Biomaterials 32 (2011) 119–127

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Extracellular matrix production by adipose-derived stem cells: Implications for heart valve tissue engineering

Francesca Colazzo [a], Padmini Sarathchandra [b], Ryszard T. Smolenski [b,c], Adrian H. Chester [b], Yuan-Tsan Tseng [d], Jan T. Czernuszka [d], Magdi H. Yacoub [b,*], Patricia M. Taylor [b]

[a] IRCCS Policlinico S. Donato, via Morandi 30, 20097, S. Donato Milanese, Milano. Italy
[b] Heart Science Centre, NHLI, ImperialCollege London, National Heart and Lung Institute, Heart Science Centre, Harefield Middx, UB9 6JH UK
[c] Department of Biochemistry, Medical University of Gdansk, 80211 Gdansk, Debinki 1, Poland
[d] Department of Materials, University of Oxford, Parks Road, Oxford OX1 3PH, UK

ARTICLE INFO

Article history:
Received 5 August 2010
Accepted 2 September 2010
Available online 11 November 2010

Keywords:
Adipose-derived stem cells
Heart valve
Extracellular matrix
Collagen
Elastin
Crosslinks

ABSTRACT

A key challenge in tissue engineering a heart valve is to reproduce the major tissue structures responsible for native valve function. Here we evaluated human adipose-derived stem cells (ADSCs) as a source of cells for heart valve tissue engineering investigating their ability to synthesize and process collagen and elastin. ADSCs were compared with human bone marrow mesenchymal stem cells (BmMSCs) and human aortic valve interstitial cells (hVICs). ADSCs and BmMSCs were stretched at 14% for 3 days and collagen synthesis determined by [^{3}H]-proline incorporation. Collagen and elastin crosslinking was assessed by measuring pyridinoline and desmosine respectively, using liquid chromatography/mass spectrometry. Three-dimensional culture was obtained by seeding cells onto bovine collagen type I scaffolds for 2–20 days. Expression of matrix proteins and processing enzymes was assessed by Real Time-PCR, immunofluorescence and transmission electron microscopy. Stretch increased the incorporation of [^{3}H]-proline in ADSCs and BmMSCs, however only ADSCs and hVICs upregulated COL3A1 gene. ADSCs produced collagen and elastin crosslinks. ADSCs uniformly populated collagen scaffolds after 2 days, and fibrillar-like collagen was detected after 20 days. ADSCs sense mechanical stimulation and produce and process collagen and elastin. These novel findings have important implications for the use of these cells in tissue engineering.

1. Introduction

Heart valve cells are key modulators of extracellular matrix (ECM) formation and remodelling that provides the basic framework for the tissue and is primarily responsible for the structural and mechanical properties of the valve [1]. Cells can remodel the ECM by producing matrix components, such as collagen and elastin, by secreting matrix enhancing or degrading products and their inhibitors, and by applying forces to the deposited fiber [2]. In the heart valve, as in many tissues, collagen is the main load-bearing protein and has a specific architecture that is crucial for the biomechanical function of the tissue. With particular regards to collagen biomechanical properties several studies have highlighted its correlation with collagen crosslinking [2], an enzymatically and non-enzymatically mediated process that enhances the strength and stiffness of tissues. Furthermore, elastin, another major component of the heart valve matrix plays an important role [3,4] in providing the tissues with resilience and elasticity. To ensure long term function, mimicking the native heart valve architecture and characteristics might be crucial in heart valve tissue engineering and for this reason the identification of a suitable cell type with phenotype and function similar to that of native VIC is essential. Interstitial cells are the most abundant cell type in the heart valve and are crucial to their function since have the ability to synthesize and process ECM and express matrix degrading enzymes that mediate matrix remodelling [5–9].

Due to initial promising reports, major basic research efforts have been undertaken to explore the potential of specific cell types in greater detail and identify their capacity to adopt the phenotypic and functional characteristics of the cells that populate heart valves.

We previously reported that BmMSCs shared many characteristics of VICs and were able to produce collagen in response to cyclic stretch [10,11]. However, MSCs represent only a small percentage of the total number of cells in bone marrow, and it is

* Corresponding author. Tel.: +44 1895828893; fax: +44 1895828900.
E-mail address: m.yacoub@imperial.ac.uk (M.H. Yacoub).

0142-9612/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.biomaterials.2010.09.003

speculated that their clinical value might be diminished as the number of MSCs decreases with age [12].

The objective of this study was to investigate for the first time the stem cell population found within adipose tissue as a source of cells for heart valve tissue engineering. ADSCs can be isolated from the patient by a less invasive method and in large quantity. They are readily available, can be cryogenically stored and can be differentiated towards osteogenic, adipogenic, myogenic, chondrogenic and neurogenic lineages [13–16]. The aim of this study was to evaluate ADSCs in terms of their ability to synthesis and process ECM proteins with particular emphasis on collagen and elastin content and crosslinking density in order to determine their suitability for use in tissue engineering heart valves.

2. Methods

2.1. Cell harvest and isolation

The study receives ethical approval from Royal Brompton and Harefield NHS Trust Ethical Committee.

2.1.1. Adipose-derived stem cells

Abdominal subcutaneous adipose tissue samples were obtained from 8 patients (36 ± 12 years, mean $\pm$ S.D.) undergoing abdominal liposuction. ADSCs were isolated by extensive washing of adipose tissue with equal volumes of 0.9% NaCl (normal saline), then digestion at 37 °C for 20 min with 0.075% collagenase A (Roche). Digested adipose tissue was centrifuged at 400 g for 5 min to obtain a pellet. The pellet was resuspended in normal saline, filtered through a 250 μm nylon mesh to remove cellular debris, centrifuged at 400 g for 5 min, then resuspended in normal saline and successively filtered through a 100 μm and 40 μm cell strainer. The resulting cell suspension was centrifuged at 400 g for 5 min, seeded at 5×10^4 cells/cm^2 in tissue culture grade flasks (Nunc) in MSC culture medium composed of low glucose (1000 ng/ml) DMEM (Sigma–Aldrich), 10% heat-inactivated foetal bovine serum (FBS, Biosera), 100 units/ml penicillin, 100 μg/ml streptomycin and 4 mM L-alanyl-L-glutamine (all Sigma–Aldrich) and maintained at 37 °C/5% CO_2 in culture medium.

2.1.2. Bone marrow stem cells

BmMSCs were isolated from 8 healthy human donors (27 ± 19 years, mean $\pm$ S.D.) and cultured as previously described [17,18]. In brief, heparinized bone marrow was mixed with an equal volume of PBS and centrifuged at 900 g for 10 min at room temperature. Cells were washed, resuspended in PBS to a final density of 4×10^7cells/ml and a 5 ml aliquot layered over a 1.073 g/ml Percoll solution (GE Healthcare) and centrifuged at 110 g for 30 min at 20 °C. Mononuclear cells collecting at the interface were recovered and washed in PBS at 900 g. The pellet was resuspended in culture medium at a concentration of 5×10^4 cells/cm^2 in tissue culture grade flasks (Nunc).

