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RAPID COMMUNICATION

Myelin protein zero (*P0*)- and *Wnt1*-Cre marked muscle resident neural crest-derived mesenchymal progenitor cells give rise to heterotopic ossification in mouse models

Heterotopic ossification (HO) describes bone formation at non-skeletal sites and results from traumatic injury, surgery, or genetic disease such as fibrodysplasia ossificans progressiva (FOP).[1,2] Although it is known that BMP signaling regulates HO, knowledge about the developmental origin of the osteogenic progenitors responsible for the BMP-associated metamorphosis is comparably less. With the use of transgenic mice and labelled neural crest-derived cell,[3] we found myelin protein zero (*P0*, or *MPZ*)- and *Wnt1*-lineage cells give rise to BMP-7 induced adult ectopic cartilage and bone. In addition, the induced expression of ACVR1(R206H), which is the major mutation found in FOP patients, in *P0*-lineage cells formed ectopic bone after cardiotoxin-induced muscle injury. We also found that the majority of muscle-resident fibro-adipogenic progenitors (FAPs), essential for muscle homeostasis and responsible for HO in skeletal muscle,[4,5] are derived from *P0*- and *Wnt1*-lineage cells. The data collectively suggest that muscle-resident neural crest-derived progenitor cells account for both nonhereditary and genetic type HO.

To determine whether neural crest-derived cells contribute to BMP-induced HO, we performed a lineage-tracing experiment. Transgenic mice containing *P0-Cre* and floxed *LacZ* reporter alleles were used to label neural crest-derived cells *in vivo* (Fig. 1A). Mice were injected with BMP-7 into the right gastrocnemius muscle to induce HO. Fourteen days after the injection, HO formation in *P0-Cre*/floxed *LacZ* mice was confirmed by X-ray and micro-computed tomography (μCT) analysis (Fig. 1B). Histological analyses for Hematoxylin and Eosin (H&E), Safranin O (acidic proteoglycan, an extracellular matrix protein of chondrocytes), and von Kossa (calcium deposition) staining indicated that the ectopic bones were formed through endochondral ossification (Fig. S1). Importantly, BMP-7-induced HO tissue was ubiquitously positive for X-gal staining (Fig. 1C). BMP-7-injected tissue expressed the osteoblast-specific factor SP7 (Fig. 1D), cartilage marker collagen II (COL2) (Fig. 1F), and bone marker collagen I (COL1) (Fig. 1G). Immunostaining Cre^{+} mice (*P0-Cre*$^{+}$) and Cre^{-} littermate (*LacZ*) controls revealed the considerable co-staining of β-galactosidase positive ($LacZ^{+}$) cells with SP7 (81.28% ± 4.05%) (Fig. 1D, E) and co-localization with the COL2 or COL1 positive site (Fig. 1F, G) only in Cre^{+} mice, suggesting that neural crest-lineage cells contributed to the formed ectopic bone. However, there was a small portion of $LacZ^{-}$ cells positive for SP7, COL2 and COL1, indicating the existence of cartilage/bone-forming progenitors derived from another source. Similar results were obtained when transgenic mice containing a *Wnt1-Cre* reporter allele, which has been widely used to label neural crest-derived cells, were used (Fig. 1A–G; Fig. S1).

Next, to investigate the role of neural crest-lineage cells in HO formation in adult FOP model mice, *Cre* transgene driven by *P0* promoter was combined with a *Rosa26-loxP-stop-loxP (LSL)-rtTA3* allele and *Col1a1-tetO-FOP-ACVR1* allele, in which *FOP-ACVR1* ($ACVR1^{R206H}$) can be induced in *P0*-lineage cells upon doxycycline (Dox) treatment (Fig. 1H). Transgenic mice were treated with Dox in the drinking water to induce *FOP-ACVR1* and treated with CTX in the right gastrocnemius muscle to initiate skeletal muscle injury and subsequent HO. Notably, HO was observed

