A method for colocalizing lineage tracing reporter and RNAscope signals on skeletal tissue section

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ABSTRACT

Fluorescent reporters have been widely used in modern biology as a powerful tool in cell lineage tracing during development and in studying the pathogenesis of diseases. RNAscope is a recently developed RNA in situ hybridization method with high specificity and sensitivity. Combined application of these two techniques on skeletal tissue is technically challenging and has not been explored; the reporter fluorophores in the tissue specimen bleach quickly, and mRNAs degrade rapidly due to the decalcification process typically used in processing skeletal samples. Therefore, we developed a method that can simultaneously detect and colocalize both the fluorescent lineage tracing reporter signal and the RNAscope signal in the same skeletal section without compromising the fidelity, sensitivity, and specificity of lineage tracing and RNAscope. This was achieved by cryosectioning bone and cartilage tissue without decalcification, thus allowing the fluorescent reporter signal and RNA in the sections to be well-preserved so that RNAscope can be carried out in situ, and these two signals can be colocalized. Our method of colocalization has versatile applications, for example, determination of gene knockout efficacy at the mRNA level in a specific cell lineage in situ, detection of alterations in target gene transcripts in reporter-positive cells caused by a specific gene mutation, and studies of the disease pathology by examining the transcript-level expression of genes of interest in the cell lineage in vivo.

Keywords: lineage tracing; RNAscope; skeletal section

INTRODUCTION

Fluorescent protein reporters have been extensively used for cell lineage tracing in developmental biology and for modeling cellular heterogeneity in cancer with minimal changes to the properties of the marked cells and their adjacent tissues (Kretzschmar and Watt 2012). Lineage tracing is usually achieved by the expression of a fluorescent reporter gene under the control of a tissue-specific promoter. Lineage tracing provides information about all founder cells and their progeny, including the location and differentiation status. However, fluorescent reporters are incapable of revealing the abundance of the transcripts of the genes of interest.

RNAscope technology is a newly developed in situ hybridization (ISH) technique (Wang et al. 2012). Compared to traditional RNA ISH methods, RNAscope has higher sensitivity and specificity, such that it can detect even a single copy of RNA in a cell on paraffin or frozen tissue sections (Wang et al. 2012; Baena-Del Valle et al. 2017; Jolly et al. 2019). RNAscope has been extensively used in studying developmental biology, oncology, neuroscience, and infectious diseases (Grabinski et al. 2015; Mirghani et al. 2016; Baltzarsen et al. 2020; Kiyama and Mao 2020). However, RNAscope can only provide limited information on cell lineage.

By combining lineage tracing and RNAscope techniques for colocalization of the reporter signal and the RNA signal, researchers can detect transcript-level alterations in a specific cell lineage. Knowing how RNA and proteins change in the specific cell lineage can provide a better understanding of cell physiology and pathology. Detecting specific protein changes in a cell lineage via colocalization of the lineage tracing reporter signal and the immunofluorescence (IF) signal is widely used. However, colocalizing the lineage tracing reporter signal and the RNA signal to detect specific RNA in a cell lineage remains a problem, especially for skeletal tissue sections due to the

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decalcification and paraffin embedding processes commonly used for histological analysis of skeletal tissue. Traditional decalcification acids, such as nitric and formic acids, reduce detectable mRNA by up to 50% (Walsh et al. 1993). Ethylenediaminetetraacetic acid (EDTA) decalcification can preserve >50% of mRNA within the tissue (Walsh et al. 1993; Singh et al. 2013), but the process requires 1–3 wk depending on the sample size and EDTA concentration. Paraffin embedding causes significant loss of fluorescence signal and has high levels of background autofluorescence, especially in skeletal sections (Jiang et al. 2005). For these reasons, a cryostat section of undecalcified skeletal tissue is required to colocalize the lineage tracing reporter signal and the RNAscope signal. One major challenge when sectioning undecalcified skeletal tissue is to preserve the structural integrity of the tissue; however, using Kawamoto's film when sectioning can solve this problem (Kawamoto and Kawamoto 2014).

Here, we report a method that combines the technological merits of both fluorescent lineage tracing and RNAscope and successfully detects and colocalizes the fluorescent reporter signal with the RNAscope signal on the same skeletal section.