2.1.3. Valve interstitial cells

VICs were cultured from human aortic valves excised from recipient hearts at time of cardiac transplantation that had no previous history of heart valve disease. Cells were isolated from valve leaflets by enzymatic digestion as previously described [8]. In brief, isolated valve leaflets were placed in a solution of DMEM containing 1000 U/ml of collagenase type II, agitated initially for 5 min to remove the endothelial cells, and then after a change of media for a further 45 min at 37 °C. The reaction was stopped by the addition of heat-inactivated FBS. Cells were then washed and seeded onto 6-well plates, where they were left in DMEM supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 4 mM L-alanyl-L-glutamine and 10% heat-inactivated FBS (VIC culture media) to adhere and grow to confluence for 3–7 days.

When ADSC and BmMSC monolayers reached 70–80% confluence, cells were passaged (0.25% trypsin/EDTA, Sigma) and resuspended in culture medium at 5×10^3 cells/cm^2. VICs were passaged when 80–90% confluent. All cell types were used between passages 3 and 6.

2.2. Phenotyping of cells

2.2.1. Flow cytometry (FACS)

For quantification of antigen expression FACS analysis was performed using antibodies against CD29, CD31, CD44, CD90 and CD105 (all Serotec); osteocalcin (Abcam); CD34, CD45, CD73 and Tie-2 (all BD Pharmingen); CD14 and CD106 (ATCC); fibroblast surface antigen (FSA, Dianova); VEGFR2 (R&D Systems Ltd); and c-Kit (Santa Cruz biotechnology). All primary antibodies were mouse anti-human and secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG Fab_2 fragments (Dako Ltd). Cells were analyzed using a BD FACSAria cell sorter (BD Biosciences, Oxford, UK).

2.2.2. Immunofluorescence

Primary antibodies at the appropriate dilution in 3% BSA in PBS were applied for 1 h to cells grown on coverslips. Antibodies included von Willebrand Factor, smooth muscle α-actin (SMα–A), CD31, vimentin and Ki67 (all Dako Ltd, UK); CD44, CD105, adiponectin and osteopontin (all Serotec); collagen type II (Southern Biotechnology); CD14, CD34, CD45 and, CD73 (all BD Pharmingen) and FSA (Dianova). Appropriate secondary antibody (Alexa goat anti-mouse 594; or Alexa goat anti-rabbit 594, Invitrogen) diluted in PBS at 1:1000 were applied for 30 min at room temperature. Cells were washed twice with DAPI (1:20,000) in PBS for 3–5 min each for nuclear staining. Slides were viewed under a light microscope (Zeiss Axioskop, Welwyn Garden City, UK) and staining was semi-quantitatively assessed. Ten fields were chosen randomly and analyzed as follows: negative (−) no positive cells, weak positive (+) < 25% cells positive, positive (++) > 25 < 75% cells positive and strong positive (+++) > 75% cells positive. When the primary antibody was omitted, or an irrelevant antibody was used as a control, there was an absence of staining.

2.3. Application of cyclic stretch to cultured cells

Cells were plated out at $2.5–3.0 \times 10^5$ cells/well into BioFlex culture plates (Dunn LabTech.) precoated with collagen type I in culture media and allowed to reach confluency. The cells were mechanically loaded on the Flexercell FX 4000T cell-straining device, as previously described [10]. Cells were subjected to square waveforms at 0.6 Hz at forces that generated 14% stretch and stretched cyclically for up to 3 days. Each stretch experiment was carried out a minimum of three times. Control group (non-stretched) was grown on BioFlex plates but not stretched.

2.4. Measurement of collagen by quantification of total [^{3}H]-proline

Culture media containing 3.55 MBq/ml [^{3}H]-proline was added to wells that had been seeded with $2.5–3.0 \times 10^5$ cells. After the appropriate experimental period, media were removed from the wells to determine the amount of soluble collagen. Trichloroacetic acid (TCA) was added to the media to give a final concentration of 10%, and left on ice for 1 h. Precipitated protein was collected by centrifugation at 3000 g for 30 min, washed with 4 ml ice-cold 10% TCA to remove any unincorporated labelled proline and centrifuged again. The supernatant was carefully removed and the pellet suspended in 0.3 ml of 0.3 M NaOH/0.3% SDS. Preparations were then warmed to 37 °C until solubilised and added to 4 ml of liquid scintillant. The cell layer was washed twice with PBS and removed from the Flexercell plates by scraping into 1 ml of ice-cold 10% TCA, to assess the amount of insoluble cell-associated collagen. Precipitated protein was collected by centrifugation at 14,000 g for 20 min. The cell layer precipitate was solubilised at 37 °C for 1 h in 0.3 ml of 0.3M NaOH/0.3% SDS and added to 4 ml of liquid scintillant. Radioactivity was counted in both the media and cell layer samples on a Packard Tricarb 1600 TR liquid scintillation analyser (Packard).

2.5. Pyridinoline and desmosine quantification

The ability of cells to crosslink collagen and elastin was assessed by measurement of pyridinoline and desmosine (respectively) together with hydroxyproline in protein hydrolysates (18 h in 6M HCl at 108 °C) using liquid chromatography/tandem mass spectrometry. The method was based on procedure described previously [19] with modification to include mass transients for hydroxyproline, pyridinoline and desmosine as well as internal standards (dehydroproline and lysyl-phenylalanine).

2.6. Gene expression analysis

Gene expression analysis of ADSCs and BmMSCs was performed before and after stretch and after 10 days seeding on collagen scaffold and compared with the gene pattern expression of hVICs. Reverse transcription (RT) and real-time polymerase chain reaction (PCR) was performed as described elsewhere [20]. TaqMan assays were purchased for COL1A1, COL2A1, COL3A1, COL4A1 and lysyl oxidase (LOX) (Hs00164004_m1, Hs00261051_m1, Hs00164103_m1, Hs00266237_m1, Hs00184700_m1 respectively; Applied Biosystems, Warrington, UK). Target gene data were normalized against 18S ribosomal RNA levels (Cat. No. 4310893E; Applied Biosystems) and analyzed using the comparative cycle threshold (Ct) method.

2.7. Collagen scaffolds

Scaffolds were provided through collaboration with Department of Materials, University of Oxford, Oxford, UK, using a modification of a previously described protocol [21]. In brief, an aqueous-based dispersion of insoluble bovine Achilles tendon collagen type I (1% w/v, gift from Devro plc, Chryston, Scotland) was cast into 13mm diameter moulds, then frozen at −20 °C for 24 h. Frozen constructs were directly chemically crosslinked in 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide/ N-hydroxysuccinimide (EDC/NHS) solution (50 mM 4-Morpholineethanesulfonic acid (MES), 33 mM EDC, 6 mM NHS, 40% ethanol, pH5.5 for 4 h at 37 °C

followed by 2 washes with a stopping solution (0.1 M sodium diphsophate pH 9.1) for 30 min. The scaffold constructs were further washed in distilled water for 4 × 30 min. Scaffolds were re-frozen at −20 °C for 24 h, followed by freeze-drying (Edwards Micro Modulyo freeze dryer) for 24 h and stored at 4 °C until use. Prior to cell seeding the scaffolds were sterilised with ethanol 70% for 30 min.

2.7.1. Cell seeding

Scaffolds were placed in 50 ml centrifuge tube containing $5 \times 10^3/mm^2$ cells and 25 ml culture media supplemented with 50 μg/ml ascorbic acid, in order to promote ECM production. The tubes were gassed with 5% CO_2/air for 20 s and rotated at 10 rpm for 2, 5, 11 and 20 days in an atmosphere of 5% CO_2 at 37 °C. Media was changed every 3 days. Experiments were performed on 3 separate occasions using 3 different isolates of ADSCs, BmMSCs and VICs. Cell distribution was analyzed using *Nikon NIS elements* software.

