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A NEW TAKE ON AN OLD STORY: CHICK LIMB ORGAN CULTURE FOR SKELETAL NICHE DEVELOPMENT AND REGENERATIVE MEDICINE EVALUATION

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Abstract

Scientific research and progress, particularly in the drug discovery and regenerative medicine fields, is typically dependent on suitable animal models to develop new and improved clinical therapies for injuries and diseases. *In vivo* model systems are frequently utilised, but these models are expensive, highly complex and pose a number of ethical considerations leading to the development and use of a number of alternative *ex vivo* model systems. The *ex vivo* embryonic chick long bone and limb bud models have been utilised in the scientific research field as a model to understand skeletal development for over eighty years. The rapid development of avian skeletal tissues, coupled with the ease of experimental manipulation, availability of genome sequence and the presence of multiple cell and tissue types has seen such model systems gain significant research interest in the last few years in the tissue engineering field. The models have been explored both as systems for understanding the developmental bone niche and as potential testing tools for tissue engineering strategies for bone repair and regeneration. This review details the evolution of the chick limb organ culture system and presents recent innovative developments and emerging techniques and technologies applied to these models that are aiding our understanding of skeletal developmental and regenerative medicine research and application.

Keywords: Chick limb organotypic culture; *ex vivo* model; embryonic femur; micro-computed tomography; osteogenesis; chondrogenesis; chorioallantoic membrane assay; bone development and regeneration.

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Introduction

Animal models have long played a crucial role in many aspects of scientific research. In particular, progress in the drug discovery and regenerative medicine fields have benefitted extensively from *in vivo* animal models in the development of new and improved clinical therapies applied to damaged and diseased tissue. However, *in vivo* model systems are expensive, highly complex and, critically, present a number of ethical concerns. These considerations have led to the development and use of a number of alternative *ex vivo* model systems. The presence of multiple cell types located within their natural extracellular matrix and organised in the requisite spatial arrangements found *in vivo*, make these models highly advanced when compared to the single or dual *in vitro* cell culture systems. This biology implies the potential to recapitulate a number of *in vivo* processes, in an experimental system that is cheaper, easier to manipulate experimentally and more acceptable ethically than *in vivo* systems.

Skeletal-based *ex vivo* models have been developed in a number of animal species. These include mouse calvarial organ cultures co-cultured with cancer cells as a model of bone metastasis (Curtin *et al.*, 2012), *ex vivo* sheep perfusion models to study fluid flow and transport processes in loaded bones (Knothe Tate and Knothe, 2000), and *ex vivo* mandibular organ cultures using either rat or mouse tissue to assess bone repair processes or inflammatory bone pathology respectively (Sloan *et al.*, 2012; Smith *et al.*, 2010). The chick embryo, which represents an economical and accessible *in vivo* and *ex vivo* model has been utilised for almost a century in the developmental biology field and is now becoming increasingly recognised as a viable model system in a number of scientific research fields, including the disciplines of skeletal tissue engineering and regenerative medicine.

The chick embryo has been a major and established model system in developmental biology for many years, indeed even since the early days of Aristotle, as reviewed in a number of papers (Stern, 2004; Tickle, 2004; Wolpert, 2004). These studies in the chick embryo suggest an archaic model, not for the modern high throughput molecular screening techniques of today. However the avian embryo is well characterised with a fully sequenced genome (Hamburger and Hamilton, 1992; Hillier *et al.*, 2004), is highly cost-effective, and experimentally accessible for manipulation *in ovo* (Rashidi and Sottile, 2009). Importantly, the *ex vivo* chick system provides a model without an immune system in early development

making it attractive for tissue/cell xenotransplantation (Boulland *et al.*, 2010; Goldstein, 2006; Wichterle *et al.*, 2008). It is also possible to utilise transgenesis techniques in embryos, one particular example being the development of green fluorescent protein (GFP)-expressing transgenic chicks (Kwon *et al.*, 2004; McGrew *et al.*, 2004; Sang, 2004). As such, the avian model system has made significant scientific contributions, not just to the field of developmental biology, but also to immunology, genetics, virology, cancer, cell biology and tissue engineering, as reviewed by Stern (2005). The rapid development of avian skeletal tissues coupled with the ease of experimental manipulation has also seen such model systems used widely in the skeletal biology research field, in particular in the development of alternative tissue engineering strategies to augment bone repair and regeneration. This article reports on the evolution of the chick limb organ culture systems and current developments in technologies and applications of the model that are currently informing skeletal development and regeneration.

Evolution of the chick limb organ culture systems

Embryonic chick long bones and limb buds were utilised over 80 years ago by Dame Honor Fell, who pioneered the development of the 'watch-glass' method of culturing whole embryonic limb buds and long bones from chick embryos in *ex vivo* organ cultures (Fell and Robison, 1929). Isolated embryonic chick limb buds and femora were cultured in media contained inside a watch-glass, placed in a Petri dish on a layer of moist cotton wool. This in essence provided a humidified chamber and enabled analysis of the growth, development and phosphatase activity of the cultured chick limbs. This model system led to seminal observations, including the functions of bone cells and the role of exogenous factors in skeletal function. A particular attraction of this system is that both cells and matrix are maintained within their *in vivo* orientation and thus the cell-cell and cell-matrix interactions, important for maintaining the differentiated state of the cells, are preserved (Hall, 1981). An additional advantage is the ability to maintain cultures in the absence of foetal calf serum (FCS), which, indeed in organotypic cultures, tends to decrease cell proliferation, increase abnormal secondary cartilage growth and ectopic mineralisation, and can also cause migration of cells out of the whole femur onto the culture apparatus (Bingham and Raisz, 1974; Roach, 1990). The absence of undefined and batch variable FCS allows much greater chemical definition, and thus this early model system of bone development was further utilised by Fell, Mellanby and Dingle (Fell, 1969) in seminal studies to analyse the impacts of vitamin A excess (Dingle *et al.*, 1961; Fell and Mellanby, 1952), complement antiserum (Fell *et al.*, 1966) and hydrocortisone (Weissmann and Dingle, 1961) in chick limb bones.

In 1969, a system was developed in which isolated chick embryonic long bones were cultured on a stainless steel mesh within small flat-bottomed dishes (Fell and Dingle, 1969). 1.5 mL media was added to each dish, sufficient to wet the mesh surface and enable the bones to be cultured at the liquid-gas interface. The air-liquid interface has since been discovered to be important for increasing oxygen tension within the tissue, promoting higher viability and capacity for bone formation (Smith *et al.*, 2010). Using this approach, bone tissue was cultured for up to 8 d to allow the analysis of sugar endocytosis in skeletal tissues. Interestingly, the bones were rotated every 48 h, which does not occur in the modern day chick limb organ culture systems, since it is thought that the forces applied to the limb through interactions with the semi-porous membrane are important in maintaining structured, ordered bone growth (Kanczler *et al.*, 2012).

Fell and Dingle's 1969 method for organotypic culture provided, for over twenty years, a method of exploring embryonic skeletal development. This was used to demonstrate the presence of cathepsin D in femur and tibia cartilage (Poole *et al.*, 1974; Weston *et al.*, 1969), as well as to assess the effects of retinol and retinol-binding protein on skeletal development in chick limb bone rudiments (Dingle *et al.*, 1972). In 1990, the model was further developed with the work of Dr. Helmtrud Roach, who altered the cultures to incorporate a semi-porous filter paper membrane on top of the stainless steel mesh and decreased the length between media changes from 48 h to 24 h, a process shown to avoid media acidification due to waste products (Roach, 1990). This approach allowed further characterisation of many aspects of the chick bone culture system, including the growth and resorption of cartilage and bone tissue, mineralisation, the effects of FCS, and the stages of growth (and subsequent deterioration) over the course of these long-term cultures (Roach, 1992a; Roach, 1990; Roach, 1997; Roach, 1992b; Roach *et al.*, 1995) (Table 1). During the first 2 days of culture, femora recovered from tissue damage sustained during dissection, while days 3-9 of culture were identified as optimal for cell proliferation and steady growth of bone and cartilage tissue. Although femur cultures can be maintained for up to 18 d *ex vivo*, a deterioration period begins after the optimal culture period resulting in cell necrosis and tissue dissolution, and therefore a standard culture period of 8-10 d is utilised in the *ex vivo* femur cultures of today.

