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Generation and Characterization of *Col10a1*-mCherry Reporter Mice

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Summary

We report here on the generation of a new fluorescent protein reporter transgenic mouse line, *Col10a1*-mCherry, which can be used as a tool to study chondrocyte biology and pathology. *Collagen, Type X, alpha 1* (*Col10a1*) is highly expressed in hypertrophic chondrocytes and commonly used as a gene marker for this cell population. The *Col10a1*-mCherry reporter line was generated using a bacterial recombination strategy with the mouse BAC clone RP23-192A7. To aid in the characterization of this animal model, we intercrossed Col10a1-mCherry mice with *Collagen, Type II, alpha 1 (Col2a1*) enhanced cyan fluorescent protein (ECFP) reporter mice and characterized the expression of both chondrocyte reporters during embryonic skeletal development from days E10.5 to E17.5. Additionally, at postnatal day 0, *Col10a1*-mCherry reporter expression was compared to endogenous *Col10a1* mRNA expression in long bones and revealed that mCherry fluorescence extended past the *Col10a1* expression domain. However, in situ hybridization for mCherry was consistent with the zone of *Col10a1* mRNA expression, indicating that the persistent detection of mCherry fluorescence was a result of the long protein half life of mCherry in conjunction with a very rapid phase of skeletal growth and not due to aberrant transcriptional regulation. Taking advantage of the continued fluorescence of hypertrophic chondrocytes at the chondro-osseus junction, we intercrossed *Col10a1*-mCherry mice with two different *Collagen, Type 1, alpha 1, (Col1a1*) osteoblast reporter mice, pOBCol3.6-Topaz and pOBCol2.3-Emerald to investigate the possibility that hypertrophic chondrocytes transdifferentiate into osteoblasts. Evaluation of long bones at birth suggests that residual hypertrophic chondrocytes and osteoblasts in the trabecular zone exist as two completely distinct cell populations. genesis 49:410–418, 2011.

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chondrocyte; cartilage; hypertrophic; endochondral; fluorescent; transgenic

INTRODUCTION

Cartilage formation occurs during endochondral ossification in the appendicular skeleton, axial skeleton and cranial base, where embryonic mesenchyme condenses and differentiates to form a cartilaginous anlagen that, in part, serves as a template for bone formation (Hall and Miyake, 2000). Signaling between the cartilaginous anlagen and outer perichondrium has been an area of great interest, where morphogenetic changes in the anlagen result in joint formation and bone formation at defined, but disparate locations. During these early stages of skeletal development, chondrocytes organize as a growth plate and undergo a well-defined program of differentiation whose characteristic stages are referred to as: resting or reserve, proliferative, prehypertrophic, and hypertrophic.

Early studies in skeletal biology revealed changes in collagen gene expression during chondrocyte maturation. *Collagen, Type 2, alpha 1* (*Col2a1*) is abundantly expressed in immature chondrocytes, but then down regulated during maturation. In contrast, *Collagen, Type 10, alpha 1* (*Col10a1*) expression is absent in immature chondrocytes, but becomes highly expressed in mature hypertrophic chondrocytes (Castagnola *et al.*, 1988; Iyama *et al.*, 1991). An increasing body of evidence has indentified many of the signaling pathways involved in regulating chondrocyte differentiation. Parathyroid hormone related protein (PTHrP) and fibroblast growth factor 18 (FGF18) signaling promote chondrocyte proliferation and block maturation (Lanske *et al.*, 1996; Liu *et al.*, 2002; Vortkamp *et al.*, 1996) while bone morphogenetic protein (BMP), Wnt, and retinoic acid signaling promote chondrocyte maturation in part through runt related transcription factor 2 (RUNX2) (Drissi *et al.*, 2003; Enomoto-Iwamoto *et al.*, 1998; Enomoto *et al.*, 2000; Li *et al.*, 2003; Yasuhara *et al.*, 2010). Additionally, Indian hedgehog (IHH) functions as a central organizer with direct and indirect roles in chondrocyte proliferation and differentiation, as well as initiating osteoblast differentiation on the outer perichondrium (Kobayashi *et al.*, 2005; Minina *et al.*, 2001, 2002; St-Jacques *et al.*, 1999; Vortkamp *et al.*, 1996).