2.7.2. Cell viability assays

Cell viability/proliferation was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) [22]. This assay was used to assess viability under two different conditions: after a stretch protocol in 2-D and when cells were seeded on collagen scaffolds (3-D). In brief, reagent and DMEM were added to BioFlex culture plates containing stretched or non-stretched cells and to culture wells containing half each of seeded or unseeded scaffolds. After a 1 h incubation at 37 °C, aliquots were taken and absorbance at 492 nm was determined with a 96-well plate reader.

2.8. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Scaffolds were seeded with BmMSCs or ADSCs for 20 days. The media was removed and specimens were washed twice in 0.1M phosphate buffer and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for at least 2 h and processed using standard SEM and TEM protocols (Roth el al 1972). Samples were analyzed with JEOL 6600 SEM microscope for SEM or JEOL 1200 EX electron microscope (JEOL UK ltd, Welwyn Garden city, Herts, UK) for TEM analysis.

2.9. Statistical analysis

All experiments were repeated a minimum of three times. All statistical analyses were performed using PRISM4 software (GraphPad Software, Inc., San Diego, CA) and $p < 0.05$ was considered significant. Data are presented as mean ± standard deviation.

3. Results

3.1. Morphology and phenotype of cells

ADSCs were found in adipose tissue in large quantities and the overall frequency in processed lipoaspirate was approximately 2×10^4/ml. When isolates from 8 donors were divided into 2 age groups (24–37 years and 38–52 years), no correlations were found between the number extracted and the age of the donor or between the age of the donor and cell proliferation. Compared over 10 days, both ADSCs and BmMSCs reached a plateau by day 10. However the ADSCs proliferation was greater, reaching $1.8 \pm 1.1 \times 10^5$ cells/well at day 10 compared with $1.2 \pm 0.3 \times 10^5$ cells/well of the BmMSCs ($p = 0.02$).

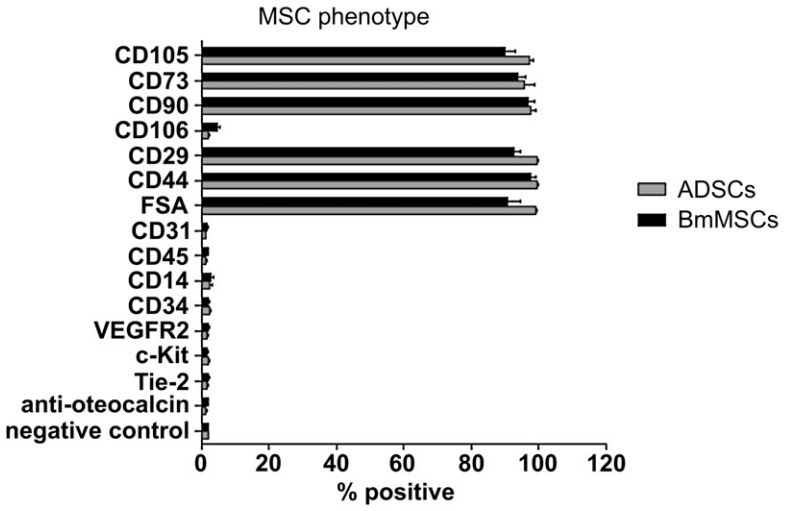
FACS analysis (Fig. 1A) revealed that ADSCs and BmMSCs were positive for CD105, CD73, CD90, CD29, CD44 and FSA; negative for hematopoietic lineage markers CD34, CD14 and CD45 and for the endothelial markers Tie-2, c-kit, VEGFR2 and CD31, indicating that they were of non-hematopoietic and non-endothelial origin respectively.

Immunofluorescence staining (Fig. 1B) of extracellular markers confirmed the results obtained with flow cytometry, the strong expression of Ki67 demonstrated their ability to proliferate. SMα-A was expressed by both populations, but to a greater extent by BmMSCs. There was no evidence that ADSCs or BmMSCs spontaneously differentiated to bone or cartilage as cells did not express osteopontin, osteocalcin or collagen type II. However, a small number of ADSCs (2%) did express adiponectin.

3.2. Response to mechanical stimuli

The analyses of [^{3}H]-proline incorporation showed that 14% stretch increased ^{3}H-proline uptake by ADSCs (Fig. 2A) and BmMSCs (Fig. 2B) to a similar extent after 3 days. Throughout the

A



B

MARKER	ADSCs	BmMSCs
vWF	-	-
CD31	-	-
CD34	-	-
SM a-A	+	++
CD14	-	-
CD45	-	-
Vimentin	+	+
CD105	+++	+++
CD73	+++	+++
FSA	+++	++
Ki67	+++	+++
Osteopontin	-	-
Adiponectin	+	-
Collagen II	-	-
Control	-	-

Fig. 1. Comparative Phenotypic analyses. (A) The figure shows representative flow cytometry analyses for MSC phenotype (mean ± S.D). (B) ADSCs and BmMSCs were examined by immunofluorescence for the expression of some intracellular and extracellular markers. Average expression ($n = 3$) is given: negative (−) no positive cells, weak positive (+) < 25% cells positive, positive (++) > 25 < 75% cells positive and strong positive (+++) > 75% cells positive.

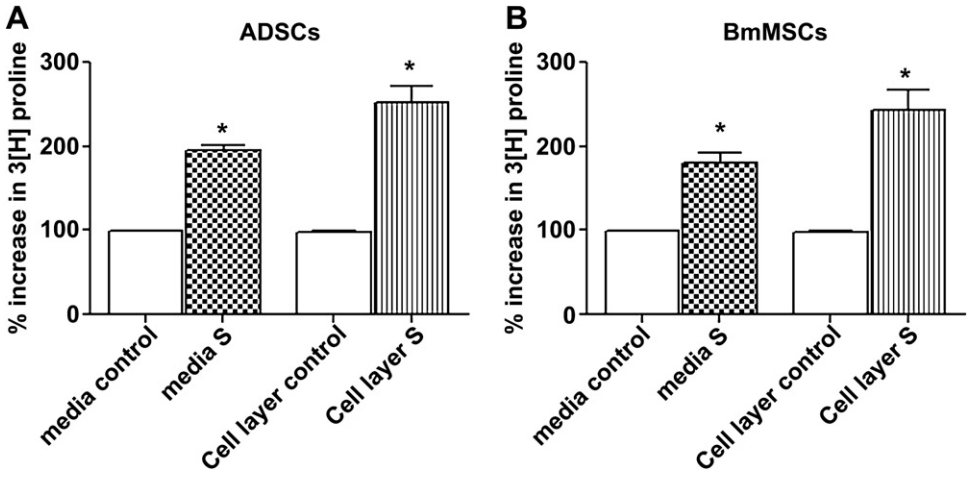


Fig. 2. [^{3}H]-proline incorporation by ADSCs and BmMSCs. Increase in incorporation of [^{3}H]-proline by ADSCs (A) and BmMSCs (B) into the medium and the cell layer in response to 14% stretch for 3 days. S = stretched. Results are expressed as mean ± S.D. of three experiments. Significance of difference according to Student's t test (* = $p < 0{,}05$).

duration of the stretching protocols there was no measurable change in either the cell number or the incidence of cell death (data not shown), indicating that changes in levels of collagen were not related to changes in numbers of cells in each well.

