A novel collagen scaffold supports human osteogenesis--applications for bone tissue engineering.

AUTHOR(S)

Michael B. Keogh, Fergal O'Brien, Jacqueline Daly, Jacqueline Daly

A novel collagen scaffold supports human osteogenesis – applications for bone tissue engineering

Journal:	*Cell and Tissue Research*
Manuscript ID:	CTR-09-0327.R1
Manuscript Type:	Regular Article
Date Submitted by the Author:	
Complete List of Authors:	Keogh, Michael; Royal College of Surgeons in Ireland, Anatomy O' Brien, Fergal; Royal College of Surgeons in Ireland, Anatomy Daly, Jacqueline; Royal College of Surgeons in Ireland, Anatomy
Keywords:	Bone Tissue Engineering, Osteoblast, Collagen, Scaffold, Transforming Growth Factor-ß

Title

A novel collagen scaffold supports human osteogenesis – applications for bone tissue engineering

M.B. Keogh[a,b*], F.J. O' Brien[b], J.S. Daly[a,b]

[a] *Division of Biology,* [b]*Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's green, Dublin 2, Ireland*

*Corresponding author. Present address: Division of Biology, Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's green, Dublin 2, Ireland. Tel.: +353(0)1-402-2147; fax: +353(0)1-402-2355

E-mail address: mkeogh@rcsi.ie

Abstract

Collagen glycosaminoglycan (CG) scaffolds have been clinically approved as an application for skin regeneration. The goal of this study was to examine if a CG scaffold is a suitable biomaterial for generating human bone tissue. Specifically, can the scaffold support human osteoblast growth and differentiation and how recombinant human transforming growth factor-beta (TGF-β_1) may enhance long term *in vitro* bone formation. We show human osteoblast attachment, infiltration and uniform distribution throughout the construct, reaching the centre within 14 days of seeding. We identified the fully differentiated osteoblast phenotype categorised by the temporal expression of alkaline phosphatase, collagen type 1, osteonectin, bone sialo protein, biglycan and osteocalcin. Mineralised bone formation was identified from 35 days post-seeding using von Kossa and Alizarin S Red staining. Both gene expression and mineral staining suggest the benefit of introducing an initial high treatment of TGF-β_1 (10ng/ml) followed by a low continuous treatment (0.2ng/ml) to enhance human osteogenesis on the scaffold. Osteogenesis coincided with a reduction in scaffold size and shape (up to 70% that of original). A notable finding was that after 49 days of culture, core degradation was identified at the centre of the tissue engineered construct. This was not observed at earlier time points therefore a maximum of 35 days in culture to be an appropriate end point for *in vitro* studies of these scaffolds. We conclude that the CG scaffold shows excellent potential as a biomaterial for human bone tissue engineering.

Key words

Bone Tissue Engineering, Osteoblast, Collagen, Scaffold, Transforming Growth Factor-ß$_1$

Introduction

Currently, there exists a need to develop new bone graft substitutes as an alternative to conventional autografting and allografting treatments due to disadvantages like cost, scarcity of tissue, multiple surgical procedures and the risk of infection (Heng *et al.*, 2004). Within bone tissue engineering, many scaffolds have been used to promote extracellular matrix formation and

mineralisation including bioactive glass, ceramics, titanium and polymers in an effort to substitute autologous bone for bone tissue engineering (Jones *et al.*, 2007; Sun *et al.*, 2006; Bokhari *et al.*, 2005; Itthichaisri *et al.*, 2007). None of which however, may be classified as a bone graft substitute gold standard. An ideal scaffold should provide a suitable environment for tissue development. It should be highly porous allowing for protein absorption to facilitate cell attachment, infiltration and differentiation, be biocompatible, biodegradable, sterilizable, easily formed, shaped and stored. The use of various scaffolds for bone tissue engineering is increasing in clinical appeal and success.

One potential substitute is type 1 collagen glycosaminoglycan (CG) based scaffolds which have been successfully used in a clinical setting as viable treatments for conjunctiva and epithelial regeneration (Yannas, 2001; Hsu *et al.*, 2000; Compton *et al.*, 1998). Much of this success is due to a number of useful properties including low antigenicity, biodegradability, high porosity and a high ligand density (O' Brien *et al.*, 2005). CG scaffolds are typically composed of type 1 collagen, an abundant connective protein in bone, and the glycosaminoglycan, chondroitin-6 sulfate: a proteoglycan commonly found in bone matrix. Both components have been shown to be important factors for cell attachment, proliferation and differentiation (Pieper *et al.*, 1999). The combination of these biologics, provide a more physiological substrate than that of synthetic polymer, ceramic and metal based scaffolds. These scaffolds are typically fabricated by lyophilising collagen based slurries to form a highly porous sterile scaffold.

Recently, the CG scaffold has been successfully shown to support attachment and proliferation of various animal cell types including fibroblasts, chondrocytes, and neurons (Freyman *et al.*, 2001a; McMahon *et al.*, 2008). Studies in this laboratory have also shown that CG scaffolds may support in vitro osteogenesis (differentiation process of a progenitor cell into a mature bone forming osteoblast) of rat mesenchymal stem cells and mouse osteogenic cell line MC3T3 (Jaasma *et al.*, 2008; Byrne *et al.*, 2008). To date however, no studies have examined the ability of this scaffold to support long term human osteogenesis.

The process of osteogenesis may be characterised by identifying temporally regulated bone formation markers; alkaline phosphatase, collagen type 1, osteopontin, bone sialoprotein,

osteonectin, biglycan and osteocalcin. Alkaline phosphates and collagen type 1 may be described as early bone formation markers and can appear in vitro within approximately 2 weeks of culturing under osteogenic conditions (Donahue *et al.*, 2000). Mid stage markers include osteopontin, bone sialo protein and osteonectin, these are often involved with the onset of crystal nucleation. Late stage markers include osteocalcin and the small leucine rich proteoglycan, biglycan, and are expressed in bones and teeth and are important in regulating mineralization (Setzer *et al.*, 2009).

