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Identifying Progenitor Cells of Heterotopic Ossification

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Approval Page
Honors Scholar Thesis

Identifying Progenitor Cells of Heterotopic Ossification

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Abstract

Identifying Progenitor Cells of Heterotopic Ossification

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University of Connecticut

2012

Heterotopic Ossification (HO) is the abnormal formation of bone within extraskeletal soft tissues. The condition can occur through both genetic and acquired means. Acquired cases of HO result from invasive surgery or traumatic injuries, with increasing prevalence of ectopic skeletogenesis as a result of combat-related blast injuries. HO has been characterized to some extent, including the histological features and the mutation underlying the genetic form, but the cells resident in skeletal muscle that represent the progenitors of heterotopic bone have yet to be determined. Only a few publications have attempted to definitively determine the progenitor cells in this disorder. Findings have been inconclusive, but cell types such as skeletal muscle satellite cells, pericytes and endothelial cells, mesenchymal progenitors, and circulating hematopoietic cells were considered attractive candidates due to accessibility and displays of osteogenic characteristics. The aim of this study was to determine the progenitor cells of HO. To accomplish this goal, lineage tracing and bioassays of heterotopic ossification were used to identify and characterize the progenitor cell type. We identified a population of Tie2+ cells that are non-endothelial (CD31-) in origin and represent a major source of progenitors for HO. The identification of the progenitor is crucial to establishing any future therapeutic agents or treatments for HO.

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Introduction

I. Heterotopic Ossification and Fibrodysplasia Ossificans Progressiva

During embryonic development, the skeleton develops from undifferentiated mesenchyme according to a precise temporal and spatial genetic plan. Postnatally, however, bone formation is restricted to fracture sites (Sharitz, et al., 1996). Heterotopic ossification (HO) results from abnormal regulation of this process.

HO is the formation of lamellar bone inside the soft-tissue structures in which bone does not normally exist (Bossche & Vanderstraeten, 2005). HO exists in both acquired and hereditary forms. The condition occurs in its acquired form as a complication following central nervous system disorders, multiple injuries, hip surgeries, burns, and in trauma and combat wounds (Bossche & Vanderstraeten, 2005; Potter 2007). Historically, HO was first documented in the medical literature in 1736 (Kaplan, et al., 2008), and has been noted in combat-related injuries in the American Civil War and World War I (Potter 2007).

Hereditary forms have also been identified, the most notable of which is a condition called fibrodysplasia ossificans progressiva (FOP) (Kaplan, et al., 2008). FOP is a rare, disabling genetic condition in which congenital skeletal malformations and progressive HO form in sites including skeletal muscle, tendon, ligament, and fascia (Kaplan, et al., 2009). FOP can be inherited in an autosomal dominant fashion, although most cases are spontaneous in origin (Kaplan, et al., 2008). This is the most disabling form of HO in humans, characterized by multiple and sporadic flare-

ups from soft-tissue injury (e.g., intramuscular injections) that ultimately lead to musculoskeletal immobility (Kaplan, et al., 2008). Clinically, FOP is characterized by a congenital malformation of the big toe observed at birth and the aforementioned heterotopic osteogenesis in predictable anatomical patterns (Kan, et al., 2004). By early adulthood, HO typically leads to ankylosis of all major joints of the axial and appendicular skeleton. As a result, movement is slowly hindered and eventually impossible, requiring lifelong assistance in performing activities of daily living (Sharitz, et al., 1996).

Soft tissue injury has been demonstrated to lead to the increased expression of BMP4 and other osteogenic cytokines, which has, in turn, lead to a proposed mechanism for HO. BMPs are a family of highly conserved extracellular signaling proteins that regulate cell differentiation fates (Kaplan, et al., 2009). One role of BMPs is as bone-inducing morphogens that participate in the developmental organization of the skeleton (Sharitz, et al., 1996). Both type I and II BMP receptors are serine/threonine kinases with similar functional domains. Following activation via ligand binding to the receptor in the GS domain of the type II receptor, the transmembrane serines and threonines of the type I receptor are phosphorylate, activating the BMP type I receptor to transmit BMP signals (Sharitz, et al., 1996). This signaling is mediated through three known type I receptors, including the activin A type I receptor/activin-like kinase 2 (ACVR1/ALK2) receptor (Sharitz, et al., 1996). Activated BMP type I receptor kinase activity phosphorylates receptor regulated Smad1, Smad5, and Smad8. These then form heteromeric complexes with Smad4 and translocate into the nucleus to regulate transcription of various target

genes (Fukuda, et al., 2009). Inhibitory Smads, including Smad6 and Smad7, are also induced by BMPs; these function as a negative feedback loop that down regulates BMP signaling through inhibition (Fukuda, et al., 2009)

FOP patients develop an ectopic skeleton because of dysregulation of BMP signaling in the presence of inflammatory triggers (Lounev, et al., 2009). Through familial studies, it was discovered that all patients who exhibit classic clinical features of FOP have the same heterozygous mutation in the ACVR1/ALK2 receptor, one of the aforementioned BMP type I receptors (Kaplan, et al., 2009). Specifically, this mutation is found in the glycine and serine residue (GS) activation domain and results in arginine replaced with histidine in codon 206, altering the receptor signaling activity (Kaplan, et al., 2009). Functional analysis has also demonstrated that this mutation induces increased BMP signaling in a ligand independent and BMP responsive manner (Fukuda, et al., 2009). This may be through Smad1 or Smad5, which increase following injury and further enhance BMP signaling that is already stimulated by a constitutively active ALK2 receptor mutation (Fukuda, et al., 2009).