3.3. Collagen and elastin crosslinking

MSCs and VICs seeded onto BioFlex culture plates and exposed to stretch remained viable for the duration of the experiments ($n = 3$), as indicated by the MTS assay results in Fig. 3A. Formation of collagen was demonstrated by the presence of hydroxyproline in protein hydrolysates and was not different between the different groups studied (not shown). Collagen (Fig. 3B) and importantly elastin (Fig. 3C) crosslinks were observed in stretched and non-stretched ADSCs and BmMSCs, but was significantly increased only in stretched ADSCs ($p < 0.05$). Pyridinoline/hydroxyproline ratio (1.27 ± 0.51 and 0.74 ± 0.30 nmol/mmol in stretched ADSCs and BmMSCs, respectively) was however lower than in VICs (4.54 ± 2.0 nmol/mmol) or in native valve cusps (5–10 nmol/mmol).

3.4. Gene expression analysis

Real Time-PCR analysis showed that collagen III gene (COL3A1) relative expression increased after stretch in ADSCs compared with cells that had received no stretch, reflecting changes in expression observed in hVICs (Fig. 4B). There was increased expression of

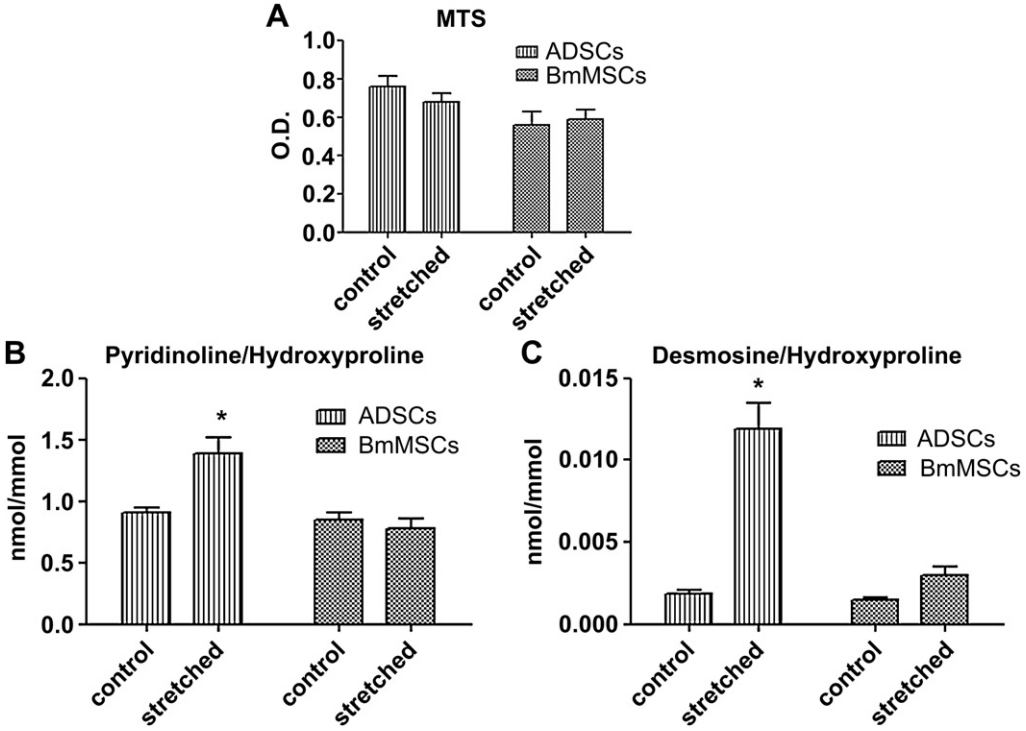


Fig. 3. Pyridinoline and Desmosine quantification. Quantification of collagen and elastin crosslinks by measurement of pyridinoline and desmosine (respectively) in protein hydrolysates using liquid chromatography/mass spectrometry. Results are expressed as nmol/mmol hydroxyproline (mean ± S.D), $n = 3$, (* = $p < 0.05$).

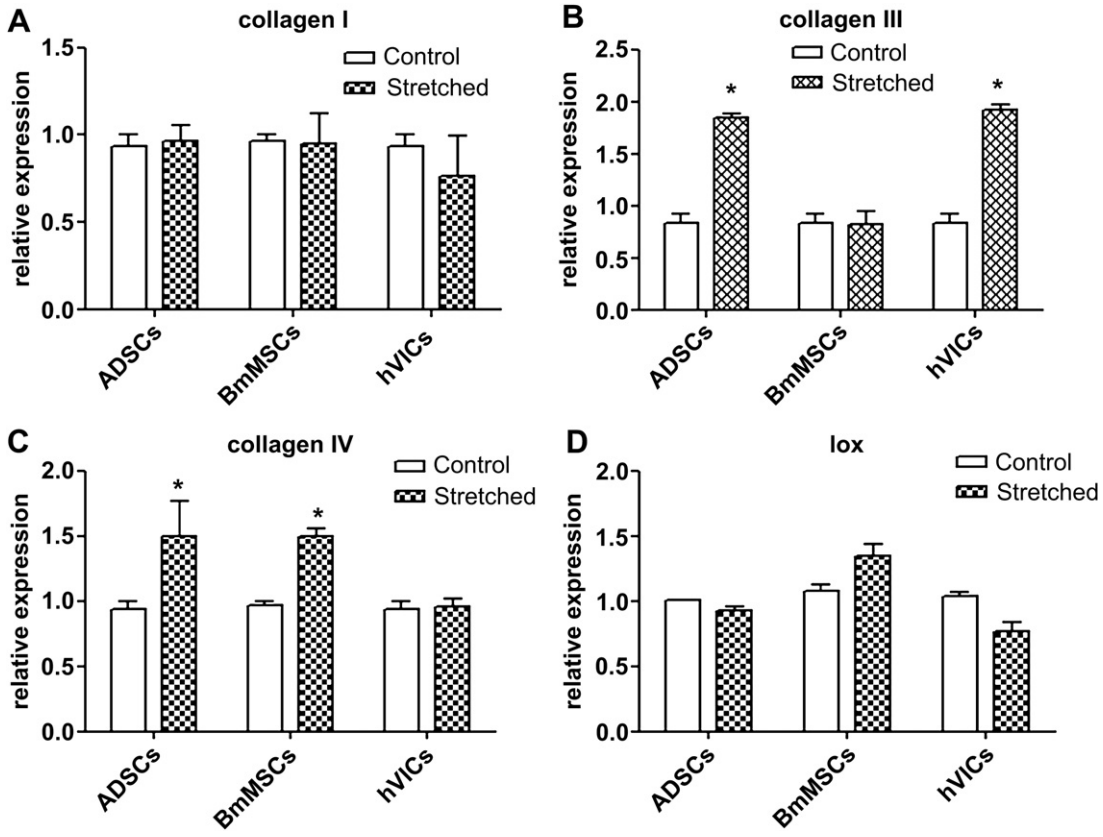


Fig. 4. Gene Expression Analysis. Real-time PCR analysis of COL1A1, COL3A1, COL4A1 and LOX in stretched and non-stretched ADSCs, BmMSCs and VICs. Results are expressed as mean ± S.D. ($n = 3$). ($^* = p < 0.05$).

collagen IV gene (COL4A1) in both stem cell populations after stretch, in contrast no expression of the collagen II gene (COL2A1) was observed in either BmMSCs or ADSCs. Collagen I gene (COL1A1) and lysyl oxidase (LOX) expression, were detectable at similar levels of intensity in stretched and non-stretched ADSCs, BmMSCs and VICs, (Fig. 4A and D).

