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# A comparison of the osteogenic potential of adult rat mesenchymal stem cells cultured in 2-D and on 3-D collagen glycosaminoglycan scaffolds

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**Abstract.** Adult mesenchymal stem cells (MSCs) have the capability to differentiate along several lineages including those of bone, cartilage, tendon and muscle, thus offering huge potential for the field of tissue engineering. The purpose of this study was to characterise the differentiation capacity of rat MSCs cultured on standard plastic coverslips in 2 dimensions and on a novel collagen glycosaminoglycan scaffold in the presence of a standard combination of osteoinductive factors. Cells were cultured for 3, 7, 14 and 21 days and several markers of osteogenesis were analysed. While the initial response of the cells in 3-D seemed to be faster than cells cultured in 2-D, as evidenced by collagen type I expression, later markers showed that osteogenic differentiation of MSCs took longer in the 3-D environment of the collagen GAG scaffold compared to standard 2-D culture conditions. Furthermore, it was shown that complete scaffold mineralisation could be evoked within a 6 week timeframe. This study further demonstrates the potential use of MSC-seeded collagen GAG scaffolds for bone tissue engineering applications.

Keywords: Mesenchymal stem cell, osteogenesis, collagen glycosaminoglycan scaffold

## 1. Introduction

The discovery of bone cell precursors in the bone marrow by Friedenstein et al. [31] and the subsequent demonstration of the ability of these cells to differentiate along several lineages including bone [32,34, 36], cartilage [33,35,36], fat [36] and tendon [29,37] has generated much hope for the use of this cell source in regenerative medicine for the repair of many muculoskeletal defects. The use of MSCs in the clinical setting for the repair of such defects will not be possible without the use of appropriate three dimensional (3-D) constructs/scaffolds. While the culture of cells in two dimensions (2-D) can yield much useful information about cellular differentiation, it is not until we move to the 3-D setting that we can generate useful tissue replacements. The scaffold environment, in which cells are cultured, must not

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only give shape to the final tissue, but also promote cell adhesion and development, be biocompatible and if necessary give structural support. According to Chapekar et al. [30], it is the combination of progenitor cells, growth factors and a suitable microenvironment that form the basis of the tissue engineering of bone and cartilage. The choice of a suitable microenvironment is as important as the choice of cells and growth factors. It is only recently that the importance of generating viable microenvironments in which to engineer tissues has been recognised [9,22]. In order for a scaffold to promote cell adhesion and growth, it must satisfy certain parameters; it must be biocompatible and degrade in the body at a rate that allows the scaffold to remain insoluble for the duration of the critical cellular processes and the products of degradation must not be toxic [18]. The chemical composition, pore size and uniformity of the scaffold are also of critical importance. It has been suggested that the presence of a 3-D scaffold can enhance cellular proliferation and differentiation by increasing cellular interactions because of the cell-cell proximity that the scaffold allows [24,32]. Whether differentiation is improved compared to 2-D culture or not is generally not assessed.

In this paper, a collagen glycosaminoglycan (collagen GAG) scaffold, previously shown to support osteogenic and chondrogenic differentiation of adult rodent MSCs [8] in 3-D was compared to standard 2-D culture for the temporal appearances of two bone related proteins (collagen type I and osteocalcin), expression of osteocalcin RNA and matrix mineralisation. This was carrried out following exposure of MSCs to an osteoinductive cocktail of ascorbic acid, $\beta$-glycerophosphate and dexamethasone, a now standard combination for the induction of osteogenesis [7]. The highly biocompatible nature of the collagen GAG scaffold, which was originally developed as a skin substitute for burn victims [4] and has also been used in regeneration of the conjunctiva and peripheral nerves [6] is a good substrate for this type of comparison. From previous work [8], it was hypothesised that the cellular response to osteoinductive factors would be slower in 3-D than 2-D. This work will further the understanding of the behaviour of adult MSCs in 3-D compared to 2-D by using the same cell source for both environments and examining widely accepted markers of osteogenesis over a period of 3 weeks.

## 2. Methods

### *2.1. Scaffold fabrication*

The collagen type I-glycosaminoglycan (GAG) scaffolds were fabricated from a collagen-GAG suspension using a lyophilization method. Recent work has led to the development of a scaffold synthesis process which controls the rate of freezing during the fabrication procedure and uses a freezing temperature of $-40^{\circ}$C to produce collagen-GAG scaffolds with a homogeneous structure characterised by a low variation in mean pore size throughout the scaffold and equiaxed pore shape. Chondroitin 6-sulfate was the glycosaminoglycan used, this was mixed with acetic acid and fibrillar collagen type I from bovine tendon. (VirTis, Gardiner, NY). After mixing, the CG suspension was degassed under vacuum (50 mTorr) for 60 minutes to remove air bubbles introduced by the mixing process. The degassed CG slurry was then poured into a 304 stainless steel tray (12.7 mm $\times$ 12.7 mm) and placed into the chamber of the freeze-dryer (VirTis, Gardiner, NY) for lyophilization. Freeze-drying the mixture at a temperature of $-40^{\circ}$C forces the coprecipitate into the spaces between the growing ice crystals to form a continuous interpenetrating network of ice, and the co-precipitate. Sublimation of the ice crystals, leads to formation of a highly porous sponge. Following the freeze-drying process, each collagen-GAG scaffold was cross-linked in a vacuum oven at a temperature of 105$^{\circ}$C under a vacuum of 50 mTorr for 24 hours in order to stiffen the collagen network. This process also sterilises the scaffold.