RESULTS

Colocalization of lineage tracing reporter signal with RNAscope signal in situ

Our goal was to develop an efficient and effective technique for visualizing both the lineage tracing reporter and RNA in the same cell of a skeletal section, using the cell lineage fluorescent reporter (ZsG) signal and the RNAscope signal, respectively. With the use of *Sox9*, *Ihh*, and *Col10a1* probes as well as the RNAscope 2.5HD Assay Red Detection Kit, RNAscope assay was applied to tibial frozen sections from 6-wk-old *Tg(Agc1-CreER; R26^{ZSG})* mice. *Agc1* gene is mainly expressed in chondrocytes and encodes aggrecan, which is a major proteoglycan in the extracellular matrix of cartilage, including articular cartilage and growth plates. The dye formed by the detection kit produced pseudored autofluorescence in situ, which is identical to the RNAscope signal under white light (Supplemental Fig. 1). As shown in Figure 1A, all Agc1$^+$ cells are marked by the R26^{ZSG} green fluorescent reporter protein and are presumed to be either epiphyseal chondroid cells or growth plate chondrocytes. The green reporter signal is weak in hypertrophic chondrocytes of growth plates because ZsG becomes diluted from the large cytoplasm volume of all the hypertrophic chondrocytes and some ZsG protein may have leaked from the section due to the multiple processing steps involved in RNAscope.

Sox9, *Ihh*, and *Col10a1* probes were used in this experiment because they are known to be expressed specifically

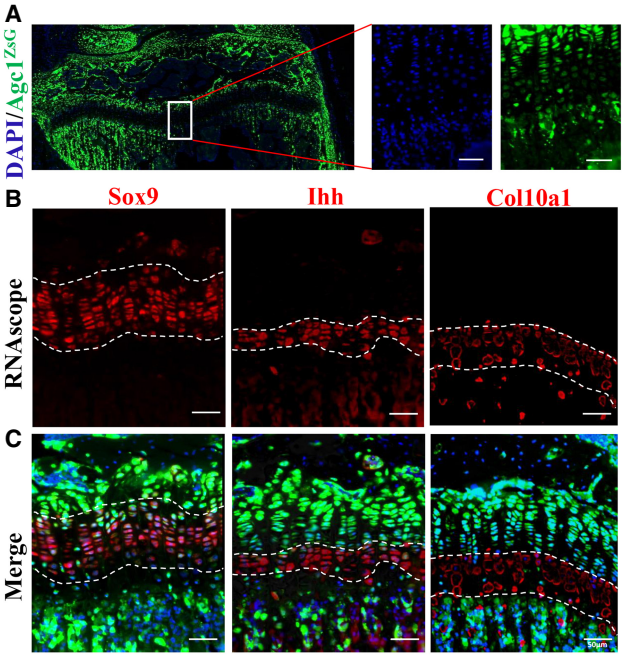

FIGURE 1. Colocalization of lineage tracing reporter signal and RNAscope signal on the same skeletal section. Tibial bones of 6-wk-old SHP2CTR$^{Agc1/ER}$ mice were used. (*A*) DAP1 (blue) and ZsG (green) reporter images of growth plate chondrocytes exhibited strong ZsG reporter signals due to Agc1-Cre. (*B*) *Sox9*, *Ihh*, and *Col10a1* mRNA (red) were respectively expressed in the proliferating, prehypertrophic, and hypertrophic chondrocytes of growth plates. (*C*) Each merged figure displays the colocalization of the ZsG reporter signal and the *Sox9*, *Ihh*, or *Col10a1* mRNA signal. The borders of the RNAscope signal-positive regions are outlined with dashed lines. The length of the scale bar represents 50 µm.

in proliferating, prehypertrophic, and hypertrophic chondrocytes, respectively, in developing growth plates. As expected, *Sox9*, *Ihh*, and *Col10a1* transcripts were detected in their respective type of chondrocytes in the growth plate (Fig. 1B). Next, we merged the images of the cell lineage reporter signal (Fig. 1A) and the RNAscope signal (Fig. 1B) to generate Figure 1C, which allowed us to simultaneously detect and colocalize the lineage reporter signal and the RNAscope signal on the same skeletal section. We applied our method to measure transcript-level expression of genes of interest in specific cell lineages for studying developmental and pathological issues in skeletal tissue.

