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The Sequential Production Profiles of Growth Factors and their Relations to Bone Volume in Ossifying Bone Marrow Explants

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Osteogenesis is a complex process that involves the synergistic contribution of multiple cell types and numerous growth factors (GFs). To develop effective bone tissue engineering strategies employing GFs, it is essential to delineate the complex and interconnected role of GFs in osteogenesis. The studies investigating the temporal involvement of GFs in osteogenesis are limited to *in vitro* studies with single cell types or complex *in vivo* studies. There is a need for platforms that embody the physiological characteristics and the multicellular environment of natural osteogenesis. Marrow tissue houses various cell types that are known to be involved in osteogenesis, and *in vitro* cultures of marrow inherently undergo osteogenesis process. Self-inductive ossification of marrow explants *in vitro* can be employed as a representative multicellular and three-dimensional model of osteogenesis. Therefore, the aims of this study were to employ the rat bone marrow explant ossification model to determine (1) the temporal production profiles of key GFs involved in osteogenesis, (2) the relation between GF production and ossification, and (3) the relations between the GF levels throughout ossification. Temporal production profiles of transforming GF β-1 (TGF-β1), bone morphogenetic protein-2 (BMP-2), vascular endothelial GF (VEGF), and insulin-like GF-1 (IGF-1) and the bone-related proteins alkaline phosphatase and osteocalcin were obtained by enzyme-linked immunosorbent assays conducted at days 2, 7, 12, 14, 19, and 21. The final amount of ossification (ossified volume [OV]) was measured by microcomputed tomography at day 21. TGF-β1, BMP-2, VEGF, IGF-1, alkaline phosphatase, and osteocalcin were produced by the ossifying marrow explants differentially over time. The early production of IGF-1 (day 2) correlated positively ($r = 0.868$) with OV; however, latent production of IGF-1 correlated negatively (day 14: $r = -0.813$; day 19: $r = -0.865$) with OV. OV also correlated with VEGF levels at day 12 ($r = 0.988$) and at day 14 ($r = 0.970$). Production of GFs also correlated to each other across time points, which indicates the complex and interconnected contribution of various GFs in osteogenesis. Therefore, tissue engineering strategies toward bone regeneration should consider the richness of GFs involved in osteogenesis and their dynamically varying participation over time.

Introduction

Osteogenesis is a complex process that involves the contribution of multiple cell types and numerous cytokines or growth factors (GFs). GFs are known to influence cell division, differentiation, and matrix synthesis and to play an important role in bone regeneration, fracture healing, and repair of other musculoskeletal tissues.[1–4] It was suggested that there is a crosstalk between the GF signaling pathways in osteogenesis, and the overall osteogenic outcome may be resulting from the synergistic contribution of numerous GFs.[1–3,5–15] To develop effective bone tissue engineering strategies that can control and modulate bone formation, it is essential to investigate the temporal and interconnected involvement of GFs in osteogenesis.[2,3,6,8,11,12,14,15] However, the studies investigating osteogenesis-related GF expression, production, and secretion are limited to two-dimensional *in vitro* studies with single cell types (i.e., osteoblasts and marrow stromal cells)[14,16–18] or complex *in vivo* studies with associated experimental hurdles.[2,3,19–21] To study the complex and interconnected involvement of multiple GFs, there is a need for a multicellular and three-dimensional *in vitro* platform that embodies the intricate physiology of natural osteogenesis. Bone marrow tissue houses multiple cell types

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(i.e., hematopoietic stem cells, mesenchymal stem cells, and accessory cells) that are known to be collaboratively involved in osteogenesis.[22–27] Therefore, bone marrow plays a critical role in bone regeneration[28] and has been shown to have osteogenic potential.[29] Marrow explants inherently ossify *in vitro*[30] without the addition of excipient osteoinductive factors (under basal conditions) and throughout the ossification process, osteoinductive factors are produced by the ossifying marrow tissue.[31] Therefore, *in vitro* bone marrow explant cultures reflect the physiological diversity of bone formation and hold the potential to be used as a platform to study osteogenesis in a more realistic and natural context. This model is conveniently situated between the two-dimensional *in vitro* culture systems employing single cell types and the complex *in vivo* animal models.

The most potent GFs known to be involved in osteogenesis are transforming GF β-1 (TGF-β1), bone morphogenetic proteins (BMPs; 2, 4, and 7), fibroblast growth factor-2, vascular endothelial GF (VEGF), insulin-like GF-1 (IGF-1), and platelet-derived growth factor.[1–4,14,32,33] BMP-2 and -7 have been introduced clinically for treatment of open tibial fractures.[13,34,35] Although there is some appreciation of sequential expression of these potent GFs in fracture healing[2,3,19,20] and in single-type cell culture models,[14,16–18] little is known about their associations with the final amount of bone formation during osteogenesis. Moreover, the knowledge on the relationships between the production levels of GFs during osteogenesis is limited as well. We hypothesized that *in vitro* bone marrow self-inductive ossification model can be used as a platform to delineate the temporal involvement of multiple GFs, and their relations with the ossification level and with each other in osteogenesis. Therefore, the aims of this study were (1) to analyze the temporal production patterns of the key GFs in osteogenesis, TGF-β1, BMP-2, VEGF, and IGF-1, using the inherently ossifying bone marrow explant model, (2) to investigate the relations between the temporal concentrations of GFs and the final ossified volume (OV) of marrow explants, and (3) to investigate relations between the production of GFs, bone-related proteins, and between the GFs themselves throughout the ossification process. These aims were accomplished by (1) measuring the concentrations of GFs in the conditioned medium via quantitative enzyme-linked immunosorbent assay (ELISA) at days 2, 7, 12, 14, 19, and 21, (2) quantifying the end point of ossification (day 21, OV) of marrow explants and correlating OV with temporal concentrations of the GFs, and (3) correlating the concentration levels of the GFs and bone-related proteins to each other at all time points and across time points.

