The scale-up of a tissue engineered porous hydroxyapatite polymer composite scaffold for use in bone repair: An ovine femoral condyle defect study

Edward Tayton, Matthew Purcell, James O. Smith, Stuart Lanham, Steven M. Howdle, Kevin M. Shakesheff, Allen Goodship, Gordon Blunn, Darren Fowler, Douglas G. Dunlop, Richard O. C. Oreffo

1Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Human Development and Health, University of Southampton Medical School, Southampton, SO16 6YD, United Kingdom
2School of Chemistry, University Park, University of Nottingham, Nottingham, NG7 2RD, United Kingdom
3School of Pharmacy, University Park, University of Nottingham, Nottingham, NG7 2RD, United Kingdom
4Institute of Orthopaedics and Musculoskeletal Science, Brockley Hill, Stanmore, HA7 4LP, United Kingdom
5Department of Histopathology, University Hospital Southampton, Tremona Road, Southampton, SO16 6YD, United Kingdom

Received 28 June 2014; accepted 3 July 2014
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.35279

Abstract: The development of an osteogenic bone graft substitute has important practical and cost implications in many branches of medicine where bone regeneration is required. Previous in vitro and small animal (murine) in vivo studies highlighted a porous hydroxyapatite/poly (L-lactic acid) composite scaffold in combination with skeletal stem cells (SSCs) as a potential bone graft substitute candidate. The aim of the current study was to scale up the bone cell-scaffold construct to large animals and examine the potential for repair of a critical-sized defect via an ovine model. SSC seeded scaffolds (and unseeded scaffold controls) were implanted bilaterally into ovine femoral condyle critical defects for 3 months. A parallel in vitro analysis of ovine SSC seeded scaffolds was also performed. Post mortem mechanical indentation testing showed the bone strengths of the defect sites were 20% (controls) and 11% (SSC seeded scaffolds) those of normal cancellous bone (p<0.01). MicroCT analysis demonstrated new bone formation within all defects with a mean increase of 13.4% in the control scaffolds over the SSC seeded scaffolds (p = 0.14). Histological examination confirmed these findings, with enhanced quality new bone within the control defects. This study highlights important issues and steps to overcome in scale-up and translation of tissue engineered products. The scaffold demonstrated encouraging results as an osteoconductive matrix; however, further work is required with cellular protocols before any human trials.

Key Words: bone regeneration, scaffold, progenitor cell, skeletal stem cell, polymer

INTRODUCTION
The last decade has seen a number of important developments in the tissue engineering field with advances in scaffold development, cell isolation, and demonstration of skeletal tissue repair in animal models. However, to date, relatively few scaffolds have made the transition from laboratory into clinical practice. Hollister et al. state that despite 25 years of research, funding totalling hundreds of millions of dollars, over 12,000 papers on bone tissue engineering and over 2,000 papers on bone tissue engineering scaffolds alone in the past 10 years, “the translation of these therapies to clinical use remains, bluntly, a failure.” This theoretical barrier to progress has been termed the “valley of death,” and is present for a number of reasons, the most pertinent including: (1) The dramatic increase in funding required to scale-up from relatively inexpensive small animal studies to large animal studies, (2) the importance of large animal studies when considering bone regenerative strategies, which introduce a multitude of other confounding factors when scaled-up, (3) the additional substantial funding required for successful large animal trials during the transition from product/technology development to regulatory approval and commercialization.

Despite the difficulties surrounding clinical translation, a number of strategies have been successfully brought to market. Recombinant human bone morphogenetic protein-2
(rhBMP-2) is the most commercially successful tissue engineered product in orthopaedics, accounting for 25% of all spinal fusion procedures. In addition, autologous cartilage implantation (ACI) is now more widely employed, especially in the treatment of cartilage defects in the knee and has also been trialled in other joints such as the ankle. The use of SSCs has also been shown to be of benefit in a cohort of patients in the treatment of fracture nonunions, as well as being a useful adjunct to allograft in patients with femoral head avascular necrosis undergoing core decompression procedures. In addition, SSCs have been combined with carrier matrices in the treatment of cartilage defects. Haleem et al. translated this into a small clinical series of five patients with full thickness cartilage defects of the femoral condyle. All patients’ symptom scores improved significantly over the follow-up period of 12 months. However, despite these encouraging case series, the combination of SSCs with carrier modalities has yet to reach widespread clinical application.

