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Ectopic implantation of juvenile osteochondral tissues recapitulates endochondral ossification

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Abstract

Subcutaneous implantation in a mouse can be used to investigate tissue maturation *in vivo*. Here, we demonstrate that this simple model can recapitulate endochondral ossification associated with native skeletal development. By histological and μ CT analysis, we investigated morphological changes of immature bovine osteochondral tissues over the course of subcutaneous implantation in immunocompromised mice for up to 10 weeks. We observed multiple similarities between the ectopic process and native endochondral ossification: (i) permanent cartilage retention in the upper zones, (ii) progressive loss of transient cartilage accompanied by bone formation at the interface, and (iii) remodeling of nascent endochondral bone into mature cancellous bone. Importantly, these processes were mediated by osteoclastogenesis and vascularization. Taken together, these findings advance our understanding of how the simple ectopic model can be used to study phenotypic changes associated with endochondral ossification of native and engineered osteochondral tissues *in vivo*.

Keywords

Endochondral ossification; Osteochondral tissue; Transient cartilage; Subchondral bone; Ectopic implantation; In vivo model; Skeletal development

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Contributions

J.N., Y.W. and G.V.N. designed research; J.N., Y.W., B.Z., and A.B. performed research; J.N., B.Z., S.B., E.G. and G.V.N. analyzed the data; J.N. and G.V.N. wrote the paper. All authors discussed the results and commented on the paper.

Competing financial interests

The authors declare no competing financial interests.

1. Introduction

The articular cartilage consists of a superficial zone that forms permanent cartilage and a transient deep zone originating from the developing epiphysis that undergoes endochondral ossification prior to skeletal maturity (Pacifci, *et al.*, 2006). During endochondral ossification, chondrocytes at the cartilage-bone interface stop proliferating, differentiate and hypertrophy before calcifying (Goldring, *et al.*, 2006). Subsequently, the calcified cartilage forms a template for bone formation by osteoprogenitors (Kronenberg, 2003). To develop a model for understanding the *in vivo* maturation of osteochondral tissues, we were interested if subcutaneous implantation in mouse can recapitulate endochondral ossification associated with native skeletal development.

Subcutaneous implantation in mouse is a model commonly used to evaluate biocompatibility and tumorigenicity (Hentze, *et al.*, 2009, VandeVord, *et al.*, 2002), and also to study the *in vivo* fate of cells in cartilage or bone (Haynesworth, *et al.*, 1992, Pelttari, *et al.*, 2006, Reinisch, *et al.*, 2015, Tsigkou, *et al.*, 2010). In this model, articular chondrocytes formed stable cartilage whereas growth plate chondrocytes underwent endochondral ossification (Dell'Accio, *et al.*, 2001, Lipman, *et al.*, 1983, Pelttari, *et al.*, 2006, Wright, *et al.*, 1985). Instead, bone marrow derived mesenchymal stem cells (MSCs) formed fibrous tissue or underwent endochondral ossification, depending on the extent of *in vitro* chondrogenic differentiation (Dell'Accio, *et al.*, 2001, Pelttari, *et al.*, 2006). Interestingly, MSCs formed cartilage that resembled a fracture callus with prolonged chondrogenic differentiation, and underwent endochondral ossification reminiscent of that seen during native long bone repair (Bahney, *et al.*, 2014, Scotti, *et al.*, 2013, Scotti, *et al.*, 2010). Such insights have motivated new strategies in bone tissue engineering for healing long bone defects (Harada, *et al.*, 2014).

Although subcutaneous implantations of cartilage and bone are well documented, few studies have yet investigated the ectopic fate of osteochondral tissues (de Vries-van Melle, *et al.*, 2014, Lolli, *et al.*, 2016). A renal capsule implantation study of mouse limb buds found that the ectopic model recapitulated endochondral ossification during fetal development (Colnot, *et al.*, 2004). However, whether subcutaneous implantation can recapitulate native endochondral ossification in postnatal osteochondral tissues remains to be determined. As an engineered osteochondral tissue typically comprises a cartilage layer and a subchondral bone layer, it resembles a postnatal osteochondral tissue more than a prenatal limb bud. Thus, insights gained from subcutaneous implantation of juvenile osteochondral tissues could motivate its use as a tool for the *in vivo* assessment of engineered osteochondral tissues.

We hypothesized that subcutaneous implantation can cause phenotypic changes in immature osteochondral tissues that recapitulate endochondral ossification associated with skeletal development. Specifically, we investigated if the following developmental features can be recapitulated: (i) the retention of permanent cartilage in the upper zone, (ii) the progressive replacement of transient cartilage by bone at the interface, and (iii) the remodeling of the nascent endochondral bone into mature cancellous bone.

