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Earliest Phases of Chondrogenesis are Dependent upon Angiogenesis during Ectopic Bone Formation in Mice

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Abstract

Endochondral ossification is the process where cartilage forms prior to ossification and in which new bone forms during both fracture healing and ectopic bone formation. Transitioning to ossification is a highly coordinated process between hypertrophic chondrocytes, vascular endothelial cells, osteoblasts and osteoclasts. A critical biological process that is central to the interactions of these various cell types is angiogenesis. Although it is well established that angiogenesis is crucial for fracture repair, less is known pertaining to the role of angiogenesis in ectopic bone formation. Furthermore, fracture repair models are complicated by extensive trauma, subsequent inflammatory responses and concurrent repair processes in multiple tissues. In order to more definitively characterize the relationship between angiogenesis and postnatal endochondral ossification, a model of ectopic bone formation was used. Human demineralized bone matrix (DBM) was implanted in immune-deficient mice (rag null (B6.129S7-Rag1^{tm1/MOM/J})) to induce ectopic bone. Inhibition of angiogenesis with either a small molecule (TNP-470) or a targeted biological (Vascular Endothelial Growth Factor Receptor type 2 [VEGFR2] blocking antibody) prevented ectopic bone formation by 83% and 77%, respectively. Most striking was that the progression of chondrogenesis was halted during very early phases of chondrocyte differentiation between condensation and prehypertrophy (TNP-470) or the proliferative phase (VEGFR2 blockade) prior to hypertrophy, while osteoclast recruitment and resorption were almost completely inhibited. Our results demonstrate angiogenesis plays a developmental role in endochondral bone formation at a much earlier phase of chondrogenesis than suggested by prior findings.

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Keywords

Developmental modeling; Chondrocytes; Cartilage Biology; Endochondral Ossification; Angiogenesis

1. Introduction

Endochondral ossification is the process by which the appendicular and axial skeleton develops during embryogenesis, and by which fracture healing and the formation of ectopic bone occur postnatally. Mesenchymal progenitor cells are recruited to the site of the future bone formation followed by their commitment to the osteo/chondro lineage marked by an increase *Sox9* expression [1–3]. Following recruitment and commitment, these cells condense into the morphological structure defining the template for eventual bone formation. At this stage, these cells are characterized by the upregulation of cell adhesion molecules such as *N-cadherin* [4]. Chondrogenesis then initiates and progresses to hypertrophic maturation of chondrocytes and cartilage mineralization. As ossification begins, the hypertrophic chondrocytes express high levels of vascular endothelial growth factor A (*VEGF-A*) leading to an increase in the morphogenesis of new vessels and their invasion into the cartilage template. As vessel formation proceeds some chondrocytes undergo apoptosis while other hypertrophic chondrocytes transdifferentiate and take on osteoblastic features. As vascular formation continues osteoclasts are recruited to assist with the removal of the mineralized cartilage and a new round of stem cells and osteoprogenitors are recruited that construct the mineralized bone. In this context it has generally been thought that the primary angiogenic signals come from hypertrophic chondrocytes and that vascular morphogenesis occurs concurrent with bone with signals from vascular cells primarily supportive of osteogenic development [5–9].

Angiogenesis is also a critical component of postnatal healing after injury or surgery. During the progression of fracture repair in mice, angiogenesis occurs early with increasing vessel density that peaks around day 7 post fracture with angiogenic factors being expressed during cartilage formation and the resorption phase of repair [10,11]. Inhibitors of angiogenesis having different mechanisms of actions have been used in multiple studies of bone repair [12–15]. The most commonly used of these inhibitors has been small molecule TNP-470. This compound inhibits methionine aminopeptidase and blocks endothelial cell proliferation [16,17]. The use of this drug showed that inhibition of angiogenesis resulted in deficiencies in the formation of woven bone, callus tissue formation, ectopic bone, and intramembranous ossification [13–15,18]. Another approach has been to use blocking antibodies that specifically target VEGFR2 (DC101) which blocks endothelial proliferation as well as endothelial migration [19]. Using this approach was shown to also inhibit angiogenesis and bone formation during distraction osteogenesis [20].

Various experiments have established that angiogenesis is crucial for both endochondral and intramembranous ossification during fracture repair. However unraveling the temporal sequences and signaling processes among the various cell types and tissues that contribute to fracture healing is complicated by the extensive inflammatory response and trauma that

occurs within the marrow and vascular tissues of the bone and surrounding muscle. The concurrent repair processes that are progressing in these multiple tissues at the site of injury makes it difficult to assess the exact role(s) that angiogenesis mediates in just the processes of skeletal cell development of the endochondral bone formation processes.

Therefore, in order to elucidate the functional role that angiogenesis plays in postnatal *de novo* endochondral bone formation ectopic bone formation was examined, since it provides a more simplified experimental format to model the interactions of the various cell types that contribute to endochondral ossification. This model largely removes contributions of the marrow, leads to the exclusive recruitment and induction of osteo/chondro stem cells with presumably no contribution of osteoprogenitors, and causes limited trauma to the surrounding vascular and muscular tissues compared to fracture models. Two different inhibitors described above were used to block angiogenesis. Comparing two independent mechanisms of angiogenesis inhibition allowed for the identification of common effects of the inhibition of angiogenesis process separate from inhibitor specific effects. The use of the Rag null mice (B6.129S7-Rag1^{tm1/MOM}/J) immune-deficient mice allowed for the use of human-derived DBM to induce ectopic bone formation Using the Rag null mice also eliminated the role that the adaptive immune activity contributes towards the endochondral bone formation [21], while leaving most of the components of the innate immune responses intact.