In order to assess human osteogenesis, a homogenous cell phenotype with controlled growth rate is beneficial which is a limitation of using primary human bone cell cultures. Expanding a primary cell population in vitro can result in them losing their ability to differentiate (Ter Brugge and Jansen, 2002). An alternative is to use osteosarcoma cell lines due to their homogeneity; however, they may be limited phenotypically by responding differently to growth hormones and factors (Subramaniam *et al.*, 2002). The clonal human osteoblast cell line hFOB offers another alternative to reflect human bone biology. hFOB is a conditionally immortalised cell line developed and described by Harris et al. (1995). The immortalized cells were transfected with a temperature sensitive plasmid (tsA58) of the SV40 large T antigen. This allows hFOB to be controlled between states of cellular growth; at 34.5^{o}C the SV40 TAg gene is optimally expressed providing a proliferative phenotype whereas at 39^{o}C it is not expressed, preventing proliferation but initiating differentiation. hFOB have been shown to express high levels of alkaline phosphatase, collagen type 1, osteopontin, osteonectin, bone-sialo protein and osteocalcin. In 2D cultures, once confluent, they can form mineralized nodules and because of this they are a model cell line for studying human osteogenesis on our support scaffold (Donahue *et al.*, 2000; Harris *et al.*, 1995).

Our bone tissue engineering triad principle consists of a CG scaffold seeded with hFOB cells exposed to osteogenic growth factors. Osteogenic growth factors typically include ascorbic acid to aid collagen synthesis and some form of phosphate donor which has been shown to be beneficial for in vitro mineralisation (Laurencin *et al.*, 1996). To this, vitamins D_3 and K_3 and recombinant human TGF-β_1, key regulators of bone formation, were added as this combination

gave optimal results in our 2D culture studies (data not shown). TGF-β_1 is the most abundant growth factor found in human bone. It alters osteoblast growth and differentiation by encouraging bone formation during maturation phases. TGF-β_1 is quite potent with studies using concentrations in the range of 0.1-20ng/ml in vitro (Lieb *et al.*, 2004). However conflicting evidence exists within the literature regarding optimal dosage of TGF-β_1 in vitro. Studies have shown that best results for human bone formation occur with either a low continuous dose e.g. 0.2ng/ml or one containing a single high concentration to induce osteogenesis e.g. 10ng/ml (Lieb *et al.*, 2004; Zang *et al.*, 2003).

As no studies have examined long term human osteogenesis on CG scaffolds; the aim of this study was to investigate the potential of the CG scaffold as a suitable biomaterial to support human osteogenesis with applications for bone tissue engineering. We examine the dose dependent response of TGF-β_1 within our tissue engineered constructs due to the aforementioned conflicting evidence. Furthermore, we identify any structural changes that occurred within our scaffold as a result of long term osteogenic cultures.

Initially an attachment, infiltration and distribution study of hFOB cells on CG scaffold was assessed up to 35 days (Study A). Subsequently, in order to determine if the scaffold can support human osteogenesis with the intention of developing a bone graft substitute hFOB cells were seeded onto CG scaffolds and cultured in osteogenic conditions up to 49 days (Study B). In this study we compare the effects of TGF-β_1 on osteogenesis using two treatment concentrations; either a continuous low dosage (0.2ng/ml) or one single high dose (10ng/ml) followed by low continuous on human osteogenesis.

Materials and methods

Scaffold fabrication

CG scaffolds were fabricated using a lyophilisation technique as described previously (O'Brien *et al.*, 2005). Briefly, CG slurry was prepared by blending 0.5 wt % bovine collagen type 1 (Integra Life Sciences, Plainsboro, NJ) with 0.05M acetic acid pH 3.2 containing 0.05 wt % shark cartilage derived chondroitin-6 sulfate (Sigma-Aldrich, Germany). The slurry was blended

(Ultra turrax) then degassed in a vacuum oven prior to lyophilisation at -40°C, 50 mTorr for 24 hours. Scaffolds were then cross linked by de-hydro thermal crosslinking at 105°C for 24 hours (VacuCell 22). Sheets of the finished product (thickness = 3.5mm; mean pore diameter = 96µm; porosity =99.5%; open interconnecting pores (O'Brien, 2005) were aseptically cut to size (10x10mm).

Cell culture

hFOB (ATCC, MA) pre-osteoblastic cells were cultured under standard conditions (5% CO_2, 37°C). Cells were routinely grown to 80% confluency in T175 culture flasks (Sarstedt, Ireland) containing culture media; a 1:1 ratio of Hams F12 and Dulbecco's modified Eagle's medium (without phenol red) (Gibco, UK), 10% fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin 10mg/ml (Sigma-Aldrich).

Study A: Attachment, infiltration and distribution up to 35 days

A total of $4x10^6$ cells were seeded onto each scaffold in a dropwise manner and cultured in 5mls of media for 24hrs. Scaffolds were replaced into new 6-well plates and cultured at 34.5°C for 35 days; replenishing 2/3 of the media every 3 days. Cellular viability and histological analysis was determined up to 35 days of culture.

Study B: Human osteogenesis study on CG scaffold up to 49 days

$4x10^6$ cells were seeded onto CG scaffolds as described above; however culturing took place at 34.5°C for 14 days to allow for proliferation to the centre of the scaffold and at 39°C thereafter to allow for optimal differentiation. After 14 days constructs were replenished with osteogenic media to induce cellular differentiation: standard media containing 100µg/ml ascorbic acid, 10mM β-glycerolphosphate, recombinant human TGF-β_1 (R&D Systems), 50nM Vitamin D_3 (Sigma-Aldrich) and 10nM Vitamin K_3 (Sigma-Aldrich).

Two treatment groups were analysed based on TGF-β_1 concentration: low continuous contained 0.2ng/ml TGF-β_1 throughout exposure period. High TGF-β_1 contained 10ng/ml TGF-β_1

for 7 days then 0.2ng/ml for the remainder of the experiment. Two time points were assessed: 35 days and 49 days (21 days and 35 days post exposure respectively). Histological analysis, chemical staining for mineralisation, gene expression and cell mediated scaffold contraction and mechanical testing was carried out on these samples.