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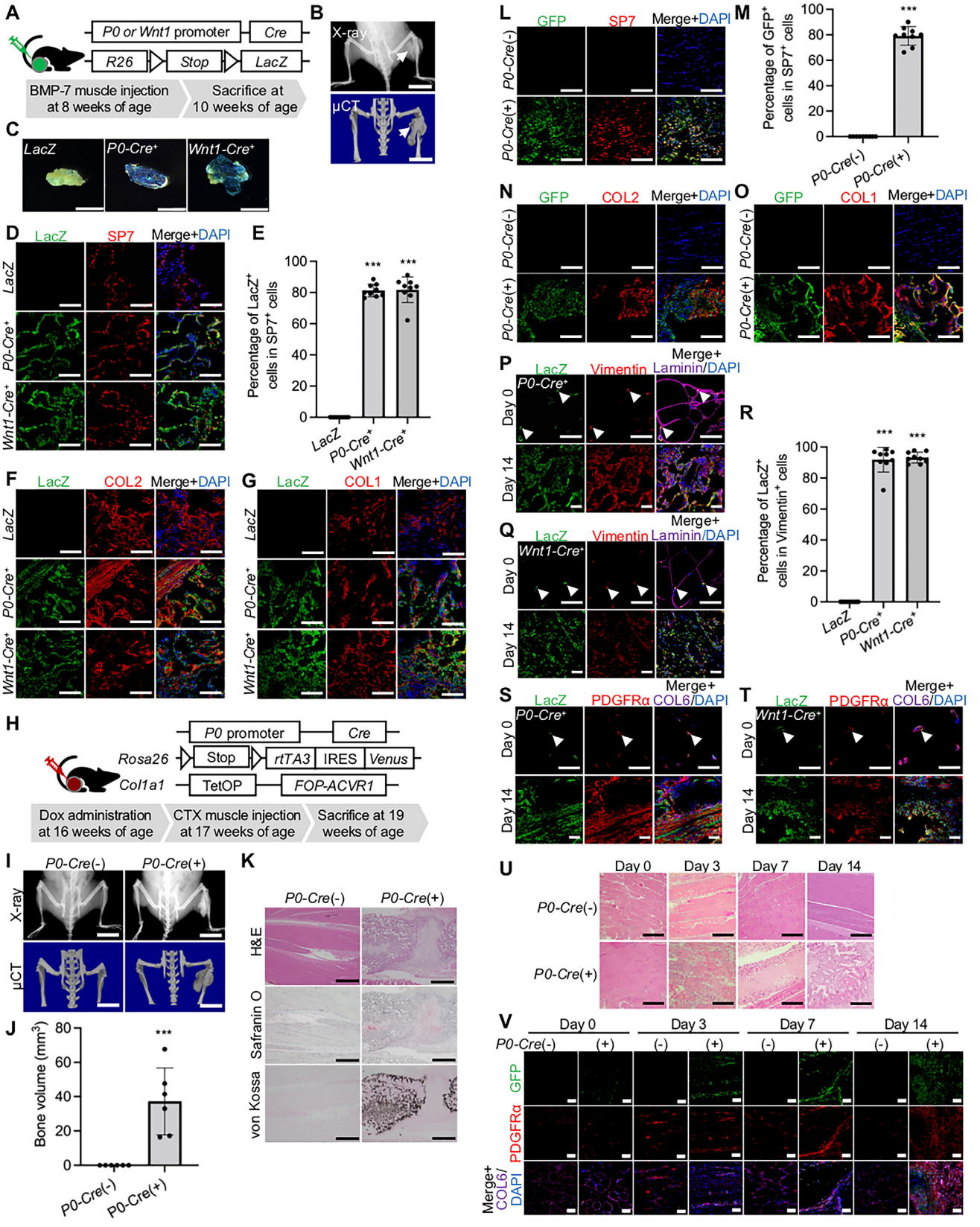

Figure 1 *P0*- and *Wnt1*-Cre marked PDGFRα^{+} mesenchymal progenitor cells give rise to adult ectopic bone in both BMP-induced and FOP mutant mice. **(A)** Schematic representation of the lineage-tracing experiment using *P0* or *Wnt1*-Cre and floxed *LacZ* reporter alleles. **(B)** Representative X-ray and μCT images of BMP-7-induced HO, $n = 5$. Scale bars: 5 mm. **(C)** Representative image of whole mount X-gal staining for HO tissue, $n = 3$. Scale bars: 2 mm. **(D–G)** Immunostaining of *P0* or *Wnt1*-Cre^{+} mice and Cre^{-} littermate control (*LacZ*) for β-galactosidase (green) and SP7 (D), COL2 (F) and COL1 (G) (red). Scale bars: 100 μm. (E) Percentage