2. Materials and Methods

2.1 Materials

Human demineralized bone matrix (DBM) (Grafton® Putty) was provided by Medtronic Inc (Minneapolis, MN). The VEGF receptor blocking antibody, DC101, was from BioXCell (West Lebanon, NH). TNP-470 was purchased from EMD Chemicals, Inc (Gibbstown, NJ). The PECAM-1 goat polyclonal antibody and BMP2 was from R&D Systems (Minneapolis, MN) and was reconstituted by following the manufacture's protocol. Primary Sox9 goat polyclonal antibody was from Santa Cruz Biotechnology. All other reagents were Thermo Fisher Scientific (Suwanee, GA) unless noted.

2.2 Animal Studies

All animal studies were approved by the Institutional Animal Care and Use Committee at Boston University. Mice were housed under standard conditions. Studies were carried out in 10- to 13-week-old male immune-deficient mice, B6.129S7-Rag1^{tm1/MOM}/J (Rag) from Jackson Laboratories (Stock number 002216, Bar Harbor, ME).

2.3 Ectopic Bone Formation Model Assessment

Two models of ectopic bone induction, DBM or an absorbable gelatin sponge (Gelfoam 09-0353-01, Pfizer) with varying amounts of BMP2 (0–0.5µg) were initially assessed by implanting them on the femoral periosteal surface (figure 1A–B). By post-operative day 16, implanted DBM induced condensed ectopic bone. At the same time point, the gelatin sponge alone and sponge with 0.1µg of BMP2 did not induce ectopic bone that was visible with plain film radiography. Ectopic bone was detected; however, with the implanted sponge with

0.3µg and 0.5µg of BMP2. Like the sponge model the DBM-induced ectopic bone was dependent upon BMP signaling. This was demonstrated by the dose response decrease in DBM-induced ectopic bone due to the BMP antagonist, Noggin (figure 1A).

A further comparison of ectopic bone formation was made at a site away from the femur, within the muscle of the upper limb with both DBM and sponge supplemented with 0.5 µg of BMP2 (figure 1C). The DBM supplemented with BMP2 induced more ectopic bone compared to the sponge supplemented with BMP2 indicating synergy between BMP2 and DBM. Implantation of DBM alone at the muscle location did not induce ectopic bone (data not shown). Even at higher amounts of BMP2 (5 µg), the sponge did not induce amounts of ectopic bone that were comparable to the DBM. Most interestingly while the BMP2 collagen sponges induced ectopic bone that showed a volume encapsulated in a shell of bone, DBM induced volumes that showed considerable trabecular bone formation within the interior of encapsulated volume as shown by the cross sectional view of the micro-computed tomography (micro-CT).

This initial analysis led us to choose the model of DBM-induced ectopic bone at the femoral surface for all of the subsequent analysis in this paper. This choice was based on the findings that the model induced the greatest amounts of mineralized tissues and that the formed tissues had both a surrounding cortical shell and developed an internal trabecular structure. The periosteal implant site was further selected for our studies since it contains the stem cell population that is most relevant in the context of clinical bone repair.

2.4 Animal Enrollments

Animals were randomly assigned to time point and treatment group. An overview of animal enrollments is shown in figure 2. A non-operated group served as a reference naïve group for mRNA gene expression (n=5). To characterize the temporal progression of DBM-induced ectopic bone formation, animals were enrolled for post-operative days (POD) 2, 4, and 8. These animals received DBM implant on the left femur. Animals enrolled for POD 12, 16, 24, and 31 received bilateral implants, and upon harvest the left implant (femur and soft tissue was removed) was isolated for mRNA gene expression analysis and the right implant (soft and hard tissue left intact) was used for micro-CT followed by histological analysis. The angiogenic studies focused on two time points, POD 8 (chondrogenesis) and POD 16 (osteogenesis). The TNP-470-treated animals received bilateral DBM implants for both day 8 and 16 time points. No adverse effects were observed with treatments. The DC101-treated animals enrolled for day 8 received unilateral DBM implant while animals enrolled for day 16 received bilateral DBM implants. An additional day-8 control group was enrolled which received unilateral DBM implant for histological analysis to compare to the blockade of angiogenesis. For micro-CT imaging of vessels, all mice were enrolled for time point day 8 and received unilateral DBM implant. Euthanasia of the mice was by CO₂ asphyxiation followed by cervical dislocation.

2.5 Surgical Implantation of DBM and Sponge

All mice received a subcutaneous injection of Buprenorphine and Baytril at time of surgery and continued for the following 48 hours.

2.5.1 Femoral Surface Implant—The femur was exposed by making an approximately 1.5-cm skin incision longitudinally along the axis of the femur, extending from the greater trochanter toward the knee joint. The femur was exposed by cutting along the fascial plane and bluntly separating the muscle from the femur. Fifty milligrams (approximately 0.04 cm³) of human DBM was then surgically implanted by flanking the periosteal surface of the mid-diaphyseal region of the femur. Where indicated, Noggin was added to the DBM right before surgery. The sponge implants consisted of cutting the gelatin sponge to be one centimeter squared followed by wetting the material with sterile PBS or sterile PBS with BMP2 (0.1 – 0.5µg). The sponge was then implanted similarly as the DBM. The facial plane was sutured with 6–0 plain gut resorbable sutures (ACE Surgical Supply Co, INC, Brockton MA) followed by the suturing of the skin with 5–0 plain gut resorbable sutures.

2.5.2 Muscle Implant—Similar to above, the skin and facial plane was cut to expose the muscle of the upper thigh. A pouch was created between the medial gluteal and femoral quadriceps muscle and the DBM or sponge was then placed within the muscle location. The BMP2 was added to either DBM or sponge right before surgery. The facial plane and skin was sutured as previously described.

