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Full Length Research Paper

Use of 3-dimensional environments to engineer osseous-like tissue

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We have previously reported enhanced aggregate formation and accelerated mineralization of human mesenchymal preosteoblastic cells (HEPM) when cultured without scaffolds in 3D-rotary wall vessel chambers. From those studies we hypothesized that, rotary wall vessel environments could be used to engineer osseous tissue. Thus, HEPM preosteoblastic cells were cultured in rotary wall vessels for up to 7 days. Progression of mineralization in 3D cultures was evident by 3 days as shown by micro-CT. A uniform ring of dense mineral on the outermost surface with an amorphous pattern of mineral within the aggregate was noted starting at 3 days. Interestingly, this expression of mineral correlated to the spatial-temporal expression of BMP2. By 7 days, when mineralization appeared complete, BMP2 expression was significantly reduced. Minimal BMP2 expression was noted in the control 2D osteoblast tissue cultures. EDAX analysis showed that, mineralization decreased significantly in aggregates treated with BMP2 antibody ($p<0.0001$). VEGF was found to be consistently expressed regardless of time in both 2D and 3D cultures. Our results suggest that osseous like tissues can be engineered in small volumes from preosteoblasts cultured 3-dimensionally in rotary wall vessels. These methods may someday translate clinically into use for engineering osseous like tissue for repair of small intraoral osseous defects.

Key words: Tissue engineering, bone, mineralization, osteoblasts, osseointegration.

INTRODUCTION

Rotary wall vessel (RWV) chambers have been used to study cellular and molecular mechanisms associated with three-dimensional (3D) tissue growth (Unsworth and Lelkes, 1998; Botchwey et al., 2001; Rucci et al., 2002; Klement et al., 2004; Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008). It has been shown that the use of rotary cell culture allows for the analysis of formation of tissue like structures in a controlled three dimensional model. This is due to alteration of the cell's perception of a continuously changing environment as a result of rotational culture conditions (Klement et al., 2004). When cells are maintained in a 3D-growth environment they tend to aggregate (Granet et al., 1998; Qiu et al., 1998; Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008) as these types of 3D-environments promote cell-cell associations (Freed et al., 1999; Botchwey et al., 2001; Rucci et al., 2002). Cells are suspended in continuous free fall with low shear stress force and turbulence (Duray et al., 1997; Freed et al., 1997; Klement et al., 2004). Culturing cells in 3D-environments provides an excellent *in vitro* system for evaluating intercellular and extracellular signaling associated with cell differentiation to model those mechanisms found in tissue (Goodwin et al., 1993; Klement et al., 2004).

Previous studies have analyzed the effect of 3D-growth environments on osteoblast differentiation using established osteoblast cell lines (Botchwey et al., 2001; Rucci et al., 2002), and demonstrated increased cellular aggregation and osteoblast differentiation (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008). The conclusions of these studies suggest that, 3D-environments may activate compensatory pathways in osteoblasts as a result of aggregate formation. We have reported previously on what appears to be normal, but accelerated aggregate formation, differentiation, and mineralization of HEPM osteoblast cells cultured in 3D environments using RWV chambers (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008). This increased rate of differentiation and mineralization in 3D-RWV environments suggests that, the cell adhesion and

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molecular pathways, such as those associated with the α2β1 integrin signaling pathway may be enhanced (Schneider et al., 1999; Schneider et al., 2001; Ko et al., 2007).

It has been reported that cell adhesion to the extracellular matrix plays a key role in regulating cell growth and differentiation (Juliano and Haskill, 1993; Yamada, 1997; Ilic et al., 1998). During osteogenesis, mineralization is related to the spatial and temporal expression, as well as the maturation of extracellular matrix proteins and the association of these ligands with their respective integrin receptor (Franceschi and Iyer, 1992; Moursi et al., 1997; Liu et al., 1997; Schneider et al., 1999). Various integrin receptors have been reported to be expressed in osteoblasts, including αVβ3, α2β1, and α5β1 (Hughes et al., 1993; Hultenby et al., 1993) and these are used by osteoblasts to interact with the extracellular matrix (Hughes et al., 1993; Saito et al., 1994; Pistone et al., 1996). Numerous bone-specific or bone related extracellular matrix proteins, such as osteopontin, collagen, fibronectin, and bone sialoprotein display differentiation-dependent expression (Malaval et al., 1994; Sommer et al., 1996; Cowles et al., 1998; Schneider et al., 1999). The spectrum of integrins expressed by osteoblasts can be defined in terms of the stage of osteoblast differentiation (Grzesik and Robey, 1994; Gronthos et al., 1999). The precise role of these integrin extracellular matrix interactions with respect to the regulation of osteoblast differentiation and mineralization is not yet fully understood.

