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Glast-Expressing Progenitor Cells Contribute to Heterotopic Ossification

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Abstract

Heterotopic ossification (HO), acquired or hereditary, is the formation of true bone outside of the normal skeleton. Although the lineages of cells contributing to bone formation during normal development are well defined, the precise lineages of cells that contribute to HO are not clear. This study utilized Cre-lox based genetic lineage tracing to examine the contribution to HO of cells that expressed either FoxD1 or Glast. Both lineages contributed broadly to different normal tissues, and FoxD1-cre labeled cells contributed to normal bone formation. Despite the similarity in labeling patterns of normal tissues, and the significant contribution of FoxD1-cre labeled cells to normal bone, only Glast-creERT labeled progenitors contributed significantly to HO at all stages, suggesting the cell populations that normally contribute to physiological bone formation, such as the Foxd1-cre labeled cells, may not participate in pathological HO. Further, identification of Glast-expressing cells as precursors that give rise to HO should help with molecular targeting of this population both for the prevention and for the treatment of HO.

Keywords

Heterotopic Ossification (HO); Fibrodysplasia Ossificans Progressiva (FOP); Glast-creERT; FoxD1-cre; genetic lineage tracing; bone morphogenetic protein (BMP)

INTRODUCTION

Heterotopic ossification (HO) is a process in which true bone tissue forms outside of the skeleton. Acquired HO is a common and costly complication of a variety of types of traumatic events including fracture, total joint replacement (TJR), traumatic brain injury (TBI), spinal cord injury (SCI), or combat-related trauma [1]. Between 16%–53% of patients with TBI (1.7 million annually) or SCI (11,000 annually) and 40–50% of patients with TJR (700,000 annually) develop HO at some point, and about 10% of HO is symptomatic resulting in limitations in range of motion. By contrast, hereditary HO (Fibrodysplasia

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Ossificans Progressiva, FOP) is a rare, progressive and potentially life-threatening disorder [2]. Mutations of ACVR1, a type I bone morphogenetic protein (BMP) receptor, cause FOP, suggesting that disturbances in the normal homeostasis of BMP signaling leads to the disorder [3]. The genesis of acquired HO has been linked to a combination of inflammatory triggers and dysregulated BMP signaling[4]. However, the identity of the precursor cells that differentiate along the osteo-chondrogenic cell lineage to form HO remains unclear [5, 6].

Previous studies have suggested various candidate precursor populations of both mesoderm and endoderm origin that could contribute to HO formation. For example, endothelium/endothelial precursors[7], circulating osteogenic precursor (COP) cells[8], satellite cells[9], perivascular mesenchymal cells[10], inducible osteogenic precursors[11], pericytes [12], multi-potential mesenchymal progenitors[13], or local mesenchymal stem/progenitor cells[14] all have been implicated in bone formation in response to BMP signaling or other situations. But most of these candidate populations have not been confirmed by more vigorous genetic lineage tracing. Cre-lox based genetic lineage tracing is a powerful technique[15] that permanently labels both cre recombinase (cre)-expressing cells and all their daughter cells, and it is commonly accepted as the gold standard in defining whether a specific lineage contributes to normal organogenesis or pathological processes, such as HO. However, the efficiency and the specificity of labeling, and the final interpretation of the data, are heavily dependent on the cre lines, which frequently label heterogeneous rather than homogenous populations. At least 10 different cre lines have been used thus far to evaluate the contribution of different candidate populations to HO [7, 13, 14, 16]. These cre lines cover a broad spectrum of populations, including hematopoietic, myogenic, somitogenic, vascular endothelium, vascular smooth muscle and pericytes population. However only one of these cre labeled subpopulations, Tie2-cre labeled cells, has been found to contribute significantly to HO by both Lounev et al.[7] and Woszczyna et al.[13]. Since Tie2 is expressed by endothelium/endothelial precursors, this initially suggested that endothelium/endothelial precursors might contribute to HO. However another cre line which specifically labels endothelium/endothelial precursors, VE-Cadherin-Cre, did not contribute to HO, indicating that the Tie2⁺ progenitors that contribute to HO may be not of endothelial origin (12). Woszczyna et al have proposed that a subpopulation of mesenchymal progenitors (Tie2⁺PDGFR⁺Sca-1⁺) that resides in the skeletal muscle interstitium contributes to HO (12). Notably, in both studies Tie2 labeled only about half of the cells indicating there are also other major unidentified contributing populations.