Viability of hFOB cells on CG scaffold

Metabolic viability on the scaffold was determined by replacing media surrounding the cell seeded constructs and replenished with that containing 10% alamar blue dye (Bioscience). Scaffolds up to 35 days of culture were incubated on an orbital shaker for 4 hours (n=6). 100µl of media was read using a spectrophotometer at 570nm and 610nm. The percentage of reduced dye was calculated in accordance with manufacturer's recommendations.

Histological and chemical staining

Hematoxylin & Eosin staining (H&E): Tissue engineered constructs were fixed in 4% paraformaldhyde for 30 minutes. Dehydration and paraffin embedding was carried out using an automated tissue processor (ASP300, Leica) and cut into 10µm sections (RM2255, Leica). All stained sections were taken in a horizontal plain between 25-50% from the surface of the scaffold. Standard H&E staining was carried out on de paraffinised sections. Images were captured on a digital microscope (NIS Elements, Nikon).

Alizarin S Red staining: In order to stain calcium deposits, 10µm sections of scaffold was deparaffinised and stained with 2% Alizarin red (Sigma-Aldrich) filtered solution for 2 minutes. Sections were rinsed several times with dH_20, dehydrated in xylene and mounted with DPX.

Von Kossa staining: To determine the presence of phosphate based mineral, 10µm sections of scaffold was deparaffinised and brought to water. Sections were stained by applying 2% silver nitrate (Sigma-Aldrich) solution for 1 hour under bright light. The reaction was stopped by adding the developing solution 1% sodium thiosulphate (Sigma-Aldrich) for 1 minute. Sections were counterstained with 0.5% Nuclear fast red (Sigma-Aldrich), dehydrated in xylene and mounted with DPX.

Gene expression

RNA isolation: Cell seeded constructs were flash frozen in liquid nitrogen at each time point and stored at -80°C (n=3). *RNA* isolation from the constructs by homogenisation in RLT lysis buffer (Qiagen) using a rotor-stator homogeniser (Omni International). Cell lysates were centrifuged using QI Shredder columns (Qiagen) and *RNA* extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. *RNA* was concentration quantified was determined using a spectrophotometer (abs 260nm).

Gene expression: Following *RNA* extraction Real time reverse transcription PCR was carried out for gene expression analysis. Trace *DNA* was removed and *RNA* reverse transcribed using 400ng total *RNA* with an RT kit (QuantiTect RT Kit, Qiagen) according to the manufacturer's instructions. Realtime PCR was then carried out using the 7500 Real-Time PCR System (Applied Biosystems). The QuantiTect SYBR Green PCR Kit (Qiagen) was used, according to the manufacturer's instructions, with QuantiTect Primers (Qiagen). Results were quantified for collagen type I, alkaline phosphatase, osteopontin/bone-sialo protein precursor, osteonectin, biglycan, and osteocalcin. Expression levels were assessed using the relative quantification $\Delta\Delta$Ct method. β-actin acted as a house keeping control.

Cell mediated scaffold contraction mechanical testing

Scaffold contraction was measured using a vernier callipers up to 49 days culture. Cell seeded constructs post 49 days culture were analysed for mechanical stiffness using a Z050 mechanical testing machine (Z050, Zwick/Reoll) fitted with a 5-N Load cell (n=6). Constructs were immersed in PBS and tested to 10% strain/minute unconfined wet compression testing. The modulus was calculated as the slope of a linear fit to the stress-strain curve over 2%-5% strain.

Statistical analysis

Statistical analysis was determined using sigma statistical software package SigmaStat 3.0. The statistical differences between 2 groups were calculated using the Students t test and

multiple groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p<0.05$.

Results

Study A: attachment, infiltration and distribution up to 35 days

hFOB cells were found to attach and infiltrate the scaffold with time (Fig. 1 a-h). Between 4 and 7 days cells resided on the scaffold edge increasing in number (Fig. 1 a-b). Cells were observed at the scaffold centre after 14 days (Fig. 1 d). Scaffold pores contained numerous cells and matrix deposition was evident at 21 days along the surface of the tissue engineered construct. Scaffold size and pore structure changed with time particularly between 28 and 35 days; a reduction in size was observed (Fig. 1 f-h). Constructs appeared confluent with cells at 35 days both at the periphery and centre of the construct (Fig. 1 g-h); extra cellular matrix (ECM) deposition increased with time which coincided with a reduction in porosity particularly at 35 days. Alamar blue results gave a consistent 15% reduction of dye at all time points up to 35 days indicating a consistent cellular metabolic viability within the tissue engineered constructs.

Study B: Human osteogenesis study on CG scaffold up to 49 days

Histological results showed clear cell attachment, infiltration and uniform distribution throughout the constructs at 35 and 49 days (Fig. 2 a-d). An external layer or capsule of cells developed on the surface of the cell seeded construct becoming more prominent with time. There was no histological difference between low continuous or high TGF-β_1 exposure between time points. ECM deposition and reduction in the original shape and porosity of the scaffold was observed at 35 and 49 days. After 35 and 49 days incubation positive mineralisation staining was observed in both TGF-β_1 treatment groups however, high TGF-β_1 treatment resulted in greater mineral staining than low continuous (Fig. 2 e, f, k, l).

A notable finding of the study was the level of degradation and contraction within the construct over time. The homogenous pore structure was obsolete after 35 days of culture, replaced with cell matrix and contracted scaffold struts. By 49 days constructs became hollow at

the centre irrespective of treatment groups (Fig. 3 a-c). Cell viable regions (Fig. 3 a) of the construct surrounded the degraded core, however, mineralization remained evident on the construct (Fig. 3 b-c).

Another interesting finding was the change in the amount of scaffold contraction with time (Fig. 4). Unseeded control scaffolds contracted in overall size by 50% of the original size however, this was more pronounced in cell seeded constructs (by 70% of the original size) at 49 days incubation. Similarly, the scaffolds shape changed over time with the construct becoming more oval at 49 days (~3.5x3mm) from their original rectangular shape (10x10x3mm). Comparable changes in scaffold size and shape were observed in both low and high TGF-β_1 treatment groups.