only in mutant mice (*P0-Cre*;*Rosa26-LSL-rtTA3*;*Col1a1-tetO-FOP-ACVR1*, hereafter referred to as *FOP-ACVR1*(*P0-Cre*(+)), 14 days after the CTX treatment, while *P0-Cre*-negative controls (*Rosa26-LSL-rtTA3*; *Col1a1-tetO-FOP-ACVR1*; designated as wild type(*P0-Cre*(−)) never developed HO, suggesting that neural crest-derived cells are a cell-of-origin for the CTX-triggered HO in FOP model mice (Fig. 1I, J). H&E, Safranin O and von Kossa staining of *FOP-ACVR1*(*P0-Cre*(+)) mutants illustrated that ectopic cartilage and bone were formed extensively throughout the CTX-injected region (Fig. 1K). The ectopic cartilage and bone robustly expressed SP7, COL2, and COL1 (Fig. 1L, N, O) in the tissue of *FOP-ACVR1*(*P0-Cre*(+)) mutants but not of wild-type(*P0-Cre*(−)) mice. In the *FOP-ACVR1*(*P0-Cre*(+)) mutants, a cluster of Venus positive cells (marked by GFP antibody) detected in the HO region was co-stained with SP7 (79.13% ± 7.33%) (Fig. 1L, M) and colocalized with COL2 or COL1 (Fig. 1N, O), showing FOP-ACVR1 in *P0*-lineage cells lead to ectopic cartilage and bone.

Previous studies have found that PDGFRα$^+$ FAPs are likely to be the major precursor of ectopic bone,[5] while their cell-of-origin remains to be fully understood. Therefore, muscle tissues from *P0-Cre*/floxed-*LacZ* mice were examined to confirm whether FAPs were derived from *P0*-lineages. Vimentin- (Fig. 1P) and PDGFRα- (Fig. 1S) expressing mesenchymal progenitors resided in the muscle interstitium, which was marked by laminin and collagen VI (COL6) in the untreated state. These cells were co-stained with β-galactosidase, indicating their origin of *P0*-expressing cells. Fourteen days after the BMP-7 treatment, majority of Vimentin- (91.86% ± 7.97%) and PDGFRα-expressing cells co-stained with β-galactosidase in the HO tissue of *P0-Cre*/floxed-*LacZ* mice were observed (Fig. 1P, R, S). Similar results were also observed in *Wnt1-Cre*/floxed-*LacZ* mice (Fig. 1Q, R, T). These data suggested that neural crest-lineage cells act as a cell-of-origin of FAPs, which contribute to BMP-7-induced HO.

Histological evaluation of the stages of the lesion formation in FOP patients has shown that a phase of tissue destruction precedes the phase of fibroblast proliferation and ectopic cartilage and bone formation.[2] To pursue the fate of neural crest-derived cells in the stages of FOP HO in mice, tissue from the CTX-injected site was collected serially and subjected to H&E staining and immunohistochemistry. In both *FOP-ACVR1*(*P0-Cre*(+)) mutants and wild-type(*P0-Cre*(−)) tissue, muscle fiber disruption, more inflammatory cells and spindle-shaped fibroblastic cells were observed in the interstitial space 3 days after the CTX injection (Fig. 1U). Highly proliferating fibroblastic cells formed the FOP lesion 7 days after the injection. Subsequently, chondrogenesis and osteogenesis occurred at 14 days in *FOP-ACVR1*(*P0-Cre*(+)) mutants. We confirmed that FAPs residing in the muscle interstitium (marked by COL6) were increased according to the proliferation of fibroblastic cells in the injected *FOP-ACVR1*(*P0-Cre*(+)) muscle and contributed to the ectopic bone formation (Fig. 1V). In *P0-Cre*(−) tissue, the population of PDGFRα$^+$ cells decreased as muscle regeneration proceeded. These results suggested that neural crest-derived PDGFRα$^+$ cells were a cell-of-origin for the proliferating cells and cartilage- and bone-forming cells that contribute to ectopic bone in FOP.