Past studies have sought to define mechanisms of *Col10a1* transcriptional regulation. Cis elements providing enhancer and repressor functions have been identified in human, mouse, and chick *Col10a1* genes (Beier *et al.*, 1997; Chambers *et al.*, 2002; Harada *et al.*, 1997; Leboy *et al.*, 2001; Riemer *et al.*, 2002; Thomas *et al.*, 1995; Volk *et al.*, 1998). Additionally, in vitro biochemical studies have revealed a host of factors that are potentially involved in the direct positive regulation of *Col10a1* transcription including: RUNX2, AP-1 factors, LEF/TCFs, and SMADs, which are involved in Wnt, BMP, and retinoic acid signaling pathways that promote chondrocyte maturation (Drissi *et al.*, 2003; Enomoto *et al.*, 2000; Gebhard *et al.*, 2008; Li *et al.*, 2003; Riemer *et al.*, 2002; Volk *et al.*, 1998; Yasuhara *et al.*, 2010). Despite the successful identification of cis and trans regulatory elements required for *Col10a1* expression, past transgenic studies that have utilized a defined *Col10a1* promoter region to drive the expression of a β-galactosidase reporter or a Cre recombinase suggested

additional cis regulatory elements were required to recapitulate endogenous *Col10a1* transcription (Gebhard *et al.*, 2004; Yang *et al.*, 2005). With this in mind, a *Col10a1* BAC clone proved sufficient to drive robust and specific expression of a β-galactosidase reporter and a Cre recombinase in hypertrophic chondrocytes (Gebhard *et al.*, 2007, 2008).

We report here on the generation and characterization of *Col10a1*-mCherry fluorescent protein reporter mice. The *Col10a1* transgenic construct was created using mouse BAC clone RP23-192A7, which was previously determined to direct accurate expression of *Col10a1* (Gebhard *et al.*, 2007, 2008). A recombinase A (RecA) bacterial recombination system was used to insert mCherry fluorescent protein just upstream of the translation start site (see Fig. 1) (Gong *et al.*, 2002). Six transgenic founder lines were initially generated. After a preliminary assessment of reporter gene expression, our two highest expressing lines were maintained thereafter.

To aid in the characterization of *Col10a1*-mCherry mice, we capitalized on the existence of *Col2a1*-ECFP and *Col1a1* fluorescent protein reporter (pOBCol3.6-Topaz and pOBCol2.3Emerald) mouse lines. Both *Col2a1* and *Col1a1* reporter mice use fluorescent protein reporters that are spectrally distinct from mCherry allowing us to simultaneously image immature chondrocytes and hypertrophic chondrocytes and/or osteoblasts and hypertrophic chondrocytes, respectively. The generation of *Col2a1*-ECFP mice was recently reported (Chokalingam *et al.*, 2009a,b) and was constructed in a fashion similar to *Col2a1*-EGFP mice (Grant *et al.*, 2000). We characterized the expression of *Col10a1*-mCherry in relation to *Col2a1*-ECFP during embryonic skeletal development from E10.5 to E17.5 (see Fig. 2). *Col2a1*-ECFP expression was detected at E10.5, well before *Col10a1*-mCherry expression in the developing somites, brachial arch 1, and craniofacial primordia covering the ventral forebrain (Fig. 2A). At E12.5, *Col2a1*-ECFP expression defines much of the early-forming axial and appendicular skeleton where condensing vertebral bodies along the axial skeleton and areas of cartilage condensations in both forelimb and hindlimb express the ECFP reporter, but *Col10a1*-mCherry expression still cannot be observed at this stage (Fig. 2B). At E14.5, (Fig. 2C,D) *Col2a1*-ECFP expression was seen in the developing ears, ribs, vertebrae, and long bones of the appendicular skeleton. However, expression of the *Col2a1* reporter was not observed in the joint spaces and in regions of the anlagen undergoing maturation where *Col10a1*-mCherry expression was now observed. Both lateral (Fig. 2C) and dorsal (Fig. 2D) views showed *Col10a1* reporter expression in the ribs and at the center of the anlagen in long bones of the appendicular skeleton where chondrocytes undergo hypertrophy. By E17.5 (Fig. 2E–H) *Col2a1* and *Col10a1* driven reporter genes sharply illuminated much of the embryonic skeleton. *Col2a1* reporter expression was seen in the ears and cranial base of the skull, in the ventral part of the rib cage and vertebral bodies of the spine, and at the proximal and distal ends of long bones of the appendicular skeleton (Fig. 2E,G,H, blue). *Col10a1* reporter expression was observed in the dorsal half of the rib cage and in symmetrical points of hypertrophy along the vertebrae (Fig. 2F,H, red). In the long bones of the appendicular skeleton, *Col10a1* reporter expression domains lay adjacent and interior to *Col2a1* expressing chondrocytes (Fig. 2F,G). To appreciate the temporal changes that occur during long bone formation, we imaged forearm development at higher magnification from E12.5 to E17.5 (Fig. 2I–K). At E12.5 *Col2a1*-ECFP expression can be observed at sites of chondrocyte condensation (Fig. 2I). At E14.5, a single *Col10a1*-mCherry

