



This is the author's version published as:

Reichert, Johannes C. and Woodruff, Maria A. and Friis, Thor and Quent, Verena M.C. and Gronthos, Stan and Duda, Georg N. and Schutz, Michael A. and Hutmacher, Dietmar W. (2010) *Ovine bone and marrow derived progenitor cells : isolation, characterization, and osteogenic potential*. Journal of Tissue Engineering and Regenerative Medicine, 4(7). pp. 565-576.

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Ovine bone and marrow derived progenitor cells: Isolation, Characterization, and Osteogenic Potential

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Key words: Mesenchymal progenitor cells, osteogenic potential, scaffold, bone tissue engineering,

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Abstract

Recently, research has focused on bone marrow derived multipotent mesenchymal precursor cells (MPC) for their potential clinical use in bone engineering. Prior to clinical application, MPC-based treatment concepts need to be evaluated in preclinical, immunocompetent, large animal models. Sheep in particular are considered a valid model for orthopaedic and trauma related research. However, ovine MPC and their osteogenic potential remain poorly characterized. In the present study, *ex vivo* expanded MPC isolated from ovine bone marrow proliferated at a higher rate than osteoblasts (OB) derived from tibial compact bone as assessed using standard 2D culture. MPC expressed the respective phenotypic profile typical for different mesenchymal cell populations (CD14⁻/CD31⁻/CD45⁻/CD29⁺/CD44⁺/CD166⁺) and showed a multilineage differentiation potential. When compared to OB, MPC had a higher mineralization potential under standard osteogenic culture conditions and expressed typical markers such as osteocalcin, osteonectin and type I collagen at the mRNA and protein level. After 4 weeks in 3D culture, MPC constructs demonstrated higher cell density and mineralization, whilst cell viability on the scaffolds was assessed >90%. Cells displayed a spindle-like morphology and formed an interconnected network. Implanted subcutaneously into NOD/SCID mice on type I collagen coated polycaprolactone-tricalciumphosphate (mPCL-TCP) scaffolds, MPC presented a higher developmental potential than osteoblasts. In summary, this study provides a detailed *in vitro* characterisation of ovine MPC from a bone engineering perspective and suggests that MPC provide promising means for future bone disease related treatment applications.

Introduction

In general, bone displays a high intrinsic regenerative capacity following insult or disease. Therefore, the majority of bone defects and fractures heal spontaneously, stimulated by well orchestrated endogenous cell populations and micro-environmental cues. Improvements in surgical techniques, implant design and peri-operative management have significantly improved treatment outcomes of complex fractures and other skeletal defects resulting from high energy trauma, disease, developmental deformity, revision surgery, and tumour resection ¹⁻⁶. However, a compromised wound environment, insufficient surgical technique or biomechanical instability can lead to formation of large defects with limited regeneration potential ⁷. Such defects pose a major surgical, socio-economical and research challenge and can significantly influence patients' quality of life ^{8, 9}. Over the years, bone grafts have advanced as the "gold standard" treatment for bone augmentation ^{1, 2, 10-16}. However, the use of autologous bone is associated with additional anaesthetic time and personnel required for graft harvesting ^{12, 14, 17}. Often, insufficient amounts of graft can be obtained while access to donor sites is limited ^{12, 13, 18, 19}. Donor site pain, nerve damage or haemorrhage can occur while donor bone is predispositioned to failure ^{4, 12, 13, 20}. To circumvent these limiting factors, there has been continuous interest in the use of synthetic and naturally derived bone graft substitutes during the past decades. More recently, the concept of tissue engineering has emerged as an important approach to bone related orthopaedic and trauma research. Tissue engineering unites aspects of cellular biology, biomechanical engineering, biomaterial sciences and trauma and orthopaedic surgery. Its general principle involves the association of cells with a natural or synthetic supporting scaffold to produce a three-dimensional, implantable construct.

A number of large animal models have been developed to biomechanically simulate human *in vivo* conditions as closely as possible, and to assess the effects of implanted bone grafts and tissue engineered constructs on segmental long bone defect regeneration. In particular, mature sheep are considered a valuable model for human bone turnover and remodelling activity since animals of 7-9 years of age show similar bone structure and composition, possess a bodyweight comparable to adult humans, and long bone dimensions enabling the use of human implants²¹⁻²⁴. However, published fracture and segmental defect models have often used considerably younger animals²⁵. Whilst, sheep are a well recognized animal model for bone related research, the molecular and cellular events surrounding fracture and bone defect healing remain poorly understood with respect to the recruitment and differentiation of osteogenic precursor cell populations.