Determining knockout efficiency of the target gene at the mRNA level in reporter-positive cells in vivo

Western blot and qPCR are widely used for determining knockout efficiency of target genes in a specific cell population from a mouse model. However, both assays are in vitro experiments. Our method can determine knockout efficiency of the target gene at the transcriptional level in specific cells in situ. We used 10-wk-old SHP2CTR$^{Agc1/ER}$

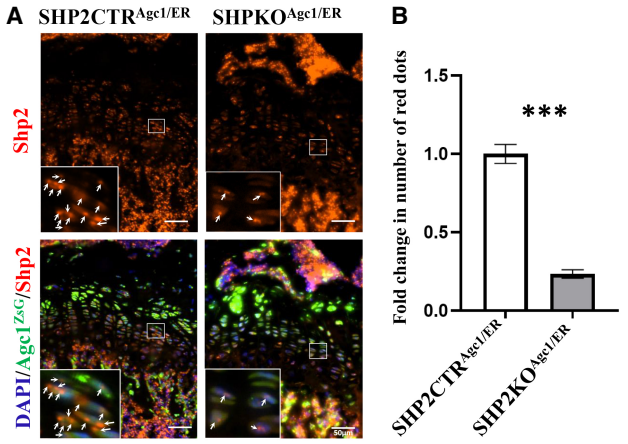

FIGURE 2. Detection of knockout efficiency of the target gene at the mRNA level in reporter-positive cells in vivo. Tibial bones from 10-wk-old mice were used. (*A*) DAPI, ZsG, and *Shp2* mRNAs are indicated by the blue, green, and red dots, respectively. A single red dot represents at least one Shp2 mRNA. (*B*) There was a significant reduction (−77%) in the number of the red dots in 100 R26^{ZsG+} chondrocytes from the middle region of growth plates in SHP2KO$^{Agc1/ER}$ mice compared to the controls ($n = 5$, [***] $P < 0.0001$). Enlarged views of the boxed areas were shown at the *bottom left* corner of each image. *Shp2* mRNAs are represented as small red dots marked by white arrows in the enlarged views. The length of the scale bar represents 50 μm.

and SHP2KO$^{Agc1/ER}$ mice to measure SHP2 (Src homology region 2-containing protein tyrosine phosphatase 2) knockout efficiency in Agc1^{+} cells from tibial growth plates. SHP2, encoded by *Ptpn11*, is a ubiquitously expressed protein tyrosine phosphatase. The red dots detected via RNAscope in the green cells of the growth plates represent *Shp2* mRNAs (Fig. 2A). Due to the low expression of the endogenous *Ptpn11* gene in chondrocytes, only a few small red dots were seen in the green cells. Counting the number of red dots in 100 green R26^{ZsG+} chondrocytes from the middle region of the growth plate revealed that the relative abundance of *Shp2* mRNA in the cell population decreased by 77% for knockout mice compared to control mice (Fig. 2B, $P < 0.001$, $n = 5$). This suggested that the SHP2 knockout efficiency was 77% at the transcriptional level in SHP2-deficient chondrocytes in growth plates.

In this way, our method can be used to detect the knockout efficiency of the target gene at the RNA level in vivo without time-consuming and complicated multistep processes, such as primary cell culture, cell purification based on the cell lineage reporter, and protein or RNA extraction.

Studying the alterations in transcripts of target genes caused by a specific gene mutation

Colocalization of the lineage tracing reporter signal and the IF signal is widely used to study the effect of a mutant gene on reporter-positive cells in vivo at the protein level. However, very few methods can detect alterations in transcript levels caused by a mutant gene in specific cells in vivo. Our method can be used to detect the effects of the target gene mutation on the transcripts of the genes of interest in specific cells in vivo by colocalizing the reporter signal and the RNAscope signal. Tibial bones from 10-wk-old SHP2CTR$^{Agc1/ER}$ and SHP2KO$^{Agc1/ER}$ mice were used to identify *Ihh* mRNA in Agc1^{+} chondrocytes in growth plates (Fig. 3). The red fluorescence indicated *Ihh* mRNAs (Fig. 3A). The thickness of the *Ihh*-expressing cell layer in the middle region of the growth plate was measured. There was a significant increase in thickness for SHP2KO$^{Agc1/ER}$ mice compared to the controls (Fig. 3B, $P < 0.001$, $n = 5$). Thus, our method can be used to detect transcript-level alterations of target genes caused by a specific gene mutation, and the results can be analyzed using semi-quantitative analysis.