Tissue regeneration therapies, like the growth and development of bone, are dependent upon the induction and transformation of progenitor cells into osteoblast-like cells capable of secreting bone matrix. Recent studies have shown that this induction and transformation, as it pertains to osseous tissues, is in large part controlled by BMP (bone morphogenic protein) signaling (Yamaguchi et al., 2000; Nakashima and Reddi, 2003; Valcourt et al., 2005; Ripamonti, 2006). BMP2, BMP7, and VEGF (vascular endothelial growth factor) are particularly noteworthy, as they have been shown to initiate both vasculogenesis and osteogenesis (Nakashima and Reddi, 2003). Regenerative treatments may provide patients with several options that were not previously available to them. Culturing osteoblasts and engineering tissues in a RWV 3D-environment, could potentially provide an important temporal advantage; and possibly a novel translational method to expedite the development of small amounts of engineered osseous tissue enabling clinicians to repair small intraoral osseous defects, or larger ones associated with the craniofacial complex.

Thus, the purpose of our study was to demonstrate enhanced osteoblast differentiation and mineralization of cells cultured in 3D-environments and to show that 3D culture environments can be used to engineer osseous like tissue. We tested the hypothesis that 3D-environments created by RWV chambers could be used to engineer small volumes of osseous like tissue.

MATERIALS AND METHODS

Cell culture

Human palatal mesenchymal preosteoblasts (HEPM 1486, ATCC) were suspended in a Synthecon RCCS D-410 10 ml cell culture vessel system (Synthecon, Inc.) as previously described (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008). Cell concentrations cultured were 10×10^6 or 5×10^6 cells/10 ml. All air bubbles were removed from the culture chamber and initially rotated at 15 rpm, after which time the rotational speed was increased to a maximum of 45 rpm. All cultures were maintained in a 37°C incubator in supplemented EMEM medium (10% FBS, 5 mM beta-GP, 50 ug/ml ascorbate).

Immunocytochemistry

Immunocytochemistry was used to detect BMP2. 5-micron serial sections of paraffin embedded osteoblast aggregate samples were placed on glass slides. Immunocytochemical staining protocols were performed as previously described (Schneider et al., 1999) using the Histostain-SP kit from Zymed Laboratories (USA). Samples were then qualitatively evaluated for the presence or absence of bone morphogenic protein 2 using polyclonal antibodies against BMP2 (Santa Cruz Biotechnology) at a labeling dilution of 1:100. Negative control samples were treated in the same manner, but only labeled with secondary antibody alone. Serial sections were used for comparison of staining and as negative controls. All samples were processed and labeled together to minimize interexperimental variation in staining. Images were examined using an Olympus BX40 microscope equipped with digital optics (40×) at final magnifications of 400x. Digital pictures were taken of the stained regions of interest via a microscope-mounted camera and the SPOT computer software program.

BMP2 perturbation mineralization assays

BMP2 Polyclonal antibodies used for immunocytochemistry labeling were also used for perturbation assays to assess the potential role of BMP2 in the regulation of differentiation of the aggregates into a mineralizing osseous like tissue. Initial antibody concentrations were based upon previous integrin perturbation studies (Schneider et al., 2001). All antibodies were purified and preservative free. Antibody free samples were used as controls. HEPM 1486 mesenchymal preosteoblasts were cultured in 3D environments as described earlier in RWVs. Anti-BMP2, 200 µg/ml in a final volume of 0.5 ml (50 µg/time point) was added at 1 and 48 h. Half (50 µg) of the final dose (100 µg) was added at each time point. Aggregate formation proceeded and was maintained in 3D environments for 3 days at which time the aggregates were collected. The media was removed; the 3D aggregates were gently rinsed with phosphate-buffered saline (PBS) and fixed for 10 min in 10% formalin. After fixation, the aggregates were rinsed with deionized water (no calcium ion) and stained for calcium with 2% Alizarin Red-S (AR-S), for 10 min at room temperature. Wells were washed 4 times with deionized water and evaluated for intensity of AR-S staining. Levels of mineralization (calcium nmol/mg) in the treated and nontreated aggregates were determined by acid hydrolysis of calcium from the treated and nontreated aggregates and read on an atomic absorption spectrophotometer.

