Developmental Cues for Bone Formation from Parathyroid Hormone and Parathyroid Hormone-Related Protein in an *Ex Vivo* Organotypic Culture System of Embryonic Chick Femora

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Enhancement and application of our understanding of skeletal developmental biology is critical to developing tissue engineering approaches to bone repair. We propose that use of the developing embryonic femur as a model to further understand skeletogenesis, and the effects of key differentiation agents, will aid our understanding of the developing bone niche and inform bone repair. We have used a three-dimensional organotypic culture system of embryonic chick femora to investigate the effects of two key skeletal differentiation agents, parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP), on bone and cartilage development, using a combination of microcomputed tomography and histological analysis to assess tissue formation and structure, and cellular behavior.

Stimulation of embryonic day 11 (E11) organotypic femur cultures with PTH and PTHrP initiated osteogenesis. Bone formation was enhanced, with increased collagen I and STRO-1 expression, and cartilage was reduced, with decreased chondrocyte proliferation, collagen II expression, and glycosaminoglycan levels.

This study demonstrates the successful use of organotypic chick femur cultures as a model for bone development, evidenced by the ability of exogenous bioactive molecules to differentially modulate bone and cartilage formation. The organotypic model outlined provides a tool for analyzing key temporal stages of bone and cartilage development, providing a paradigm for translation of bone development to improve scaffolds and skeletal stem cell treatments for skeletal regenerative medicine.

Introduction

DEVELOPMENT OF ALTERNATIVE tissue engineering strategies to augment bone repair and regeneration requires a comprehensive understanding of the developing tissue niche. Bone formation occurs in a complex environment, influenced by a number of chemical and physical cues, and involves the complex interaction of multiple cell types.^{1–4} Enhancement of our knowledge of these processes can be hampered by limitations in model systems available for the study of bone development and repair. *In vitro* cell culture models typically use only a single or dual cell system, and are unable to recapitulate *in vivo* spatial arrangement. *In vivo* animal models, although able to provide considerable information on developmental and reparative processes,^{5–7} are expensive, with large numbers of animals often required, and systemic influences can often complicate the data obtained. *Ex vivo* organ cultures provide an alternative model system,

whereby collective populations of cells present in their natural extracellular matrix can be cultured and manipulated, thus providing information on growth, differentiation, and developmental processes, as well as the numerous growth factors therein that influence and modulate such processes.⁸ Such models can further address ethical issues by complying with the 3Rs of reduction, refinement, and replacement.

Skeletal *ex vivo* organ cultures and embryonic limb rudiment cultures have been developed to study a range of developmental, pathological, and reparative bone processes.^{9–12} One such model is the embryonic chick bone organ culture system, where isolated whole-bone rudiments are cultured in an organotypic setup at the liquid–gas interface.¹³ Such organotypic culture systems, where tissue is cultured on a semiporous membrane over liquid media, promote high oxygen tension within the cultured tissues, thus promoting viability and the capacity for bone formation.¹² The use of chick embryos in such a system is highly cost-effective, enabling large numbers of

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experimental parameters/repeats, and advantageous due to the rapid development of the chick, enabling detailed analysis of tissue responses over a relatively short culture period. We have previously developed an *ex vivo* organotypic culture model of embryonic chick femurs, and have demonstrated and evaluated the embryonic development of bone and cartilage tissue, using a combination of immunohistochemical techniques and microcomputed tomography (μ CT).¹⁴ The current study characterized the differentiation potential of the embryonic chick femora cultured in the presence of exogenous bioactive growth factors to differentially modulate bone and cartilage formation. Embryonic day 11 chicks were chosen as an early model of bone development, as *in ovo* these femurs display a paucity of bone tissue and are predominantly cartilaginous in nature, with a high cellular differentiation potential. In addition, embryonic day 13 chicks were also analyzed, as a model of later bone development, with femurs displaying a developed and significant bone collar, differentiated cells, extensive bone marrow and, importantly, blood vessels that have started to invade the bone.