2.6 Inhibition of angiogenesis

TNP-470 was dissolved in ethanol that had been diluted to 10% stock solution in PBS [13]. The TNP-470 (25mg/kg) solution was administered by subcutaneous injections daily, beginning within three hours post-surgery. DC101 (350µl at 1.5mg/ml) was intraperitoneally injected following surgery (within three hours post-surgery) for every other day until euthanasia [20]. Animals did not receive an injection on day of euthanasia.

2.7 Radiological Analysis

2.7.1 Plain Film Radiography—Plain-film radiographs were taken of the entire limb containing the implant at the time of euthanasia using a Faxitron at 30 kv for 40 seconds, magnification 3X (Tucson AZ).

2.7.2 Micro-CT—Soft tissues were left intact surrounding the femur/implant for micro-CT. Samples were fixed with 4% paraformaldehyde for one week followed by storage at 4°C in PBS. Samples were scanned at a nominal resolution of 20 µm/voxel, voltage 70 kVp, current 114 µA, and integration time 200 ms using a desktop micro-CT system (micro-CT40; Scanco Medical, Brüttisellen, Switzerland). Scans included the entire volume of the mineralized implant and the volume containing the implant was segmented from the femur. Global thresholding was used with thresholds determined by an iterative method (Scanco Medical). It was determined that the average threshold was 597 mg HA/ccm and it was applied to all samples.

2.7.3 Contrast-Enhanced Imaging of Vascular Tissues—Following euthanasia, the vasculature was perfused in a similar manner as Mastsubara et al substituting a barium/sulfate/gelatin mixture as the contrast agent for the Microfil [22,23]. The barium sulfate/gelatin perfusate consisted of a mixture of 20% w/v gelatin/PBS solution and 250% w/v barium sulfate (98% w/w, E-Z-EM Canada Inc). The two solutions were heated (~100°C) in

a water bath for up to one hour prior to use. The final concentration of the perfusate was 12% w/v gelatin and 100% w/v barium sulfate which was made by appropriately diluting the two solitons with each other. The perfusate was then allowed to cool briefly prior to perfusion. The vasculature was first perfused with 10ml of 10% buffered formalin followed by approximately 3ml of barium sulfate (250% w/v) and then approximately 9ml of the barium sulfate/gelatin perfusate. The hind limbs were then removed and stored at 4°C overnight to allow the perfusate to form a vascular cast. Samples were fixed again in 10% buffered formalin for three days after which the samples were stored in PBS at 4°C.

Perfused samples were embedded in 2% agarose/PBS and imaged with μ CT40 (μ CT40; Scanco Medical) both before and after decalcification (14% EDTA at 4°C). The nominal resolution of the scans was 10 μ m/voxel, voltage 70 kVp, current 114 μ A, and integration time 300 ms. The post-decalcified images were registered (Bookstein algorithm, Amira 5.2, Visage Imaging) against the corresponding pre-decalcified images in order to recover the original anatomical position of the vessels and to identify the region of interest (ROI) [24]. ROI was first defined using controls and determined to start at the mid third trochanter and continued distally for 300 slices (3mm). Component labeling was performed to remove small islands of perfusate followed by vessel volume and thickness quantification using Scanco.

2.8 Histology

After completion of the micro-CT scans, samples were decalcified in 14% w/v EDTA at 4°C. Samples were embedded in paraffin and 7.5- μ m-thick sections were cut across the sample. Sections were stained with Fast Green and Safranin O or stained for tartrate-resistant acid phosphatase (TRAP) positive cells. Immunohistochemistry was performed on slides of paraffin-embedded, 7.5 mm-thick sections by standard protocol on an IntelliPATH Automated Slide Staining System from Biocare Medical. Briefly, the slides were heated for 15 minutes at 60°C oven followed by deparaffinization starting with xylene and rehydrated through grading of alcohol to distilled water. Slides were incubated with Biocare Peroxidase 1 solution for 10 minutes at RT, washed with TBST, then were retrieved via trypsin digestion for 15 min. The samples were blocked with Biocare Rodent Block M for 30 minutes, and washed. Primary antibody Sox 9 goat polyclonal (1:100) and PECAM-1 goat polyclonal (1:100) was diluted in Biocare Da Vinci Green Diluent and incubated for 2 hours at RT followed by washing in TBST. Incubation in Goat Probe and Goat HRP Polymer was then performed for 30 minutes followed by washing in TBST. DAB was diluted in DAB substrate buffer and applied to slide for 5 minutes followed by washing in DI-H₂O. A light hematoxylin stain was applied, followed by dehydration, air drying, and mounting with EcoMount with a coverslip. Microscopy was carried out on an Olympus BX51 light microscope using the cellSens Dimension software, Olympus Corp. Histology controls were performed following the same procedure with the exception that the primary antibody was absent (data not shown). For the DBM implant control and TNP-470 treated animals no background was observed at the implant site or growth plate. However, the DC101 treated samples did show high levels of background and positive regions could not be determined from background.

2.9 Quantitative real time PCR

For mRNA analysis the implant was separately collected, flash frozen, and stored at -80°C. Total RNA was extracted and quantified [20]. Two micrograms of total RNA were used for synthesis of cDNA using random hexamer priming. 18S rRNA was used for normalization of each target sequence. All qPCR reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Calsbad CA): Hot-Start activation at 95 °C for 2 min, 40 cycles of denaturation (95°C for 15 sec) and annealing/extension (55°C for 60 seconds). The cDNA from individual implants were analyzed in duplicate. Each plate contained the non-operated naïve group. Primers were from Applied Biosystems and labeled with FAM. Relative gene expression was determined using the Ct method [20].

2.10 Statistics

Statistical analyses were performed with Prism 5 (GraphPad Software Inc) and JMP10. Means are graphed and standard error of means are represented by error bars unless noted otherwise. One-factor ANOVA followed by Tukey or Dunnett test was used to analyze data. A significance level of 0.05 was used for all statistical analyses.