### 2.2. Culture of rat MSCs

Three-month old Wistar rats (250–350 g) were obtained from the Bioresources Unit, Trinity College Dublin. Animals were sacrificed by $CO_2$ asphyxiation in accordance with local guidelines. The femur was dislocated from the tibia and placed in sterile pre-warmed Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, England) supplemented with 10% fetal bovine serum; 100U/ml penicillin/streptomycin; 2 mM Glutamax; 1 mM L-glutamine and 1% non-essential amino acids. The femur and tibia were cut at both epiphyses and marrow was flushed into a 50 ml tube using 5 ml supplemented DMEM and a 25-gauge needle. The suspension was centrifuged (650 x g, 5 min, 20 °C), resuspended in 10 ml of DMEM and passed sequentially through a 16-gauge, 18-gauge and 20-gauge needle. The suspension was passed through a 40 $\mu$m nylon mesh and incubated for 30 min in a tissue culture flask in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. The supernatant was removed and split between 2 T75 flasks at a cell density of $5 \times 10^7$ nucleated cells per flask; following a 24 hour period the culture medium was replaced to remove non-adherent cells. Upon reaching 80–90% confluence the cells were passaged and replated onto 2 T175 tissue culture flasks. The purity of the preparation was determined as $>$ 90% MSCs, as verified by positive surface endoglin expression. The medium was replaced every 3 days and cells were maintained in a humidified atmosphere of 95% air/5%$CO_2$ at 37°C until required (up to 21 days). To induce osteogenesis in this 2-D environment the cells were treated with DMEM supplemented with 0.68 nM dexamethasone, 10 mM ß-glycerophosphate and 50 $\mu$M ascorbic acid for 14 days. For timecourse studies, cells were cultured on plastic coverslips at a density of $1 \times 10^6$ cells/ml.

### 2.3. Seeding onto collagen-GAG scaffold

Cells were rinsed with phosphate-buffered saline (PBS; 100 mM NaCl; 80 mM $Na_2HPO_4$; 20 mM $Na_2H_2PO_4$) and detatched with trypsin-EDTA (Sigma-Aldrich, England). The resulting suspension was centrifuged (2000 x g, 5 min at 20°C), resuspended in supplemented DMEM and aspirated through a 20-gauge needle to obtain a single cell suspension of $1 \times 10^6$ cells/ml. Collagen-GAG scaffolds (8 $mm^2$) were seeded with 150 $\mu$l of cell suspension and incubated for 30 min. The scaffolds were then overturned onto agar-coated wells and a further 150 $\mu$l of cell suspension was placed onto the scaffold. After 30 min, 2 ml of supplemented DMEM was added to each well and the scaffold was submerged. To induce osteogenesis the scaffold was placed in DMEM supplemented with 0.68 nM dexamethasone, 10 mM ß-glycerophosphate and 50 $\mu$M ascorbic acid. At least 5 scaffolds were treated with osteoinductive factors (OF), with corresponding controls (maintained in DMEM). Half of the respective media were replaced every 3 days. Scaffolds were treated for 3–21 days and were maintained in a humidified atmosphere of 95% air/5%$CO_2$ at 37°C.

### 2.4. Immunohistological detection of osteocalcin and collagen I

To assess differentiation along the osteogenic route, the seeded scaffolds were assessed for collagen I and osteocalcin immunoreactivity, early and late-stage protein markers of osteogenesis respectively. Scaffolds were fixed in 4% paraformaldehyde for 1 h, embedded in liquid paraffin wax and cut into 10 $\mu$m sections using a microtome (Ernst Leitz Ltd, Germany). The sections were placed onto subbed glass microscope slides for immunocytochemistry, dewaxed by soaking sequentially in xylene, absolute alcohol, spirit and 70% alcohol. For samples grown on 2-D, cells were fixed in 4% paraformaldehyde. Cells or scaffold sections were permeabilised in trisbuffered saline (TBS) containing 0.1% Triton-X100

for 10 min and refixed with 4% paraformaldehyde for 30 min. Non-reactive sites were blocked using 20% horse serum/2% bovine serum albumin (2 h at room temperature) and slices were incubated overnight with an anti-osteocalcin antibody purified from goat serum (DSL Diagnostic Systems Lab; 1:1000 dilution) or an anti-collagen I antibody purified from goat serum (Santa Cruz Biotechnology Inc, CA; 1:500 dilution). Scaffolds were washed in TBS, incubated with an anti-goat IgG conjugated to biotin (1:50 dilution; 2 h at room temperature), followed by incubation in ExtrAvidin-FITC (1:50 dilution; 1 h at room temperature). The scaffold was washed several times in TBS and viewed by fluorescence microscopy (490 nm excitation; 520 nm emission) using the Improvision OpenLab software. Non-specific immunoreactivity was assessed by incubating cells with the secondary antibody alone, and subtracting the resultant level of fluorescence.

### *2.5. Histological staining for mineralization*

Extracellular matrix mineralisation was determined by Von Kossa staining. Wax-embedded scaffolds sections were de-waxed, incubated in 5% silver nitrate and placed under an electric lamp (100W) for 1h. The sections were then washed ($5 \times 1$ min) in distilled water and placed in 5% sodium thiosulphate for 2 min. Sections were washed in distilled water, counterstained in toludine blue (1: 5 dilution in distilled water) for 10 sec and then washed in distilled water. Sections were dehydrated, mounted with DPX and viewed under a light microscope. Mineralization of the extracellular matrix was determined by Alizarin red S staining. De-waxed sections were placed in Alizarin red ($C_{14}H_7O_7S.Na$, 2%) for 2 min, washed in distilled water ($5 \times 1$ min), dehydrated and mounted in DPX for observation under light microscopy.