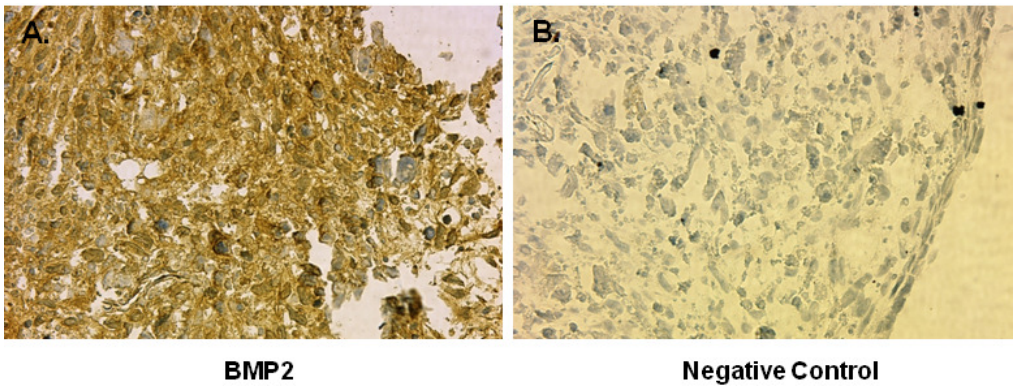

Figure 1. Bone morphogenic protein expression. Osseous like aggregates expressed BMP2 throughout the 3D aggregate (A) as compared to samples labeled with a negative control (B).

Micro-CT analysis

Micro-CT was used for structural comparison, 3D reconstruction, and evaluation of relative mineral density. Each aggregate, 3 and 7 day, was scanned on a Siemens Micro-CAT II imaging system. Image projections were gathered over 720 steps while moving 200° around the specimen. A source to detector distance was set to achieve 32.8 micron spatial resolution while reconstructing 1024 slices over a 4 cm sample area.

RT-PCR analysis

RT-PCR was used to detect BMP2 and VEGF expression. For RT-PCR, total RNA was isolated from 3 or 7 day old cells cultured in standard 2D tissue culture plastic or in 3D rotary wall vessels. Total RNA was quantified by spectrophotometer at 260λ. Primers corresponding to human BMP2 (forward GACCTGTATCGCAGGCACTCA and reverse CACTCGTTTCTGGTAGTTCTTCCA), human VEGF (forward CCCACTGAGGAGTCCAA CATC and reverse ACATTTGTTGTGCTGTAGGAAGCT), and a control gene human GAPDH (forward GAAGGTGAAGGTCGGAGT and reverse GAAGATGGTGATGGGA TTTC) were used for RT-PCR. RNA and downstream primer cDNA were reverse transcribed and subsequently amplified with the addition of upstream primer. PCR products were examined by 3% agarose gel electrophoresis and visualized by ultraviolet transillumination of ethidium bromide-stained DNA.

Statistical analysis

Statistical analysis (N=3) was performed by one-way analysis of variance (ANOVA) with a Tukey's Multiple Comparison Test to a confidence level of $P < 0.05$.

RESULTS

BMP2 pathways utilized by 3D-aggregates during mineralization development

HEPM cells were cultured in a rotary vessel 3D-environment for up to 7 days using a starting cell number of 10×10^6 cells/10 ml. Aggregates were then fixed and sectioned, then labeled for BMP2 (Figure 1). BMP2 is a known promoter of *in vivo* bone formation, functioning as an initiator signal in bone differentiation and mineralization (Yamaguchi et al., 2000) (Ripamonti and Renton, 2006). BMP2 was uniformly detected throughout the aggregate (Figure 1A) in comparison to the control (Figure 1B). Elemental analysis of aggregates treated with or without αBMP2 showed Ca^{2+} levels were significantly lower in aggregates treated with anti-BMP2 ($p<0.0001$; N=6, Figure 2) suggesting that BMP2 pathways are necessary for HEPM preosteoblast differentiation and mineralization processes when cultured in 3D RWV environments.

