Modeling human limb skeletal development using human pluripotent stem cell-derived skeletal assembloids

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SUMMARY

Despite recent advances in pluripotent stem cell-based approaches to induce skeletal cells, recapitulating human limb skeletal development in terms of structure and longitudinally oriented growth remains an unresolved challenge. Here, we report a method to differentiate human pluripotent stem cells into region-specific skeletal organoids harboring $GDF5^{+}PRG4^{+}$ interzone/articular chondrocyte progenitors (IZ/ACPs) and $SP7^{+}$ growth plate chondrocytes (GPCs) via $PRRX1^{+}$ limb-bud mesenchymal cells. Comparative analysis demonstrated marked similarities of IZ/ACP and GPC organoids to the human embryonic limb, and graft fate and regenerative capacity *in vivo* were further characterized. We also mimicked the limb skeletal developmental process in a spatially structured manner by vertically positioning two IZ/ACP organoids at both ends of a GPC organoid to generate a human skeletal assembloid. Notably, this human skeletal assembloid recapitulated endochondral ossification with longitudinal skeletal growth upon transplantation. In summary, our study provides a novel research platform for human limb skeletal development and disease.

Keywords
Human pluripotent stem cells, organoid, assembloid, skeletal development, endochondral ossification, GDF5, PRG4, SP7, Interzone, growth plate, articular chondrocyte

INTRODUCTION

Understanding human skeletal development is essential not only to advance bone physiology, e.g., providing a weight-bearing framework, regulating endocrine functions, and supporting hematopoiesis [1], but also to address clinical challenges, including osteoarthritis [2,3], nonunion fractures [4] in the elderly, and congenital skeletal disorders [5], which remain unresolved by conventional therapies. Although ethical constraints on research into human skeletal development have long existed, advancements in human pluripotent stem cells (hPSCs) have transformed this field. With their self-renewal and pluripotency, hPSCs provide experimental models that mimic skeletal development by navigating the brachiating landscape and efficiently differentiating intermediate stem/progenitor cells into desired cell types for regenerative medicine and recapitulation of congenital skeletal disorders [6]. In mammals, the skeleton is derived from three distinct embryonic sources according to the anatomical region. The axial skeleton (e.g., vertebrae and ribs) is specified from the paraxial mesoderm, the cranial skeleton (e.g., facial and cranial bone) is derived from neural crest, whereas the appendicular skeleton (e.g., limbs) arises from the lateral plate mesoderm (LPM) [7]. Moreover, ossification during skeletal development is mediated by two distinct processes: intramembranous ossification and endochondral ossification. In intramembranous ossification, mesenchymal cells directly differentiate into osteoblasts that are responsible for bone formation [8]. Conversely, in endochondral ossification, a cartilaginous template (anlagen) is initially established, which is subsequently replaced by bone [9]. Recent studies employed hPSCs to recapitulate skeletal lineages, successfully inducing skeletal cells or organoids (three-dimensional (3D) structures grown from stem cells that mimic certain aspects of organs) that participate in intramembranous [10–14] or endochondral [15–21] ossification. However, replicating processes that emulate skeletal morphology with skeletal growth using hPSCs remains an unresolved challenge.

Development of limb skeletal structures is initiated by condensation of PRRX1^{+} limb-bud mesenchymal (LBM) cells, followed by formation of skeletal anlagen composed of immature chondrocytes [22]. The joint interzone develops at the position of the presumptive joint and precedes articular cartilage differentiation and joint cavitation. Immature chondrocytes outside the interzone undergo hypertrophy at the mid-diaphysis as the first step of endochondral ossification [22]. Vasculature invades hypertrophic chondrocytes and subsequently forms the bone marrow cavity and growth plate chondrocytes (GPCs)/cartilage, which support longitudinal skeletal growth of limbs until puberty [9,22]. To replicate these complex human limb developmental processes, induction of LBM cells from hPSCs is essential. Recently, we successfully generated PRRX1^{+} LBM cells from hPSCs in a stable and scalable manner by employing PRRX1-tdTomato reporter hPSCs [23,24]. This achievement laid the foundation for induction of

intermediate structures, such as the interzone and growth plate cartilage, from hPSCs. In this study, we developed a protocol to generate distinct intermediate organoids, characterized by interzone/articular chondrocyte progenitors (IZ/ACPs) or GPCs, from ontogenetically-defined PRRX1^{+} LBM cells derived from hPSCs via establishing lineage-specific tdTomato reporter hPSC lines. Additionally, by employing a 3D assembly strategy for these intermediate tissue constructs, we successfully recapitulated spatial developmental processes, thereby reproducing skeletal growth along the longitudinal axis.

RESULTS

Generation of ontogenetically-defined IZ/ACP and GPC organoids from hPSCs

In our previous study, we induced and expanded PRRX1^{+} limb-bud-like mesenchymal cells (expandable limb-bud-like mesenchymal cells, ExpLBM cells) from hPSCs [23]. This protocol begins with differentiation into the mid-primitive streak and LPM, followed by induction of LBM cells, which have chondrogenic capacity in the presence of BMP4, TGFβ1, and GDF5 (Figure S1A). The expression level of each marker gene (pluripotency, LPM, LBM, and chondrocyte markers) clearly revealed the sequential induction of genes representative of each differentiation stage (Figure S1B). Chromatin accessibility, which is epigenetically regulated by histone modifications such as acetylation and methylation, determines the access of transcription factors and underlies cell type-specific gene expression [25,26]. Therefore, we investigated cell specificity during the stepwise induction protocol by analyzing chromatin accessibility using ATAC-seq and histone modifications using CUT&Tag. *NANOG, HAND1*, and *PRRX1* are specific markers of pluripotent, LPM, and LBM cells, respectively, and mRNA expression of these genes was specific to each developmental stage. When the 5' flanking regions of these genes were analyzed using ATAC-seq and CUT&Tag with antibodies against H3K27Ac and H3K27me3, peaks of ATAC-seq and H3K27Ac were observed at time points corresponding to the mRNA expression peaks. By contrast, H3K27me3 peaks were inversely correlated with these patterns (Figure S1C). Furthermore, we assessed whether our ontogenetically-defined induction protocol for ExpLBM cells recapitulates formation of the forelimb or hindlimb. Reanalysis of single-cell RNA-sequencing (scRNA-seq) data of human forelimb and hindlimb buds [27] revealed that *TBX5* was highly expressed in forelimb buds, while *PITX1* and *ISL1* were enriched in hindlimb buds, consistent with previous mouse studies [28] (Figure S1D). *PITX1* and *ISL1*, but not *TBX5*, were expressed during stepwise induction processes from hPSCs to ExpLBM cells (Figure S1D). Under these conditions, high peak patterns of ATAC-seq and H3K27Ac were found in *ISL1* and *PITX1*, but not in *TBX5*. On the other hand, H3K27me3 peaks were found in *TBX5*, but not in *ISL1* and *PITX1* (Figure S1E).

These results indicate that our induction protocols generate ontogenetically-defined LBM cells that recapitulate hindlimb bud initiation.

Limb skeletal development begins with condensation of LBM cells, forming a cartilage template. This template subsequently differentiates into two distinct lineages: articular permanent cartilage, which develops through intermediate GDF5-expressing interzone cells, and growth plate cartilage comprising resting, proliferative, and SP7-expressing hypertrophic chondrocytes, which drives longitudinal bone growth. Our previous studies generated cartilage tissues from ExpLBM cells by 30 ng/mL BMP4 and 10 ng/mL TGFβ1 supplementation *in vitro* [23,24]. However, RNA-seq analysis of cartilage generated using this protocol revealed expression of markers for both interzone/articular cartilage and growth plate cartilage (Figure S1B). In addition, subcutaneous transplantation of this cartilage into NOD-SCID mice revealed that some transplanted cartilage maintained hyaline cartilaginous-like tissues, but some showed ossification with columnar chondrocytes and spongy bone at 8 weeks (Figure S1F). Thus, our previous chondrogenic induction protocol from ExpLBM cells does not seem to selectively induce each lineage for interzone/articular cartilage or growth plate cartilage. To develop a lineage-selective induction protocol for ExpLBM cells into interzone/articular cartilage or growth plate cartilage, we established GDF5-tdTomato reporter human induced pluripotent stem cells (GDF5$^{3'tdTomato}$ hiPSCs) and SP7-tdTomato reporter human embryonic stem cells (SP7$^{3'tdTomato}$ hESCs) to visualize GDF5 (interzone marker)$^{+}$ or SP7 (hypertrophic chondrocyte marker)$^{+}$ cells. hPSCs were electroporated with the targeting vector harboring a homology arm and IRES-tdTomato-PGK-Neo cassette, and the PX459-gRNA genome-editing vector. Electroporated cells were selected with puromycin and G418, followed by cell cloning to isolate 3'tdTomato reporter hPSCs. Single colonies were expanded, and genomic DNA was extracted to identify correct insertion of the knock-in reporter cassette. PCR results showed that the selected clone harbored a homozygous knock-in reporter cassette (Figure S2A). These reporter clones of GDF5$^{3'tdTomato}$ hiPSCs or SP7$^{3'tdTomato}$ hESCs were chosen for induction experiments.

Next, ExpLBM cells derived from GDF5$^{3'tdTomato}$ hiPSCs or SP7$^{3'tdTomato}$ hESCs were condensed into aggregates (step 1) and subsequently differentiated into chondrocytes using BMP4, TGFβ1, and FGF2 (step 2). This was followed by treatment with 18 combinations of chemicals in the presence of 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 10 ng/mL GDF5 for 14 days (Figure 1A). Among the combinations tested, addition of PD0325901 (a MEK inhibitor) in the presence of BMP4 and TGFβ1 was necessary to induce GDF5-tdTomato expression. By contrast, high concentrations of BMP4 and TGFβ1 (300 and 100 ng/mL, respectively) effectively induced SP7-tdTomato expression (Figure 1B and S2B). Principal component analysis revealed distinct differential gene expression profiles between pluripotent cells (day 0), LPM cells (day 2), LBM cells (day 4), ExpLBM cells, and cell aggregates treated with 30 ng/mL BMP4 and 10

ng/mL TGFβ1 (conventional); 30 ng/mL BMP4, 10 ng/mL TGFβ1, and PD0325901 (hereafter referred to as the IZ/ACP organoid strategy); or 300 ng/mL BMP4 and 100 ng/mL TGFβ1 (hereafter referred to as the GPC organoid strategy) (Figure 1C). Notably, RNA-seq analysis clearly demonstrated that interzone/articular cartilage markers (e.g., *GDF5, PRG4,* and *BARX1*) were enriched in IZ/ACP organoids, whereas growth plate cartilage markers (e.g., *RUNX2, SP7, IHH,* and *COL10A1*) were highly expressed in GPC organoids (Figure 1D). We next assessed the expression pattern of each marker by immunohistochemistry in ExpLBM cell aggregates and IZ/ACP and GPC organoids. While SOX9 and aggrecan (ACAN) were consistently expressed in all tested aggregates, PRG4 and type I collagen (COL1) were detected exclusively in IZ/ACP organoids. By contrast, RUNX2, type X collagen (COL10), and SP7 were observed only in GPC organoids (Figure 1E). We also confirmed that two different types of hPSCs (802-3G hiPSCs and SEES4 hESCs) could established similar expression profiles by performing real-time quantitative RT-PCR (RT-qPCR) analysis (Figure S2C).

To further analyze lineage specification and its heterogeneity, we performed scRNA-seq of 9741 cells from ExpLBM cell aggregates, 5090 cells from IZ/ACP organoids, and 4660 cells from GPC organoids. Uniform manifold approximation and projection (UMAP) dimensionality identified two clusters in ExpLBM cells [LBM cells (non-dividing) and MKI67$^+$ LBM cells (dividing)], two clusters in IZ/ACP organoids (GDF5$^+$ interzone cells and PRG4$^+$ articular chondrocyte progenitors), and two clusters in GPC organoids (IHH$^+$ pre-hypertrophic chondrocytes and SP7$^+$ hypertrophic chondrocytes) based on gene expression patterns (Figures 1F and 1G,). To evaluate the similarity between these hPSC-derived cells and cells from human embryos, we integrated their scRNA-seq expression profiles with data from the human limb developmental process [27]. Our analysis revealed that ExpLBM cells, IZ/ACP organoids, and GPC organoids most closely resembled limb-bud mesenchyme (LimbMes1), articular chondrocytes, and hypertrophic chondrocytes observed during human limb development, respectively (Figure 1H). Dot plots from scRNA-seq analyses of ExpLBM cells, IZ/ACP organoids, and GPC organoids confirmed their identities as limb-bud mesenchyme, interzone and articular chondrocytes, and hypertrophic chondrocytes, respectively, by comparing the expression of cell type-specific genes in each cluster with those observed in scRNA-seq analyses of human embryonic limb cells (Figure 1I). Furthermore, KEGG pathway enrichment analysis was performed of genes exhibiting significant differential expression identified through pseudotime trajectory analysis using scRNA-seq datasets of human embryonic limbs, with LimbMes1 designated the initial state. This analysis revealed that, unlike differentiation into GPCs, the developmental transition from LBM cells to articular chondrocytes was characterized by suppression of MAPK signaling (Figures S3A, S3B, S3C and S3D). These findings indicate that our differentiation strategy from hPSCs into two distinct chondrocyte

lineages, including IZ/ACP and GPC organoids, recapitulates the human skeletal developmental process of the hindlimb bud.