Chick limb organ culture systems for tissue engineering

The current chick limb organ culture system remains comparable to established models, although the stainless steel mesh of previous experiments has been replaced with cell culture inserts – plastic pre-made sterile dishes containing a semi-porous polycarbonate membrane (Fig. 1). In addition, current experiments primarily focus on femora from embryonic day 11 (E11) chicks, rather than older aged embryos, since these immature, cartilaginous femora display a high number of undifferentiated progenitor cells and thus offer far greater potential for experimental manipulation, particularly in terms of inducing formation of skeletal tissue. As indicated above, to study skeletal development an optimal standard culture period of 8-10 d is utilised. The immature embryonic day 11 femora, which are undergoing rapid growth at this

Table 1. Summary of the characterisation of the *ex vivo* chick organ culture system; adapted from (Roach, 1990; Roach, 1997; Roach *et al.*, 1995).

Test	Observation
Ex vivo bone growth and resorption	• New osteoid matrix secreted by osteoblasts in trabecular spaces and beneath the periosteum. • Multidirectional apposition rather than the unidirectional observed *in ovo*. • Lack of bone resorption (due to lack of osteoclast number and / or activity).
Ex vivo cartilage growth and resorption	• Proliferation and maturation occurred *ex vivo*, similar to observed *in ovo*. • Abnormal formation of secondary cartilage was occasionally observed, increased in presence of foetal calf serum (FCS) and in submerged non-organotypic cultures. • Some chondrocyte populations were able to 'switch' to osteogenic cells. • Cartilage resorption lost over culture period, due to loss of mononuclear phagocytes.
Ex vivo mineralisation	• Only effective way to induce mineralisation *ex vivo* was to add calcium β-glycerophosphate (but this could cause ectopic calcification).
Effects of FCS in culture	• Osteogenic and chondrogenic cells proliferated and differentiated throughout culture in serum-free media. • FCS decreased rates of [^{3}H]thymidine uptake, and increased abnormal growth of secondary cartilage and ectopic calcification. • Therefore FCS considered disadvantageous in the *ex vivo* chick model.
Ex vivo stages of growth and deterioration	Three stages: • Adaptation: Femurs adjusted to culture conditions following trauma of dissection. Lasted approximately 2 days. • Steady growth: Low tissue breakdown with occurrence of cellular proliferation and differentiation. Lasted at least 9 days. • Deterioration: Cell necrosis and tissue dissolution.

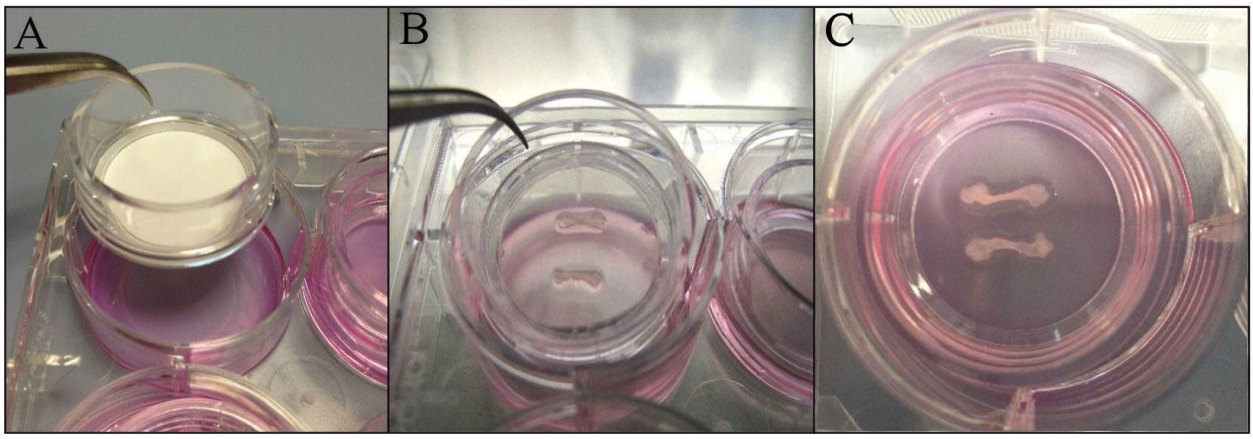

Fig. 1. Setup of the organotypic chick femur culture system. The figure shows the semi-porous (pore size 0.4 μm) polycarbonate membrane with 1 mL media beneath **(A)** onto which freshly isolated paired chick femora are placed **(B)** and cultured at the liquid-gas interface for 10 d **(C)**.

time *in ovo*, show high levels of cellular proliferation and subsequent tissue formation, and thus the culture period is sufficient to demonstrate significant formation of both bone and cartilage matrix in response to exogenous stimuli. Characterisation of the tissue and cellular behaviour in response to chondrogenic and osteogenic media components indicated this capability of the *ex vivo* femora to respond differentially to set stimuli. Osteogenic media compounds, such as dexamethasone, significantly increased bone tissue and associated markers, while chondrogenic compounds increased markers of chondrogenesis and reduced the amount of bone tissue within the femora, as assessed by micro-computed tomography (μCT) and histological/immunohistochemical analysis (Kanczler *et al.*, 2012). The use of virtual microscopy scanning technology enables detailed examination of histological sections at the macroscopic, microscopic and single cell level (Fig. 2). The responsiveness of the tissue in combination with relatively low cost (allowing for a large number of experimental repeats), the ease of experimental manipulation, and the potential in terms of the 3Rs of replacement, reduction and refinement has resulted in the wide application of the chick femur model system as detailed below.

Ex vivo chick femur applications for toxicology

Screening and toxicology testing

The capabilities of the *ex vivo* chick femur model to respond differentially and uniquely to exogenous stimuli provides an attractive model for testing growth factors and screening small molecules. The model has been used to demonstrate the negative feedback loop that exists between Indian hedgehog and parathyroid hormone-related protein

