



3D mesenchymal stem/stromal cell osteogenesis and autocrine signalling

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ABSTRACT

Mesenchymal stem/stromal cells (MSC) are rapidly becoming a leading candidate for use in tissue regeneration, with first generation of therapies being approved for use in orthopaedic repair applications. Capturing the full potential of MSC will likely require the development of novel *in vitro* culture techniques and devices. Herein we describe the development of a straightforward surface modification of an existing commercial product to enable the efficient study of three dimensional (3D) human bone marrow-derived MSC osteogenic differentiation. Hundreds of 3D microaggregates, of either 42 or 168 cells each, were cultured in osteogenic induction medium and their differentiation was compared with that occurring in traditional two dimensional (2D) monolayer cultures. Osteogenic gene expression and matrix composition was significantly enhanced in the 3D microaggregate cultures. Additionally, BMP-2 gene expression was significantly up-regulated in 3D cultures at day 3 and 7 by approximately 25- and 30-fold, respectively. The difference in BMP-2 gene expression between 2D and 3D cultures was negligible in the more mature day 14 osteogenic cultures. These data support the notion that BMP-2 autocrine signalling is up-regulated in 3D MSC cultures, enhancing osteogenic differentiation. This study provides both mechanistic insight into MSC differentiation, as well as a platform for the efficient generation of microtissue units for further investigation or use in tissue engineering applications.

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1. Introduction

Discovered in 1980 [1] but only fully recognised in 1994 [2], mesenchymal stem/stromal cells (MSC) have recently garnered significant acclaim for their potential in regenerative medicine. There are currently 198 registered MSC clinical trials (www.clinicaltrials.gov), as well as allogeneic and autologous MSC products [3] already approved for bone repair in various international jurisdictions (Osteocel by Osiris therapeutics, Inc.) [4]. The ability to manipulate MSC *in vitro* has enabled these rapid advancements. However, full realization of MSC therapeutic potential will likely require the development of more cost effective expansion technologies, as well as *in vitro* platforms that facilitate investigation into their biology. Given the propensity of MSC to differentiate into osteoblasts, and their already proven clinical potential in bone repair, considerable research has been focused on the identification of factors that direct osteogenesis *in vitro* and *in vivo* [5].

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Matrix elasticity has the capacity to direct MSC fate decisions *in vitro* [6]. Specifically, MSC commit to an osteogenic lineage when cultured on two dimensional (2D) substrates having a relatively rigid modulus of 25–40 kPa. This is intuitively pleasing, as this range correlates with the measured stiffness for the cross-linked collagen found in osteoids [6–8]. Lower modulus surfaces selectively drove alternative lineage selections in these experiments. Whilst MSC lineage selection in response to tissue matrix elasticity appears to be a logical and elegant explanation for cell fate decisions, the actual mechanism is likely not so simplistic. Cell spread area correlates with matrix stiffness [6], and differential spread area on a fixed modulus surface also appears to influence MSC fate decisions [9]. In this case, a large spread area promotes osteogenesis, whilst a reduced spread area on the same rigid surface promotes adipogenesis. Collectively, these studies suggest that allowing MSC to spread unhindered on rigid 2D surfaces should maximize osteogenic differentiation [6,9]. These same observations appear to be valid when MSC are cultured in three dimensional (3D) gels, although matrix stiffness appears to influence MSC fate decisions more so than the resulting cell shape or “spread area” [10]. In both 2D and 3D matrix elasticity studies a low cell density was maintained to maximize cell interaction with

the tailored artificial microenvironment and to minimize the potentially confounding influence of adjacent cells [6,9]. However, this may be a confounding factor itself as cell behaviour in or on biomaterial surfaces can be perturbed [11]. There is increasing awareness in cancer biology [11] and tissue engineering [12–14] that cell-only microaggregates often provide a far more realistic *in vivo* mimic than traditional 2D monolayer cultures on biomaterial surfaces or even those performed in 3D gels. It has long been appreciated that 3D cell aggregation enhances the chondrogenic potential of MSC [15], whilst 3D aggregation has only recently been shown to enhance MSC maintenance [16], as well as their adipogenic [17] and osteogenic differentiation [18].