Assessment of the graft fate and regenerative capacity of IZ/ACP and GPC organoids

Next, we investigated graft fate by subcutaneously transplanting these organoids into NOD-SCID mice. Transplanted IZ/ACP organoids remained Safranin O staining^{+} hyaline cartilaginous-like tissues without ossification up to 3 months post-transplantation, whereas transplanted GPC organoids exhibited ossification, with columnar chondrocytes and spongy bone formation observed at 3 months (Figure 2A). Furthermore, cells of mouse articular cartilage exhibit smooth surfaces, as observed via scanning electron microscopy (SEM), and possess simple intracellular organelle structures, as observed via transmission electron microscopy (TEM). This phenotype closely resembled that of transplanted IZ/ACP organoid-derived cartilaginous tissues. By contrast, cells of mouse growth plate cartilage are characterized by an abundance of cellular projections (observed via SEM) and are rich in rough endoplasmic reticulum and Golgi apparatus (observed via TEM). This appearance was strikingly similar to that of transplanted GPC organoid-derived cartilaginous tissues (Figure 2B). Indeed, immunohistochemical analysis revealed that transplanted IZ/ACP organoid-derived cartilaginous tissues were positively stained for type II collagen (COL2) and ACAN but did not express pre-/hypertrophic chondrocyte markers, such as RUNX2 and SP7, or the endothelial cell marker endomucin (EMCN). By contrast, transplanted GPC organoid-derived cartilaginous tissues expressed not only COL2 and ACAN but also RUNX2 and SP7, and contained EMCN^{+} cells (Figure 2C).

To demonstrate the regenerative efficacy of IZ/ACP organoids *in vivo*, they were transplanted into osteochondral defects created in knee joint articular cartilage of X-linked severe combined immunodeficiency (SCID) rats. At 1 and 3 months post-transplantation, IZ/ACP organoids were successfully engrafted into osteochondral defects, as evidenced by human vimentin (hVIM) expression. These organoids produced extracellular matrix that was positive for Safranin O staining and immunohistochemical staining for COL2 and ACAN. Notably, PRG4, a marker of the superficial zone of articular cartilage, was specifically expressed in the joint-side surface of the graft (Figure 2D). During fracture healing, the cartilaginous callus that forms at the injury site undergoes endochondral ossification, facilitating bone repair [29]. To evaluate the regenerative capacity of GPC organoids, we transplanted them into critical-sized femur defects of SCID rats. Periodic observation of mineralized bone using micro-computed tomography (μCT) revealed that, in the absence of transplantation, bone union did not occur, resulting in a condition resembling nonunion. By contrast, transplantation of GPC organoids led to bone formation at the defect site, which was observed by day 28 post-transplantation, with

complete bone union achieved by day 56 (Figure 2E). Histological analysis further confirmed active bone formation at the transplantation site, with remnants of Safranin O-stained hyaline cartilaginous-like matrix (Figure 2F). Human-specific nucleoli staining demonstrated that newly formed bone was predominantly graft-derived (Figure 2G). Finally, bone strength at the fracture site was assessed 3 months post-transplantation using a three-point bending test. Although the values did not reach those of intact femurs without defects, significant recovery of ultimate stress (N), extrinsic stiffness (N/mm²) and failure energy (N mm) was observed in GPC organoid-transplanted femurs (Figure 2H). Collectively, these results demonstrate that IZ/ACP organoids exhibit characteristics of IZ/ACPs, which generate permanent articular cartilage, whereas GPC organoids display characteristics of GPCs, driving endochondral ossification.

Generation of skeletal assembloids with IZ/ACP and GPC organoids to recapitulate human limb skeletal development

The process of skeletal formation begins with condensation of LBM cells, leading to formation of cartilage anlagen. Subsequently, this structure develops into the cartilage containing growth plate chondrocytes, contributing to elongation of bone along its longitudinal axis through endochondral ossification, which is flanked by the interzone, the region that eventually gives rise to joints (Figure 3A) [22]. The generation of two types of organoids with characteristics of IZ/ACPs or GPCs enabled reconstruction of this skeletal complex *in vitro*. To this end, we fused IZ/ACP and GPC organoids to produce a skeletal assembloid. To facilitate visualization, we inserted a ubiquitous GFP or Kusabira Orange (KuO) marker into hiPSCs using a piggyBac transposon system, followed by generation of IZ/ACP or GPC organoids, respectively (Figure 3B). Two GFP-labeled IZ/ACP organoids were positioned vertically at both ends of a KuO-labeled GPC organoid within a low-melting point agarose gel. By day 7, the three organoids had fused within the wells (Figure 3C), forming a skeletal complex, which was subsequently subcutaneously transplanted into NOD-SCID mice. At 1 and 6 months post-transplantation, excised tissues retained expression of GFP and KuO (Figure 3D). Notably, significant longitudinal growth of the skeletal assembloid was observed over time post-transplantation (Figure 3E). Furthermore, μCT analysis at 6 months post-transplantation revealed ossification in the central region (Figure 3F). Histological analysis of transplanted skeletal assembloids at 1 month post-transplantation demonstrated that GFP^+ regions were observed at the peripheral edges of the tissue, while KuO^+ regions were localized in the central area. ACAN was detected throughout the entire tissue, whereas PRG4 was predominantly observed in the peripheral regions. COL10 and RUNX2 were detected in the KuO^+ central region of the tissue. By contrast, expression of CTSK or EMCN was not observed (Figure 3G). At 6 months post-transplantation, GFP^+ regions remained localized at the peripheral edges of the transplanted tissue, as

observed at 1 month post-transplantation. These regions were positive for Safranin O staining, ACAN, and PRG4. Meanwhile, in KuO^+ regions at 6 months post-transplantation, a columnar structure characteristic of growth plate cartilage positive for COL10 and RUNX2 was observed. Additionally, bone marrow-like structures were identified in the central region of the tissue construct, along with $CTSK^+$ cells and vascular-like structures expressing EMCN (Figure 3H). Overall, our results demonstrate that the skeletal assembloid, which comprises IZ/ACP and GPC organoids, recapitulates longitudinal bone growth upon transplantation due to induction of primary endochondral ossification in its center.

DISCUSSION

In this study, we successfully developed a strategy to selectively induce two distinct chondrocyte lineage organoids, namely, IZ/ACP and GPC organoids, from hPSCs via ontogenetically-defined ExpLBM cells. Several conventional chondrogenic protocols using BMP2/4 and TGFβ1/3 [18,30,31], including ours [23,24], have been reported previously and yielded mixtures of both articular chondrocyte and GPC phenotypes (Figure S1B). Here, we discovered that addition of the MEK inhibitor PD0325901 (IZ/ACP organoid strategy) preferentially drives cells toward the "interzone/articular cartilage" lineage capable of forming permanent hyaline cartilaginous tissue without evidence of ossification, whereas elevation of the BMP4 and TGFβ1 concentrations (GPC organoid strategy) promotes generation of the "growth plate cartilage" lineage capable of endochondral ossification, giving rise to bone tissue with vasculature and osteoclasts.

MAPK signaling regulates chondrocyte growth, differentiation, and hypertrophy during endochondral ossification [32–34]. Although no evidence has previously demonstrated its importance in generating interzone or articular cartilage progenitors, unbiased chemical screening using GDF5-tdTomato reporter hPSCs in this study unveiled this aspect (Figure 1B). In fact, scRNA-seq datasets of the human embryonic limb clearly indicated that MAPK signaling is attenuated during developmental processes from LBM cells to articular cartilage (Figure S3B). Therefore, in conjunction with scRNA-seq data indicating that our organoids exhibit similar signatures as embryonic limb constituent cells (Figures 1H and 1I), we confirmed that this stepwise induction from hPSCs to ExpLBM cells and each differentiated organoid faithfully recapitulates the human developmental program of articular cartilage and growth plate cartilage. Furthermore, in a rat knee defect model, IZ/ACP organoids engrafted into the joint defect area and secreted an extracellular matrix containing ACAN and PRG4, which are characteristics of articular cartilage. Meanwhile, GPC organoids transplanted into a femoral defect model formed cartilage calluses that underwent endochondral ossification, eventually leading to bone union and partial recovery of mechanical strength. Thus, our protocol not only faithfully recapitulates

the *in vivo* signaling pathways that drive lineage bifurcation during human limb development but also sheds light on how a promising cell source can be provided for regenerative medicine.

An assembloid is an advanced 3D culture model created by combining or fusing multiple organoids. By integrating different types of organoids or cell populations, assembloids aim to recapitulate more complex tissue and organ-like structures and functions than traditional single-lineage organoids [35,37]. A further highlight of our study is the generation of a skeletal assembloid by fusing IZ/ACP and GPC organoids. When transplanted into immunodeficient mice, the assembloid exhibited longitudinal growth and endochondral ossification at its center, while maintaining cartilage-like tissues at both ends. This arrangement mimics the limb developmental organization, where growth plate cartilage regions are flanked by articular cartilage regions. While several studies have recapitulated endochondral ossification in cartilage tissue derived from hPSCs [10–14], this is the first study in which the process has been faithfully reproduced in a directional manner along the skeletal longitudinal axis. This model holds great promise for investigating complex human skeletal development as well as for studying pathological conditions affecting both cartilage and bone, such as osteoarthritis and skeletal dysplasia.

Limitations of the study

While this study presents an innovative assembloid approach with hPSC-derived IZ/ACP and GPC organoids to recapitulate human limb skeletal development, it is important to note certain limitations. Longitudinal skeletal growth of the assembloid in this study was observed through subcutaneous transplantation into immunodeficient mice; thus, functional integration of the transplanted tissue within a normal immune environment and under external factors such as mechanical forces has not yet been determined. In fact, we could not observe a secondary ossification center, which typically forms postnatally in the epiphysis of mammals [36]. Furthermore, given the potential of skeletal assembloids, it is important to utilize this system in future studies to model diseases that cause growth retardation due to impaired limb endochondral ossification.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takeshi Takarada (takarada@okayama-u.ac.jp).

Materials availability

tdTomato reporter hPSCs generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

All raw sequencing data were deposited in the GEO database. These data can be accessed using the GSE accession number GSExxxxx (in progress). This study also analyzed publicly available data. Accession numbers of these datasets are listed in the STAR Methods and key resources table. Any additional information and materials will be made available upon request.

ACKNOWLEDGEMENTS

We thank Ayume Nitta, Kokoro Furutani, Aoba Nakajima, Yukio Hitsumoto, Masayuki Okamoto, Yuka Yamamoto, and Naoshige Ono for technical assistance, and Ziyi Wang and Mitsuaki Ono for their invaluable comments and advice. Preparation of slides and staining were supported by the Central Research Laboratory, Okayama University Medical School. Computation was performed using the General Projects on the supercomputer “Flow” at the Information Technology Center, Nagoya University. We thank the members of the Department of Animal Resources, Advanced Science Research Center, and Okayama University for maintaining mice. This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (17H04399, 21H02643, and 24KK0206 to T.Takarada; and 23K08677 to T.Takao), AMED (JP24bm1123059 to T.Takao), the JST FOREST Program (JPMJFR225H to T.Takarada), the Astellas Foundation for Research on Metabolic Disorders (to T.Takao), and the Cooperative Research Program of the Institute for Frontier Life and Medical Sciences, Kyoto University (to T.Takarada and J.T.). These funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

T.Takao and T.Takarada conceived the project, designed experiments, and prepared the manuscript. T.O. analyzed sequencing data. K.S. transplanted organoids into critical-sized femur defects in SCID rats. D.Y. and Y.F. performed experiments. M.H. provided critical advice on producing a skeletal assembloid. E.N., T.O., and J.T. discussed the data and provided critical advice.

DECLARATION OF INTERESTS

T.Takao and T.Takarada have filed a patent for the small-molecule combinations reported in this paper.

STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-SOX9	Sigma-Aldrich	Cat# AB5535 RRID: AB_2239761
Rabbit polyclonal anti-ACAN	Proteintech	Cat# 13880-1-AP RRID: AB_2722780
Mouse monoclonal anti-COL1	SouthernBiotech	Cat# 1441-01 RRID: AB_2794758
Mouse monoclonal anti-COL2	Thermo Fisher Scientific	Cat# MA1-37493 RRID: AB_2082337
Mouse monoclonal anti-COL10	Thermo Fisher Scientific	Cat#14-9771-80 RRID: AB_2573017
Rabbit polyclonal anti-PRG4	Novus Biologicals	Cat# NBP1-19048 RRID: AB_2169241
Rabbit monoclonal anti-RUNX2	CST	Cat# 12556 RRID: AB_2732805
Mouse monoclonal anti-SP7	R&D	Cat# MAB7547 RRID: AB_3658831
Rat anti-mouse CD107b (LAMP2)	BD Pharmingen	Cat# 550292 RRID: AB_393587
Rabbit monoclonal anti-cathepsin K	Abcam	Cat# ab19027 RRID: AB_2261274
Rat monoclonal anti-EMCN	Santa Cruz	Cat# sc-65495 RRID: AB_2100037
Rabbit monoclonal anti-hVIM	Abcam	Cat# ab16700 RRID: AB_443435
Mouse monoclonal anti-human nucleoli	Abcam	Cat# ab190710 RRID: AB_2937055
Rabbit polyclonal anti-KuO	MBL	Cat# PM051M RRID: AB_2876863
Rabbit polyclonal anti-histone H3K27ac	Active Motif	Cat# 39134 RRID: AB_2722569
Rabbit polyclonal anti-histone H3K27me3	Active Motif	Cat# 39155 RRID: AB_2561020
Goat anti-mouse IgG (H+L), F(ab')2 fragment (Alexa Fluor 647 conjugate)	CST	Cat# 4410S
Goat anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor 488 conjugate)	CST	Cat# 4412S
Goat anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor 647 conjugate)	CST	Cat# 4414S
Goat anti-rat IgG (H+L) (Alexa Fluor 488 conjugate)	CST	Cat# 4416S
Goat anti-rat IgG (H+L) (Alexa Fluor 647 conjugate)	CST	Cat# 4418S
Chemicals, peptides, and recombinant proteins		
CHIR99021	Axon Medchem	Cat# Axon1386
PIK-90	Selleck	Cat# S1187
A 83-01	Tocris	Cat# 2939
Wnt-C59	Cellagen Technology	Cat# C7641-2s
LDN-193189	REPROCELL	Cat# 04-0074
GDC-0449 (Vismodegib)	Cellagen Technology	Cat# C4044-5
Y-27632	MedChemExpress	Cat# MCE HY-10583
SAG 21k	R&D	Cat# 5282
ATRA	Sigma-Aldrich	Cat# R2625
XAV-939	Selleck	Cat# S1180
SF1670	Cayman	Cat# 15368
MK-2206	Cayman	Cat# 11593
PD0325901	Selleck	Cat# S1036

VO-OHpic	Cayman	Cat# 10009965
MHY1485	Cayman	Cat# 18008
DAPT	Wako	Cat# 043-33581
Rapamycin	Selleck	Cat# S1039
Recombinant human activin A	R&D	Cat# 338-AC
Recombinant human BMP4	R&D	Cat# 314-BP
Recombinant human TGFβ1	PEPROTECH	Cat# 100-21C
Recombinant human GDF5	PEPROTECH	Cat# 120-01
Recombinant human FGF2	Katayama Chemical	Cat# 161-0010
Recombinant human EGF	STEMCELL TECHNOLOGIES	Cat# ST-78006
Critical commercial assays		
RNeasy Mini Kit	QIAGEN	Cat# 74106
ReverTra Ace qPCR RT Master Mix with gDNA Remover	TOYOBO	Cat# FSQ-301
Luna Universal qPCR Master Mix	NEB	Cat# M3003X
KAPA RNA HyperPrep Kit with RiboErase (HMR)	Nippon Genetics	Cat# KK8561
SeqCap Adapter Kit (Set A/B)	Roche	Cat# 07141530001 Cat# 07141548001
Chromium Next GEM Single Cell 3′ GEM Kit v.3.1	10x Genomics	Cat# 1000269
Chromium Next GEM Single Cell G Tip Kit	10x Genomics	Cat# 1000127
Dual Index Kit TT Set A	10x Genomics	Cat# 1000215
High sensitivity D5000 screen tape	Agilent	Cat# 5067-5592
Illumina Tagment DNA Enzyme and Buffer	Illumina	Cat# 20034197
MinElute PCR Purification Kit	QIAGEN	
Nextera Index Kit	Illumina	Cat# 15055290
CUT&TAG-IT Assay Kit, Anti-Rabbit	Active Motif	Cat# 53160
RNAscope Multiplex Fluorescent Reagent Kit ver. 2	Advanced Cell Diagnostics	Cat# 323100
GFP probe (RNAscope)	Advanced Cell Diagnostics	Cat# 538851
TSA Vivid Fluorophore 570 (RNAscope)	Advanced Cell Diagnostics	Cat# 323272
Deposited data		
Raw and analyzed data (bulk RNA-seq, scRNA-seq, ATAC-seq, and CUT&Tag)	This study	GSExxxxx (in progress)
Human limb-bud data (scRNA-seq)	Zhang et al. [27]	E-MTAB-8813
Stepwise differentiation data from hiPSCs into cartilage (bulk RNA-seq)	Yamada et al. [23]	GSE165620
Experimental models: Cell lines		
414C2 hiPSCs	CiRA, Kyoto University	N/A
SEES4 hESCs	RIKEN BRC	HES0017
802-3G hiPSCs	REPROCELL	RCRP004N
GDF5$^{3'tdTomato}$ reporter 414C2 hiPSCs	This paper	N/A
SP7$^{3'tdTomato}$ reporter SEES4 hESCs	This paper	N/A
CAG-GFP hiPSCs	This paper	N/A
CAG-KuO hiPSCs	This paper	N/A
Experimental models: Organisms/strains		
NOD-SCID mouse (NOD.Cg-*Prkdc*^{scid}/J)	The Jackson Laboratory, Japan	N/A
C57BL/6J mouse	CLEA Japan	N/A
SCID rat (F344-*Il2rg*^{em1Iexas})	Laboratory Animal Research, The University of Tokyo	Cat# 12076-002
Oligonucleotides		
Primers for RT-qPCR and genotyping, see Table S1	This paper	N/A

Recombinant DNA		
pPB-CAG-DEST-pA-pgk-bsd	Addgene	Cat# 74918
KW158 (PCAG-PBase)	Kim et al. [38]	Kindly provided by Dr. Knut Woltjen
pSpCas9(BB)-2A-Puro (PX459) V2.0	Addgene	Cat# 62988
PX459-GDF5 gRNA	This paper	N/A
PX459-SP7 gRNA	This paper	N/A
pUCFa-GDF5 3′HA HA-IRES-tdTomato-PGK-Neo	This paper	N/A
pTA2-SP7 3′HA HA-IRES-tdTomato-PGK-Neo	This paper	N/A
Software and algorithms		
GraphPad Prism 8	GraphPad software	https://www.graphpad.com/
Fastp (version 0.23.4)	OpenGene	https://github.com/OpenGene/fastp
Kallisto (version 0.48.0)	Pachter laboratory	https://pachterlab.github.io/kallisto/
Bowtie2 (version 2.2.5)	Quinlan laboratory	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Gopeaks (version 1.0.0)	Maxson Braun laboratory	https://github.com/maxsonBraunLab/gopeaks
Bedtools (version 2.30.0)	Quinlan laboratory	https://github.com/arq5x/bedtools2
Homer (version 4.11)	Benner laboratory	http://homer.ucsd.edu/homer/
deeptools (version 3.5.1)	Max Planck Institute	https://github.com/deeptools/deepTools
Cell Ranger (version 7.0.1)	10x Genomics	https://www.10xgenomics.com/jp/support/software/cell-ranger/latest
R (version 4.1.3)	R Foundation	https://www.r-project.org/
edgeR (version 4.0.3)	Smyth laboratory	https://github.com/OliverVoogd/edgeR
NOISeq (version 2.46.0)	Conesa laboratory	https://github.com/ConesaLab/NOISeq
Seurat (version 5.0.3)	Satija laboratory	https://satijalab.org/seurat/
DoubletFinder (version 2.0.4)	Git Hub	https://github.com/chris-mcginnis-ucsf/DoubletFinder
Other		
StemFit	Ajinomoto	Cat# AK02N
DMEM	Wako	Cat# 048-29763
IMDM, GlutaMAX_ supplement	Thermo Fisher Scientific	Cat# 31980097
Ham's F-12 Nutrient Mix, GlutaMAX_ supplement	Thermo Fisher Scientific	Cat# 1765092
Polyvinyl alcohol	Sigma-Aldrich	Cat# P8136-250G
Chemically defined lipid concentrate (CD lipid concentrate)	Thermo Fisher Scientific	Cat# 11905031
Monothioglycerol	Sigma-Aldrich	Cat# M6145
Insulin	Sigma-Aldrich	Cat# 11376497001
Transferrin	Roche	Cat# 10652202001
L(+)-Ascorbic acid	Wako	Cat# 014-04801
Insulin-transferrin-selenium (ITS) 100×	Thermo Fisher Scientific	Cat# 41400045
Fetal bovine serum (FBS)	Gibco	Cat# 10270106 (Lot: 42F5094K)
iMatrix-511 silk (iMatrix)	MATRIXOME	Cat# 892021
Human plasma fibronectin (1 mg/mL)	Sigma-Aldrich	Cat# FC010
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat# 15140122
G418	Life Technologies	Cat# 076-05962
TrypLE Select	Thermo Fisher Scientific	Cat# 12563029
Accutase	NACALAI TESQUE	Cat# 12679-54
Collagenase, type II, powder	Gibco	Cat# 17101015
0.5 M EDTA (pH 8.0) solution	NIPPON GENE	Cat# 311-90075
96-well clear ultra-low attachment microplates	CORNING	Cat# 7007
Agarose, low gelling temperature	Sigma-Aldrich	Cat# A9414-5G

4',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific	Cat# D3571
Calcein-AM	DOJINDO	Cat# C326
In-Fusion HD Cloning Kit	Takara	Cat# Z9648N
KOD FX NEO Kit	Takara	Cat# KFX-201
pTA2 vector (TArget Clone™)	TOYOBO	Cat# TAK-101
4% paraformaldehyde (PFA) Hematoxylin	Wako Wako	Cat# 163-20145 Cat# 131-09665
Eosin	Wako	Cat# 051-06515
Safranin O	Wako	Cat# 530-00592
Fast green	Wako	Cat# 061-00031
Silver nitrate Entellan™ new	Wako Merck	Cat# 195-09382 Cat# 1079610100
Normal goat serum (NGS)	Vector Laboratories	Cat# S-1000-20
Fluoromount-G	SouthernBiotech	Cat# 0100-01

EXPERIMENTAL MODEL

Animals

The experiments involving mice and rats were approved by the Okayama University Animal Care and Use Committee. Immunodeficient NOD-SCID mice and SCID rats were purchased from The Jackson Laboratory (Japan) and Laboratory Animal Research (The University of Tokyo), respectively. The animals were housed under a 12-h light/12-h dark cycle (06:00–18:00) in a temperature-controlled room maintained at 22 ± 1°C with 40–60% humidity. Food and water were provided ad libitum.

Cell lines

hPSC lines and maintenance

hPSCs were cultured and maintained with StemFit (Ajinomoto). Before reaching subconfluency, cells were dissociated with TrypLE Select (Thermo Fisher Scientific)/0.25 mM EDTA and suspended in StemFit containing 10 μM Y-27632 (MedChemExpress). Cells (1×10^4) were suspended in StemFit containing 10 μM Y-27632 and 8 μL of iMatrix (human laminin-511 E8 fragment, MATRIXOME) and added to a 6-cm dish. The next day, culture media were replaced with fresh StemFit lacking Y27632. Culture media were changed every 2 days until the next passage. The following hPSC lines were used: 414C2 hiPSCs (provided by the Center for iPS Cell Research and Application, Kyoto University, Japan) and 802-3G hiPSCs (REPROCELL). SEES4 hESCs were donated by RIKEN BRC.