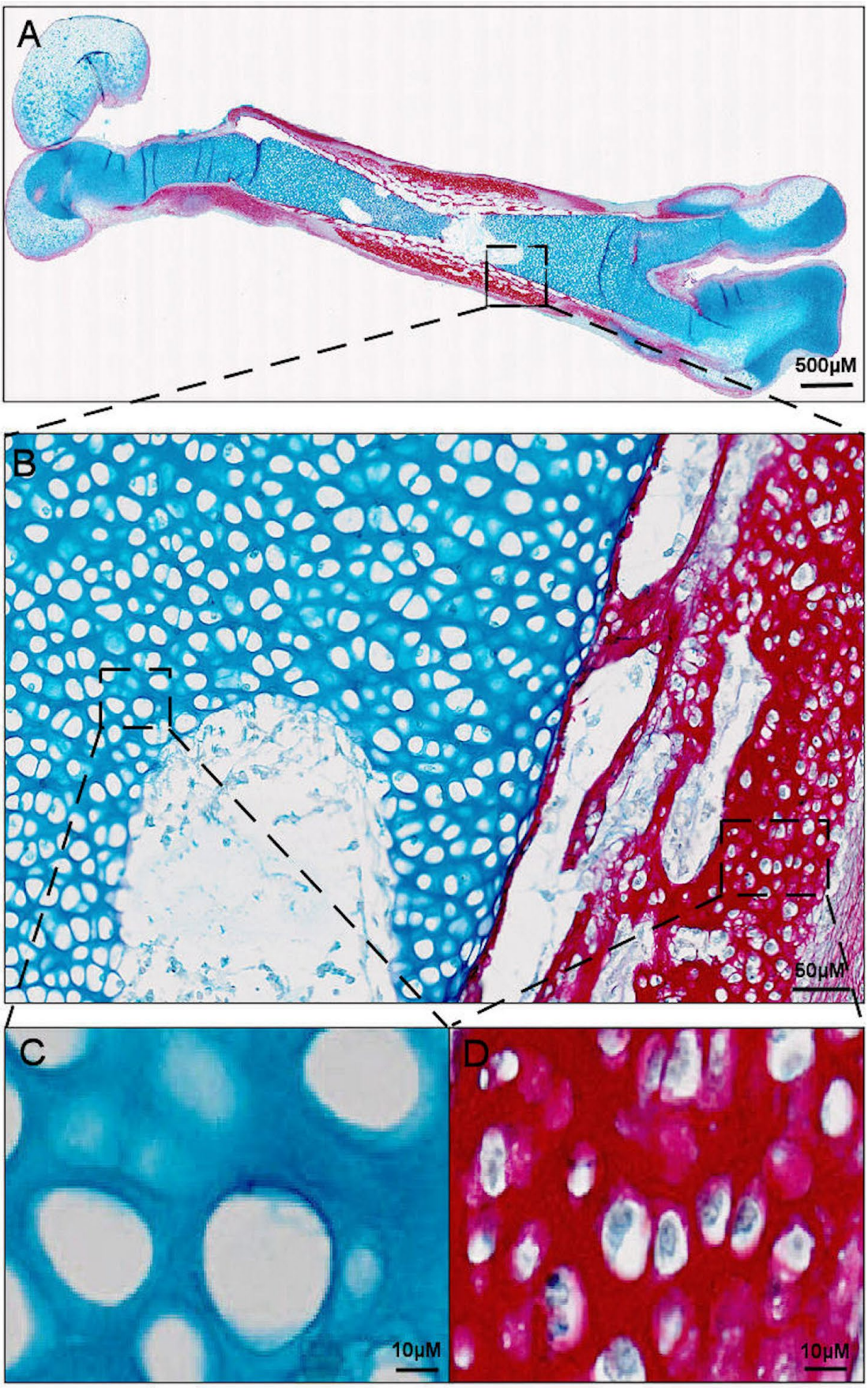

Fig. 2. Digital virtual scanning microscopy enables detailed histological analysis of chick femur tissue sections at the macroscopic, microscopic and single cell level. **(A)** Whole mount femur section. **(B)** Microscopic detail of bone, cartilage and marrow spaces. **(C)** Single chondrocyte lacunae within the hypertrophic cartilage region. **(D)** Osteocytes within the bone matrix.

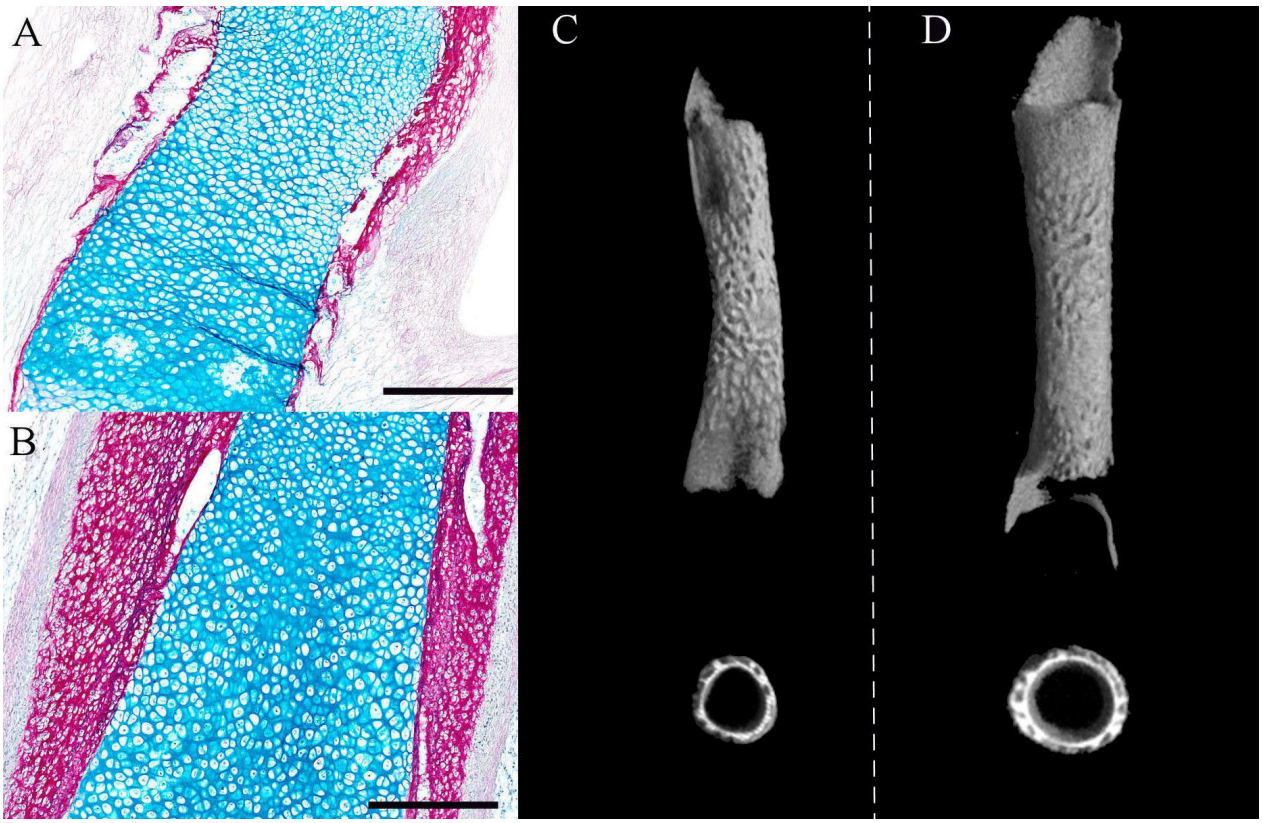

Fig. 3. Responsiveness of *ex vivo* chick femora to exogenous stimuli. **(A)** Bone matrix formation was significantly reduced in E11 chick femora cultured with 15 ng/mL TGF-β3 compared to **(B)** femora cultured in basal media alone. μCT data indicated **(C)** a shorter, thinner bone collar in TGF-β3 stimulated femora compared to **(D)** basal cultured femora. Scale bar = 200 μm.

(Minina *et al.*, 2001) and to analyse transforming growth factor (TGF) signalling in limb development (Lorda-Diez *et al.*, 2010). Recent studies have also assessed the effects of exogenous growth factors on skeletal tissue formation. Initial stimulation of *ex vivo* chick femora with exogenous growth factors alone demonstrated the chondrogenic effects of TGF-β3 (Fig. 3) (Smith *et al.*, unpublished data). There was a significant reduction in the amount of bone tissue within the femora treated with TGF-β3 compared to basal cultured controls, as assessed by μCT which demonstrated a reduction in a number of bone volume and structural parameters and histological analysis which demonstrated decreases in bone matrix and bone marker expression such as collagen type I. The data correlated with subsequent increases in markers of chondrogenesis, including collagen type II and tissue glycosaminoglycan content. In contrast to the chondrogenic effects of TGF-β3, addition of exogenous parathyroid hormone and parathyroid hormone-related protein to the femur cultures in a non-continuous manner significantly enhanced osteogenesis, with μCT demonstrating significant increases in bone tissue and histological analysis revealing increases in associated marker expression, such as collagen type I and STRO-1, together with increased cell proliferation (Smith *et al.*, 2012). The model therefore has potential to provide crucial information on appropriate growth factor application for developing and informing clinical skeletal regeneration strategies.