Materials and Methods

Extraction and culture of bone marrow explants

Bone marrow was isolated from the tibiae of 80–90-day-old male Long-Evans rats (Harlan) under Purdue Animal Care and Use Committee approval with a centrifugation-based extraction technique. Briefly, one of the diaphyseal end of the bones was cut with a high-speed circular saw, the medullary components (marrow) were exuded with a brief centrifugation of the cut bone, and the centrifugate was gently pipetted onto the culture inserts (Transwell, Corning) at a 7 μL volume with a low protein binding pipette tip. Bone marrow isolation procedure did not involve dispersion of the marrow contents in a solution, and therefore entailed minimal manipulation and processing of the tissue to preserve the cellular integrity (both adherent and nonadherent) and structural composition of the extracted marrow explant. The growth medium was added underneath the culture insert (below the membrane with 0.4 μm pore size), which resulted in an air–medium interface culture system (Fig. 1). The membrane allowed the attachment and the growth of cells. Explants were cultured under serum-free conditions to identify the baseline ability of marrow explants to ossify. The serum-free growth medium was modified from Lennon *et al.*[36] and composed of 60% Dulbecco's modified Eagle's medium, 40% MCDB-201 supplemented with 1% ITS+ 1 (Sigma), 50 μg/mL ascorbic acid, 5 mM Na-β-glycerophosphate, 3.5 mg/mL glucose, 40 U/mL penicillin, 40 μg/mL streptomycin, and 1.5 μg/mL Fungizone. No osteoinductive factors (e.g., dexamethasone and BMP-2) were added into the culture medium at any point in time. The explants were cultured for 21 days and the medium was changed on days 2, 5, 7, 10, 12, 14, 17, 19, and 21. Since the inserts carrying the explants were set aside during culture medium change, the cellular and compositional integrity of the marrow explants were maintained, which kept the adherent and nonadherent cells together throughout the culture period (Fig. 1). Due to the small pore size (0.4 μm) of the membrane above which the marrow explants were cultured, the cells were not able to migrate through the pores and hence were contained within the insert. This feature of the culture system kept the bone marrow cells above the membrane and allowed the secreted products to be released

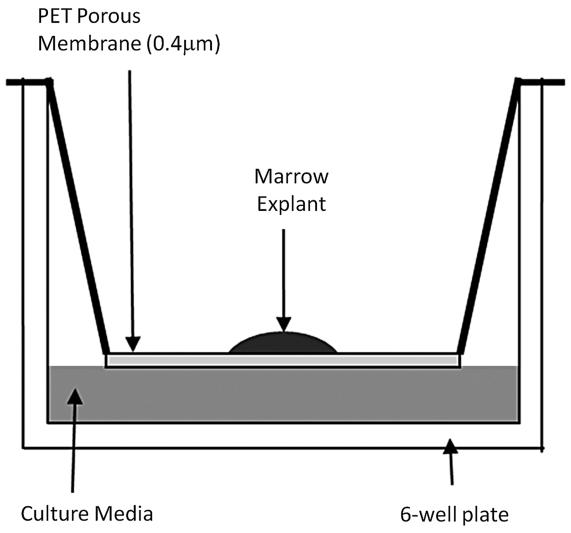


FIG. 1. Cross-sectional view of air–medium interface culture system designed to preserve the adherent and nonadherent cellular composition of marrow tissue throughout the culture period. Marrow explants were placed on PET porous membrane (0.4 μm pore size) of culture inserts and supplemented with a sufficient amount of culture medium underneath the membrane. Culture medium was not added above the membrane, therefore marrow explants were not in direct contact nor were they dispersed in the medium, which prevented the nonadherent marrow cells from being washed away during medium changes.

into the culture medium. The medium conditioned by bone marrow explants was collected from each ossifying explant before each fresh medium addition and stored in sterile low protein binding tubes (LoBind; Eppendorf) separately. The samples were stored at −80°C freezer for ELISA measurements, which were performed at the end of the experiment collectively. Repeated freezing and thawing of the collected conditioned medium was eliminated with appropriate aliquoting.

To test the effect of dispersion or disruption of the cellular integrity of marrow explants on their self-inductive ossification potential, an additional experimental group was included in which marrow explants above the porous membrane were dispersed with the addition of the culture medium inside the culture insert (Fig. 1). In this group, same volume of marrow tissue (7 μL) was utilized in the same culture setting with the culture medium added above the membrane (Fig. 1) submerging and disrupting the marrow explants. This group was similar to the ones used in regular marrow culture studies, in which nonadherent marrow cells are gradually washed away with each medium change, purifying the adherent marrow stromal cells. In this experimental group, the same culture conditions and the same culture medium were used for the same duration as the regular intact marrow explant culture described above. The mineralization of submerged (dispersed) marrow culture samples was evaluated with Raman microspectroscopy and microcomputed tomography. Raman microspectroscopy analysis (LabRam, Horiba Jobin-Yvon) was performed on the randomly selected nodule-like structures (eight samples, six nodule-like structure per sample) observed in the dispersed marrow culture samples. The presence of apatitic mineral peak (located at 959 cm^{-1}) was evaluated by performing a scan in the wavenumber range of 250–1800 cm^{-1}.[37]

Microcomputed tomography of ossifying marrow explants

The ossified marrow samples and the dispersed marrow cultures were fixed with 10% formalin and kept in the fixative before and throughout the scans. The OV of the marrow explants was measured by microcomputed tomography (μCT 40; SCANCO Medical AG) with a 16 μm voxel resolution ($I = 145$ μA, $E = 55$ kVp, integration time = 200 ms). The porous membranes supporting the dispersed marrow cultures were cut out of the culture inserts and scanned with μCT with the same settings. The scanned images were reconstructed and analyzed with commercial software (SCANCO evaluation software), and the segmentation parameters of 0.8 (sigma), 1 (support), and 100 (threshold) were used.[38–40] The total bone volume (mm^3) calculated by software was used and reported as the final OV of the marrow explants.