In the present study, we hypothesized that ovine marrow derived cells of 7-8 year old animals are equivalent to those previously described for humans. The aims of the project were to isolate, characterize and compare populations of marrow cells derived from the iliac crest with cells from compact bone. Initial characterization of cell properties, phenotype and genotype was performed using techniques already established for human MPC. The potential of *ex vivo* expanded ovine marrow and bone derived cells to produce tissues with properties consistent with those of mature bone was further assessed *in vitro* and *in vivo*. The purpose of this study was to provide a detailed characterisation of ovine MPC and OB prior to their use in a preclinical ovine large animal model in order to evaluate bone tissue regeneration following implantation of autologous marrow or bone derived cells into surgically created tibial bone defects.

Materials and Methods

Isolation of ovine MPC and OB

Ovine osteoblast explants were obtained from 6-7 year old Merino sheep undergoing experimental surgery as approved by the animal ethics committee of the Queensland University of Technology, Brisbane, Australia (ethics number 0700000915). Compact tibial bone samples were collected under sterile conditions, minced, washed with PBS (Invitrogen) and vortexed prior to being incubated with 10 ml 0.25% trypsin/EDTA (Invitrogen) for 3 min at 37°C, 5% CO₂. After trypsin inactivation with 10 ml low glucose Dulbecco's Modified Eagle Media (DMEM) containing 10% foetal bovine serum (FBS) (Invitrogen), samples were washed once with PBS and transferred to 175 cm² tissue culture flasks (Nunc). Samples were topped-up with 12 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin. Osteoblast outgrowth could be observed after 5-7 days. Cells were expanded to the second or third passage for subsequent experiments.

Bone marrow aspirates were obtained from the iliac crest under general anaesthesia. Total bone marrow cells ($0.5-1.5 \times 10^7$ cells/ml) were plated at a density of $1-2 \times 10^7$ cells/cm² in complete medium comprising low glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subsequently plated at a density of 10^3 cells/cm².

Flow cytometric analysis

Ex vivo expanded populations were used at passage three of culture for immunophenotypic analysis. *Ex vivo* expanded MPC or OB were treated with trypsin/EDTA and resuspended in blocking buffer for 30 min. Individual tubes containing 1×10^5 cells were incubated with murine monoclonal IgG antibodies reactive to either ovine CD14, CD31, and CD45 (Serotec), ovine and human CD29, CD44 (Clone H9H11; Division of Haematology, IMVS, Adelaide, SA, Australia), and CD166 (BD Biosciences), or isotype matched controls, 1B5 (IgG₁), and 1A6.11 (IgG_{2b}) at a concentration of 10 µg/ml for 1 h on ice. After washing, cells were incubated with secondary detection reagents, goat anti-mouse IgG-FITC or IgM-FITC conjugated antibodies (1:50; Southern Biotechnology Associates, Inc., Birmingham, AL) for 45 min on ice. Following washing, samples were analysed using a Cytomics FC 500 flow cytometry system (Beckman Coulter).

Cell proliferation assay

Adherent passage three MPC and OB were seeded in triplicates at 3000/cm² in flat bottomed 24-well plates (Nunc) and maintained in 1 ml standard culture medium consisting of low glucose DMEM supplemented with 10% FBS for 1, 3, 5 or 7 days in a humidified atmosphere (37°C, 5% CO₂). At each time point, cells were washed twice with PBS and stored at -80°C until analysis. For analysis, samples were digested overnight with 0.5 mg/ml proteinase K in 1 x TE at 55°C. DNA content for 100 µl of each sample in triplicate were measured and quantified using a Quant-iT PicoGreen dsDNA assay kit according to the protocol supplied by the manufacturer (Invitrogen). An equal volume of the Quant-iT PicoGreen aqueous working solution was added to each triplicate and incubated for 3 min on a rocking

plate. Fluorescence was measured with a Polar Star Optima plate reader (BMG Labtech, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

CFU-F clonogenic assay

CFU-F assays were performed as described previously^{26 27}. Briefly, passage two sheep osteoblasts (OB) and bone marrow derived, mesenchymal progenitor cells (MPC) were plated at a density of 0.25×10^4 in six-well plates. Cells were maintained in low glucose DMEM supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% or 20% FBS respectively, at 37°C with 5% CO₂ for 6 days. To enumerate colonies, cultures were washed with PBS, fixed in ice cold methanol for 15 min, and stained with 0.05% w/v crystal violet in dH₂O for 15 min. Stained aggregates of greater than 50 cells were scored as CFU-colonies under a light microscope (TS100-U, Nikon, Melville, NY).

2D differentiation in vitro

Passage three MPC and OB were seeded in triplicate into 6-well plates (Nunc) at a density of 3000 cells/cm² and expanded in low glucose DMEM/10% FBS (Invitrogen) until confluent. Osteogenic induction was then performed over the following 28 days using DMEM/10% FBS supplemented with 50 µg/ml ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.1 µM dexamethasone (Sigma-Aldrich). Controls were cultured in standard expansion medium (DMEM/10% FBS).