To test our hypothesis, we studied the morphological changes in juvenile bovine osteochondral tissues implanted subcutaneously in immunocompromised mice for up to 10 weeks. We determined that subcutaneous implantation in mouse caused the replacement of cartilage with endochondral bone at the cartilage-bone interface and extensive endochondral bone remodeling. Furthermore, we identified osteoclastogenesis and vascularization as important mediators, and noted some interesting differences in the fate of cartilage implanted with and without subchondral bone components. Thus, we show that subcutaneous implantation in mouse recapitulates some aspects of native endochondral ossification. We propose that this simple ectopic model can be an inexpensive and accessible way to study joint maturation and the *in vivo* development of engineered osteochondral tissues

2. Materials and Methods

2.1 Sample Preparation

Juvenile bovine osteochondral explants (4 mm in diameter) were obtained from the femoral condyles of 2 month old bovine calves (Green Village Packing Company) by drilling and rinsing with sterile phosphate buffer solution (PBS). Excess subchondral bone was trimmed from the explants so that the length of the explants (cartilage and bone inclusive) was approximately 9 mm. Explants were incubated in PBS with 2% antibiotic-antimycotic at 37°C for an hour to ensure sterility. Subsequently, two identical axial sections measuring 4 mm × 1 mm × 9 mm were sectioned from each osteochondral cylinder (Fig. 1). Prior to implantation, the sections were incubated for 2 days to ensure sterility in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Inspection of the media after incubation found no signs of contamination and some consumption of the media, indicating that the explants were viable. Due to a lack of age-matched specimens from calves 4 to 10 weeks older than those used in the experiments, osteochondral tissues were obtained from skeletally mature cows (2 year old) by slicing with a band saw for histological comparison.

For comparison between pre and post implantations, one section from each explant was fixed in 10% formalin and kept for subsequent analysis while the other one was rinsed in PBS just before implantation. To investigate the role of the subchondral components, subchondral bone marrow was removed from some osteochondral tissues by sterile flushing as described previously, and cartilage explants were also prepared by removing the whole subchondral bone (Grayson, *et al.*, 2008). Cellularity in the subchondral space was determined by visual inspection of histological stains.

2.2 Subcutaneous Implantation in SCID Mice

The animal study was carried out following an IACUC approved protocol at Columbia University. Explants were implanted into subcutaneous pouches of female SCID mice (ages 8–10 weeks; Jackson Laboratory, Maine, U.S.A.). Two incisions were prepared on the backs of the mice, and a blunt forceps was used to create a pocket in the subcutaneous space inserting the explant sections. After implantation, wounds were closed with two sutures and mice were monitored daily. No signs of discomfort were observed following surgery in any

of the experimental animals throughout the study. Samples were explanted at 4 weeks and 10 weeks, fixed in 10% formalin and analyzed. All animal experiments followed federal guidelines and were conducted under a protocol approved by the Columbia University Animal Care and Use Committee.

2.3 Histologic Evaluation and Analysis

All samples were fixed in 10% formalin for 24 hours, and (except for cartilage only samples) decalcified using Immunocal (Fisher Scientific, U.S.A.). Subsequently, samples were dehydrated in ethanol, embedded in paraffin and sectioned to 5 μm thick. Sections were stained using (i) Hematoxylin and Eosin (H&E) for cell nuclei and extracellular matrix, (ii) Movat's Pentachrome for cartilage and bone, (iii) Von Kossa with Nuclear Fast Red for mineralization, or (iv) tartrate resistant acid phosphatase (TRAP) with 1% Alcian Blue (pH 1.0) for osteoclasts. All reagents unless otherwise specified were from Sigma, U.S.A.

Immunohistochemical analyses were performed using the following antibodies: type X collagen (Abcam, U.S.A.), PDGF-BB (Abcam, U.S.A.), and CD31 (BD Pharmingen, U.S.A.). Dilutions of antibodies were prepared as recommended by manufacturers (1/1000 for type X collagen, and 1/100 for PDGF-BB and CD31). Antigen retrieval was performed with pronase E (1 mg/mL) and hyaluronidase (2 mg/mL) treatment for type X collagen, and 10 mM citrate buffer (pH 6.0) for all other markers. Sections were blocked with serum in PBS and incubated with primary antibody solution overnight at 4 °C. Immunobinding was detected with biotinylated secondary antibodies using the Vectastain ABC kit and sections were counterstained with hematoxylin. All images were acquired using an Olympus FSX-100 microscope. To quantitatively compare TRAP, PDGF-BB, and CD-31 staining, corresponding images were balanced, thresholded and analyzed using ImageJ (National Institutes of Health, U.S.A.) (Fig. S3). Cells with positive expression were counted as particles in three representative frames per sample (n=4).

