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Lineage tracking of mesenchymal and endothelial progenitors in BMP-induced bone formation

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ABSTRACT

To better understand the relative contributions of mesenchymal and endothelial progenitor cells to rhBMP-2 induced bone formation, we examined the distribution of lineage-labeled cells in *Tie2-Cre:Ai9* and *αSMA-creERT2:Col2.3-GFP:Ai9* reporter mice. Established orthopedic models of ectopic bone formation in the hind limb and spine fusion were employed. *Tie2*-lineage cells were found extensively in the ectopic bone and spine fusion masses, but co-staining was only seen with tartrate-resistant acid phosphatase (TRAP) activity (osteoclasts) and CD31 immunohistochemistry (vascular endothelial cells), and not alkaline phosphatase (AP) activity (osteoblasts). To further confirm the lack of a functional contribution of *Tie2*-lineage cells to BMP-induced bone, we developed conditional knockout mice where *Tie2*-lineage cells are rendered null for key bone transcription factor *osterix* (*Tie2-cre:Osx*$^{fx/fx}$ mice). Conditional knockout mice showed no difference in BMP-induced bone formation compared to littermate controls. Pulse labeling of mesenchymal cells with Tamoxifen in mice undergoing spine fusion revealed that *αSMA*-lineage cells contributed to the osteoblastic lineage (*Col2.3-GFP*), but not to endothelial cells or osteoclast populations. These data indicate that the *αSMA* + and *Tie2* + progenitor lineages make distinct cellular contributions to bone formation, angiogenesis, and resorption/remodeling.

1. Introduction

Bone is a tissue that developmentally arises from the mesoderm. This is regulated by a variety of signals, however expression of the bone morphogenetic proteins (BMPs) is key factors that modulate this process [1]. Recombinant human BMPs (rhBMPs) are utilized in orthopedic medicine as potent inducers of new bone formation and are approved for the treatment of open fractures and non-unions [2]. When used clinically, cells are exposed to super-physiological concentrations of these factors, which have the potential to transform cells beyond those of the mesenchymal lineages.

In 2009 a study by Lounev et al. examined the contribution of different cell lineages to the bone in a model of rhBMP-2 induced ectopic bone formation and in the Nse-BMP4 transgenic mouse [3]. A variety of transgenic mouse strains employing the Rosa26R (R26R) reporter were used to assess the relative input of *MyoD*-lineage, *Tie2*-lineage, and *SMMHC*-lineage cells. These were intended to reflect the contributions of myogenic, endothelial and mesenchymal progenitors respectively. Ectopic bone formed readily in the muscle, a tissue that is often adjacent to the bone and has been speculated to contain numerous types of cellular progenitors that could contribute to bone formation and repair [4,5].

Lounev et al. reported that *MyoD*-lineage cells made a negligible contribution to rhBMP-2 induced ectopic bone formation [3]. This was later contrasted by studies showing greater *MyoD*-lineage contribution in a variant of the ectopic bone formation model featuring rhBMP-7 as well as increased tissue trauma [6]. Intriguingly, Lounev et al. found that up to 50% of cells in the ectopic bone were of the *Tie2*-lineage. In a subsequent study, the group described a mechanism by which *Tie2*-lineage cells could undergo an endothelial–mesenchymal transition (EMT) to form osteoblasts [7]. While this study focused on describing the genetic disease *fibrodysplasia ossificans progressiva*, it was unclear whether EMT could also occur when endothelial cells were exposed to high concentrations of rhBMPs.

The potential of endothelial *Tie2*-lineage cells to contribute to bone repair has been further implied by a range of cell transplantation studies. Purified endothelial progenitor cells (EPCs) have been repeatedly demonstrated to enhance orthopedic repair [8–12], although the precise mechanism for this is unclear. EPCs have been shown to

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enhance angiogenesis, potentially by secretion of pro-angiogenic growth factors [13], or by direct contribution to new vessels. Transplanted EPCs have also been shown to enhance tissue mineralization [9], raising the possibility of a direct contribution of these cells to bone repair.

In 2012 Wosczyna et al. published a report indicating that the *Tie2*-lineage cells included multiple sub-populations of cells, some capable and some incapable of contributing to rhBMP-2 induced cartilage and bone formation [14]. The populations that formed the cartilage and bone were not of endothelial origin, suggesting that EMT was not the underlying mechanism in this model. Both the Lounev and Wosczyna studies utilized a *Tie2*-cre line that demarks endothelial and hematopoietic, progenitors as well as a subset of mesenchymal-like progenitors [15].

In this study we report the contribution of endothelial cells to rhBMP-2 induced ectopic bone formation and spine fusion using an alternative *Tie2*-cre mouse, which shows greater specificity for the endothelial lineages [16,17]. These cell tracking studies employ a Cre-dependent fluorescent tdTomato reporter that allows for co-labeling with chondrocyte, osteoblastic, osteoclastic, and vascular markers [18]. Conditional knockout mouse models were also employed where *Tie2*-lineage cells selectively inactivated the key osteogenic transcription factor *osterix*. This approach allowed for the functional assessment of the contribution of cells of the *Tie2*-lineage to new bone formation. To complement these experiments we examined the contribution of mesenchymal progenitors using mice with an inducible *αSMA*-creERT2 transgene. Prior studies have shown that this cell lineage directly contributes to bone formation and repair [19–22].