3.5. Collagen scaffolds

MTS analyses (Fig. 5A) showed that MSCs remained viable for the duration of the experiment, however VIC viability decreased when compared with cells cultured in tissue culture plates (controls). Fluorescent microscopy (Fig. 5E) revealed a progressive increase in the percentage of ADSCs that migrated into the scaffolds during the time of investigation. The majority of BmMSCs in contrast remained densely packed on the collagen surface unable to migrate towards the center of the scaffold matrix.

Comparison of the number of ADSCs, BmMSCs and hVICs (Fig. 6) determined by image processing software showed a different distribution confirming these data. After 5 days seeding, ADSCs were uniformly distributed and BmMSCs remained near the surfaces. The analyses of hVIC scaffolds showed a normal distribution but a decrease in viability as demonstrated by MTS analysis.

SEM analysis of ADSCs seeded for 20 days showed that ADSCs had migrated across the scaffold (Fig. 7A) and there were many processes radiating from the cells and forming a meshwork of fibrils resembling collagen fibres (Fig. 7B). BmMSCs in contrast were localised near the surface and did not show any fibrils associated with cells (Fig. 7C and D).

In TEM we noted that after seeding and culturing for 20 days, ADSC exhibited the presence of typical newly formed collagen fibers (Fig. 7E and F) similar in diameter to native valve collagen fibres [23] (around 30–40 nm). These newly formed fibres were morphologically different to the scaffold collagen that was thicker, resembling its origin – tendon collagen fibres (around 200 nm). Abundant rough endoplasmic reticulum cisternae were visible (Fig. 7H arrow) indicative of active protein synthesis. BmMSCs even after 20 days were located near the surfaces and showed some collagen fibres associated with the cells (Fig. 7G and H).

Real-time PCR analysis of ADSCs seeded for 5 or 11 days on collagen scaffolds (3-D) compared with ADSCs cultured in tissue culture plates (2-D, control) is given in Fig. 8. Evaluation of newly formed collagen by ADSCs using real-time PCR showed that the expression of COL3A1 transcript and LOX were significantly increased after 11 days in a 3-D environment ($p < 0.05$) and that COL1A1 gene was also detectable. In addition, increased expression of SM α-actin, indicative of a myofibroblast (activated, migratory, contractile) phenotype was observed in ADSCs after 11 days in the scaffolds ($p < 0.05$). Real-time PCR analysis of BmMSCs seeded for 5 or 11 days did not show any significant difference in the examined genes.

4. Discussion

This study demonstrates for the first time that ADSCs have the ability to synthesize and process ECM components suitable for tissue engineering a heart valve. In our experiments ADSCs were isolated in large quantities which could potentially eliminate the need for extensive *in vitro* expansion and expressed many phenotypic markers common to BmMSCs and VICs [11]. The evaluation of the responsiveness of ADSCs to mechanical stress and their ability to mediate matrix remodelling showed the expression of two major

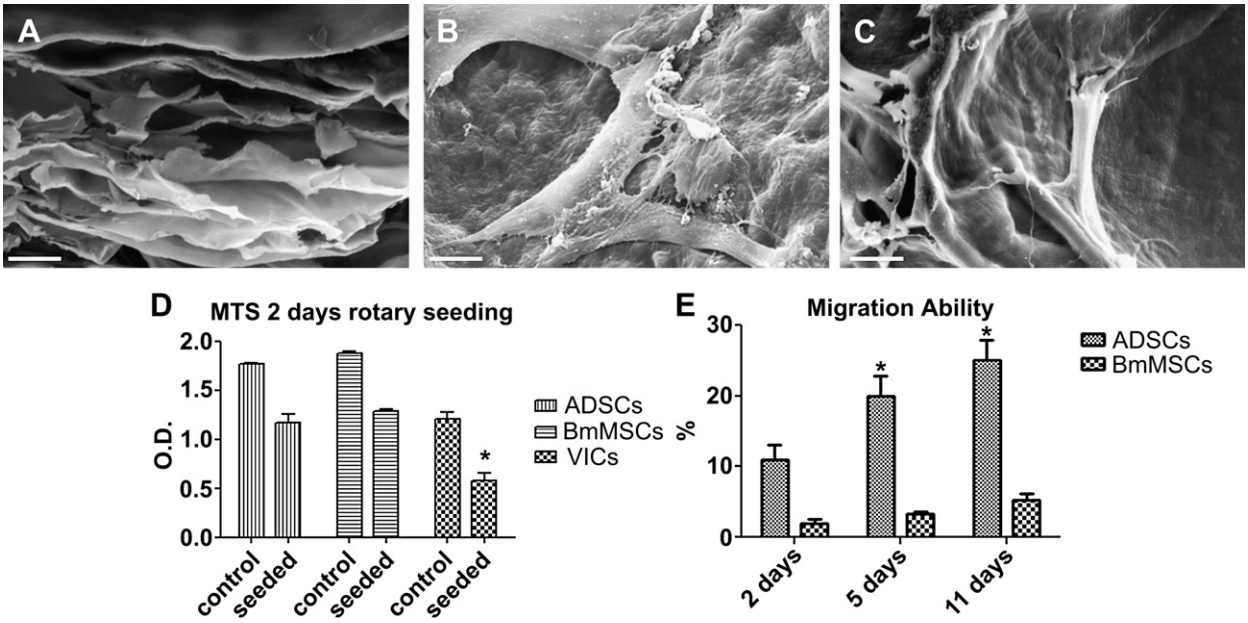


Fig. 5. Scaffold Seeding: Cell Viability and Migration. Scanning electron micrographs of (A) unseeded-, (B) ADSC- and (C) BmMSCs-seeded scaffold showing adherent cells. (D) MTS results demonstrating cells remained viable for the duration of the experiment: number of viable hVICs decreased when compared with cells cultured in tissue culture plates ($* = p < 0.05$). (E) Quantification of the percentage of ADSCs and BmMSCs that migrated into the scaffolds with time. Scale bar = 10 μm.