2.4 Micro CT imaging (μCT) and morphological analysis

All samples were fixed in 10% formalin for 24 hours at room temperature. Following fixation, the samples were rinsed in PBS. High-resolution three-dimensional (3D) images were obtained for each sample using a μCT system with a 14 μm resolution (VivaCT 40; SCANCO Medical AG, Bassersdorf, Switzerland). The grayscale images were binarized using a global thresholding technique with a lower threshold of 220 Hounsfield units. Standard morphological parameters such as the bone volume fraction (BVF) and bone mineral density (BMD) were measured for each sample using the standard morphological analysis software on the VivaCT 40 system. To obtain axial profiles of the BVF and BMD, 200 μm thick segments were evaluated across the entire axial length of the tissue samples (Fig. S2).

2.5 Individual Trabecular Segmentation (ITS)-based morphological analyses

A complete volumetric decomposition technique was applied to segment the trabecular bone network into individual trabecular plates and rods, as in our previous studies (Liu, *et al.*, 2006, Stauber and Muller, 2006). Briefly, the surface and curve skeleton of the trabecular bone microstructure were first extracted through an iterative thinning process, while

preserving the topology and morphology of the original microstructure. Then, the entire skeleton was decomposed into individual plate and rod skeletons, with each skeletal voxel uniquely identified as either a plate or a rod, based on digital topological classification, using an iterative reconstruction method.

The plate- and rod-specific morphological parameters of the trabeculae were measured as follows. Plate and rod tissue fraction (pBV/BV and rBV/BV) were calculated as the total volume of plate and rod bone volume divided by the total bone volume. The number of plates and rods was quantified by the corresponding number densities (pTb.N and rTb.N, mm⁻¹). The average size of plates and rods was quantified as the plate and rod thicknesses (pTb.Th and rTb.Th, mm), plate surface area (pTb.S, mm²), and rod length (rTb.L, mm). The intactness of the trabecular network was characterized by the plate-plate, plate-rod, and rod-rod junction density (P-P, P-R, and R-R Junc.D, mm⁻³), calculated as the total number of junctions between trabecular plates or rods per unit volume.

2.6 Statistical Analysis

The results of quantitative μ CT analysis and semi-quantitative image analysis were presented as a mean $\pm$ SEM (n=4). Statistical analysis was performed with Prism (GraphPad, U.S.A.) using the Student t-test with the Holm-Sidak correction for multiple comparisons, one-way ANOVA with Tukey post analysis, or two way ANOVA with Bonferroni post analysis ($\alpha=0.05$). The p-values were reported for statistically significant differences between the groups.

3. Results

3.1 Native osteochondral tissues undergo endochondral ossification during ectopic implantation

Native juvenile osteochondral tissues were harvested from bovine femoral condyles and implanted subcutaneously in immunocompromised mice for 4 weeks and 10 weeks; cartilage-only tissues and osteochondral tissues without marrow cells were studied as controls (Fig. 1). Following implantation, the osteochondral tissues displayed cartilage retention in the upper zone and endochondral bone formation in the deep zone, in continuity with the pre-existing subchondral bone (Fig. 2A). The replacement of cartilage by endochondral bone at the interface progressed axially from the subchondral bone towards the articular surface. Notably, there was no significant change in the total length of the graft, suggesting a lack of interstitial cartilage growth during the implantation period (Fig. 2B).

We included 4 weeks as a time point as it was commonly chosen in previous studies to evaluate engineered bone and cartilage tissues. Interestingly, most endochondral bone formation occurred over the first 4 weeks of implantation, by which time the cartilage depth decreased and the bone depth increased by approximately 1.2 mm each. In comparison, the corresponding change in the cartilage depth (decrease) and bone depth (increase) were 2 mm each over the total of 10 weeks of implantation (Fig. 2C). As bone and cartilage turnover from 4 to 10 weeks was limited in comparison to the first 4 week, we did not conduct a longer study. Morphological analysis revealed that hypertrophic chondrocytes were initially