Quantification of baseline levels of alkaline phosphatase, osteocalcin, BMP-2, IGF-1, VEGF, and TGF-β1 in freshly isolated bone marrow tissue at day 0

Two male Long-Evans rats, 80–90 days old, were euthanized under Purdue Animal Care and Use Committee approval. Bone marrow was removed from the tibiae using a centrifugation-based extraction technique explained in the previous section. Extracted marrow tissue (7 μL) was diluted in tubes that allow minimal protein binding (protein LoBind; Eppendorf) with the same serum-free growth medium described above. Marrow extracts were incubated for 30 min at 37°C to allow the soluble factors to solubilize in the medium. The suspension was then centrifuged for 10 min at 300 *g* to precipitate the cells as a pellet at the bottom of the tube. The supernatant was aspirated and filtered through a 0.2 μm filter using a syringe to remove the remaining cells. The solubilized bone marrow deficient of cells was then aliquoted and stored at −80°C for the quantitative ELISA assays described below. Appropriate conversion of the quantified concentrations of the factors was performed based on the dilution ratios employed.

Histology of ossified marrow explants for matrix typification

At the end of the culture period, the ossified explants were fixed in 10% formalin. Samples were decalcified in formic acid solution (1:1 solution of 50% aqueous formic acid and 20% sodium citrate) for 8–12 h, washed in tap water for 30–45 min, embedded in paraffin, sectioned, and dried overnight in 37°C oven. For all staining procedures, the sections were deparaffinized and hydrated in gradually decreasing percentages of alcohol solutions (100%, 95%, 70%, and water). The sections were stained with safranin-O/fast green for assessing the presence of proteoglycans and hematoxylin and eosin according to standard procedures. Safranin-O/fast green staining is a common method used for staining cartilage–bone interface. Alkaline phosphatase (AP) activity in the histological sections was stained with naphthol AS-MX–based commercial AP staining kit (85L1; Sigma). After staining, the sections were dehydrated, cleared in xylene, and cover-slipped.

Quantification of AP, osteocalcin, BMP-2, IGF-1, VEGF, and TGF-β1

The quantification of these factors was performed on the medium conditioned by ossifying explants (days 2, 7, 12, 14, 19, and 21) and the fresh marrow tissue (day 0). AP level was measured with the colorimetric *p*-nitrophenyl phosphate substrate AP assay kit (SensoLyte™; Anaspec Corp.). Fifty microliters of samples and standards was added to each well of a 96-well plate. Fifty microliters of *p*-nitrophenyl phosphate reaction mixture was added to each well and incubated 2–3 h until color developed. Optical density was determined using a microplate reader (Spectramax M5; Molecular Devices) set to 405 nm. Absorbance values were converted to AP concentration with the utilization of the calibration curve. Osteocalcin (OC) levels were measured using a Rat Osteocalcin EIA kit (Biomedical Technologies). One hundred microliters of samples and standards were added to a 96-well plate precoated with OC capture antibody, incubated for 20 h at 4°C, and washed three times with phosphate saline wash buffer, and 100 μL of OC antiserum was added to each well and incubated at 37°C for 1 h. After another set of washes, 100 μL of diluted donkey anti-goat IgG peroxidase was added to each well, incubated for 1 h at room temperature, and rerinsed, and then 100 μL of substrate mix (1:1 of hydrogen peroxide solution and tetramethyl benzidine) was added and incubated at room temperature for 30 min, avoiding direct light. One hundred microliters of

stop solution was added to each well, and the absorbance was measured using a microplate reader set at 450 nm with a wavelength correction set at 540 nm. The concentrations of BMP-2, VEGF, TGF-β1, and IGF-1 in the conditioned medium were measured by quantitative ELISA development kits (BMP-2: PeproTech; VEGF, IGF-1, and TGF-β1: R&D Systems). Briefly, 96-well microplates (MaxiSorp; Nalge) were coated with capture antibody, the wells were blocked for at least 1 h, and 100 μL of samples or standards was added to wells followed by incubation for 2–3 h at room temperature. After thorough washing, detection antibody was added at the specified concentration for each kit and incubated for 2 h at room temperature. The peroxidase substrate solution was added (protected from direct light) and incubated at room temperature for 20 min, and the enzyme reaction was stopped with 2 N HCl solution. The color product was detected by a microplate reader set at 450 nm with wavelength correction set at 540 nm. Quantification was also carried out on the nonconditioned serum-free medium to determine baseline levels of the GFs, OC, and AP. TGF-β1 in the samples was activated to its immunoreactive form using 1 N HCl followed by the addition of 1.2 N NaOH/0.5 M HEPES before being used in ELISA.

Statistical analysis

The measured concentration profiles were analyzed statistically with Kruskal–Wallis one-way analysis of variance followed by a *post hoc* Mann–Whitney *U*-test with Bonferroni correction for multiple comparisons. Statistical significance threshold was set at $p < 0.05$, and the p-value obtained for each test was adjusted based on the number of comparisons according to Bonferroni correction (p-value obtained from the test multiplied by number of comparisons). Error bars in the figures were displayed as standard error. Relations between the concentrations and the final OV and between the measured concentrations themselves were analyzed by calculating the Pearson product moment correlation coefficient. The statistical significance between the day 0 baseline concentrations ($n = 10$–12) of the bone-related proteins and GFs and their day 2 levels ($n = 6$) produced by the ossifying explants was tested with a Mann–Whitney *U*-test with the significance threshold set at $p < 0.05$.

Results

Bone marrow explants inherently ossified without the addition of any osteoinductive factors

Bone marrow explants cultured under serum-free conditions without any osteoinductive factors (dexamethasone, BMP-2, etc.) inherently ossified to form a matrix that was visible through low magnification light microscopy (Fig. 2A). μCT scans of ossified explants revealed a plate-like ossified structure (Fig. 2B). The absence of orange-red stain in safranin-o/fast green–stained sections was an indication of proteoglycan deficiency, indicating the absence of cartilaginous matrix (Fig. 2C). Hematoxylin and eosin staining displayed viable cells embedded in the matrix (Fig. 2D). Naphthol AS-MX–based AP staining indicated that AP activity (dark purple-red regions, Fig. 2E) was concentrated in the lower and the upper surfaces of the ossified matrix, indicating the locations of the actively ossifying regions in the marrow explant. However, when the marrow explants were dispersed and submerged in the culture medium, which gradually eliminated the nonadherent cell population with each medium change, no indication of mineralization was observed as per Raman microspectroscopy (absence of apatitic mineral peak) and μCT (no detectable mineralized volume).