Alkaline phosphatase activity

At day 14 and 28, ALP enzyme activity was quantified using a colorimetric assay. Triplicates were washed with PBS, incubated with 0.1% Triton X in 0.2 M Tris buffer at -20°C for 10 min. Cells were harvested and centrifuged at 10000 rpm for 10 min at 4°C and 100 µl of the cell extraction supernatant was incubated with 125 µl p-Nitrophenylphosphate (1mg/ml) in 0.2 M Tris buffer (Sigma-Aldrich) in 96 well plates (Nunc) and OD was measured after 30 min at 405 nm in a Polar Star Optima plate reader. ALP activity was normalized against the sample DNA content determined using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen).

Alizarin red staining

To determine matrix mineralization, at day 14 and 28, triplicate samples were washed twice with PBS and fixed with ice cold methanol for 10 min at room temperature. Samples were then washed twice with ddH₂O and incubated with 1% alizarin red s (Sigma-Aldrich) in ddH₂O, pH 4.1 for 10 min with gentle shaking. After aspiration of the unincorporated dye, samples were washed three times with ddH₂O and air dried. Stained monolayers were documented using inverted phase microscopy (TS100-U, Nikon, Melville, NY). For quantification of staining, 800 µl 10% (v/v) acetic acid was added to each well, and the plate incubated at room temperature for 30 min with shaking. The monolayer was then scraped from the plate with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5 ml microcentrifuge tube. After vortexing for 30 s, the slurry was overlaid with 500 µl mineral oil (Sigma-Aldrich), heated to 85°C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 x g for 15 min and 500 µl of the supernatant was removed to a new 1.5 ml microcentrifuge tube. Then 200 µl of 10%

(v/v) ammonium hydroxide was added to neutralize the acid. Aliquots (150 µl) of the supernatant were read in triplicate at 405 nm in 96-well format using opaque-walled, transparent-bottomed plates (Nunc). Obtained values were normalized against the DNA content of separate samples since treatment with acetic acid was expected to cause denaturation of DNA.

Immunohistochemistry

For immunohistochemistry, OB and MPC were cultured on Thermanox coverslips to fit 24-well plates (Nunc). Media was removed; samples were washed twice with PBS and fixed in 4% paraformaldehyde for 1 h on ice. Cells were then permeabilized with 0.1% Triton X in PBS for 5 min and quenched with 0.15 M glycine in PBS for 15 min (Sigma-Aldrich). Samples were blocked with 1% BSA (Sigma-Aldrich) in PBS for 60 min and incubated with primary mouse anti-human type I collagen (1:100) (MP Biomedicals, Irvine, CA) and mouse anti-bovine osteocalcin (1:500) (Takara Bio Inc., Japan) antibodies in 1% BSA in PBS for 1 h at room temperature. Samples were then washed three times with 0.1% BSA in PBS for 5 min each wash and incubated with a FITC-conjugated goat anti-mouse secondary antibody (Invitrogen) at a concentration of 1:200 for 30 min. Cover slips were then mounted on glass microscope slides and visualized using a fluorescent microscope (TE2000-U, Nikon, Melville, NY).

Total RNA isolation, primer design and qRT-PCR

Total RNA was harvested from triplicate wells, from both differentiated and control cells, on days 7, 14, 21 and 28. Cells were washed twice with PBS and lysed in 1 ml Trizol reagent (Invitrogen) and RNA isolated following the manufacturer's instructions. cDNA was synthesized from 1µg of total RNA using SuperScript III (Invitrogen) according to the manufacturer's instructions. Sheep specific oligonucleotides (Geneworks, TheBarton, SA, Australia) were designed according to these parameters: 20-30 nt in length; melting temperature 60°C, +/- 2°C; at least one primer spanning an exon boundary; amplicon length 150 nt, +/- 50 nt; GC content between 40 and 60%; and at the 3' end a C, G, CG or GC. In those cases where an *Ovis aries* mRNA transcripts was not available, a BLAST search was performed on the International Sheep Genome Consortium database (<https://isgdata.agresearch.co.nz/>) using the equivalent human mRNA transcript. To date, the exon boundaries of sheep transcript have not been annotated; these boundaries were therefore based on exon boundary information for human and mouse mRNA transcripts. Quantitative RT-PCR was performed on an Applied Biosystems 7900HT FAST Real Time PCR system (Applied Biosystems, Scoresby, VIC, Australia) using a 384-well plate layout; templates and reagents were aliquoted using an Eppendorf 5075 epMotion pipetting robot (Quantum Scientific, Murarrie, QLD, Australia). The reaction volumes per well were as follows: 5 µl 2X SYBR Green (Roche, Castle Hill, NSW, Australia), 1 µl forward and reverse primers at 1 µM final concentration, 1 µl water, 2 µl cDNA template diluted 1:10 from stock. The thermocycling conditions were as follows: 1 cycle of 10 min at 95°C for activation of the polymerase, 40 cycles of 10 sec at 95°C and 1 min at 60°C for amplification. Dissociation curve analysis was carried out to verify the absence of primer dimers

and/or non-specific PCR products. The expression of the genes of interest was normalized against the GAPDH housekeeping gene.