abundant at the interface, but their presence was markedly reduced by 4 weeks following implantation and was barely visible after 10 weeks of implantation. Similarly, the expression of type X collagen at the interface was observed at day 0 and after 4 weeks but attenuated after 10 weeks of implantation (Fig. 2D). Using osteochondral tissue explanted from a skeletally mature animals (2 year old cows) as a benchmark, we found that ectopic implantation did not progress to the extent of native skeletal maturity within the time point investigated. The final cartilage thickness (~4 mm) did not reach that of a fully mature native articular cartilage (~2 mm), suggesting ectopic implantation recapitulated the early but not the final stages of endochondral ossification reminiscent of native skeletal development. High turnover of cartilage and bone over the first 4 weeks was followed by bone remodeling between 4 weeks and 10 weeks of implantation. Bone trabeculation was nascent after 4 weeks of implantation, progressing throughout the mature subchondral region, as evidenced at the 10-week time point by cancellous bone interspersed with fatty marrow spaces (Fig. 2A).

3.2 Ectopic endochondral ossification recapitulates tissue properties of juvenile subchondral bone

The newly formed endochondral bone and subchondral bone were further investigated by the Movat's pentachrome staining (Fig. 3A) and quantitative μ CT imaging (Fig. 3B), to reveal differences in bone architecture in the subchondral region. To quantify these differences, 200 μ m segments of the subchondral bone at different time points were analyzed lengthwise by μ CT. Bone in the deeper subchondral region that has been remodeled exhibited higher mineral density (MD) in the range of 700 – 800 HA/cm³ whereas bone closer to the interface had a lower MD in the range of 550 – 700 HA/cm³ (Fig. 3C). Interestingly, the newly formed endochondral bone at the interface seen at 4 weeks and 10 weeks following implantation displayed similar MD profiles to those of the native bone. Also, the MD of subchondral bone in the deeper region that originated from the pre-existing subchondral bone increased significantly at 10 weeks. Conversely, the deeper subchondral region exhibited a lower bone volume fraction (BVF = 0.1 – 0.2) relative to the interface (BVF = 0.2 – 0.3). Although the BVF profiles were similar, the newly formed endochondral bone at the interface had BVF values below those measured for the initial native bone (BVF = 0.4 – 0.55) (Fig. 3C).

In addition to standard morphological analysis, ITS-based analysis highlighted key differences between the subchondral bone before and after ectopic implantation. Whereas plates (shown in green) were initially predominant, rods (shown in red) were vastly more abundant at 10 weeks due to extensive bone remodeling (Fig. 3D). This was documented by the decreasing bone volume fraction of plates (pBV/BV = 0.76 to 0.22) and increasing volume fractions of rods (rBV/BV = 0.24 to 0.78). Similarly, the density of plates decreased from 3.79 mm⁻¹ to 2.24 mm⁻¹ while the density of rods increased from 3.32 to 4.17 mm⁻¹ (Fig. 3E). Besides substantial decrease in the density of junctions between plates and between plates and rods (P-P and P-R), the junction density between rods (R-R) also increased (from 16 to 62 mm⁻¹). Consistently, the plate surface area (pTb.S) and the trabecular thickness (pTb.Th and rTb.Th) also decreased, whereas the rod length (rTb.l) increased (Fig. S2).

3.3 Osteoclastogenesis, vascularization and inflammation mediate ectopic endochondral ossification

TRAP staining revealed a vast presence of TRAP+ cells after 4 weeks of implantation that was attenuated by 10 weeks (Fig. 4A). By comparing multiple regions across multiple samples, we determined that the number of TRAP+ cells in the subchondral bone decreased significantly by more than 70% from 4 weeks to 10 weeks following implantation (Fig. 4B). As mononuclear TRAP+ cells (pre-osteoclasts) form larger multinuclear cells (osteoclasts) during osteoclastogenesis, we counterstained TRAP with DAPI and compared pseudo-colored stains to detect increased presence of large multinuclear osteoclasts (Blumer, *et al.*, 2012). Corresponding to the greater number of TRAP+ cells, the TRAP-stained areas that are indicative of the osteoclast maturation were significantly larger at 4 weeks than at 10 weeks following implantation (Fig. 4B). Notably, the numbers of large TRAP+ cells at the interface were also higher at the 4-week than 10-week time point. Taken together, these data suggest the early osteoclastogenesis mediated greater cartilage resorption and bone turnover after 4 weeks than after 10 weeks of implantation.