Developmental biology applications: microinjection of distinct cell populations

Micromanipulation and microinjection techniques have been utilised in a number of chick *in ovo* and *in vitro* models, primarily to examine and delineate mechanisms of embryonic development (Rashidi and Sottile, 2009). *In ovo* microinjection techniques were used as early as 1981 to assess the formation of embryonic chick mesonephric nephrons (Friebova-Zemanova, 1981) and, more recently, to assess formation of the precisely patterned axonal connections that are required for proper movement of the vertebrate eye (Lance-Jones *et al.*, 2012). Microinjections of specific substances, to either enhance or inhibit a particular signalling or differentiation pathway, have also been used in a number of studies. Microinjection of exogenous platelet-derived growth factor (PDGF), or a PDGF inhibitor, demonstrated the role of this growth factor in derivation of the peripheral nervous system of the head (McCabe and Bronner-Fraser, 2008). Microinjection of glycosaminoglycan-degrading enzymes enabled assessment of the role of GAGs in the morphology of the embryonic mesoblast, to demonstrate that hyaluronate is involved in preserving the mesenchymal aspect of the middle layer during lateral cell migration (Van Hoof *et al.*, 1986). Ganan *et al.* (1993) examined the microinjection of substances that induce interdigital chondrogenesis in order to assess the phenomenon of embryonic extra digit formation. Microinjection techniques have also

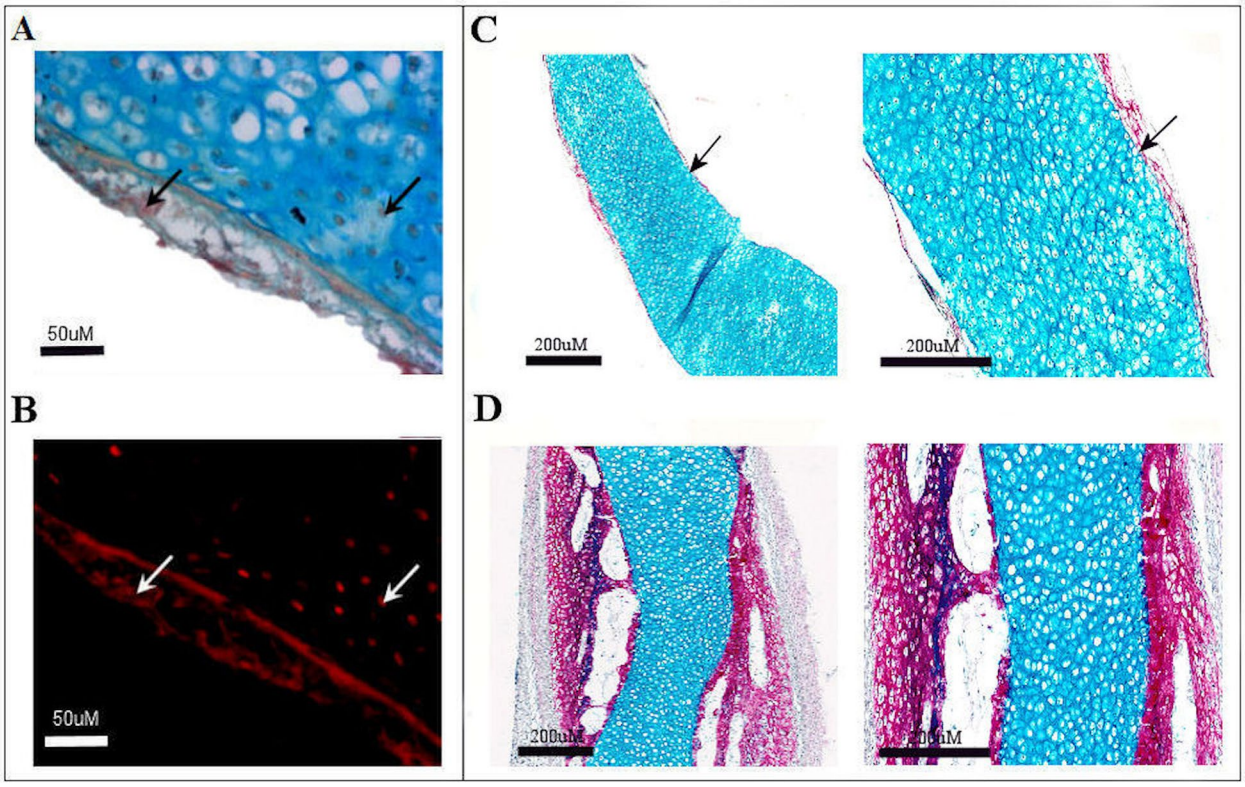

Fig. 4. Microinjection of preosteoclast cell populations into *ex vivo* chick femora. **(A)** Tartrate-resistant acid phosphatase (TRAP) activity of injected preosteoclast cells and **(B)** corresponding PKH26 fluorescent cell tracker. Arrows indicate TRAP activity and corresponding PKH26 dye. Scale bars = 50 µm. **(C)** Bone resorption in chick femora injected with preosteoclasts and stimulated with PTH compared to **(D)** femora with sham PBS injection. Arrows indicate areas of decreased bone matrix. Scale bars = 200 µm.

been combined with RNA technologies, such as injection of short hairpin RNA (shRNA) to produce targeted knockdown of specific proteins (Nagchowdhuri *et al.*, 2012), or injection of heterospecific messenger RNAs to study their translation in living cells (Stacey and Allfrey, 1976). In addition, McKinney and Kulesa (2011) used *in ovo* micromanipulation techniques to examine calcium dynamics within the neural crest – a novel application using the genetically encoded calcium indicator GCaMP3 that has the potential to elucidate mechanisms underlying complex cell migration and patterning events that occur during embryogenesis.

The facile nature of avian embryo culture and manipulation *in ovo* makes micromanipulation an attractive tool. Indeed, microinjection techniques can also be applied to the *ex vivo* femur model. The ability to inject a distinct population of cells, that would not normally reside in the femur tissue, presents a unique opportunity to assess tissue developmental processes. We have recently demonstrated that injection of chick preosteoclast cell populations (not normally present in the immature chick tissue *in ovo*) can induce significant bone resorption and modelling processes within the femur tissue, when stimulated with factors known to be involved in bone resorption and remodelling *in vivo* such as PTH and PTHrP (O'Brien *et al.*, 2008; Schlüter, 1999; Silva *et al.*, 2011) (Fig. 4) (Smith *et al.*, unpublished data). Injection of other distinct cell types enable assessment of alternative developmental processes within an *ex vivo* setting, such as injection of endothelial or inflammatory cells to assess vascularisation or inflammation, respectively. In addition, application of microinjection can be further extended to biomaterials such as, for example, scaffold microparticles which can be injected into distinct localised sites within the chick femora, in combination with cell types or exogenous growth factors. This approach enables analysis of parameters such as toxicity, cell response and tissue response to a biomaterial, alongside an internal control constituted by biomaterial-free regions of the tissue.