II. Animal Models of Heterotopic Ossification

Animal models of HO are necessary in order to best represent the pathophysiology in a practical setting for experimentation and laboratory manipulation. As research and knowledge progress, animal models of HO will continue to be important for the opportunity to better understand the biology of

these conditions and to study the effectiveness and safety of currently available and emerging therapies, prior to human application (Kaplan, et al., 2008).

BMPs induce heterotopic bone formation through the classic endochondral ossification pathway. In HO lesions, this pathway begins with an early fibroproliferative phase, which is then followed by chondrocyte differentiation. Next, vascularization occurs, followed by osteoblast differentiation with bone matrix formation, and finally mineralization of the osteoid (O'Connor, 1996). The osteogenic response that occurs in HO has been characterized in detail by multiple groups (Lounev, et al., 2009). At present, animal models exist that emulate the induction of HO through this same endochondral ossification pathway—BMP injection (Lounev, et al., 2009) and over-expression of BMPs using the Nse transgenic mouse line (Kan, et al., 2004). Both models of HO recapitulate characteristic features of common acquired forms of HO (Lounev, et al., 2009). A genetic model of FOP has just recently been published that recapitulates this genetic form (Chakkalakal, et al., 2012).

The BMP injection model recapitulates the phenotype of acquired HO via a lesion that follows the classical endochondral ossification pathway. Growth factor-reduced Matrigel is impregnated with recombinant human BMP2 and either injected directly into the leg musculature, or implanted into subcutaneous sites of adult mice (Lounev, et al., 2009). At physiological temperatures, the BMP2 infused-Matrigel solidifies to form a localized source of BMPs (Lounev, et al., 2009). The heterotopic lesion is recovered for analysis 4 days to 2 weeks following implantation.

In the Nse transgenic mice, the BMP4 gene is ectopically expressed at the neuromuscular junction under the control of the neuron-specific enolase (Nse) promoter, leading to progressive HO (Kan, et al., 2004). HO is induced in these mice via muscle injury by injecting cardiotoxin into the quadriceps muscle (Kan, et al., 2004). Analysis can be performed on the tissue at various points after injection.

The most recent animal model is a genetic model, which used gene-targeting methods to develop a knock-in mouse model with the R206H mutation in *ACVR1* found in FOP patients. This mouse's phenotype recapitulates identifying characteristics of FOP in humans, including malformed first digits in the hind limbs and post-natal extra-skeletal bone formation (Chakkalakal, et al., 2012). In addition to providing another mechanism for future research into FOP, this mouse provides the first in vivo evidence that the mutation in the BMP type I receptor *ACVR1/ALK2* is the direct genetic cause of FOP (Chakkalakal, et al., 2012).

III. Potential Progenitor Cells

Ultimately, HO research aims to develop treatments that will prevent, halt, or someday even reverse the progression of the condition (Kaplan, et al., 2008). Although the mutation responsible for FOP is known, this is merely the proximate genetic cause—the cells that respond by forming bone in acquired and genetic forms remain unidentified. Determination of the lineage of cells responsible for HO will provide cellular and molecular mechanisms relevant to this condition. An understanding of the cellular basis of these conditions is necessary to further research.

Although the particular cells have remained elusive, a number of groups have speculated as to a variety of potential progenitor cells of the HO. Speculation of potential progenitors has included tissue-resident skeletal muscle stem cells (satellite cells), endothelial precursors, vascular smooth muscle, circulating osteoprogenitors, and multipotent mesenchymal cells (Lounev, et al., 2009).

In order to trace and model the contributions of these various cells, many groups employ the Cre/loxP system. Both the BMP injection and Nse transgenic mouse models can be used in combination with the Cre/loxP system. Mice expressing Cre recombinase under a cell-specific promoter are crossed to mice in which a reporter gene is separated from a constitutively active promoter by stop sequences, surrounded by loxP sites. LoxP sites are DNA sequences containing specific binding sites where Cre cuts and recombines the DNA, which ensures the reporter gene is transcribed, providing permanent, Cre-dependent expression and cell-specific labeling (Lounev, et al., 2009). This is a cell tracing method in mice used to identify cell lineages; in HO experiments, the contribution of labeled cells to fibroproliferative lesions, cartilage, and bone can be evaluated using histological methods (Lounev, et al., 2009). According to recent characterizations in the literature, cell-specific promoters of interest include the following: Tie2-Cre, to label vasculature and hematopoietic stem cells; MyoDiCre, to label muscle and muscle satellite cells; and SM22Cre, to label pericytes and smooth muscle (Lounev, et al., 2009; Medici, et al., 2010).