Collagen type 1 a marker for early stage osteogenesis was expressed in all cultured constructs. Low continuous TGF-β_1 groups at day 35 and day 49 gave greater expression levels than treatments with high TGF-β_1; this difference was significant at 35 and 49 days reducing by 54% and 60% ($p= 0.023$; $p= 0.026$) (Fig. 5 a). Another marker of early osteogenesis, alkaline phosphatase showed similar trends to that of collagen type 1. There was a statistically significant difference in alkaline phosphatase expression between treatment groups; at 35 days an initial high treatment of TGF-β_1 reduced expression levels relative to low treatment of TGF-β_1 by 60% ($p<0.024$). This trend followed at 49 days where both treatments were statistically significantly reduced by 80% (low TGF-β_1) and 100% (high TGF-β_1) when compared to day 35 low TGF-β_1 ($p= 0.021$, $p= 0.005$) (Fig. 5 b).

Osteonectin an important matrix protein expressed during osteoblast maturation shows similar trends to that of the early stage markers alkaline phosphatase and collagen type 1. Expression levels of osteonectin decrease by 70% and 84% with high TGF-β_1 over low TGF-β_1 treatment at 35 and 49 days respectively ($p= 0.012$; $p= 0.025$) (Fig. 5 c). Both bone-sialo protein and the TGF-β_1 regulated matrix protein biglycan showed trends of increasing expression levels by 38% and 57% respectively, for high TGF-β_1 treatment over low continuous treatment at 49 days (Fig. 5 d-e). Osteocalcin, a marker of late stage osteogenesis and mineralisation, showed a statistically significant increase in expression with time ($p< 0.05$). The higher dose of TGF-β_1

resulted in a 40% increase in the expression of osteocalcin over that of the low dose at 49 days and a 700% increase between day 35 and day 49 with high TGF-β_1 treatment (Fig. 5 f).

Mechanical testing after 49 days of culture found a 5 fold increase in the stiffness of the tissue engineered constructs for both low ($p= 0.01$) and high ($p< 0.0291$) treatments of TGF-β_1 when compared to unseeded control scaffold (Fig. 6).

Discussion

The aim of this study was to investigate the potential of the CG scaffold as suitable biomaterial to support human osteogenesis with applications for bone tissue engineering. Furthermore, we examined the effects of recombinant human TGF-β_1 within our tissue engineered constructs.

In study A we showed hFOB cell infiltration from the scaffold edge to the centre by 14 days post-seeding with uniform distribution throughout the construct. As a result of this we incorporated a 14 day preculture in our subsequent long term study to provide the construct with the best start prior to the induction of osteogenesis. A fully confluent construct was found at 35 days of culture with maintained cell viability. Previous studies examining animal osteoblasts on scaffold have not shown such levels of confluency throughout the scaffold (Farrell *et al.*, 2006; Tierney *et al.*, 2008). We noted structural changes within our scaffold, such as a reduced porosity and overall scaffold size. This appeared to increase with higher cellular density and matrix deposition but did not affect cellular viability. This study illustrated that our CG scaffold is a good support structure for 3D osteoblast attachment and growth.

In study B, we examined the potential of our scaffold to support human osteogenesis. As osteogenesis proceeds following the onset of confluency; it was therefore decided to examine hFOB cells under osteogenic conditions containing TGF-β_1 within the scaffold up to 49 days.

TGF-β_1 is linked with embryogenesis, tissue repair and bone regulation. Studies have shown its importance in bone formation by increasing osteoblast populations and their maturation (Zang *et al.*, 2003). Given the conflicting literature pertaining to concentrations of TGF-β_1 for human cultures, we assessed human osteogenesis on the scaffold using a previously optimised

osteogenic based media containing either a low continuous treatment of TGF-β_1 (0.2ng/ml) or high TGF-β_1 treatment which consisted of an initial stimulating treatment (10ng/ml) followed by low continuous treatment.

The hFOB cell line has been described as a model cell line for osteogenesis with an ability to form mineralised nodules (Harris *et al.*, 1995). However, not all studies using the hFOB cell line have produced mineral staining (Dhurjati *et al.*, 2006; Subramaniam *et al.*, 2002). Our histological and chemical staining identified cell distribution and mineralization in the CG construct up to 49 days. We found that osteogenesis on the scaffold proceeded under both TGF-β_1 treatments; however, high TGF-β_1 yielded a more mature osteoblastic phenotype capable of mineralising our scaffold in vitro.

An important finding of this study for people trying to engineer bone *in vitro* with any scaffold was identifying core degradation at 49 days culture. The issue of core degradation of *in vitro* engineered tissues has been observed in other studies (Shea *et al.*, 2000). In this study it was possibly due to a lack of diffusion of sufficient nutrients from the surrounding media as a result of extensive matrix and mineral deposition that formed *in vitro.* As this effect was not observed at the earlier time points, it suggests that 35 days might serve as an end point for *in vitro* cultures of these scaffolds. In order to overcome capsule formation and the development of core degradation, custom designed bioreactors may be used to encourage cellular infiltration at early stages of culture so that scaffolds may become confluent with cells earlier, thus shortening the duration for the onset of osteogenesis. Perfusion flow bioreactors can also be used to enhance nutrient diffusion throughout the scaffold (Jaasma *et al.*, 2008).

A significant reduction in scaffold size was observed during long term incubations. Scaffold contraction may result in loss of contact between the implanted graft and the surrounding host tissue making integration of the repair tissue difficult (Lee *et al.*, 2001). Contraction therefore could be limited by increasing the initial stiffness of the scaffold using crosslinking methods which should reduce scaffold contraction during culture. It should be noted that some scaffold contraction may however, be beneficial to promote osteogenesis by providing mechanical stimuli to cells during contraction (Freyman *et al.*, 2001b).

In accordance with our mineral staining, we further identified stages of osteogenesis using Real Time PCR. Osteogenic markers were expressed in a temporal manner, similar to that found in both *in vitro* and *in vivo* studies (Stein *et al.*, 1993; Bilezikian *et al.*, 2001). Early markers of bone formation like collagen type 1 and alkaline phosphatase reduced in expression with time. Similarly, the late stage marker osteocalcin increased in expression with time. These expression patterns are consistent with the accepted model of osteogenesis indicating the development of a fully differentiated osteoblast phenotype on the CG scaffold (Stein *et al.*, 1993). The timing of the expression of these osteogenic markers mimicked that more of *in vivo* osteogenesis than *in vitro* studies (Donahue *et al.*, 2000; Stein *et al.*, 1993). We expect this to be associated with levels of confluency in 3D structures and hypothesise this should be more relevant for in vivo clinical studies.