expression zone was seen at the center of anlagen in the humerus, radius, ulna, and digits (Fig. 2J). By E17.5 the *Col10a1* expression zone has separated into two domains with a gap in between where hypertrophic chondrocytes are undergoing apoptosis, promoting the formation of a marrow cavity, and leaving a mineralized template upon which trabecular bone will form (Fig. 2K). Tissue sections through the forearm of E17.5 mice (Fig. 2L–O) showed some overlapping expression between *Col2a1* and *Col10a1* reporters in the growth plate (Fig. 2O). *Col2a1* reporter expression can be seen throughout the resting and proliferative zones, but was down regulated in the hypertrophic zone (Fig. 2L). *Col10a1* reporter expression is absent from the resting and proliferative zones, but robustly expressed in chondrocytes undergoing hypertrophy (Fig. 2M).

To further characterize the expression of *Col10a1*-mCherry reporter mice, we compared reporter gene expression to endogenously expressed *Col10a1* mRNA at postnatal day 0 (P0) in long bones (Fig. 3, compare A with B). As anticipated, endogenous *Col10a1* mRNA was largely restricted to the hypertrophic zone (HZ) (Fig. 3B, tibia). Interestingly, while *Col10a1*-mCherry reporter expression was detected in the hypertrophic zone, the fluorescence of the reporter actually intensified as hypertrophic cells became part of the bone marrow (BM) (Fig. 3A). To clarify whether this was aberrant transcriptional regulation of our transgenic DNA construct, we carried out an in situ for mCherry mRNA (Fig. 3D, fibula). However, mCherry mRNA expression was largely restricted to the hypertrophic zone like *Col10a1* mRNA, while mCherry protein fluorescence extended from the hypertrophic zone into the bone marrow (Fig. 3C). On the other hand, at adult ages mCherry fluorescence is largely restricted to the hypertrophic zone and substantially less is observed in the bone marrow trabecular region (data not shown). Therefore, we conclude that the transcriptional regulation of our reporter is accurate, but the long protein half-life of mCherry (~24 hours) allows for the continued marking of hypertrophic chondrocytes at a very active stage of skeletal growth. Consistent with hypertrophic chondrocytes undergoing programmed cell death, much of the mCherry fluorescence we observed in the bone marrow appeared as cell debris particularly as the distance from the growth plate into the bone marrow space increased (Fig. 3E, white arrows). However, we also observed a diverse range of cellular phenotypes that we speculate are hypertrophic cells at different stages of cell death. Among this complex environment, we occasionally observed mCherry positive cells within the trabecular region that looked relatively intact, retained nuclei as determined by DAPI staining, and showed high mCherry fluorescence (Fig. 3E, and imaged at lower exposure in Fig. 3F, cells marked by an asterisk). Previous studies have also commented on the existence of hypertrophic chondrocytes that seem to evade apoptosis (Gebhard *et al.*, 2007). While the reason for increased mCherry fluorescence as hypertrophic chondrocytes meet and mix with bone marrow cells is not clear, we speculate that it is related to the changing physiology of hypertrophic chondrocytes.