3. Results

3.1 DBM-induced Ectopic Bone Progresses through Endochondral Bone Formation

A model for DBM-induced ectopic bone formation was established by implanting human DBM onto the femoral periosteal surface of the immune-deficient Rag mice and confirming that well-developed mineralized tissue formed at the implant site (Figure 3). The progress of the ectopic bone development was followed by sequential radiographic and histological analysis at post-operative days (POD) 2, 4, 8, 12, 16, 24, and 31. POD 12 was the earliest time point at which mineralized tissue was observed. Therefore time points after day 12 were chosen to follow the quantitative gain of bone volume using micro-CT. The induced ectopic bone was approximately 1.5mm³ in volume at day 12 and steadily increased to 2.5mm³ by day 31. Following micro-CT scans, samples were processed for histology and sections were stained with fast green and safranin O in order to identify cartilage tissues. Figure 3D shows the histological developmental progression of ectopic bone. At POD 12, regions of cartilage were present followed by the formation of bone tissue at day 16 and development of trabecular-like structures. A well-defined second marrow space began to develop by 24 that continued to be present at day 31 which was encapsulated between a new outer cortical shell and the original cortical surface (denoted with arrows in the figure). By POD 31 marrow also had a larger amount of adipocytes contained with the encapsulated space that was formed by the ectopic bone than seen in the original marrow space of the bone. The molecular progression of endochondral bone formation was followed by assessing the mRNA expression of key markers for the stages of vessel, cartilage and bone tissue development (Figure 4A–C). Well defined temporal patterns of mRNA expression were observed during the formation of ectopic bone, with angiogenesis markers peaking at PODs 4 and 8, and markers of chondrogenesis peaking at POD 8. Markers of osteogenesis were upregulated during the period of maximal expression of chondrogenic markers but peaked later, at POD 16. These results together with histology suggest that DBM induces bone formation that progresses through endochondral ossification rather than intramembranous

ossification. The expression patterns of two sets of genes encoding two member of the BMP family and two members of the Wnt family are seen in Figure 4D. Both GDF5 and BMP2 followed very similar expression patterns showing very strong initial induction followed by two peaks of expression corresponding to periods of initial bone replacement and secondary bone formation during remodeling. In contrast, Wnt5a showed a very narrow window of expression at day 4, while Wnt 5b had a broad period of elevated expression from PODs 8 to 16 days.

3.2 Varying Effect of Angiogenic Inhibitors on Vessel Morphogenesis during DBM-Induced Endochondral Bone Formation

In order to identify the role that angiogenesis carries out in DBM-induced ectopic bone formation, angiogenesis was blocked with two different known anti-angiogenic agents (TNP-470) a small molecule inhibitor or a biological inhibitor (antibody blocking the VEGFR2, [DC101]) [13,20,25]. The effect of these differing inhibitors on vasculature tissue development was first assessed structurally by micro-CT analysis of barium sulfate vascular casts (Figure 5a–d). Compared to the controls, DC101 showed a 50% decrease in vessel volume ($p=0.030$) while no change was observed in mean vessel thickness ($p=0.2245$). The distribution of thickness measurements on individual vessels were averaged for each treatment group. The control samples showed a strong biphasic pattern. Few thickness measurements were seen between 0.21 mm and 0.35 mm in the control samples. Both TNP-470 and DC101 treatments significantly decreased the large size vessels (>0.35 mm) (TNP-470 $p=0.022$, DC101 $p=0.012$), while only DC101 significantly decreased the small vessels (<0.21 mm) ($p=0.006$). Interestingly TNP-470 increased the number of vessels with intermediate thickness (between 0.22 and 0.34 mm) creating a triphasic distribution.

Immunohistochemistry was next carried out to complement the microCT analysis of the vasculature. Sections were labeled with Pecam1 at POD 8 and 16. Because of the technical difficulty of obtaining reactions with DC101 samples that lacked a uniform background due to the reaction of anti-mouse antibodies with the DC101, these studies could not be carried out in these samples. At day 8, control samples clearly had observable large vessels and many very small capillary sized vessels within the ectopic bone areas. In contrast the TNP-470 sections had a large number of intermediate sized vessels but very few capillary-sized vessels. By day 16 the majority of staining in the control sections was seen in vessels that formed throughout the new marrow space that formed in the ectopic bone, while the tissues of the TNP-470 remained as fibrous tissues devoid of small vessels.

The final analysis that was carried out assessed the mRNA expression of *PECAM1* and *VEGFR2*. While TNP-470 significantly decreased *Pecam1* and *VEGFR2* expression by 87% ($p=0.001$) and 83% ($p=0.026$) respectively, at POD 8, the peak of chondrogenesis and angiogenesis, interestingly the DC101 treated samples showed no change in the expression for either *Pecam1* or *VEGFR2* at either time point.

3.3 Inhibition of Angiogenesis Does Not Block Osteo/Chondro Progenitor Cell Recruitment or Commitment but Blocks Endochondral Progression

At POD 16 both TNP-470 and DC101 decreased bone volume from 2 mm³ to 0.34 mm³ and 0.46 mm³, respectively (Figure 6). Histology was performed to assess cellular differences with inhibition of angiogenesis. Blocking angiogenesis did not disrupt cell recruitment or infiltration into the DBM material. Samples treated with DC101 also contained pockets of chondrocytes as shown by the red Safarin O stain. Assessing the gene expression profile for chondrogenesis (*Col2A*, *Aggrecan*, *Col10a1*) and osteogenesis (*Osterix*, *Osteocalcin*, *DMP1*) showed that while control samples showed peak chondrogenesis at POD 8 followed by peak osteogenesis at POD 16, this pattern was not observed with inhibition of angiogenesis. Early steps of chondrogenesis are marked by the expression of the lineage commitment marker, *Sox9*, followed by condensation and differentiation [1,2].