Importantly, cell seeded constructs increased in mechanical strength; this increase in stiffness during culturing may be attributed to a) matrix formation following the induction of osteogenesis on the scaffold and b) contraction of the scaffold assisted by the action of the osteoblasts.

Conclusions

The CG scaffold supports attachment, infiltration and viability of human osteoblast cells. We have successfully demonstrated the ability of a CG scaffold to support human osteogenesis and form mineralised tissue in vitro. We show the importance of cellular infiltration and confluency of the scaffold as an important factor to allow osteogenesis to proceed, we identify the benefit of introducing an initial high dose of TGF-β_1 for one week over a continuous low dose to enhance human osteogenesis and we determine 35 days *in vitro* culture as an end point for these scaffolds where later timepoints result in construct core degradation. Given these findings we conclude that CG scaffolds show excellent potential as a biomaterial for human bone tissue engineering applications.

Acknowledgements

The authors acknowledge the RSCI Research committee and President of Ireland Young Researcher Award (PIYRA) for funding.

References

Bilezikian JP. (2001) Principles of Bone Biology. Second edition ed. Academic press London (1) 59-83 .

Bokhari MA, Akay G, Zhang S, Birch MA. (2005) The enhancement of osteoblast growth and differentiation in vitro on a peptide hydrogel-polyHIPE polymer hybrid material. Biomaterials 26(25):5198-208.

Byrne EM, Farrell E, McMahon LA, Haugh MG, O'Brien FJ, Campbell VA, et al. (2008) Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation. J Mater Sci Mater Med 19(11):3455-63.

Compton CC, Butler CE, Yannas IV, Warland G, Orgill DP. (1998) Organized skin structure is regenerated in vivo from collagen-GAG matrices seeded with autologous keratinocytes. J Invest Dermatol 110(6):908-16.

Dhurjati R, Liu X, Gay CV, Mastro AM, Vogler EA. (2006) Extended-Term Culture of Bone Cells in a Compartmentalized Bioreactor. Tissue Engineering 12(11):3045-3054.

Donahue HJ, Li Z, Zhou Z, Yellowley CE. (2000) Differentiation of human fetal osteoblastic cells and gap junctional intercellular communication. Am J Physiol Cell Physiol 278(2):C315-322.

Farrell E, O'Brien FJ, Doyle P, Fischer J, Yannas I, Harley BA, et al. (2006) A collagen-glycosaminoglycan scaffold supports adult rat mesenchymal stem cell differentiation along osteogenic and chondrogenic routes. Tissue Eng 12(3):459-68.

Freyman TM, Yannas IV, Gibson LJ. (2001) Cellular materials as porous scaffolds for tissue engineering. Progress in Materials Science 46(3-4):273.

Freyman TM, Yannas IV, Yokoo R, Gibson LJ. (2001) Fibroblast contraction of a collagen-GAG matrix. Biomaterials 22(21):2883-91.

Harris SA, Enger RJ, Riggs BL, Spelsberg TC. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 1995;10(2):178-86.

Heng BC, Cao T, Stanton LW, Robson P, Olsen B. (2004) Strategies for directing the differentiation of stem cells into the osteogenic lineage in vitro. J Bone Miner Res 19(9):1379-94.

Hsu WC, Spilker MH, Yannas IV, Rubin PA. (2000) Inhibition of conjunctival scarring and contraction by a porous collagen-glycosaminoglycan implant. Invest Ophthalmol Vis Sci 41(9):2404-11.

Itthichaisri C, Wiedmann-Al-Ahmad M, Huebner U, Al-Ahmad A, Schoen R, Schmelzeisen R, et al. (2007) Comparative in vitro study of the proliferation and growth of human osteoblast-like cells on various biomaterials. J Biomed Mater Res A 82(4):777-87.

Jaasma MJ, O'Brien FJ. (2008) Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A 14(7):1213-23.

Jones JR, Tsigkou O, Coates EE, Stevens MM, Polak JM, Hench LL. (2007) Extracellular matrix formation and mineralization on a phosphate-free porous bioactive glass scaffold using primary human osteoblast (HOB) cells. Biomaterials 28(9):1653-63.

Laurencin CT, Attawia MA, Elgendy HE, Herbert KM. (1996) Tissue engineered bone-regeneration using degradable polymers: the formation of mineralized matrices. Bone 19(1 Suppl):93S-99S.

Lee CR, Grodzinsky AJ, Spector M. (2001)The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials 22(23):3145.

Lieb E, Vogel T, Milz S, Dauner M, Schulz MB. (2004) Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. II: Osteoblastic differentiation. Tissue Eng 10(9-10):1414-25.

McMahon LA, Reid AJ, Campbell VA, Prendergast PJ. (2008) Regulatory effects of mechanical strain on the chondrogenic differentiation of MSCs in a collagen-GAG scaffold: experimental and computational analysis. Ann Biomed Eng 36(2):185-94.

O'Brien FJ, Harley BA, Yannas IV, Gibson LJ. (2005) The effect of pore size on cell adhesion in collagen-GAG scaffolds. Biomaterials 26(4):433-41.

Pieper JS, Oosterhof A, Dijkstra PJ, Veerkamp JH, van Kuppevelt TH. (1999) Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. Biomaterials 20(9):847.

Setzer B, Bachle M, Metzger MC, Kohal RJ. (2009) The gene-expression and phenotypic response of hFOB 1.19 osteoblasts to surface-modified titanium and zirconia. Biomaterials 30(6):979-90.

Shea LD, Wang D, Franceschi RT, Mooney DJ. (2000) Engineered bone development from a pre-osteoblast cell line on three-dimensional scaffolds. Tissue Eng 6(6):605-17.

Spilker MH, Asano K, Yannas IV, Spector M. (2001) Contraction of collagen-glycosaminoglycan matrices by peripheral nerve cells in vitro. Biomaterials 22(10):1085-93.

Stein GS, Lian JB. (1993) Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14(4):424-42.