Previous in vitro and small animal in vivo studies highlighted a composite polymer hydroxyapatite scaffold in combination with SSCs for potential use as a tissue engineered bone graft substitute. Scaffold characterization via microCT and scanning electron microscopy (mean porosity 63.4 ± 5.4%, pore size 192 ± 51 μm), cellular viability studies and shear testing found high molecular weight porous poly (α-lactic acid) hydroxyapatite (PDLA + 10% HA) composite to display both the mechanical, osteogenic capabilities and tissue compatibility required for clinical use. The use of large animal models is essential when investigating bone regenerative strategies, circumventing issues of translation, scale, nutrient requirements, and lack of relevance of skeletal form and forces present in small animal models. The current study set out to investigate the osseointegration and bone regenerative capacity of the scaffold both with and without the addition of SSCs in a scaled-up ovine critical sized femoral condyle defect model.

MATERIALS AND METHODS
A timeline depicting the stages of the study is given in Figure 1.

Sheep selection
Six healthy and skeletally mature (>2 years old) welsh mule ewes weighing between 60 and 90 kg were obtained, vetted, and acclimatized in a barn for 4 weeks before use following approval under the Home Office Animals (Scientific Procedures) Act 1986 (project licence 30/2880).

General anaesthesia
All sheep underwent general anaesthesia before any interventional procedure, which followed a routine protocol. Premedication consisted of 0.1 mg/kg intramuscular xylazine injection (Rompun, Bayer PLC, Bury St Edmunds, UK) and the prophylactic antibiotic 5 mg sodium cefalexin (Ceporex, Schering-Plough, Welwyn Garden City, UK).

Anaesthesia was induced using 2.5 mg intravenous (IV) Midazolam (Hypnovel, Roche, Welwyn Garden City, UK) and 2 mg/kg IV ketamine hydrochloride (Ketaset, Fort Dodge Animal Health Limited, Southampton, UK). The sheep were...
then intubated, and general anaesthesia was maintained using inhaled isoflurane, oxygen, and nitrous oxide.

**Harvest of skeletal stem cells**

Once fully anaesthetized the sheep were placed in the left lateral position. An area measuring ~ 20 × 20 cm² directly over the iliac crest was shaved and thoroughly cleaned using 10% povidone-iodine (betadine) solution followed by 2% chlorhexidine gluconate solution. A trochar was then inserted through the cortical bone and 10 mL aspirated. The aspirate from each sheep was then placed into a separate universal container into a cool box for transportation to the laboratory. The sheep were then allowed to recover and given analgesia as required. Sheep were housed in a barn, penned as a group and were fed according to standard practice, with free access to water from troughs until further use.

**Expansion of SSC populations**

Ten millilitres of basal media (α-MEM with 10% fetal calf serum with additional 1% penicillin and streptomycin) was added to the universal tubes containing the aspirates, and the resultant solutions were passed through 70 μm filter cell strainers and into a separate universal containers [All reagents used for tissue culture were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated]. The resultant cell solutions were then centrifuged at 1100 rpm for 4 min. The supernatants were carefully removed and the cell pellets re-suspended in 40 mL basal media. These solutions were then placed into four T75 tissue culture flasks (10 mL per flask), incubated at 37 °C for 4 min. The supernatants were removed and the cell pellets resuspended in basal media before determining total cell counts using a haemocytometer. Appropriate dilution with basal media was then achieved to obtain a concentration of 5 × 10⁵ cells/mL.