3D cultures

Fused deposition modelling was used to fabricate circular mPCL-TCP scaffolds of 5 mm diameter and 3 mm thickness. Type I rat tail collagen (Vitrogen 100, Cohesion, Palo Alto, CA) was lyophilized into the pore space forming a microporous mesh throughout the polymer. For 3D cultures, 120.000 ovine MPC or OB suspended in 60 μ l of basal medium were seeded onto each type I collagen coated mPCL-TCP scaffolds and placed in an incubator. After 1 h, 1 ml of medium was added to each 24-well. Cell scaffold constructs were cultured in DMEM/20% FBS supplemented with 50 μ g/ml ascorbate-2-phosphate, 10 mM beta-glycerophosphate, 0.1 μ M dexamethasone on a rocking plate ($f=0.125$ Hz) for up to 4 weeks.

SEM

Cell scaffold constructs were fixed with 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer solution (pH 7.3) for 1 h at 4°C. Fixed specimens were then dehydrated through a series of alcohols; two changes each of 50%, 70%, 90%, and 100% ethanol and were incubated for 10 min between each change. Specimens were then critical point dried (Denton Vacuum, Moorestown, NJ) and gold coated in a SC500, Bio-Rad sputter coater (Bio-Rad) before examination using a FEI Quanta 200 scanning electron microscope (FEI, Hillsboro, OR).

Confocal laser microscopy

To assess cell viability and morphology of MPC and OB seeded onto type I collagen coated mPCL-TCP scaffolds, samples were stained with fluorescein diacetate (FDA) and propidium iodide (PI)(Invitrogen) or rhodamine conjugated phalloidin and 4',6-diamidino-2-phenylindole (DAPI)(Invitrogen). For FDA-PI staining, samples were rinsed 3 times with PBS and incubated with FDA staining solution (2 µg/ml) at 37°C for 15 min in the dark. FDA is a cell-permeant esterase substrate which is hydrolysed by living/viable cells to give green fluorescence. Samples were then rinsed 3 times with PBS and incubated with PI staining solution (20 µg/ml) at room temperature for 2 min in dark. PI is actively excluded by live cells thus dead cells in a population are stained red. Samples were again rinsed 3 times with PBS and visualised with a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

For phalloidin-DAPI staining, samples were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature with gentle rocking. Samples were then washed twice for 5 min with 1 ml PBS at room temperature. 700 µl rhodamine-conjugated phalloidin (0.8 U/ml in 1% BSA in PBS) was added to each sample and incubated for 1 h at room temperature with gentle rocking. Phalloidin binds to F-actin. Samples were then washed twice for 5 min with 1 ml PBS at room temperature and nuclei were stained with DAPI staining solution (1.0 µg/ml in PBS) for 40-50 min at room temperature. Samples were washed twice for 5 min with 1 ml PBS at room temperature and visualised with a Leica SP5 confocal microscope.

In vivo transplantation studies

Ovine MPC and OB were seeded onto type I collagen coated mPCL-TCP scaffolds at a density of 120.000 cells/scaffold and cultured for 4 weeks in DMEM/20% FBS supplemented with 50 µg/ml ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.1 µM dexamethasone on a rocking plate (f=0.125 Hz). The cell scaffold constructs were then transplanted subcutaneously into both left and right side pockets formed in the dorsal surface of 10-week-old immunocompromised NOD/SCID mice (ARC, Perth, WA, Australia). Implants were recovered after 8 weeks and fixed in 4% paraformaldehyde.

µCT analysis

Micro CT analysis was performed on both the *in vitro* constructs and the *in vivo* constructs. After 4 weeks of *in vitro* culture, constructs were carefully removed from each well and inserted into polycarbonate sleeves for micro-CT analysis. *In vitro* mineralization within the constructs was quantified using a Micro-CT 40 scanner (Scanco Medical, Brüttisellen, Switzerland) at a voxel size of 6 µm. Samples were evaluated at a threshold of 72, a filter width of 3.0 and filter support of 5.0. *In vivo* transplanted constructs were scanned at a voxel size of 16 µm and were evaluated at a threshold of 140, a filter width of 1.0 and filter support of 2. X-ray attenuation was correlated to sample density using a standard curve generated by scanning hydroxyapatite phantoms with known mineral density. Mineralized matrix volume or

bone volume fraction, and mineral density were quantified throughout the entire construct.