### *2.6. Quantitation of RNAs from specific genes*

Total RNA was extracted using an RNeasy® Mini Kit and RNase-Free DNase Set (Qiagen) according to the manufacturers' instructions; scaffolds were first disrupted in 600 $\mu$l of kit lysis buffer using an Omni electric homogeniser. Reverse transcriptions (10 $\mu$l) were performed on 100 ng of RNA by random hexamer priming using TaqMan® Reverse Transcription Reagents (Applied Biosystems) in the conditions recommended by the manufacturers for "all amplicons except 18S". Real-time PCR reactions (10 $\mu$l) were performed in duplicate on 0.5 ng (18S), or 10 ng (Osteocalcin) cDNA. Reactions were carried out with TaqMan® Universal PCR Master Mix (Applied Biosystems) according to the manufacturers' instructions, using primers at 900 $\mu$M, except for 18S (300 $\mu$M), and 5'-Fam-/3'-Tamra-labelled probes at 250 $\mu$M, on a Prism 7700 Sequence Detection System (Applied Biosystems). Relative quantities of each RNA were calculated from standard curves and then osteocalcin values were normalised to 18S as endogenous reference [to control for RNA quality and reverse transcription efficiency].

Primers and probes (MWG) were designed using Primer Express software (Applied Biosystems); underlined dinucleotides represent the positions of exon-exon junctions:

| | | |
|---|---|---|
| 18S | forward | 5'-ACATCCAAGGAAGGCAGCAG-3' |
| | reverse | 5'-TCGTCACTACCTCCCCGG-3' |
| | probe | 5'-CGCGCAAATTACCCACTCCCGA-3' |
| OCN | forward | 5'-AGGACCCTCTCTCTGCTCACTCT-3' |
| | reverse | 5'-TTTGCA<u>CC</u>TGCCAGGTCAG-3' |
| | probe | 5'-CTGGCCCTGACTGCATTCTGCCTC-3' |

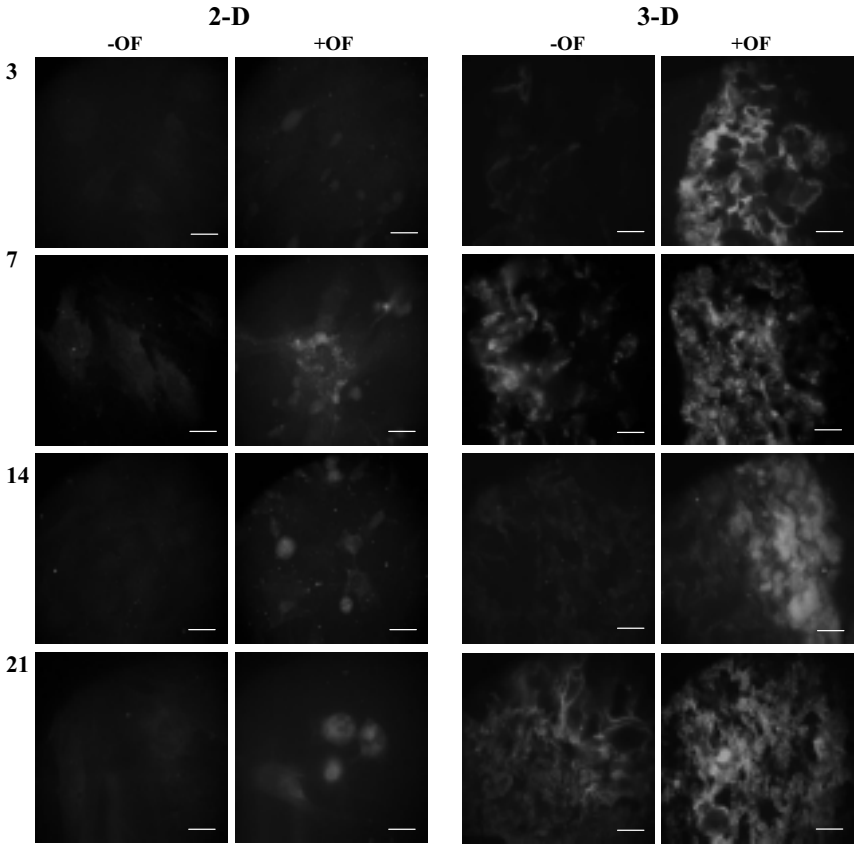


Fig. 1. Timecourse of type I collagen expression in 2-D and 3-D. Expression of type I collagen in monolayer MSC cultures and MSC seeded collagen GAG scaffolds was assessed by immunofluorescent staining. Following 3 days in culture, cells exposed to OF (dexamethasone, 0.68 nM; $\beta$-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) exhibited positive immunoreactivity in collagen GAG scaffolds and were only weakly immunoreactive in 2-D monolayer cultures. By day seven, collagen I expression was observed extracellularly in both 2-D and 3-D. Positive staining for collagen I continued up to day 21 in 2-D and 3-D in the presence of OF. Results are representative of 5 individual cell culture preparations at each timepoint.

## 3. Results

### *3.1. Expression of collagen type I over a 3 week period in 2 and 3 dimensions*

To assess the differentiation potential of adult MSCs in monolayer culture (2-D) and collagen GAG scaffolds (3-D) during culture in an osteogenic medium, expression of collagen type I, an early marker of osteogenesis, was assessed at 3, 7, 14 and 21 days (Fig. 1). 2-D cultures were grown on plastic coverslips in normal culture medium (control) or medium supplemented with osteoinductive factors (OF; dexamethasone, 0.68 nM; $\beta$-glycerophosphate, 10 mM; ascorbic acid, 0.05 mM). For 3-D cultures, MSCs were seeded onto novel collagen GAG scaffolds and treated in the same manner. Collagen type I expression was observed as early as 3 days post seeding in 3-D samples treated with OF, but only weak immunoreactivity was observed until day 7 in 2-D OF treated samples. At day seven collagen I immunoreactivity was also observed extracellularly in 2-D and 3-D, and this expression continued up to day 21. Results are representative of 5 individual cell culture preparations used for both 2-D and 3-D cultures to exclude inter-animal variation.