To determine whether lineage commitment and condensation occurs with inhibited angiogenesis, expression of *Sox9* and *N-Cadherin* was probed (figure 7). There was no change in *Sox9* expression with TNP-470 treatment, while there was a significant two- fold increase in expression with DC101 treatment at POD 8. These results are supported by immuno-histological labeling of Sox9-positive cells. Labeling was apparent in both control and TNP-470 samples at POD 8 but then decreased in the control and increased in the TNP-470 samples at POD 16. Most interesting was the expression of *N-cadherin*. Inhibiting angiogenesis with TNP-470 decreased *N-cadherin* expression at both POD 8 and 16. However, blocking angiogenesis with DC101 did not show similar results; at POD 8 *N-cadherin* expression was similar to the control while it significantly doubled expression at POD 16. These results suggest TNP-470 and DC101 may inhibit bone formation differently.

The next studies that are presented focused on how the blockade of angiogenesis affected the interconnected series of autocrine/paracrine factors that regulates endochondral bone formation (figure 8). One of the key regulatory mechanisms for chondrogenesis is the PTHrP/Ihh negative feedback loop. Pre-hypertrophic chondrocytes express Ihh which upregulates PTHrP resulting in increased proliferation and decreased progression to prehypertrophic chondrocytes. Working in parallel with PTHrP/Ihh negative feedback loop is Wnt5a/5b. Lastly, BMP2 and GDF5 are involved with progression of differentiation [26–31]. Both TNP-470 and DC101 treatment increased *PTHrP* expression by 85- and 180-fold at POD 8 and 16, respectively. Expression of *Wnt5a* also increased with the inhibition of angiogenesis, while that of *GDF5* and chondrogenesis to progress from proliferation, pre-hypertrophic chondrocytes to hypertrophic *BMP2* was decreased. These results indicate that angiogenesis may be needed for chondrocytes. Interestingly, TNP-470-treated groups also showed decreased expression of *Ihh* and *PTH1r*, both of which are expressed by pre-hypertrophic chondrocytes, as well as increased expression of *Wnt5a* and *Wnt5b*; indicating highly restricted progression to hypertrophy. These results are consistent with the actions of TNP-470 blocking progression at the earlier condensation stage.

3.4 Recruitment of Osteoclasts is Dependent on Vascular Tissue Development

The final studies that are presented examined the relationship between endochondral progression, angiogenesis and resorption. The transition between cartilage and bone is a

coordinated process. Angiogenesis is occurring while osteoclasts are recruited for resorption of cartilage and osteoblasts mineralize the cartilage template [32,33]. Osteoclast recruitment is present in the ectopic bone model as shown in figure 9. TRAP stain was used to identify the osteoclasts recruited to the site of ectopic bone. Inhibiting angiogenesis independent of treatment type decreased osteoclast recruitment demonstrated by both histology and expression of *TRAP5b*. However, osteoclasts were identified within the regions of cartilage that were able to form in the DC101-treated groups. Growth plate cartilage was included as the control for these studies.

4. Discussion

4.1 Ectopic bone formation model

Bone repair in response to either fracture or osteotomy leads to concurrent intramembranous bone formation with the direct recruitment of osteoprogenitors in response to the injury of the cortical bone as well as endochondral bone formation that develops through the recruitment of skeletal stem cells that first form cartilage. Comparing patterns of gene expression between DBM induced ectopic bone formation on the periosteal surface to that seen after fracture suggests that no intramembranous bone formation or osteoprogenitor recruitment takes place in this ectopic bone formation model. This conclusion is based both on the lack of the induction of any genes associated with osteoblast matrix during POD 2–7 and the much sharper and very discrete temporal phases of expression of genes associated with cartilage and bone cells that were seen in this model in comparison to fracture [10,34]. Fracture and osteotomy models also induce both an extensive inflammatory response, as well as a marrow response both of which will affect angiogenesis. Thus an ectopic bone formation model provides a means to address the role of angiogenesis in postnatal endochondral bone formation, with less confoundment from these other variables. It should be noted there are a few limitations to this study. The use of the Rag mouse is a “non-leaky” immune-deficient mouse that results in no adaptive immune system (no mature B or T lymphocytes) [21]. The contribution to ectopic bone formation by the innate immunity is still present however even though the adaptive immune system is lost. The study focuses on only male mice. And there may be limited confounding effects with unilateral and bilateral implants. Following the formation of ectopic bone formation, the study included animals that received unilateral implant at the earlier time points while later time points the animals received bilateral implants.

4.2 Role of Angiogenesis

Only one study to date has examined the mechanistic role of angiogenesis during postnatal ectopic bone development. In that study by Mori et al, ectopic bone formation was induced by the implantation of BMP2 with a collagen carrier and was decreased when TNP-470 was used to block new vessel formation [18]. Data presented here confirms the results of this prior study and extends these data by systemically characterizing the temporal progression of both chondrocyte and osteoblast differentiation and coupled remodeling during endochondral ossification in an ectopic bone model. In the study presented here, TNP-470 was additionally compared to a targeted biological, DC101. This comparison provided a

means to gain further insight into the exact stages of skeletal cell differentiation that were effected by inhibition of angiogenesis during ectopic endochondral bone formation.