Histology

For histological examination, specimens were fixed in 4% paraformaldehyde, and dehydrated using an ethanol gradient (30 min in 70%, 1h in 90%, 95% and 100% ethanol). The samples were then processed through xylenes for 40 min three times, infiltrated with MMA for 3h and embedded in MMA containing 3% PEG. Seven micrometre sections were cut with an osteomicrotome (SM2500; Leica Microsystems, Wetzlar, Germany), stretched with 70% ethanol onto a polylysine coated microscope slide (Lomb Scientific), overlaid with a plastic film and slides were clamped together before being dried for 12 h at 60°C. Sections were then stained using combined von Kossa and van Giesen ²⁸ stains to visualise the mineralised bone and connective tissue respectively.

Image analysis

Histology sections were quantified using Image J software to quantify the amount of mineralisation in a given area of section. Briefly, a JPEG image of the entire tissue section was selected, converted to grayscale and a scale bar was calibrated onto the image. The entire tissue section area was then calculated by segmenting the entire tissue region from the background, and then measuring the area. Next, only the mineralised (black) area was segmented from the entire tissue

area, and measured. The total mineralised area was then calculated as a percentage of the total section area. Six sections were analysed per sample group.

Statistical analysis

Statistical analysis was carried out using the student's *t* test and *p* values < 0.05 were considered significant.

Results

MPC show a higher proliferation rate than OB in vitro

Cells isolated from ovine bone marrow showed a significantly higher proliferative potential after 1,3 and 5 days in culture ($p < 0.05$) when compared to ovine tibial osteoblasts as represented by the higher DNA content per 24-well for MPC (Fig. 1 A). After 6-7 days both MPC and OB entered a plateau phase indicative for contact inhibition of cells reaching confluency ($p = 0.0502$).

MPC and OB exhibit a similar immunophenotype

Fluorescence-activated cell sorting (FACS) analysis was performed to characterise the phenotype of *ex vivo* expanded ovine bone marrow derived MPC and OB. The two cell populations exhibited similar expression patterns for CD29 (β_1 -Integrin), CD44, CD166 (ALCAM) and CD14 (LPS-R). CD44 and CD166 have previously been identified as markers associated with human bone marrow stromal,

adipose, and dental pulp stem cells ^{26, 29-31}. Importantly, both populations did not react with hematopoietic markers CD45 (common leukocyte antigen) and CD31 (PECAM-1, endothelial) (Fig. 2).

Clonogenic efficiency of MPC

The mean frequency of CFU-F derived from marrow aspirates was 2.5 ± 0.5 per 0.25×10^4 mononuclear cells (MNC) in both DMEM/10% FBS and 20% FBS, with the incidence of CFU-F forming MSC within the marrow MNC population being approximately 0.08-0.12%. The mean frequency of CFU-F derived from tibial bone explants was 2 ± 0.6 in DMEM/10% FBS and 1.8 ± 0.7 in DMEM/20% FCS respectively (incidence of colony forming cells 0.04-0.12%)(data not shown).

2D differentiation potential of ovine MPC and OB in vitro

The potential of bone marrow derived MPC to differentiate into osteoblasts and of bone derived osteoblasts to secrete a mineralised extracellular matrix was investigated by culturing cells in the presence of L-ascorbic-2-phosphate, dexamethasone, and β -glycerophosphate ³¹. After 4 weeks of induction, cultured MPC and OB had formed extensive amounts of alizarin red-positive mineral deposits throughout the adherent layers. However, OB consistently formed significantly fewer mineralized nodules ($p < 0.05$) compared to bone marrow derived cells (Fig. 1 C, Fig. 3 B and H). Extracellular matrix produced by both MPC and OB stained positive for type I collagen and osteocalcin (Fig. 3 C-F and I-L). ALP activity measured at day 14 and day 28 displayed a typical rise-fall pattern ^{32, 33} and was significantly increased in

osteogenically induced OB and MPC ($p < 0.05$) when compared to their respective controls (Fig. 1 B). Under osteogenic conditions a significant increase in type I collagen expression of 31% could be observed over the course of 4 weeks for MPCs whilst an increase of only 9% was found in the control culture without osteogenic supplements (Fig. 4 A). Osteocalcin expression was up-regulated around day 21 and further increased towards the end of week 4 (23%, 11% in control)(Fig. 4 C). An increase in osteopontin expression could be detected between day 7 and 14 (26%, 5% in control)(Fig. 4 E). For OB, no significant changes in type I collagen expression (5%, 5% in control) were found (Fig. 4 B), only a small increase in osteocalcin (9%, 6% in control) expression (Fig. 4 D). Osteopontin levels slightly increased between day 0 and 7 (12%, 7% in control) to further stay on that level (Fig. 4 F). All percentages compare to base level activity on day 0.