We also determined that TRAP+ cells expressed PDGF-BB, in agreement with a previous study highlighting that preosteoclasts secrete PDGF-BB and thereby promote vascularization (Xie, *et al.*, 2014). Similar to the TRAP staining, large numbers of PDGF-BB+ cells were detected at the 4-week time point and only few at the 10-week time point (Fig. 4C, Fig. 4E). Correspondingly, CD-31 staining for endothelial cells revealed vascular lumens seen after 4 weeks but not after 10 weeks of implantation (Fig. 4D, Fig. 4E). Therefore, osteoclastogenesis seems to couple bone turnover to bone vascularization via PDGF-BB secretion, and mediate the early progression of endochondral ossification.

To investigate the progression and resolution of inflammation, we stained samples at 4 weeks and 10 weeks for markers associated with classically (M1) and alternatively (M2) activated macrophages, inducible nitric oxide synthase (iNOS) and arginase 1 (Arg-1) (Fig. S4). Corresponding to the greater turnover and number of TRAP+ cell, both iNOS+ and Arg-1+ cells were more abundant at 4 weeks than at 10 weeks. In particular, iNOS+ cells were seen extensively in the subchondral bone region at 4 weeks but not at 10 weeks. Arg-1+ cells were also extensive at 4 weeks, especially near the interface where deep zone cartilage was being replaced by endochondral bone. In the subchondral region, Arg-1+ cells lined the surface the trabecular bone.

3.4 Host cells populate the subchondral space and drive endochondral ossification

To decouple the roles of host and graft cells, similar implantation studies were conducted with osteochondral tissues stripped of marrow cells in the subchondral bone prior to implantation, and using cartilage-only tissues. Earlier comparisons revealed that pre-implanted tissues had residual graft-derived marrow cells in the subchondral bone, and it was unclear if these cells or the host cells were driving endochondral ossification. Thus, the cells were completely removed from the subchondral bone using saline buffer and the implantation outcomes were evaluated. Prior to implantation, it was evident that the cells, except for osteocytes, were not present in the subchondral bone. The vacant subchondral bone was infiltrated by the host cells over 4 weeks of implantation (Fig. 5A). In line with the

other data, endochondral ossification was apparent as the replacement of cartilage by bone progressed from the interface (Fig. 5B). Bone length at 4 weeks increased and cartilage length decreased by a similar amount (Fig. 5C).

Notably, the cartilage-only tissues did not undergo endochondral ossification after 4 weeks of implantation when the onset of endochondral ossification was already apparent in the other groups. Instead, the deep zone hypertrophic chondrocytes at day 0 mineralized to form a distinct zone of calcified cartilage at 4 weeks without visible bone formation (Fig. 5D). Our results suggest that host cells populate the subchondral space and drive endochondral ossification, and confirm that native cartilage is resistant to endochondral ossification despite ectopic implantation (Fig. 6).

4. Discussion

While several studies investigated the fate of cells in cartilage or bone during ectopic implantation, the present study is the first to look at phenotypic changes in immature osteochondral tissues over the course of subcutaneous implantation. Motivated by the findings from developmental studies, we hypothesized that subcutaneous implantation in mouse can recapitulate endochondral ossification associated with native skeletal development. The results we obtained support our hypothesis, as immature osteochondral tissues underwent endochondral ossification reminiscent of that seen during skeletal development and characterized by the progressive loss of cartilage and concomitant endochondral bone formation at the cartilage-bone interface. Cartilage retention was evident in the upper zone whereas all cartilage turnover occurred at the interface and progressed axially towards the surface. Instead, previous studies reported that ectopic implantation of callus-like cartilage formed from MSC resulted in bone formation at the surface and cartilage loss that progressed into the bulk of the tissue.

We also noted several interesting differences between the ectopic and the native processes. Despite the steady progression of endochondral ossification, there was no increase in the graft size, suggesting a lack of interstitial cartilage growth. During skeletal development, cartilage thickens as chondrocytes proliferate and deposit matrix prior to terminal differentiation. This process is regulated by the signaling crosstalk between multiple pathways involving bone morphogenetic protein (BMP) and Wingless (Wnt), parathyroid hormone-related peptide (PTHrP) and Indian Hedgehog (Ihh) (Goldring, *et al.*, 2006, Kronenberg, 2003). Spatial restriction of BMP signaling resulting from a band of Noggin expressing cells allows proliferating chondrocytes to differentiate under the influence of Wnt signaling (Ray, *et al.*, 2015). The feedback loop between PTHrP in the periarticular chondrocytes and Ihh in the prehypertrophic chondrocytes also tightly regulate the proliferation and differentiation of proliferating chondrocytes. Thus, the lack of cartilage growth could be due in part to the disruption of signaling crosstalk and feedback in the ectopic environment.