In the prior study by Mori et al [18], it was suggested that TNP-470 inhibition of angiogenesis affected skeletogenic stem cells since both cartilage and bone were absent. In our study, however, we observed comparable levels of *Sox9* expression, an early marker of osteochondral lineage commitment [1,2], in both control and TNP-470-treated samples. We verified the presence of these cells immunohistologically. While TNP-470 has been shown to have effects on osteosarcoma cell growth [18], others have also shown that TNP-470 treatment has no effects on non-transformed osteoblast proliferation or differentiation [14]. Interestingly, TNP-470 did lead to a 75% decrease in N-cadherin expression, almost ten-fold diminishment in *Col2a* and *Aggrecan*, expression, and a near absence of *Col10a* expression. In contrast, VEGFR2 inhibition showed two- to three-fold elevated levels of both *Sox9* and *N-cadherin* expression relative to controls, but showed a very similar inhibition of all of the downstream markers of chondrogenic differentiation as TNP-470. Independent of the method used, both inhibitors decreased DBM-induced ectopic bone formation, osteogenic cell differentiation, and coupled remodeling, consistent with current literature demonstrating that the progression of bone formation from the cartilage phase is dependent on angiogenesis [13–15,18,20,35].

Although bone formation was severely impacted with the inhibition of angiogenesis, these results suggest that angiogenesis carries out a functional role at a much earlier stage in the postnatal endochondral bone formation than is currently hypothesized. The role of angiogenesis during an early phase of chondrogenesis is supported by data that show that when an angiogenesis blockade is initiated after the cell-recruitment and commitment stages of chondrogenesis but prior to osteogenesis, mature ectopic bone still forms [18]. This conclusion is also consistent with the expression of the primary regulator of angiogenesis *VEGFA*, which showed early expression at POD 4 preceding the maturation of chondrocytes. In a model of BMP-2 induced ectopic bone, *VEGFA* expression was also detected prior to chondrogenesis, suggesting vessel-dependent patterning of cartilage [36]. Embryological data is also supportive of a very early role for vessel formation during limb bud development since blood vessels are present and form a vascular plexus prior to endochondral ossification. Vessel regression allows for mesenchyme to condense at the avascular areas, although the developing skeleton appears to regulate vessel regression. It is very likely that there is a reciprocal role for the vascular tissues to also regulate the developing skeletal elements [37,38]. Dilling et al. and Zelzer et al. also showed a biphasic expression of *VEGFA* during ectopic bone formation and embryogenesis, respectively [8,36]. The second peak correlates with the transition between cartilage and bone. While a strong biphasic pattern of *VEGFA* was not observed in the present model of ectopic bone, *VEGFA* expression was present during chondrogenesis at POD 8 to a lesser extent than at POD 4. Other markers of angiogenesis did demonstrate a biphasic expression: *Ve-cadherin*, and to a lesser extent, *VEGFR2* showed peaks at both POD 2 and 8. The early role of angiogenesis is less studied and understood, and our observations suggest that such studies would be very informative to its developmental role in bone formation.

Consistent with our interpretation that TNP-470 blocked the chondrogenesis at the cell condensation stage was the strong inhibition of *Ihh* and *PTH1r* observed in our data. Interestingly, expression of *Wnt5b* was increased in samples treated with TNP-470. The overexpression of *Wnt5b* at the growth plate directly delays chondrocyte hypertrophy by promoting proliferation and inhibiting prehypertrophic chondrocyte differentiation [30]. Inhibiting angiogenesis also altered the other known autocrine/paracrine mechanisms that control chondrocyte progression including members of the TGF β superfamily (GDF5 and BMP2), Wnt, and PTHrP [26–31,39–41]. Although GDF5 is well known to be expressed by joint progenitors, it has also been observed in condensing mesenchyme and in regulation of chondrocyte proliferation and cell adhesion during early chondrogenesis [40]. PTHrP inhibits the progression to prehypertrophic chondrocytes while overexpression of Wnt5a at the growth plate indirectly delays chondrocyte hypertrophy [27,28,30]. Chondrocyte maturation is also dependent on BMP2: conditional BMP2 knockout in early chondrocytes or limb bud cells leads to chondrodysplasia and a delay in chondrocyte hypertrophy [31]. Both *GDF5* and *BMP2* expression was decreased, while *PTHrP* and *Wnt 5a* expression were increased, suggesting angiogenesis is key to coordinating events of multiple growth factors that regulate chondrocyte maturation.

4.3 Comparison between inhibitors

This is to our knowledge, one of first studies that directly compare two different approaches of inhibiting angiogenesis during endochondral ossification. This comparison allowed us to both independently validate how inhibiting the processes angiogenesis commonly effect endochondral bone formation while identifying differences that are mediated through the separate molecular mechanisms of actions of the TNP-470 and DC101 treatments.

It is worth noting how TNP-470 and DC101 affect vessel morphogenesis in very different manners. TNP-470 is a synthetic analog of Fumagillin whose actions on angiogenesis are mediated by the selective inhibition of methionine aminopeptidase-2, which effects vascular endothelial cell proliferation through its selective actions on various MAP kinase activities [25]. On the other hand VEGFR2 blocking antibodies prevent VEGFR2 signaling and have been shown to have effects on both vascular endothelial cell proliferation, migration as well as the expression of a multitude of selective genes within endothelial cells. While both TNP-470 and VEGFR2 antibodies equally inhibited small vessel formation, it is interesting to note that TNP-470 resulted in fewer larger vessels (>0.35 mm) but more mid-size vessels (0.21 –0.35 mm). The causes of this shift of vessel size are not clear but could explain the lack of an effect on average vessel thickness and total vessel volume with TNP-470 treatment. On the other hand, DC101 treatment resulted in fewer smaller (<0.21 mm) and fewer larger (>0.35 mm) vessels as well as lower total vessel volume.

One possible difference in the molecular actions of these inhibitors may be mediated thorough TNP-470 effects on the Wnt planar cell polarity (PCP) signaling pathway. In this regard it is noteworthy that recent studies have shown that TNP-470 disrupts PCP signaling in vascular endothelial cells [42,43]. Interestingly recent data has shown that PCP signaling specifically alters vascular endothelia migration, which this is an essential process in angiogenesis. Two recent studies have shown that PCP signaling is essential for patterning of

arterial vessel morphogenesis[44,45]. Strikingly our observed difference in the patterns of vessel morphogenesis that was seen between the two inhibitors is very similar to the patterns of arteriole morphogenesis that are observed with the targeted ablation of the Fzd4 receptor, which mediates PCP signaling.