In this study, we identified PDGFRα-expressing mesenchymal progenitor cells in the *P0* and *Wnt1*-lineages as a cell type that gives rise to adult ectopic bone. Our results also showed that the expression of FOP mutated R206H ACVR1 (FOP-ACVR1) in *P0*-lineage cells gives rise to ectopic bone induced by CTX injection. Although it is also known that *P0* or *Wnt1* alone is not exclusive to neural crest cells, our observation strongly suggests that ectopic bones were neural crest cell-origin since ectopic bone was marked in both *Wnt1*-cre and *P0*-cre lineages, which mark different neural crest populations (*P0*-cre: migrating neural crest cells vs *Wnt1*-cre: the dorsal neural tube prior to the emigration of the neural crest).

In skeletal muscle, muscle-resident FAPs are essential for muscle homeostasis and regeneration. Our study highlights that the PDGFRα$^+$ FAPs responsible for BMP-7-induced HO originate from the neural crest. In FOP tissue, *P0*-lineage PDGFRα$^+$ cells contributed to the assembly of mesenchymal progenitors, which caused the FOP lesion and ectopic cartilage/bone tissue in damaged muscle. These findings will contribute the study of FAP behavior in HO.

In conclusion, our results strongly suggest that neural crest-lineage cells give rise to adult ectopic bone, targeting neural crest-lineage progenitors may open new

of LacZ$^+$ cells in SP7$^+$ cells. Data represent the mean ± SD of representative sections from three independent experiments. ***$P < 0.001$, by Student's *t* test compared with the *LacZ* group. **(H)** Schematic illustration of the genetic construct for Dox-inducible FOP-ACVR1 in neural crest derivatives. **(I)** Representative X-ray and μCT images of CTX-induced HO. **(J)** Average ectopic bone volume induced by CTX. Data represent the mean ± SD of $n = 6$/group. **$P < 0.001$, by Student's *t* test compared with the *P0-Cre*(−) group. Scale bars: 5 mm. **(K)** Histological analysis of the CTX-injected region by H&E, Safranin O, and von Kossa staining. Scale bars: 500 μm. (L–O) Immunostaining of *P0-Cre*(+) mice and *P0-Cre*(−) littermate control for GFP (green) and SP7 **(L)**, COL2 **(N)** and COL1 **(O)** (red). Scale bars: 100 μm. **(M)** Percentage of GFP$^+$ cells in SP7$^+$ cells. Data represent the mean ± SD of representative sections from three independent experiments. ***$P < 0.001$, by Student's *t* test compared with the *P0-Cre*(−) group. Muscle sections of *P0* or *Wnt1*-Cre; floxed LacZ mice were subjected to immunofluorescence staining for β-galactosidase, Vimentin, laminin **(P–R)**, PDGFRα and COL6 **(S, T)**. Arrows indicate β-galactosidase and PDGFRα co-stained cells, or β-galactosidase and vimentin co-stained cells. Scale bars: 100 μm. (R) Percentage of LacZ$^+$ cells in vimentin$^+$ cells (day 14). Data represent the mean ± SD of representative sections from three independent experiments. ***$P < 0.001$, by Student's *t* test compared with the *LacZ* group. **(U, V)** Localization and behavior of *P0*-lineage FOP-ACVR1-expressing cells in CTX-treated muscle. At the indicated time points (days 0, 3, 7, and 14) after the CTX injection, muscle sections were subjected to H&E staining (U) and immunofluorescence staining for GFP, PDGFRα and COL6 (V). Scale bars: 500 μm (U) and 100 μm (V).

opportunities for HO treatment, and targeting FAPs induced through the neural crest lineage could be a basis for designing therapeutic strategies to treat several muscle related diseases.

Author contributions

C.Z.: conception and design, data collection and assembly, data analysis and interpretation, manuscript writing, and final approval of the manuscript; Y.I., K.S., K.H., and M.N.: data collection and assembly; Y.Y.: provision of materials and critical discussion; S.M., and J.T.: administrative support; M.I.: conception and design, financial support, administrative support, data interpretation, manuscript writing, and final approval of the manuscript.

Conflict of interests

K. Hino is an employee of Sumitomo Pharma Co., Ltd. All other authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.09.002.

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