Parathyroid hormone (PTH) and the parathyroid hormone-related protein (PTHrP) were chosen in this study as bioactive molecules known to have significant roles in bone physiology, providing an opportunity to assess the potential and efficacy of the *ex vivo* chick osteogenic/chondrogenic responses to exogenous bioactive molecules. PTH, as well as functioning as a calcium regulator, is critically involved in skeletal remodeling processes, exerting both catabolic and anabolic effects on bone tissue.¹⁵ Furthermore, studies have shown that intermittent administration of PTH enhances bone formation.^{16,17} PTHrP has been shown to control cellular behavior and differentiation in chondrocytes, osteoblasts, and bone marrow cells, and is critically involved in maintaining chondrocyte proliferation within long bones, via the PTHrP/Indian hedgehog feedback loop, which controls both chondrocyte hypertrophy and proliferation and can be influenced by other growth factors, including the bone morphogenetic proteins.^{18–22}

The *ex vivo* chick femur culture model enables a variety of cell types to be cultured in their 3D matrices, in the orientation present in an *in vivo* system. The system contains cells of both the osteoblast and the chondrocyte lineage, including their precursors, providing analysis of intricate cell–cell and cell–matrix interactions, without the complex systemic influences of an *in vivo* system that can often complicate the data obtained. This study aimed to demonstrate and assess the responsiveness of the chick femur organ culture model to differentiation agents, by analyzing both the osteogenic and chondrogenic tissue responses. The hypothesis under test is that exogenous osteotropic factors PTH and PTHrP will accelerate endochondral bone formation in *ex vivo* chick embryonic femurs. Resolution of this hypothesis will validate and provide efficacy that the chick femur organotypic model is an appropriate model system to examine bone development and growth factor/stem cell/scaffold therapies for bone or cartilage regeneration.

Methods

Organotypic culture of embryonic chick femurs

Organotypic cultures of embryonic day 11 (E11) and 13 (E13) chick femurs were performed as described previously.¹⁴ Control noncultured femurs were placed into 4% paraformaldehyde (PFA) for fixation, while cultured femurs were placed

onto Millicell inserts (0.4- μ m pore size, 30-mm diameter; Millipore) in six-well tissue culture plates (Greiner Bio-One Ltd), containing 1 mL media per well at the liquid/gas interface. Culture media was basal (minimum essential medium alpha [α -MEM] containing 100 units penicillin, 100 μ g/mL streptomycin, and 100 μ M L-ascorbic acid 2-phosphate (Sigma-Aldrich), alone or supplemented with human recombinant PTH (50 ng/mL or 100 ng/mL) (Abcam) or human recombinant PTHrP (50 ng/mL or 100 ng/mL) (Sigma-Aldrich) ($n=12$, per condition and dose). Femurs were cultured for 10 days at 37°C, 5% CO₂ in air, with media changed every 24 h.

Analysis of tissue glycosaminoglycan content

Selected noncultured and organotypic cultured femurs ($n=4$ per condition and dose) were weighed and digested overnight at 60°C in 1.06 mg/mL papain solution (Sigma-Aldrich). Following digestion, samples were added to 1,9-dimethylmethylene blue solution (DMMB) (Sigma-Aldrich) in a 96-well flat-bottomed plate (Greiner Bio-One Ltd), with 0 to 100 μ g/mL standards prepared using chondroitin-4-sulfate from shark cartilage (Sigma-Aldrich). Absorbance of standards and samples were read at 540 nm on a microplate spectrophotometer, and the absorbance curve of the standards was used to calculate the quantity of glycosaminoglycan (GAG) in samples, as a percentage of tissue weight.

Faxitron x-ray analysis

Following the culture period, chick femurs were fixed in 4% PFA for at least 24 h. Femurs were then imaged radiographically using a Faxitron[®] Specimen Radiography System (MX-20) (Qados Ltd) and the lengths measured ($n=8$ for each condition and each dose).

Micro-computed tomography

Quantitative 3D analysis of fixed noncultured and organotypic cultured femurs was performed using an Xtek BenchTop 160Xi CT scanning system for μ CT (X-TEK Systems Ltd) equipped with a Hamamatsu C7943 x-ray flat panel sensor (Hamamatsu Photonics) ($n=4$ per condition and dose) as described previously.¹⁴ Reconstructed femurs were selected for quantification of bone, and analyzed for macro- and microscopic structural properties using Volume Graphics (VG) Studio Max 1.2.1 software package (VG, GmbH). Bone Volume (BV), Bone Surface/Bone Volume (BS/BV), Bone Volume/Total Volume (BV/TV), Trabecular Thickness (Tb.Th), Trabecular Number (Tb.No), and Trabecular Spacing (Tb.Sp.) were calculated using X-Tek computational algorithms.