AP and OC were produced by in vitro *ossifying marrow explants*

The baseline levels of AP and OC in day 0 marrow tissue were quantified as 14.8 ng/mL (SD: 3.8 ng/mL) and 514 pg/mL (SD: 212 pg/mL), respectively (Fig. 3). Both of these baseline concentrations were significantly lower than the day 2 production levels of these bone-related proteins by *in vitro* ossifying marrow explants as seen in Figure 3. AP concentration measured in the conditioned medium displayed a high level at the beginning of the culture period and decreased significantly at day 7 and further decreased after day 12 (Fig. 3A). The decrease in AP concentration was significant by day 21 relative to day 12. OC concentration profile displayed a similar pattern at the beginning of the culture period, which was significantly greater on day 2 than all the following time points (Fig. 3B). A significant decrease in OC concentration was observed on day 7. There was no statistically significant decrease in OC concentration between day 12 and day 21.

Osteoinductive GFs (BMP-2, IGF-1, TGF-β1, and VEGF) were produced by ossifying marrow explants

The baseline levels of BMP-2, IGF-1, TGF-β1, and VEGF in day 0 marrow tissue were significantly lower than the day 2 production levels of these factors by *in vitro* ossifying marrow explants (Fig. 4). BMP-2 concentration in the conditioned medium was at a high level early on at days 2 and 7 (Fig. 4A). BMP-2 concentration decreased significantly by day 12 and displayed further significant decrease on days 19 and 21 (Fig. 4A). IGF-1 concentration was significantly higher on day 2 than the later time points, which decreased significantly and stayed around 50 pg/mL between days 7 and 14 (Fig. 4B). A significant increase in IGF-1 concentration was observed in the later stage, starting day 19 and beyond. TGF-β1 concentration displayed a significantly high level on days 2, 7, and 12 than all of the subsequent time points (Fig. 4C). TGF-β1 concentration decreased significantly by day 14 and stayed constant around 50 pg/mL till the end of the culture period. Similarly, VEGF concentration was significantly higher at the early and mid phase of the culture period (days 2, 7, 12, and 14) than all the following time points (Fig. 4D). VEGF concentration displayed a significant steady decrease during the entire culture period.

Levels of OC, AP, BMP-2, IGF-1, TGF-β1, VEGF, and final OV display significant correlations at specific time points

The rightmost column of Table 1 displays the correlation between the levels of measured GFs and the bone markers (OC and AP) at different time points and the final OV measured at day 21. VEGF concentration in the conditioned medium displayed a high correlation (Table 1) with the final OV on days 12 (0.988) and 14 (0.970). IGF-1 concentration