Subramaniam M, Jalal SM, Rickard DJ, Harris SA, Bolander ME, Spelsberg TC. (2002) Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ER alpha cells: bone formation in vivo and karyotype analysis using multicolor fluorescent in situ hybridization. J Cell Biochem 87(1):9-15.

Sun H, Wu C, Dai K, Chang J, Tang T. (2006) Proliferation and osteoblastic differentiation of human bone marrow-derived stromal cells on akermanite-bioactive ceramics. Biomaterials 27(33):5651.

Ter Brugge PJ, Jansen JA. (2002) In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. Tissue Eng 8(2):321-31.

Tierney CM, Jaasma MJ, O'Brien FJ. (2008) Osteoblast activity on collagen-GAG scaffolds is affected by collagen and GAG concentrations. J Biomed Mater Res A 2 (2) 202.

Yannas I. (2001) Tissue and Organ Regeneration in Adults. illustrated ed. New York: Springer;.

Zhang H, Ahmad M, Gronowicz G. (2003) Effects of transforming growth factor-beta 1 (TGF-beta1) on in vitro mineralization of human osteoblasts on implant materials. Biomaterials 24(12):2013-20.

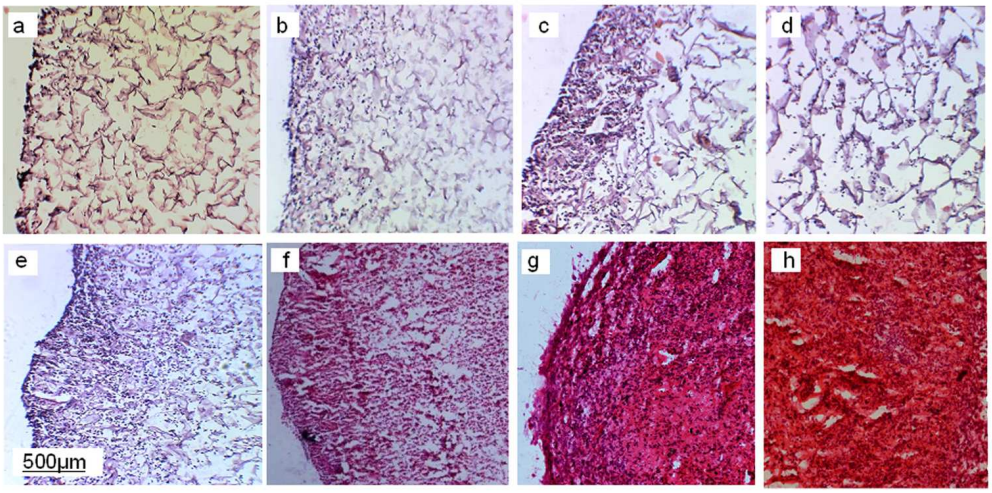

Fig. 1 H&E staining of the cell seeded CG scaffold shows a clear infiltration and augmentation of hFOB cells within the scaffold over time (a-h) (10x magnification). At 4 and 7 days (a, b) cells reside along the scaffold edge. By 14 days (c) cells increase in number along the edge infiltrating into the centre of the construct (d); further cell proliferation and distribution was observed at 21,28 and 35 days (e-g) resulting in a fully confluent construct from edge (g) to centre (h)
64x32mm (600 x 600 DPI)