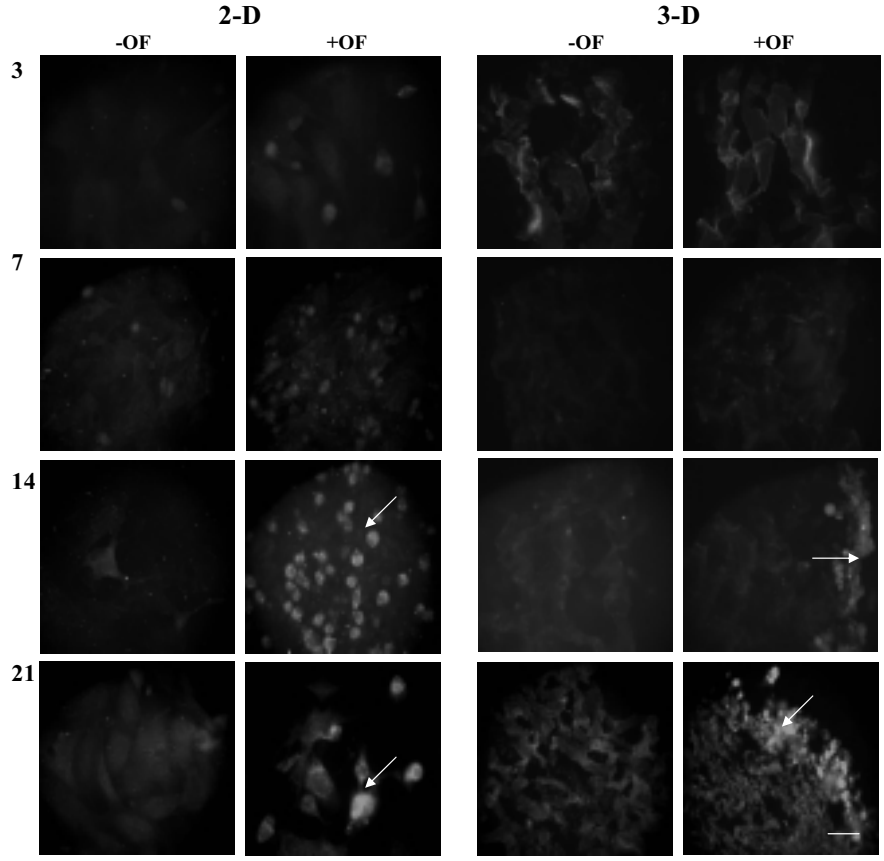


Fig. 2. Timecourse of osteocalcin expression in 2-D and 3-D. Expression of osteocalcin, a post proliferative osteoblastic marker, in monolayer MSC cultures and MSC seeded collagen GAG scaffolds was assessed by immunofluorescent staining. In 2-D, weak immunoreactivity was observed by day 7 in the presence of OF (dexamethasone, 0.68 nM; $\beta$-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). By day 14 strong osteocalcin immunoreactivity was observed throughout cultures. In contrast, osteocalcin expression was only weakly evident by day 14 and not before with much stronger staining evident by day 21. At no timepoint in 2-D or 3-D was any osteocalcin immunoreactivity observed in untreated control cultures. Results are representative of 5 individual cell culture preparations at each timepoint. Positive immunoreactivity is indicated by arrows.

### *3.2. Expression of osteocalcin in 2-D and 3-D for 21 days*

The expression of osteocalcin, a later stage marker of osteogenesis, being expressed by osteoblasts in the post proliferative phase of osteogenic differentiation, was also analysed immunofluorescently in MSCs cultured on coverslips and collagen GAG scaffolds (Fig. 2). In 2-D OF treated cultures, weak osteocalcin immunoreactivity was observed at day 7 and was much more evident by day 14 in pre-nodular cellular aggregations. Quantitative western immunoblot demonstrated that treatment with osteogenic factors for 14 days significantly increased osteocalcin expression from 0.50 $\pm$ 0.02 (mean $\pm$ s.e.m; arbitrary units) to 6.3 $\pm$ 0.10 ($p < 0.05$, Student's paired t-test, $n = 6$). In 3-D the expression of osteocalcin was slower. No immunoreactivity was observed until day 14 and then weakly at the very periphery of the scaffold, as had been observed previously [8]. By day 21 3-D expression of osteocalcin was much more evident. Results are representative of 5 individual cell culture preparations used for both 2-D and 3-D cultures to exclude inter-animal variation.