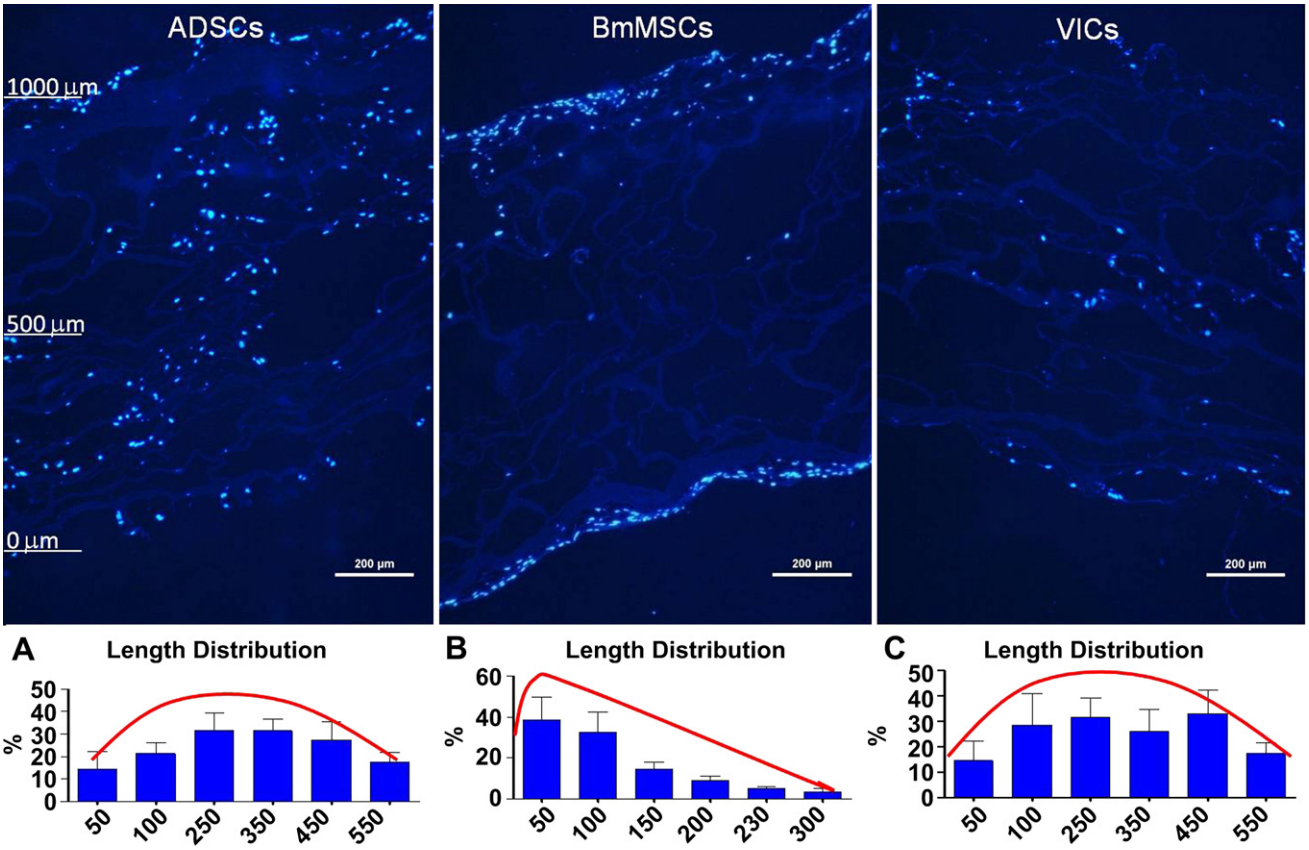


Fig. 6. Distribution of cells in collagen scaffolds. Quantification of the percentage distribution of (A) ADSCs-, (B) BmMSCs- and (C) hVICs after 5 days seeding with DAPI labelled cells using histomorphometric analysis. The comparison in cell number determined by image processing software, showed a different distribution by the different cells. Scale bar = 200 μm.

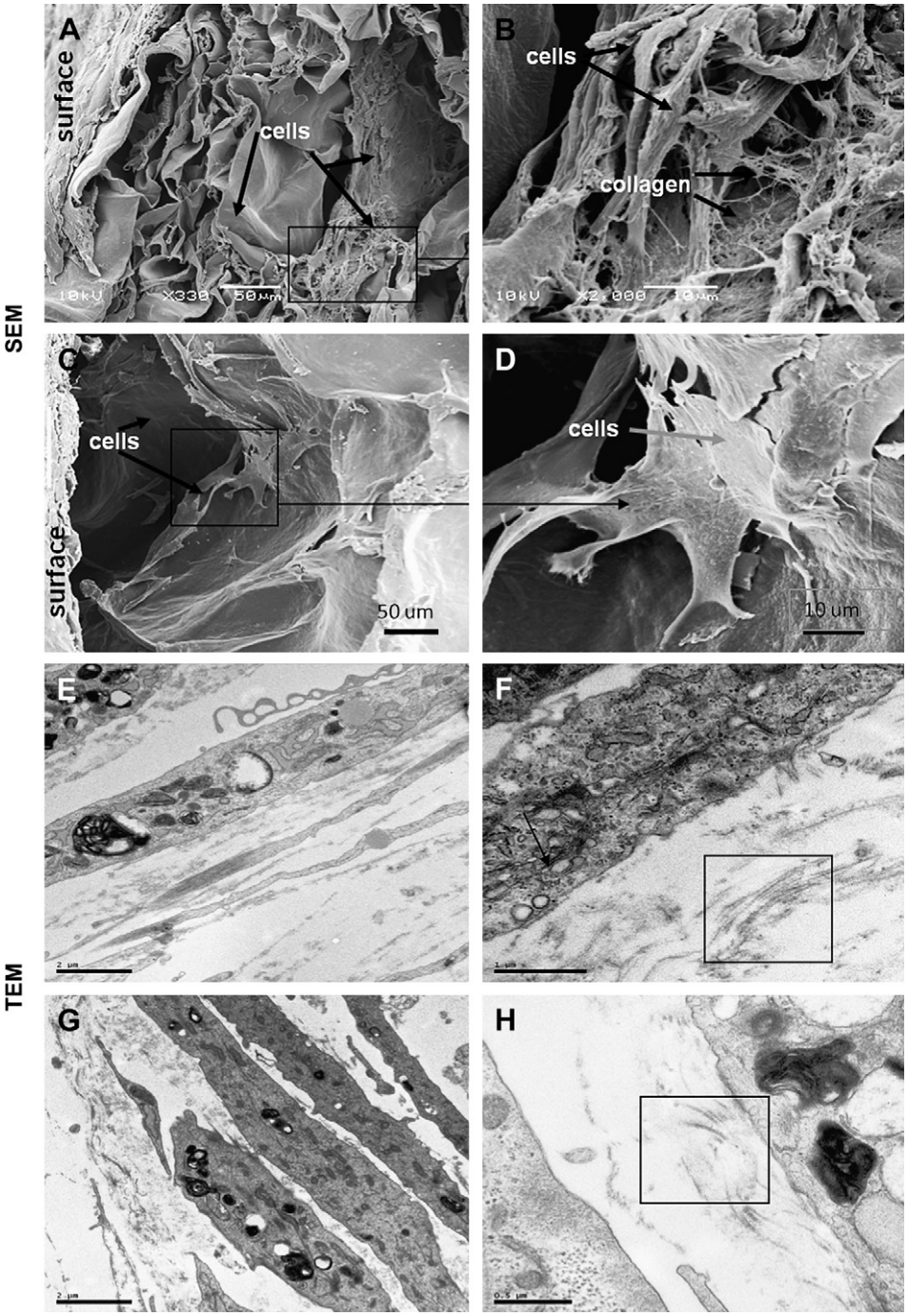


Fig. 7. Scaffold Seeding: Collagen Production. Scanning electron micrographs of ADSCs (A, B) and BmMSCs (C,D) show cell migration and collagen production after 20 days seeding. The surface of the scaffold is indicated in A & C. A shows ADSCs had migrated through the scaffold and B (higher magnification of boxed area of A) shows the meshwork of fibers associated with cells indicating newly formed collagens fibres. C show that BmMSCs are localised near the surface of the scaffold and D shows a higher magnification of the boxed area of C. Transmission electron micrographs of ADSCs (E,F) and BmMSCs (G,H) demonstrating secretion of collagens fibers (box in F, H). Arrow in F shows active RER. Scale bar = A, C = 50 μm; B, D = 10 μm 10 E,G = 2 μm; F = 1 μm; H = 0.5 μm.

collagen genes, COL1A1 and COL3A1, at the level of the mRNA in response to 14% stretch and demonstrated that ADSCs respond to their mechanical environment in a similar manner to VICs. In this respect, the ability to secrete collagen represents a fundamental mechanism whereby the cusp can continue to maintain the load-bearing structures that allow the valve to function adequately. The potential for mesenchymal stem cells to reproduce the function of VICs is important for the replication of native valve function in a tissue engineered heart valve.