Whilst a wealth of biological potential can be unlocked through simply manipulating MSC or other cells in 3D microaggregates [11], such cultures have historically been tedious and rate limiting [11–14]. Historically, microaggregates were formed individually in tubes or using hanging-drop cultures. However, recently developed platforms [17,19] and products (Aggrewell™, STEMCELL Technologies) facilitate the rapid manufacture of hundreds to thousands of uniform 3D microaggregates. The Aggrewell™ product is essentially a polymer insert having approximately 600 microwells/cm² on its surface. This insert is seated at the bottom of a 24-well plate, allowing approximately 1200 uniform microaggregates to be formed simultaneously within a single well. The ability to generate hundreds of microaggregates of a uniform size is a feature virtually exclusive to the Aggrewell™ product. The Aggrewell™ works seamlessly when cultures are maintained in serum-free medium [19], with cells preferentially aggregating with each other rather than adhering to the insert's reasonably inert surface. Any cellular adhesion to the polymer surface compromises microaggregate formation. Herein we outline a simple surface modification strategy that enhances the functionality of the Aggrewell™ product by further limiting cell attachment to the insert's polymer surface, even in the presence of serum. We used this modified high-throughput platform to investigate MSC differentiation, and the temporal expression of bone morphogenic protein-2 (BMP-2) gene expression, evaluating microaggregates formed from either ~42 or ~168 cells. Microaggregate cell numbers span those shown previously to support enhanced 3D MSC osteogenesis (50 cells/microaggregate) [17], as well as numbers that we have previously shown to support enhanced MSC chondrogenesis (168 cells/microaggregate) [20].

2. Materials and methods

2.1. Human MSC isolation and expansion

Human bone marrow aspirates were obtained from iliac crest of fully informed healthy volunteer donors in the Mater Hospital (Brisbane, Australia). Ethical approval for this study was granted through the Mater Health Services Human Research Ethics Committee and the Queensland University of Technology in accordance with the Australian National Health and Medical Research Council's Statement on Ethical Conduct in Research Involving Humans. Mononuclear cells were enriched by density gradient centrifugation and cultured as described previously [20].

2.2. Microwell surface modification

To make the AggreWell™ insert's surface resistant to protein adsorption, and to minimize cell attachment, we utilized an electrostatic multilayering technique. Firstly, the Aggrewell™ microwell polymer insert was chemically modified using a high frequency plasma generator (Model BD-20, ETP) for approximately 30 s to yield a surface with a net negative charge [21]. A layer of

electropositive poly-L-lysine (50 µg/mL, Sigma) in MES buffer (pH 5.5) was adsorbed to the negatively charged surface for 30 min at room temperature (RT). Five bilayers of electronegative hyaluronic acid (HA, 1.78 MDa, Lifecore Biomedical) and electropositive chitosan (CHI, Sigma) were then deposited on top of the poly-L-lysine coated Aggrewell™. This HA and CHI multilayer (ML) protocol was based on our work [22], as well as a previous study that characterised the ML's ability to effectively resist protein adsorption [23]. In brief, the ML was formed through sequential layer-by-layer deposition of either CHI or HA at 50 µg/mL MES buffer (pH 5.5) until 5 bilayers were established. In our hands, >3 bilayers were required to effectively generate the desired surface properties. Each bi-layer was permitted to adsorb for 15 min at RT, and then the surface was rinsed with 1 mL of MES buffer before treating the surface with the next electrostatic solution. The ML structure was stabilized by covalent cross-linking of the amine groups on the CHI with the acid groups on the HA via classical NHS-EDAC chemistry (*N*-Hydroxysuccinimide/*N*-Ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride) [22]. The cross-linking step was performed during the adsorption of each HA layer. The HA solution was spiked with a 1:100 dilution of 50 mg/mL EDAC and 70 mg/mL NHS in dimethyl sulfoxide. Following ML assembly, the wells were thoroughly rinsed with PBS and sterilized in 70% ethanol for 20 min at RT. Wells were then rinsed thoroughly with sterile PBS and stored in PBS at 4 °C until use.