**Scaffold seeding**

The polymer composite (PDLA + 10% HA) was produced, milled, and sterilized as described previously.²¹ Twenty millilitres of each of the cell solutions was added to 20 mL of the polymer in a universal container and incubated (37 °C, 5% CO₂) for 2 h, with gentle agitation every 30 min, to allow cell adhesion. The solution was then aspirated and discarded. The seeded scaffold was then placed into individual wells of a six-well plate, and cultured in osteogenic media (basal media + 100 μM ascorbate-2-phosphate, and 10 nM dexamethasone) for 14 days, with PBS washes every 3–4 days. The control scaffold was treated in exactly the same manner, but without the addition of the SSCs.

At the end of the incubation period the scaffold was divided in half for parallel *in vivo* and *in vitro* testing.

**In vivo ovine study**

**Operative procedure.** Twelve hours before surgery the sheep were given two analgesic Fentanyl patches (75 mcg/h) and were randomized to receive scaffold + SSCs or scaffold alone to either the right or left side. The sheep underwent full general anaesthesia as detailed earlier. Under full aseptic precautions, 10 cm longitudinal incisions were made centred over the palpable bony protuberances located on the medial sides of the medial femoral condyles of each leg, with careful dissection through subcutaneous tissue, fascia, muscle, and periosteum to expose the underlying bone. A customized 8 mm drill bit was used to create a hole with a consistent depth of 15 mm into the underlying cancellous bone [Fig. 2 (A,B)]. The SSC seeded scaffolds and control scaffolds were packed tightly into the holes on their allocated sides such that the holes were filled [Fig. 2(C,D)]. Two guide wires were placed superior and inferior to the holes for radiographic location purposes at a later stage. The periosteum and other layers were closed over the filled holes using 2/0 Vicryl® suture in order to contain the scaffold and the skin was closed with interrupted 2/0 Ethilon® sutures and OpSite spray (Smith and Nephew, Hull, UK). The wounds were dressed with betadine soaked gauze and wool and crepe bandage. The sheep received a five day post-operative antibiotic regime (Ceporex 5 mL OD) with Fentanyl patches and buprenorphine (0.6 mg) for 72 h after application of the patches (for analgesia) and regular wound checks. After surgery, the sheep were housed individually in pens for 5 days, followed by grouped pens for the remainder of the study. No control (empty defect) sheep were utilized in this study as the critical defect nature of the model had been previously demonstrated.¹⁵

**Retrieval of specimens.** The sheep were euthanized with a barbiturate overdose (0.7 mg/kg) 13 weeks post implantation of the specimens. The femoral condyles were harvested together with the popliteal lymph nodes to assess any potential inflammatory reactions associated with *in vivo* polymer use, as indicated by previous studies.¹⁶,¹⁷

**Preparation of condyles.** All condyles were prepared and mechanically tested within 6 h of harvest. The condyles were placed in a jig and carefully orientated using radiographs and marker wires, such that when cut with a diamond-edged band saw (EXAKT, Hamburg, Germany), the specimen-filled drill holes were transected longitudinally in the coronal plane at the midpoints. One half of each specimen was subsequently taken for immediate mechanical testing and μCT evaluation, and the other half was prepared for histology.

**Mechanical testing**

The specimens for mechanical testing were placed onto the stage of the Z005 static materials testing machine (Zwick/ Roell, Leominster, Herefordshire, UK), and a flat ended metal indentation rod (diameter 4 mm) was placed in opposition to the desired test area, with a preload of 10 N applied at a speed of 5 mm/min. The test area are shown in Figure 3.
where areas 1 and 2 correspond to the superficial and deep regions of the scaffold within the drill hole. Areas A and B correspond to proximal and distal regions of normal cancellous bone. The indentation rod was advanced at a rate of 10 mm/min, and the force required at each time point recorded using a load cell.