Generation of GDF5$^{3'tdTomato}$ and SP7$^{3'tdTomato}$ reporter cell lines

The targeting strategy for knock-in of the IRES-tdTomato-PGK-Neo cassette at the *GDF5* 3′-UTR or *SP7* 3′-UTR or to achieve bicistronic expression of GDF5/SP7 and tdTomato is shown in

Figure S2A. The *GDF5* 3′ homology region (GDF5 3′HA) was obtained by an artificial gene synthesis service (pUCFa-GDF5 3′HA vector) (FASMAC). To construct the targeting vector (pUCFa-GDF5 3′HA HA-IRES-tdTomato-PGK-Neo), IRES-tdTomato-PGKNeo fragments were inserted into the pUCFa-GDF5 3′HA vector using an In-Fusion HD Cloning Kit (Takara). Guide RNAs (gRNAs) were designed to target the PAM sequence located 161 bp downstream from the stop codon of GDF5 (CTGAGTGTGACTTGGGCTAAAGG, underlining indicates the PAM sequence). gRNA oligonucleotides (Table S1) were designed, phosphorylated, annealed, and subcloned into the PX459 vector (Addgene) harboring a Cas9 expression cassette (PX459-GDF5 gRNA). The *SP7* 3′ homology region (SP7 3′HA) was synthesized using PCR with a KOD FX NEO Kit (Takara). Genomic DNA extracted from 414C2 hiPSCs, which served as a template, was ligated with the pTA2 vector (TOYOBO) by TA cloning (pTA2-SP7 3′HA vector). To construct the targeting vector (pTA2-SP7 3′HA HA-IRES-tdTomato-PGK-Neo), IRES-tdTomato-PGKNeo fragments were inserted into the pTA2-SP7 3′HA vector using an In-Fusion HD Cloning Kit (Takara). gRNAs were designed to target the PAM sequence located 104 bp downstream from the stop codon of *SP7* (CCCATGCATGCCATCCTTCGGGG, underlining indicates the PAM sequence). gRNA oligonucleotides (Table S1) were designed, phosphorylated, annealed, and subcloned into the PX459 vector (Addgene) harboring a Cas9 expression cassette (PX459-SP7 gRNA).

To generate the GDF5$^{3'tdTomato}$ or SP7$^{3'tdTomato}$ reporter line, 1 × 10^6 414C2 hiPSCs (GDF5$^{3'tdTomato}$ reporter) or SEES4 hESCs (SP7$^{3'tdTomato}$ reporter) were electroporated with the targeting vector (7.5 μg) and Cas9/gRNA expression vector (2.5 μg). Selection with G418 (Life Technologies) was maintained for 10 days until stable colonies appeared. Colonies with diameters >500 μm were selected using a P10 pipette tip with the aid of a microscope and used to inoculate larger volumes of media. To verify precise integration of the IRES-tdTomato-PGKNeo cassette into the GDF5/SP7 3′UTR locus, the 5′ or 3′ ends between GDF5/SP7 and the IRES-tdTomato-PGKNeo cassette were amplified for nucleotide sequence analysis. The PCR primers used are listed in Table S1.

Generation of CAG-GFP and CAG-KuO reporter cell lines

To generate the CAG-GFP hiPSC and CAG-KuO hiPSC reporter lines, 1 × 10^6 single cells dissociated from 414C2 hiPSCs were electroporated with 5 μg of the piggyBac Transposase expression plasmid (PCAG-PBase, kindly provided by Dr. Knut Woltjen) [38] and 5 μg of the donor plasmid (pPB-CAG-GFP or pPB-CAG-KuO) using Super Electroporator NEPA21 Type II (NEPAGENE), and then plated onto iMatrix-coated plates. Three days after electroporation, cells were treated with puromycin for 1 week and allowed to recover for another 5–7 days. Then, GFP$^+$ or KuO$^+$ clones were picked and expanded.

METHOD DETAILS

Generation of ExpLBM cells from hPSCs

For stepwise differentiation into LBM cells [23,24], hPSCs (3×10^4) were suspended in 1 mL of StemFit (Ajinomoto) containing 10 µM Y-27632 (MedChemExpress), and 4 µL of iMatrix was added to a 3.5-cm culture dish. The next day, the culture medium was replaced with fresh StemFit lacking Y27632. After 2 days of culture, cells were washed with phosphate-buffered saline (PBS) and differentiation was induced by changing to different culture medium (based on chemically defined CDM2 medium) at each time point as described previously [24]. In brief, cells were treated with mid-primitive streak (MPS) medium (CDM2 basal medium containing 30 ng/mL activin A, 40 ng/mL BMP4, 6 µM CHIR99021, 100 nM PIK-90, and 10 µM Y27632) for 1 day to induce MPS cells (day 1), LPM medium (CDM2 basal medium containing 1 µM A 83-01, 30 ng/mL BMP4, 1 µM C59, and 10 µM Y-27632) for 1 day to induce LPM cells (day 2), and finally LBM medium (CDM2 basal medium containing 1 µM A 83-01, 0.5 µM LDN-193189, 3 µM CHIR99021, 150 nM Vismodegib, and 10 µM Y-27632) for 2 days to induce LBM cells (day 4). The composition of CDM2 basal medium was 50% IMDM (with GlutaMAX, HEPES, and sodium bicarbonate; Gibco) and 50% F12 (with GlutaMAX) containing 1 mg/mL polyvinyl alcohol (Sigma-Aldrich), 1% CD lipid concentrate (Gibco), 450 µM monothioglycerol (Sigma-Aldrich), 7 µg/mL insulin (Sigma-Aldrich), 15 µg/mL transferrin (Roche), and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific). To generate ExpLBM cells, LBM cells were dissociated using accutase (NACALAI TESQUE), and 2×10^5 cells were suspended in ExpLBM medium [CDM2 basal medium containing 3 µM CHIR99021, 1 µM A 83-01, and 20 ng/mL FGF2 (Katayama Chemicals); 20 ng/mL EGF (STEMCELL TECHNOLOGIES); and 10 µM Y-27632] and cultured on a 6-cm dish coated with 4 µg/mL human plasma fibronectin (Sigma-Aldrich). The culture media was replaced with fresh ExpLBM medium every 2 days. Before reaching subconfluency, the cells were passaged as described above. The details of the human recombinant proteins and small-molecule agonists and inhibitors are provided in the key resources table.

Chondrogenic induction (generation of IZ/ACP organoids, GPC organoids, and skeletal assembloids)

To perform chondrogenic induction of ExpLBM cells under pellet culture conditions (3DCI), 2 × 10 ExpLBM cells were suspended in 200 µL of step 1 medium [CDM2 medium containing 3 µM CHIR99021, 10 ng/mL FGF2, and 50 µg/mL ascorbic acid (Wako) and 1× ITS (Thermo Fisher Scientific)] and plated into 96-well ultra-low U-bottom plates (CORNING). The cells were

pelleted by centrifugation at 2,000 rpm for 5 min. After 6 days of culture, the culture medium was changed to step 2 medium [CDM2 medium containing 50 μg/mL ascorbic acid, 1× ITS, and 30 ng/mL BMP4 (R&D); 10 ng/mL TGFβ1 (PEPROTECH); 10 ng/mL GDF5 (PEPROTECH); and 10 ng/mL FGF2] and cultured for an additional 6 days. Subsequently, the medium was replaced with step 3 medium (CDM2 medium containing 50 μg/mL ascorbic acid, 1× ITS, 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 10 ng/mL GDF5) and maintained for 14 days in the presence of various tested chemicals. To generate IZ/ACP organoids, step 3 medium supplemented with 500 nM PD0325901 was used (#16 condition, Figure 1B). GPC organoids were generated using CDM2 medium containing 50 μg/mL ascorbic acid, 1× ITS, 300 ng/mL BMP4, 100 ng/mL TGFβ1, and 10 ng/mL GDF5 during the 14-day step 3 period (#2 condition, Figure 1B). The culture medium was refreshed with fresh medium every 3 days. To generate skeletal assembloids, two IZ/ACP organoids were positioned vertically at both ends of a GPC organoid within low-melting point agarose gel (Sigma-Aldrich) and then cultured in DMEM (Wako) supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific) for 7 days to allow spontaneous fusion of these samples. The media were changed every other day.

Bulk RNA-seq and data processing

Total RNA was extracted using a RNeasy Mini Kit (QIAGEN), and sequencing libraries were prepared using a KAPA RNA HyperPrep Kit with RiboErase (HMR) (Nippon Genetics) and a SeqCap Adapter Kit (Set A or Set B) (Roche) following the manufacturers' instructions. Sequencing was performed using NovaSeq 6000 (Illumina) with a pair-read sequencing length of 150 bp. All sequence reads were extracted in FASTQ format using the CASAVA 1.8.4 pipeline. Fastp (v0.23.4) was used to remove adapters and filter raw reads with < 60 bases in addition to leading and trailing bases with a read quality of less than 30. For pseudoalignment of the filtered reads to GRCh38, Kallisto (v0.48.0) was used. GeTMM normalization was performed using edgeR (v4.0.3) to account for sample variation. Differentially expressed genes were identified by NOISeq (v2.46.0) analysis with a threshold of $p > 0.8$ and abs(Log2FC) > 1. For conversion of hiPSCs to ExpLBM cells, our previous data were reanalyzed (GSE165620).

ATAC-seq and data processing

ATAC-seq was conducted following a previously described method [39]. In brief, 4000 cells were collected and lysed using lysis buffer containing 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% IGEPAL CA-630. The Tn5 transposase reaction using Illumina Tagment DNA Enzyme and Buffer (Illumina) was performed at 37°C for 30 min. The reacted DNA was purified using a MinElute PCR Purification Kit (QIAGEN) and amplified for 8–15 cycles using a Nextera Index Kit

(Illumina) to produce libraries for sequencing. Sequencing was performed using HiSeq1500 (Illumina) with a pair-read sequencing length of 150 bp. Two or three biological replicates were analyzed. Sequences were aligned to human genome sequences (hg38) using Bowtie2 with default parameters. Gopeaks was used to call peaks using default parameters. Regions where overlapping peaks were identified by Bedtools that intersected between biological replicates were designated as high-confidence peaks. The read count matrix of each sample was used to detect differentially accessible peaks using edgeR. To generate a heatmap, normalized read counts obtained using edgeR were z-score-scaled and plotted. For motif analysis, findMotifsGenome.pl of Homer was used with the -size200-mask option. For annotation of peaks, annotatePeaks.pl of Homer was used with default settings. For visualization, BAM files were converted to bigwig format using deeptools with the command “bamCoverage -binSize 1 -normalizeUsing RPGC --extendReads”.

CUT&Tag analysis of histone modifications and data processing

A total of 20,000 cells were collected as input, and libraries were prepared using a CUT&Tag-IT Assay Kit (Active Motif) following the manufacturer’s protocol. Anti-H3K27ac (Active Motif) and anti-H3K27me3 (Active Motif) antibodies were used for reactions. Three biological replicates were analyzed. Sequencing was performed using HiSeq1500 (Illumina) with a pair-read sequencing length of 150 bp. Sequences were aligned to human genome sequences (hg38) using Bowtie2 (parameters: --end-to-end, --very-sensitive, --no-mixed, --no-discordant, --phred33, -I 10, -X 700). For peak calling, gopeaks and bedtools were utilized as described in the previous section. BAM files were converted to bigwig format using deeptools with the command “bamCoverage -binSize 1 -normalizeUsing RPGC --extendReads”. A heatmap was created using the “computeMatrix” and “plotHeatmap” functions of deeptools. To separate promoter and non-promoter regions, the “annotatePeaks.pl” function of Homer was utilized, and a promoter was defined as one that was annotated as a promoter-TSS in the default setting.

***In vivo* transplantation**

For subcutaneous transplantation, organoids and assembloids were transplanted into immunodeficient NOD-SCID mice. For transplantation into osteochondral defects created in knee joint articular cartilage, the joint capsules of the knee joints of 10-week-old SCID rats (F344-Il2rgem1Iexas) were opened. A drill hole with a 1-mm diameter and a 0.5-mm depth was created at the femoral groove. Organoids were transplanted into the osteochondral defect of articular cartilage via press fitting. The joint capsules were closed, and rats were sacrificed 3 months later. For transplantation into critical-sized femur defects of SCID rats, a critical-size defect measuring 5 mm was created in the midshaft of the femur using a drill. The created

defect was stabilized using an external fixator, and large GPC organoids were implanted into the defect. For postoperative evaluation of bone regeneration, radiographic imaging and histological analysis were performed at 1 and 3 months after surgery.

Transplanted tissues were harvested and fixed with 4% PFA (Wako) for 24 h. Tissue samples were paraffin-embedded at the Central Research Laboratory, Okayama University Medical School. For hematoxylin and eosin (HE) staining, deparaffinized sections were stained with hematoxylin (Wako) for 20 min and with eosin (Wako) for 1 min. For Safranin O staining, deparaffinized sections were stained with 0.05% Fast Green (Wako) solution for 5 min and then with 0.1% Safranin O (Wako) solution for 5 min. For von Kossa staining, fixed samples were incubated in 5% silver nitrate (Wako) under sunlight for 60 min and then treated with 5% sodium thiosulfate for 3 min. After staining, samples were dehydrated and embedded with Entellan new reagent (Merck). Images were acquired using a BZ-X710 microscope (Keyence).

Flow cytometry

Following dissociation with accutase, 1×10^5 cells were suspended in 100 µL of PBS containing 2% FBS and incubated with an antibody (diluted 1:200) for 1 h on ice. Fluorescence was detected using a CytoFLEX S flow cytometer (Beckman Coulter), and data were analyzed using FlowJo software (FlowJo LLC). The antibodies used are listed in the key resources table.