2.3. Osteogenic culture

MSC microaggregates of approximately 42 or 168 cells each were formed by suspending 50,000 or 200,000 cells in modified Aggrewells™, respectively. Each 24-well Aggrewell™ insert has approximately 1200 microwells, thus the number of cells collected in each microwell can be estimated by dividing the total cell number (50,000 or 200,000) by the number of microwells (1200). Freshly seeded plates were spun in a plate centrifuge for 5 min at 400g to facilitate aggregation. Control 2D cultures were initiated with 20,000 MSC per well (10,000 cells/cm²) in a standard tissue culture 24-well plate (BD Falcon). Cells were cultured in osteogenic induction medium containing DMEM-high glucose (DMEM-HG, Gibco-Invitrogen) supplemented with 10% FBS, 1% P/S, 100 nM dexamethasone (Sigma), 50 µM L-ascorbic acid 2-phosphate (Sigma), 10 mM β-glycerol phosphate (Sigma) and 4 mM CaCl₂ (Merck). Microaggregates were harvested at either day 7 or 14 of culture.

2.4. Relative gene expression analysis

At culture endpoints, RNA was isolated in order to determine the relative osteogenic gene expression. RNA was extracted using an RNeasy Mini Kit (Qiagen) and then reverse transcribed using SuperScript III RT (Gibco-Invitrogen) according to the manufacturers' instructions. qPCR master mix, containing Platinum SYBR Green qPCR SuperMix-UDG (Gibco-Invitrogen) and primers, was dispensed into 384-well reaction plates and combined with cDNA template using an EpMotion 5057 liquid handling robot (Eppendorf). The PCR amplification was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Product size and primer sequences are described in Table 1 [20,24,25]. The results were analysed using the 2^{-ΔΔCt} method normalized against the geometric mean of two housekeeping genes, cyclophilin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5. Calcium content and alkaline phosphatase assays

Alkaline phosphatase (ALP) activity was quantified at day 7 and 14 of culture. The cells were lysed in a solution of 0.1%

Table 1
Primer sequences used for qPCR.

Genes	Primers sequences	Amplicon size (bp)	References
F: GAPDH	ATGGGGAAGGTGAAGGTCTG	119	[20]
R: GAPDH	TAAAAGCAGCCCTGGTGACC		
F: Cyclophilin A	CTCGAATAAGTTTGACTTGTGTTT	164	[20]
R: Cyclophilin A	CTAGGCATGGGAGGGAACA		
F: Collagen I	CAGCCGCTTCACCTACAGC	83	[20]
R: Collagen I	TTTTGTATTCAATCACTGTCTTGCC		
F: Osteonectin	AGGTATCTGTGGGAGCTAATC	224	[25]
R: Osteonectin	ATTGCTGCACACCTTCTC		
F: Osteocalcin	GAAGCCCAGCGGTGCA	70	[20]
R: Osteocalcin	CACTACCTCGCTGCCCTCC		
F: BMP-2	AAAACGTCAAGCCAACACAAA	72	[24]
R: BMP-2	GTCCACGTACAAAGGGTCTCTCT		
F: BMP-4	GCCAAGCGTAGCCCTAAGC	65	[24]
R: BMP-4	GTGGCGCCGGCAGTT		
F: ALP	CGTGGCTAAGAATGTCATCATGTT	89	[24]
R: ALP	TGGTGGAGCTGACCCTTGA		
F: Runx2	GGAGTGGACGAGGCAAGAGTTT	133	[20]
R: Runx2	AGCTTCTGTCTGTGCCTTCTGG		

Triton X100 in 0.2 M carbonate buffer (pH 10.1), and para-nitrophenyl phosphate (1 mg/mL; Sigma) was used as the ALP substrate. The ALP activity in each culture was normalized to the total DNA content in individual cultures. DNA content was determined using a Quant-iT PicoGreen (Gibco-Invitrogen) DNA quantification kit as per the manufacturer's instructions. Standard curves were generated using λ Phage DNA (Gibco-Invitrogen) as a reference.