2. Materials & methods

2.1. Mouse lines and genotyping

The *Ai9* reporter line (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J) was purchased from Jackson Laboratories [18]. The *Tie2-cre* line (B6.Cg-Tg(Tek-cre)12Flv/J) was sourced from the Garvan Institute for Medical Research with permission from the original laboratory [23]. The $Osx^{fx/fx}$ line was sourced from Benoit de Crombrugghe (MD Anderson Cancer Center, Houston, TX, USA) [15]. The *Tie2-cre* × *Ai9* line was generated in-house by cross breeding and showed no adverse phenotype; the line specifically labels Tie2-lineage with a fluorescent tdTomato reporter. The *Tie2-cre* × $Osx^{fx/fx}$ line was generated by crossing for two generations to create homozygous conditional double knockout mice; in this line there is targeted disruption of the key osteoblastic gene *osterix (Osx)* in all cells of the *Tie2*-lineage. Mice were genotyped from ear biopsies using real time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN, USA). Experiments using the *Tie2-cre* cross strains were approved by the CHW/CMRI Animal Ethics Committee (Protocol K248, K303). All strains were on the C57BL6/J background, and hemizygous strains were maintained on wild type mice purchased from the Animal Resources Centre (ARC, Perth, Australia).

αSMA-creERT2 (smooth muscle α-actin promoter) × *Ai9* mice, which enable labeling of mesenchymal progenitors [19,20] that also incorporated a GFP transgene under the control of the upstream 2.3 kb region of the *Col1a1* promoter (*Col2.3-GFP* transgene) to label osteoblasts [24] were sourced from in-house colonies at the University of Connecticut (Farmington, CT, USA). Experiments using these mice were approved by the UConn animal ethics committees.

2.2. Surgical models

Implants were manufactured by adding 20 μl sterile saline containing 5 μg recombinant human BMP-2 (Medronic Australasia, North Ryde, Australia) onto collagen-hydroxyapatite sponge prior to surgery. For the ectopic bone formation model, uniform discs manufactured using a surgical tissue punch were surgically implanted into the hind limbs of mice using published methods [25]. For the spine fusion model, twin porous collagen sponges (Medronic Australasia) were inserted parallel to the vertebrae as previously described [26]. All mice were aged 8–12 weeks. Anesthesia was induced with inhaled isoflurane (hind limb model) or using Ketamine/Xylazine (35 mg/kg; 5 mg/kg) (spine fusion model). Buprenorphine (0.05–0.1 mg/kg) was administered subcutaneously (s.c.) preoperatively and every 12 h as required for pain management. Post-surgery saline was given s.c. to prevent dehydration. Mice of the *αSMA-creERT2* × *Ai9* cross received tamoxifen at a dose of 75 mg/kg on the day of surgery to induce lineage labeling. Mice were euthanized at 7, 14, or 21 days (ectopic bone) or 2, 10, or 17 days (spine fusion) for radiological and/or histological analyses.

2.3. Radiological imaging

The formation of rhBMP-2 induced bone was confirmed and visualized by digital X-ray (25 kV, 2× magnification; Faxitron X-ray Corp, Illinois, USA). For studies where ectopic bone was quantified, samples were scanned by micro-computed tomography (microCT) using a SkyScan 1174 compact microCT scanner (Kontich, Belgium) at a pixel resolution of 14.8 μm. All samples were scanned in 70% ethanol, using a 0.5 mm aluminum filter, 50 kV X-ray tube voltage, and 800 μA tube electric current. A global threshold to define bone tissue in pellets was set at a mineral density of 0.3 g/cm^3. Images were reconstructed using NRecon, version 1.6.1.7 (SkyScan), and analyzed using CTAnalyser software, version 1.11.8.0 (SkyScan).

2.4. Immunofluorescence and microscopy

The fluorescent signal from the tdTomato reporter strain was captured using either an epi-fluorescent microscope or a Leica TCS SP5 confocal laser scanning microscope. The sections were cover-slipped and the intrinsic fluorescent signal imaged directly. ELF97 Phosphatase Substrate (Molecular Probes) was used to detect alkaline phosphatase (AP) and tartrate-resistant acidic phosphatase (TRAP). AP assay was done according to the manufacturer's instructions. For TRAP: The classical TRAP buffer (110 mM acetate buffer, pH 5.2, 1.1 mM sodium nitrite, 7.4 mM tartrate) was used followed by 200 μM concentration of ELF97 incubation for 5-min. Slides were washed with an EDTA containing wash buffer. The fluorescence was visualized with a DAPI/Hoechst longpass filter set.

For examining co-labeling between tdTomato signal and immunofluorescent staining for lineage markers, the following methods were used. Slides were rehydrated in PBS for 20 min and treated with 0.5% Triton X-100 for 20 min. The sections were blocked in 10% goat serum PBS for 1 h at room temperature prior to incubation with a primary antibody (SOX9, Millipore AB5535; CD31, BD Pharmingen MEC13.3) in blocking buffer overnight at 4 °C. After washing the sections were incubated with Alexa-Fluor-647 conjugated secondary antibody (Molecular Probes) diluted in PBS. Tissue was counter stained with 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI, molecular probes) for 1 min and washed in PBS and mounted in Aqueous Mounting Medium (DAKO). Images were captured using an epi-fluorescent microscope with appropriate filters or using the Leica TCS SP5 confocal laser scanning microscope.

2.5. Statistical analyses

Statistical analysis of microCT data was conducted with non-parametric testing as amount of bone formed by these assays has not been determined to follow a normal distribution. Significant differences in bone volume were determined using a Mann Whitney *U* test with a cutoff of $\alpha < 0.05$ (Graphpad Prism, La Jolla, CA, USA).