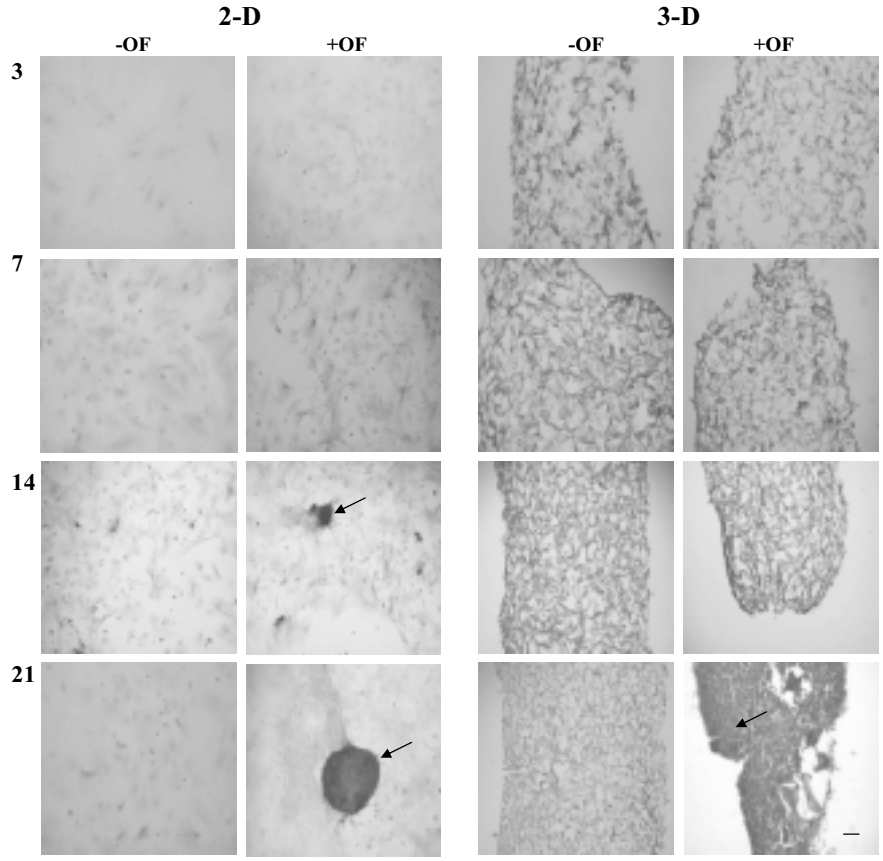


Fig. 3. Timecourse of mineralisation in 2-D and 3-D. Mineralisation, the final marker of osteogenesis, was assessed by Alizarin Red S staining for calcium. MSCs were cultured in 2-D and 3-D in the presence or absence of OF (dexamethasone, 0.68 nM; $\beta$-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for up to 21 days. By day seven, a small amount of positive staining for calcium was observed in 2-D cultures, with large mineralised nodules evident by day 14. In the collagen GAG scaffold however, mineralisation was not observed in any scaffold samples until day 21, suggesting a slower rate of MSC differentiation in 3-D. Results are representative of 5 individual cell culture preparations at each timepoint.

### *3.3. Histological analysis of mineralisation of 2-D and 3-D cultures*

The final marker of osteogenic differentiation is the mineralisation of osteoid, detectable by the presence of calcium/calcium phosphate. Adult MSCs were cultured in monolayer on plastic coverslips or seeded onto collagen GAG scaffolds in the presence or absence of OF for 3, 7, 14 and 21 days (Fig. 3). Mineralisation was assessed by Alizarin Red S staining for calcium. The osteogenic response of cells as assesed by the presence of calcium was much faster in 2-D than 3-D. By day 7, some calcium positive cells were observed in 2-D, with large bonelike nodules stained by day 14. In contrast, no mineralisation was observed in collagen GAG scaffolds until 21 days in any samples. Results are representative of 5 individual cell culture preparations used for both 2-D and 3-D cultures to exclude inter-animal variation.

### *3.4. RNA expression of osteocalcin in 2-D and 3-D over 21 days*

To further confirm the results obtained above, the levels of osteocalcin mRNA at 0, 3, 7, 10, 14 and 21 days were assessed. As can be seen in Fig. 4, when MSC seeded collagen GAG scaffolds were cultured

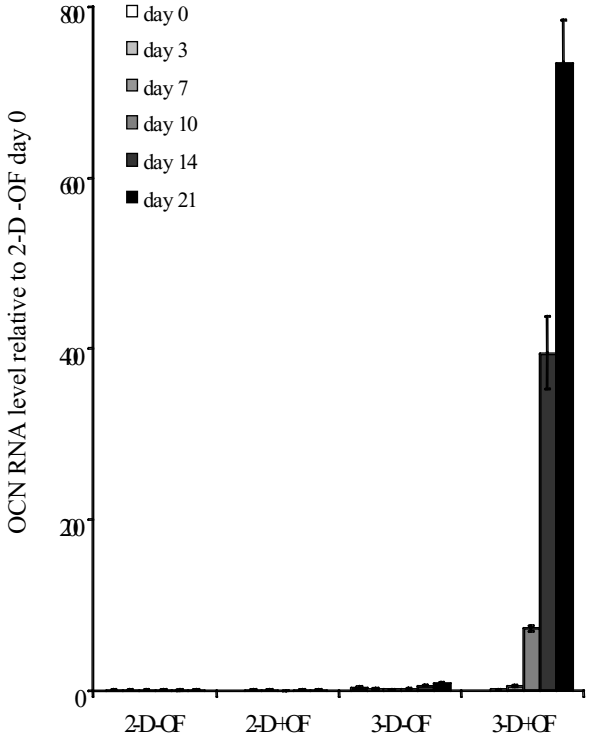


Fig. 4. Timecourse of osteocalcin RNA expression in 2-D and 3-D. Levels of osteocalcin RNA from MSC-seeded plastic or CG scaffold substrates incubated for 0, 3, 7, 10, 14 or 21 days in the presence or absence of OF, relative to the day 0 2-D culture. Day 0 samples were taken two days after seeding, before osteogenic treatment. Each point represents an average derived from duplicate* real-time PCR measurements of target cDNA, normalised to the average value for 18S rRNA derived in the same way (*With the exception of osteogenically treated scaffold samples from days 10 and 14, where there was only one valid measurement for OCN and 18S respectively.) Osteocalcin RNA levels were observed to increase from day 10 onwards in collagen GAG scaffolds. Error bars represent standard deviations derived from the replicates of both target and normalising gene measurements.

in the presence of OF, osteocalcin RNA levels began to rise from day ten onwards, slightly preceeding the expression at the protein level as would be expected. Unexpectedly, no detectable osteocalcin mRNA was observed in cultures in 2-D in the presence of OF. This is possibly due to the transient nature of osteoaclcin mRNA expression which might have been missed.