The data was transferred to a spreadsheet (Excel 2007, Microsoft, Redmond, WA) and load deformation curves were generated. The peak value corresponded to the failure of the test area. The force required at this peak was recorded for each area under test, and converted into a stress value. Mean stress values (along with standard deviations) were calculated for each area of the femora implanted with the scaffold alone, and those implanted with the scaffold + SSCs.

Micro computed tomography (µCT) evaluation
After indentation the specimens were frozen until µCT evaluation. Samples were scanned using a SkyScan 1176 µCT. The scans were performed with minimized ring artefacts to give an 18 µm voxel resolution. Analysis of the reconstructed images was performed using CTAn (Skyscan, Belgium). The region of interest (corresponding to the volume within the original drill hole) was extracted manually using the software, and via comparison with standard phantoms,
new bone was deemed to correspond to all density values over 0.25. Mean volume of new bone within the region of interest was calculated for the femora implanted with the scaffold alone, and those implanted with the scaffold + SSCs.

**Histological examination**

*Femoral condyle.* The other half of the femoral condyle samples were fixed in buffered formal saline (pH 7.2) for 1 week followed by dehydration through an ascending alcohol series over a 3-week period. The samples were then infiltrated with JB4 resin (Ted Pella, Inc., Redding, CA) for 3 weeks, refreshing the solution weekly, and were then embedded in the same resin. Using a band saw, 5 mm thick longitudinal slices were taken through the specimens and from these slices thinner sections measuring between 50 and 80 μm thick were prepared. The sections were attached to an acrylic slide using UV curable cyano-acrylate glue, before being ground and polished on an EXAKT grinding machine. Sections were stained with toluidine blue to stain the soft tissue followed by paragon to stain the bone, before being viewed by light microscopy.

*Popliteal lymph node.* The lymph nodes were fixed in formaldehyde, and dehydrated in graded ethanol solutions before wax embedding on a mounting plate. Seven micrometer sections were cut, transferred to a water bath and mounted on slides, before transferring to an oven set to 37°C for 4 h. Samples were subsequently stained using a standard Haematoxylin and Eosin (H + E) stain, and assessed for the presence of abnormal inflammatory cells.

**Parallel in vitro study**

At the start of the study, the remaining SSC seeded scaffold was kept for parallel in vitro evaluation. The seeded scaffold from each sheep was weighed out into multiple, separate 1 gram portions, and each portion was placed into an individual well of a 24-well plate and cultured at 37°C, 5% CO₂ in osteogenic media with PBS washes every 3–4 days.

**WST-1 cell viability assay.** After 14 days in vitro incubation (day 1 of ovine experiment), and 8 weeks in vitro incubation (midpoint of ovine experiment) three portions from each sheep SSC sample (18 in total) were assessed for cell viability via a WST-1 assay.

Each portion was submerged in 1 mL 1:10 dilution WST-1 substrate (Roche, Welwyn Garden City, UK). Unseeded scaffolds were run in parallel (controls). At 4 h, samples (3 × 100 μl) were removed from each portion and analyzed via a Bio-Tek KC4 microplate fluorescent reader (Bio-Tek) at 410 nm. An increase in absorbance value indicated increased cell number and viability. The mean absorbance value was calculated and the mean control WST-1 absorbance value subtracted.

**Alkaline phosphatase specific activity/DNA assays.** Alkaline phosphatase (ALP) specific activity was used as a measure of osteoblastic differentiation on the scaffolds following 2 weeks and 8 weeks of in vitro incubation. Three portions of SSC seeded scaffold from each sheep were tested at each time point. The scaffolds were individually washed in PBS, and fixed in 90% ethanol before air drying. Samples were then re-washed in PBS, and subsequently suspended in 2 mL of 0.5% Triton X-100. Samples were subjected to three freeze thaw cycles with vigorous agitation between each freeze. Lysate was measured for ALP activity using p-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (Sigma, Poole, UK) and DNA content was measured using Pico Green (Molecular Probes, Paisley, UK) according to routine manufacturer protocol. Ten microlitres of lysate was run in triplicate for each cell seeded sample and control samples on a plate against two standards, read in triplicate on an ELx 800 and FLx-800 microplate fluorescent reader (Bio-Tek) and mean and standard deviations calculated. Specific activity was expressed as nanomoles of p-nitrophenyl phosphate/hr/ng DNA.