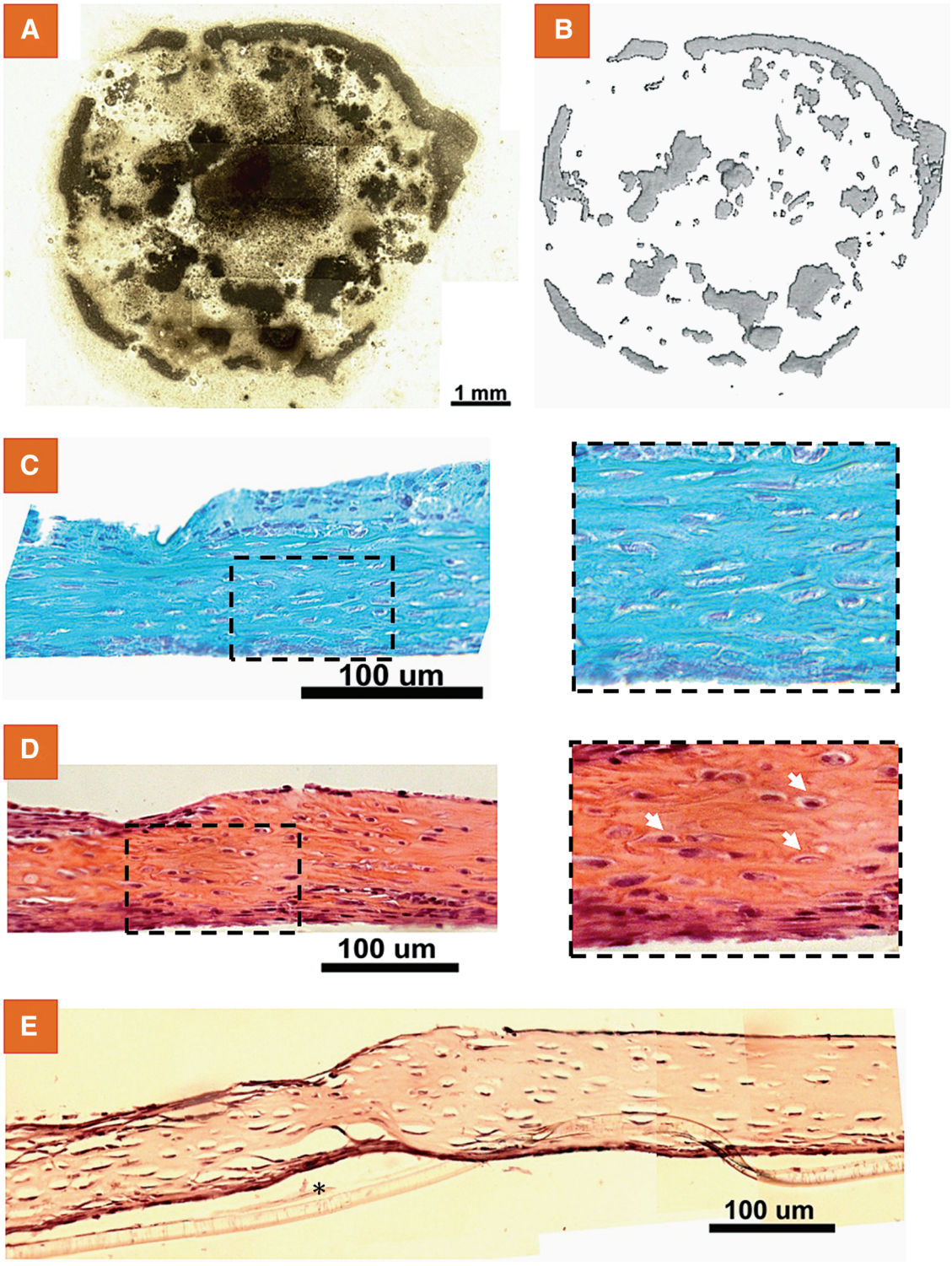


FIG. 2. The appearance of a marrow explant cultured for 21 days. (**A**) Light microscope image of the ossified explant. (**B**) The reconstructed three-dimensional view of the ossified regions of the explant from microcomputed tomography scan indicates a plate-like ossified structure. (**C**) Safranin-O/fast green–stained histological section image of an ossified region in the marrow explant and the magnified inset. The absence of orange-red stain indicates that the extracellular matrix does not contain proteoglycans, and thus it is not a cartilaginous matrix. (**D**) Hematoxylin and eosin–stained histological section image and the magnified inset, displaying viable osteocyte-like cells (arrows). (**E**) Alkaline phosphatase (AP)–stained (dark purple-red regions; counter stain, hematoxylin) histological section image. AP activity is observed in the upper and lower sections of the ossified plate-like structures. The membrane (*) is visible in the image, which lines the bottom of the ossified matrix. Color images available online at www.liebertonline.com/ten.