Based on this observation, we propose to test additional cre lines so that we can cover broader lineage range, and also further narrow down the potential target populations. We focused on two promising cre lines, Glast-creERT [17] and the FoxD1-cre[18], because we found that these two cre lines label potential contributing populations in tissues where HO is often first found i.e., in subcutaneous connective tissue and in the interstitium of skeletal muscles of hind limbs.

Glast-creERT is a BAC transgenic line that expresses inducible CreERT under the control of the Glast (glial high affinity glutamate transporter, also called the Slc1a3) promoter. In neural tissues, Glast is expressed primarily by astrocytes[19] but has also been detected in ventricular ependymal cells[19, 20], Bergmann glia[21], specific layers of the meninges, satellite cells of the dorsal root ganglion[22], and neural stem cells[23]. In non-neural tissues, Glast expression has been detected in epithelial cells, cells of the macrophage-lineage, lymphocytes, fat cells, interstitial cells in connective tissue, salivary gland acini [24] and pericytes[17].

FoxD1-cre is a knock-in line that expresses an eGFP-Cre fusion protein from the endogenous FoxD1 allele. FoxD1(also called brain factor 2, BF-2) is a forkhead family

transcription factor that has been implicated in variety processes including specification of the temporal retina[25], formation of the optic chiasm[26], coordination of NF- κ B and NFAT antagonism[27], and cellularity in the renal capsule and renal development[28]. Consistent with these functions, FoxD1 is expressed in ventrotemporal retina, rostral diencephalon, and stromal cells in kidney. The FoxD1-cre line has been used only to label the mesenchymal population (stromal cells/pericytes) in the kidney [29], and the labeling pattern of FoxD1-cre in other tissues has not been explored.

In this study, we first further characterized the labeling pattern of these two cre lines with a new conditional reporter line, Rosa-CAG-LSL-ZsGreen1-WPRE (hereafter Zsgreen) which conditionally expresses ZsGreen1, one of the brightest fluorescent proteins currently available [30]. We found that these two cre lines have different, broad labeling activities, but both cre lines labeled similar mesenchymal populations in our target tissues. Remarkably, even though FoxD1-cre labeled cells contributed significantly to normal endogenous bone formation, the contribution of FoxD1-cre labeled population to HO was negligible. By contrast, even though Glaxt-creERT labeled cells do not contribute significantly to normal bone formation, they contribute significantly to HO at all stages. Identification of this specific subpopulation of cells as a major contributor to HO may provide a cellular/molecular target for possible therapeutic intervention to prevent or treat HO.

MATERIALS AND METHODS

Animals and Injury Models

The Nse-BMP4 transgenic mice used in this study have been described previously [4, 14, 31]. This line overexpresses BMP4 under the control of neuron-specific enolase (Nse) promoter and develops injury induced HO robustly. The Glaxt-creERT (Tg(Slc1a3-cre/ERT)1Nat/J) [17] and FoxD1-cre (B6;129S4-Foxd1tm1(GFP/cre)Amc/J)[18] lines, and the Zsgreen reporter line (B6.Cg-Gt(ROSA)26Sortm6(CAG-ZsGreen1)Hze/J)[30] were from the Jackson Laboratory (Bar Harbor, ME). The Zsgreen reporter mouse has a loxP-flanked STOP cassette that prevents transcription of the downstream enhanced green fluorescent protein (ZsGreen1) in the absence of cre. However, when bred to mice that express cre, the STOP cassette is permanently deleted in cre-expressing cells resulting in constitutive expression of ZsGreen1 in those cells and all their progeny. To induce Glaxt-cre expression, tamoxifen was injected intraperitoneally into adult (>1 month) mice as previously reported [17]. In the FoxD1-cre mice, cre is expressed as an eGFP-cre fusion protein; however, since the fluorescence signal from eGFP-cre protein is so much weaker than ZsGreen1 that its contribution to the fluorescence signal is insignificant. All animal experiments in this study were approved by the Animal Care and Use Committee at Northwestern University.