Understanding angiogenesis utilising chorioallantoic membrane cultures

A relatively recent modification of the *ex vivo* chick femur model has been to couple the model with the chick chorioallantoic membrane (CAM) culture system (Green *et al.*, 2004; Salem *et al.*, 2003; Takahashi *et al.*, 1991; Yang *et al.*, 2004). The CAM, an extraembryonic membrane that mediates both gas and nutrient exchanges within the chick embryo, is highly vascularised with a dense capillary network. In addition, the CAM model allows exogenous materials to be implanted without issues of material rejection, since the immune system of the chick embryo is not fully developed. As such, it has been used extensively as a model system to study angiogenesis in a variety of scientific research fields, including oncology, obstetrics, pharmacology/pharmaceuticals and even veterinary oncology. A large number of studies have utilised the CAM system as a model for angiogenesis

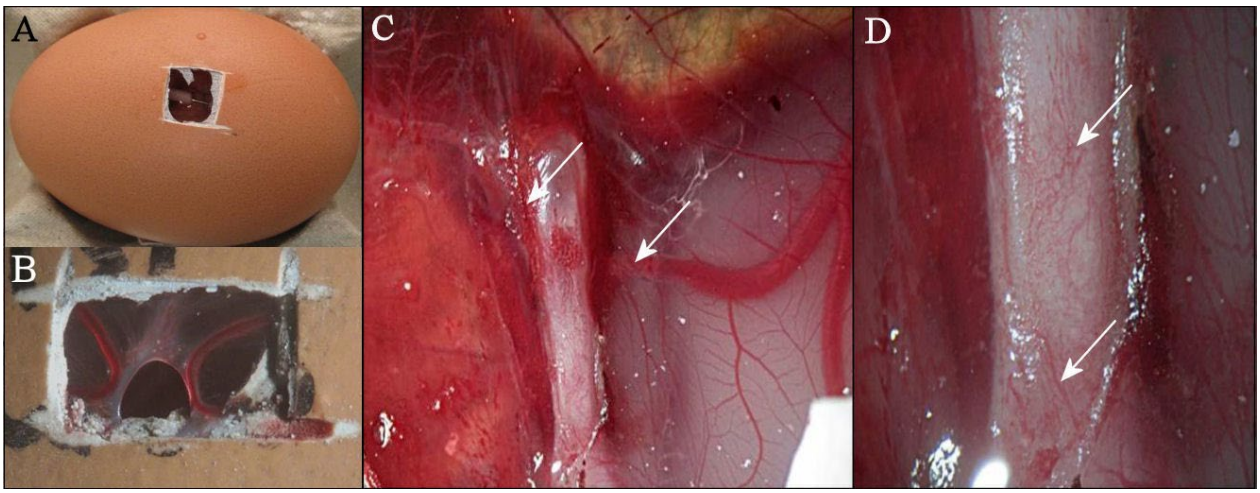

Fig. 5. Setup of the CAM culture system. **(A)** Window prepared in embryonic chick egg shell into which the *ex vivo* chick femur is placed onto the chorioallantoic membrane beneath. **(B)** Demonstration of chick membranes covering the window after 7 d of incubation in a 38 °C Hatchmaster incubator. **(C, D)** Blood vessels surrounding the chick femur (indicated by arrows) after 7 d in CAM culture.

Supplementary Video S1 (To be found on the web page of the paper). Demonstration of viable chick embryo movement and blood vessel presence during 7 d CAM culture.

and/or anti-angiogenesis in the context of cancer, for the development or testing of anti-angiogenic drugs aimed at treating certain cancers where enhanced angiogenesis is a key feature (Chen *et al.*, 2010; Ribatti, 2008a; Ribatti, 2008b; Tufan and Satiroglu-Tufan, 2005). Two such studies have examined the use of antibodies to block the angiogenic endocrine gland-related vascular endothelial growth factor (EG-VEGF), which led to disruption of the vasculature and extravasation of red blood cells (Feflea *et al.*, 2012) and the potent anti-angiogenic effects of a heparin modified endostatin (Ning *et al.*, 2012).

The CAM assay has also been utilised as a culture method and screening tool. Culturing of xenotransplanted sarcomas on the membrane surface illustrates the potential for the use of CAM as a prognostic and predictive preclinical model (Sys *et al.*, 2012), while CAM culture of ovarian cancer cells enabled assessment of their metastatic properties as well as the effects on the cells of potential therapeutic agents (Lokman *et al.*, 2012). In addition, culture of cryopreserved ovarian tissue on the CAM has been used to assess the quality of cryopreservation of ovarian tissue before cancer therapy and prior to re-implantation (Isachenko *et al.*, 2012). The CAM culture system has also been used in the veterinary field, in a novel model to derive and cultivate a feline vaccine-associated sarcoma cell line (Zabielska *et al.*, 2012).

Within skeletal tissue engineering, the CAM culture system has been widely used to assess angiogenesis, since insufficient vascularisation within bone grafts leading to a necrotic core remains a central unmet challenge for bone tissue regeneration. The CAM system has proved to be an effective method of confirming the angiogenic properties of novel scaffold materials, with or without cells or angiogenic factors, for potential application in critical size bone grafts as well as for assessing the susceptibility of these materials to vessel invasion (Baiguera *et al.*, 2012; Borges *et al.*, 2003; Kanczler *et al.*, 2007). Exemplar studies include: the testing of 45S5 Bioglass-derived glass-ceramic scaffolds for biocompatibility and bone induction (Vargas *et al.*, 2009); spheroidal osteoblast and endothelial cell cocultures for induction of perfused blood vessel networks into scaffolds (Steffens *et al.*, 2009); porous polycaprolactone (PCL) scaffolds incorporating VEGF for stimulation of angiogenesis (Singh *et al.*, 2012); and DegraPol® foam scaffolds seeded with human osteoblasts and endothelial cells for angiogenic induction (Buschmann *et al.*, 2011). The CAM model has also been used to demonstrate the potential of functionalised self-assembling peptide scaffolds, to provide microenvironments for migration of endothelial cells leading to increased angiogenesis (Liu *et al.*, 2012), and the osteoconductivity and vascularisation of an electrospun nanocomposite based on poly-lactic-co-glycolic acid and amorphous calcium phosphate nanoparticles (PLGA/a-CaP) seeded with human adipose-derived stem cells (Buschmann *et al.*, 2012).

Bio-imaging technology development and organ culture

The development of additional imaging techniques, such as magnetic resonance imaging (MRI), allow better resolution and quantification of angiogenic responses (Chesnick *et al.*, 2011). Combination of the CAM model system with the *ex vivo* chick femur model as described above, allow for longitudinal assessments of angiogenesis and vessel invasion into the femora in response to exogenous growth factors or scaffold materials using time-lapse photography (Fig. 5, Supplementary video S1 – available on the paper's webpage). This provides a closer approximation of the *in vivo* situation and enables angiogenic processes and blood vessel formation to be studied in a dynamic *ex vivo* setting, reducing the requirement for large numbers of *in vivo* experimental studies (Eder *et al.*, 2006; Falkner *et al.*, 2004).