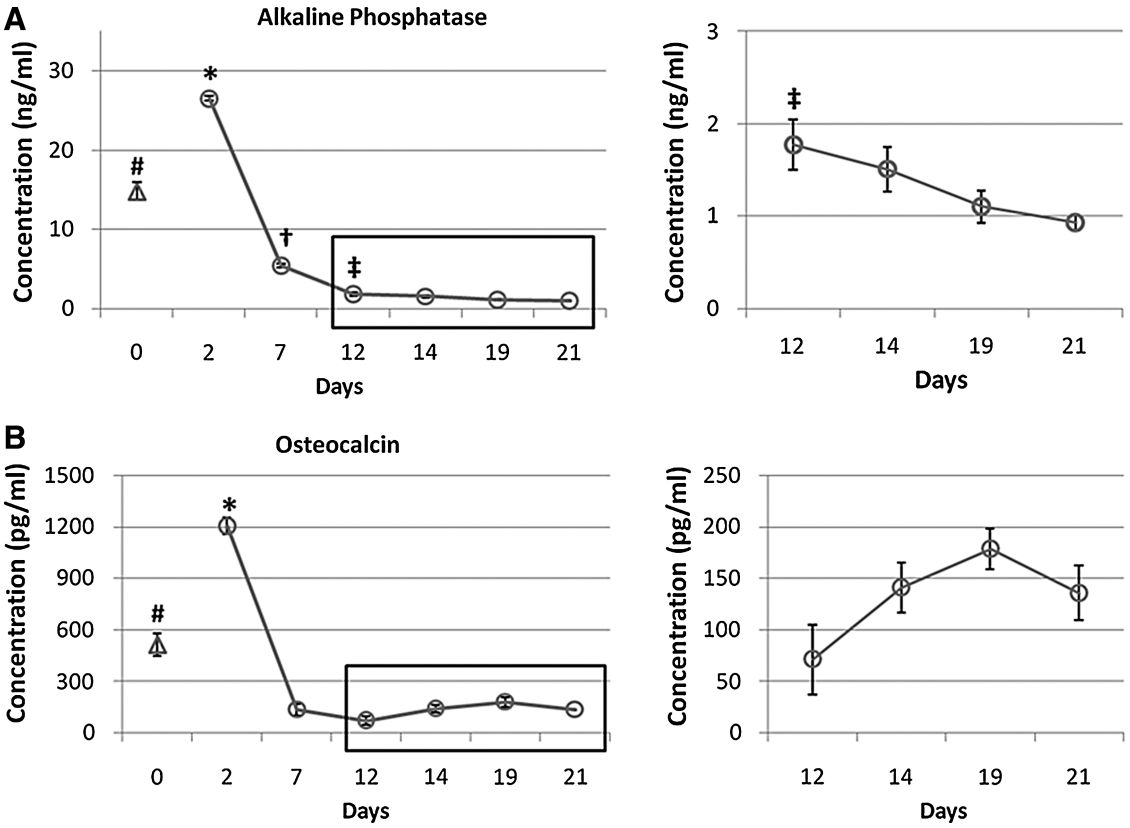


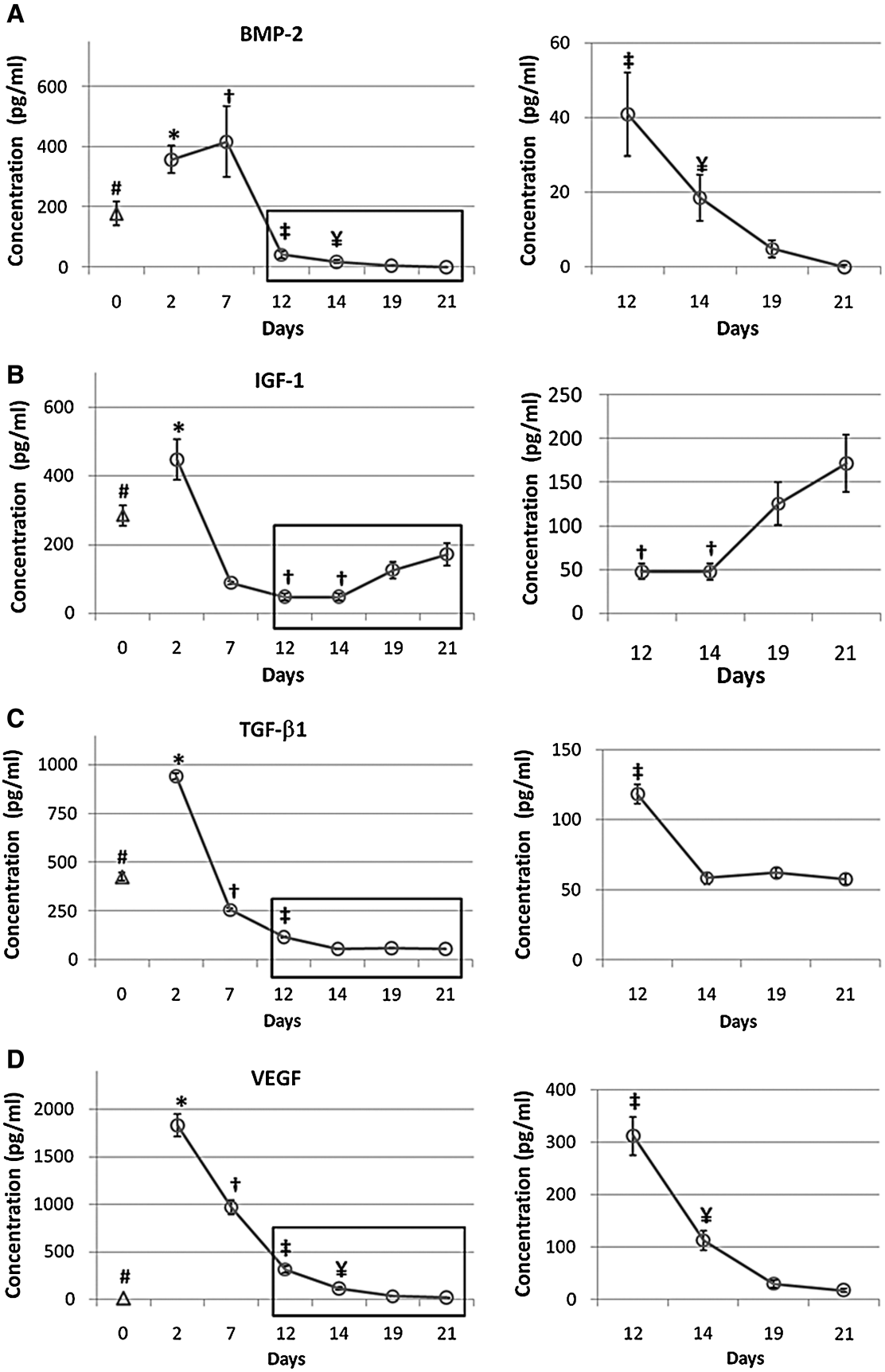
FIG. 3. Concentration profiles of AP and osteocalcin measured in the conditioned medium ($n = 6$ per time-point). A close-up view of days 12–21 highlighted by insets is displayed on the right. The triangular marker at day 0 indicates the baseline concentration in bone marrow tissue at day 0 ($^{\#}p < 0.05$ between the baseline concentration in bone marrow tissue at day 0 and expression of that factor on day 2). (**A**) AP concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus day 21. (**B**) Osteocalcin concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21.

in the conditioned medium early on in the culture (day 2) correlated positively with OV (0.868; Table 1), whereas latent IGF-1 concentration correlated negatively on days 14 (−0.813) and 19 (−0.865) with OV. OC level displayed a high negative correlation with OV on day 19 (−0.931; Table 1).

Discussion

The importance of the GFs included in this study (BMP-2, VEGF, TGF-β1, OC, and IGF-1) and their synergistic combinatorial role on bone regeneration is widely accepted.[1–3,14,16,17,19–21,32,34,41,42] On the other hand, data on sequential expression of GFs in osteogenesis are either limited to two-dimensional single-type cell culture studies[14,16,17] or complex animal models of fracture healing.[2,3,19–21] The current model of marrow explant cultures is situated in between single-type cell culture studies and animal models and present several unique advantages. First, it encompasses multiple cell types (adherent and nonadherent, hematopoietic, and mesenchymal stem cells). Second, it displays a natural osteogenic potential (under serum-free conditions without any excipient osteoinductive factors), which results in a significant volume of bone formation. Third, it presents a platform in which the protein production can be quantified

FIG. 4. Concentration profiles of potent osteoinductive factors (bone morphogenetic protein-2 [BMP-2], insulin-like growth factor-1 [IGF-1], transforming growth factor β-1 [TGF-β1], and vascular endothelial growth factor [VEGF]) measured in the conditioned medium ($n = 6$ per time-point). A close-up view of days 12–21 highlighted by insets is displayed on the right. The triangular marker at day 0 indicates the baseline concentration in bone marrow tissue at day 0 ($^{\#}p < 0.05$ between the baseline concentration in bone marrow tissue at day 0 and expression of that factor on day 2). (**A**) Bone morphogenetic protein-2 concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus days 19 and 21; $^{¥}p < 0.05$ for day 14 versus day 21. (**B**) Insulin-like growth factor-1 concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 12 versus days 19 and 21, and for day 14 versus days 19 and 21. (**C**) Transforming growth factor β-1 concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus days 14, 19, and 21. (**D**) Vascular endothelial growth factor concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus days 14, 19, and 21; $^{¥}p < 0.05$ for day 14 versus days 19 and 21.


A
BMP-2
Concentration (pg/ml)
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IGF-1
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VEGF
Concentration (pg/ml)
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1500
1000
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0
0 2 7 12 14 19 21
Days
400
300
200
100
0
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Days

Table 1. Correlation Between the Measured Concentrations Across Time Points and the Final Ossified Volume of the Marrow Explants

Days		*2*		*7*				*12*			*14*			*19*			*21*				*Ossified volume (day 21)*
	Factors	BMP-2	VEGF	BMP-2	VEGF	TGF-β1	OC	BMP-2	VEGF	IGF-1	BMP-2	IGF-1	OC	VEGF	IGF-1	OC	BMP-2	VEGF	IGF-1	OC	
2	BMP-2		0.893				0.986					0.864	0.896			0.872					
	VEGF	0.893		0.877		0.923	0.866	0.888			0.817										
	IGF-1			-0.833					0.918							-0.914					0.868
	OC	0.845	0.977	0.847		0.961												0.848			
	AP	-0.817					-0.861	-0.814		-0.948	-0.841	-0.931	-0.895		-0.859	-0.910					
7	BMP-2						0.957									0.830					
	OC			0.957				0.945			0.926	0.856									
	AP																			-0.814	
12	BMP-2											0.897	0.944			0.831					
	VEGF											-0.829			-0.874	-0.942					0.988
	IGF-1												0.896			0.837					
	OC																				
	AP																			-0.922	
14	BMP-2											0.946	0.977		0.847	0.870					
	VEGF														-0.845	-0.890					0.970
	IGF-1										0.946					0.906					-0.813
	OC										0.977			-0.890							
	AP																			-0.828	
19	BMP-2																	0.812			
	VEGF																0.842				
	IGF-1																				-0.865
	OC																				-0.931
	AP																			-0.957	