Genetic Lineage Tracing

Genetic lineage tracing was performed as described previously [14]. Briefly, Zsgreen conditional reporter mice were first mated with Nse-BMP4 mice. The Zsgreen; Nse-BMP4 double transgenic mice were selected and mated with the two cre lines, separately (suppl. Fig. 1). For the purpose of further characterizing the cre-labeled cells, adult (>1 month old) double transgenic mice FoxD1-cre; Zsgreen and tamoxifen-induced adult Glaxt-creERT; Zsgreen mice were selected from the second round of crossing. After the tamoxifen injections, tissues were harvested and fixed in 4% PFA overnight for histological studies. To evaluate the contributions of labeled cells to HO, skin injuries were performed on adult triple transgenic mice, as described previously [14]. Briefly, adult (> 1 month old) transgenic mice were shaved first, and then a 5-mm sharp, shallow cut was made through the skin but sparing the muscles. The injured and control hind legs were harvested at different time points (1, 2, 3 and 4 weeks after injury) after the injuries for further histological and

immunohistological examinations. Uninjured triple transgenic mice (Nse-BMP4; FoxD1-cre; Zsgreen and tamoxifen-induced Nse-BMP4; Glst-creERT; Zsgreen) were also selected to study the patterns of labeled cells.

Histology and Immunohistochemistry

Alkaline phosphatase and Alcian blue staining and Immunostaining for different markers were performed as previously described [14]. Briefly, sections were pre-fixed with 4% paraformaldehyde in PBS. Nonspecific binding was blocked with 10% normal serum diluted in 1% bovine serum albumin (BSA; Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.25% Triton X-100 (Sigma) for 1 hour in room temperature. The sections were then incubated with primary antibodies diluted with 1% BSA + 0.25% Triton X-100 at 4°C overnight. The sections were then incubated with appropriate secondary antibodies (Cy3 or Cy2 conjugated antibodies (Jackson ImmunoResearch Laboratories) diluted with 1% BSA + 0.25% Triton X-100 or Alexa Fluor 488, Alexa Fluor 594, and Alexa 647 (1:1000, Invitrogen) in the dark at room temperature for 2 hours. Counterstaining was then performed with 4,6-diamidino-2-phenylindole (1:5000). Primary antibodies against S100A4 (mIgG1, Novus, 1F12-1G7), von Willebrand factor (mIgG1, abcam, ab68545), α -smooth muscle actin (SMA, clone 1A4, mIgG2a, sigma, A5228), STRO1 (mIgM, Santa Cruz, sc-47733), PDGFR (Rb, Cell Signaling, #5241), PDGFR (Rb, Abcam, ab32570), ALP (Rb, Abcam, ab65834), ColII (mIgG2a, Developmental Studies Hybridoma Bank, CII1) and Tie2 (Rb, Santa Cruz, sc-9026) were used in this study.

RESULTS

Glast-creERT and FoxD1-cre mediated recombination in all tested tissues

The Glast-creERT line has been used in Cre-lox based studies in the central nervous system [23], and the FoxD1-cre line has been used to label renal stromal cells/pericytes of the kidney [28], but the labeling activities in other tissues are largely unknown. We therefore first characterized the labeling patterns of these two lines with Zsgreen to explore whether these lines might be useful for labeling cell populations that potentially contribute to HO. To achieve a relative unbiased conclusion, we examined the Glast-creERT labeled cells in many different adult tissues of Glast-creERT; Zsgreen double and uninjured Glast-creERT; Zsgreen; Nse-BMP4 triple transgenic mice, generated according to previous report [14] (suppl. Fig. 1). Similarly, we also examined the FoxD1-cre labeled cells in adult tissues of FoxD1-cre; Zsgreen double and uninjured FoxD1-cre; Zsgreen; Nse-BMP4 triple transgenic mice. The tested tissues included brain, spinal cord, spleen, thymus, liver, kidney, heart, lung, tail, ear, skin and muscles (hind limbs). Even though the pattern of both Glast-creERT labeled cells and FoxD1-cre were generally consistent with the previous reported expression patterns of endogenous Glast and FoxD1 respectively [19–24], we found labeled cells in most tested tissues (suppl. Fig. 2&3 and data not shown). This suggests that both cre lines have broader tissue labeling activities than indicated by the original studies [18, 23]. The sections from Glast-creERT; Zsgreen double and uninjured Glast-creERT; Zsgreen; Nse-BMP4 without tamoxifen induction displayed no detectable fluorescence signal, confirming that the expression of Glast-CreERT transgene was strictly tamoxifen dependent (data not shown).