Many groups have debated whether the progenitor cells are local, residing within the skeletal muscle and associated soft tissues, or are from a more widely distributed cell progenitors that are osteogenic within the conditions provided by the muscle. Although there is much speculation into circulating osteogenic progenitors, bone marrow transplantation has contributed conflicting data regarding the contribution of circulating cells. In addition, lineage tracing in transgenic mice did not detect a direct cellular contribution of cells of a hematopoietic lineage (Kaplan, et al., 2007). However, these findings conflict with those of other groups, such as Suda, et al. (Suda, et al., 2009), previous lineage tracing (Otsuro, et al., 2007), and parabiosis (Otsuro, et al., 2008) studies which have found that the osteogenic progenitor cells were blood-derived. These blood-derived progenitors were shown to contribute to heterotopic bone in BMP2-induced osteogenesis, as well as to exist as osteogenic progenitors in culture experiments (Otsuro, et al., 2007). The same group confirmed their results further in parabiotic experiments, demonstrating that ~50% of all osteoblasts were derived from osteogenic progenitors that were marrow-derived (Otsuro, et al., 2008).

On the other hand, a tissue resident cell of interest, muscle-specific stem cells, has also been a target as a progenitor for heterotopic lesions, as they have demonstrated osteogenic capabilities in cell culture. Once again though, lineage-tracing experiments by Lounev, et al. have demonstrated that satellite cells do not significantly contribute to HO (Lounev, et al., 2009).

Vascular endothelium has become the leading candidate for the progenitor cell source of heterotopic lesions. In recent work by Medici et al., endothelial cells have demonstrated both osteogenic activity and multipotency (Medici, et al., 2010). However, it is important to note that the Cre used in these experiments lacked stringent lineage specificity. In past studies, cells expressing Tie2 have been demonstrated to contribute to all stages of BMP2-induced heterotopic lesions, though the endothelial origin must be evaluated further because, although endothelium is the predominant cell type labeled by transgenic Tie2Cre; *R26^{NG/+}* mice, Tie2 is expressed in many non-endothelial cell types (Lounev, et al., 2009; Medici, et al., 2010).

Lineage tracing and bioassays were used to identify the progenitor cell of HO. In these experiments, we demonstrate that endothelial cells do not significantly contribute to HO in mouse models, and that Tie2⁺, non-endothelial cells resident in the skeletal muscle interstitium are the predominant source of progenitor cells of heterotopic lesions.

Materials & Methods

I. Mouse Models and Genotyping

Tie2-Cre transgenic mice, SCID transgenic mice, and *R26^{NG}* Cre-dependent GFP reporter mice were obtained for use in these experiments. Experimental mice carried the Cre transgene and were heterozygous for the necessary reporter allele. Genotypes were verified via PCR and through observation of reporter fluorescence. Tissue-specific recombination was verified at the time of tissue harvesting. All animal procedures were reviewed and approved by the University of Connecticut's Institutional Animal Care and Use Committee under the Goldhamer laboratory protocol.

Tails were obtained by taking a small tail clip with the mice under general anesthesia using isoflurane. Tail clippings were processed for DNA extraction by initially digesting them in a mixture of 20 mg/ml of proteinase K and tail buffer overnight at 55°C. Each sample was then incubated at 37°C for one hour after adding 10 mg/ml of RNase A. After spinning down the samples, the lysate suspension was added to a mixture of QX1 buffer and diatomaceous earth. After two minutes, Merlin V was added, the samples were spun down, and the supernatant was removed. This was repeated, and the particulate was allowed to dry. The purified DNA particle was finally resuspended in 65°C TE buffer and stored at 4°C for further use. DNA samples were genotyped by PCR amplification and visualized using agarose gel electrophoresis, with an ~ 479 bp product expected if

the Tie2 promoter driven Cre gene was present. Primers used for Tie2Cre PCR were: forward 5'- CCCTGTGCTCAGACAGAAATGAGA- 3', and reverse 5'- CGCATAACCAGTGAAACACGATTGC- 3'.

II. Tissue Preparation

Muscles and lesional tissue were isolated via dissection and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 3-6 hours at 4°C with gentle agitation, washed with PBS, and then cryo-protected overnight in 30% sucrose at 4°C overnight with gentle agitation. The samples were then embedded in O.C.T. and frozen in cooled pentanes. Tissue samples were cryostat sectioned in 12µm sections and collected on glass slides. Samples and subsequently sectioned tissues were stored at -80°C until further use.

III. Immunofluorescence and Histochemistry (IHC)

For CD31, Osterix, and Sox9 staining, sections were rehydrated in PBS, permeabilized in 0.1% Triton in PBS, blocked in 1% bovine serum albumin (BSA), 10% goat serum, and 0.1% Tween in PBS. They were then stained with primary antibody in PBS containing 10% goat serum and 1% BSA overnight at 4°C. The samples were then washed in PBS, stained with a fluor conjugated secondary antibody at room temperature for 1.5-2 hours, washed in PBS, stained with DAPI and cover-slipped.