Histological examination

Fixed noncultured and organotypic cultured femurs were dehydrated through a series of graded ethanols, cleared in chloroform, and embedded in low-melting point paraffin wax using an automated Shandon Citadel 2000. Seven-micrometer sections were cut and stained with Alcian blue/Sirius red to assess proteoglycan and collagen production, or von Kossa to assess bone mineralization, as described previously.¹⁴ Briefly, sections were stained following deparaffinization and rehydration through graded alcohols and water. For Alcian blue/Sirius red, sections were stained with Weigert's hematoxylin, differentiated in acid/alcohol, and

stained with 0.5% Alcian blue (for proteoglycan-rich cartilage matrix) and 1% Sirius red (for collagenous bone matrix). For von Kossa, sections were incubated in silver nitrate under ultraviolet light and sodium thiosulfate, with Alcian blue and Van Gieson's counterstains. All sections were subsequently dehydrated and cleared before mounting in DPX. Images were captured with an Olympus BX-51/22 dotSlide digital virtual microscope, and created using OlyVIA 2.1 software (Olympus Soft Imaging Solutions, GmbH) ($n=8$ per condition and dose, with a total of six sections from each femur for each histological stain).

Immunohistochemical examination

Paraffin wax-embedded femur sections were immunohistochemically stained to analyze bone and cartilage matrix, by assessing expression of collagen type I and II, respectively, and to analyze expression of the skeletal stem cell marker STRO-1 and the proliferation marker proliferating cell nuclear antigen (PCNA), as described previously.¹⁴ Briefly, sections were deparaffinized and rehydrated, and incubated with 3% hydrogen peroxide to quench an endogenous peroxidase activity. Cell permeabilization with 0.5% Triton-X was performed before PCNA immunostaining, and hyaluronidase digestion before type II collagen immunostaining. Nonspecific binding was blocked with 1% bovine serum albumin (BSA), followed by overnight 4°C incubation with primary antibodies diluted appropriately in 1% BSA: type I collagen (polyclonal rabbit antibody diluted 1:1000, kind gift from Larry Fisher, NIH), type II collagen (polyclonal rabbit antibody diluted 1:500, Calbiochem), PCNA (mouse monoclonal antibody diluted 1:100, Abcam), or STRO-1 (undiluted culture supernatant obtained from the STRO-1 hybridoma provided by Dr J. Beresford, University of Bath, UK.). Sections were subsequently incubated with biotinylated secondary antibodies diluted in 1% BSA: goat anti-rabbit IgG (1:100, collagen type I and II), goat anti-mouse IgG (1:100, PCNA), and goat anti-mouse IgM (1:100, STRO-1) (Sigma-Aldrich). ExtrAvidin Peroxidase and 3-amino-9-ethylcarbazole (AEC) substrate solution (Sigma-Aldrich) was used to visualize the brown immune complex reaction product, with light green and Alcian blue counterstains. Negative controls, either primary antibody exclusion or replacement with a nonimmune IgG or IgM, showed absence of any positive staining. Images were captured using the Olympus BX-51/22 dotSlide digital virtual microscope

($n=8$ per condition and dose, with a total of six sections from each femur for each immunohistochemical stain).

Histological quantification of bone formation and PCNA expression

Quantification of Sirius red-stained bone matrix in histological sections was performed using ImageJ analysis software.²³ The area of bone matrix was calculated as a proportion of the total diaphyseal area, based on color thresholds (red bone matrix versus blue Alcian blue-stained cartilage matrix). Bone matrix area in treated femurs was normalized to control basal-cultured femurs. Quantification of cell number, and the proportion of cells expressing the proliferation marker PCNA, was performed using CellProfiler image analysis software.²⁴ Automated cell counts were performed within the whole diaphysis and epiphysis of imaged femur sections. The number of cells positive for PCNA was separately counted, based on color (brown positivity versus green counterstain) and size (based on a calculated minimum and maximum cell size) thresholds, and expressed graphically as a proportion of the total cell number. For each femur, six sections were quantified for each stain, chosen at set depth intervals to ensure an accurate representation of the whole femur.

Statistical analysis

All measurements were calculated as mean $\pm$ standard error of the mean. Statistical analyses were performed using GraphPad InStat3 v3.06 software. Differences among groups were determined by one-way analysis of variance with a *post hoc* Tukey's test, and statistical differences were considered to be significant if $p \leq 0.05$.