Our target tissues in this study for injury with subsequent HO formation were the hind limbs. Glast-creERT labeled cells were observed in hind limbs in subcutaneous connective tissue (Fig. 1A&B) and in the interstitium of skeletal muscles (Fig. 1C&D), where HO is often first found. Furthermore, in the interstitium of skeletal muscles most Glast-creERT were closely associated with vasculature (Fig. 1C&D). In contrast, in skin and subcutaneous connective tissue, there were many scattered fibroblast-like single cells or cells clusters, but

they were not randomly distributed; for example, in subcutaneous connective tissue, labeled cells formed band-like structures (Fig. 1A). Importantly, we found that Glax-creERT labeled cells, did not contribute significantly to normal endogenous bone formation of tail vertebrae (Fig. 1E&F) and long bone (Fig. 1G&H).

Similar to Glax-creERT, FoxD1-cre labeled cells were also found in our target tissues, i.e., in subcutaneous connective tissue (Fig. 2A&B) and in the interstitium of skeletal muscles (Fig. 2C&D). Interestingly, FoxD1-cre labeled cells were also found to be closely associated with vasculature (Fig. 2C&D). However, unlike Glax-creERT, FoxD1-cre labeled cells, contributed significantly to normal endogenous bone formation in thoracic vertebrae (Fig. 2E&F) tail vertebrae (Fig. 2G&H) and the articular cartilage at the end of long bone (Fig. 2I&J). This data strongly suggested that, unlike Glax-creERT, the FoxD1-cre cells labeled population contributed to normal skeletogenesis.

Both Glax-creERT and FoxD1-cre labeled mesenchymal cells predominately in target tissues

Based on morphology and anatomic context, both FoxD1-cre and Glax-creERT predominately labeled mesenchymal populations in our target tissues (Fig. 1&2). To further clarify the identity of these labeled cells, we examined the co-localization in the cre-labeled cells of endothelial and mesenchymal cell and other available markers. The specificity of these primary antibodies was first tested by comparing the signals of adjacent sections that incubated with primary antibodies and with specie matched normal serum (suppl. Fig. 4), and data supported that all the primary antibodies used in this study are specific. Further co-localization study found that neither Glax-creERT nor FoxD1-cre co-localized with von Willebrand factor (an endothelial marker) or α -smooth muscle actin (SMA, a smooth muscle cell marker) (Fig. 3). Notably, Glax-creERT labeled cells extensively co-labeled with S100A4 (also called FSP1), a mesenchymal progenitors/stem cell marker, while there was no appreciable co-labeling of the FoxD1-cre cells with S100A4 (Fig. 4A&C), while both co-localized with STRO1, another mesenchymal progenitor/stem cell marker (Fig. 4D&F). Another interesting finding was that the colocalization of Glax-creERT labeled cells with mesenchymal progenitors/stem cell marker observed in uninjured animals was remarkably diminished once the animals were injured, suggestion the loss of stemness of Glax-creERT labeled cells (comparing Fig. 4A&B, also Fig. 4D&E). In addition, the co-localization of Glax-creERT labeled cells with Tie2 is very limited in all tested tissues (Fig. 4G, showed the co-labeling in the subcutaneous tissue, and suppl. Fig. 5A–D, showed the co-labeling in the lesional tissues). Similarly, the co-localization of FoxD1 labeled cells with Tie2 is also very limited (suppl. Fig. 5E&F and data not shown). In addition, subsets of Glax-creERT labeled cells also co-labeled with PDGFR β (Fig. 4H in small vessels) and PDGFR α (Fig. 4I in muscle).