The continued marking of intact hypertrophic chondrocytes within the trabecular region of bone by the mCherry reporter suggested the possibility that these cells may be a rare population of hypertrophic chondrocytes that further differentiate into osteoblasts as an alternative to apoptosis. The topic of hypertrophic chondrocytes differentiating into osteoblasts has been speculated upon for years (Kahn and Simmons, 1977; Moskalewski and Malejczyk, 1989; Thesingh *et al.*, 1991) and has been the subject of different review articles

(Adams and Shapiro, 2002; Shapiro *et al.*, 2005; Wlodarski *et al.*, 2006). To explore the possibility of chondrocyte to osteoblast transdifferentiation, we crossed *Col10a1*-mCherry mice with two different osteoblast reporter mouse lines, pOBCol3.6-Topaz and pOBCol2.3-Emerald (see Fig. 4), which have been extensively used to study osteoblast biology (Bilic-Curcic *et al.*, 2005; Kalajzic *et al.*, 2002, 2005; Ushiku *et al.*, 2010). We reasoned that the persistent detection of *Col10a1*-mCherry fluorescence in cells present within the trabecular bone region may be used in a developmental fate mapping-like scheme and allow us to detect skeletal cells that express both chondrocyte and osteoblast reporters. However, examination of long bones at P0 from triple (*Col2a1*-ECFP, *Col10a1*-mCherry, and pOBCol3.6-Topaz) (Fig. 4A–D) and dual (*Col10a1*-mCherry and pOBCol2.3-Emerald) (Fig. 4E–H) transgenic mice showed no evidence of cell populations expressing both chondrocyte and osteoblast reporters. While *Col10a1*-mCherry positive cells (Fig. 4B,F, yellow arrow heads) and pOBCol3.6-Topaz (Fig. 4C) or pOBCol2.3-Emerald (Fig. 4G) positive osteoblasts (white arrow heads) could be seen amongst each other (Fig. 4D,H), both cell populations were entirely distinct. Since this is not a true genetic fate mapping scheme, we cannot completely rule out the possibility of transdifferentiation. However, given the clear distinction of chondrocyte and osteoblast cell types at this very active stage of skeletal development, we believe that transdifferentiation is unlikely to be a natural process.

Numerous pathological conditions exist that directly involve cartilage, including genetically inherited disorders, sports induced injuries, and age related deterioration. Genetic manipulation of the mouse is a common strategy to study gene function, to model different pathological conditions, and to test regenerative therapies. Here we have introduced the generation *Col10a1*-mCherry transgenic reporter mice that will serve as a valuable tool to study chondrocyte development, damage, and repair. To aid in the characterization of this animal model and increase our understanding of chondrocyte biology, we crossed *Col10a1*-mCherry mice with *Col2a1*-ECFP, pOBCol3.6-Topaz, and pOBCol2.3-Emerald mice. The combinatorial use of spectrally-distinct fluorescent protein reporters enables the intercrossing of different fluorescent protein reporter lines to further resolve cell types, mechanisms of development, and tissue regeneration.

MATERIALS AND METHODS

DNA Constructs

BAC Clone RP23-192A7 was obtained from Children's Hospital Research Institute (CHORI). PLD53.SC2 and PSV1.RecA vectors were kindly provided by Shiaoching Gong (Gong *et al.*, 2002).

Assembly of the *Col10a1*-mCherry Reporter

A 453bp homology arm was PCR amplified from purified RP23-192A7 using primers: Col10a1 5′Not1 (Sense) 5′-TCTCGCGGCCGCGTACCCAGACAGGTTCATTTGT ATTGATCT-3′ and Col10a1 3′ BamH1 (antisense) 5′-CTCTGGATCCTTTCAGATAGATTCTGAAAAGCAGAAGA GAATC-3′. The PCR amplicon was cloned into Not1 and BamH1 sites of the pLD53.sc2-mCherry shuttle vector using standard cloning methods. Recombinase A was introduced into RP23-192A7 host

bacteria by transformation with pSV1.RecA vector (100 ng) and selected for on chloramphenicol (12.5 ug/ml)/tetracycline (10ug/ml) LB agar plates. RP23-192A7 host bacteria containing RecA were then transformed by electroporation with 1 μg (1–2 μl) of the pLD53.sc2-mCherry containing the *Col10a1* homology arm. SOC medium (1 ml) was added and transformed bacteria were incubated with shaking at 200 rpm for one hour at 30°C. Recombinants were first selected for by adding 5 ml of LB medium containing chloramphenicol (12.5 μg/ml), ampicillin (50 μg/ml), and tetracycline (10 μg/ml) to bacteria and grown overnight at 30°C with shaking at 200 rpm. Further selection for recombinant clones was carried out by plating 100 μl of overnight culture on to chloramphenicol (12.5 μg/ml), ampicillin (50 μg/ml) LB agar plates and incubated overnight at 42°C. Chloramphenicol/ampicillin resistant colonies were screened by colony PCR using primers that flank the *Col10a1* homology arm: *Col10a1* 5′ Recom (sense) 5′-CCTGCCAGCAGGCTTGGAAACAGGCTTCA-3′ and mCherry 3′ (antisense) 5′-GCACCTTGAAGCGCATGAACTCCTTGATGA-3′. Colony PCR identified candidate recombinants were further verified by diagnostic restriction enzyme digestion with Sal1 and Pvu1 and field inversion gel electrophoresis.