Alternatively Wnt PCP signaling can regulate the migration and aggregation of chondroprogenitor cells [46]. Wnt5b is known to signal through the PCP signaling pathway leading to migration and decreased aggregation and *N-cadherin* expression [46]. Although TNP-470 treatment showed increased expression of *Wnt5b* and decreased expression of *N-cadherin*, it might be possible that TNP-470 could inhibit the Wnt PCP signaling pathway [47] and cell migration within chondrocytes as well. It is thought that cellular migration is needed for condensation which could explain the differences between TNP-470 and DC101 inhibitors.

Similarly, it cannot be ruled out that DC101 might have a direct effect on chondrocytes since they also express the VEGF receptors [48,49]. Although the direct role of VEGF has on the mesenchyme and early chondrocytes is not clear. *In vitro* micromass culture of chick limb mesenchymal cells showed chondrogenesis is not affected by VEGF stimulation [38]. It suggests that timing of angiogenesis/VEGF expression is extremely important for proper endochondral ossification to proceed.

4.4 Conclusion

A summary of how the two inhibitors specifically affected the progression of endochondral ossification is presented in figure 10. In conclusion our data show the ectopic bone formation model can be used to study endochondral ossification. Inhibition of angiogenesis blocked endochondral ossification early during chondrogenesis, well before the stage of chondrocyte hypertrophy. This indicates not only osteogenesis but chondrogenesis is dependent on angiogenesis. Furthermore, the blockade in angiogenesis led to a complete inhibition of osteoclastogenesis and greatly diminished secondary bone formation. Finally, the method by which angiogenesis is inhibited should be taken into account when interpreting results.

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Abbreviations

C	Cortical bone
D	DBM
DBM	Demineralized Bone Matrix
M	Muscle
Micro-CT	Micro-Computed Tomography
PCP	Planar Cell Polarity
POD	Post-Operative Day
R	Response
ROI	Region of Interest
UD	Undetectable

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Highlights

- Angiogenesis is needed for the earliest phase of chondrogenesis.
- Blockade of angiogenesis led to complete inhibition of osteoclastogenesis.
- Direct comparison between two different antiangiogenic agents, TNP-470 and DC101.
- DBM-induced ectopic bone model can be used to study endochondral ossification.

Figure 1. DBM Induce Ectopic Bone Formation

A. Faxitron analysis depicts mineralized DBM-induced ectopic bone with increasing amounts of Noggin. Increasing amount of Noggin results in decreased visualized mineralized ectopic bone. White arrow points to the location of the DBM implant. **B.** Plain film radiography depicts mineralized BMP2-induced ectopic bone at the periosteal surface of the femur. Only the higher concentration of BMP2 (0.3µg and 0.5µg) induced ectopic bone. **C.** Left panels show the 3D micro-CT rendering of the ectopic bone resulting from DBM supplemented with BMP2 (0.5µg) and the sponge supplemented with BMP2 (0.5µg). Both are located in the muscle of the hind limb. Right panels show the cross section through the ectopic bone induced by DBM with BMP2 (5.0µg) and sponge with BMP2 (5.0µg).

Figure 2. An overview of animal enrollments

The temporal progression of DBM-induced ectopic bone formation was characterized (A–B). Animals were enrolled for post-operative days (POD) 2, 4, and 8. These animals received DBM implant on the left femur. Animals enrolled for POD 12, 16, 24, and 31 received bilateral implants, and upon harvest the left implant was isolated for mRNA gene expression analysis and the right implant was used for micro-computed tomography (micro-CT) followed by histological analysis. The angiogenic studies focused on two time points (C–E), POD 8 (chondrogenesis) and POD 16 (osteogenesis). The TNP-470-treated animals received bilateral DBM implants for both day 8 and 16 time points. The DC101-treated animals enrolled for day 8 received unilateral DBM implant while animals enrolled for day 16 received bilateral DBM implants. An additional day-8 control group was enrolled which received unilateral DBM implant for histological analysis to compare to the blockade of angiogenesis. For micro-CT imaging of vessels, all mice were enrolled for time point day 8 and received unilateral DBM implant (E). * Indicates 5 animals were enrolled per time point, however too low amounts of RNA was isolated for analysis. At POD 2 and POD 4; the final analysis is based on n=3 and n=4 respectively.

Figure 3. Progression of DBM-induced ectopic bone on periosteal surface

A. Faxitron analysis depicts the progression of mineralized DBM-induced ectopic bone through POD 31. DBM was not implanted in the control. POD 12 shows earliest time point where ectopic bone is visible (white arrow). **B.** Representative 3D renderings from the micro-CT showing specifically the newly formed DBM-induced mineralized tissue across time. The femur is not visible. Bar represents 1.0 mm. **C.** Quantified bone volume of the DBM-induced ectopic bone shows a non-significant increase over time (n=5). **D.** Representative histological assessment of ectopic bone development across time. Sections were stained with fast green and safranin O (red represents cartilage). Low magnification images (a–d) and high magnification images are shown (e–h). Selective areas of cartilage formation and cortical and trabecular bone are depicted (i and j). Arrow head indicates chondrocyte enriched area at POD 12. Panel i shows further magnification of the cartilage in e. By day 16, cartilage is absent and replaced by bone (arrow shows formation of trabeculae like structures). Panel j shows further magnification of new marrow space containing a high adipocyte content. Arrows denote the original and new cortical surfaces. Original marrow (OM) and new marrow (NM) are shown. Scale bar represents 200 µm.