Overall, these observations indicate that Glax-creERT and FoxD1-cre labeled cells in our target tissues are of mesenchymal rather than endothelial origin, but that the Glax-creERT and FoxD1-cre labeled populations are not the same.

Glax-creERT labeled but not FoxD1-cre labeled cells contribute significantly to HO at all stages

To directly test if Glax-creERT labeled mesenchymal progenitors contribute to HO, we first induced cre expression in adult triple transgenic mice (Nse-BMP4; Glax-creERT; Zsreen) by tamoxifen injection [17], and we then performed skin injuries on these mice according to previously established procedures [14]. The mice were then sacrificed at different time points (1, 2, 3 and 4 weeks) to trace the contribution of labeled cells at different stages of HO. The consistent finding was that Glax-creERT labeled progenitors contributed

significantly to HO at all stages (early fibroproliferative lesions, chondrocytes, and later bone formation), even though the efficiency of tamoxifen dependent labeling varied among different animals (Fig. 5&6G and data not shown). Alkaline phosphatase (ALP) and Alcian blue staining with adjacent sections suggested that these regions were a heterotopic osteogenic region (Fig. 5B&C). Further double staining of Glast-creERT cells with ALP (an osteoblast marker) and ColIII (a chondrocyte marker), using sections from different time points, confirmed that Glast-creERT labeled cells were observed in different stages of HO (Fig. 5D–F). A substantial subset of these cre labeled cells co-labeled with osteoblast or chondrocyte markers indicated that Glast-creERT labeled cells truly contribute to HO. H&E staining with adjacent sections further supported the conclusion (Fig. 5G–I). We also performed similar injuries to Nse-BMP4; FoxD1-cre; Zsgreen triple transgenic mice and sacrificed mice at the same time points as the Glast-creERT mice. Surprisingly, even though FoxD1-cre labeled cells contribute significantly to normal endogenous bone formation (Fig. 2), FoxD1-cre labeled progenitors contributed negligibly to HO at any stage (Fig. 6).

DISCUSSION

Although there is substantial debate about the cellular origins of HO, it has generally been thought that the processes leading to formation of normal bone and HO are largely the same, since HO produces bone that faithfully replicates the appearance of normal bone. However, we found that FoxD1-cre labeled cells contributed to normal skeletogenesis but not HO at any stage whereas Glast-creERT cells were major contributors to HO. The most parsimonious interpretation of this data are that Glast-creERT and FoxD1-cre labeled cells are different populations of cells, and there may be some fundamental differences between normal and pathological bone formation. In support of this concept, we notice that pathological bone formation is often closely associated with inflammation, while normal bone formation is not. Alternatively, it is possible that, adult Glast-creERT labeled populations may be more plastic, while FoxD1-cre labeled cells have mostly committed or terminally differentiated (lost stemness) in the adult, even though they may have osteogenic potential during the developmental stage. It is worthy to mention that the lack of FoxD1-Cre-positive cells in HO could not be due to lack of FoxD1-Cre expression in adult mice, simply because that the cre-induced labeling is permanent.

Tie2-cre and Glast-creERT labeled progenitors are the only cre labeled populations (out of 12 tested – see Table 1) that have been shown to contribute significantly to HO. There are a number of similarities between these two populations of cells, but there are also some significant differences. Both labeled populations are closely associated with the vasculature (fig. 1, 3&4 and [32]), non-randomly distributed single cells or cell clusters were observed in both cases, and subsets of both populations co-labeled with mesenchymal progenitors markers (fig. 4 and [17, 33]). However Tie2 is known to be expressed by at least 3 distinct cell types: endothelial cells of endoderm origin, proangiogenic monocytes cells of hematopoietic origin, and pericyte precursors of mesenchymal origin [33]. In contrast, neither our study nor previous ones have shown any evidence that Glast is expressed by endothelial populations (fig. 3), and Glast-creERT labeled populations were predominately mesenchymal in our target tissues. Even though the exact relationship between the previously identified Tie2-cre⁺ and the Glast-creERT⁺ cells is still unclear, our data strongly suggested that they are not the same population (fig. 4G and supplementary fig. 5A–D). However, our findings are consistent with the idea that local multipotential mesenchymal progenitors/stem cells contribute to HO[13].