BMP2 expression correlates to mineralization of 3D-aggregates

It has been reported that BMP2, a member of the TGF-β superfamily of growth factor proteins, helps to stimulate the induction of bone formation (Deckers et al., 2002; Ripamonti, 2006; Dai and Rabie, 2007). As such, RT-PCR analysis was performed on HEPM cells cultured under standard 2D tissue culture methods, or on 3D-aggregates cultured in RWVs. RNA was collected at 3 or 7 days (Figure 3). BMP2 expression was barely detected in osteoblast cells cultured for 0 h or grown for 3 days using standard 2D tissue culture techniques (Figure 3, lanes 1 and 2). However, BMP2 expression was significantly increased and peaked by 3 days in aggregates, cultured in 3D rotary wall vessels (Figure 3, lanes 3 and 4). By 7 days, BMP2 expression was slightly elevated in the 2D cultures (Figure 3, lane 6), whereas in the 3D aggregate cultures BMP2 expression was decreased (Figure 3, lanes 7 and 8). Results also showed that, VEGF was constitutively expressed at both 3 and 7 days in both the 2D and 3D cultures (Figure 4, lanes 1 to 8).

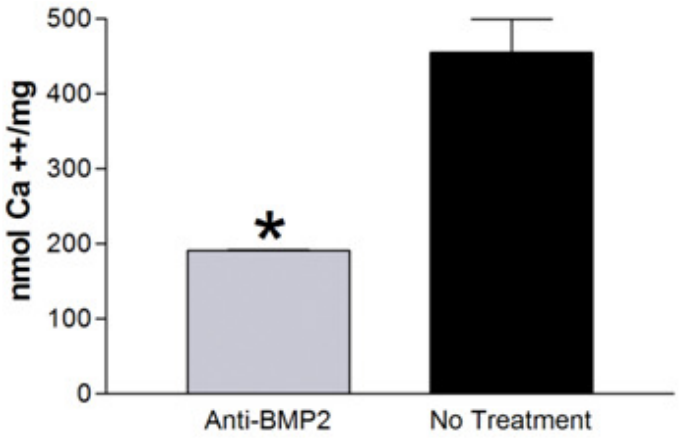

Figure 2. Changes in mineralization with BMP2 antibody treatment. When 3D osseous like aggregates were treated with BMP2 antibody there was a significant (*) decrease in level of mineralization ($p<0.0001$, N=6).

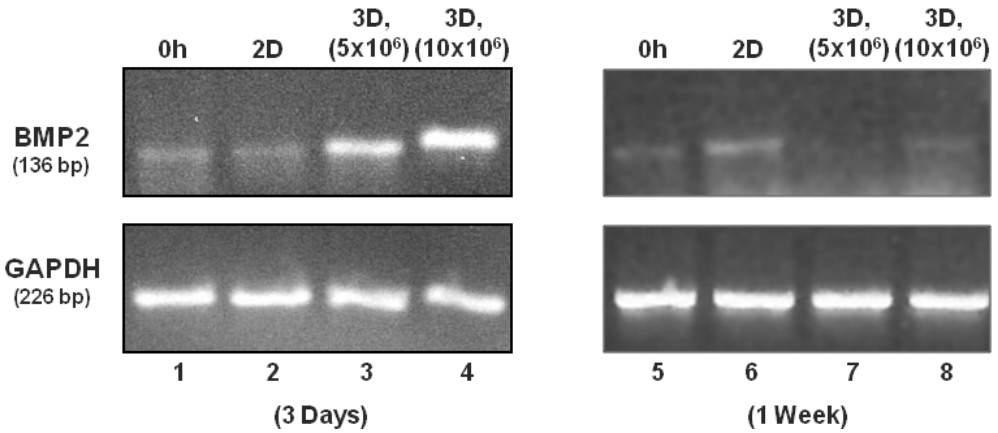

Figure 3. Mineralization of osseous like aggregates correlated to BMP2 expression. RT-PCR analysis demonstrated that BMP2 expression peaked by day 3 (lane 3) at a time that correlated to elevation of aggregate mineralization, then decreased by day 7 (lane 7) once mineralization had occurred in the 3D aggregates regardless of cell number.