**Immunostaining.** SSC seeded specimens from two sheep were subjected to immunostaining after 8 weeks in vitro incubation time. Samples were removed from containers, washed in PBS and fixed for 24 h by submersion in 4% paraformaldehyde. This was followed by a 10 minute incubation in 1% bovine serum albumin (BSA) and overnight incubation in 1 mL of primary antibody (e.g., LF68 (whole rabbit serum) collagen 1 antibody [1:300 dilution with PBS/BSA]), followed by three washes in 0.1% PBS Tween. Appropriate secondary antibody [anti-rabbit antibody (alex-fluor® 594)] was added for 1 h at room temperature, followed again by three 0.1% PBS Tween washes. Finally, SYTOX® blue (1:100 dilution, 10 minute incubation) was added as a nuclear counterstain. To ensure specificity of the secondary antibody a portion of the samples were treated without addition of the primary antibody. Primary antibodies were type-1 collagen, osteocalcin and bone sialoprotein. In order to preserve the polymeric architecture, the sections were not wax embedded. Confocal microscopy (Leica SP5 Laser Scanning Confocal Microscope and software, Leica Microsystems, Wetzlar, Germany) allowed 3D topographical visualization of the surfaces.

**Statistics.** Mean percentage bone volume in the regions of interest as measured by μCT in the control group versus the cell seeded group were compared using a Mann-Whitney U Test (Prism version 6.03, GraphPad Software, La Jolla, CA). The biochemical assays at the 2 weeks and 8 weeks time points were also compared with the same software using a Mann–Whitney U Test.

**RESULTS**

*In vivo ovine study*  

**Mechanical testing.** The mean mechanical strength of the defect area containing the scaffold controls was 20% that of the mean strength of the cancellous bone areas (11.80 ± 7.15 n/mm² vs. 59.43 ± 27.46 n/mm², U test: p < 0.01). The mean mechanical strength of the defect area containing the scaffold + SSCs was 11% that of the mean strength of
the cancellous bone areas (4.61 ± 4.80 n/mm² vs. 43.05 ± 23.10 n/mm², U test: \( p < 0.01 \)) as illustrated in Figure 4. Comparison of the mean mechanical stress at failure of the defects containing the scaffold to those containing the scaffolds + SSCs produced a 41% reduction in stress at position 1 (7.88 ± 4.84 n/mm² vs. 4.63 ± 6.07 n/mm², U test: \( p < 0.01 \)), and a 72% reduction in stress at position 2 (16.49 ± 6.92 n/mm² vs. 4.58 ± 3.40 n/mm², U test: \( p < 0.01 \)) with the addition of SSCs.

\textbf{\mu CT evaluation.} Representative images of the \mu CT reconstructions of the defects containing scaffold controls and scaffold + SSCs are shown in Figure 5. The 3D reconstruction images [Fig. 5(A,C)] showed that the defects were still visible in both samples after the 3 month incubation period. This was to be expected as not all of the scaffold would be fully resorbed within this timeframe. However, axial slices demonstrated a sclerotic margin around the samples seeded with SSCs, indicating that full integration would be limited. In contrast within the defects filled with the scaffold alone there was evidence of normal trabecular bone, indicating that with extended time, the defect would be completely filled.

The mean percentage bone volume content of the defects filled with scaffold alone and scaffold + SSCs was calculated from the raw \mu CT data. There was a reduction in percentage bone volume within the defect of 13.4% (38.17% ± 19.08% vs. 24.81% ± 7.72%) on comparison of scaffold with the scaffold + SSCs although this was not significant (\( U \) test: \( p = 0.13 \)).