Pericytes are among the populations of cells in the central nervous system that express Glast-creERT[17]. The original definition of a pericyte was based on anatomical features, and it is generally accepted that pericytes have an intimate relationship with mesenchymal

stem cells. But more recently, it has become appreciated that these cells themselves have stem/progenitor cell-like features [34, 35]. However, it is still unclear if pericytes are all mesenchymal stem cells, or vice versa [17, 36, 37]. The characteristic anatomic distribution and the labeling pattern of Glast-creERT labeled cells in our study (fig. 1C&4H) are similar to those of the Glast-expressing pericytes that contributed to scar-forming stromal cells in response to spinal cord the injuries in a previous study[17]. Interestingly, however, previous lineage tracing study showed no significant contribution of pericytes to HO (Table 1), using a NG2-Cre line.

Since there are no other specific markers of pericytes, it is unclear whether some or all of the Glast-creERT labeled cells in this study are pericytes. This highlights the point that cre lines generally label more than one cell population which limits the conclusions that can be drawn [7, 17, 18, 23, 38, 39]. Nevertheless our identification of Glast-expressing cells as precursors that give rise to HO should help with molecular targeting of this population both for the prevention and for the treatment of HO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Both FoxD1-cre and Glast-creERT labeled cells contribute broadly to different normal tissues.
- FoxD1-cre labeled cells contribute to only normal bone.
- Glast-creERT labeled progenitors contributed only to HO.
- The cell populations that contribute to physiological bone formation may not participate in pathological HO.
- Identification of Glast-expressing cells as precursors that give rise to HO should help with therapeutic interventions.

Figure 1. Glast-creERT labeled cells were found in target tissues but not in normal bones
A–D) cross-section images from hind limbs of Glast-creERT; Zsgreen mice show general patterns of labeled cells in target tissues: scattered labeled cells in skin, more labeled cells in subcutaneous connective tissue, and a few within muscle fiber bundles, but more in interstitium. A) Many Glast-creERT labeled cells in subcutaneous connective tissue form a band-like structure. C) within muscles of hind limbs of Glast-creERT; Zsgreen mice, labeled cells are mostly found in the interstitium of skeletal muscles. More interestingly, many labeled cells are closely associated with vascular structures. B&D) show the corresponding histological images (H&E staining of adjacent sections of A&C, respectively). The black arrows in D) point to blood vessels in muscle tissue. E–H) show that Glast-creERT labeled

cells within normal skeleton are negligible, even though many of these cells were closely associated with normal bones, enriched in periosteum. E) Glast-creERT labeled cells were found in periosteum of tail vertebrae, but very few labeled cells were found within the osseous tissue of tail vertebrae. F) shows the corresponding histological image of (E). G&H) Glast-creERT labeled cells were found in periosteum and surrounding connective tissues of long bones (femur shown here), but very few labeled cells were found within the osseous tissue. G) is a high power image of white box in H). H) is a low power image of femur. The white broken line in (E&F) indicated surface contour of the articular cartilage and the boundary between the tail vertebrae and the surrounding connective tissue. The white broken line in G) indicated the boundary between the long bone and the surrounding connective tissue. Bar=80 μ m.

Figure 2. FoxD1-cre labeled cells were found both in target tissues and normal bones
A–D) cross-section images from hind limbs of FoxD1-cre; Zsgreen mice show general patterns of labeled cells: scattered labeled cells in skin, more labeled cells in subcutaneous connective tissue, and a few within muscle fiber bundles, but more in interstitium. A) FoxD1-cre labeled cells in subcutaneous connective tissue form a less obvious band-like structure. C) within muscles of hind limbs of FoxD1-cre; Zsgreen mice, labeled cells are mostly found in the interstitium of skeletal muscles, and many labeled cells are closely associated with vascular structures. E, G&I) show FoxD1-cre labeled cells in normal skeleton. Representative images of cross section of thoracic vertebrae (E), longitudinal section of tail vertebrae (F), and articular cartilage of knee (G) are shown. B, D, F, H&J) show the

corresponding histological images (H&E staining of adjacent sections of A, C, E, G&I, respectively). The black arrows in D) point to blood vessels in muscle tissue. The broken lines in (G&H) indicated the surface contour of the articular cartilage of the tail vertebrae. Note that, in cancellous bone (E), the FoxD1-cre labeled cells were enriched in trabeculae (T), not in bone marrow (BM). Bar=80µm.