BMP2 expression correlates to mineralization of 3D-aggregates

To confirm that the changes in BMP2 expression noted in the BMP2 perturbation assays and RT-PCR experiments described in Figures 2 and 3 correlated to aggregate mineralization, aggregates grown for 3 or 7 days were subjected to micro-CT analysis (Figure 5). Horizontal (Figure 5A) and vertical (Figure 5B) 3D reconstructions were fabricated from micro-CT slices (Figure 5C). At 3 days, levels of mineralization were noted in aggregates cultured from 10 million cells. Levels of mineralization increased from 3 to 7 days, as noted in the micro-CT images (Figure 5A and B), and in the cross sectional slices seen in Figure 5C. The pattern of mineral correlated to increases in the level of BMP2 expression, that was seen in the aggregates at 3 days in Figure 3 (lanes 3 and 4). In addition, micro-CT slices (Figure 5C) demonstrated a pattern of mineralization throughout the aggregate, indicating a non-necrotic center. There appeared to be a more concentrated pattern of mineral expressed in a ring like nature around the periphery of the aggregate which correlated to previous reported macroscopic findings (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008).

DISCUSSION

The demand for tissue engineering as a result of loss and/or failure of tissues can be estimated to account for as much as half of all medical and dental related

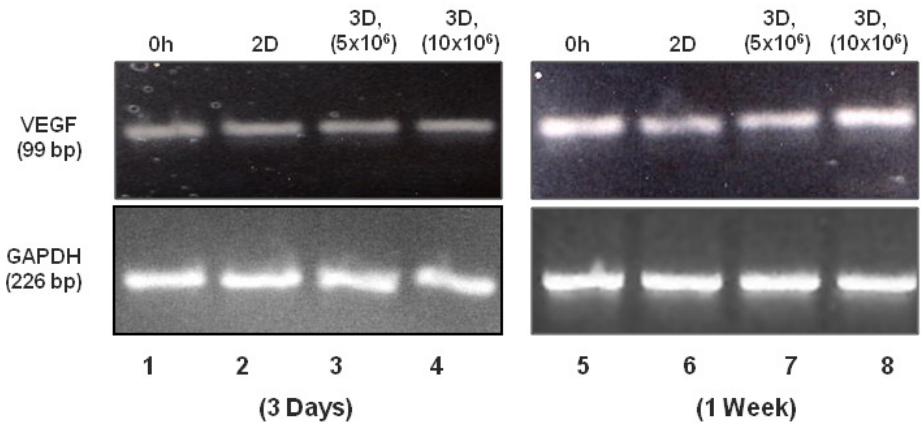

Figure 4. VEGF was consistently expressed during osteoblast differentiation in 3D environments. RT-PCR analysis showed that VEGF was constitutively expressed in 3D cultures during aggregate formation regardless of cell number.

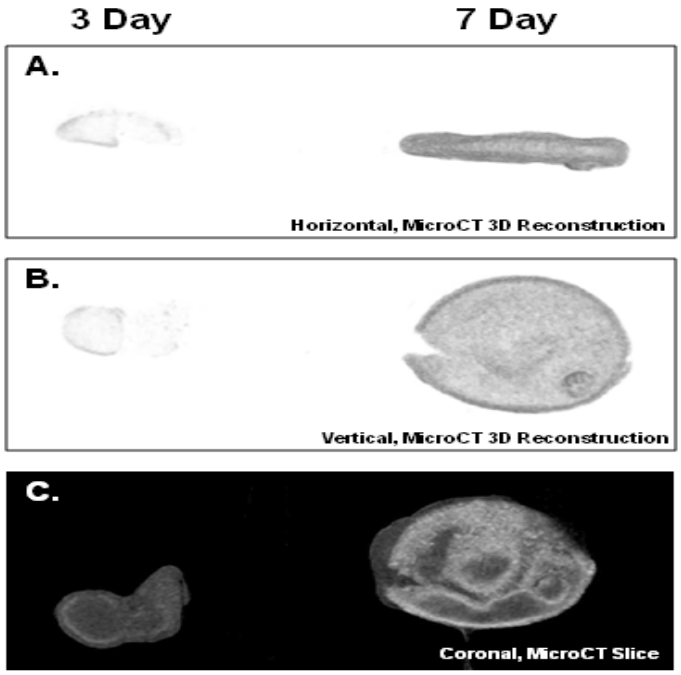

Figure 5. MicroCT analysis confirmed changes in BMP2 expression (as seen in figure 3) correlated to mineralization initiation at day 3. Micro-CT analysis also demonstrated a pattern of mineralization throughout the aggregate by day 7, indicating a non-necrotic center. There appeared to be a more concentrated pattern of mineral expressed in a ring like nature around the periphery of the aggregate.