Figure 4. Endochondral bone formation is induced by DBM

Relative gene expression for angiogenic (A), chondrogenic (B) and osteogenic (C) markers as determined by RT-qPCR is shown for the DBM-induced ectopic bone across time. * represents significance at indicated time points and # represents significance compared to all time points ($p < 0.05$, $n = 3-5$). Significance was determined by ANOVA followed by Tukey test. Values that were undetectable are labeled (UD).

Figure 5.

A. 3D renderings of micro-CT scans of perfused vasculature at POD 8 where vessels (red), implant (purple), and bone (gray) are shown. **B–D.** Vessel measurements as determined by micro-CT (n=4). Error bars represent stand deviation. **E.** Representative histological sections of POD 8 and 16 for control and TNP-470 treated group. Pecam1 was immunohistochemically labeled. Scale bar represents 125 μ m. **F.** Relative gene expression for angiogenic markers POD 8 and 16. * indicates significance which was determined by ANOVA followed by Dunnett test where each treatment was compared to control at each time point ($p < 0.05$, $n = 3-5$).

Figure 6. Inhibition of angiogenesis decreases endochondral bone development

A. 3D renderings of micro-CT scans of the femur (gray) and DBM-induced ectopic bone formation (purple) from control, TNP-470 and DC101 treated groups POD 16. Black arrow indicates the approximate location of cross-sectional view of the femur (white) and surrounding implant (gray). **B.** Bone volume as quantified by micro-CT is shown to be significantly decreased for both anti-angiogenic treatments, TNP-470 and DC101. Significance is represented by * ($p < 0.05$, $n = 5$). **C.** Histological sections of the femur, DBM implant, and surrounding muscle for control, TNP-470, and DC101 at POD 16. Sections were stained with fast green and safranin O for cartilage (red). The top panel shows the cortical bone (C), the response to DBM (R), and DBM (D). The box indicates the location where high magnification images were taken and shown below. Trabecular-like structures are present indicated by * and the arrow head points to regions of chondrocytes. Scale bar represents 125 μm . **D.** Relative gene expression for chondrogenic and osteogenic markers as determined by RT-qPCR is shown for the DBM-induced ectopic bone at POD 8 and 16. Significance is denoted by * which was determined by ANOVA followed by Dunnett test where each treatment was compared to control at each time point ($p < 0.05$, $n = 3-5$).

Figure 7. Commitment and condensation of chondrocytes is not associated with angiogenesis
A. The relative gene expression of *Sox9* and *N-Cadherin* as determined by RT-qPCR at POD 8 and 16. * indicates significance which was determined by ANOVA followed by Dunnett test where each treatment was compared to control at each time point ($p < 0.05$, $n = 3-5$). **B.** Representative histological sections where Sox9 was immunohistochemically labeled. Scale bar represents 100 μm .

Figure 8. Relative gene expression of key regulators of chondrogenesis

Relative gene expression was evaluated by RT-qPCR from the ectopic bone collected from the control, TNP-470, and DC101 treated groups at POD 8 and 16. * indicates significance which was determined by ANOVA followed by Dunnett test where each treatment was compared to control at each time point ($p < 0.05$, $n = 3-5$).

Figure 9. Recruitment of osteoclasts at DBM implant site

A–B. Shown are representative histological sections of control, TNP-470, and DC101 treated samples at POD 16. Sections were stained for TRAP positive cells indicating osteoclasts. **A.** Regions of interest are indicated by box. Muscle (M), DBM (D), and cortical bone (C) are designated. Response to DBM is outlined by dotted line. **B.** The DC101 treated group showed minimum TRAP positive cells in areas of ectopic bone; however, areas of cartilage showed TRAP positive cells. The growth plate was used as a control for the stain. Left column are images of the fluorescence produced from TRAP stain. Right column is the overlay between TRAP stain and bright field. Arrows indicate osteoclasts. Scale bar represents 50 μ m. **C.** Relative gene expression for the marker for osteoclasts, TRAP as determined by RT-qPCR is shown for the DBM-induced ectopic bone at POD 8 and 16. Significance is denoted by * which was determined by ANOVA followed by Dunnett test where each treatment was compared to control at each time point ($p < 0.05$).

Figure 10. Schematic overview of angiogenesis and known regulatory factors during endochondral bone formation

Results are summarized using a modified schematic of traditional chondrogenesis at the growth plate [27,50,51]. The stages of endochondral bone formation are shown on the far left. The expression of known markers is on the near left and expression of signaling molecules is on the right. Angiogenesis occurs early during development along with the expression of *BMP2*, *GDF5*, and *VEGFA*. This is followed by cartilage formation. Progression of chondrogenesis begins with recruitment and lineage commitment shown by expression of *Wnt5a* and *Sox9*. Condensation then follows with expression of *N-Cadherin* and continued expression of *BMP2*, *GDF5*, and *PTHrP*. Chondrocytes proliferate and become prehypertrophic and hypertrophic. During this phase *GDF5* is expressed, there is decreased expression of *PTHrP* with increased expression of *Ihh*. Similarly, the expression of *Wnt5a* decreases and *Wnt5b* continues to be expressed. The hypertrophic chondrocytes express *VEGFA* which initiates angiogenesis and cartilage degradation begins (osteoclasts express *Trap5b*). Lastly, osteoblasts are recruited for bone formation demonstrated by increased expression of *Osteocalcin*. Angiogenesis blockade inhibited chondrogenic progression at commitment/condensation with TNP-470 treatment and at proliferation/prehypertrophy with DC101 treatment. Growth factors, *BMP2* and *GDF5*, showed increased expression while there was increased expression of *PTHrP* and *Wnt5a*. It is possible that angiogenesis is key to coordinating events of multiple growth factors that regulate chondrocyte maturation.