Similarly, the osteochondral tissue could have been subjected to supranormal oxygen tension in the vascularized subcutaneous environment across different cartilage zones, which in turn could have disrupted cartilage growth (Murphy, *et al.*, 2009). In addition, the extent of

endochondral ossification was also limited as its progression attenuated after 10 weeks of subcutaneous implantation. The limited turnover between 4 weeks and 10 weeks suggests that endochondral ossification mostly occurred by 10 weeks of implantation. Notably, the diminished presence of hypertrophic chondrocytes, as indicated by cell morphology and type X collagen expression, at the interface with prolonged implantation suggests that terminal differentiation of chondrocytes was inhibited in the absence of native gradients of trophic factors. Consequently, the final cartilage thickness did not reach that of mature native tissue. As mice are known to reach skeletal maturity around 5 months of age, it is unlikely that the skeletal maturity of the host affected morphogenesis of osteochondral grafts (Henderson, *et al.*, 2011).

There were also similarities and differences between the bone turnover during the native and ectopic processes. Morphological comparisons (by μ CT imaging and analysis) revealed that the new endochondral bone exhibited similar BVF and MD profiles as the native juvenile subchondral bone after bone remodeling. Just like the initial subchondral bone, endochondral bone at the interface had a higher BVF and a lower MD than cancellous bone in the deeper subchondral regions. These similarities suggest that endochondral bone formed during ectopic endochondral ossification was also progressively remodeled into mature cancellous bone.

Still, there were important differences in the quality of the cancellous bone and the composition of the marrow within the trabeculae. ITS-based analysis revealed that unlike the initial subchondral bone that was mostly composed of plates, the remodeled bone following subcutaneous implantation was mostly composed of rods. The prevalence of rods is reminiscent of osteoporosis and indicative of pathological bone remodeling. As mechanical loading affects bone maturation, it is likely that the lack of physiologic loading adaptation resulted in adverse bone remodeling and the formation of cancellous bone of subnormal properties (Zaidi, 2007). Trabecular bone microstructure and mechanical properties vary among different skeletal sites (Amling, *et al.*, 1996, Hildebrand, *et al.*, 1999, Lochmueller, *et al.*, 2008). We confirmed in our earlier studies such differences in trabecular plate-rod microstructures among different anatomic locations, and found their dependence on local mechanical environment (Zhou, *et al.*, 2014a). In addition, the marrow that filled the spaces between the trabeculae during later stages of implantation was fatty and not hematopoietic. Because adipocytes limit hematopoiesis and cause fatty marrow formation, it is likely that the access of cells from the adipocyte-rich hypodermis enabled fatty marrow formation and inhibited hematopoiesis (Naveiras, *et al.*, 2009, Torisawa, *et al.*, 2014).

Besides morphological comparisons, we also identified osteoclastogenesis and vascularization as important mediators of ectopic endochondral ossification. Initial progression of endochondral ossification was characterized by a vast presence of mononuclear pre-osteoclasts and mature multinuclear osteoclasts. TRAP staining was evident at 4 weeks throughout the subchondral bone, particularly at the interface where cartilage was resorbed and endochondral bone was formed. Concomitantly, vascularization was evident at site of bone formation. Attenuation of endochondral ossification at 10 weeks post-implantation correlated with the diminished presence of pre-osteoclasts, osteoclasts and vasculature. Motivated by recent studies showing that PDGF-BB couples bone formation

and angiogenesis, we studied PDGF-BB expression and found that the results mirrored those of TRAP staining (Kreja, *et al.*, 2010, Xie, *et al.*, 2014). Our data suggest that PDGF-BB mediated early endochondral ossification during ectopic implantation by coupling osteoclastogenesis with vascularization.

To investigate the role of inflammation, we stained for markers associated with different macrophage phenotypes. Macrophages are broadly classified into classically activated (M1) and alternatively activated (M2) phenotypes. M1 macrophages kill intracellular pathogens by converting arginine into nitric oxide (NO) via iNOS. Instead, M2 macrophages inhibit NO production by converting arginine into ornithine, a precursor of hydroxyproline and polyamines, via Arg-1 (Briken and Mosser, 2011). The abundant expression of iNOS and Arg-1 at 4 weeks agreed well with the extensive cartilage and bone turnover, and mirrored the results of TRAP and PDGF-BB staining. Interestingly, NO has been found to directly regulate the expression of MMP-13, a marker of endochondral ossification, during bone formation (Zaragoza, *et al.*, 2006).