IV. Fluorescence Activated Cell Sorting (FACS) and Cell Transplantation

Total hind limb muscle was isolated via dissection and minced for 7 minutes with scissors. The tissue was then transferred to a conical containing Collagenase and Dispase in DMEM. The conical was incubated in a 37°C water bath for 60-85 minutes with trituration every 15 minutes. To end the digestion, 20% FBS in DMEM was added. The sample was filtered through 100 µm and 70 µm cell strainers. After centrifugation, the sample was washed with PBS and re-suspended in 10% fetal bovine serum (FBS) in PBS. The sample cells were then incubated with fluorescently conjugated antibodies (CD31) at 4°C for 30 minutes. The cells were washed with PBS, collected by centrifugation, and re-suspended in 2% FBS. Cells were placed on ice until analysis and sorting. The cells were then filtered through 30µm cell strainer and propidium iodine was added. Sorting and analysis was then performed using a FACS machine.

Cell populations from the FACS sort were washed with PBS chilled on ice. These were collected via centrifugation and resuspended in Matrigel containing BMP2. The cell suspension was injected into the tibialis anterior (TA) hindlimb muscle of SCID mice using an insulin syringe. At 10.5 days post-injection, the TA muscle was isolated and fixed as described.

Results

I. Mouse Models

The primary aim of this study was to determine the progenitor cell type in HO. This required the Tie2-Cre transgenic mice, which labels vasculature and hematopoietic stem cells, because past studies have shown cells expressing this marker contributing to heterotopic lesions (Lounev, et al., 2009; Medici, et al., 2010; Wosczyzna, et al., 2012). In addition, this required the *R26^{NG}* Cre-dependent GFP reporter mice, and immunodeficient (SCID) mice. We employed the Cre/loxP system to label cells and trace the contribution of Tie2+ cells to HO lesional tissue, providing permanent, Cre-dependent expression and cell-specific labeling (Lounev, et al., 2009). Using a bioassay of HO, reporter cell contribution to the cartilage and bone of fibroproliferative lesions was evaluated using histological methods described in later subsections.

Experimental mice possessed the Cre transgene and were heterozygous for the necessary reporter allele. Genotypes were verified via PCR analysis of DNA from tail clippings (Fig. 1) and through observation of reporter fluorescence of the same tail clippings, prior to DNA extraction (Fig. 2). A ~ 479 bp product was present in the gel electrophoresis of the PCR products when the Tie2 promoter driven Cre gene was present, as demonstrated by the columns in the example in Fig. 1. These are labeled with a +, consistent with the known positive control (labeled), while a negative example is listed, labeled with a -, and has no band present. The mice determined to be positive for both transgenes were used in further experiments.

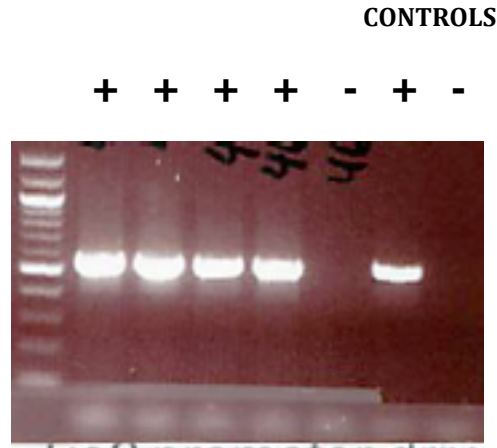


Fig. 1 Image of genotyping results by PCR and gel electrophoresis to verify Tie2Cre; *R26^{NG/+}* recombination. Columns with a + represent mice positive for the reporter, as they have a band at ~479 bp consistent with the known Tie2-Cre control, also marked with a +. The column marked with a - represents a mouse negative for the reporter, consistent with the known negative control.