Studying the pathology of diseases by analyzing the expression of the genes of interest in a specific cell lineage

There are limited methods for identifying transcript changes of the genes of interest in specific cells to study the pathology of skeletal diseases. Our method offers the ability to detect changes in the transcripts of the genes of interest in a specific cell lineage of skeletal lesions in vivo in order

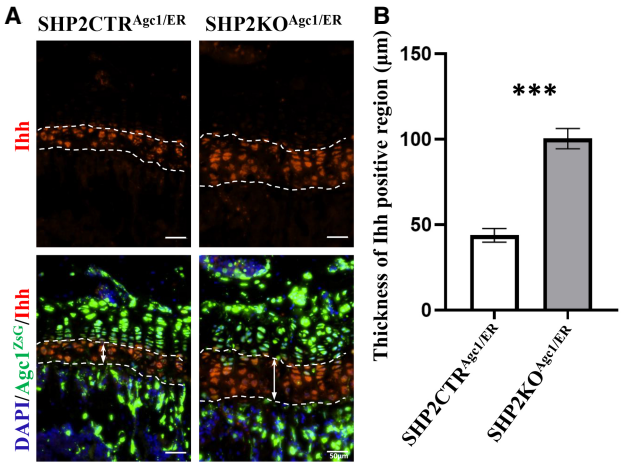

FIGURE 3. The effect of SHP2 deletion on growth plate development. (*A*) Colocalization of the lineage tracing reporter signal and the RNAscope signal identified the *Ihh* (red) mRNA expressed in the prehypertrophic zone of growth plates of tibias from 10-wk-old SHP2CTR$^{Agc1/ER}$ and SHP2KO$^{Agc1/ER}$ mice. (*B*) Measurement of the thickness of *Ihh*-expressing cell layer (indicated by the double-headed arrow) in the middle region of the growth plates demonstrated significant increase in *Ihh* mRNA expression in the growth plates of mutant mice compared to those of the controls ($n = 5$) (***) $P < 0.001$). The borders of the RNAscope signal-positive region were outlined with dashed lines. The length of the scale bar represents 50 μm.

to study their pathological changes. Exostoses on the articular surface of tibial bones from 10-wk-old SHP2KO^{Ctsk} mice were used to identify mRNAs of *Sox9*, *Col2a1*, *Ihh*, and *Col10a1* genes. These four genes are known biomarkers for proliferating, prehypertrophic, and hypertrophic chondrocytes, respectively, in growth plates. Therefore, they were used to examine the differentiation status of the cells in the lesions. Ctsk (Cathepsin K) is expressed in osteoclasts and the perichondral grooves of Ranvier cells. The merged images showing the colocalization of the lineage tracing reporter signal and the RNAscope signal revealed that the cells in the exostoses originated from Ctsk$^+$ cells and expressed *Sox9*, *Col2a1*, *Ihh*, and *Col10a1* mRNA in their respective regions of the lesions (Fig. 4), indicating a benign ecchondroma. This experiment showed that our method can be used to study the pathology of skeletal diseases by analyzing the transcript-level expression of the genes of interest in a specific cell lineage.

DISCUSSION

Both lineage tracing and RNAscope have shortcomings despite their popularity in biological research. Lineage tracing cannot detect the transcripts of the genes of interest in reporter-positive cells, and RNAscope can only provide limited information on cell lineage. To overcome the limitation of each technique, a method that combines these two techniques in the same tissue section, especially for skeletal tissue, needs to be developed.

Despite the challenges in histological techniques, we successfully developed a method to colocalize the lineage tracing reporter signal and the RNAscope signal on the same murine skeletal section. Kawamoto's film was used to section undecalcified skeletal tissue to maintain its morphology. A commercially available RNAscope was used to detect transcripts of the genes of interest due to its high specificity and sensitivity (Wang et al. 2012). ZsG was used as the lineage fluorescent reporter protein. Our method has broad application prospects, including determining knockout efficiency of a target gene at the mRNA level in reporter-positive cells in situ, detecting transcript-level alterations of the genes of interest caused by a specific gene mutation, and studying the pathology of diseases by analyzing the transcript-level expression of the genes of interest in a specific cell lineage.