Results

Analysis of the modulatory effects of PTH on cultured embryonic chick femurs

μ CT analysis of embryonic day 11 (E11) chick femurs stimulated with 50 or 100 ng/mL PTH did not demonstrate any significant changes in length or mineralized bone structural parameters compared to basal-cultured femurs (Fig. 1a, b), although (BS/BV) and trabecular thickness was decreased between the 50 ng/mL and the 100 ng/mL dose. An osteogenic response to PTH was demonstrated with histology (Fig. 2a). Alcian blue/Sirius red staining demonstrated that addition of PTH increased diaphyseal bone formation,

FIG. 1. Microcomputed tomography (μ CT) analysis of E11 chick femurs following 10-day organotypic culture in basal or parathyroid hormone (PTH)-stimulated conditions. (a) μ CT images (segmented mineralized bone, sagittal sections, and cross-sectional sections of the central diaphysis region). (b) μ CT structural morphometric indices of E11 embryonic chick femurs. *ns*=no significant differences; E11, embryonic day 11. * $p < 0.05$.

FIG. 2. Histological analysis of E11 chick femurs following 10-day basal or PTH organotypic culture. **(a)** Histological staining for alcian blue/Sirius red, von Kossa, and expression of collagen type I & II, proliferating cell nuclear antigen (PCNA) and STRO-1⁺ (scale bar=100 μ m, arrows indicate areas of increased or decreased expression compared to basal controls). **(b)** Diaphyseal quantification of Sirius red-stained bone matrix. **(c)** Glycosaminoglycan (GAG) content of whole-chick femurs (NC = noncultured). **(d)** Quantification of diaphyseal cell number and proportionate PCNA positivity (▨ = total cell count ■ = number of PCNA-positive cells). **(e)** Quantification of epiphyseal cell number and proportionate PCNA positivity (▨ = total cell count ■ = number of PCNA-positive cells). * $p < 0.05$. ** $p < 0.01$. Color images available online at www.liebertpub.com/tec

with formation of a thicker bone collar. Quantification of Sirius red-stained diaphyseal matrix indicated a significant dose-dependent increase in the bone matrix area (Fig. 2b). This correlated with increased collagen I deposition along the thickened bone collar, although von Kossa stain indicated that the new bone was not mineralized. Expression of STRO-1, typically used to identify osteoprogenitor/skeletal stem cell populations, was increased within bone matrix areas and the inner diaphyseal region. PTH decreased collagen II expression, and DMMB assays confirmed a significant reduction in GAG levels (Fig. 2c). In addition, PTH decreased proliferation within diaphyseal regions, as evidenced by a significant decrease in PCNA expression (Fig. 2d), but had no effect on epiphyseal proliferation (Fig. 2e).

Older embryonic day 13 (E13) femurs also demonstrated osteogenic responses to PTH, but only to the higher dose.

Femur length was not affected, but μ CT analysis revealed increased BV and trabecular number (Appendix Fig. A1a,b). Histological analysis also indicated an increase in diaphyseal bone matrix, with increased mineralization and a thinner chondrocyte-rich region, although other markers were not affected (Appendix Fig. A2a). Quantification of Sirius red-stained matrix confirmed an increase in bone tissue (Appendix Fig. A2b), with a corresponding decrease in GAG levels (Appendix Fig. A2c). Proliferation was not altered (Appendix Fig. A2d, e).

Analysis of the modulatory effects of PTHrP on cultured embryonic chick femurs

μ CT analysis of E11 chick femurs cultured with PTHrP did not indicate any significant changes in length or mineralized bone structural parameters (Fig. 3a, b). However, histological

FIG. 3. μ CT analysis of E11 chick femurs following 10-day organotypic culture in basal or parathyroid hormone-related protein (PTHrP)-stimulated conditions. **(a)** μ CT images (segmented mineralized bone, sagittal sections, and cross-sectional sections of the central diaphysis region). **(b)** μ CT structural morphometric indices. ns = no significant differences.

analysis indicated an osteogenic response at the cellular level (Fig. 4a). Addition of 50 or 100 ng/mL PTHrP increased bone formation within the diaphyseal bone collar, and the higher dose employed also increased bone and marrow formation within the inner diaphyseal region. Quantification of Sirius red-stained matrix confirmed a significant increase in bone formation with both doses of PTHrP (Fig. 4b). This correlated with increased expression of collagen I and STRO-1, although von Kossa staining did not indicate increased mineralization. Inhibition of chondrogenesis was also observed following PTHrP stimulation, with a reduction in collagen II expression and GAG levels (Fig. 4c). There was a significant decrease in proliferation, within diaphyseal regions only (Fig. 4d, e).