Molecular analysis suggested that an increased expression of COL3A1 gene was a factor in the enhanced incorporation of [3H]-proline observed in ADSCs and VICs. Collagen is the most abundant protein in cardiovascular tissue and is secreted by cells to provide tensile strength, as an organizational scaffold. Type I and III collagens are present in valve cusps in the ratio 3:1. In addition, about 2% of the collagen present is collagen V. These are fibrillar collagens that form banded fibrils and provide tissues with tensile strength, as well as influence cell attachment and migration. Type I collagen is

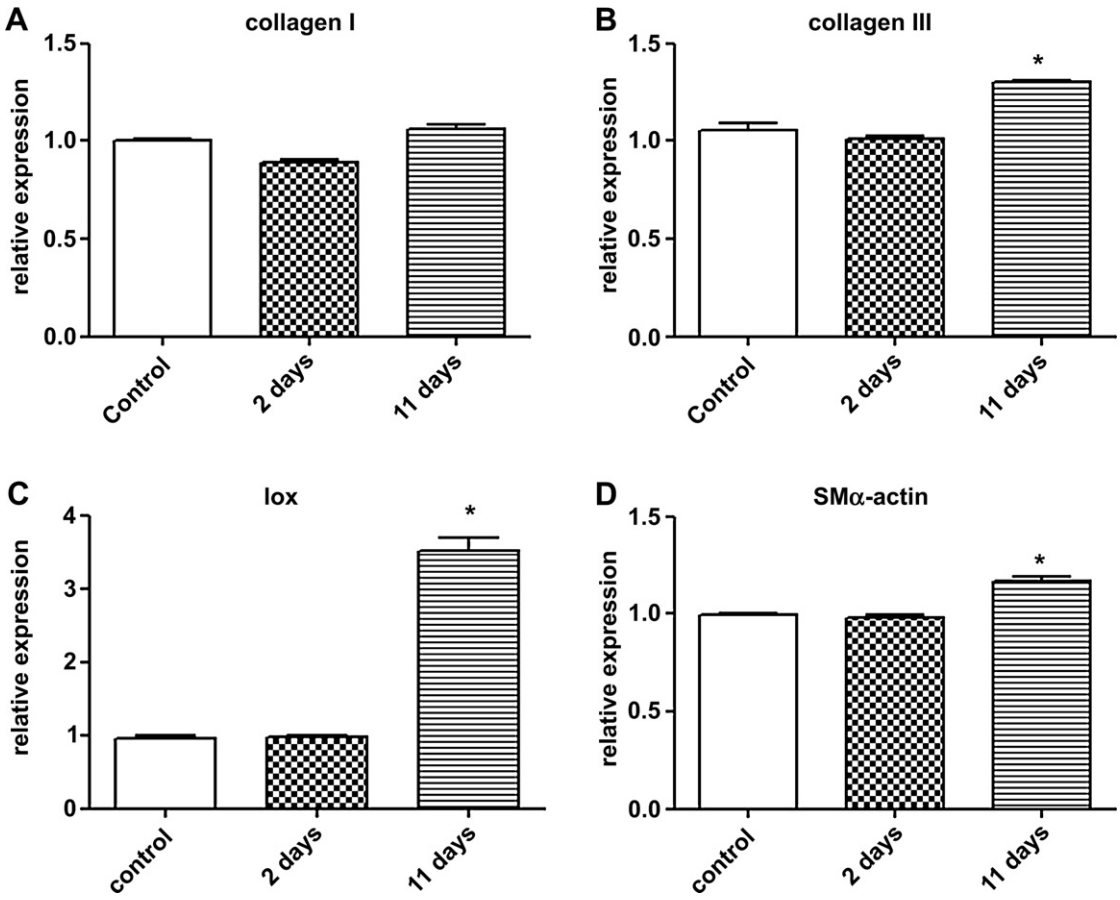


Fig. 8. Scaffold Seeding: Gene Expression Analysis. Real-time PCR analysis of COL1A1, COL3A1, LOX and SMα-actin in ADSCs seeded for 2 or 11 days on collagen scaffold (3-D) compared with ADSCs cultured in tissue culture plates (2-D, control). Results are expressed as mean ± S.D. ($n = 3$). ($^* = p < 0.05$).

the most abundant and widely distributed collagen in the body and is synthesised in response to injury. Type III collagen is similar in structure to type I, but less abundant, and usually occurs in the same fibril with type I collagen. The ability to secrete collagen represents a fundamental mechanism whereby the cusp can continue to maintain the load-bearing structures that allow the valve to function optimally. Interestingly, type III collagen is often encountered in areas of rapid new collagen synthesis [24]. Our results with ADSCs are in agreement with studies using VICs and cardiac fibroblasts that have reported an increased level of collagen type III mRNA, but not of collagen type I in response to cyclic mechanical stretch [25].

In our study we also took into consideration the maturation of collagen since crosslinking is essential for tensile strength and mechanical stability of the collagen microfibrils and protects them against enzymatic degradation [26]. Crosslinking is either enzymatically or non-enzymatically mediated. The enzymatic process, mediated by lysyl oxidase (LOX), results in the formation of pyridinoline and deoxypyridinoline trivalent collagen crosslinks [27,28]. Non-enzymatic collagen crosslinking occurs via spontaneous condensation of arginine, lysine and free sugars. LOX is also responsible for catalysing the crosslinking and stabilisation of elastin resulting in the formation of desmosine, which is essential for the development of functional elastic fibres. Mass spectrometry analysis showed that ADSCs were more efficient than BmMSCs in crosslinking collagen and elastin in response to stretch suggesting also that mechanical stimulation in a 2-D environment enhanced crosslinking of deposited matrix proteins. However expression of LOX mRNA by ADSCs was not increased by stretch and was expressed by both cell types to a similar extent, suggesting that other factors (e.g. LOX activity regulators) may be responsible for the increased crosslinking after stretch. Elastic fibers are a critical structural component in heart valve and several studies illustrate the complexity of elastogenesis, which encompasses gene transcription, post-transcriptional regulation, and coordinated assembly of multiple molecules within a receptive extracellular milieu [29]. However in our experiments, mass spectrometry evaluation demonstrated that ADSCs indeed had the ability to produce and process elastin when stimulated.