Unlike PCR, ISH is a technique that integrates histology and molecular biology to precisely locate specific segments of nucleic acids within a histologic section. RNAscope is a recently developed RNA ISH technique (Wang et al. 2012) that is used in soft tissue samples, but it is not commonly used in skeletal samples due to technical difficulties. Decalcification is commonly used for histological analysis of skeletal tissue. However, there are disadvantages of decalcification, such as its lengthy process as well as significant RNA degradation in the tissue sample (Walsh et al. 1993; Belluoccio et al. 2013). Therefore, decalcification should not be recommended for preparing skeletal samples used in RNAscope.

Due to various technical difficulties, RNAscope is rarely used for the histological analysis of skeletal samples. To date, no method has been developed to colocalize the lineage tracing reporter signal and the RNAscope signal on the same skeletal section. Nonetheless, colocalization of these signals is useful in histological analysis because it can provide information about the founder cells and their progeny, such as location, differentiation status, and transcript changes of the genes of interest in vivo. In the literature, there have been reports of the combined use of RNAscope and immunohistochemistry (IHC) to detect transcripts of the genes of interest in specific cell populations that express certain proteins in soft tissue (Grabinski et al. 2015); however, this combination lacks the ability to precisely trace founder cells and all of their progeny since they express different proteins at various stages of differentiation. Therefore, it would be advantageous to use lineage tracing, instead of IHC, with RNAscope.

Paraffin embedding is commonly used for histological analysis of skeletal samples, but it results in high background autofluorescence as well as significant loss of the lineage tracing fluorescent signal (Jiang et al. 2005). Instead, optimal cutting temperature compound (OCT)-embedding should be used in skeletal samples to maintain the lineage tracing reporter signal and prevent background autofluorescence.

Furthermore, it is challenging to preserve the morphology of undecalcified skeletal tissues when sectioning. Kawamoto's film was developed to maintain tissue

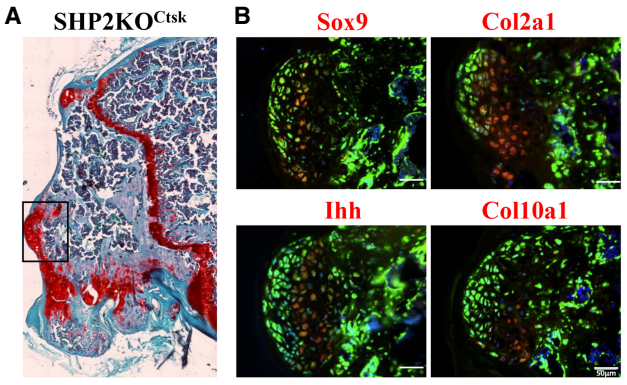

FIGURE 4. Pathology of the tibial exostoses from SHP2KO^{Ctsk} mice. (*A*) Safranin O and Fast Green staining of the tibias from SHP2KO^{Ctsk} mice indicated cartilaginous exostoses. (*B*) RNAscope images of the magnified area (black box in *A*) revealed that the exostoses originated from Ctsk$^+$ cells and expressed *Sox9*, *Col2a1*, *Ihh*, and *Col10a1* in different regions of the exostoses. This suggested that the exostoses were benign ecchondromas. The length of the scale bar represents 50 μm.

morphology and facilitate the process of preparing cryostat sections of OCT-embedded undecalcified skeletal samples (Kawamoto and Kawamoto 2014). The usage of this film does not require decalcification; therefore, the preparation process is shortened, and a substantial amount of RNA is preserved (Belluoccio et al. 2013). Kawamoto's film prevents skeletal tissue from falling off the slides during RNAscope and remains transparent after it is treated with RNAscope reagents, thus reducing background noise when capturing fluorescent images.