Calcium was extracted from the monolayer and the microaggregate cultures by incubating the cultures overnight in 10% acetic acid at RT. Diluted extracts were then mixed with *o*-cresolphthaleincomplexone (Sigma) reagent, which interacts with calcium in a linear concentration-dependent manner. The absorbance was read at 570 nm on a Polar Star Optima reader (BMG labtech).

2.6. Histological analysis

For histological analysis, the microaggregates were fixed in 4% paraformaldehyde (PFA) and then harvested with a pipette by flushing PBS over the microwell surface. Microaggregates were collected and centrifuged at 350g for 5 min and snap-frozen in Tissue-Tek OCT compound (Sakura Finetek). Sections (7 μ m thick) were generated using a CM1850 cryostat (Leica). Sections were collected onto polylysine-coated slides (Thermo Scientific) and air-dried. Calcium depositions were stained with Alizarin red S stain and subsequently visualized using a Nikon Eclipse TE2000-U phase contrast fluorescent microscope (Nikon Coolpix 4500). Collagen I content was identified by first labelling with a monoclonal mouse anti-collagen I antibody (Abcam) overnight at 4 °C, and then for 1 h at RT with a secondary anti-mouse IgG1 antibody (Superlight-allophycocyanin; Abcam). After washing, coverslips were mounted using ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) mounting media (Gibco-Invitrogen). Images were acquired using an Eclipse TE2000-U fluorescence microscope and NIS Elements (F3.2) software.

2.7. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA). Results were deemed statistically different if $P < 0.05$. All continuous data were expressed as mean \pm standard deviation (SD). All experiments had a minimum of $n = 4$ and were repeated using a second MSC donor.

3. Results and discussion

3.1. Surface modification enhances microaggregate formation

The Aggrewell™ platform facilitated the consistent distribution of approximately 42 or 168 cells/microwell when the well was loaded with either 50,000 or 200,000 cells, respectively. However, microaggregate formation on the unmodified surface was compromised by cell adhesion to the underlying polymer insert (Fig. 1A). The Aggrewell™ platform was originally developed for embryoid body formation, or the aggregation of human embryonic stem cells (hESC), which is traditionally performed in serum-free medium [19]. In these studies the authors did not indicate that the attachment of hESC to the polymer surface was problematic. However, in our hands, human bone marrow-derived MSC initially attached to the unmodified Aggrewell™ surface even when cultures are performed in serum-free medium [20]. As our osteogenic medium contained serum, cell attachment to the polymer insert became increasingly problematic, prompting the development of ML surface modification (Fig. 1B) to make the surface more resistant to protein adsorption [23]. As a result of this surface modification MSC attachment to the Aggrewell™ polymer insert was reduced (Fig. 1C), and microaggregate formation was significantly enhanced at 24 h of culture. This simple ML modification strategy enabled reliable manufacture of microaggregates having diameters of 55 or 90 μ m composed of \sim 42 and \sim 168 cells, respectively (Fig. 1D–F). Microaggregates were easily harvested from this surface by pipette aspiration.

3.2. 2D and 3D calcium mineralization and alkaline phosphatase activity

MSC osteogenesis was investigated via quantification of calcium mineralization and alkaline phosphatase activity, both of which are indicative of MSC osteogenesis [26]. At day 7 there was significantly greater calcium deposition in the microaggregates formed from 168 cells, relative to either the 2D cell monolayer or 42 cell microaggregates (Fig. 2A). Calcium content continued to increase in all cultures over the subsequent week. By day 14 the calcium content in the microaggregate cell cultures was statistically equivalent between the 3D cultures, and both of these cultures had significantly greater calcium content than in the 2D monolayer controls. The cellular ALP activity increased relative to day zero MSC for all three culture conditions tested, although this activity