problems in the United States (Baum and Mooney, 2000). In order for bone engineering to be possible, there must be a source of cells. It has been suggested that progenitor cells are present in the mature skeleton and dental tissues (Mao et al., 2006). These self-renewing cells, also termed mesenchymal stem cells, are capable of postnatal growth, repair and regeneration. Depending upon their environment, these cells have the ability to differentiate into a number of different cell lineages. Macroscopic and gene analysis have shown differences in osseous-like tissue generation between cells cultured in either conventional 2D or 3D rotary wall vessels (Facer

et al., 2005; Ko et al., 2007; Boehrs et al., 2008). In addition, potential integrin mediated pathways that may modulate enhanced differentiation seen in aggregate cultures have been reported (Schneider et al., 2001; Ko et al., 2007).

We have shown that aggregates share similar mineralization patterning regardless of starting cell number, even with a sequential decrease in amount of calcium correlating to cell number (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008). Qualitative assessment of calcium dispersion showed differences in calcium levels between aggregates from different cell starting numbers, yet there appeared to be uniform expression of mineral throughout the aggregate (Boehrs et al., 2008). This may be attributed to mineral density differences throughout the areas of mineral expression between aggregates generated from different cell numbers. Knowing the size of aggregates that can be produced from different starting cell numbers may prove useful, in determining the amount of cells needed to fill different sized bony defects for tissue engineering. We have previously demonstrated that different sized macroscopic aggregates developed in 3D-RWV environments could be formed corresponding to the different starting cell numbers (Ko et al., 2007; Boehrs et al., 2008).

In these studies, it was also shown that 3D environments enhanced differentiation and mineralization of osteoblast cell aggregates, and that regardless of the starting cell number mineralization patterns were similar. In addition, aggregates were found to share common differentiation proteins, such as collagen, BSPII (bone sialoprotein II), and osteopontin. Results from our current study also show that, aggregates developed in 3D environments express the important *in vivo* osseous mediator BMP2 in a spatial and temporal fashion related to mineralization initiation in osteoblasts cultured in a 3D fashion. The correlation of BMP2 expression and mineralization of the 3D aggregate also suggests that, the environment created by the RWV appears to enhance our ability to engineer small quantities of osseous like tissue.

This in turn may one day translate into the repair of small intraoral osseous defects. With the increasing number of studies demonstrating osteoblastic differentiation in 3D-rotary wall vessel environments, there has been increased interest in new bone tissue engineering techniques. These 3D environments may be one of the most realistic environments to study the development of tissue as would occur *in vivo*. The main advantage of this system comes from the idea that, it creates an environment that tends to aggregate individual cells into a tissue mass and allows it to differentiate at an increased rate compared to conventional tissue culture (Granet et al., 1998; Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008). Previous studies have shown that osteoblastic differentiation and the formation of a mineralized matrix can occur by 1 week in 3D cultures, as compared to nearly 4 weeks time in 2D (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008).

Several studies have found that scaffolds are needed for 3D cellular aggregation (Botchwey et al., 2001; Botchwey et al., 2003; Botchwey et al., 2004). Interestingly, our studies have shown the development of viable osseous aggregates in 3D environments in the absence of scaffold. While the importance of scaffolds for tissue development and support cannot be understated, we find it interesting that we have been able, along with others (Rucci et al., 2002; Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008) to develop small quantities of osseous like tissue aggregates in the absence of scaffold. These small aggregates would be more than ample to help repair small osseous like defects associated with the oral cavity or between the roots of teeth where bone resorption has occurred due to periodontal disease or tooth extraction.

Our previous studies, with 5 and 10 million cells, have also shown that a tissue-like mass could be seen as early as 5 h after being cultured (Facer et al., 2005). In addition, the phenotype of this tissue-like mass was analyzed macroscopically and revealed that, it has a general appearance similar to that of a bone (Ko et al., 2007; Boehrs et al., 2008). Microscopically, there also appears to be evidence of higher organization structurally among aggregates as shown by SEM (Boehrs et al., 2008). Our observations (Facer et al., 2005; Ko et al., 2007; Boehrs et al., 2008), as well as others (Botchwey et al., 2001; Rucci et al., 2002) also suggests that osteogenic genes, such as RUNX2, related to osteoblast differentiation and mineralization are generally expressed at higher levels in osteoblastic cells cultured in 3D environments, as compared to those cultured 2D. This implies that the 3D environment may enhance cell to extracellular matrix signaling pathways as previously described for 2D systems (Schneider et al., 1999; Schneider et al., 2001).