RT-qPCR

Total RNA was extracted using a RNeasy Mini Kit, and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's protocol. cDNAs were then used as templates for qPCR analysis with Luna Universal qPCR Master Mix (NEB) and gene-specific primers. qPCR was performed using an AriaMX Real-Time PCR System (Agilent). The cycle parameters were as follows: denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and elongation at 72°C for 30 sec. The expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are provided in Table S1.

Immunohistochemistry

Tissues were fixed with 4% PFA, and paraffin-embedded samples were sectioned (4 µm thick). Tissue samples were deparaffinized, and antigens were activated by heating slides in 10 mM citrate buffer (pH 6.0). Samples were incubated with blocking solution [1× PBS (−) containing 3% NGS (Vector Laboratories) and 0.1% Triton X-100]. Samples were treated with a primary antibody (diluted 1:200) overnight at 4°C and were incubated with blocking solution [1× PBS (−) containing 3% NGS and 0.1% Triton X-100], followed by a secondary antibody (diluted 1:400) for 1 h at room temperature. Samples were embedded with Fluoromount-G (SouthernBiotech)

after staining with DAPI (Thermo Fisher Scientific), and images were acquired using a BZ-X710 camera. The antibodies are listed in the key resources table.

scRNA-seq and data processing

ExpLBM cells were washed with PBS (−) and dissociated with accutase at 37°C for 5 min, while IZ/ACP and GPC organoids were washed with PBS (−) and then treated with 900 U/mL collagenase (type II) (Gibco) for 60 min at 37°C, followed by gentle pipetting using a serological pipette to dislodge and dissociate aggregates. Cells were sorted on a FACSAria III Cell Sorter (BD Biosciences) to remove dead cells and debris via forward scatter, side scatter, and DAPI/calcein-AM staining. A total of 10,000 cells were analyzed. A Chromium Next GEM Single Cell 3′ GEM Kit v3.1 (10x Genomics) was used for library preparation. Dissociated cells were separated into single droplets with unique molecular barcodes and then processed following the manufacturer's protocol. Quality checks of the samples were performed using high sensitivity D5000 screen tape (Agilent) and the Agilent 4150 TapeStation system (Agilent). Sequencing was performed using DNBseq (MGI).

To obtain count matrices, reads were aligned to the human genome GRCh38 using Cell Ranger (v7.0.1) with default parameters. Downstream analysis was performed using the R package Seurat (v5.0.3) [40]. For quality control, cells with more than five detected genes, a UMI count higher than 5, and <10% mitochondrial genes were kept as high-quality cells. Doublets were detected using DoubletFinder (v2.0.4) [41] and removed. Counts were normalized, and then the top 3000 highly variable genes were detected via the SCTransform and FindVariableFeatures functions of Seurat.

For data integration, the batch effect was removed by the LeverageScore method via SketchData in Seurat. After scaling gene expression and performing dimensional reduction, cells were visualized using UMAP embedding. Cluster annotations were labeled with canonical markers.

Transcriptome similarity was compared with published developing human limb [5–9 postconceptional weeks (PCW)] scRNA-seq data using anchor-based label transfer implemented in Seurat. To this end, anchors between the query and reference dataset were identified and filtered using the FindTransferAnchors function with the top 14 dimensions. Then, the prediction score was calculated using the TransferData function. The cell type and region similarity (defined as the mean prediction score of each cell type) were visualized in a heatmap.

Trajectory analysis was performed using the monocle3 package (v1.3.7) [42] with default parameters, and the Mes1 cluster was chosen as the root node to order cells. A pseudotime heatmap was plotted using the ComplexHeatmap (v2.18.0) package.

SEM and TEM

For SEM, samples were fixed overnight at 4°C in 0.1 M phosphate buffer (pH 7.4) containing 2% PFA and 2% glutaraldehyde. After washing, samples were post-fixed with 2% osmium tetroxide for 90 min at room temperature. Following washing, tissues were dehydrated using a series of ascending ethanol solutions (30%, 50%, 70%, 90%, and two changes of 100%) for 10, 10, 10, 30, 30, and 30 min, respectively. Dehydrated tissues were immersed in t-butyl alcohol for 30 min and freeze-dried using an EIKO ID-2 freeze dryer. Dried samples were cracked with a razor blade to expose the photoreceptor layer and mounted on the aluminum sample stage. Sample surfaces were coated with osmium using HPC-1SW (vacuum device). Subsequently, samples were analyzed using a Hitachi S-4800 scanning electron microscope. For TEM, cartilaginous particles were fixed with 4% PFA and 2% glutaraldehyde, followed by post-fixation with 2% osmium tetroxide. After dehydration, embedding, and polymerization, ultra-thin sections were stained with 2% uranyl acetate and observed using a Hitachi H-7100S transmission electron microscope at an acceleration voltage of 80 kV.

***In situ* hybridization**

Paraffin sections were stained for GFP using an RNAscope Multiplex Fluorescent Reagent Kit ver. 2 (Advanced Cell Diagnostics). More precisely, sections were pretreated with RNAScope hydrogen peroxidase for 10 min at room temperature, with RNAScope Target Retrieval for 15 min at 98°C, and with protease plus treatment for 30 min at 40°C. Sections were then hybridized with a GFP probe (Advanced Cell Diagnostics) for 2 h at 40°C and amplified through six sequential steps according to the manufacturer's instructions. The probe signal was visualized with TSA Vivid Fluorophore 570 (Advanced Cell Diagnostics). Then, slides were observed using a BZ-X710 camera.

µCT analysis

To analyze critical-sized femoral defects in SCID rats, imaging was performed using a Latheta LCT-200 (Hitachi Aloka Medical) µCT system with a voltage of 50 kV and a current of 200 µA. To analyze skeletal assembloids, scans were performed on ScanXmate-L090HH (Comscantecno) with a voltage of 74 kV and a current of 100 µA. The resolution was 11.906 µm per pixel. 3D images were reconstructed using VXRE, VX3D (3D Industrial Imaging), and TRI/3D-BON-FCS (RATOC System Engineering).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using Prism 8. All data were acquired by performing biological replicates of two or three independent experiments and are presented as the mean ± standard error of the

mean. Statistical significance was determined using a two-tailed Student's t-test and an ordinary one-way analysis of variance with correction for multiple testing using Tukey's method.

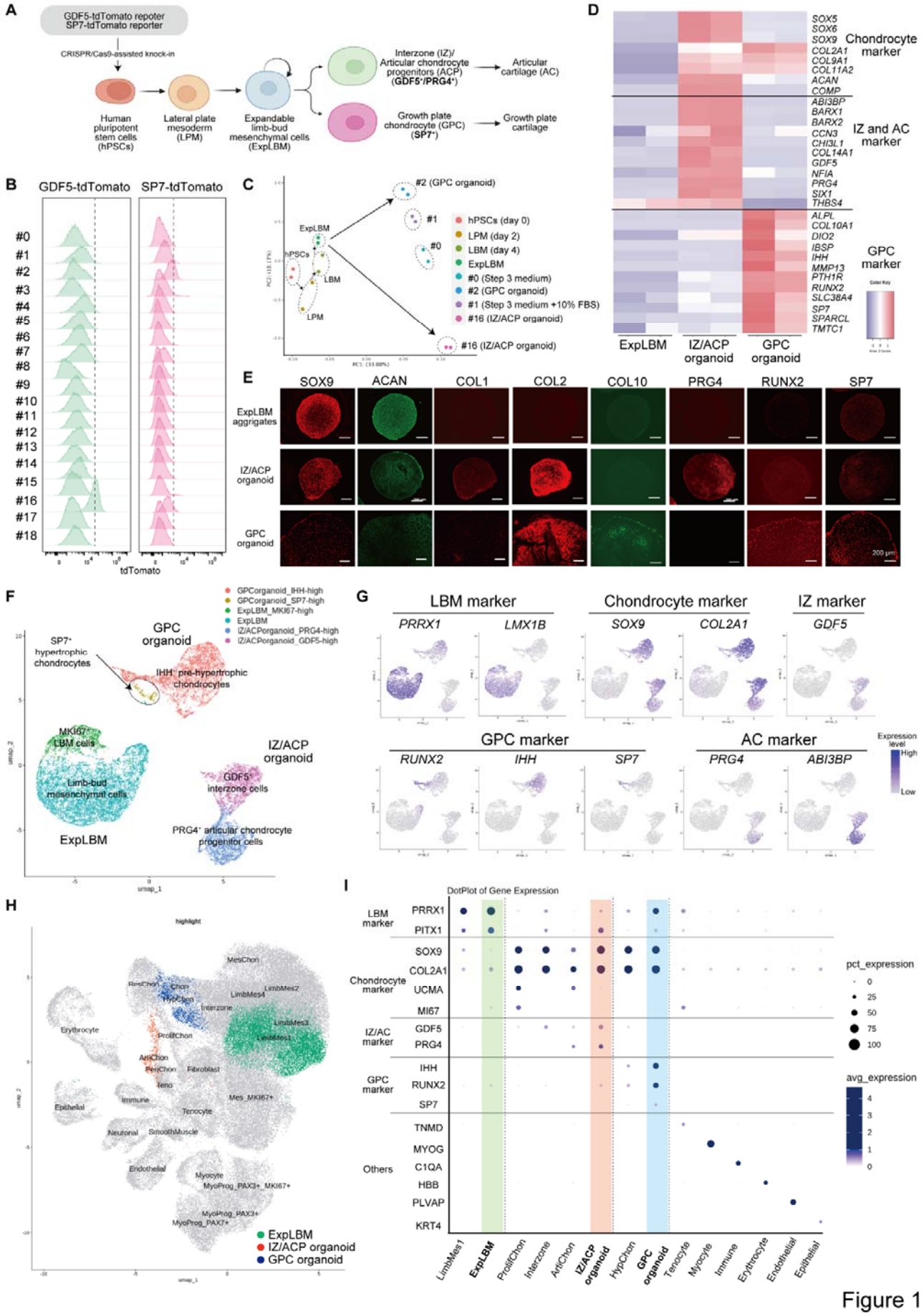
A
GDF5-tdTomato repoter
SP7-tdTomato reporter
CRISPR/Cas9-assisted knock-in
Human pluripotent stem cells (hPSCs)
Lateral plate mesoderm (LPM)
Expandable limb-bud mesenchymal cells (ExpLBM)
Interzone (IZ)/ Articular chondrocyte progenitors (ACP) (GDF5+/PRG4+)
Articular cartilage (AC)
Growth plate chondrocyte (GPC) (SP7+)
Growth plate cartilage
B
GDF5-tdTomato
SP7-tdTomato
tdTomato
C
#2 (GPC organoid)
#1
#0
#16 (IZ/ACP organoid)
ExpLBM
hPSCs
LBM
LPM
hPSCs (day 0)
LPM (day 2)
LBM (day 4)
ExpLBM
#0 (Step 3 medium)
#2 (GPC organoid)
#1 (Step 3 medium +10% FBS)
#16 (IZ/ACP organoid)
D
Chondrocyte marker
IZ and AC marker
GPC marker
ExpLBM
IZ/ACP organoid
GPC organoid
E
SOX9
ACAN
COL1
COL2
COL10
PRG4
RUNX2
SP7
ExpLBM aggrigates
IZ/ACP organoid
GPC organoid
200 μm
F
GPCorganoid_IHH-high
GPCorganoid_SP7-high
ExpLBM_MKI67-high
ExpLBM
IZ/ACPorganoid_PRG4-high
IZ/ACPorganoid_GDF5-high
GPC organoid
SP7+ hypertrophic chondrocytes
IHH+ pre-hypertrophic chondrocytes
MKI67+ LBM cells
Limb-bud mesenchymal cells
ExpLBM
IZ/ACP organoid
GDF5+ interzone cells
PRG4+ articular chondrocyte progenitor cells
umap_1
umap_2
G
LBM marker
PRRX1
LMX1B
Chondrocyte marker
SOX9
COL2A1
IZ marker
GDF5
GPC marker
RUNX2
IHH
SP7
AC marker
PRG4
ABI3BP
Expression level
High
Low
H
highlight
MesChon
ResChon
Chon
HypChon
LimbMes2
LimbMes4
Interzone
LimbMes3
LimbMes1
Erythrocyte
ProliferChon
AntiChon
PeriChon
Fibroblast
Teno
Immune
Mes_MKI67+
Epithelial
Tenocyte
Neuronal
SmoothMuscle
Endothelial
Myocyte
MyoProg_PAX3+_MKI67+
MyoProg_PAX3+
MyoProg_PAX7+
ExpLBM
IZ/ACP organoid
GPC organoid
umap_1
umap_2
I
DotPlot of Gene Expression
LBM marker
PRRX1
PITX1
Chondrocyte marker
SOX9
COL2A1
UCMA
MI67
IZ/AC marker
GDF5
PRG4
GPC marker
IHH
RUNX2
SP7
Others
TNMD
MYOG
C1QA
HBB
PLVAP
KRT4
LimbMes1
ExpLBM
ProliferChon
Interzone
AntiChon
IZ/ACP organoid
HypChon
GPC organoid
Tenocyte
Myocyte
Immune
Erythrocyte
Endothelial
Epithelial
pct_expression
0
25
50
75
100
avg_expression
4
3
2
1
0

Figure 1

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Figure 1: *In vitro* stepwise induction of hPSC-derived LBM cells into IZ/ACP and GPC organoids.