In this article, we report three applications of our method. First, we detected the SHP2-knockout efficiency in Agc1^{+} chondrocytes using SHP2CTR$^{Agc1/ER}$ and SHP2KO$^{Agc1/ER}$ mice. Only a few small red dots (indicating *Shp2* mRNA) were seen in the green chondrocytes due to the low expression of the endogenous *Ptpn11* gene. Some large red dots were observed in the chondrocytes, likely caused by aggregation of *Shp2* transcripts. We counted the number of the red dots in 100 green R26^{ZsG+} chondrocytes in growth plates, and there was a 77% decrease in the relative abundance of *Shp2* mRNA in the growth plates of mutant mice. The lack of 100% knockout efficiency could be explained by the size of DNA between the two LoxP sites (Zheng et al. 2000), the mosaic expression of Cre-ER (Sandlesh et al. 2018), the incomplete excision of the floxed loci (Bao et al. 2013), and transcription of the *Ptpn11* gene before Tamoxifen injection and Cre recombinase induction. Secondly, we discovered that there was a significant increase in the thickness of the *Ihh*-expressing cell layer in the middle region of tibial growth plates from SHP2KO$^{Agc1/ER}$ mice compared to that of the control mice. Our finding is consistent with a previous publication that claimed that SHP2 deficiency increases the amount of *Ihh* mRNA transcripts in chondrocytes (Bowen et al. 2014). Lastly, we found that the exostoses on the tibial bones of SHP2KO^{Ctsk} mice expressed different biomarkers at various differentiation stages of chondrocytes. Our findings were consistent with a prior report of these lesions by Yang et al. (2013). In addition to our three applications, there may be other potential applications of our method that have yet to be investigated.

A potential limitation of our method is the use of Fast Red. During the signal detection procedure, Fast Red is a chromogenic substrate that is catalyzed by alkaline phosphatase (ALP), which is labeled on the probes. Fast Red enables RNA molecules to be visualized as red chromogenic dots under white light. On the other hand, under fluorescent light, Fast Red will be visualized as red pseudofluorescence (Supplemental Fig. 1), which, when in excess, can partially mask other fluorescence. Therefore, when there is too much Fast Red deposit in the cell due to an abundance of target RNAs, the lineage tracing reporter signal will be partially masked. There are ways to overcome this limitation. One method is to conduct RNAscope after capturing the image of the DAPI signal and the lineage tracing reporter signal. An easier method is to dilute the probe solution or reduce the reaction time for the last step of RNAscope.

In summary, our method offers the ability to colocalize the lineage tracing reporter signal and the RNA signal on the same skeletal tissue section. Our unique method allows researchers to detect transcripts of the genes of interest in reporter-positive cells in vivo, even one RNA per cell. There are also three applications of our method, for example, to determine knockout efficiency of the target gene at the mRNA level in a specific cell lineage, to investigate the alterations in transcripts of the genes of interest caused by a specific gene mutation in vivo, and to study the pathology of diseases by analyzing the transcript-level expression of the genes of interest in a specific cell lineage. Further experiments should be done to investigate additional applications of our method.

MATERIALS AND METHODS

Animals

Tg(Ptpn11$^{fl/+}$) (Yang et al. 2013), *Tg(Rosa26^{ZSG})* (Madisen et al. 2010), *Tg(Agc1-CreER)* (Henry et al. 2009), and *Tg(Ctsk-Cre)* (Nakamura et al. 2007) mice and genotyping conditions were previously described in the literature. *Ptpn11* is a ubiquitously expressed gene encoding a protein tyrosine phosphatase, SHP2. Agc1 (Aggrecan) is a proteoglycan expressed and secreted by chondrocytes from articular cartilage and growth plates. Ctsk (Cathepsin K) is a lysosomal cysteine protease expressed in osteoclasts and the perichondral grooves of Ranvier cells. Mice with *Ptpn11* floxed alleles were bred to *Tg(Agc1-CreER) or Tg(Ctsk-Cre)* mice to generate control offspring and mutant offspring in which SHP2 was deleted in *Agc1*- or *Ctsk*-expressing cells. *Tg (Rosa26^{ZSG})* served as a reporter for Cre expression and activation. The genotypes and shorthand nomenclature of these mice are listed in Supplemental Figure 2. To induce *Tg(Agc1-CreER)* activity, 4-OH Tamoxifen (TM; Sigma, MO) was dissolved in dimethyl sulfoxide (DMSO)-ethanol-corn oil mixture (4:6:90) at a concentration of 10 mg/mL and intraperitoneally injected into SHP2CTR$^{Agc1/ER}$ and SHP2KO$^{Agc1/ER}$ mice (1 mg/mouse/dose, 3 doses at 2-wk-old, 1 dose/2 d). Control and SHP2 mutant mice were sacrificed at 6-wk-old or 10-wk-old for the study. All mice were maintained on a C57BL/6J background. This study was reviewed and approved by the Rhode Island Hospital Institutional Animal Care and Use Committee.