3D differentiation potential of MPC and OB in vitro

Viability, morphology and osteogenic potential of ovine MPC and OB in a three dimensional environment was assessed by FDA/PI staining, phalloidin-DAPI staining, SEM, and micro computed tomography. After 28 days of osteogenic induction under dynamic conditions, cell viability was assessed $>90\%$ (Fig. 5 A and D). Phalloidin-DAPI staining (Fig. 5 B and E) and SEM analysis (Fig. 5 C and F) revealed elongated, spindle-shaped, osteoblast-like cell morphology for both OB and MPC. However, MPC seemed to have proliferated at a higher rate on the type I collagen coated mPCL-TCP scaffolds forming a dense, interconnected three dimensional network. Micro CT analysis displayed mineral deposition throughout the entire thickness of OB and MSC-constructs, compared to control constructs (Fig. 6

A-F). Scaffolds seeded with MPC showed a significantly higher mineral volume fraction (MVF) compared to scaffolds seeded with OB ($p=0.000197$) (Fig. 6 G) while no significant difference in mineral density could be found between MPC and OB-constructs (Fig. 6 H).

Differentiation potential of MPC and OB in vivo

The developmental potential of culture-expanded, ovine MPC and OB was assessed *in vivo* following transplantation into NOD/SCID mice in association with type I collagen coated mPCL-TCP scaffolds. Transplants were recovered after 8 weeks, subjected to micro CT analysis and then processed for histology. Micro CT analysis revealed a significantly higher degree of ectopic bone formation for the scaffolds seeded with MPC prior to implantation (Bone volume fraction: 20.15%) when compared to OB (6.12%) or the respective cell free controls (0.55 %) (Fig. 7 A-C, M). However, no significant difference could be found with regard to the mineral density of newly formed bone matrix (Fig. 7 N).

Histological examination of ectopic explants, using von Kossa staining revealed no mineralisation for the control (no cell) constructs (Fig. 7 D,G,J) whereas both MPC (Fig. 7 E, H, K) and OB (Fig. 7 F, I, L) seeded mPCL-TCP scaffolds formed extensive ectopic bone within the implants, over a course of 8 weeks *in vivo*. Mineral nodules containing calcium, stain black with the von Kossa staining by virtue of silver ions (positive charge) binding with the mineralised tissue (negative portion of the calcium salt) forming a silver salt which is black in colour. The amount of ectopic

bone formed was significantly higher for MPC seeded tissue engineered constructs compared with OB-seeded constructs (Fig. 7 O)($p < 0.05$). Residual mPCL-TCP scaffold was evident within all transplants (Fig. 7 D) evidenced by voids in the tissue from longitudinal and transverse sectioning of the scaffold struts. The infiltration of haematopoietic cells together with associated adipose elements was reminiscent of native bone marrow (Fig. 7). The formation of different tissue types within the transplanted constructs included mineralised bone (mb), fat (f) and fibrous connective tissue (c) with clear blood vessel (bv) formation. The predominantly formed tissue type in all both MSC and OB samples however, was bone with mature osteocytes enclosed in characteristic lacunae surrounded by the bone extracellular matrix.

Discussion

Orthopaedic research frequently necessitates the utilisation of large animal models. Animal models in bone repair research include representations of normal fracture-healing, segmental bone defects, and fracture non-unions³⁴. The selection of a specific animal species as a model system involves the consideration of a number of factors. Physiological and pathophysiological analogies are essential in respect to the scientific question under investigation. It must be manageable to operate and observe a multiplicity of study objects over a relatively short period of time^{22, 35, 36}. Further selection criteria include costs for acquisition and care, animal availability, acceptability to society, tolerance to captivity and ease of housing²³. Mature sheep and goats possess a bodyweight comparable to adult humans and long bone dimensions enabling the use of human implants²¹. The mechanical

loading environment occurring in sheep is well understood ^{37, 38}. Since no major differences in mineral composition ³⁹ are evident and both metabolic and bone remodelling rates are akin to humans ⁴⁰, sheep are considered a valid model for human bone turnover and remodelling activity ⁴¹ and show comparable bone healing potential and bone blood supply ⁴². On a cellular and molecular level however, sheep as a well recognized animal model for bone related research remain poorly characterized and understood, and the aim of this study was to begin to address the shortfalls in literature on this subject.

As described previously, bone marrow aspirates collected from adult sheep contain a proportion of CFU-F forming cells with an incidence and morphology highly similar to human MSC ^{27, 31, 43-46}. The incidence of ovine CFU-F exceeds those described for most other animals, but lie within the normal range reported for human bone marrow CFU-F ^{27, 47-50}.