Figure 3. Glaxt-creERT and FoxD1-cre labeled cells were not appreciably co-labeled with SMA or with vW in hind limbs

A) Glaxt-creERT labeled cells were closely associated with α -smooth muscle actin (SMA)-expressing cells but no co-labeling was observed. Insert shows a typical high power image of semi-longitude section of a small blood vessel. B) FoxD1-cre labeled cells also closely associate with SMA but no co-labeling was observed. Insert shows a typical high power image of semi-cross section of a small blood vessel. C) Glaxt-creERT labeled cells were closely associated with von Willebrand factor (vW) expressing cells but no co-labeling was observed. Insert shows a typical high power image of a small cluster of small blood vessels. D) FoxD1-cre labeled cells were closely associated with vW but no co-labeling was

observed. Insert shows a typical high power image of semi-longitude section of a small blood vessel. E&F) show the corresponding histological images of A&C, and B&D, respectively. The black arrows in E&F) point to blood vessels in muscle tissue. Bar=100 μ m.

Figure 4. Glaxt-creERT and FoxD1-cre labeled cells show different patterns of co-labeling with markers of mesenchymal precursors/stem cells

A–A'') Glaxt-creERT labeled cells were extensively co-labeled with S100A4, a mesenchymal stem cell marker, in uninjured subcutaneous connective tissue. A' & A'' show the split channel of Glaxt-creERT and S100A4, respectively. B–B'') in contrast, in lesion tissues in triple Glaxt-creERT; Zsgreen; Nse-BMP4 mice, only a few labeled cells in the edge of lesion still keep high levels of S100A4 expression (perhaps suggesting the loss of stemness). B' & B'' show the split channel of Glaxt-creERT and S100A4, respectively. C–C'') FoxD1-cre labeled cells, however, have no co-labeling with S100A4 even in uninjured subcutaneous connective tissue. C' & C'' show the split channel of FoxD1-cre and S100A4, respectively. D–D'') Glaxt-creERT labeled cells were extensively co-labeled with STRO1, a marker of mesenchymal stromal cell precursors, in uninjured subcutaneous connective tissue. D' & D'' show the split channel of Glaxt-creERT and STRO1, respectively. E–E'') similar to (B), but in contrast to (D), in lesion tissues in triple Glaxt-creERT; Zsgreen; Nse-BMP4 mice, only a few labeled cells at the edge of lesion had high levels of STRO1 expression. E' & E'' show the split channel of Glaxt-creERT and STRO1, respectively. F–F'') unlike (C), many FoxD1-cre labeled cells co-labeled with STRO1 in uninjured subcutaneous connective tissue. F' & F'' show the split channel of FoxD1-cre and STRO1, respectively. G–G'') Glaxt-creERT labeled cells were closely associated with Tie2⁺ cells in many tissues (uninjured subcutaneous connective tissue showed here), but co-labeled cells were rare. G' & G'' show the split channel of Glaxt-creERT and Tie2, respectively. H–H'') a subset of

Glast-creERT labeled cells that co-labeled with PDGFR- β is closely associated with small blood vessels in muscle tissue. H'&H'' show the split channel of Glast-creERT and PDGFR- β , respectively. I-I'') a subset of Glast-creERT labeled cells in normal muscle tissue co-labeled with PDGFR- β . I'&I'' show the split channel of Glast-creERT and PDGFR- β , respectively. J-L) images of H&E staining show the typical contexts of tissue in (A-I): J) shows the typical tissue context of (A, C, D, F&G), K) shows the typical tissue context of (B&E), and L) shows the typical tissue context of (H&I). White arrows in all the panels point to the co-labeled cells, and broken lines in (B, E&K) indicate the edges of the lesions. Note the obvious "inside-out" maturation pattern in (K). Bar=60 μ m.