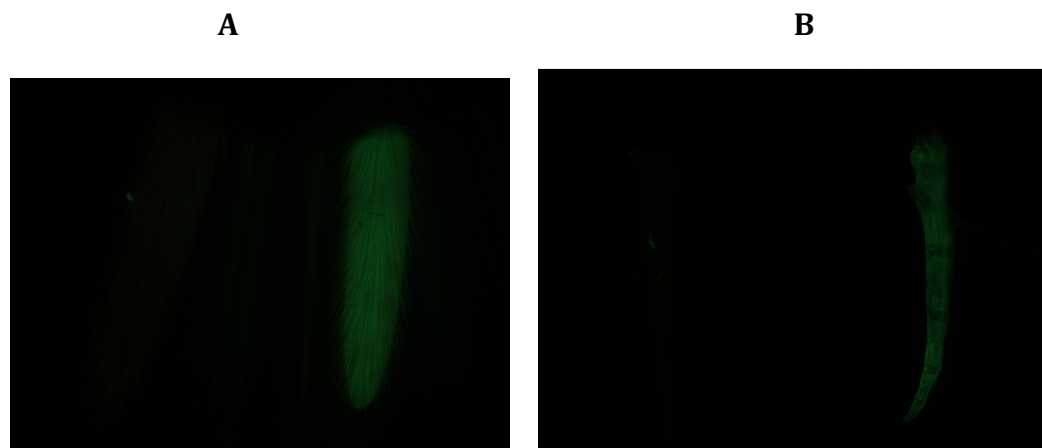


Fig. 2 Fluorescence microscopy image of a Tie2Cre; *R26^{NG/+}* - mouse-tail clip. In both images, the left side is a negative mouse, and the positive is on the right in green. (A) A mouse-tail clip with skin and (B) a mouse-tail clip with the skin removed for better visibility of fluorescence.

II. Endothelial Labeling in the Tie2-Cre;Reporter Mouse

Past studies have demonstrated cells expressing Tie2 contribute to all stages of BMP2-induced heterotopic lesions, but have conflicted in their determination of the

endothelial origin of these potential progenitor cells (Lounev, et al., 2009; Medici, et al., 2010; Wosczyzna, et al., 2012). Although endothelium is the predominant cell type labeled by transgenic Tie2Cre; *R26^{NG/+}* mice, Tie2 is expressed in many non-endothelial cell types.

We first established the efficiency of the Cre reporter to label endothelium using immunohistochemistry (IHC). Using the endothelial marker, CD31, we estimated the labeling efficiency by co-localization with GFP cells. Nearly 100% of the CD31+ cells were also GFP+ (Fig. 3), proving highly efficient Cre-dependent endothelial cell labeling.

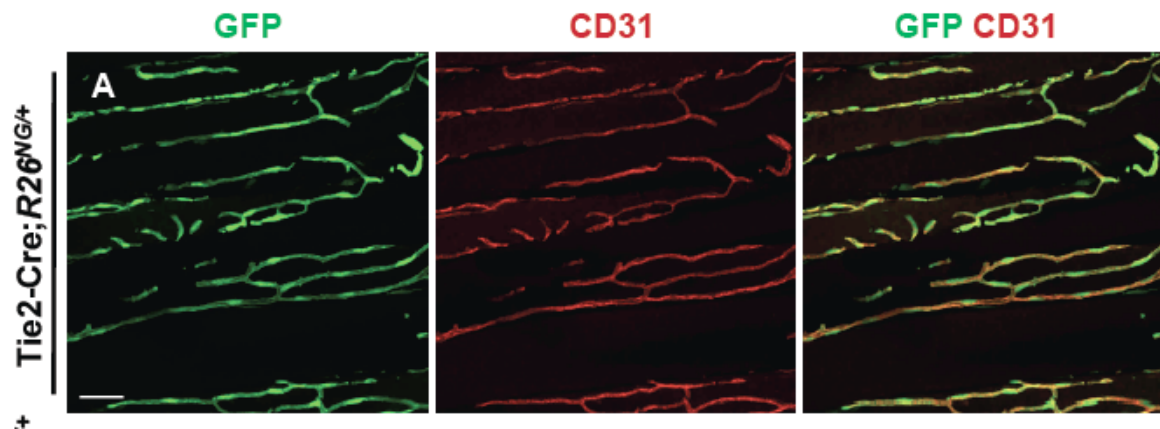


Fig. 3 Cre/loxP labeling with the Tie2 reporter efficiently labels endothelial cells.

Immunohistochemistry on TA muscle from Tie2-Cre; *R26^{NG/+}* mice using an endothelial marker (CD31) demonstrates this, as nearly 100% of the cells that are labeled with CD31 co-localize with GFP fluorescence. (Wosczyzna et. al, 2012)

Next, cells were obtained from the total hind limb musculature of Tie2-Cre; *R26^{NG/+}* mice by fluorescence-activated cell sorting (FACS); they were sorted for the expression of GFP and CD31. The cell populations were isolated in two groups,

GFP+/CD31+ (Fig. 4, population A in blue) and GFP+/CD31- (Fig. 4B, population B in red), representing Tie2+ cells that are endothelial and non-endothelial in origin, respectively.

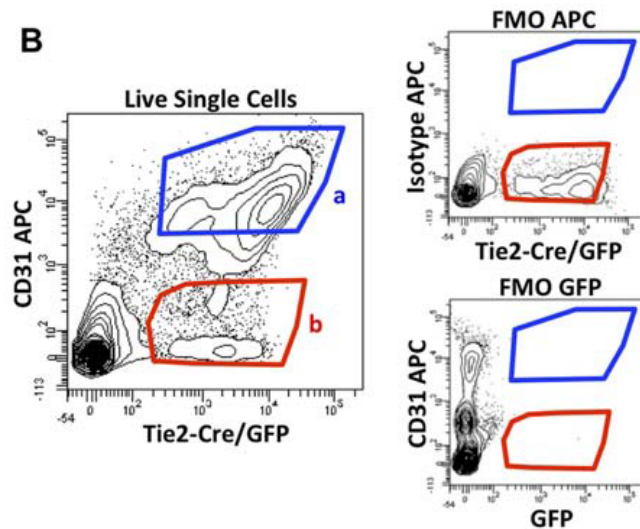


Fig. 4 FACS analysis sorting the total hind limb muscles from Tie2-Cre; *R26^{NG/+}* mice into two populations: (A) GFP+/CD31+ (endothelial) and (B) GFP+/CD31- (non-endothelial). These populations were collected for analysis using a heterotopic osteogenesis bioassay to determine the extent of their contribution to the lesional tissue. (Wosczyzna et. al, 2012)