In our current study we demonstrate that the level of BMP2, as shown by RT-PCR, is expressed earlier in 3D cultures when compared to 2D. These results correlated to the earlier mineralization levels detected by MicroCT analysis of aggregates grown in 3D rotary wall vessel systems. In contrast, by adding BMP2 antibody to cells maintained in 3D environments, it was shown that mineralization decreased. In order for bone tissue to survive *in vivo*, it is necessary to have vasculature to carry nutrients throughout the tissue to prevent necrosis. One way that osteoblastic-like cells promote endothelial growth is through production of VEGF. Our studies found VEGF to be constitutively expressed constantly in the 3D cultures suggesting that there is possible mechanisms of neovascularization of the aggregate osseous like tissue.

In conclusion, this data and our previous studies support the hypothesis that 3D RWV environments can be used to engineer small quantities of osseous like tissue. This may one day translate into novel osseous

tissue engineering strategies for the repair of small intraoral osseous defects.

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REFERENCES

Baum BJ, Mooney DJ (2000). The Impact of Tissue Engineering on Dentistry. J. Am. Dent. Assoc., 131: 309-318.

Boehrs JK, Zaharias RS, Laffoon JE, Ko YJ, Schneider GB (2008). Three-Dimensional Culture Environments Enhance Osteoblast Differentiation. J. Prosthodont., 17(7): 517-521.

Botchwey EA, Pollack SR, Levine EM, Laurencin CT (2001). Bone tissue engineering in a rotating bioreactor using a microcarrier matrix system. J. Biomed. Mater. Res., 55(2): 242-253.

Botchwey EA, Dupree MA, Pollack SR, Levine EM, Laurencin CT (2003). Tissue engineered bone: Measurement of nutrient transport in three-dimensional matrices. J. Biomed. Mater Res., 67A(1): 357-67.

Botchwey EA, Pollack SR, Levine EM, Johnston ED, Laurencin CT (2004). Quantitative analysis of three-dimensional fluid flow in rotating bioreactors for tissue engineering. J. Biomed. Mater. Res., 69A(2): 205-215.

Cowles EA, DeRome ME, Pastizzo G, Brailey LL, Gronowicz GA (1998). Mineralization and the expression of matrix proteins during *in vivo* bone development. Calcified Tissue Int., 62(1): 74-82.

Dai J, Rabie AB (2007). VEGF: an essential mediator of both angiogenesis and endochondral ossification. J. Dent. Res., 86(10): 937-950.

Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE (2002). Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology, 143(4): 1545-1553.

Duray PH, Hatfill SJ, Pellis NRIPNR (1997). Tissue culture in microgravity. Sci. Med., 4(3): 46-55.

Facer SRZ, Andracki M, Lafoon J, Hunter S, Schneider GB (2005). Rotary culture enhances preosteoblast aggregation and mineralization. J. Dent. Res., 84(6): 542-547.

Franceschi RT, Iyer BS (1992). Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J. Bone Miner. Res., 7(2): 235-246.

Freed LE, Langer R, Martin I, Pellis NR, Vunjak-Novakovic G (1997). Tissue engineering of cartilage in space. Proc. Natl. Acad. Sci. USA., 94(25): 13885-13890.

Freed LE, Pellis N, Searby N, de Luis J, Preda C, Bordonaro J (1999). Microgravity cultivation of cells and tissues. Gravit. Space Biol. Bull.: Publ. Am. Soc. Gravit. Space Biol., 12(2): 57-66.

Goodwin TJ, Schroeder WF, Wolf DA, Moyer MP (1993). Rotating-wall vessel coculture of small intestine as a prelude to tissue modeling: Aspects of simulated microgravity. Proc. Soc. Exp. Biol. Med., 202(2): 181-192.

Granet C, Laroche N, Vico L, Alexandre C, Lafage-Proust MH (1998). Rotating-wall vessels, promising bioreactors for osteoblastic cell culture: Comparison with other 3D conditions. Med. Biol. Eng. Comput., 36(4): 513-519.

Gronthos S, Zannettino ACW, Graves SE, Ohta S, Hay SJ, Simmons PJ (1999). Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J. Bone Miner. Res., 14(1): 47-56.