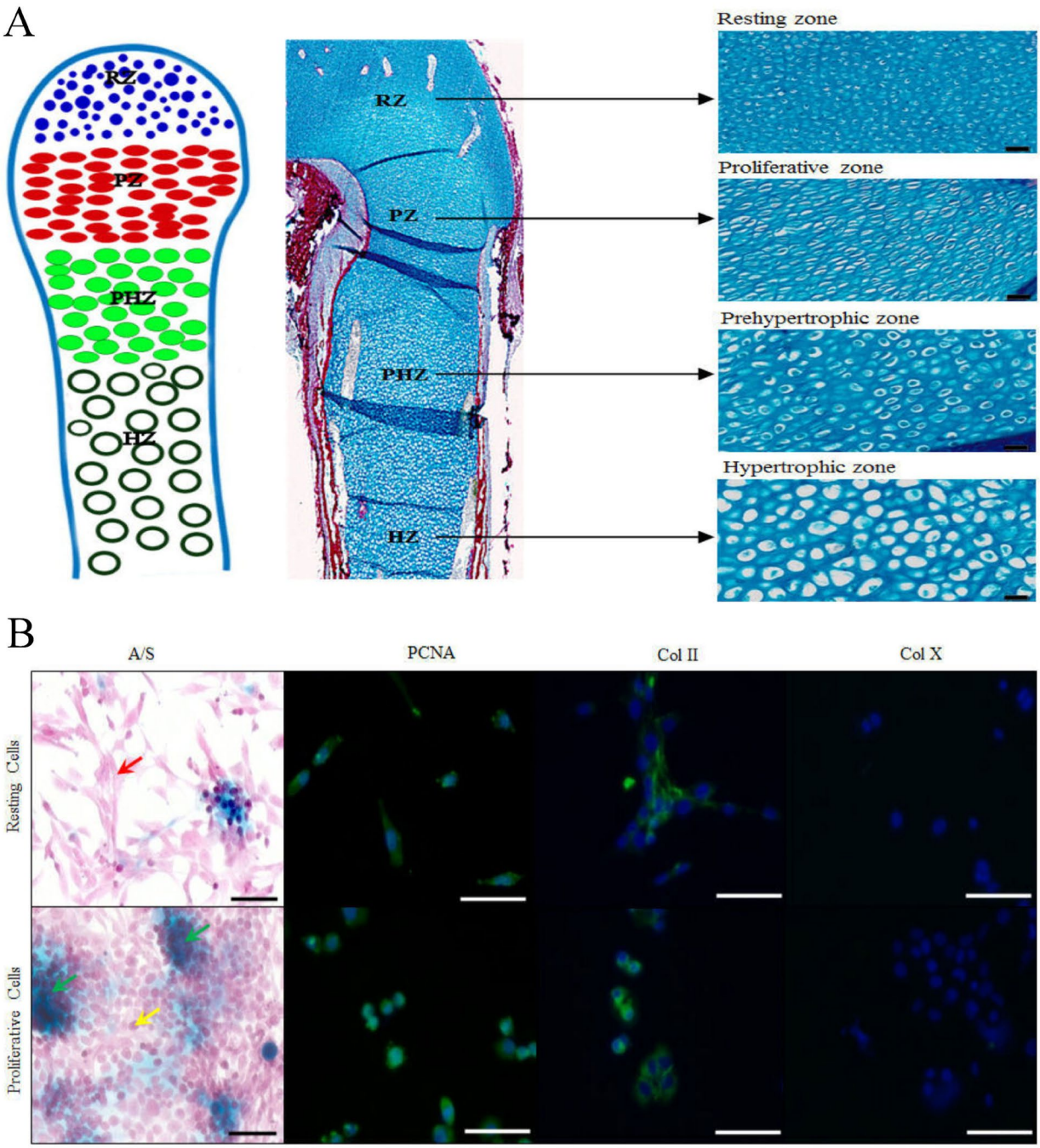

Fig. 6. (A) Representation of the embryonic day 16 avian growth plate structure consisting of resting zone (RZ), proliferative zone (PZ), prehypertrophic zone (PHZ) and hypertrophic zone (HZ) cells. Scale bar = 20 μm. **(B)** Expression profile of *in vitro* cells isolated from the resting zone (top) or proliferative zone (bottom) stained for alcian blue/Sirius red (A/S), proliferating cell nuclear antigen (PCNA), collagen type II and collagen type X. Red arrows indicate fibroblastic morphology of resting zone cells, while yellow and green arrows indicate small, round proliferative zone cells expressing material staining with alcian blue. Scale bar = 50 μm.

Mechanobiology

Assessment of mechanical forces using bioreactor technology

One obvious drawback to using an *ex vivo* model system over *in vivo* approaches for bone development and repair processes is the absence of the mechanical forces which are so crucial for bone development *in vivo*. Cells are permanently subjected to a wide variety of mechanical, chemical and electrical stimuli *in vivo* that can influence their behaviour in a variety of ways. The absence of these forces in static *in vitro* and *ex vivo* conditions can alter natural cellular behaviour and, in the context of tissue engineering, impede the development of a functional tissue (Bilodeau and Mantovani, 2006; Salgado *et al.*, 2004).

The development of bioreactor technology has enabled the simulation of *in vivo* mechanical forces in *in vitro* and *ex vivo* model environments, as well as improving the

perfusion of nutrients throughout the tissue (Bilodeau and Mantovani, 2006; Butler *et al.*, 2000; David *et al.*, 2008; El Haj and Cartmell, 2010; Mauney *et al.*, 2004; Yu *et al.*, 2004). A wide variety of bioreactor types exist that include rotating oxygen-diffusing vessels, filled with culture media, and perfusion systems incorporating controlled flow back and forth within the construct. These systems have been shown to increase cell proliferation and enhance osteoblast differentiation/osteogenesis (Botchwey *et al.*, 2001; Granet *et al.*, 1998; Kavlock and Goldstein, 2008; Milan *et al.*, 2009; Pound *et al.*, 2006; Sikavitsas *et al.*, 2003). In addition, perfusion systems can be combined with application of mechanical forces using compression bioreactors to maintain viable bone explants (El Haj *et al.*, 1990) and significantly increase osteogenesis in cell-seeded scaffolds (Bolgen *et al.*, 2008). Recent developments have seen the use of magnetic force bioreactors, which apply forces directly to the cell membrane itself, rather than the surrounding scaffold, through the use of cell-attached magnetic nanoparticles. Magnetic force bioreactors are capable of increasing expression of bone matrix proteins and enhancing osteogenic differentiation (Bock *et al.*, 2010; Cartmell *et al.*, 2002; Dobson *et al.*, 2006; Hughes *et al.*, 2007; Kanczler *et al.*, 2010). As well as mechanically stimulating scaffolds, cells and bone explants, bioreactor technologies can be adapted for use in the chick femur system, enabling application of forces across the *ex vivo* tissue to provide a closer approximation of the model to the *in vivo* environment. A recent study using a custom-made bioreactor system, developed to apply forces across the organotypic culture system, demonstrated an increase in bone growth and mineralisation within *ex vivo* chick femora that had cyclic, but not static, hydrostatic pressure applied to them (Henstock *et al.*, 2013).

Tissue Regeneration

Assessment of bone repair using a chick defect model

The *ex vivo* chick femur model can be manipulated to provide a tissue reparation model by applying defects to the bone or cartilage tissue. These could include wedge defects, drill defects and critical sized defects (Gellynck *et al.*, 2007; Green *et al.*, 2004; Salem *et al.*, 2003). The creation of such defects allows the response of cells and tissues proximal to the defect site to be assessed and the efficacy of regenerative interventions such as scaffolds, cells, growth factors etc. for facilitating the skeletal repair processes.

As discussed above, a particular strength of this model is the presence of multiple cell and tissue types that enables simultaneous analysis of the three key processes underlying skeletal repair: osteogenesis, chondrogenesis and, in combination with the CAM model, angiogenesis. For example, Salem *et al.* (2003) used a chick femur wedge defect model to demonstrate the biocompatibility of a self-assembling injectable porous scaffold that had potential uses in bone tissue engineering therapies. The same wedge defect model was also utilised to demonstrate the biocompatibility and angiogenic activity of calcium carbonate microspheres seeded with human bone marrow stromal cells (Green *et al.*, 2004). Gellynck *et al*. (2007) also demonstrated the potent osteogenic effects of the bone agonist purmorphamine, an activator of the Hedgehog pathway, by seeding chick femur drill-defects with purmorphamine-coated hydroxyapatite beads.