III. Heterotopic Osteogenesis Bioassay

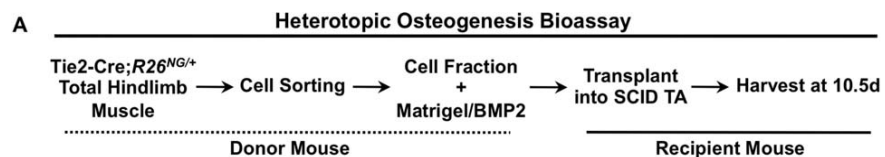


Fig. 5 Schematic of the intramuscular transplantation experimental design. (Wosczyzna et. al, 2012)

The capacity of GFP+/CD31+ and GFP+/CD31- cells to participate in HO was assessed using cell transplantation experiments, followed by immunofluorescence analysis by IHC. Figure 5 is a schematic of the experimental design. To test for osteogenic activity, the two populations of cells were each mixed with BMP2/Matrigel and injected into the mid-belly of the TA muscle of SCID mice. The lesional tissue was harvested 10.5d post-injection. Fig 6 displays an example of the histology of a BMP2-induced heterotopic lesion, both at 8 and 15 days post-injection. These correspond to known time points in which there are both chondrogenic and osteogenic cells, which is why 10.5d was the most convenient time point to harvest tissue to analyze Tie2 reporter cell contribution to cartilage and bone within the lesion. The results of GFP+ cells of endothelial origin versus GFP+ cells of non-endothelial origin are discussed in the next section.

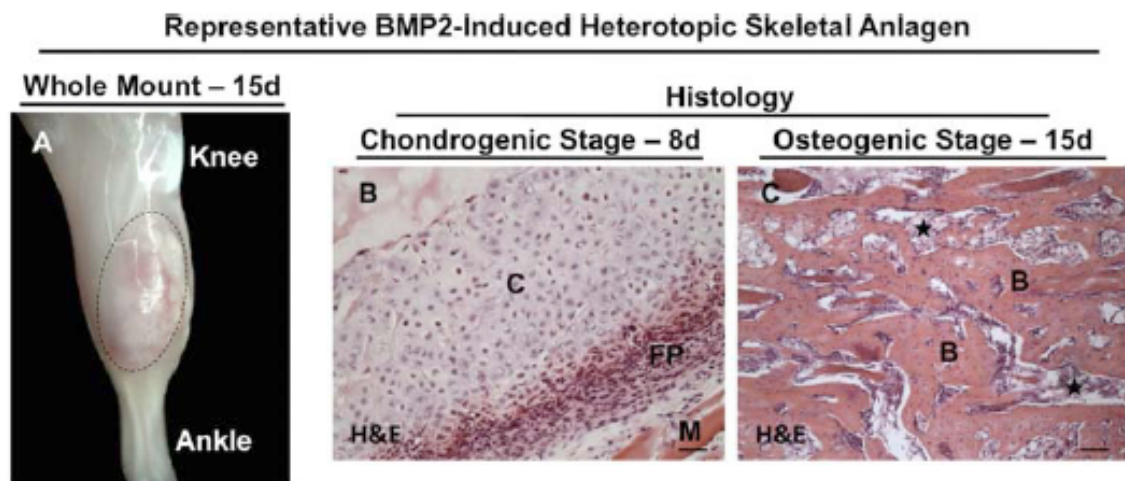


Fig. 6 BMP2-induced HO in a mouse TA. Two time points, 8d and 15d, demonstrate the lesion's progression through the classic endochondral ossification pathway (A) A significant heterotopic lesion (dashed oval) is visible, shown here 15d after ossification was induced. (B,C) Hemotoxylin and eosin (H&E) stained sections to show histology of a lesion 8d and 15d after ossification was induced.