(A) Overview of the stepwise induction and differentiation of human LBM cells, followed by chondrocyte lineages, which closely mimics embryonic development. The optimal conditions were determined for ExpLBM cells to induce IZ/ACP and GPC organoids using GDF5- and SP7-tdTomato reporters, respectively.

(B) tdTomato fluorescence intensity in GDF5$^{3'tdTomato}$ or SP7$^{3'tdTomato}$ reporter-derived ExpLBM cells treated with 18 combinations of chemicals and cytokines for 14 days in the presence of 30 ng/mL BMP4 and 10 ng/mL TGFβ1. #0, none; #1, 10% FBS; #2, 300 ng/mL BMP4, 100 ng/mL TGFβ1, and 10% FBS; #3, CHIR99021 (GSK3β inhibitor and WNT activator); #4, C59 (PORCN inhibitor and WNT inhibitor); #5, XAV-939 (tankyrase 1 inhibitor and WNT inhibitor); #6, LDN-193189 (ALK2/3 inhibitor); #7, A 83-01 (ALK4/5/7 inhibitor); #8, SAG 21k (hedgehog signaling activator); #9, Vismodegib (hedgehog signaling inhibitor); #10, MHY1485 (mTOR activator); #11, VO-OHpic (PTEN inhibitor and PI3K/mTOR activator); #12, SF1670 (PTEN inhibitor and PI3K/mTOR activator); #13, rapamycin (mTOR inhibitor); #14, ATRA (RA signaling activator); #15, MK-206 (AKT inhibitor); #16, PD0325901 (MEK inhibitor); #17, PIK-90 (PI3K inhibitor); and #18, DAPT (γ-secretase inhibitor and Notch inhibitor).

(C–D) Bulk RNA-seq transcriptome analysis of hPSCs (day 0), LPM cells (day 2), LBM cells (day 4), ExpLBM cells (passage numbers 4–5), and aggregates treated under the following conditions: (i) 30 ng/mL BMP4 and 10 ng/mL TGFβ1 (condition #0), (ii) 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 10% FBS (condition #1), (iii) 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 500 nM PD-0325901 (condition #16, referred to as IZ/ACP organoids), and (iv) 300 ng/mL BMP4, 100 ng/mL TGFβ1, and 10% FBS (condition #2, referred to as GPC organoids). n = 2 biologically independent experiments. Principal component analysis (C) and a heatmap of the gene expression level of each marker (D).

(E) Immunohistological analysis of ExpLBM aggregates, IZ/ACP organoids, and GPC organoids with antibodies against SOX9, ACAN, COL1, COL2, COL10, PRG4, RUNX2, and SP7.

(F) UMAP embedding of scRNA-seq data from ExpLBM cells (9741 cells), IZ/ACP organoids (5090 cells), and GPC organoids (4660 cells). UMAP clustering identified six clusters, including LBM cells (non-dividing) and MKI67^{+} LBM cells (dividing) in ExpLBM cells, GDF5^{+} interzone cells and PRG4^{+} articular chondrocyte progenitors in IZ/ACP organoids, and IHH^{+} pre-hypertrophic chondrocytes and SP7^{+} hypertrophic chondrocytes in GPC organoids.

(G) Feature plot of each marker gene in UMAP of integrated scRNA-seq datasets from ExpLBM cells, IZ/ACP organoids, and GPC organoids.

(H) UMAP of integrated scRNA-seq profiles of ExpLBM cells, IZ/ACP organoids, and GPC organoids with human embryonic limb cells at PCW 5–9 (119,870 cells). Cells are colored by

origin: human embryonic limb cells (gray), ExpLBM cells (green), IZ/ACP organoids (red), and GPC organoids (blue). The original datasets of human embryonic limb cells are from Zhang et al. [27].

(I) Dot plots showing expression of cell type-specific genes in each cluster of scRNA-seq analysis of human embryonic limb cells and scRNA-seq profiles of ExpLBM cells, IZ/ACP organoids, and GPC organoids.

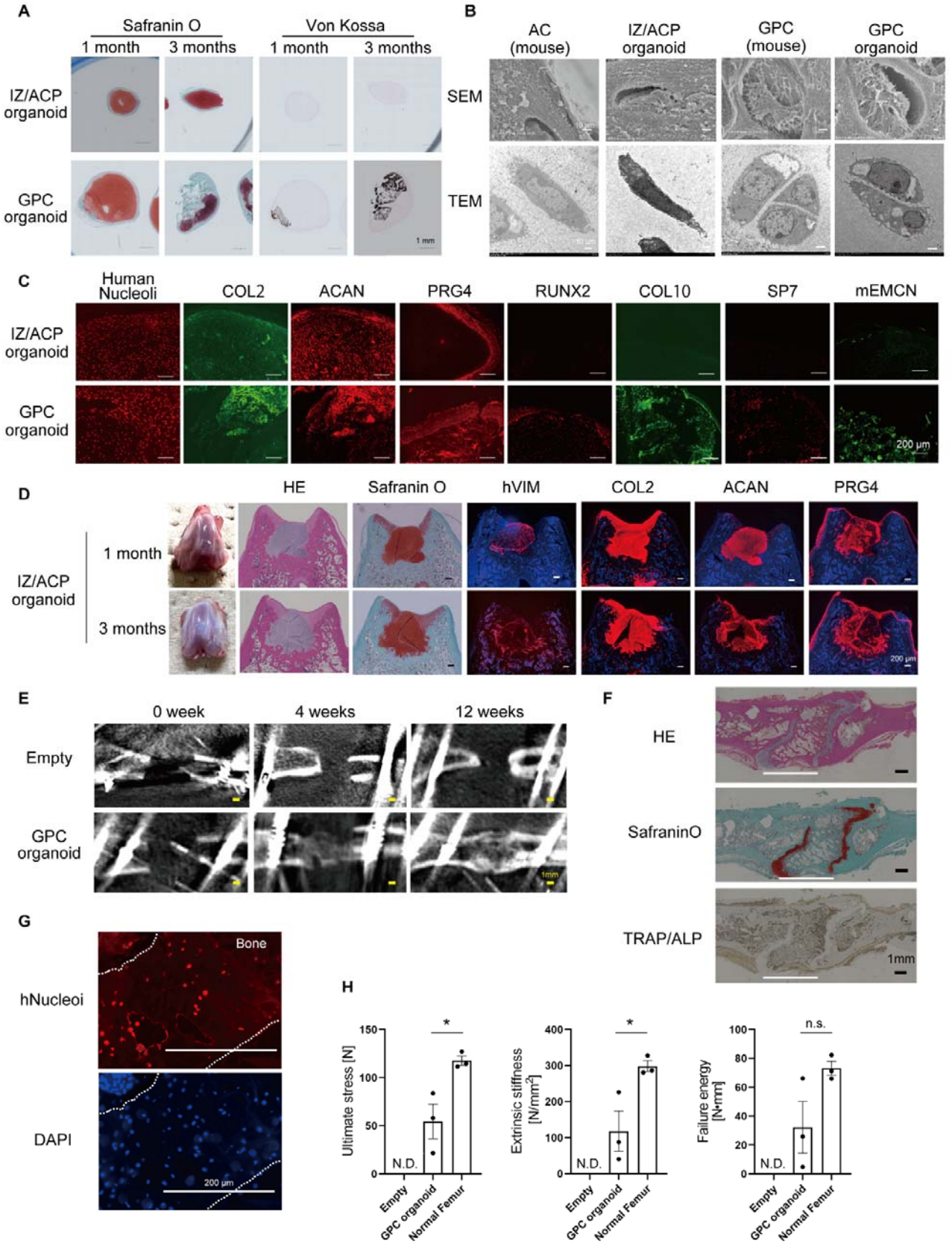
A
Safranin O
Von Kossa
1 month
3 months
1 month
3 months
IZ/ACP organoid
GPC organoid
1 mm
B
AC (mouse)
IZ/ACP organoid
GPC (mouse)
GPC organoid
SEM
TEM
C
Human Nucleoli
COL2
ACAN
PRG4
RUNX2
COL10
SP7
mEMCN
IZ/ACP organoid
GPC organoid
200 μm
D
HE
Safranin O
hVIM
COL2
ACAN
PRG4
IZ/ACP organoid
1 month
3 months
200 μm
E
0 week
4 weeks
12 weeks
Empty
GPC organoid
1mm
F
HE
SafraninO
TRAP/ALP
1mm
G
hNucleoi
DAPI
Bone
200 μm
H
Ultimate stress [N]
Extrinsic stiffness [N/mm²]
Failure energy [N·mm]
N.D.
*
n.s.
Empty
GPC organoid
Normal Femur

Figure 2

688

Figure 2: Assessment of the graft fate and regenerative capacity of IZ/ACP and GPC organoids.

(A–C) Subcutaneous transplantation of IZ/ACP or GPC organoids into NOD-SCID mice. Their engraftment was histologically assessed at 12 weeks post-transplantation by Safranin O and Von Kossa staining (A), SEM and TEM (B), and immunohistological analysis (C).

(D) Transplantation of ExpLBM-derived IZ/ACP organoids into osteochondral defects of articular cartilage in SCID rats. The knee joints were harvested at 1 and 3 months post-transplantation. Histological examination was performed of osteochondral defects engrafted with IZ/ACP organoids. At 1 and 3 months post-transplantation, knee joints were fixed, and tissue sections were stained with HE or Safranin O or an antibody against hVIM, COL2, ACAN, or PRG4. Representative images are displayed.

(E) Engraftment of GPC organoids into critical-sized femoral defects of SCID rats was assessed at 4 and 12 weeks post-transplantation using μCT analysis.

(F–G) Engraftment of GPC organoids into critical-sized femoral defects of SCID rats was histologically assessed at 8 weeks post-transplantation using HE, Safranin O, and ALP/TRAP staining (F), as well as immunostaining with an anti-human nucleoli antibody (G).

(H) Bone strength in critical-sized femoral defects was assessed at 12 weeks post-transplantation using a three-point bending test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. N.D., not detected.