BAC DNA Preparation for Pronuclear Injection

The *Col10a1*-mCherry BAC construct was purified from a 200-ml culture using a Maxi Kit (Qiagen) with minor modifications. During alkaline lysis, 2M potassium acetate was used in place of the 3M solution. Additionally, after elution with QF buffer, two phenol/chloroform extractions followed by one chloroform extraction were carried out to further clean up the BAC DNA. BAC DNA (10 μg) was then linearized with PI-SceI restriction enzyme for 2 hours and loaded onto a CL-4B Sepharose (Sigma) column pre-equilibrated with injection buffer (10 mM Tris, pH7.5, 0.1 mM EDTA, 100 mM NaCl). Ten 300 μl fractions were collected from the column and DNA concentrations were measured with a Nanodrop Spectrophotometer (Thermo-Scientific). Thirty microliters of each fraction were also run on a field inversion gel to assess the DNA quality. Pronuclear injection was carried out at the UCONN Health Center Gene Targeting and Transgenic Facility.

Probe Synthesis for In Situ Hybridization

BAC clone RP23-192A7 containing the *Col10a1* gene and PLD53.sc2-mCherry plasmid were used to synthesize a *Col10a1* probe (733 bp) and mCherry probe (741 bp), respectively. PCR primers for *Col10a1* were: sense, 5′-GTAGGAGCTAAAGGAGTGCCTGGACAC-3′; anti-sense, 5′-GTGAGGAAACTTGGTCCCAGAGTGCAC-3′ and nested oligo, anti-sense, 5′-ACGAGTGGACGTACTCAGAGGAGTAG-3′. PCR primers for mCherry were: sense, 5′-GGAG GATAACATGGCCATCATCAAGG-3′; anti-sense, 5′-CGTAC TGTTCCACGATGGTGTAGTCCTCG-3′. The final PCR reaction for probe synthesis was done only with the antisense oligo to generate single-stranded DNA (ssDNA) probes in the presence of digoxigenin-11-dUTP (Roche) as previously described (O'Connell, 2002). Synthesized probes were further cleaned up using PCR purification columns (Qiagen).

In Situ Hybridization

Frozen tissue sections were first fixed in 4% paraformaldehyde (PFA) at room temperature for 10 min and then incubated in 3% H_2O_2/PBS for 10 min to quench endogenous

peroxidase activity. After proteinase K treatment (1 μg/ml in Tris-HCl/0.05M EDTA) for 10 min, sections were further permeabilized in 0.1% Triton X-100 for 10 min. Sections were then refixed in 4% PFA for 5 min. Sections were washed three times (5 min/wash) with PBS and dehydrated in 70% ethanol for 5 min followed by brief washes in 80%, 90%, and 100% ethanol before hybridization. Hybridization buffer: (50% formamide, 10% dextran sulfate, 1% Denhardt's, 250 μg/ml yeast tRNA, 0.3M NaCl, 20mM Tris-Cl (pH 8.0), 5mM EDTA (pH 8.0), 10mM $NaPO_4$ (pH 8.0), 1% Sarkosyl). Single-stranded DNA probes were diluted in hybridization buffer (1:200). Hybridization was then carried out in a humidified chamber at 55°C for 18 hours. After hybridization sections went through sequential wash steps: 30 min in 50% formamide in 2× SSC at 65°C, 15 min in 2× SSC at 37°C, 15 min in 0.1× SSC at 37°C, and 15 min in TNT buffer (0.1M Tris-Cl pH 7.5, 0.15M NaCl, and 0.05% Tween-20). After blocking in 0.5% blocking reagent (PerkinElmer, FP1020) in TNT buffer for 1 hour, the hybridized ssDNA probes were detected by incubating 30 min with antidigoxigenin-POD, Fab fragment (1:100) (Roche). Digoxigenin labeled probe was detected by incubating 10 min in 1:50 TSA plus Fluorescein (PerkinElmer) and washed three times in TNT buffer before visualization.