The principle of tissue engineering is to seed and culture cells from the recipient onto a carrier material, the scaffold, which provides support for the 3-D growth of cells in an organized way. In this study we also observed that ADSCs were able to populate 3-D type I collagen scaffolds and had the potential to produce and remodel their own matrix within the scaffold. When seeded onto the scaffold, ADSCs showed not only a more favourable propensity to attach and to populate it in exponential manner for the duration of the experiment compared with BmMSCs, but also the ability to produce matrix. With particular regards to collagen, cells after 11 days expressed COL1A and displayed a significant increase in COL3A genes, also the increase in LOX expression suggested that the cells were able to remodel the newly formed collagens fibers that became visible by TEM analysis both intracellularly and extracellularly after 20 days on the scaffold. In particular the newly formed fibers observed extracellularly aligned along the cell axes that seemed to direct their position and orientation. When cultured on the scaffold, cells showed a change in phenotype as seen from the increase in SMα-actin expression suggesting cells were becoming activated, migratory and myofibroblast-like. The superior

migration of the ADSCs into the scaffolds may be a consequence of this increased SMα-actin expression. Since migratory cells require enzymes such as MMPs to allow them to migrate through the matrix, experiments are in progress to assess if these migratory ADSCs would possess a greater activity of those enzymes. In addition, because the optimization of the collagen and elastin architecture is essential for the creation of a functional load-bearing valve replacement, supplementary longer-time studies are required to investigate the expression and activity of enzymes involved in collagen and elastin synthesis and processing as well as enzymes such as matrix metalloproteases and cathepsins that regulate collagen breakdown.

5. Conclusions

In summary these results present for the first time ADSCs as candidate cells for heart valve tissue engineering. In particular because of their capacity to synthesize and remodel extracellular matrix and to respond to a change in biophysical and biochemical stimuli we here defined a potential role for these cells in tissue engineering. These experiments provide useful information for the development of tissue engineered heart valves and highlight the importance of appropriate mechanical and environmental cues during *in vitro* conditioning. However in order to fully understand the role of ADSCs in cardiovascular tissue engineering, additional studies possibly involving animal models will be required to assess their efficacy.

Acknowledgements

This research was supported in part by IRCCS Policlinico San Donato and in part by the Magdi Yacoub Institute. Authors thank Basim A. Matti M.D. from the Division of Plastic Surgery at Harley Street Clinic, London (UK) for providing adipose tissue samples; Dr Ivan Carubelli from IRCCS Policlinico San Donato, Milan (IT) and Dr. Puspa Batten for their technical assistance.

Appendix

Figure with essential color discrimination. Fig. 6 in this article is difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.09.003.

References

[1] Sacks MS, Schoen FJ, Mayer JE. Bioengineering challenges for heart valve tissue engineering. Annu Rev Biomed Eng 2009;11:289–313.
[2] Baaijens F, Bouten C, Driessen N. Modeling collagen remodeling. J Biomech 2010;43(1):166–75.
[3] Vesely I. The role of elastin in aortic valve mechanics. J Biomech 1998;31(2):115–23.
[4] Adamczyk MM, Lee TC, Vesely I. Biaxial strain properties of elastase-digested porcine aortic valves. J Heart Valve Dis 2000;9(3):445–53.
[5] Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, et al. Human semilunar cardiac valve remodeling by activated cells from fetus to adult: implications for postnatal adaptation, pathology, and tissue engineering. Circulation 2006;113(10):1344–52.
[6] Dreger SA, Thomas P, Sachlos E, Chester AH, Czernuszka JT, Taylor PM, et al. Potential for synthesis and degradation of extracellular matrix proteins by valve interstitial cells seeded onto collagen scaffolds. Tissue Eng 2006;12(9):2533–40.
[7] Mulholland DL, Gotlieb AI. Cell biology of valvular interstitial cells. Can J Cardiol 1996;12(3):231–6.
[8] Taylor PM, Allen SP, Yacoub MH. Phenotypic and functional characterization of interstitial cells from human heart valves, pericardium and skin. J Heart Valve Dis 2000;9(1):150–8.
[9] Taylor PM, Batten P, Brand NJ, Thomas PS, Yacoub MH. The cardiac valve interstitial cell. Int J Biochem Cell Biol 2003;35(2):113–8.
[10] Ku CH, Johnson PH, Batten P, Sarathchandra P, Chambers RC, Taylor PM, et al. Collagen synthesis by mesenchymal stem cells and aortic valve interstitial cells in response to mechanical stretch. Cardiovasc Res 2006;71(3):548–56.
[11] Latif N, Sarathchandra P, Thomas PS, Antoniw J, Batten P, Chester AH, et al. Characterization of structural and signaling molecules by human valve interstitial cells and comparison to human mesenchymal stem cells. J Heart Valve Dis 2007;16(1):56–66.
[12] Bieback K, Kern S, Kocaomer A, Ferlik K, Bugert P. Comparing mesenchymal stromal cells from different human tissues: bone marrow, adipose tissue and umbilical cord blood. Biomed Mater Eng 2008;18(1 Suppl.):S71–6.
[13] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13(12):4279–95.
[14] Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM, et al. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem 2006;99(5):1285–97.
[15] Seo MJ, Suh SY, Bae YC, Jung JS. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun 2005;328(1):258–64.
[16] Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy 2003;5(5):362–9.
[17] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284(5411):143–7.
[18] Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112(8):1128–35.
[19] Slominska EM, Adamski P, Lipinski M, Swierczynski J, Smolenski RT. Liquid chromatographic/mass spectrometric procedure for measurement of NAD catabolites in human and rat plasma and urine. Nucleosides Nucleotides Nucleic Acids 2006;25(9–11):1245–9.
[20] Felkin LE, Birks EJ, George R, Wong S, Khaghani A, Yacoub MH, et al. A quantitative gene expression profile of matrix metalloproteinases (MMPS) and their inhibitors (TIMPS) in the myocardium of patients with deteriorating heart failure requiring left ventricular assist device support. J Heart Lung Transpl 2006;25(12):1413–9.
[21] Taylor PM, Sachlos E, Dreger SA, Chester AH, Czernuszka JT, Yacoub MH. Interaction of human valve interstitial cells with collagen matrices manufactured using rapid prototyping. Biomaterials 2006;27(13):2733–7.
[22] Taylor PM, Allen SP, Dreger SA, Yacoub MH. Human cardiac valve interstitial cells in collagen sponge: a biological three-dimensional matrix for tissue engineering. J Heart Valve Dis 2002;11(3):298–306.
[23] Balguid A, Driessen NJ, Mol A, Schmitz JP, Verheyen F, Bouten CV, et al. Stress related collagen ultrastructure in human aortic valves–implications for tissue engineering. J Biomech 2008;41(12):2612–7.
[24] Dawes KE, Cambrey AD, Campa JS, Bishop JE, McAnulty RJ, Peacock AJ, et al. Changes in collagen metabolism in response to endothelin-1: evidence for fibroblast heterogeneity. Int J Biochem Cell Biol 1996;28(2):229–38.
[25] Carver W, Nagpal ML, Nachtigal M, Borg TK, Terracio L. Collagen expression in mechanically stimulated cardiac fibroblasts. Circ Res 1991;69(1):116–22.
[26] Riem Vis PW, Kluin J, Sluijter JP, van Herwerden LA, Bouten CV. Environmental regulation of valvulogenesis: implications for tissue engineering. Eur J Cardiothorac Surg, 2010, doi:10.1016/j.ejcts.2010.05.032.
[27] Buckley A, Hill KE, Davidson JM. Collagen metabolism. Methods Enzymol 1988;163:674–94.
[28] Rucker RB, Kosonen T, Clegg MS, Mitchell AE, Rucker BR, Uriu-Hare JY, et al. Copper, lysyl oxidase, and extracellular matrix protein cross-linking. Am J Clin Nutr 1998;67(5 Suppl.):996S–1002S.
[29] Long JL, Tranquillo RT. Elastic fiber production in cardiovascular tissue-equivalents. Matrix Biol 2003;22(4):339–50.