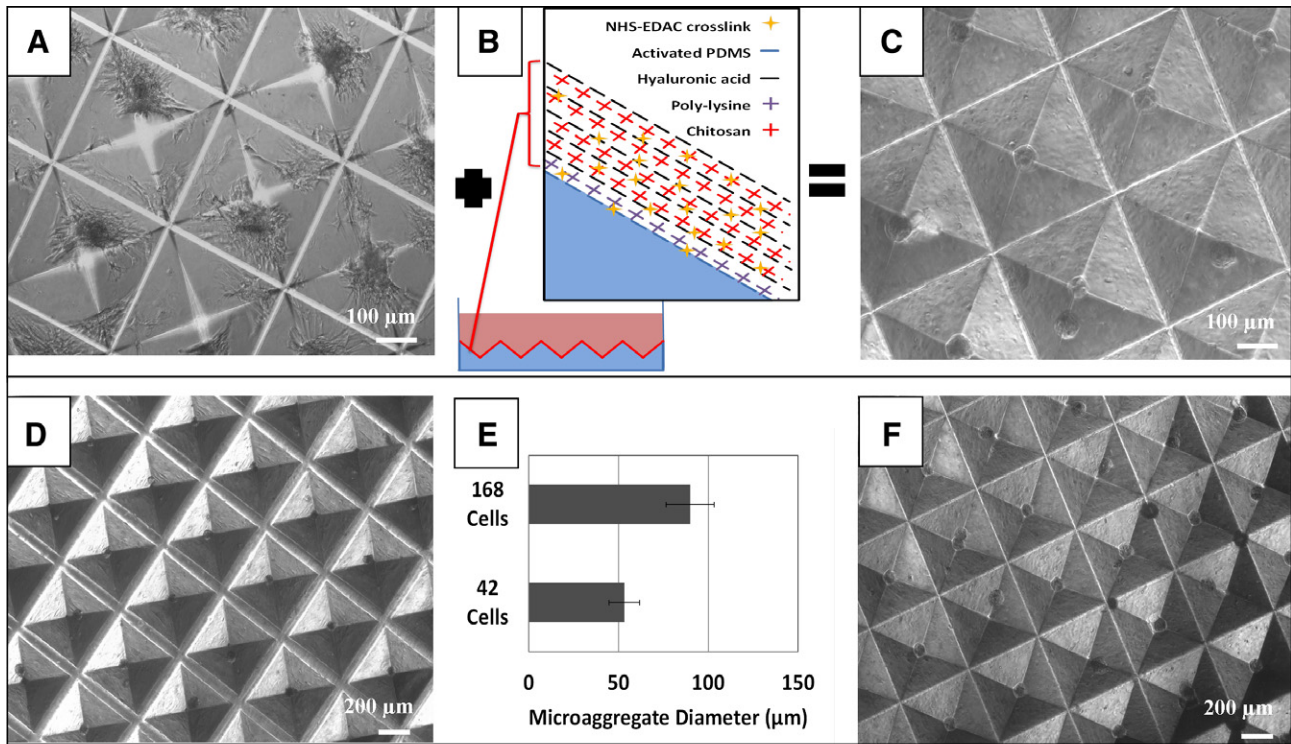


Fig. 1. Surface modification of Aggrewwells™ and microaggregte size distribution. (A) On unmodified surfaces microaggregte formation was compromised by cellular adhesion to the insert's polymer surface. (B) The schematic shows the sequential deposition of the CHI:HA ML onto the microwell surface. (C) Cells grown in a ML surface modified Aggrewwell™ do not adhere to the surface, and instead form consistent microaggregates. (D–F) Microaggregates formed from approximately 42 or 168 MSC each. (D) Microaggregates of ~42 cells each. (F) Microaggregates of ~168 cells each. (E) Diameter of the microaggregates ($n = 50$).

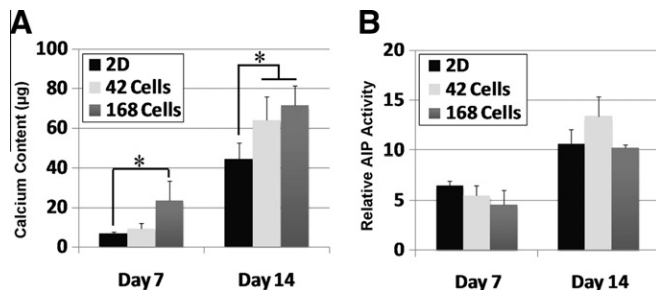


Fig. 2. Calcium content and alkaline phosphatase (ALP) activity in 3D microaggregates and 2D monolayer cultures at day 7 and 14. (A) Calcium content was greater in the 168 cell microaggregates at day 7 relative to 2D and 42 cell microaggregates. At day 14 calcium content in both 3D microaggregate cultures was greater than in the 2D control cultures. (B) ALP activity was similar in both 2D and 3D systems. (Displayed values are averages and SD, $n = 4$).

was found to be statistically equivalent in all culture conditions (Fig. 2B). In summary, initial ALP activity indicated that both 2D and 3D platforms supported MSC osteogenesis, and calcium content assessment indicated that 3D cultures outperformed 2D cultures.