Application of cell populations to enhance skeletal repair in chick femora

As well as injecting exogenous populations of cells into the chick femora to examine specific processes, it is also possible to isolate endogenous cell populations from within the femora themselves. Isolated cell populations can be examined *in vitro* or, in combination with micromanipulation techniques, re-implanted into different loci within the femur tissue providing an opportunity to dissect specific mechanisms in cell behaviour, interactions and signalling. We have examined cell isolation from the growth plate regions of chick femora, specifically looking at the resting and proliferative zone regions where highly proliferative progenitor cells reside (Fig. 6A). Isolated cell**s** from these individual zones of the avian growth plate exhibit unique morphologies and protein expression (Fig. 6B). Resting zone cells exhibit a fibroblastic morphology, while cells isolated from the proliferative zone display a small, rounded phenotype and express alcian blue-staining material. Both cell types express the proliferation marker PCNA (proliferating cell nuclear antigen) and the cartilage marker collagen type II, but neither expresses collagen type X, a marker of chondrocyte hypertrophy. Furthermore, implantation of whole growth plate regions into a chick-femur defect model indicates the presence of directional cues within the growth plate that enable ordered directional bone growth *in vivo*. For example, implantation of either the resting zone region or the proliferative zone region into a central chick-femur defect initiates host tissue integration on only one side of the defect depending on the orientation of the implanted growth plate region (Fig. 7). Removal of growth plate cells from within their natural extracellular matrix disrupts this directional response with host tissue integration at both sides and a strong host periosteal induction (Smith *et al.*, unpublished data).

The chick femur model also presents an opportunity for studying mechanisms of niche development and stem cell fate. Analysis of the mesenchymal stem cell marker STRO-1 indicates a defined and ordered spatio-temporal expression pattern throughout embryonic development of the chick femur (Fig. 8). STRO-1 expression is virtually absent within immature cartilaginous embryonic day 10 femora, but begins to appear within the diaphyseal bone collar at embryonic day 11. Expression increases significantly within the developing bone and marrow spaces over the next few days of development until embryonic day 15, after which expression is again reduced and is predominantly restricted to marrow spaces.

Limitations of chick femur organ culture

Despite the many advantages of *ex vivo* chick femur organ cultures, including low cost, ease of experimental manipulation, rapid development and the presence of

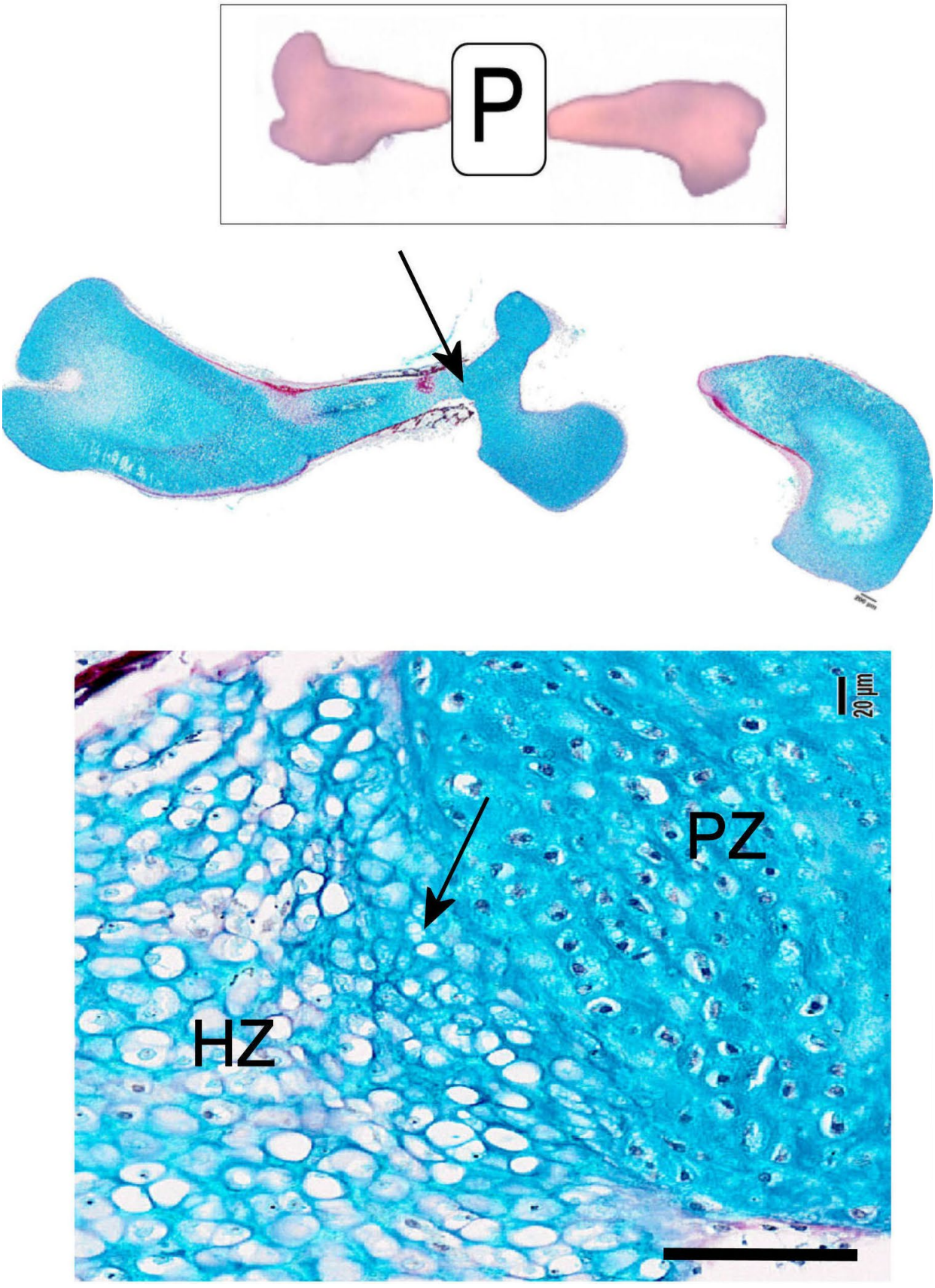

Fig. 7. Directional cues in chick growth plate regions. Implantation of proliferative zone region (P) favours integration on the left hand side of the host femur tissue, depending on orientation. HZ = hypertrophic region of host femur tissue, PZ = proliferative cells of implanted region. Arrows indicate areas of integration between implanted and host tissue. Scale bar = 100 µm.

