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PERSPECTIVES

Transgenic Mouse Strains for Conditional Gene Deletion During Skeletal Development

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Abstract

The development of the Cre-*lox* recombination system has dramatically altered studies in skeletal development by allowing for both temporal and cell-specific mutations of essential genes. Localizing these mutations to the skeleton allows for a more direct study of the gene's role in skeletal development while minimizing the effect of the mutation on other critical tissues. As our understanding of the biological processes in skeletal development expands, so too does the need for a wider range of Cre strains to interrogate issues of bone development, osteoporosis, and skeletal metastasis. Osteoblast-, osteoclast-, chondrocyte-, and osteocyte-specific Cre expression has been achieved with various promoters regulating expression. In this review, we will provide an overview of the Cre-*lox* system and the principal cell types of the skeleton, and then focus on the development of the major Cre-expressing strains of the skeleton and the cell-specific expression patterns of each strain. *IBMS BoneKEy*. 2008 May;5(5):151-170.

Like many areas of biomedical research, studies of skeletal development and osteoporosis have been dramatically impacted by the ability to selectively delete genes in defined cell lineages and at specific stages of skeletal differentiation. These advances were facilitated by the development of Cre-*lox* recombination systems. In this review, we will summarize the development and characteristics of many of the major Cre-expressing strains that have been used successfully to gain insight into the genetic mechanisms underlying skeletal development.

Overview of the Cre-*lox* System

The Cre-*lox* system was identified in bacteria in the early 1980s (1). The P1 bacteriophage protein called cyclization recombination (Cre) is 38 kDa and catalyzes recombination between two of its sequence recognition (*loxP*) sites. A *loxP* (locus of X-over P1) site is a 34-base-pair consensus sequence containing a core domain of 8 base pairs flanked on each side by a 13-base-pair palindrome sequence (2). Cre-mediated recombination results in the elimination of sequences flanked by the *loxP* sites. The utility of this system in eukaryotic cells was first demonstrated in the late 1980s (3-5), and further confirmation of its activity in transgenic mice was shown in 1992 (6;7). This led to the development of numerous mouse strains in which essential portions on the gene are flanked by *loxP* sites (so-called "floxed" strains). If the floxed alleles are properly designed, Cre-mediated recombination leads to creation of a null gene.

Overview of Cell Types Discussed

Using promoters that specifically target the skeletal system has allowed for a wide range of conditional studies in bone development. Inserting Cre recombinase into the genome of an organism under the transcriptional control of one of these promoters creates a specific Cre expression in a particular cell type and at a particular stage in skeletal development. We have focused our discussion on four of the principal types of cells that regulate the

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growth of the skeleton: chondrocytes, osteoblasts, osteocytes, and osteoclasts. These four cell types are responsible for maintaining the two main components of the skeleton: cartilage and bone.

Chondrocytes are cells found within the matrix of cartilage. By secreting proteins such as collagen and elastin, chondrocytes establish a tough but flexible extracellular matrix (8). Chondrocyte activity is often associated with the formation of growth plates. During skeletal formation, the cartilaginous growth plate establishes the preliminary skeletal structure. Mineralized bone is then deposited along this cartilaginous scaffolding in the process of endochondral ossification. Furthermore, cartilage is used by the skeleton as the primary structural element in regions of the body where strength and flexibility are required, for example, the ears and nose, as well as serving as a cushion between bones at the joints (9).

Osteoblasts are the bone-forming cells of the body. They are responsible for secreting osteoid and further mineralization of this osteoid in the process of bone formation (8). Osteoblasts are found on the exterior surface of developing bone. Once the osteoblast has deposited enough bone to trap itself inside the ossified matrix, it is then referred to as an osteocyte. Osteocytes no longer secrete osteoid, but rather regulate osteoblast activity and likely respond to mechanical transduction (10).

Part of maintaining bone homeostasis is controlling the resorption of mineralized bone and calcium. This process is crucial in establishing a stable serum Ca^{2+} ion concentration and is performed by bone cells called osteoclasts. Osteoclast-mediated bone resorption occurs at the surface of the bone and is regulated in balance with osteoblast activity (11).