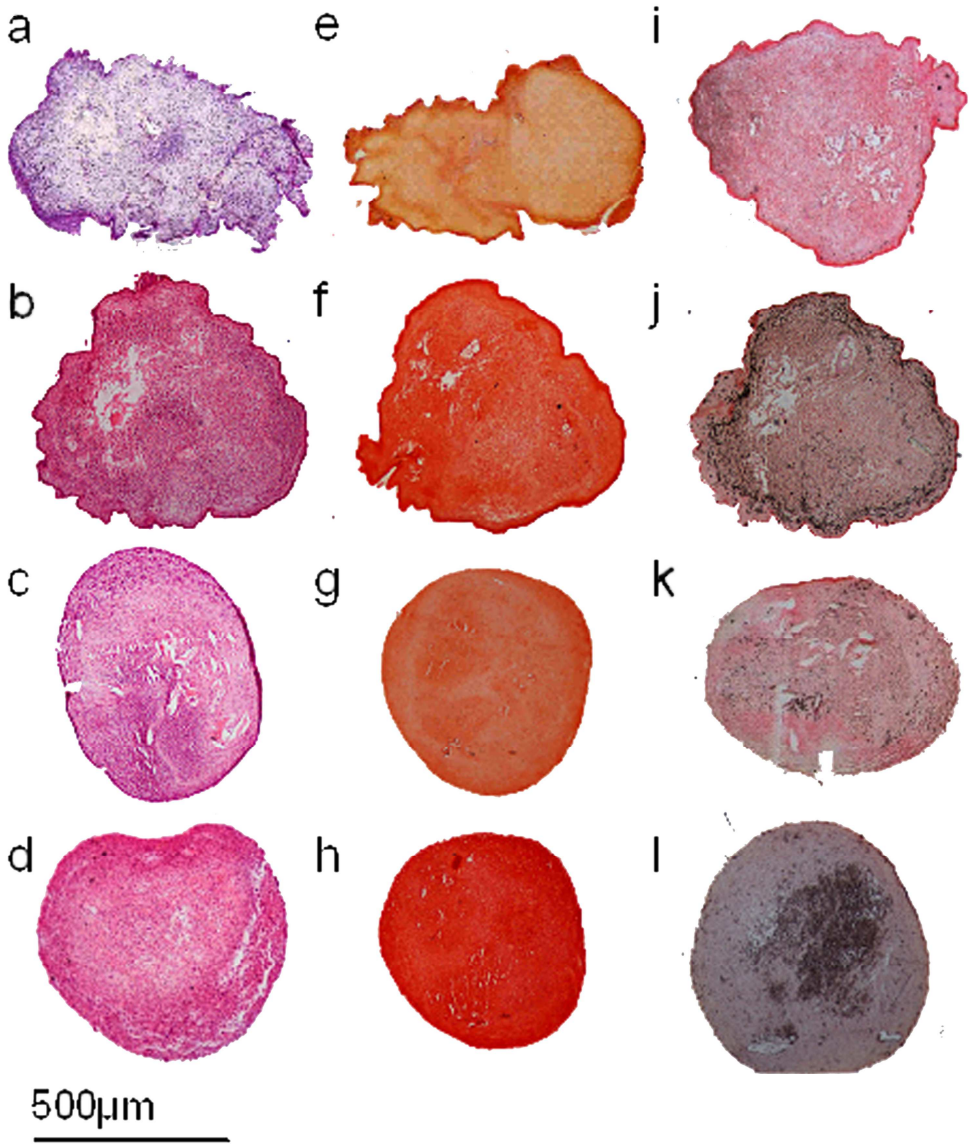

Fig 2. Histological and mineral staining of the cell seeded collagen GAG scaffold (magnification 4x). Sections illustrate (a-d) H&E staining (e-h) Alizarin red and (i-l) von Kossa staining for mineralisation. Low TGF-β1 (a, e, i) and high TGF-β1 (b, f, j) represent 35 day cultures with 49 days represented as low TGF-β1 (c, g, k) and (d, h, l) high TGF-β1.
H&E staining (a-d) illustrate highly infiltrated cell seeded constructs at all time points. Alizarin red and von Kossa staining showed greater staining for groups containing high treatment of TGF-β1 (f, j at 35 days and h, l at 49 days respectively)
128x156mm (600 x 600 DPI)

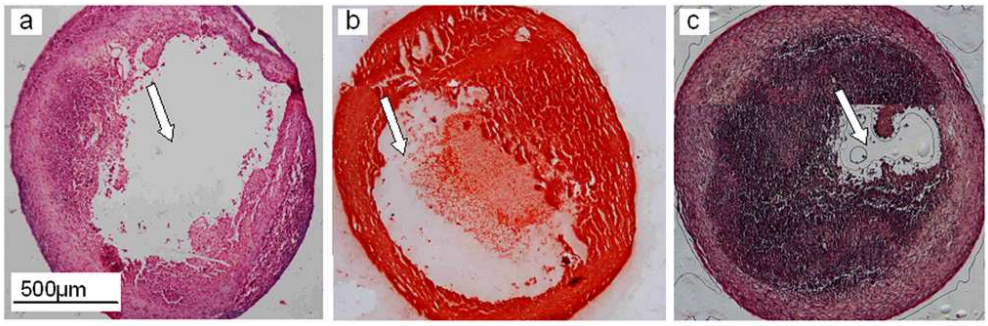

Fig. 3 Sections of the scaffold centre following a 49 day culture under high TGF-β1 conditions showing necrotic core region (illustrated by white arrows) surrounded by (a) H&E stained cellular capsule that stained positive for mineralised tissue formation (b) Alizarin red and (c) Von Kossa (magnification 4x).
43x14mm (600 x 600 DPI)

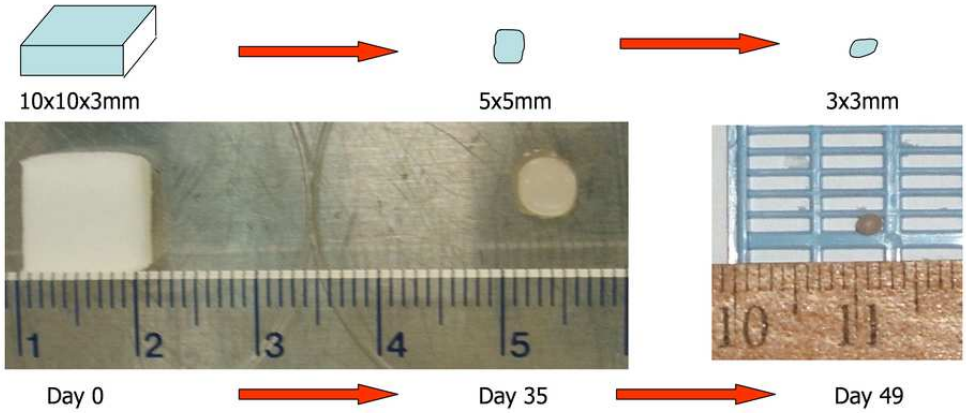

Fig. 4 Cells seeded onto scaffolds and cultured under high TGF-β1 osteogenic conditions up to 49 days reduced in size by ~70%. Low and high TGF-β1 treatments showed similar levels of contraction
39x18mm (600 x 600 DPI)