Grzesik WJ, Robey PG (1994). Bone matrix RGD glycoproteins: Immunolocalization and interaction with human primary osteoblastic bone cells *in vitro*. J. Bone Miner. Res., 9(4): 487-496.

Hughes DE, Salter DM, Dedhar S, Simpson R (1993). Integrin expression in human bone. J. Bone Miner. Res., 8(5): 527-533.

Hultenby K, Reinholt FP, Heinegard D (1993). Distribution of integrin subunits on rat metaphyseal osteoclasts and osteoblasts. Eur. J. Cell Biol., 62(1): 86-93.

Ilic D, Almeida EA, Schlaepfer DD, Dazin P, Aizawa S, Damsky CH (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. J. Cell Biol., 143(2): 547-560.

Juliano RL, Haskill S (1993). Signal transduction from the extracellular matrix. J. Cell Biol., 120(3): 577-585.

Klement BJ, Young QM, George BJ, Nokkaew M (2004). Skeletal tissue growth, differentiation and mineralization in the NASA Rotating Wall Vessel. Bone, 34(3): 487-498.

Ko YJ, Zaharias RS, Seabold DA, Lafoon J, Schneider GB (2007). Osteoblast Differentiation is Enhanced in Rotary Cell Culture Simulated Microgravity Environments. J. Prosthodont., 16(6): 431-438.

Liu F, Malaval L, Aubin JE (1997). The mature osteoblast phenotype is characterized by extensive plasticity. Exp. Cell Res., 232(1): 97-105.

Malaval L, Modrowski D, Gupta AK, Aubin JE (1994). Cellular expression of bone-related proteins during *in vitro* osteogenesis in rat bone marrow stromal cell cultures. J. Cell Physiol., 158(3): 555-572.

Mao JJ, Giannobile WV, Helm JA, Hollister SJ, Krebsbach PH, Longaker MT (2006). Craniofacial Tissue Engineering by Stem Cells. J. Dent. Res., 85(11): 966-979.

Moursi AM, Globus RK, Damsky CH (1997). Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation *in vitro*. J. Cell Sci., 110: 2187-2196.

Nakashima M, Reddi AH (2003). The application of bone morphogenetic proteins to dental tissue engineering. Nat. Biotechnol., 21(9): 1025-1032.

Pistone M, Sanguineti C, Federici A, Sanguineti F, Defilippi P, Santolini F, et al. (1996). Integrin synthesis and utilization in cultured human osteoblasts. Cell Biol. Int., 20(7): 471-479.

Qiu Q, Ducheyne P, Gao H, Ayyaswamy P (1998). Formation and differentiation of three-dimensional rat marrow stromal cell culture on microcarriers in a rotating-wall vessel. Tissue Eng., 4(1): 19-34.

Ripamonti U (2006). Soluble osteogenic molecular signals and the induction of bone formation. Biomaterials, 27(6): 807-822.

Ripamonti U, Renton L (2006). Bone morphogenetic proteins and the induction of periodontal tissue regeneration. Periodontology, 2000(41): 73-87.

Rucci N, Migliaccio S, Zani BM, Taranta A, Teti A (2002). Characterization of the osteoblast-like cell phenotype under microgravity conditions in the NASA-approved Rotating Wall Vessel bioreactor (RWV). J. Cell. Biochem., 85(1): 167-179.

Saito T, Albelda SM, Brighton CT (1994). Identification of integrin receptors on cultured human bone cells. J. Orthop. Res., 12(3): 384-394.

Schneider GB, Whitson SW, Cooper LF (1999). Restricted and coordinated expression of beta3-integrin and bone sialoprotein during cultured osteoblast differentiation. Bone, 24(4): 321-327.

Schneider GB, Zaharias R, Stanford C (2001). Osteoblast integrin adhesion and signaling regulate mineralization. J. Dent. Res., 80(6): 1540-544.

Sommer B, Bickel M, Hofstetter W, Wetterwald A (1996). Expression of matrix proteins during the development of mineralized tissues. Bone, 19(4): 371-380.

Unsworth BR, Lelkes PI (1998). Growing tissues in microgravity. Nature Med., 4(8): 901-907.

Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A (2005). TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol. Biol. Cell., 16(4): 1987-2002.

Yamada KM (1997). Integrin signaling. Matrix Biol., 16(4): 137-141.

Yamaguchi A, Komori T, Suda T (2000). Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr. Rev., 21(4): 393-411.