Preparing frozen sections of skeletal tissues without decalcification

The mice were sacrificed at 6-wk-old or 10-wk-old. Their tibial bones were collected and fixed in 4% formaldehyde overnight and were then dehydrated in 30% sucrose solution overnight. Finally, they were embedded in OCT compound for cryostat section. Frozen sections were cut into 7 µm sections using a freezing stage and sliding microtome (Leica CM1950). During the process of sectioning samples, Kawamoto's film (SECTION-LAB Co. Ltd.)

was used to preserve the morphology of the skeletal sections. Sections were immediately transferred to a −80°C freezer and were stored for no more than 6 mo. The key steps for cutting skeletal frozen sections on Kawamoto's film include film preparation, film adhesion, tissue sectioning, and film transferring onto glass slides (Supplemental Fig. 3). The combined use of the cryostat section of undecalcified skeletal tissue and Kawamoto's film can maximize the preservation of mRNA integrity and tissue morphology for RNAscope ISH assays and reduce tissue autofluorescence while retaining the fluorescent reporter signal.

RNAscope procedures

Customized and commercially available RNA probes (*Ptpn11*, *Sox9*, *Col2a1*, *Ihh*, and *Col10a1*) were purchased from Advanced Cell Diagnostics (ACD), and detailed information about the probes was provided in Supplemental Figure 4. RNAscope signal was detected using RNAscope 2.5HD Assay Red Kit per the manufacturer's instructions available online at: https://acdbio.com/documents/product-documents. Some steps of the protocol were modified to save reagents and to prevent the tissue from falling off from the slide. The sections were boiled in Pretreatment 2 solution at 80°C–85°C, not 100°C, in order to prevent tissue from falling off. The protocol recommends preparing a 700 mL Pretreatment 2 solution; however, it is not necessary to use so much. Less volume can be used as long as all of the slides can be fully immersed in the Pretreatment 2 solution. The protocol also recommends using four drops of the following solutions: probe solution, signal amplification solution, and signal detection solution. However, less drops can be used as long as the entire section is covered by those solutions.

Image analysis

Phase contrast and fluorescent images were acquired using the Nikon Eclipse E800 microscope system at 200× (10× eyepiece and 20× objective) magnification and were analyzed with the NIH ImageJ software. Blue, green, and red fluorescence represent nuclei stained with DAPI, the $R26^{ZSG}$ reporter, and the RNAscope signal, respectively. To determine knockout efficiency of *Ptpn11* at the mRNA level, the number of red dots was counted in 100 $R26^{ZsG+}$ cells from the middle region of proximal tibial growth plates of $SHP2CTR^{Agc1/ER}$ and $SHP2KO^{Agc1/ER}$ mice. To study the effect of SHP2 deletion on growth plate development, we measured the thickness of the *Ihh*-expressing cell layer in the middle region of the growth plates of $SHP2CTR^{Agc1/ER}$ and $SHP2KO^{Agc1/ER}$ mice.

Statistical analysis

Statistical analysis comparing the two groups was performed using Student *t*-test. A *P*-value <0.05 was considered to be significant. Data analysis was performed using Prism 3.0 (GraphPad) and Excel (Microsoft).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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REFERENCES

Baena-Del Valle JA, Zheng Q, Hicks JL, Fedor H, Trock BJ, Morrissey C, Corey E, Cornish TC, Sfanos KS, De Marzo AM. 2017. Rapid loss of RNA detection by in situ hybridization in stored tissue blocks and preservation by cold storage of unstained slides. *Am J Clin Pathol* **148:** 398–415. doi:10.1093/ajcp/aqx094

Baltzarsen PB, Georgsen JB, Nielsen PS, Steiniche T, Stougaard M. 2020. Detection of mRNA of telomerase protein in benign naevi and melanomas using RNAscope. *Appl Immunohistochem Mol Morphol* **28:** 36–41. doi:10.1097/PAI.0000000000000690

Bao J, Ma HY, Schuster A, Lin YM, Yan W. 2013. Incomplete Cre-mediated excision leads to phenotypic differences between *Stra8-iCre; Mov10l1*$^{lox/lox}$ and *Stra8-iCre; Mov10l1*$^{lox/\Delta}$ mice. *Genesis* **51:** 481–490. doi:10.1002/dvg.22389

Belluoccio D, Rowley L, Little CB, Bateman JF. 2013. Maintaining mRNA integrity during decalcification of mineralized tissues. *PLoS One* **8:** e58154. doi:10.1371/journal.pone.0058154