\textbf{Histological examination}

\textit{Femoral condyle.} Representative images displaying the histological appearance of the femoral condyle defects filled with scaffold controls and scaffold + SSCs are shown in Figure 6 (A–D). As noted with the \mu CT findings, there was evidence of new bone formation in both the groups, although the new bone in the scaffold alone defects appeared more diffuse throughout the defect. The new bone appeared to integrate more closely with the remaining scaffold material.

Examination under higher magnification of the areas of new bone formation it was possible to identify the hydroxyapatite particles that were originally from within the polymer scaffold and, evidence of the osteoconductive nature of these particles [Fig. 6(F)].

\textit{Popliteal lymph node.} The popliteal lymph nodes serving the region including the operative site were examined histologically. Representative examples of H+E stains of the lymph nodes are shown in Figure 7 (A+B). All lymph nodes presented an essentially normal appearance, although occasional discrete small circular spaces, probably representing polymer material which had been lost through processing, were observed; critically, there was no evidence of an overt inflammatory response. This was similar to the histology seen around the surgical biodegradable sutures, and hence was likely to be of negligible clinical significance.

\textbf{Parallel in vitro study}

\textit{WST-1 assay.} WST-1 assays were used as a measure of cell viability on the scaffolds at 2 weeks and 8 weeks time
points (Fig. 8). Significant cell growth and viability at both time points was observed as demonstrated by the large change in optical density over the 4 h assay period. There was however a decrease in the final optical density achieved when comparing the 8 week sample to the 2 week sample (although this was not significant ($p = 0.16$)), possibly reflecting a decrease in viable cell number at this time point or a reduction in cell metabolic activity, as occurs on differentiation.

**Biochemical analysis.** The mean ALP specific activity at the 2 and 8 week time points was used as a measure of osteoblastic differentiation within the SSC population (Fig. 9). Negligible ALP specific activity was observed at the 2 week time point (control value $0.0015 \pm 0.0015$ nmols PNPP ALP/hr vs. scaffold + SSC $0.006 \pm 0.002$ nmols PNPP ALP/hr), and even though there was a significant increase in alkaline phosphatase specific activity after 8 weeks ($U$ test: $p < 0.05$), the value obtained remained low, indicating minimal osteoblastic differentiation.

**Immunostaining.** Scaffolds were examined for type-1 collagen, osteocalcin and bone sialoprotein (BSP) immunocytochemistry using confocal microscopy, as potential markers of osteoblastic differentiation and bone matrix formation (Fig. 10). There was limited evidence for good bone formation from the *in vitro* studies as evidenced by the moderate positivity for BSP, but only minimal positive staining for both type-1 collagen and osteocalcin.

**DISCUSSION**

The use of large animal models is essential when investigating bone regenerative strategies. Whilst small animal studies are warranted when investigating the osteogenic potential of constructs, as well as allowing an array of substances to be tested relatively cheaply and quickly, there are a number of essential factors which are severely compromised and need careful consideration when interpreting any subsequent research findings. Small animal models typically hold limitations in the consideration force and its impact on skeletal form and bone remodelling. Furthermore, the amount of new bone produced, or distance of any bone defect bridged in a small animal model typically does not translate proportionately in a larger animal model. Finally, other issues surrounding distance and time for ingrowth of blood vessels, and the related survival of any preseeded cells are not typically addressed by small animal models.