(B) 8d lesions are well representative of cartilage (C), while (C) 15d lesions are well representative of bone (B). (Wosczyzna et. al, 2012)

IV. Tie2+ Progenitors are a Major Contributor to Heterotopic Ossification and are of Non-Endothelial Origin

The heterotopic lesions within the TA muscles of the SCID mice were isolated by dissection, frozen for preservation, and sectioned onto slides. Sections were analyzed via immunofluorescence to determine if either cell population contributed significantly to ossification in the lesion. Sox9 and Osterix staining was employed to label cartilage and bone, respectively. In addition, CD31 staining was used to identify reporter cell contribution to vasculature of the lesion. The GFP+/CD31- population contributed to both chondrogenic (Sox9) and osteogenic (Osterix) cells of the BMP2-induced lesions (Fig. 7F-H, L-N). It is important to note that nearly half of the cartilage and bone cells of the lesion remained unlabeled. On the other hand, transplanted GFP+/CD31+ cells did not contribute to heterotopic cartilage or bone (Fig. 7C,E, I-K). However, the GFP+/CD31+ cells did contribute to the vasculature of the lesion, consistent with their endothelial origin (Fig. 7C,E, I-K). The evidence presented here demonstrates that there is a population of Tie2+ cells that are a significant progenitor of induced HO and are of non-endothelial origin.

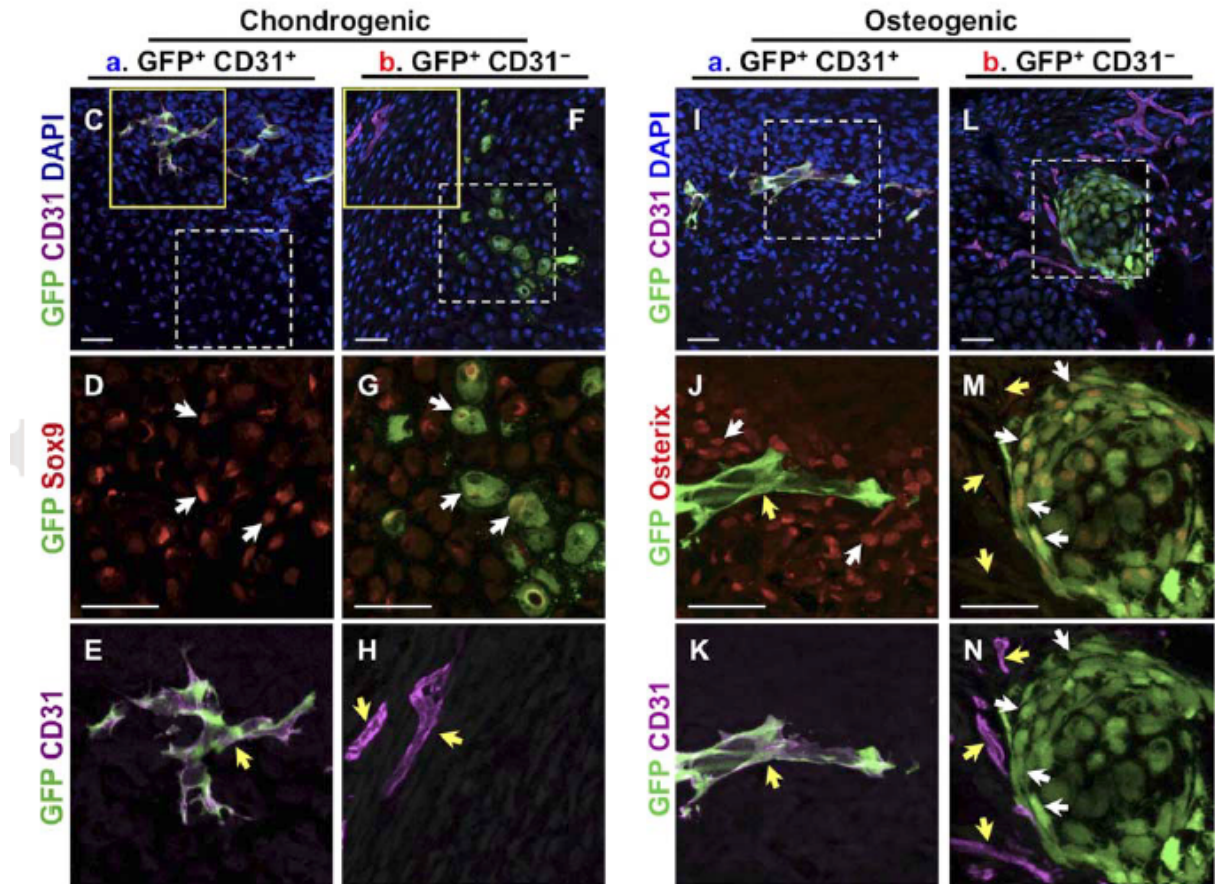


Fig. 7 Confocal images of Sox9 (cartilage) and Osterix (bone) staining of induced heterotopic lesions 10.5d post-injection demonstrate that GFP⁺/CD31⁻ cells from Tie2-Cre; *R26^{NG/+}* mice contribute to heterotopic lesions, while GFP⁺/CD31⁺ cells do not. (**C-E, I-K**) GFP⁺/CD31⁺ cells contribute to the vasculature of the lesion, evident from co-localization with CD31 staining, but do not co-localize with Sox9 or Osterix staining. (**F-H, L-N**) GFP⁺/CD31⁻ cells co-localize with both Sox9 and Osterix staining. (Wosczyzna et. al, 2012)