Imaging of Reporter Gene Mice

Whole embryo imaging was carried out on a Zeiss Lumar stereomicroscope. Tissues sections were imaged using a Zeiss Imager Z.1 upright microscope. Filter sets (Chroma Technology) to image fluorescent protein expression were as follows: ECFP: ET436/20× (excitation), ET480/40 m (emission), T455LP beam splitter, mCherry: ET577/20× (excitation), ET640/40 m (emission), Q595lp beam splitter, Emerald: ET470/40× (excitation), ET525/50 m (emission), T495 beam splitter, Topaz: ET500/20× (excitation), ET535/30 m (emission), T515LP beam splitter. Beam splitters were not part of the optics for stereomicroscope imaging.

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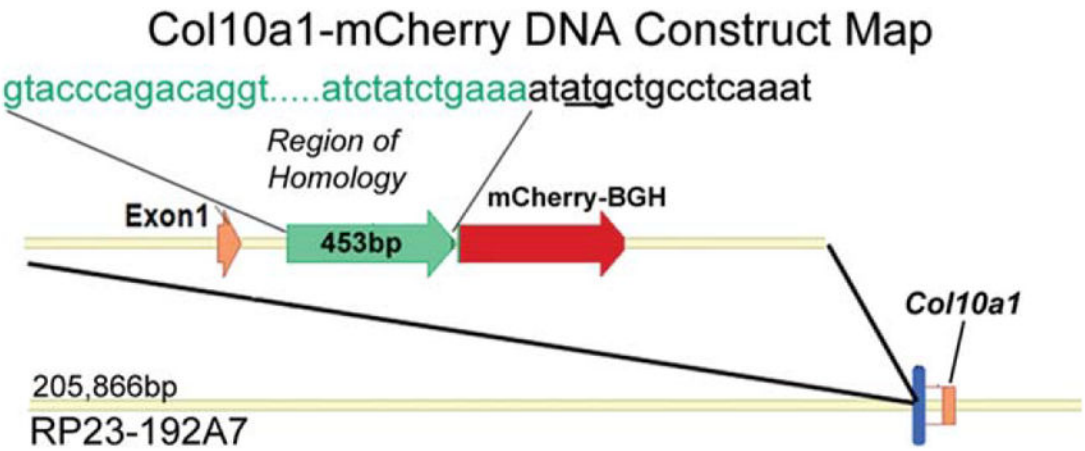

FIG. 1.

DNA construct map for *Col10a1*-mCherry. The *Col10a1*-mCherry reporter was made in BAC clone RP23-192A7. The fluorescent protein mCherry was inserted two nucleotides upstream of the *Col10a1* translation start site (underlined sequence). A 453 bp homology arm (shown in green) was used to direct the recombination of mCherry into the BAC clone by homologous recombination in bacteria.