To date, there is limited information available on the cell surface characteristics of ovine bone marrow derived MPC and bone derived OB. This can mainly be attributed to the limited availability of antibodies specific for and cross-reacting with equivalent sheep antigens ^{27, 47, 51}. However, to validate sheep as a model system for research on a cellular and molecular level, and to provide insight into fundamental processes such as haematopoiesis, cell migration and homing, injury repair, differentiation, and proliferation, the availability of suitable antibodies plays a key role. The cell surface expression profile of ovine MPC and OB showed high and uniform levels of cell surface CD29, CD44 and CD166 which have previously been identified as markers associated with human bone marrow stromal, adipose, and dental pulp cells ^{26, 29-31}. Characteristic for ovine MPC and OB cultures was the absence of expression of the endothelial associated adhesion marker CD31 and

haematopoietic marker CD45 consistent with expression patterns previously described for human bone marrow derived MSC ^{26, 27}.

When compared to ovine OB isolated from compact tibial bone, the cells isolated from the bone marrow showed a higher proliferative potential (Fig. 1 A) indicative for immature progenitor cells. Both MPC and OB followed a normal growth curve reported for different human cell types consisting of a lag phase followed by a log phase of exponential cell growth, ending with a plateau phase in which the growth rate declined ⁵²⁻⁵⁵. The steeper slope of the MPC growth curve between day 1 and 3 after seeding results in a higher density of cells before the rate of growth begins to decline. A high proliferation capacity is desirable when it comes to the application of cell based tissue engineering strategies in preclinical models since large cell numbers generated over a relatively short period of time may be required for various clinical applications.

After 28 days of culture under osteogenic conditions both MPC and OB were shown to produce mineralized extracellular matrix positive for alizarin red, osteocalcin and type I collagen. We showed that MPC and OB can be induced to form mineral in culture by treatment with osteogenic medium *in vitro*. Osteogenic medium contains a source of phosphate, ascorbic acid, and dexamethasone in a rich medium such as α -MEM containing fetal bovine serum (FBS) ⁵⁶. In a chelation process, alizarin red S forms complexes with calcium and therefore allows simultaneous evaluation of mineral distribution and inspection of fine structures by phase contrast microscopy. It is particularly versatile in that the dye can be extracted from the stained monolayer and readily assayed with low variability and a much wider linear detection range than traditional calcium detection methods ^{57, 58}. To monitor inorganic phosphate deposition may be problematic due to the high levels of

contaminating phosphate associated with other components of the cell and the high levels of free phosphate in the cytosol.

The extracellular matrix produced by ovine MPC and OB was shown to stain positive for type I collagen and osteocalcin. Type I type I collagen comprises approximately 95% of the entire collagen content of bone and about 80% of the total proteins present in bone ^{59, 60}. The increase in type I collagen expression on the gene level in MPC and its presence in the extracellular matrix deposited by MPC under osteogenic conditions is therefore consistent with osteogenic differentiation processes. Osteocalcin is synthesized and secreted by normal maturing osteoblasts. It is one of the major non-collagenous bone matrix proteins, with osteocalcin comprising 1% to 2% of the total proteins in the skeleton ⁶¹. Osteocalcin binds with high affinity to hydroxyapatite crystals, the key mineral component of bone, and regulates bone crystal formation ⁶². Osteocalcin can also act as a chemo-attractant in the recruitment of osteoblasts and osteoclasts, contributing to the dynamics of new bone formation and bone resorption ⁶³. Ducy et al. ⁶⁴ reported that osteocalcin-null mice exhibit increased bone formation without impaired bone resorption, suggesting a more complex interaction between recruited osteoblasts and osteoclasts in this process. In the MPC cultures, osteocalcin expression was up-regulated around day 21 and further increased towards the end of week 4 which is consistent with osteocalcin being a late osteogenic marker. Osteocalcin protein was additionally detected immunohistochemically in the ECM produced by both ovine MPC and OB further suggesting a bone like composition. Osteopontin is biosynthesized by a variety of tissue types including pre-osteoblasts, osteoblasts, osteocytes, and bone marrow cells and is considered an early osteogenic marker and was shown to be up-regulated in the ovine MPC cultures early during differentiation. It has further been

implicated as an important factor in bone remodelling ⁶⁵. Specifically, research suggests it plays a role in anchoring osteoclasts to the mineral matrix of bones ⁶⁶. For the OB, no significant changes in type I collagen expression were found, only a small increase in osteocalcin expression. Osteopontin levels increased between day 0 and 7 and remained at this level. The findings suggest that while MPC undergo a differentiation process when supplemented with osteogenic factors, OB – as they are mature cells already – very much maintain their gene expression pattern. Alkaline phosphatase (ALP) is considered a marker for osteoblastic activity *in vitro*. ALP activity measured at day 14 and 28 displayed a typical rise-fall pattern ^{32, 33} and was significantly increased in osteogenically induced OB and MPC ($p < 0.05$) when compared to their respective controls (Fig. 1 B).