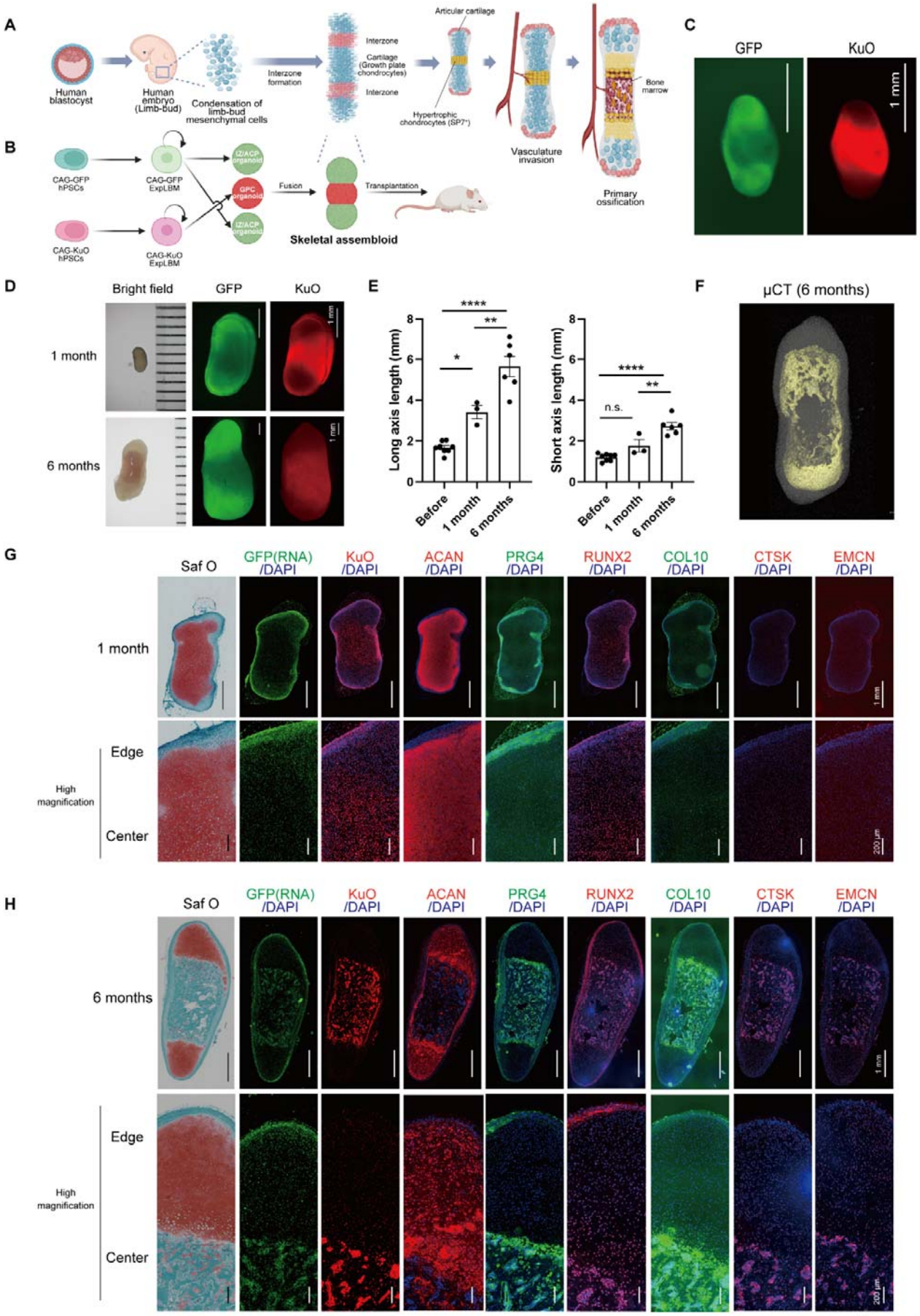
A
Human blastocyst
Human embryo (Limb-bud)
Condensation of limb-bud mesenchymal cells
Interzone formation
Interzone
Cartilage (Growth plate chondrocytes)
Interzone
Articular cartilage
Hypertrophic chondrocytes (SP7+)
Vasculature invasion
Bone marrow
Primary ossification
B
CAG-GFP hPSCs
CAG-GFP ExpLBM
CAG-KuO hPSCs
CAG-KuO ExpLBM
IZ/ACP organoid
GPC organoid
IZ/ACP organoid
Fusion
Skeletal assembloid
Transplantation
C
GFP
KuO
1 mm
D
Bright field
GFP
KuO
1 month
6 months
1 mm
E
Long axis length (mm)
Short axis length (mm)
Before
1 month
6 months
n.s.
F
μCT (6 months)
G
Saf O
GFP(RNA) /DAPI
KuO /DAPI
ACAN /DAPI
PRG4 /DAPI
RUNX2 /DAPI
COL10 /DAPI
CTSK /DAPI
EMCN /DAPI
1 month
High magnification
Edge
Center
1 mm
200 μm
H
Saf O
GFP(RNA) /DAPI
KuO /DAPI
ACAN /DAPI
PRG4 /DAPI
RUNX2 /DAPI
COL10 /DAPI
CTSK /DAPI
EMCN /DAPI
6 months
High magnification
Edge
Center
1 mm
200 μm

Figure 3

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Figure 3: Generation of skeletal assembloids from IZ/ACP and GPC organoids to recapitulate human limb skeletal development.

(A) Schematic drawing of skeletal development in the limb. Skeletogenesis begins with LBM cell condensation, followed by formation of skeletal anlagen composed of immature chondrocytes. The joint interzone develops at the position of the presumptive joint and precedes articular cartilage differentiation and joint cavitation. Immature chondrocytes outside the interzone undergo hypertrophy at the mid-diaphysis as the first step of endochondral ossification. Vasculature invades hypertrophic chondrocytes and subsequently forms the bone marrow cavity and growth plate cartilage, which support growth and elongation of the limbs until puberty, when their closure halts any further skeletal growth.

(B) Graphical illustration of the approach to generate assembloids from GFP-labeled IZ/ACP organoids and KuO-labeled GPC organoids.

(C) Fluorescent images of a typical example of a skeletal assembloid fabricated with a KuO-labeled GPC organoid sandwiched by two GFP-labeled IZ/ACP organoids, followed by culture for 7 days.

(D) Subcutaneous transplantation of a skeletal assembloid into NOD-SCID mice. Representative bright-field and fluorescent images of the assembloid at 1 and 6 months post-transplantation.

(E) Long and short length of the skeletal assembloid at 1 and 6 months post-transplantation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

(F) µCT image of the skeletal assembloid at 6 months post-transplantation.

(G) Engraftment was histologically assessed at 1 month post-transplantation by Safranin O staining and immunohistological analysis.

(H) Engraftment was histologically assessed at 6 months post-transplantation by Safranin O staining and immunohistological analysis.

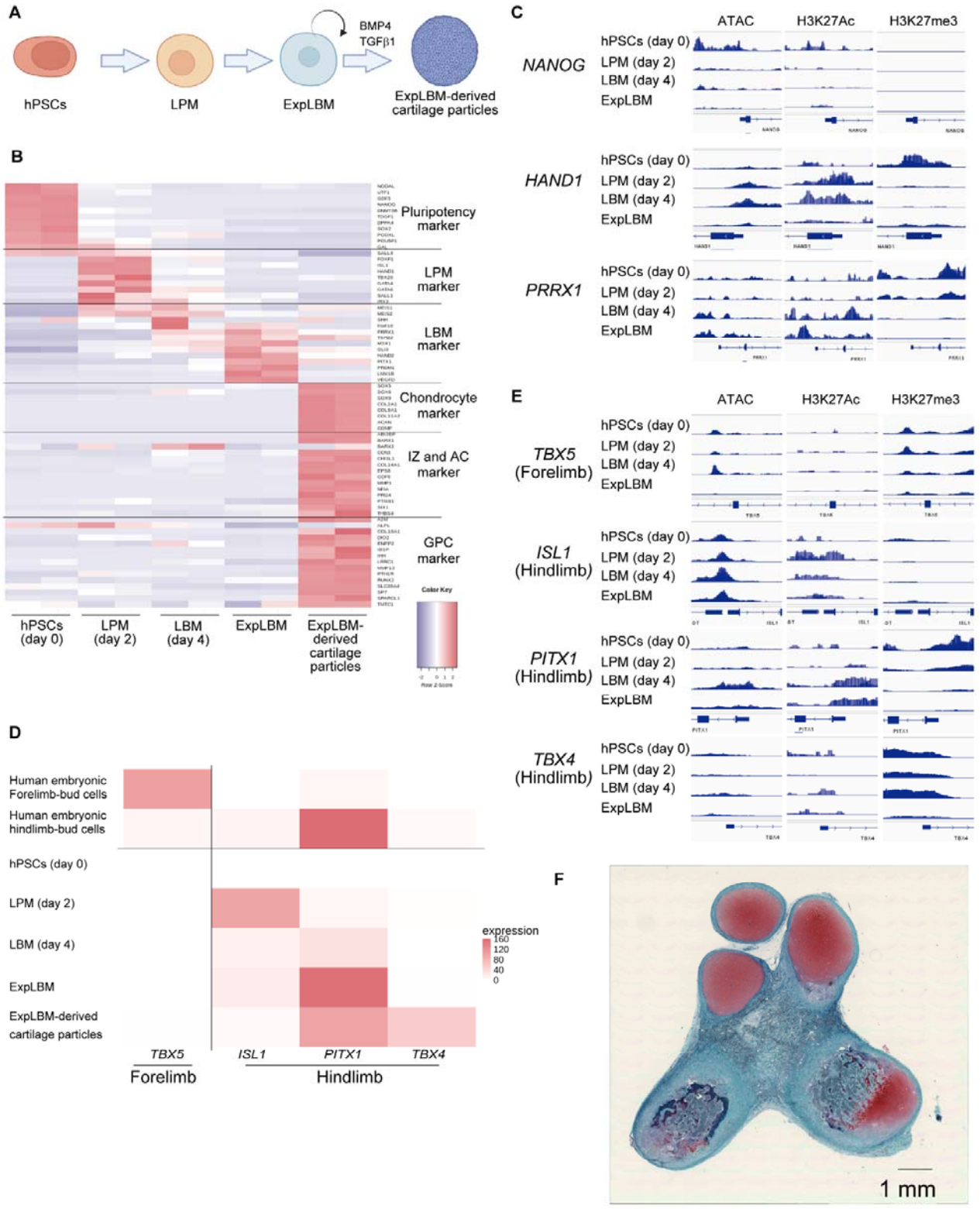
A
BMP4
TGFβ1
hPSCs
LPM
ExpLBM
ExpLBM-derived cartilage particles
B
Pluripotency marker
LPM marker
LBM marker
Chondrocyte marker
IZ and AC marker
GPC marker
Color Key
hPSCs (day 0)
LPM (day 2)
LBM (day 4)
ExpLBM
ExpLBM-derived cartilage particles
C
ATAC
H3K27Ac
H3K27me3
NANOG
HAND1
PRRX1
hPSCs (day 0)
LPM (day 2)
LBM (day 4)
ExpLBM
E
TBX5 (Forelimb)
ISL1 (Hindlimb)
PITX1 (Hindlimb)
TBX4 (Hindlimb)
D
Human embryonic Forelimb-bud cells
Human embryonic hindlimb-bud cells
hPSCs (day 0)
LPM (day 2)
LBM (day 4)
ExpLBM
ExpLBM-derived cartilage particles
expression
160
120
80
40
0
TBX5
ISL1
PITX1
TBX4
Forelimb
Hindlimb
F
1 mm

Figure S1

Figure S1: *In vitro* stepwise induction of hPSCs into LBM cells in a manner consistent with hindlimb specification.

(A) Schematic view of the hierarchical induction of ExpLBM cells and subsequent differentiation into hyaline cartilaginous-like tissues.

(B) Heatmap of gene expression levels of markers in bulk RNA-seq transcriptome analysis of hPSCs (day 0), LPM cells (day 2), LBM cells (day 4), ExpLBM cells (passage numbers 4–5), and ExpLBM-derived cartilage particles generated with 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 10 ng/mL GDF5. n = 2 biologically independent experiments.

(C) Integrative Genomics Viewer (IGV) screenshots showing peaks from ATAC-seq and CUT&Tag analyses around marker genes in hPSCs (day 0), LPM cells (day 2), LBM cells (day 4), and ExpLBM cells (passage numbers 4–5).

(D) Heatmap showing expression of the forelimb-specific gene *TBX5* and the hindlimb-specific genes *ISL1*, *PITX1*, and *TBX4* in human embryonic forelimb and hindlimb bud cells from scRNA-seq analysis of human embryonic limb cells [27], and in bulk RNA-seq transcriptome analysis of hPSCs (day 0), LPM cells (day 2), LBM cells (day 4), ExpLBM cells (passage numbers 4–5), and ExpLBM-derived cartilage particles treated with 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 10 ng/mL GDF5. Data represent the mean of n = 2 biologically independent experiments.

(E) IGV screenshots showing peaks from ATAC-seq and CUT&Tag analyses of forelimb and hindlimb marker genes in hPSCs (day 0), LPM cells (day 2), LBM cells (day 4), and ExpLBM cells (passage numbers 4–5).

(F) Subcutaneous transplantation of ExpLBM-derived cartilage particles treated with 30 ng/mL BMP4, 10 ng/mL TGFβ1, and 10 ng/mL GDF5 into NOD-SCID mice. Their engraftment was histologically assessed at 5 months post-transplantation by Safranin O staining.