In E13 femurs, PTHrP did not influence length or bone morphometric parameters (Appendix Fig. A3a, b). Furthermore, PTHrP did not affect tissue composition or marker expression, compared to basal controls (Appendix Fig. A4a). There were no significant changes in the bone matrix area (Appendix Fig. A4b), GAG content (Appendix Fig. A4c), or diaphyseal proliferation (Appendix Fig. A4d), although epiphyseal proliferation was increased (Appendix Fig. A4e). All immunohistochemical stains were confirmed with negative controls, either primary exclusion or replacement with a nonimmune IgG or IgM, which all showed absence of any positive staining (Appendix Fig. A5).

Discussion

This study examined the potential and efficacy of the chick organotypic femur culture system to examine and delineate osteogenic and chondrogenic responses to growth factors and small molecules. The study demonstrated that PTH increased bone matrix formation within cultured femurs. PTH primarily functions to increase blood calcium concentration,²⁵ but in addition is critically involved in skeletal remodeling processes, via its abilities to couple the osteoblast and osteoclast activity, and signal in osteocytes.²⁶ PTH has recorded some success in the clinical arena, with the recombinant form of PTH (1–34), Teriparatide, gaining Food and Drug Administration (FDA) approval for the treatment of severe osteoporosis.^{27,28} PTH increased the diameter within E11 chick femurs, with formation of a thicker collagen I-rich diaphyseal bone collar. Interestingly, this osteogenic response was only demonstrated with histochemical analysis, with μ CT demonstrating no significant changes in BV param-

eters. μ CT analyzes only the mineralized bone content and does not distinguish nonmineralized osteoid. In contrast, histological analysis measures bone formation in terms of collagen deposition, and can assess both mineralized bone and nonmineralized osteoid. Since histological staining demonstrated an increase in nonmineralized bone deposition in response to PTH, this explains the lack of significant changes observed with μ CT analysis, and demonstrates the requirement for a combination of techniques when analyzing *ex vivo* models of bone development. μ CT analysis did, however, reveal an increase in mineralized BS, with thinner trabeculae, after stimulation with the higher dose of PTH, in all likelihood due to influences on remodeling. As well as increasing bone formation, PTH inhibited chondrogenesis in E11 femurs. Collagen II expression and tissue GAG levels were reduced, known to correlate with the development and mineralization of bone matrix.²⁹ Proliferation of diaphyseal chondrocytes was decreased, which together with decreased collagen II is indicative of chondrocyte hypertrophy, essential for blood vessel invasion and bone formation.^{30,31} The data obtained using cultured chick femurs are consistent with previous observations that PTH preferentially stimulates differentiation of more immature cells of the osteoblast lineage.³² Interestingly, the repeat administration of PTH into the cultures, given its rapid half-life, also contributed to the measured increase in bone, correlating with previous studies demonstrating that intermittent PTH administration increases bone mass compared to continuous administration.^{16,17}

Histological analysis indicated a similar osteogenic response following PTHrP stimulation of E11 femurs, with increased bone matrix and marker expression, although there were no significant differences in μ CT bone parameters. As with PTH, this can be attributed to the deposition of new nonmineralized bone matrix. PTH and PTHrP are structurally similar, sharing a common receptor, and are known to initiate, in part, comparable effects in osteoblasts, chondrocytes, and osteoclasts *in vivo*.^{33–36} Ablation of PTHrP in mice or humans leads to a lethal skeletal dysplasia, caused by abnormal chondrocyte proliferation and differentiation,^{37,38} and PTHrP has been implicated in the progression and bone metastases of a number of cancers.^{39,40} In E11 femurs, higher dose of PTHrP resulted in slightly different effects to PTH, with increased formation of bone and marrow cavities within the inner diaphyseal region as well as the bone collar. This may be due to the ability of PTHrP to influence proliferation, differentiation, and apoptosis of multiple cell types, including osteoblasts, chondrocytes, and marrow cells.^{19,20,41} PTHrP inhibited

