysis using t-test; * p≤0.05.
Fig S3. (a) Cell yield and (b) cell size distribution of adult mesenchymal stem cells cultured on various commercially xenogenic-free substrates in comparison to TCPS (red). Notations for the
various conditions (A - B): A implies the substrate (CELLStart™ from Life Technologies; Laminin isoforms 111, 211, 332, 411, 421, 511, 521 from BioLamina; MesenCult-XF Attachment Substrate from STEMCELL Technologies; Synthemax II from Corning) while B denotes the type of media used (DLGD10; MesenCult®-XF Medium, StemPro®MSC SFM CTS™ Medium). Xeno-free substrates were prepared according to the manufacturers' instructions. Cells on all laminin isoforms were cultured on DLGD10 media. Statistical analysis using 1-way ANOVA with Tukey's Multiple Comparison Test; ** p≤0.01, *** p≤0.001