By stripping osteochondral tissues of the subchondral bone marrow and comparing their implantation outcome with that of intact osteochondral tissues, we found that cells populated the subchondral space to drive endochondral ossification with or without pre-existing bone marrow. Interestingly, we also implanted cartilage-only tissues and found that they did not undergo endochondral ossification but instead mineralized in the deep zone. This suggests that the subchondral bone was necessary for the recruitment of hematopoietic and vascular cells in the ectopic environment to initiate endochondral ossification via a mineralized cartilage template. Recent developmental studies using tamoxifen-inducible genetic recombination and in situ hybridization challenged the paradigm that hypertrophic chondrocytes undergo apoptosis during endochondral ossification, by showing that they can become osteoblasts via transdifferentiation (Yang, *et al.*, 2014, Zhou, *et al.*, 2014b). Still, based on the findings from this study, we propose that the host cells populate the subchondral space and potentiate ectopic endochondral ossification as there has been no evidence that chondrocytes or osteocytes are capable of transdifferentiating into cells of hematopoietic or vascular origin.

Despite showing the usefulness of the ectopic model for investigating endochondral ossification of osteochondral tissues, the present study has limitations. For example, we did not study the expression of MMP-13. Although some of our findings are supported by mechanistic evidence from native developmental studies, we showed only correlation but not causality, which would require rigorous gain and loss of function experiments. We also did not directly compare cartilage and bone turnover in osteochondral tissues with and without bone marrow to determine if the bone marrow affected the extent of endochondral ossification. Although we showed that endochondral ossification progressed in osteochondral tissues without bone marrow but not without the subchondral bone after 4 weeks of implantation (Fig. 6), we did not investigate the full extent of endochondral ossification in the former or show the lack of endochondral ossification with prolonged implantation in the latter. Also, we did not track cell lineages and thus could not accurately define the contribution of host and graft cells towards phenotypic changes.

In summary, we demonstrate here for the first time that subcutaneous implantation of osteochondral tissues in a mouse recapitulates endochondral ossification resembling that occurring during native skeletal development. We observed the cartilage retention in the upper zone, and the formation of endochondral bone progressing from the cartilage-bone interface towards the cartilage surface. We show that the endochondral bone remodeled into mature cancellous bone resembling the native subchondral bone. However, we also noted several important differences between the ectopic and native processes, which could be attributed to the inability of the ectopic process to recapitulate the native trophic and mechanical factors. The lack of physiologic stimuli likely disrupted chondrocyte differentiation and bone maturation. Future studies could further explore these relationships using the ectopic model to gain insights into the progression of endochondral ossification. For example, the reversibility of the adverse subchondral bone remodeling from plate to rod morphology can be probed by administering the mice with bisphosphonates. In addition, *in situ* hybridization studies could clarify the origin and fate of different cell types during ectopic endochondral ossification of osteochondral tissues. Whether the lack of cartilage growth is a result of the disruption of BMP/Noggin/Wnt crosstalk and Ihh/PTHrP feedback requires a deeper mechanistic investigation that is outside of the scope of this study. With further mechanistic understanding, the utility of this model can also be extended towards the evaluation of engineered osteochondral tissues *in vivo* prior to their orthotopic implantation in large animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental design

4mm diameter cylindrical cores (approximately 9 mm in length) were harvested from juvenile (2 month old) bovine femoral condyles and sectioned in the axial direction into 1 mm slices. One slice from each core was fixed for analysis and one slice was implanted subcutaneously in immunocompromised mouse for 4 weeks or 10 weeks.

Figure 2. Juvenile osteochondral tissues undergo endochondral ossification during ectopic implantation

(A) Representative H&E stains of osteochondral sections at day 0, and after 4 weeks and 10 weeks of subcutaneous implantation, and of a skeletally mature cow (2 year old). Articular cartilage and subchondral bone are denoted as AC and SB. Deep zone cartilage was replaced by endochondral bone at the interface. Trabeculation was nascent in the subchondral bone at 4 weeks and well established at 10 weeks. Scale bar: 500 μ m. (B) Length of cartilage determined from representative histologies, and length of bone determined by μ CT analysis, showing osteochondral tissue composition before and after implantation. Cartilage length decreased and bone length increased apparently by 4 weeks, and significantly at 10 weeks, respectively ($n=4$). (C) Representative photographs of whole tissue sections at day 0, after 4 weeks and 10 weeks of implantation. Scale bar: 1 mm (D) Representative type X collagen stains of osteochondral sections at day 0, after 4 weeks and 10 weeks of implantation. Hypertrophic chondrocytes (indicated by red arrowheads) were observed initially at the interface, but diminished over time. Scale bar: 200 μ m.