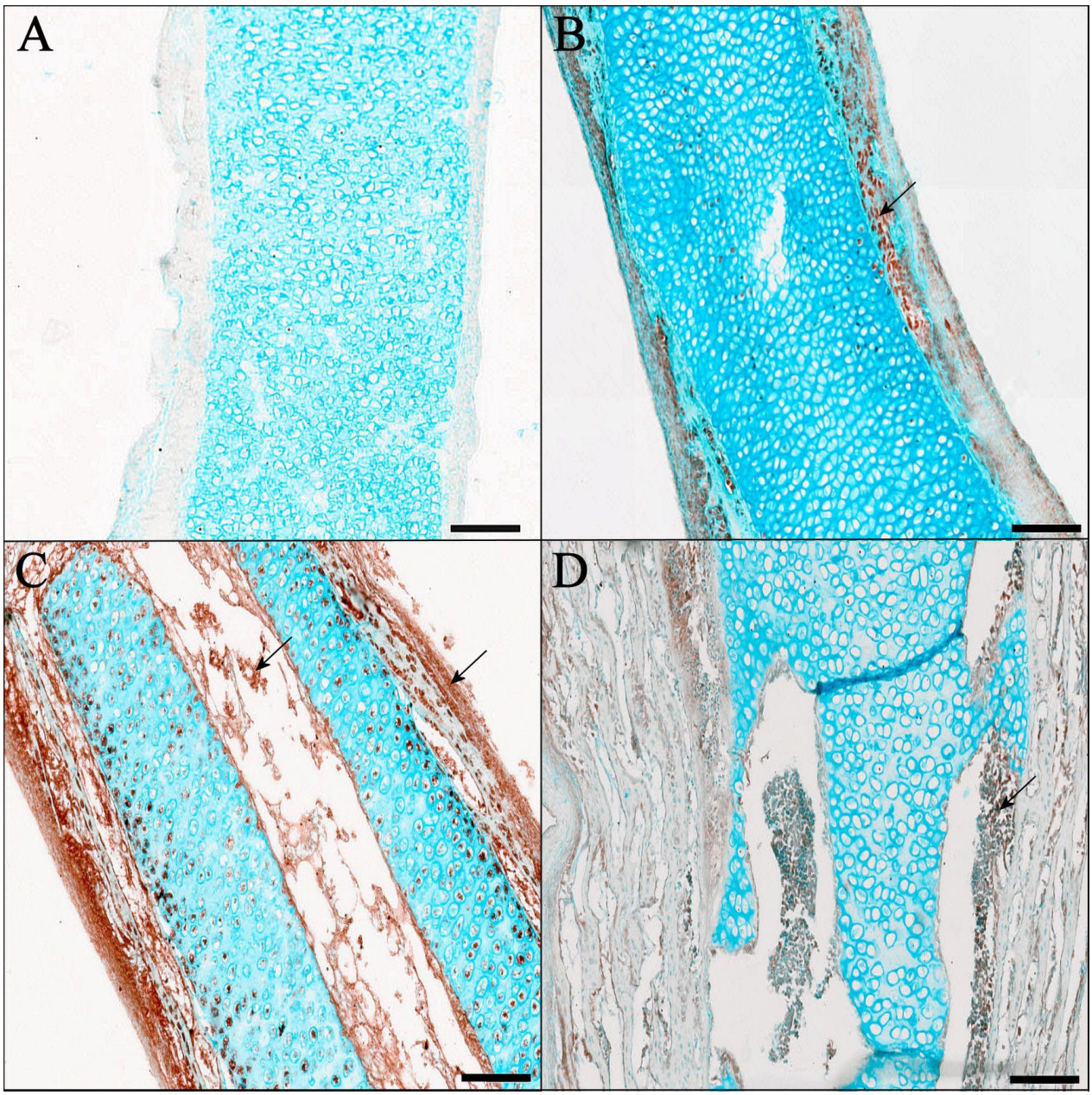

Fig. 8. Developing chick femora exhibit defined temporal and spatial expression patterns of the mesenchymal stem cell marker STRO-1. Expression of STRO-1 is absent from embryonic day 10 femora **(A)**, but begins to appear within the diaphyseal bone collar at embryonic day 11 **(B)**. Expression significantly increases within newly developing bone and marrow from embryonic day 12 to day 15 **(C)**, but decreases in later development, with the limited expression restricted to the marrow spaces **(D)**. Arrows indicate STRO-1 expression. Scale bar = 100 **µ**m.

spatially-orientated multiple cell types in their natural matrix, there are also, as with all model systems, disadvantages. The lack of a blood supply *ex vivo* remains a clear drawback, since invasion of blood vessels is critical for nutrient diffusion, cell delivery and invasion of bone tissue into hypertrophic cartilage during the processes of endochondral ossification (Dai and Rabie, 2007; Mackie *et al.*, 2008; Ortega *et al.*, 2004). However, the combination of the *ex vivo* chick femur system with the CAM model enables blood vessel invasion into the *ex vivo* tissue, thus bringing the model one-step closer to an approximation of an *in vivo* situation. In addition, the lack of a blood supply provides a unique opportunity, as it enables the study of bone/cartilage development and formation processes without the complicated and additional processes initiated by the cells and factors of the vasculature.

Further disadvantages of the chick femur model are the differences in development and growth between avian and human bone, which raises questions about the relevance of these experimental observations to clinical strategies. Specific differences include the lack of a secondary ossification centre in embryonic avian femora, until after hatching and the absence of vascularisation of the primary cartilage prior to mineralisation (Nowlan *et al.*, 2007). In mammals, the growth plate remains relatively constant in thickness due to similar rates of vascular invasion and resorption, whereas the chick growth plate markedly increases in thickness during embryonic development.

Furthermore, the chick growth-plate is not as distinctly ordered as its mammalian counterpart and specialised regions not found in mammals can be identified within the hypertrophic zone (Roach, 1997). It should be noted, however, that cellular and signalling events occurring during bone growth and development are similar across both species. There is also the possibility to extend this model system into human foetal femora to confirm the relevance of observation from the chick model in a human situation. Overall, the advantages of the chick femur model outweigh the disadvantages of the system, and it remains a useful, cheap and easily manipulated model system.

Future directions

Chick limb organ culture systems have been used in the scientific research field since the 1920s and continue to be used throughout a wide number of disciplines. Future directions for the model system include the introduction of exogenous cell populations into the chick femora, to enable the study of processes that would not normally be able to be examined in the system. For example, the microinjection of endothelial cells in combination with CAM technologies could be used to dissect the mechanisms of vascular formation and invasion in an *ex vivo* setting. Exogenous cells can be labelled with fluorescent trackers to permit assessment of cell migration within the tissue and the availability of GFP-expressing transgenic chick embryos would also allow assessment of host *versus* graft cell responses. The model also presents an opportunity for studying the mechanisms underlying niche development and stem cell fate, as demonstrated above with analysis of the spatial and temporal expression patterns of STRO-1. Utilising such approaches it is possible to employ the chick femur model to examine and delineate the processes governing stem cell differentiation and to further elucidate the identity of mesenchymal/skeletal stem cells.

Use of the chick femur as a model of bone repair, by creating critical sized defects within the tissue, offers significant potential as a testing system for novel scaffold biomaterials and for development of niche microenvironments. Such scaffolds seeded with cells and/or growth factors can be implanted into the chick femur defects and the cellular/tissue behaviour of the surrounding bone and cartilage assessed, thus enabling the study of skeletal regeneration *ex ovo*. Thus, the model serves to bridge the gap between simpler *in vitro* testing systems and complex, expensive *in vivo* models. In addition, the exciting developments of bioreactor technology provide a unique opportunity for simulating the mechanical forces experienced by skeletal tissue *in vivo*, bringing the model another step closer to mimicking the skeletal growth and regeneration processes that occur *in vivo*.

Conclusions

The *ex vivo* chick femur organotypic culture system represents a useful model with potential applications in a number of scientific fields. This approach to culturing and manipulating collective populations of cells within their natural extracellular matrix can provide crucial information on developmental and repair processes. Combination with other techniques, such as CAM systems, creation of bone defects, micromanipulation, microinjection and bioreactor technologies, further enhance the capacity of the model to recapitulate and manipulate *in vivo* processes. The chick-femur organ culture models have informed our knowledge on the complex processes of bone development and repair, and provide a high throughput, facile, inexpensive screening model for novel scaffold biomaterials free from the ethical concern surrounding *in vivo* models. Thus, the model offers significant potential as a test bed for scaffold, cell and growth factor therapies, while addressing the 3Rs of reduction, refinement and replacement and bridging the gap between facile *in vitro* cell systems and complex *in vivo* models. These many advantages of the *ex vivo* chick system, combined with recent and emerging technical developments in the field that further enhance and improve the model, suggest the embryonic chick model will continue to offer important contributions to the growing field of tissue regenerative medicine over the coming years.

Acknowledgements

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Discussion with Reviewer

Reviewer I: Influence of mechanical forces is clearly a contributing factor in natural cell function in bone development and mimicking that, through the use of bioreactors for cell cultures, is well established. It is not clear however, from the manuscript how a bioreactor design can be developed for the chick femur system. Would such a bioreactor design be the same as that used with cells/scaffolds or bone explants or would specific changes need to be made? Which type would be preferable?

Authors: A recent study by Henstock *et al*. (2013) used a custom-designed bioreactor to apply hydrostatic forces across the organotypic chick femur cultures, and indicated an increase in bone formation and mineralisation in response to cyclical, but not static, pressures. The cycling frequency was critical to inducing increased skeletal tissue formation, whereas the magnitude of the force applied did not significantly influence the amount of bone. Therefore it appears that a cycling hydrostatic bioreactor is most suited to stimulating osteogenesis in the *ex vivo* chick system.