Discussion & Future Directions

Vascular endothelium recently emerged as the best candidate progenitor cell of HO. Other experiments have previously demonstrated the contribution of Tie2⁺ cells to induced heterotopic lesions (Lounev et al., 2009; Wosczyzna et. al, 2012). In order to trace this cell type, we employed the Cre/loxP lineage tracing system. Mice were genotyped to determine which mice were successfully recombined by PCR and detection of fluorescence in tail clippings. We confirmed the efficiency of this reporter by IHC, demonstrating the majority of cells labeled with GFP from the reporter mouse were also positive for an endothelial marker, CD31. We successfully isolated two cell populations by FACs: Tie2⁺/CD31⁺ (endothelial) and Tie2⁺/CD31⁻ (non-endothelial), introduced the cell populations into SCID mice, and induced HO by BMP2 injection. These lesions were analyzed by immunohistochemistry for both chondrogenic (Sox9) and osteogenic (Osterix) marker co-localization with the Tie2 reporter cells.

We demonstrated that a population of Tie2⁺, non-endothelial cells contributed to the heterotopic lesions in multiple phases of the classic endochondral pathway. The same methods of lineage tracing and analysis demonstrated that Tie2⁺, endothelial cells do not contribute to any phases of heterotopic skeletogenesis, but that these cells did contribute to the vascularization of the lesional tissue, consistent with their endothelial origin. This data supports that there is a Tie2⁺, non-endothelial progenitor cell in acquired HO.

These data contradict that of recent studies by Medici, et al that demonstrated progenitor cells of induced heterotopic cartilage and bone were of an endothelial origin (Medici, et al., 2010). Although our work has demonstrated that both endothelial cells in their native in vivo context and FACS-purified endothelial cells do not contribute to heterotopic cartilage or bone, a level of ambiguity remains regarding conclusions to be drawn and require further investigation. Cells expressing Tie2 have been demonstrated to contribute to all stages of BMP2-induced heterotopic lesions, including the present study, but results presented by Lounev, et al. and Wosczyzna, et al. conflict with that presented by Medici, et al. regarding the endothelial origin of these cells (Lounev, et al., 2009; Medici, et al., 2010; Wosczyzna, et al., 2012). Although endothelium is the predominant cell type labeled by transgenic Tie2Cre; *R26^{NG/+}* mice, Tie2 is expressed in many non-endothelial cell types. The Medici, et al. experiments did not delineate between Tie2+ cells of endothelial and non-endothelial origin, assuming that all cells expressing Tie2 were of endothelial origin (Medici, et al., 2010). In addition, an alternative transgenic mouse that labeled endothelium, VE-Cadherin-Cre, verified results that endothelial cells do not contribute to heterotopic cartilage and bone (Wosczyzna, et al., 2012), leading us to conclude that endothelial cells are not a progenitor of bone and cartilage of HO.

The determination of a major resident progenitor cell for HO provides valuable insight into this condition, but also opens the door to research to expand on this knowledge. In order to facilitate further characterization of the cellular and molecular mechanisms by which these cells proceed, further work must be done to

characterize this cell population. Others in our laboratory have already continued this work, determining this cell population expresses markers also expressed by mesenchymal progenitors (PDGFR α and Sca-1), showing a multipotent nature for these cell, and defining the anatomical location of these cells (Wosczyzna, et al., 2012). These progenitor cells were found to be local residents of the skeletal muscle interstitium. The same work in our laboratory has suggested these cells are not restricted to skeletal muscle though, as cells with a similar marker profile were isolated from mouse lung and kidney tissue (Wosczyzna, et al., 2012). The marker profile and anatomical location further distinguished these cells from many other potential progenitors, including pericytes and muscle-derived stem cells (Wosczyzna, et al., 2012).

Despite contribution by the Tie2⁺, non-endothelial cell population to the lesion, nearly half of the cartilage and bone cells were unlabeled. The lack of GFP expression suggests that there may be two or more progenitor cell populations in addition to the one identified in the present study. However, this is not definitive because the unlabeled cells could also be due to inefficient Cre-mediated recombination. Further studies must be done to determine if any other cell populations are contributing to the heterotopic lesion, but the similar anatomical location, multipotent nature, and cell marker characterization of the GFP⁺ cells from the lesional tissue to our population suggests that the inefficient Cre-mediated recombination is the most likely scenario (Wosczyzna, et al., 2012).

Currently, all of the work to identify this cell type has been performed in a model of induced HO, most similar to acquired forms of the disease. Similar studies must be done in a form that recapitulates the genetic forms, which would first require the mouse model with the mutation in the ACVR1/ALK2 receptor to be further established. Use of this model would delineate any potential differences and could strengthen the results of this work. However, the identification of the progenitors is a crucial step in establishing any future therapeutic agents or treatments for HO. Eventually, further knowledge from this research into the initiation of these states will aid in the development of targeted therapies for the treatment and prevention of HO.

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