Since the extrapolation from results in 2D to cell behaviour in 3D is rather difficult, 3D *in vitro* cultures of ovine MPC and OB were established on medical grade PCL-TCP scaffolds produced via fused deposition modelling. Polymer–calcium phosphate composites confer favourable mechanical and biochemical properties for bone tissue engineering, including strength (ceramic phase), toughness and plasticity (polymer phase), more favourable degradation and resorption kinetics, and graded mechanical stiffness ^{67, 68}. Results obtained from 3D *in vitro* culture confirmed the findings from 2D differentiation studies with MPC demonstrating a higher proliferative and osteogenic potential (Fig. 5 and 6). Micro CT analysis revealed no significant difference in matrix mineral density suggesting that the MPC had undergone an osteogenic differentiation process towards osteoblast like cells actively secreting mineralized matrix.

Preliminary analysis of the *in vivo* osteogenic developmental potential of ovine MPC and OB was undertaken by subcutaneous transplantation into immune-

compromised NOD/SCID mice. Both MPC and OB demonstrated osteogenic potential upon transplantation with type I collagen coated mPCL-TCP composite scaffolds, as indicated by the presence of extensive deposits of ectopic bone (Fig. 7). The observation of ectopic bone, fibrous tissue, and haematopoiesis is analogous with studies using these scaffolds seeded with porcine bone marrow derived progenitor cells⁶⁸.

Conclusion

In summary, in the present study, ovine MPC isolation from bone marrow aspirates and the isolation of OB from cortical bone explants have been standardized. It was found that ovine MPC exhibit morphological, immunophenotypical and multipotential characteristics similar to those in human bone marrow derived MPC. The study represents an essential first step towards the detailed characterization of ovine MPC and OB in translational studies towards the establishment of preclinical *in vivo* models. However, further studies are required to verify the regenerative potential of MPC and OB *in vivo*.

Acknowledgements:

This work was supported by Prof. Hutmacher's QUT start up grant, the Wesley Research foundation and the ARC (grant number 241402-0122.51). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Figure legends

Figure 1: Proliferation capacity, ALP activity and matrix mineralization of MPC and OB. When compared to OB, MPC displayed a higher proliferative potential (A). In osteogenically induced MPC and OB, ALP activity showed a typical rise fall pattern (B). After 28 days of culture in osteogenic media, MPC cultures showed a significant higher degree of mineralization (C).

Figure 2: Surface antigen expression for MPC and OB. The two cell populations exhibited similar expression patterns for CD29 and CD44. CD166 and CD14 was >50% MPC while only low levels were detected on OB. Both populations did not react with the hematopoietic markers CD45 and CD31.

Figure 3: Alizarin red, osteocalcin (OC) and type I collagen staining for MPC and OB cultures after 28 days. Under osteogenic conditions, both MPC and OB secreted a mineralized matrix (B, H) that stained positive for OC (D, J) and type I collagen(F, L).

Figure 4: Quantitative RT-PCR for osteogenic markers. RT-PCR revealed significant increases in type I collagen and osteocalcin expression over 4 weeks for MPC under osteogenic conditions and an increase in osteopontin expression between day 7 and 14. For OB, no significant changes in type I collagen, osteopontin and osteocalcin expression were found.

Figure 5: SEM, live-dead and phalloidin-DAPI staining of MSC and OB on mPCL-TCP scaffolds. Cell viability was assessed >90% for both cell types (A and D).

Phalloidin-DAPI staining (B and E) and SEM analysis (C and F) revealed elongated, spindle-shaped, osteoblast-like cell morphology for both OB and MPC forming a dense, interconnected three dimensional network.

Figure 6: μ CT analysis of 3D *in vitro* cultures. μ CT displayed mineral deposition throughout the entire thickness of all constructs. Scaffolds seeded with MPC (C, D) showed a higher mineral volume fraction (MVF) (G) compared to OB seeded (E, F) or cell free scaffolds (A, B). No significant difference in mineral density was found (H).

Figure 7: Micro CT of *in vivo* specimens revealed significantly more bone formation for MPC compared with OB and control (B, C, A, M). No significant difference in mineral density was observed (N). Histology revealed extensive ectopic bone formation for MPC and OB (E, F, H, I, K, L). Tissue types: mineralised bone (*mb*), muscle (*m*) fat (*f*), blood vessels (*bv*) and fibrous connective tissue (*c*).