3.3. Characterization of 2D and 3D osteogenic gene expression

qPCR was used to quantify the relative expression of osteogenic markers including Osteonectin, Osteocalcin, ALP, Runx2, and Collagen I, as well as potential autocrine factors including BMP-4 and BMP-2 [17,27]. Osteogenic gene expression was similar between the 2D monolayer cultures and both of the 3D microaggregate cultures at day 7 (Fig. 3). However, at day 14 osteogenic gene expression was significantly higher in both 3D microaggregate cultures, relative to the 2D monolayer controls. Osteogenic gene expression

was similar in the 42 cell and 168 cell microaggregates at both day 7 and 14. Collagen I gene expression appeared to be greater in the 42 cell microaggregates at day 14, however, this difference was not statistically significant.

BMP-2 autocrine signalling has been shown to enhance MSC osteogenic potential [28]. Blocking BMP-2 autocrine signalling with antibodies compromised Runx2 controlled transcription and its ability to effectively drive MSC osteogenic differentiation [28]. In an earlier investigation of MSC microaggregate differentiation a modest increase in BMP-2 gene expression (2–3.5-fold) was observed in 3D cultures (~50 cells/microaggregate) at days 1 and 6 of culture and this increase coincided with enhanced osteogenesis [17]. In the study reported here the temporal expression of BMP-2 was monitored in 2D and 3D differentiation cultures at days 7 and 14. At day 7, there was an approximate 30-fold increase in BMP-2 gene expression in the 3D cultures relative to 2D control cultures (Fig. 3), suggesting that autocrine signalling was indeed up-regulated in the 3D cultures. However, by day 14 BMP-2 gene expression had equalized in all 2D and 3D cultures, suggesting that both 2D and 3D cultures reached maximum BMP-2 expression by day 14. In contrast, BMP-4 gene expression was equivalent in all 2D and 3D cultures until day 7, and then marginally reduced in 3D cultures at day 14 (Fig. 3).

The observed increase in BMP-2 gene expression may have arisen as a direct result of 3D cell aggregation, or it could have been dependent on exposure of the cell population to osteogenic induction medium. To distinguish between these two possibilities, the relative BMP-2 gene expression in 2D monolayers and 3D microaggregates was assayed after 3 days in cultures that were maintained in either MSC expansion medium (DMEM-LG + 10% FBS) or osteogenic induction medium. The medium composition did not significantly influence BMP-2 gene expression (data not shown). However, the organization of the cells into 3D