The ovine femoral condyle defect provides a useful comparative model to analyze new bone formation because of the size of the defect, the surrounding tissue coverage and blood supply, as well as the nature of the surrounding trabecular bone being comparable in nature to human trabecular bone. Given their size, sheep also potentially provide
models whereby constructs can be exposed to loads and forces comparable to the human clinical setting. However, the current model was only minimally loaded (subjected to the normal physiological load of condylar trabecular bone), and hence, provided a transitional model to a more complex loaded model, before a human clinical trial.20 This limitation does however provide a less complicated, robust model subject to fewer variables, providing a cost effect method to screen, and hence further improve and enhance constructs and experimental protocols prior to evaluation in loaded (and more expensive) ovine models. This factor has proved important in this study, whereby the scaffolds examined

FIGURE 6. Histological sections showing defect areas of the sheep condyles stained with toluidine blue and paragon stain. For images A-E scale bar = 500 μm. Images show area packed with polymer (PDLLA HA) alone (A,B) and polymer + SSCs (C,D). New bone formation is shown in purple (thick white arrows), with fibrous tissue formation in the samples containing SSCs (thick black arrows). The thin white arrows (C) depict the external margin of the defect, and showed negligible osseointegration had occurred in this region. The thin black arrows represent the remaining polymer. (E) Section of normal sheep condylar cancellous bone. (F) Section taken from within an area of new bone formation (scale bar = 100 μm). A hydroxyapatite particle (thick white arrow) is shown completely encompassed by new bone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 7. (A,B) Micrographs displaying H+E stains of lymph nodes after the study period. (A) Occasional small circular spaces are visible in otherwise normal tissue (arrows) (scale bar = 1000 μm), (B) higher magnification of small circular space, showing no increased inflammatory response around it (scale bar = 50 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
have provided encouraging results and, has also introduced important factors for investigation surrounding the potential of the SSCs and the protocols for their use.

The scaffold, consisting of porous high molecular weight PDLLA in combination with 10% HA has shown, after a 3 month in vivo incubation period, the potential to stimulate new bone formation within a critical sized defect of cancellous bone. Both histological and μCT analysis provided good evidence for new bone formation around and within the constructs, although this was incomplete in all cases. Mechanical indentation tests confirmed these findings, with structural integrity within the defects, although the bone was approximately only one fifth of the strength of that of the neighboring cancellous bone. This finding is encouraging and it is likely that with further time the mechanical strength would further improve, but further studies would be required to include these time points and may not be of clinical relevance. This structural issue is of paramount importance when considering a fully bioresorbable scaffold for use in a loaded clinical situation. Previous in vitro mechanical tests demonstrated the composite to have comparable strength to milled allograft (the current gold standard for large bone defect filling) although the comparison in this model is to native cancellous bone. A control defect group containing milled allograft, would allow an accurate comparison to the current gold standard for bone grafting procedures (not undertaken because of fiscal constraints on experimental group numbers) but remains for future studies. It would appear over time, in vivo, the breakdown of the polymer reduced mechanical integrity. The ideal scaffold solution should achieve a balance whereby the bioresorption and hence loss of strength of the scaffold is matched by the ingrowth and increase in strength provided by new bone formation. Shikinami et al. investigated this process using PDLA/HA composites in a rabbit model, concluding that composites containing 30% and 40% HA particles are clinically effective for application in high-strength bioactive, bioresorbable bone-fixation devices with the capacity for total bone replacement.21

Consistently throughout the in vivo analyses, this study has shown that the addition of SSCs was less effective in the generation of both the volume and quality of new bone formation in the defect site, as well as the mechanical strength of the construct/new bone composite. This was inconsistent with a number of other in vitro and in vivo studies, where the addition of SSCs was shown to generate additive effects in terms of enhanced bone formation when combined with a host of different scaffolds in murine, lapine, and ovine models.22–24