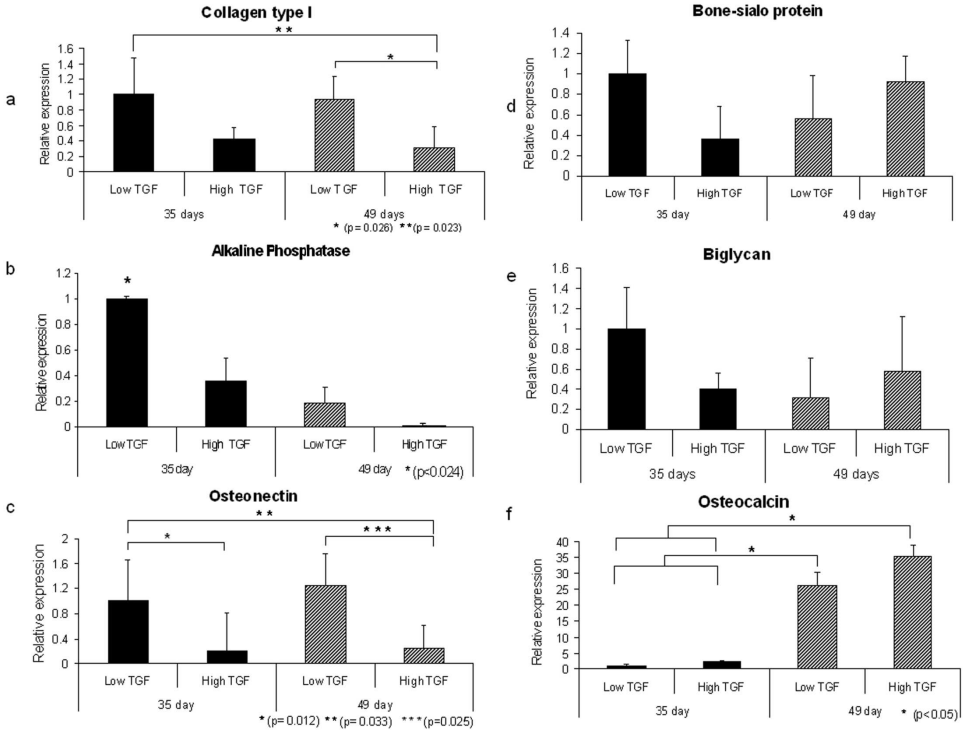

Fig. 5 Gene expression levels of osteogenic markers for cells seeded on CG scaffold at 35 and 49 days culture (a-f). Higher TGF-β1 treatment resulted in greater expression of late stage osteogenic markers than low TGF-β1 treatments. A reduction in expression was observed for early markers of bone formation collagen type 1 ($P<0.026$) and alkaline phosphatase ($p<0.024$) with dosage and duration. Whereas mid and late stage markers increased with high TGF-β1 dosage at 49 days indicating a more differentiated osteoblastic phenotype; osteocalcin shows a 7 fold increase in expression between 35 and 49 days high TGF-β1 treatment groups
96x72mm (600 x 600 DPI)

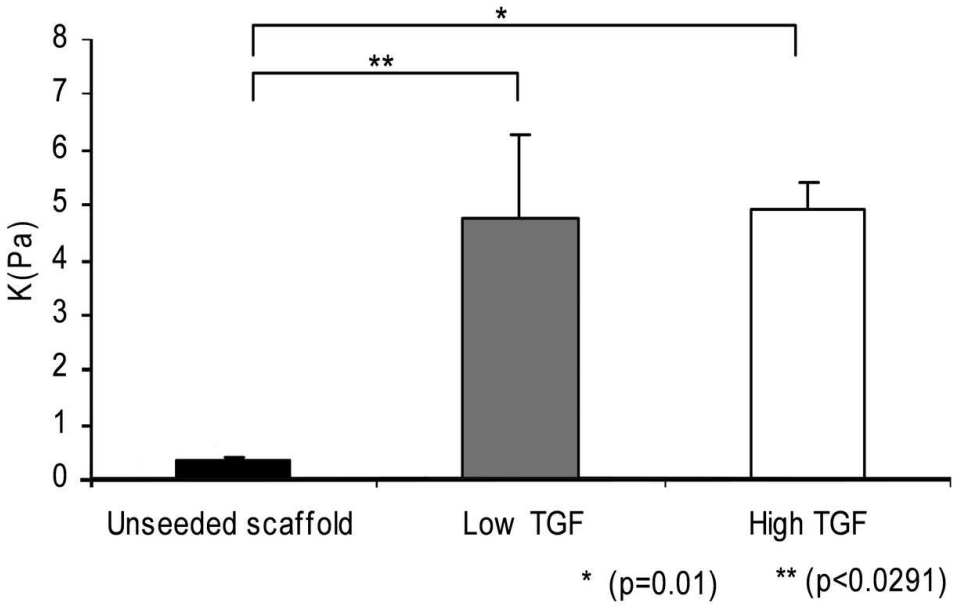

Fig. 6 Mechanical stiffness of cells seeded on scaffolds for 49 days under high osteogenic TGF-β1 conditions. A 5 fold increase in stiffness was observed in low and high TGF-β1 treatment groups when compared to unseeded scaffolds (*p=0.01, **p<0.0291). No difference was observed between treatment groups of TGF-β1
54x34mm (600 x 600 DPI)

Figure captions

Fig. 1 a-h H&E staining of the cell seeded CG scaffold shows a clear infiltration and augmentation of hFOB cells within the scaffold over time (10x magnification). **a** At 4 and **b** 7 days cells reside along the scaffold edge. **c** By 14 days cells increase in number along the edge infiltrating into **d** the centre of the construct; **e-f** further cell proliferation and distribution was observed at 21 and 28 days. **g** At 35 days a fully confluent construct was observed from the edge to **h** the centre of the construct

Fig. 2 a-l Histological and mineral staining of the cell seeded collagen GAG scaffold (magnification 4x). Sections illustrate **a-d** H&E staining **e-h** Alizarin red and **i-l** von Kossa staining for mineralisation. Low TGF-β_1 **a, e, i** and high TGF-β_1 **b, f, j** represent 35 day cultures with 49 days represented as low TGF-β_1 **c, g, k** and high TGF-β_1 **d, h, l**.
H&E staining **a-d** illustrate highly infiltrated cell seeded constructs at all time points. Alizarin red and von Kossa staining showed greater staining for groups containing high treatment of TGF-β_1; **f, j** at 35 days and **h, l** at 49 days respectively

Fig. 3 a-c Sections of the scaffold centre following a 49 day culture under high TGF-β_1 conditions showing necrotic core region (illustrated by white arrows) surrounded by **a** H&E stained cellular capsule that stained positive for mineralised tissue formation, **b** Alizarin red and **c** Von Kossa (magnification 4x).

Fig. 4 Cells seeded onto scaffolds and cultured under high TGF-β_1 osteogenic conditions up to 49 days reduced in size by ~70%. Low and high TGF-β_1 treatments showed similar levels of contraction

Fig. 5 a-f Gene expression levels of osteogenic markers for cells seeded on CG scaffold at 35 and 49 days culture. Higher TGF-β_1 treatment resulted in greater expression of late stage osteogenic markers than low TGF-β_1 treatments. A reduction in expression was observed for

early markers of bone formation collagen type 1 ($p<0.026$) and alkaline phosphatase ($p<0.024$) with dosage and duration. Whereas mid and late stage markers increased with high TGF-β_1 dosage at 49 days indicating a more differentiated osteoblastic phenotype; osteocalcin shows a 7 fold increase in expression between 35 and 49 days high TGF-β_1 treatment groups

Fig. 6 Mechanical stiffness of cells seeded on scaffolds for 49 days under high osteogenic TGF-β_1 conditions. A 5 fold increase in stiffness was observed in low and high TGF-β_1 treatment groups when compared to unseeded scaffolds (*$p=0.01$, **$p<0.0291$). No difference was observed between treatment groups of TGF-β_1