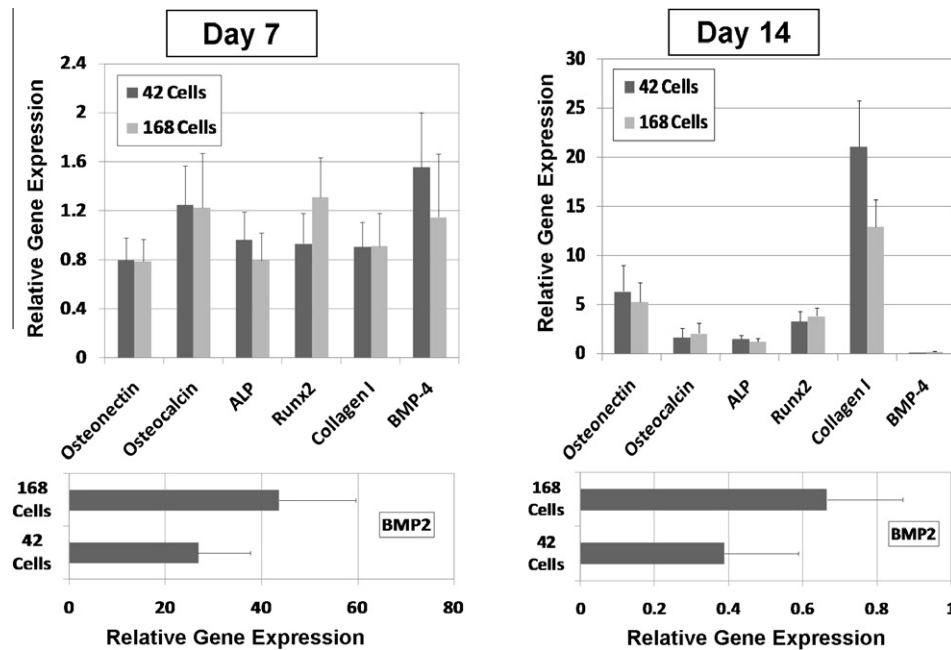


Fig. 3. Relative osteogenic gene expression at day 7 and 14. Osteogenic gene expression was similar in 2D and 3D cultures at day 7. BMP-2 gene expression was significantly up-regulated in 3D cultures at day 7. Osteogenic gene expression was up-regulated at day 14 in both 3D cultures, relative to 2D. All bars represent gene expression in the 3D culture system relative to 2D monolayer cultures. Error bars represent one SD ($n = 4$).

microaggregates, regardless of medium composition, resulted in a 25-fold ($P < 0.01$, $n = 4$) increase in BMP-2 gene expression relative to 2D control cultures.

In summary, BMP-2 gene expression was up-regulated in MSC 3D microaggregates at day 3, and this up-regulation persisted through the first week of osteogenic induction. This differential gene expression was lost as the differentiation of the cultures progressed, and at day 14 BMP-2 gene expression was equivalent in all 2D and 3D cultures. Our results are consistent with the previously proposed theory that the up-regulation of BMP-2 in 3D MSC cultures likely enhances the osteogenic potential of the culture through autocrine signalling [17]. However, the high relative BMP-2 gene expression appears to be dependent on the stage of MSC differentiation and is not maintained in 3D cultures as the cells mature into osteoblasts. This would suggest that this is an MSC phenomenon and not an osteoblastic one.

3.4. Characterization of 2D and 3D osteogenic matrix deposition

To assess the “tissue-like” properties of the bone microaggregates, immunohistochemistry was performed (Fig. 4). Calcium staining was greater in the 3D microaggregates than in the 2D monolayers at both day 7 and 14. This qualitative visual assessment is consistent with quantitative calcium measurements shown in Fig. 2. Collagen I immunoreactivity was more intense in the 3D microaggregates than in the 2D monolayer controls, and this too was consistent with day 14 qPCR results indicating greater collagen I gene expression in 3D microaggregate cultures. Cumulatively, calcium and collagen I matrix content was greater in the 3D microaggregates relative to the 2D controls. Additionally, this matrix material appeared to be relatively uniformly distributed throughout the microaggregates as evidenced by the uniform staining shown in Fig. 2. Adequate mass transport and uniform matrix distribution would be expected, given the small diameter ($<100 \mu\text{m}$) of the microaggregates [12,20].

The ability to efficiently and consistently generate bone 3D microaggregates is important for subsequent utilization as bone mimics in other *in vitro* investigations or as building blocks in tis-

sue engineering applications. In fact, MSC and osteoblast microaggregates have been used as mimics to investigate haematopoietic stem cell (HSC) homing *in vitro* [29] and microaggregates have been assembled into larger, and potentially clinically relevant macroscopic tissue dimensions [30]. Current methodologies used to generate microaggregates, such as the hanging drop or non-adhesive 96-well plates (generating a single aggregate per well), are labour intensive. The high throughput microaggregate assembly strategy described here, utilizing the ML surface modified Aggrewell™ enables the generation of hundreds of microaggregates in a single well, and facilitates their easy harvest. Additionally, the Aggrewell™ surface geometry, which functions to focus the cell population into the bottom of each microwell, will facilitate the controlled and accurate seeding of a secondary cell population onto individual aggregates. This provides an efficient platform to study *in vitro* homing or metastasis. Similar concepts have been tried previously, but only using single aggregates in 96-well plates [29].