### *3.5. Long term mineralisation analysis of MSC seeded collagen GAG scaffolds*

In MSC seeded collagen GAG scaffolds cultured in the presence of OF, complete mineralisation of the scaffold did not always occur, as was previously observed [8]. It was hypothesised, based on observations and the findings of others [10,15], that the lack of mineralisation was due to insufficient time allowed for complete mineralisation to occur. To verify this, MSC seeded collagen GAG scaffolds were cultured in the presence or absence of OF for 4, 5, 6 and 7 weeks using cells from the same culture preparation for each timepoint to exclude inter-animal variation (Fig. 5). Scaffold mineralisation was assessed by the Von Kossa technique to stain calcium phosphate. It was observed that over time, the level of scaffold mineralisation increased until scaffolds were almost completely mineralised after 6 weeks in culture. Results are representative of 3 individual cell culture preparations for 4, 5, and 6 weeks and 1 at 7 weeks.

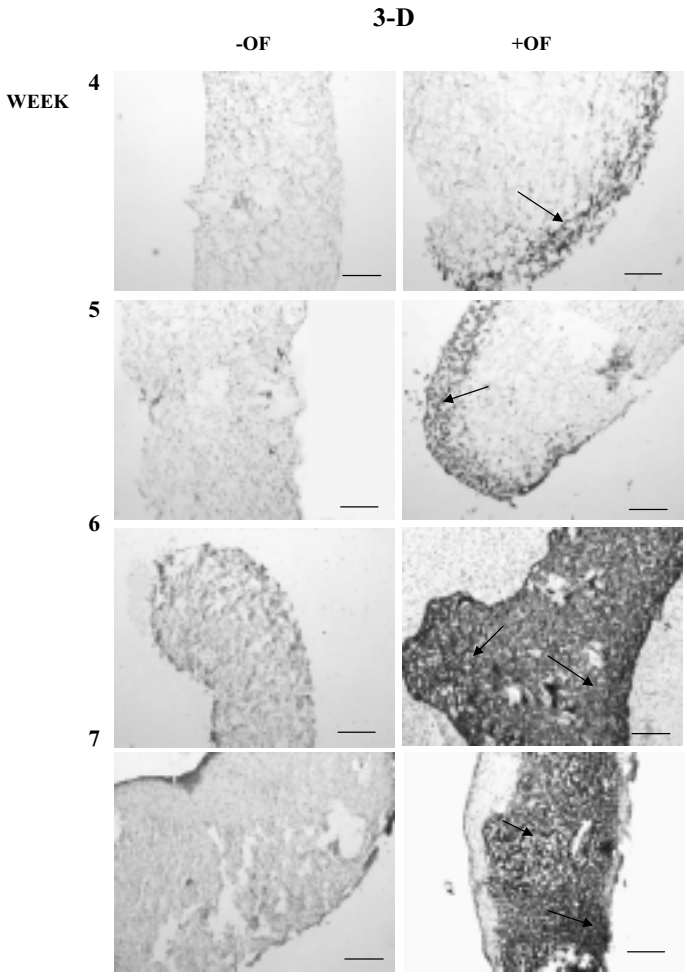


Fig. 5. Timecourse of scaffold mineralisation from 4–7 weeks. Scaffolds were seeded with MSCs and cultured in control medium in the absence or presence of OF (dexamethasone, 0.68 nM; $\beta$-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 4, 5, 6 and 7 weeks. The level of mineralisation was assessed at each timepoint by Von Kossa staining for calcium phosphate. The level of mineralisation (arrows) was observed to increase with time until complete mineralisation at 6 weeks. No core degradation was observed. The same 3 individual cell culture preparations were used at each timepoint to rule out inter-animal variation except at week seven where only one sample was available for analysis.

## 4. Discussion

The purpose of this study was to compare the rate of osteogenic differentiation of adult rat MSCs in culture on plastic (2-D) and in collagen GAG scaffolds (3-D) as assessed by analysis of several markers of osteogenic differentiation. The sustained expression of collagen I from day 3 onwards mirrored previous findings [1]. The potent expression of osteocalcin from day 10 onwards also matched temporal expression of osteocalcin found in other laboratories [5,12,23]. In accordance with Bianco et al. [2], 2-D cultures treated with osteoinductive factors were observed to form colonies with the subsequent formation of nodules as evidenced from day 7 onwards. Satomura et al. [21] also observed similar morphological characteristics, with MSCs appearing as large flattened cells with many processes and osteoblast-like cells being smaller and more cuboidal in shape. Maniatopoulos et al. [17] additionally

observed clusters of aggregating cells by day 8.

Having previously demonstrated the ability of these collagen GAG scaffolds to maintain MSC viability for up to 3 weeks [8], the expression of osteogenic markers and the appearance of mineralisation was assessed, compared to 2-D cultured samples. As the major organic component of bone [16], and one of the first proteins expressed in the osteogenic process [1], collagen type I is a very important protein in the osteogenic process. By day 3, some collagen I expression was evident in 3-D demonstrating a comparable initial cellular response to osteoinductive factors to that observed in 2-D. Positive collagen I immunofluorescence was observed from day 3 until the last timepoint of 21 days in both 2-D and 3-D. This staining was confined to the edges of the scaffolds where, as previously shown [8], the cells remain until at least day 14. By day seven, collagen I was seen to be produced extracellularly forming a matrix within the scaffold structure. This is a very important step since collagen I is known to be necessary for osteogenesis, and has been shown to stimulate bone formation [25]. Xiao et al. [27] suggested the possibility that the stimulation of the pre-osteoblast cell surface integrin $\alpha_2\beta_1$ leads to the activation of the core binding factor $\alpha 1$ (Cbf-$\alpha 1$) gene, that is necessary for osteogenesis to occur, via a MAP kinase signalling cascade. Activation of this surface integrin is by contact with type I collagen, demonstrating the importance of collagen I production and the possible benefit of using a scaffold primarily composed of same material. Again, the appearance of extracellularly produced collagen I at day 7 coincided with the 2-D expression. From these results it would appear that cells respond at a similar rate in 3-D as they do in monolayer cultures, perhaps with collagen I expression in 3-D prempting the 2-D expression. This might be due to the presence of collagen type I in the scaffold.