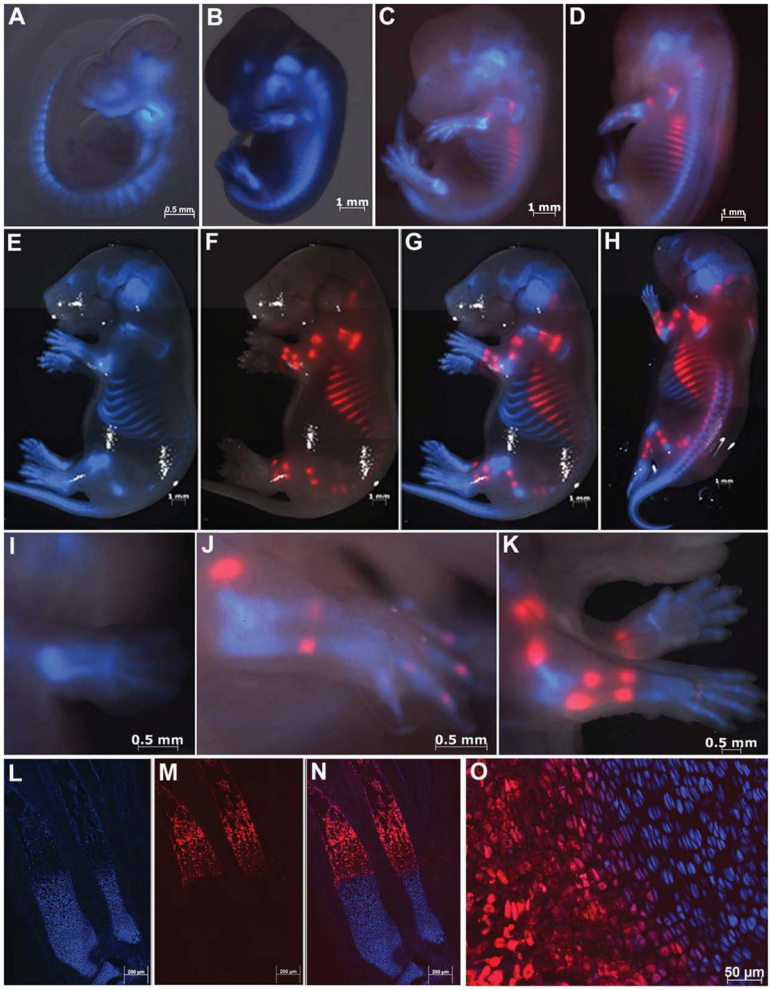

FIG. 2.

Reporter gene expression of *Col2a1*-ECFP (blue) and *Col10a1*-mCherry (red) transgenic mice during embryonic skeletal development. (**A**–**H**) Whole embryo imaging of reporter gene expression from E10.5 to E17.5. (A) *Col2a1*-ECFP expression at E10.5 in the developing somites, brachial arch1, and surface of the ventral forebrain. (B) *Col2a1*-ECFP expression at E12.5 in chondrocyte condensations of the limb bud and vertebral bodies. (C and D) *Col2a1*-ECFP and *Col10a1*-mCherry reporter expression at E14.5. *Col2a1* reporter expression continues to be expressed in growing condensations. Initial expression of *Col10a1* reporter expression was detected in the developing ribs adjacent to the spine and at the center of the anlagen in long bones of the forearm. (E–H) *Col2a1*-ECFP and *Col10a1*-mCherry reporter expression at E17.5. *Col2a1* and *Col10a1* are expressed in largely distinct zones of cartilage maturation with *Col2a1* reporter expression (E, G, H) being expressed in the proximal and distal ends of long bones and the ventral half of the rib cage and *Col10a1* reporter expression (F, G, H) occurring adjacent and interior to *Col2a1* expression domains in long bones and the dorsal half of the rib cage. **(I–K)** Imaging of reporter gene expression in the forelimbs at E12.5, E14.5, and E17.5 days of development. (I) *Col2a1* reporter expression initially appears throughout the cartilaginous anlagen of the forearm, but as chondrocytes mature *Col2a1* expression was down regulated and *Col10a1* reporter

expression was initiated (J). (K) By E17.5 *Col2a1* expression was restricted to the proximal and distal ends of long bones and the single zone of hypertrophy marked by *Col10a1* reporter expression was also separated into two regions. (**L–O**) Tissue sections through the radius and ulna at E17.5 days of development. (L) *Col2a1* reporter expression was seen in the resting and proliferative zone. (M) *Col10a1* reporter expression was seen in the hypertrophic zone. (O) *Col2a1* and *Col10a1* reporter expression slightly overlap in the growth plate.