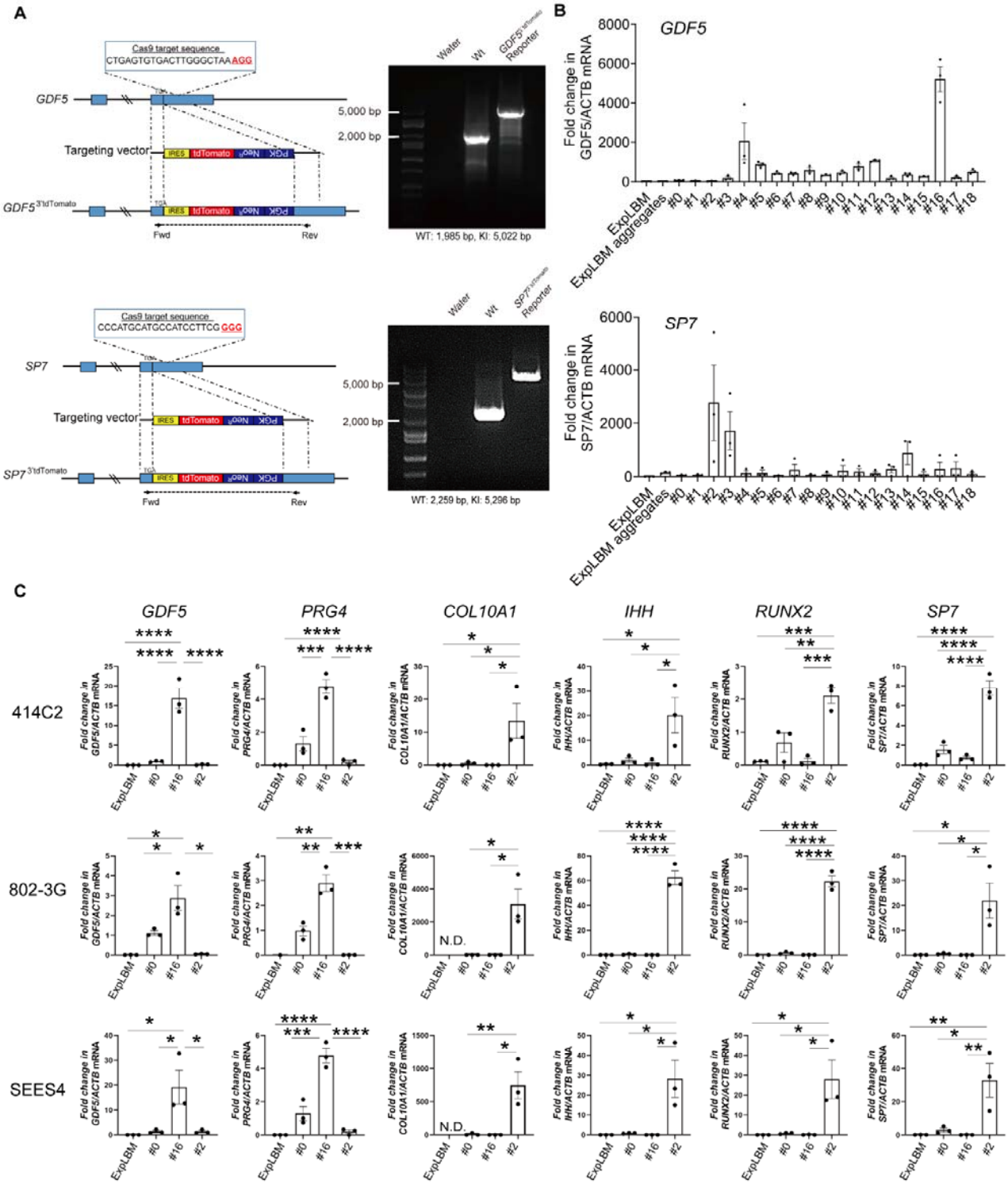

Figure S2

759

Figure S2: Generation of ontogenetically-defined IZ/ACP and GPC organoids from hPSCs, related to Figure 1.

(A) Targeting cassettes of the GDF5$^{3'tdTomato}$ and SP7$^{3'tdTomato}$ knock-in alleles. PAM sequences are highlighted in red. Agarose gel of genotyping PCR of genomic DNA extracted from parental hPSCs and each reporter hPSC line. Targeting cassette sequences were detected using forward and reverse primers that recognize sequences outside the homology arms. WT, wild-type allele; KI, knock-in allele.

(B) RT-qPCR analysis of each marker gene in GDF5$^{3'tdTomato}$ and SP7$^{3'tdTomato}$ reporter-derived ExpLBM cells treated with 18 combinations of chemicals and cytokines for 14 days in the presence of 30 ng/mL BMP4 and 10 ng/mL TGFβ1. #0, none; #1, 10% FBS; #2, 300 ng/mL BMP4, 100 ng/mL TGFβ1, and 10% FBS; #3, CHIR99021 (GSK3β inhibitor and WNT activator); #4, C59 (PORCN inhibitor and WNT inhibitor); #5, XAV-939 (tankyrase 1 inhibitor and WNT inhibitor); #6, LDN-193189 (ALK2/3 inhibitor); #7, A 83-01 (ALK4/5/7 inhibitor); #8, SAG 21k (hedgehog signaling activator); #9, Vismodegib (hedgehog signaling inhibitor); #10, MHY1485 (mTOR activator); #11, VO-OHpic (PTEN inhibitor and PI3K/mTOR activator); #12, SF1670 (PTEN inhibitor and PI3K/mTOR activator); #13, rapamycin (mTOR inhibitor); #14, ATRA (RA signaling activator); #15, MK-206 (AKT inhibitor); #16, PD0325901 (MEK inhibitor); #17, PIK-90 (PI3K inhibitor); and #18, DAPT (γ-secretase inhibitor and Notch inhibitor).

(C) RT-qPCR analysis of each marker gene in various clones of hPSCs, including 414C2 hiPSCs, 8023-G1 hiPSCs, and SEES4 hESCs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

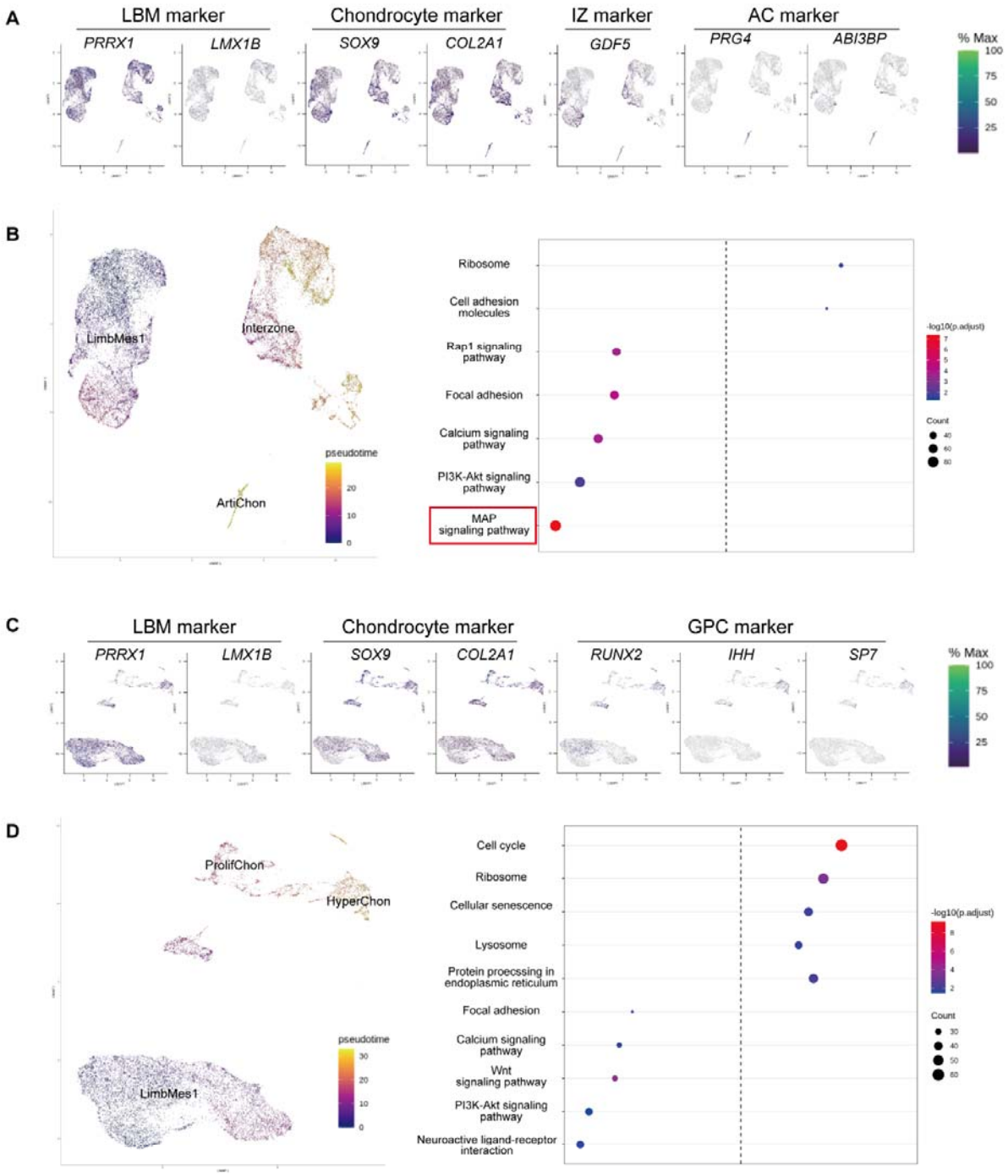

Figure S3

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Figure S3: scRNA-seq analysis of human embryonic limb cells, related to Figure 1.

(A) Feature plot of each marker gene in the UMAP representation of LimbMes1, Interzone, and ArtiChon clusters, obtained from scRNA-seq datasets of the human embryonic limb.

(B) Pseudotime trajectories of subpopulations in the UMAP representation of LimbMes1, Interzone, and ArtiChon clusters, and KEGG pathway enrichment analysis of these subpopulations.

(C) Feature plot of each marker gene in the UMAP representation of LimbMes1, ProlifChon, and HyperChon clusters, obtained from scRNA-seq datasets of the human embryonic limb.

(D) Pseudotime trajectories of subpopulations in the UMAP representation of LimbMes1, ProlifChon, and HyperChon clusters, and KEGG pathway enrichment analysis of these subpopulations.

Table S1: Oligonucleotides used in this study, related to STAR Methods.

Oligonucleotides for PX459-GDF5 gRNA

Purpose	Name	Sequence
KI of GDF5 3'UTR	GDF5(3prime)-CRPs2 (#6141)	caccgCTGAGTGTGACTTGGGCTAA
	GDF5(3prime)-CRPa2 (#6142)	aaacTTAGCCCAAGTCACACTCAGc

Oligonucleotides for PX459-SP7 gRNA

Purpose	Name	Sequence
KI of SP7 3'UTR	hOSX-CRPs1 (#5709)	caccgCCCATGCATGCCATCCTTCG
	hOSX-CRPa1 (#5710)	aaacCGAAGGATGGCATGCATGGGc

Oligonucleotides for pUCFa-GDF5 3′HA HA-IRES-tdTomato-PGK-Neo

Purpose	Name	Sequence
IRES-tdTomato-PGKNeo (In-fusion)	GDF5_F1_FW2 (#6151)	AGTGTGACTTGGGCTGGTACCGAGCTCGGATCC
	GDF5_F1_RV2 (#6152)	TAAAAGGGGGCCTTTGGTCTAGACTCGAGGCGG
pUCFa-GDF5 3`HA (In-fusion)	GDF5_Vec_FW2 (#6153)	AAAGGCCCCCTTTTATCCAC
	GDF5_Vec_RV2 (#6154)	AGCCCAAGTCACACTCAGAG

Oligonucleotides for pTA2-SP7 3′HA HA-IRES-tdTomato-PGK-Neo

Purpose	Name	Sequence
SP7 3'UTR Homology arm	OSX3UTRHA-F1 (#5186)	ATATGCACCCTGGAGGCAAC
	OSX3UTRHA-R1 (#5187)	CAGTGGAGGAACCTGCTCTG
IRES-tdTomato-PGKNeo (In-fusion)	fragment 1 FW for OSXHA (#5743)	ATGCATGCCATCCTTGGTACCGAGCTCGGATCCGC
	Fragment 1 RV Primer for OSXHA (#5744)	AGGGAGAGAGCCCCGGGTCTAGACTCGAGGCGGCC
pTA2-SP7 3`HA (In-fusion)	Vector Primer FW for OSXHA (#5745)	CGGGGCTCTCTCCCTCTGTC
	Vector Primer RV for OSXHA (#5746)	AAGGATGGCATGCATGGGG

Genotyping primers for GDF5-tdTomato reporter

Purpose	Name	Sequence
Knock-in check (WT: 1985 bp, KI: 5628 bp)	GDF5KI-5-F1 (#6197)	ATCTTGCGGAAGAAGCCCT
	GDF5KI-3-R2 (#6204)	AAAGGCAGGGGACTCCGATT

Genotyping primers for SP7-tdTomato reporter

Purpose	Name	Sequence
SP7 knock-in check (WT: 2259 bp, KI: 5296 bp)	OSXreporter_OUT(F) (#5842)	TCTGCGGGACTCAACAACTC
	OSXreporter_OUT(R) (#5843)	CCTGATGGGGTCATGGTGTC

RT-qPCR primers

Gene	Forward primer sequence	Reverse primer sequence
ACTB (#4793/#4794)	AGAAAATCTGGCACCACACC	AGAGGCGTACAGGGATAGCA
GDF5 (#6155/#6156)	TCTCGCTGTACAGGACGCTG	GACCTCGGTCATCTTGCCCT
PRG4 (#6327/#6328)	TGCAGGGTACCCCAAACCAA	TGCTGAATGCTGCCACCTCT
RUNX2 (#4751/#4752)	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
COL10A1 (#5474/#5475)	CCCAGCACGCAGAATCCATC	AGTGGGCCTTTTATGCCTGT
IHH (#5472/#5473)	CGGTGGACATCACCACATCA	CGTGGGCCTTTGACTCGTAA
SP7 (#5246/#5247)	ATCCAGCCCCCTTTACAAGC	TAGCATAGCCTGAGGTGGGT

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