The post-proliferative osteoblastic marker osteocalcin was also used to assess the differentiation progress of MSCs in 2-D and 3-D. Osteocalcin is involved in matrix mineralisation [11,13] and as was expected, its expression closely mirrored the mineralisation timecourse in 2-D. Expression occurred weakly from day 3 onwards in 2-D, but this was probably related to a small number of partially differentiated MSCs in culture, as staining was of a low intensity and not very widespread. By day 10 (result not shown) osteocalcin expression was much more evident, with expression taking place in large nodule-like clusters of cells by day 14. This timecourse of events in 2-D is in accordance with the findings of several others [5,12,23]. By day 14, osteocalcin expression was only weakly evident at the periphery of the collagen GAG scaffold and was not observed at earlier timepoints. Localisation of bone specific osteocalcin was confined to the regions of the scaffolds in which cells were evident at 14 days, suggesting that although the cells had not penetrated the scaffold, differentiation was taking place. Taking into account the late stage expression of osteocalcin during osteogenesis [3], it appears that the cells had progressed along the differentiation pathway before migration into the centre of the scaffolds took place. Osteocalcin expression was more intense in 21 day treated samples compared to 14 day samples. This expression profile appeared to be slightly slower than was observed in 2-D. As shown by Laurencin et al. [15], cells take longer to respond to a 3-D environment, as is evident from this study.

Despite expression of osteocalcin at the periphery of scaffolds at day 14, no mineralisation was observed in any scaffolds as evidenced by Alizarin Red S staining for calcium. This is most likely due to insufficient time for the cells to attach to the scaffolds and move far enough along the differentiation pathway to mineralise osteoid in 14 days partly because the response time of these cells increases in the 3-D setting [15]. The difference between 14 and 21 days in culture was marked in all cases, with substantial mineralization occurring in osteoinductive factor-treated scaffolds after 21 days. Mineralisation was 14 days later in scaffolds compared to 2-D samples, occurring at day 21 rather than day 7. It appears that while cells initially respond at the same rate in a 3-D setting, with early expression of collagen I, it takes longer for each step on the osteogenic differentiation process to occur compared to 2-D cultures.

Despite this apparent delay in osteogenesis, Xiao et al. [26] observed a similar timecourse of activity with collagen scaffolds, seeded with osteoblasts. Considering these were already differentiated cells, matching the duration of time required for mineralisation with adult stem cells is promising and of interest regarding the use of this cell population for the repair of defects *in vivo*. It also illustrates the biocompatible nature of the collagen GAG scaffold and its suitability as a 3-D medium for support of osteogenic differentiation of MSCs. The presence of collagen I in the scaffold, with which the MSCs had close contact, appears to be a suitable factor to aid the induction of differentiation of these cells along the osteogenic pathway, as has been demonstrated by Xiao et al. [25].

It was observed that matrix mineralisation did not always take place throughout the entire scaffold by day 21, often only occurring at the periphery. Since full scaffold penetration had not even occurred by day 14 [8], one would not expect complete mineralisation to occur within a week. In addition to the findings of Freed et al. [10], Yang et al. [28] also observed that it took 6 weeks for the internal matrix of PLGA scaffolds to be penetrated by MSCs. In this study, we observed complete scaffold penetration after only 21 days. It is interesting to note that the scaffold employed by Freed et al. was only 2 mm thick whereas the one used in this study was 3–4 mm when wet. Unfortunately Yang et al. do not provide the dimensions of their scaffold for similar comparison. Additionally Laurencin et al. [15] demonstrated the increased time required for cells to respond in a 3-D environment. As a result of these findings, and the lack of consistency of complete mineralisation of the scaffolds in this study, experiments were carried out to determine if this was caused by insufficient time for mineralisation to take place or lack of nutrients in the centre of the scaffolds. Scaffolds were cultured for 4, 5, 6 and 7 weeks in control and osteoinductive medium. Scaffold mineralization was subsequently assessed. The results clearly demonstrate that, while mineralisation was a slow process, if given enough time the entire scaffold will be mineralised. As discussed by Kelly and Prendergast [14] and demonstrated by Pei et al. [19], a current problem with scaffolds and tissue engineered constructs is core degradation. This can be as a result of insufficient nutrient delivery and waste removal, particularly in static cultures. The results from this study are promising. They demonstrate complete mineralisation of a scaffold 3–4 mm thick without dynamic stimulation or any other means of nutrient delivery by 6–7 weeks. Importantly, this study also demonstrated the ability to maintain these scaffolds in culture for extended periods without matrix degradation or resorption by the cells. The glycosaminoglycan content in the scaffold would have played a role in the maintenance of scaffold integrity as shown by Pieper et al. [20].

Following 21 days culture within the collagen GAG scaffold, MSCs show the ability to differentiate fully along the osteogenic lineage, slightly slower than when cultured on coveslips in 2-D, as evidenced by collagen type I and osteocalcin expression and osteocalcin RNA levels. Furthermore, matrix mineralisation was evident in those scaffolds cultured in the presence of osteoinductive factors for 21 days. As stated, complete mineralisation of scaffolds did not always occur at 21 days, but if cultured for longer periods, would take place. This demonstrates the long term viability of MSC seeded scaffolds and the ability of this scaffold to support osteogenic differentiation of MSCs for up to 7 weeks. This paper provides some important data concerning the activity of adult rodent MSCs when cultured in 2 different environments and further illustrates the potential use of collagen GAG scaffolds for the generation of tissue engineered bone constructs using adult stem cells.