Pearson product moment correlation coefficient is presented in the cells (all the presented correlations are significant with $p < 0.05$). The cells corresponding to the correlations between the same factor at the same time points and reverse correlations in time were shaded due to the lack of physical significance. BMP-2, bone morphogenetic protein-2; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; TGF-β1, transforming growth factor beta 1; AP, alkaline phosphatase; OC, osteocalcin.

via the conditioned medium. In the future, this model would also allow interrogation with silencing-RNA or gene transfection to control GF production to assess their ultimate effect on the amount of bone formation. Therefore, *in vitro* bone marrow explant culture model presented here is a useful model for studying the temporal production profiles of the osteogenic factors and their synergistic combinatorial roles on bone generation/regeneration process.

The importance of preserving the cellular integrity of marrow tissue in terms of osteogenic potential was verified by including an experimental group in which marrow explants were dispersed in the culture medium. This condition allowed the gradual elimination of nonadherent cells (i.e., hematopoietic cells) with each medium change and left the adherent cells (i.e., marrow stromal cells) in the culture. The absence of mineralization (as per Raman microspectroscopy and μCT) in this group indicated that the multicellular nature of marrow tissue needs to be preserved to achieve self-inductive ossification. On the other hand, bone marrow explants whose integrity was maintained ossified to varying extents. Therefore, the final OV of the marrow explants was used as an indicator of the osteogenic capacity of individual samples, which may be directly related to levels of osteoinductive factors produced. The varying ossification levels of the samples allowed us to evaluate the correlation between the final OV and the concentration levels of individual factors at different time points (Table 1). To the best of our knowledge, the correlation between the levels of osteoinductive factors at different time points, across time points, and the final OV in an *in vitro* ossification model is being reported for the first time in this study. This information can be used to determine the most critical osteoinductive factors, as well as the most critical time of application of these factors to obtain a more efficient and natural ossification mechanism. In addition, the correlation between the levels of factors at different time points and across time points can be used to delineate the synergistic involvement of multiple osteoinductive factors in the ossification process.

GFs present in the serum that is commonly used as a supplement in *in vitro* cell culture studies confound the picture and make it hard to study the involvement of GFs in various processes such as osteogenesis. Therefore, we confirmed the ability of marrow explants to ossify in serum-free culture conditions and without application of any excipient osteoinductive factors. This property facilitates carrying out more comprehensive *in vitro* analysis of various proteins involved in osteogenesis without the inhibiting and/or interfering effects of serum supplements.

The histochemical analysis on the *in vitro* ossified marrow explants with safranin-o/fast green staining displayed (Fig. 2C) the absence of proteoglycans, which can be found abundantly in cartilaginous matrix. In addition, the presence of AP activity on the lower and upper surfaces of the ossifying explants indicated the presence of differentiated osteoblast-like cells forming a mineralized matrix. Therefore, the absence of a cartilaginous matrix and the presence of AP activity indicate that the ossification mechanism in this *in vitro* bone marrow culture model resembles the intramembranous ossification mechanism.

The baseline levels of AP, OC, BMP-2, IGF-1, TGF-β1, and VEGF in day 0 marrow tissue have been quantified, and all of them have been observed to be significantly lower than the day 2 production levels of these proteins by ossifying marrow explants (Figs. 3 and 4, triangular markers at day 0). Therefore, the presented concentration profiles of these quantified proteins could not be related merely to the baseline concentrations in fresh marrow tissue.

AP and OC are commonly accepted bone markers.[18] AP is expressed by many cell types to some extent. AP is also associated with osteoblast differentiation and its production is high in preosteoblasts and osteoblasts.[43] Using AP as an ossification marker with a heterogeneous population of marrow cells is complicated since only a small population of the cells in marrow stroma are AP-positive osteoblast precursors and many other cell types in marrow express AP, such as adipocytic cells.[44] The marrow explants employed in this study were handled minimally, which preserved the natural components together, including all the resident cell types, the extracellular matrix, as well as the soluble proteins and factors. Therefore, the early high production of AP in this study (Fig. 3A) may be attributed to the other cell types in the marrow tissue that are in high concentration, such as adipocytes. Due to the difficulties with quantifying ossification-related AP activity, a secondary ossification marker (OC) was used to assess ossification. OC is a specific marker of mature osteoblast phenotype.[18] Previously, OC level was observed to be increasing after about 10 days with *in vitro* mineralization models that employed marrow cells.[14,18] A peak in OC level around day 14 was also reported when the marrow stromal cells were stimulated with dexamethasone and $1,25(OH)_2D_3$.[18] However, the high OC level observed early on in this study (Fig. 3B) is not in agreement with the previous findings. At the earlier stage of the marrow explant culture, the high level of OC at day 2 decreased dramatically by day 7 and did not increase significantly between day 12 and day 21 (Fig. 3B). This observation may be attributed to the complex nature of the current model due to the presence of multiple cell types.