Figure 5. Glast-creERT labeled cells contribute significantly to HO at all stages

A) Typical low power image of an early HO lesion in Glast-creERT; Zsgreen; Nse-BMP4 mice showed substantial numbers of Glast-creERT labeled cells almost exclusively located in the lesion area. White broken line outlines the lesion region. B) ALP staining in the section adjacent to (A) confirmed that majority of cells in the lesion site express high level of ALP (dark blue staining), a sign of osteogenic commitment. C) Consistently, Alcian blue staining of the adjacent section confirmed the up-regulation of sulfated and carboxylated acid mucopolysaccharides or sialomucins that are specifically enriched in cartilage and chondrocytes, in the lesion site. D-F) showed typical higher power images from early (D), middle (E) and late (F) stage of endochondral HO, which demonstrated the morphologies and osteogenic or chondrogenic commitment of Glast-creERT labeled cells at different stages. D) At the early fibroproliferative stage, there are many Glast-creERT labeled cells in the lesion; many of these labeled cells showed elongated fibroblast-like morphologies, and many of them were also ALP⁺. E) At the chondrocyte/hyperchondrocyte stage, there are also many Glast-creERT labeled cells; interestingly, many of these labeled cells have lost the elongated fibroblast-like morphology, but are ColIII⁺. F) At the final stage, in mature trabeculae, there are also many Glast-creERT labeled cells. The morphology of labeled cells varied greatly at this stage, but most of them were ALP⁺. Note that Glast-creERT labeled cells were enriched in mature trabeculae, not in bone marrow. Also note that Glast-creERT labeled cells normally account for less than 50% of total cells within the lesions, regardless of the stage. White arrows in (D-F) point to the co-labeled cells. G-I) H&E staining of

adjacent sections show the typical morphological features of early (G, adjacent to D), middle (H, adjacent to E) and late (I, adjacent to F) stage of endochondral HO. Bar=80 μ m.

Figure 6. FoxD1-cre labeled cells do not contribute significantly to HO

A) Typical low power image of early lesion in FoxD1-cre;Zsgreen;Nse-BMP4 mice showed no detectable FoxD1-cre labeled cells in the lesion area. B) Typical low power image of a late lesion (mature stage) in FoxD1-cre; Zsgreen; Nse-BMP4 mice showed no substantial FoxD1-cre labeled cells in the lesion area even though some scattered labeled cells were observed in areas surrounding the lesion. C&D) ALP staining in the adjacent section to A) and B), respectively, confirmed that many cells in the lesion site express high levels of ALP (dark blue staining), a sign of osteogenic commitment. White broken line outlines the lesion regions in (A&B). E&F) H&E staining of adjacent sections show the typical morphological features of early (E, adjacent to A), and late (F, adjacent to B) stage of endochondral HO. G)

quantification the contribution of *Glast*-creERT and *FoxD1*-cre labeled cells to different stages of HO. The percentages of Zsreen positive cells over the total cells in the area (total counts of DAPI) are plotted. Since most of cells within the well-defined bone barrow of mature HO are presumably hematopoietic cells (not skeletal cells), the DAPI⁺ nuclei of those cells were excluded from the total cell numbers (this was designed to prevent the underestimating the skeletal contribution of labeled cells). Bar=80μm.

Table 1

Summary of currently available genetic lineage tracing data

Cre line	Intended target population	Contribution to HO (Ref.)
CD19-cre	B-cells	No [14]
LCK-cre	T-cells	No [14]
Lyz-cre	monocytes/macrophages	No [14]
Myf5-cre	myogenic lineage	No [14]
Nestin-cre	somite-derived cells	No [14]
MyoD-Cre	skeletal muscle stem cells	No [7]
Tie2-Cre	endothelium/mesenchymal lineage	Yes [7, 13]
SMMHC-Cre	vascular smooth muscle	No [7]
NG2-Cre	pericytes	No [16]
VE-Cadherin-Cre	endothelium/endothelial precursors	No [13]
FoxD1-cre	mesenchymal lineage	No (this study)
Glast-creERT	mesenchymal lineage	Yes (this study)