Figure 3. Newly formed endochondral bone recapitulates native subchondral bone, and undergoes extensive remodeling

(A) Representative Movat's pentachrome stains (scale bar: 500 μm) and (B) μCT images (scale bar: 1 mm) of osteochondral sections at day 0, and after 4 weeks and 10 weeks of implantation. Both assays document the formation of new endochondral bone remodeling into subchondral bone. (C) Spatial profiles of the mineral density (MD) and bone volume fraction (BVF) of the subchondral bone at day 0, after 4 weeks and 10 weeks of implantation. The 4 weeks group is shown in a lighter shade for ease of comparison between day 0 and 10 weeks. Newly formed bone at the interface exhibited higher BVF and lower MD than native bone, whereas the bone in the deeper region exhibited significantly higher MD and lower BVF after remodeling ($*p<0.05$; $**p<0.01$; $n=4$). Schematics showing the profiles of MD (increasing from interface to subchondral region) and BVF (decreasing from interface to subchondral region). (D) Visualization of plates (green) and rods (red) identified from μCT images of osteochondral tissues at day 0 and 10 weeks. Plates were predominant at day 0, whereas rods were predominant at 10 weeks. (E) ITS-based analysis showed significant decrease in the plate volume and density, increase in the rod volume and density, and increase in the rod-rod junction density ($****p<0.001$; $n=4$).

Figure 4. Ectopic endochondral ossification is mediated by osteoclastogenesis and vascularization

(A) Representative stains of TRAP with Alcian Blue of osteochondral sections at day 0, and after 4 weeks and 10 weeks of implantation. Magnified images with pseudo-colored nuclear counterstains (DAPI) are shown in the right panels. TRAP⁺ cells were more abundant at 4 weeks than at 10 weeks following implantation. Large, multi-nuclear TRAP⁺ cells (indicated by red arrowheads) seen at 4 weeks indicate the presence of mature osteoclasts mediating cartilage resorption and bone remodeling. Scale bars: 200 μm (main images) and 50 μm (magnified images). (B) The number of TRAP⁺ cells and average stain area of TRAP⁺ cells were significantly higher at 4 weeks than at 10 weeks following implantation ($n=4$). (C) Representative PDGF-BB stains of osteochondral sections at day 0, and after 4 weeks and 10 weeks following implantation. Magnified images are shown in the right panels. PDGF⁺ cells were seen at 4 weeks and 10 weeks following implantation, and were more abundant at 4 weeks. Scale bars: 200 μm (main images) and 50 μm (magnified images). (D) Representative CD-31 stains of osteochondral sections at 4 weeks and 10 weeks. Vascular lumens (indicated by red arrowheads) were seen at 4 weeks but not 10 weeks following implantation. Scale bar: 50 μm . (E) The numbers of PDGF-BB⁺ cells and CD-31⁺ area were significantly greater at 4 weeks than at 10 weeks ($n=4$).

Figure 5. Host cells populate the subchondral bone and drive endochondral ossification

(A) Representative H&E stains of osteochondral sections at day 0 and at 4 weeks following implantation. Scale bar: 500 μ m. Sections were stripped of bone marrow (BM) cells in the subchondral bone prior to implantation by sterile rinsing. Magnified images are shown in the right panels. Scale bar: 200 μ m. The subchondral bone was densely populated with cells at 4 weeks, indicating host involvement. (B) Representative μ CT images of osteochondral sections at day 0 and at 4 weeks following implantation showing new endochondral bone formation and subchondral bone remodeling. (C) The lengths of cartilage (determined from representative histologies) and bone (determined by μ CT analysis) showing osteochondral tissue composition before and after implantations. Cartilage length decreased and bone length increased markedly by 4 weeks following implantation ($n=4$). (D) Implantation outcomes of cartilage-only grafts. H&E and Von Kossa stains at day 0 and after 4 weeks of implantation. Hypertrophic chondrocytes mineralized during implantation without visible endochondral bone formation. Scale bar: 200 μ m.

Figure 6. Summary of ectopic implantation outcomes

Schematics illustrating that osteochondral tissues comprising articular cartilage (AC) and subchondral bone (SB) with or without bone marrow (BM) underwent endochondral ossification (EO) characterized by the replacement of cartilage by bone at the interface. Instead, tissues without subchondral bone (full-thickness cartilage) mineralized in the deep zone but did not undergo endochondral ossification at 4-week time point.