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## References


[1] J.E. Aubin et al., Osteoblast and chondroblast differentiation, *Bone* **17**(2 Suppl) (1995), 77S–83S.
[2] P. Bianco et al., Bone marrow stromal stem cells: nature, biology, and potential applications, *Stem Cells* **19**(3) (2001), 180–192.
[3] J. Bilezikian, L. Raisz and G.A. Rodan, *Principles of Bone Biology*, 2001.
[4] J.F. Burke et al., Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury, *Ann Surg* **194**(4) (1981), 413–428.
[5] P. Cassiede et al., Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-beta 1 or PDGF-BB as assayed *in vivo* and *in vitro*, *J Bone Miner Res* **11**(9) (1996), 1264–1273.
[6] L.J. Chamberlain et al., Collagen-GAG substrate enhances the quality of nerve regeneration through collagen tubes up to level of autograft, *Exp Neurol* **154**(2) (1998), 315–329.
[7] M.J. Coelho and M.H. Fernandes, Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation, *Biomaterials* **21**(11) (2000), 1095–1102.
[8] E. Farrell et al., A collagen glycosaminoglycan scaffold supports adult rat mesenchymal stem cell differentiation along the osteogenic and chondrogenic routes, *Tissue Eng* (2005).
[9] M.M. Fedewa et al., Chondrocytes in culture produce a mechanically functional tissue, *J Orthop Res* **16**(2) (1998), 227–236.
[10] L.E. Freed et al., Chondrogenesis in a cell-polymer-bioreactor system, *Exp Cell Res* **240**(1) (1998), 58–65.
[11] P.V. Hauschka et al., Presence of osteocalcin and related higher molecular weight 4-carboxyglutamic acid-containing proteins in developing bone, *J Biol Chem* **258**(1) (1983), 176–182.
[12] A. Herbertson and J.E. Aubin, Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures, *Bone* **21**(6) (1997), 491–500.
[13] S. Kasugai et al., Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells *in vitro*: inductive effects of dexamethasone on the osteoblastic phenotype, *J Cell Physiol* **147**(1) (1991), 111–120.
[14] D.J. Kelly and P.J. Prendergast, Effect of a degraded core on the mechanical behaviour of tissue-engineered cartilage constructs: a poro-elastic finite element analysis, *Med Biol Eng Comput* **42**(1) (2004), 9–13.
[15] C.T. Laurencin et al., Tissue engineered bone-regeneration using degradable polymers: the formation of mineralized matrices, *Bone* **19**(1 Suppl) (1996), 93S–99S.
[16] H. Lodish, A. Berk and S.L. Zipursky, *Molecular Cell Biology*, New York: W.H. Freeman and Co., 2000.
[17] C. Maniatopoulos, J. Sodek and A.H. Melcher, Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats, *Cell Tissue Res* **254**(2) (1988), 317–330.
[18] F.J. O'Brien et al., Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds, *Biomaterials* **25**(6) (2004), 1077–1086.
[19] M. Pei et al., Bioreactors mediate the effectiveness of tissue engineering scaffolds, *Faseb J* **16**(12) (2002), 1691–1694.
[20] J.S. Pieper et al., Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats, *Biomaterials* **21**(16) (2000), 1689–1699.
[21] K. Satomura, K. Hiraiwa and M. Nagayama, Mineralized nodule formation in rat bone marrow stromal cell culture without beta-glycerophosphate, *Bone Miner* **14**(1) (1991), 41–54.
[22] J.S. Temenoff and A.G. Mikos, Review: tissue engineering for regeneration of articular cartilage, *Biomaterials* **21**(5) (2000), 431–440.
[23] H. Tsuchida et al., Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats, *J Orthop Res* **21**(1) (2003), 44–53.
[24] M. Weber et al., Formation of cartilage matrix proteins by BMP-transfected murine mesenchymal stem cells encapsulated in a novel class of alginates, *Biomaterials* **23**(9) (2002), 2003–2013.
[25] G. Xiao et al., Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor, *J Biol Chem* **273**(49) (1998), 32988-32994.
[26] Y. Xiao et al., Tissue engineering for bone regeneration using differentiated alveolar bone cells in collagen scaffolds, *Tissue Eng* **9**(6) (2003), 1167–1177.
[27] Z.S. Xiao, T.K. Hinson and L.D. Quarles, Cbfa1 isoform overexpression upregulates osteocalcin gene expression in non-osteoblastic and pre-osteoblastic cells, *J Cell Biochem* **74**(4) (1999), 596–605.
[28] X.B. Yang et al., Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification, *Bone* **29**(6) (2001), 523–531.
[29] H.A. Awad et al., Autologous mesenchymal stem cell-mediated repair of tendon, *Tissue Eng* **5**(3) (1999), 267–277.
[30] M.S. Chapekar, Tissue engineering: challenges and opportunities, *J Biomed Mater Res* **53**(6) (2000), 617–620.
[31] A.J. Friedenstein, S. Piatetzky II and K.V. Petrakova, Osteogenesis in transplants of bone marrow cells, *J Embryol Exp Morphol* **16**(3) (1966), 381–390.

[32] N. Jaiswal et al., Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*, *J Cell Biochem* **64**(2) (1997), 295–312.
[33] B. Johnstone et al., *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells, *Exp Cell Res* **238**(1) (1998), 265–272.
[34] S. Kadiyala et al., Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential *in vivo* and *in vitro*, *Cell Transplant* **6**(2) (1997), 125–134.
[35] A.M. Mackay et al., Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow, *Tissue Eng* **4**(4) (1998), 415–428.
[36] M.F. Pittenger et al., Multilineage potential of adult human mesenchymal stem cells, *Science* **284**(5411) (1999), 143–147.
[37] R.G. Young et al., Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair, *J Orthop Res* **16**(4) (1998), 406–413.