FIG. 4. Histological analysis of E11 chick femurs following 10-day basal or PTHrP organotypic culture. **(a)** Histological staining for alcian blue/Sirius red, von Kossa, and expression of collagen type I & II, PCNA and STRO-1⁺ (scale bar = 100 μ m, arrows = areas of increased/decreased expression compared to basal controls). **(b)** Diaphyseal quantification of Sirius red-stained bone matrix. **(c)** GAG content of whole-chick femurs (NC = noncultured). **(d)** Quantification of diaphyseal cell number, and proportionate PCNA positivity (▨ = total cell count ■ = number of PCNA-positive cells). **(e)** Quantification of epiphyseal cell number and proportionate PCNA positivity (▨ = total cell count ■ = number of PCNA-positive cells). * $p < 0.05$. ** $p < 0.01$. Color images available online at www.liebertpub.com/tec

diaphyseal chondrogenesis, decreasing collagen II expression, GAG levels, and proliferation. The proportion of proliferating PCNA-positive cells in femurs cultured with 100 ng/mL PTHrP was lower than in femurs cultured with 100 ng/mL PTH (10% compared to 13%, respectively), which may explain the increase in diaphyseal bone and marrow formation. Interestingly, it would appear that this observation contradicts previous studies demonstrating that PTHrP, via its interactions with Indian hedgehog, increases chondrocyte proliferation, and prevents hypertrophy, keeping a pool of proliferative cells within the prehypertrophic region,^{18,42} however, this observation is only true within the central diaphyseal region, where the majority of bone formation occurs. Chondrocytes within this area are hypertrophic and PTHrP would not normally be expressed here *in vivo*,⁴² whereas if the

epiphyses are examined, where highly proliferative PTHrP-expressing chondrocytes reside *in vivo*, proliferation is increased, correlating with the previous studies above.

Interestingly, there were varied responses to growth factor stimulation in E13 femurs. Analysis of E13 femurs stimulated with the higher dose of PTH indicated osteogenesis, with an increase in diaphyseal bone matrix and mineralization, as well as significant increases in μ CT bone structural parameters. Since the amount of bone and marrow formed *in ovo* is significantly increased between embryonic day 11 and 13, this may be attributed to the mature, more differentiated state of the bone tissue within these femurs, as well as the increased deposition of mineralized bone matrix, which is able to be quantified with μ CT. However, there were no discernible differences in STRO-1 expression, cellular

proliferation, or cartilage marker expression, and there were no significant changes in femurs stimulated with the lower dose of PTH. In regard to PTHrP, E13 chick femurs failed to respond to either dose, with no observed differences in bone or cartilage, although interestingly there was an increase in epiphyseal proliferation, due to the ability of PTHrP to maintain epiphyseal chondrocytes in a proliferative state.^{18,42} Together, these results confirm E11 as the optimal experimental age as indicated in previous studies.¹⁴

This study has demonstrated that the chick femur *ex vivo* model system, staged at E11, provides a valuable tool in testing osteogenic and chondrogenic responses to growth factors. Development of the chick embryo is rapid both *in ovo* and within our *ex vivo* system, enabling detailed analysis of tissue responses over a relatively short culture period. The use of chick embryos is highly cost-effective, enabling large numbers of experimental repeats, and the model also has potential to provide information on repair mechanisms, by formation of critical-sized bone defects.^{43,44} A distinct advantage of the model system is the presence of multiple cell types in the system, *in situ* in their natural extracellular matrix, which has been shown to be a crucial factor in both osteogenic and chondrogenic differentiation and subsequent tissue formation. However, as with all model systems, there are limitations to the chick femur *ex vivo* system. The major limitation remains the absence of a blood supply, important for nutrient and oxygen delivery, and growth factor and signaling cascade modulation in the tissue. Thus, the organotypic model has time limitations and cannot be cultured indefinitely, although the 10-day culture period utilized is sufficient for analysis of bone and cartilage tissue in response to external stimuli over the culture period.¹⁴ The absence of a blood supply also negates analysis and evaluation of the interaction of a number of cell types that would be traditionally provided by this system, including a hematopoietic compartment containing immune cells and bone-resorbing osteoclasts. It is important to note, however, that there is the potential to further develop the chick femur model system, by combining with the highly vascularized chick chorioallantoic membrane, potentially allowing blood vessel invasion into the tissue. Other refinements include microinjection of distinct cell populations into the femur tissue to analyze cell types not present in the *ex vivo* system. A further limitation of the *ex vivo* chick femur model is the lack of the mechanical forces applied to the tissue, known to be a critical component of bone formation and remodeling. However, there is again the potential to further develop the model, in combination with *in vitro* bioreactors capable of applying relevant (*in vivo*) mechanical forces. We have demonstrated previously that the application of chick femurs to Millicell PTFE membranes will confer a modest degree of mechanical force to the tissue, as femurs in submerged cultures display markedly different patterns of bone formation (poorly structured in direction and order).¹⁴ The potential of the chick femur model system may be further enhanced by the possibility of applying similar culture techniques to mammalian tissues, including human fetal femurs, which will further widen the clinically relevant information obtained from such a system.