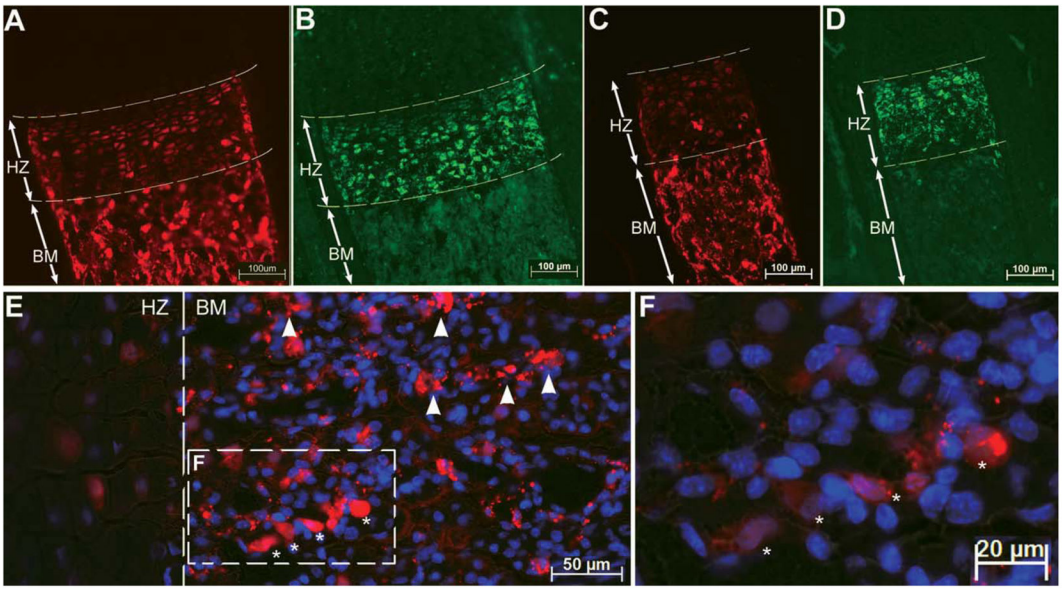

FIG. 3.

Comparison of *Col10a1*-mCherry protein fluorescence to endogenous *Col10a1* mRNA and mCherry mRNA. *Col10a1*-mCherry protein fluorescence **(A)** was compared to endogenous *Col10a1* mRNA **(B)** in the proximal tibia at postnatal day 0. **(C)** Comparison of *Col10a1*-mCherry protein fluorescence to mCherry mRNA **(D)** in the distal fibula at P0. **(E)** Visualization of mCherry fluorescence and DAPI stained nuclei in the bone marrow (BM) below the hypertrophic zone (HZ). mCherry fluorescent cell debris (white arrow heads) was scattered below the HZ, but an area containing mCherry positive cells with intact nuclei can been seen (boxed in region, marked by an asterisk). **(F)** Higher magnification of boxed region shown in E, mCherry positive cells contain nuclei (marked by an asterisk). (F) mCherry fluorescence was imaged at lower exposures than shown in (E) to better visualize individual cells with DAPI stained nuclei.

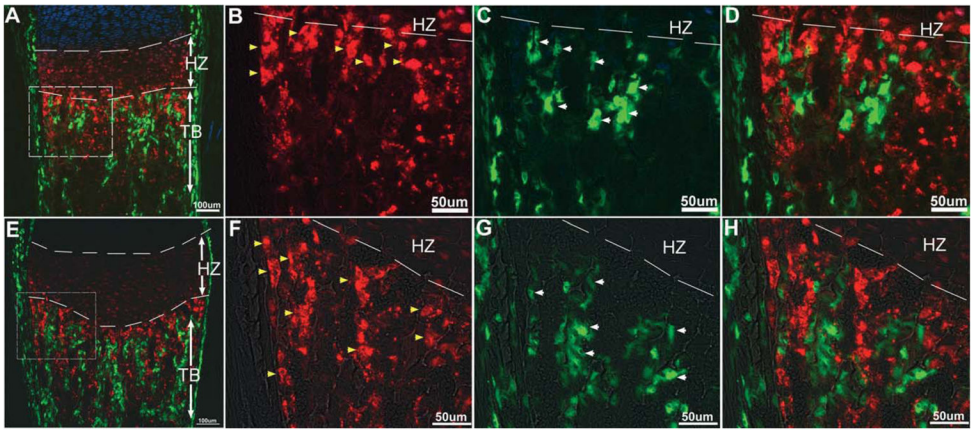

FIG. 4.

No evidence for chondrocyte to osteoblast transdifferentiation. To determine if hypertrophic chondrocytes transdifferentiate into osteoblasts, femur **(A–D)** and tibia **(E–H)** section were examined from triple transgenic (A–D, *Col2a1*-ECFP, *Col10a1*-mCherry, and pOBCol3.6-Topaz) and dual transgenic (**E–H**), *Col10a1*-mCherry and pOBCol2.3-Emerald) mice, retaining chondrocyte and osteoblast reporter genes. (A, E) Detection of chondrocyte and osteoblast reporters at low magnification showing a region of interest (boxed in area) magnified in the corresponding panels, (B–D for A, and F–H for E). Both regions are located at the boundary where the hypertrophic zone (HZ) ends and osteoblasts make trabecular bone (TB). While hypertrophic chondrocytes [mCherry positive—red (yellow arrow heads, panels B and F)] and osteoblasts [Topaz or Emerald positive—green (white arrow heads, panels C and G)] cell types were in close proximity to each other, they existed as two distinct cell populations and no cells expressed both chondrocyte and osteoblast reporters.