In vivo studies indicate that BMP-2 expression in the fracture site displays an early increase during the phase in which mesenchymal stem cells are recruited to the injury site.[2,4,45] The high level of BMP-2 production in the early phase of fracture healing is followed by a decrease as it was also observed in this study (Fig. 4A). A similar trend, in which upregulation of BMP-2 around day 4 followed by downregulation around day 12 was previously observed during mineralization of osteoprogenitors *in vitro*.[14] BMP-2 level measured at day 14 highly correlated with the IGF-1 level on day 14 (Table 1; Fig. 4C), which may be an indication of interaction or a similarity in terms of source cells for these two factors at this phase. Similarly, it was previously shown that early application of BMP-2 (day 1) followed by later application of combination of BMP-2 and IGF-1 (after day 5) resulted in the highest amount of cell number and AP activity in pluripotent C3H10T1/2 cells.[15] The high positive correlation between the OC level and the BMP-2 concentration at days 14 and 19 may be an indication on the role of BMP-2 in mature osteoblast function in mineralization. On the other hand, BMP-2 production on day 2 was observed to correlate negatively with AP level on day 2 (Table 1). This could be due to the early high levels of AP, which suppressed the production of BMP-2, which later peaked at day 7 when AP level decreased significantly. The

early level of BMP-2 (days 2–7) also correlated with later levels of OC (at days 7, 14, and 19) and IGF-1 (at day 14). This observation suggests that an early involvement of BMP-2 has repercussions on the latent stages of osteogenesis. The early involvement of BMP-2 in osteogenesis has recently been shown in sequential growth factor delivery studies with BMP-2 and BMP-7.[46,47] In these studies, early release of BMP-2 and latent release of BMP-7 were achieved with nanocapsules in an *in vitro* study with MSCs. Superior osteoinductive effects of the sequential application of BMP-2 and BMP-7 were demonstrated over individual and simultaneous applications based on elevated AP activity on days 14 and 21.

IGF-1 production by *in vitro* ossifying marrow explants in this study was bimodal. It displayed a high level at the early stage, followed by a decrease during days 7–14, and then a latent increase during days 19–21 (Fig. 4B). A similar pattern of IGF-1 production in the later stage was observed in fracture healing in an *in vivo* model by Wildemann *et al.*[33] As the earliest time point included in the study by Wildemann *et al.* was day 5, it is not possible to compare the early level of IGF-1 to that was observed in our study. However, in an *in vitro* mineralization model utilizing osteoprogenitor cells, a high level of IGF-1 was observed early on, followed by a decrease between days 5–12, and then followed by an increase starting day 13.[14] Therefore, the results presented in this study in terms of IGF-1 concentration profile agree with the previous findings in the literature. The high IGF-1 level at day 2 was observed to correlate positively with OV (Table 1), which may be an indication that early application of IGF-1 is critical in bone regeneration. On the other hand, we observed that IGF-1 levels at later stages (days 14–19) correlated negatively with OV (Table 1), which may be an indication that IGF-1 application in the later stages may have a deterring role in ossification. IGF-1 concentration was also observed to correlate with OC level positively on days 14 and 19 (Table 1). When this information is combined with the findings presented above (IGF-1 correlated negatively with OV on days 14–19) and the fact that OC correlated negatively with OV on day 19 (Table 1), it is reasonable to suggest a connection between IGF-1 and OC in curbing further ossification of the marrow explants starting day 14 and allowing the mineralization to reach a steady state. In attestation, OC is known to curb mineralization[48] and current results imply that IGF-1 may be associated in this pathway.

Early involvement of TGF-β1 during the proliferation phase in fracture-healing process has been shown previously.[2–4,33] Similarly, TGF-β1 was observed at a high level early on in the marrow explant culture model and decreased continually to reach a steady-state level by day 14 (Fig. 4C). Therefore, TGF-β1 production profile presented here agrees with the previous findings. However, TGF-β1 levels at various time points displayed a limited number of correlations with other factors. The only TGF-β1 level that has displayed a significant correlation was day 7 level, which correlated positively with day 2 levels of VEGF and OC. Therefore, a link between VEGF and TGF-β1 involvement could be present between days 2 and 7 during ossification.

VEGF is considered to play its most important role in fracture healing in the earlier stages.[49] Therefore, the high concentration of VEGF observed in this study during day 2, which gradually decreased starting day 7 up to day 21 (Fig. 4D), agrees with the previous findings. A similar trend of VEGF expression during mineralization by osteoprogenitors was also observed in an *in vitro* study.[14] Even though VEGF concentration was high at the earlier stage of ossification and decreased gradually till day 21 (Fig. 4D), VEGF concentration and OV was observed to highly correlate only during days 12–14 (Table 1). This high correlation between VEGF concentration and OV indicates that the presence of VEGF during days 12–14 was associated with final amount of ossification. Importance of VEGF at the mid-phase of fracture repair was also shown and emphasized before.[4]

The correlations that were reported between the GF levels and the final OV are useful for identifying the temporal involvement of the GFs in osteogenesis. However, these correlations do not necessarily imply causations. The correlations observed in this study require further analysis with studies employing targeted inhibition of GFs at specific time points to assess the overall effect in the ossification of marrow tissue.

There are many GFs involved in osteogenesis, and they are not limited to the four factors (BMP-2, VEGF, IGF-1, and TGF-β1) studied here. Other factors that are known to be actively involved in osteogenesis are BMPs (4, 6, 7, and 13), fibroblast growth factor-2, Wnt, growth/differentiation factor-5, and platelet-derived growth factor.[14,21,50–54] A detailed characterization of the involvement of many GFs and signaling molecules is necessary, and proteomic analysis could be used for high-throughput screening of all the proteins. Therefore, we are currently conducting experiments to analyze a myriad of proteins and their temporal expression profiles during inherent *in vitro* ossification of bone marrow explants.

Conclusions

It was shown that BMP-2, IGF-1, TGF-β1, and VEGF are expressed differentially over time by the ossifying marrow explants and the concentration of IGF-1 and VEGF correlate at different time points with the final OV. IGF-1 has dichotomous effect on the final OV, which is indicated by a positive correlation on day 2 and negative correlations on days 14 and 19. The GF levels and the production of bone markers (AP and OC) have been shown to be highly interdependent due to correlations to each other at same time points as well as across various time points. The results presented in this study provide a more robust understanding of the osteogenesis process in terms of the involvement of BMP-2, IGF-1, TGF-β1, VEGF, AP, and OC in marrow explants and the secretion sequence and the amounts of key osteoinductive factors involved in this osteogenesis model. The information obtained from the marrow ossification model can be used to develop multifactor and multiphase GF delivery strategies for fracture healing and bone tissue engineering applications.

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Disclosure Statement

No competing financial interests exist.

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