Taken together, this model system of cultured embryonic femurs offers significant potential as a first-line testing system for scaffold/cell/growth factor therapies, providing for more complex interactions to be studied, while addressing the 3Rs of replacement, refinement, and reduction of ani-

mals, thus bridging the gap between more simple *in vitro* systems and complex, expensive *in vivo* models. The current techniques employed (μ CT and histology) provide a multi-dimensional technical approach to enhance our understanding of bone development and repair within a 3D structural format and this, together with the potential to combine the chick femur model with CAM, bioreactor, and cell microinjection techniques, provides a model system that has the potential to yield considerable information on the processes in normal and diseased conditions, and, critically, thus inform skeletal tissue reparation strategies.

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Disclosure Statement

No competing financial interests exist.

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Appendix

APPENDIX FIG. A1. Microcomputed tomography (μ CT) analysis of E13 chick femurs following 10-day organotypic culture in basal or parathyroid hormone (PTH)-stimulated conditions. **(a)** μ CT images (segmented mineralized bone, sagittal sections, and cross-sectional sections of the central diaphysis region). **(b)** μ CT structural morphometric indices. ns=no significant differences, ** $p < 0.01$, *** $p < 0.001$.

APPENDIX FIG. A2. Histological analysis of E13 chick femurs following 10-day basal or PTH organotypic culture. **(a)** Histological staining for alcian blue/Sirius red, von Kossa, and expression of collagen type I & II, proliferating cell nuclear antigen (PCNA) and STRO-1⁺ (scale bar=100 μ m, arrows=areas of increased/decreased expression compared to basal controls). **(b)** Diaphyseal quantification of Sirius red-stained bone matrix. **(c)** Glycosaminoglycan (GAG) content of whole-chick femurs (NC=noncultured). **(d)** Diaphyseal quantification of cell number, and proportionate PCNA positivity (▣=total cell count ■=number of PCNA-positive cells). **(e)** Epiphyseal quantification of cell number and proportionate PCNA positivity (▣=total cell count ■=number of PCNA-positive cells). * $p < 0.05$. Color images available online at www.liebertpub.com/tec

APPENDIX FIG. A2. (Continued)

APPENDIX FIG. A3. μ CT analysis of E13 chick femurs following 10-day organotypic culture in basal or parathyroid hormone-related protein (PTHrP)-stimulated conditions. (a) μ CT images (segmented mineralized bone, sagittal sections, and cross-sectional sections of the central diaphysis region). (b) μ CT structural morphometric indices. ns=no significant differences.

APPENDIX FIG. A4. Histological analysis of E13 chick femurs following 10-day basal or PTHrP organotypic culture. **(a)** Histological staining for alcian blue/Sirius red, von Kossa, and expression of collagen type I & II, PCNA, and STRO-1⁺ (scale bar = 100 μ m, arrows = areas of increased/decreased expression compared to basal controls). **(b)** Diaphyseal quantification of Sirius red-stained bone matrix. **(c)** GAG content of whole-chick femurs (NC = noncultured). **(d)** Diaphyseal quantification of cell number, and proportionate PCNA positivity (▨ = total cell count ■ = number of PCNA-positive cells). **(e)** Epiphyseal quantification of cell number and proportionate PCNA positivity (▨ = total cell count ■ = number of PCNA-positive cells). *** $p < 0.001$. Color images available online at www.liebertpub.com/tec

APPENDIX FIG. A5. Representative negative controls for collagen I, collagen II, PCNA, and STRO-1 immunostains (scale bar = 100 μ m). Color images available online at www.liebertpub.com/tec