Bowen ME, Ayturk UM, Kurek KC, Yang W, Warman ML. 2014. SHP2 regulates chondrocyte terminal differentiation, growth plate architecture and skeletal cell fates. *PLoS Genet* **10:** e1004364. doi:10.1371/journal.pgen.1004364

Grabinski TM, Kneynsberg A, Manfredsson FP, Kanaan NM. 2015. A method for combining RNAscope in situ hybridization with immunohistochemistry in thick free-floating brain sections and primary neuronal cultures. *PLoS One* **10:** e0120120. doi:10.1371/journal.pone.0120120

Henry SP, Jang CW, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. 2009. Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage. *Genesis* **47:** 805–814. doi:10.1002/dvg.20564

Jiang X, Kalajzic Z, Maye P, Braut A, Bellizzi J, Mina M, Rowe DW. 2005. Histological analysis of GFP expression in murine bone. *J Histochem Cytochem* **53:** 593–602. doi:10.1369/jhc.4A6401.2005

Jolly S, Lang V, Koelzer VH, Sala Frigerio C, Magno L, Salinas PC, Whiting P, Palomer E. 2019. Single-cell quantification of mRNA expression in the human brain. *Sci Rep* **9:** 12353. doi:10.1038/s41598-019-48787-w

Kawamoto T, Kawamoto K. 2014. Preparation of thin frozen sections from nonfixed and undecalcified hard tissues using Kawamot's film method (2012). *Methods Mol Biol* **1130:** 149–164. doi:10.1007/978-1-62703-989-5_11

Kiyama T, Mao CA. 2020. Ultrasensitive RNAscope in situ hybridization system on embryonic and adult mouse retinas. *Methods Mol Biol* **2092:** 147–158. doi:10.1007/978-1-0716-0175-4_11

Kretzschmar K, Watt FM. 2012. Lineage tracing. *Cell* **148:** 33–45. doi:10.1016/j.cell.2012.01.002

Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, et al. 2010. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13:** 133–140. doi:10.1038/nn.2467

Mirghani H, Casiraghi O, Guerlain J, Amen F, He MX, Ma XJ, Luo Y, Mourareau C, Drusch F, Lakdhar AB, et al. 2016. Diagnosis of HPV driven oropharyngeal cancers: comparing p16 based algorithms with the RNAscope HPV-test. *Oral Oncol* **62:** 101–108. doi:10.1016/j.oraloncology.2016.10.009

Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, et al. 2007. Estrogen prevents bone loss via estrogen receptor α and induction of Fas ligand in osteoclasts. *Cell* **130:** 811–823. doi:10.1016/j.cell.2007.07.025

Sandlesh P, Juang T, Safina A, Higgins MJ, Gurova KV. 2018. Uncovering the fine print of the CreERT2-LoxP system while generating a conditional knockout mouse model of Ssrp1 gene. *PLoS One* **13:** e0199785. doi:10.1371/journal.pone.0199785

Singh VM, Salunga RC, Huang VJ, Tran Y, Erlander M, Plumlee P, Peterson MR. 2013. Analysis of the effect of various decalcification agents on the quantity and quality of nucleic acid (DNA and RNA) recovered from bone biopsies. *Ann Diagn Pathol* **17:** 322–326. doi:10.1016/j.anndiagpath.2013.02.001

Walsh L, Freemont AJ, Hoyland JA. 1993. The effect of tissue decalcification on mRNA retention within bone for in-situ hybridization studies. *Int J Exp Pathol* **74:** 237–241.

Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y. 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn* **14:** 22–29. doi:10.1016/j.jmoldx.2011.08.002

Yang W, Wang J, Moore DC, Liang H, Dooner M, Wu Q, Terek R, Chen Q, Ehrlich MG, Quesenberry PJ, et al. 2013. Ptpn11 deletion in a novel progenitor causes metachondromatosis by inducing hedgehog signalling. *Nature* **499:** 491–495. doi:10.1038/nature12396

Zheng B, Sage M, Sheppeard EA, Jurecic V, Bradley A. 2000. Engineering mouse chromosomes with Cre-*loxP*: range, efficiency, and somatic applications. *Mol Cell Biol* **20:** 648–655. doi:10.1128/MCB.20.2.648-655.2000