The previous papers in this series detail both the in vitro and small animal in vivo work surrounding the production, optimization, and choice of this composite scaffold. All previous work was performed with the addition of human SSCs, including evaluation within a murine model (immunocompromized mice). It has however been shown that the osteoinductivity of certain substances or culture conditions is different between human and ovine SSCs.25 The parallel in vitro component of this study examined this theory, and offers some indication as to why the SSCs may have been less efficacious in this ovine study. The WST-1 assay showed excellent cell viability on the scaffolds at 2-week and 8-week incubation times indicating biocompatibility although, the ALP assay was negative at 2 weeks and only weakly positive at 8 weeks. These observations indicated a lack of osteoblast differentiation amongst the SSC population a finding reinforced with a paucity of type-1 collagen and osteocalcin expression following 8-weeks incubation. Possible explanations include; either the scaffold was not osteoinductive to ovine SSCs, or the culture conditions were not conducive to osteoblast differentiation of the SSC population. These factors could potentially favor differentiation along less osteogenic populations (fibroblasts), which could reduce osteoconduction or osteoinduction of surrounding host cells when implanted in vivo. Given the observation that control scaffolds performed well during the in vivo study, indicating at least osteoconductivity, it is likely that it is the initial SSC isolation and seeding protocols which need to be addressed in order to achieve potential SSC benefits. Future work will therefore concentrate on this area, with culture expansion of ovine SSCs in enhanced osteogenic conditions (e.g., in the presence of BMP-2), and scaffold seeding at the time of in vivo implantation, such

![FIGURE 8](Image) Graphical representation of WST-1 assay showing mean increase in optical density (minus controls) of the scaffolds seeded with sheep SSCs at the 2 week (in vivo implantation) and 8 week (in vivo mid-point) stages (± SD, n = 6, *p = 0.16).

![FIGURE 9](Image) Mean ALP specific activity of the sheep SSC population on the scaffolds at 2 week and 8 week time points (± SD, n = 6, *p < 0.05).
that the cells are immediately exposed to the regional growth factors and stimulatory microenvironments.

Interestingly, a number of studies have shown enhanced osteoinductivity of biodegradable scaffolds containing increasing HA concentrations. Zannettino et al. found that a 15% concentration of HA was required, whereas Shikinami et al. found that over 30% was effective in inducing bone formation.21,25 In a previous murine \textit{in vivo} study performed with the PDLLA/HA composite scaffold, \textmu CT analysis showed enhanced bone formation in areas in contact with the HA particles.12 This therefore suggests additional approaches to improve the scaffold prior to a second ovine study: (1) enhancement of HA content and, (2) increasing the availability of the HA to the SSCs. The first issue could be addressed by increasing the proportion of HA to PDLLA during the initial foaming process, whereas the recent development of nano HA particles suggests a potential solution to enhancement of availability to skeletal cells. The use of PDLLA with nano HA in the foaming process offers the potential of a matrix with HA distributed diffusely throughout the structure and thus a synthetic bone graft with PDLLA as collagen and the nano HA as the inorganic components of bone.

CONCLUSIONS

The current studies demonstrated that bone regeneration occurred in the defects filled with both the scaffold/SSC composites and the scaffold controls. However, there was significantly more bone regeneration in the scaffold controls.

These studies offer further insight toward the successful translation of a tissue engineered product previously shown to have encouraging capability in both \textit{in vitro} and small animal \textit{in vivo} studies. The potential of this fully bioresorbable composite scaffold to stimulate bone regeneration in a critical defect has thus been demonstrated in a scaled-up large animal study. However, there are areas for improvement including: optimization of cell seeding protocols, including predifferentiation of SSCs or inclusion of osteoinductive stimuli within the scaffolds, the need to address vascularization of the graft or techniques to enhance cell survival before host vascular ingrowth, and the investigation of HA concentration and bioavailability. Further research in these areas is on-going such that future studies should improve the regenerative capacity of the scaffold, and clinical use of a tissue engineered product is one step closer.
ACKNOWLEDGMENTS

The authors like to thank Miss E. Ralph for technical support, and the Imaging and Microscopy Centre for assistance with confocal microscopy (University of Southampton). The authors also like to thank Miss Gillian Hughes and the Royal veterinary team for their care with the animal husbandry.

REFERENCES