Herein we have described a ML methodology for making the polymeric Aggrewell™ surface, or any other polydimethylsiloxane (PDMS) surface, resistant to protein adsorption and subsequent cell attachment. This surface modification enhances the formation of MSC microaggregates in the Aggrewell™ and facilitates their easy harvest for subsequent analysis or use in tissue engineering applications. The protocol is straightforward and uses a commercial product, making it widely accessible to other laboratories. Using the modified Aggrewell™ we studied the osteogenic differentiation of MSC in 3D microaggregates, and compared the results to traditional 2D monolayer controls. The 3D microaggregates exhibited greater calcium and collagen I matrix content, both of which are measures of MSC osteogenesis, as well as significantly greater osteogenic gene expression at day 14. This is perhaps counterintuitive, as previous reports suggested that stiff surfaces [6] and maximal cell spread areas [9] best support MSC osteogenesis. However, our data also revealed that BMP-2 signalling, which is known to promote MSC osteogenesis [28], is up-regulated approximately 25-fold in 3D cultures at day 3, regardless of whether cells are maintained in MSC expansion medium or in osteogenic induction medium. This is consistent with the previous hypothesis that

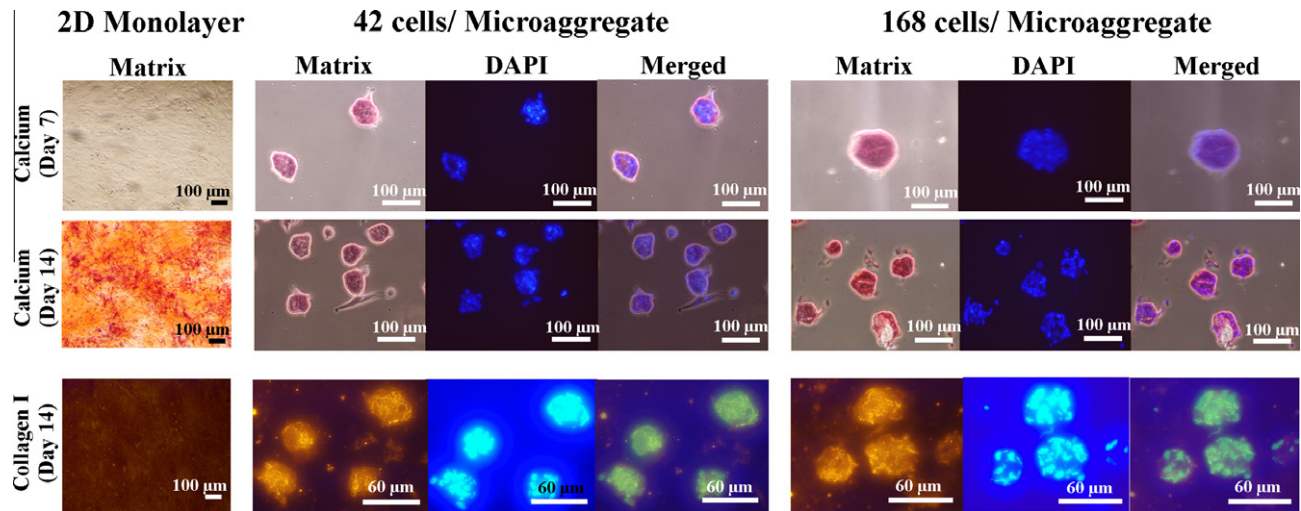


Fig. 4. Histological evaluation of osteogenic microaggregates. Cross sections of 42 and 168 cell microaggregates and 2D monolayer cultures were stained to visualize calcium mineralization and Collagen I. Both calcium and collagen I staining were more intense in the 3D microaggregates than in the 2D monolayers. The staining pattern for collagen I at day 7 in all the cultures was very similar to day 14 immunostaining (data now shown).

autocrine BMP-2 signalling might be up-regulated when MSC osteogenic differentiation cultures were performed in 3D [17]. The modified Aggrewell™ product enabled the generation of hundreds of microaggregate bone mimics resulting in enhanced 3D osteogenesis.

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