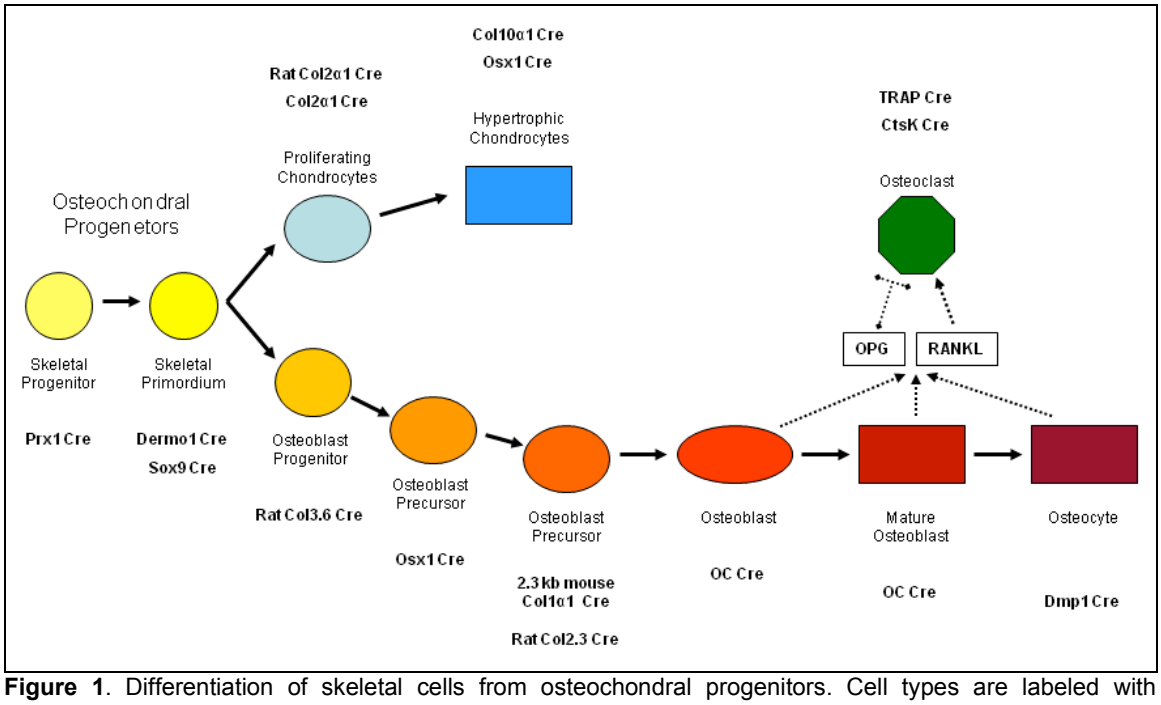

Figure 1. Differentiation of skeletal cells from osteochondral progenitors. Cell types are labeled with corresponding Cre strains based on the noted Cre expression. Proposed signal pathway via ligands RANK and OPG to stimulate or inhibit osteoclasts are also presented (11). Figure adapted from Rodda *et al.* (9).

Many genes involved in skeletal development are also crucial in the development of other organs and tissues. Due to the importance of these skeletal genes in general development, often a global knock-out of the gene of interest

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cannot be achieved in a viable mouse. In these situations, the conditional knock-out of a gene only in the skeleton can allow for a living mouse with the desired phenotype. Using the Cre-*lox* system under transcriptional regulation of promoters that are only active in the skeleton of the mice allows for such conditional knock-outs. Below, we will review the different Cre promoters used in skeletal studies and cover the cell type or types in which these promoters are active (Figure 1).

Osteochondral Progenitors

During skeletal cell development, osteoblasts and chondrocytes differentiate from the same progenitor cells. Activating the Cre gene in these osteochondral progenitors results in the conditional knock-out of the gene(s) of interest early in skeletal system development, in *both* the cartilage and the mineralized bone. Importantly, these strains have allowed the interrogation of the role of specific genes in the process of commitment to the osteoblast and chondrocyte lineages. Several types of Cre-expressing strains are used to target these osteochondral progenitors, including *Prx1-Cre*, *Dermo1-Cre,* and *Sox9-Cre*.

Prx1-Cre

The *Prx1-Cre* strain was created by Malcolm Logan and colleagues in the Department of Genetics of Harvard Medical School (12). The Cre recombinase gene is under the transcriptional control of the 2.4-kb Paired Related Homeobox gene 1 (Prx1) enhancer (Figure 2). The *Prx1* gene was originally shown to be essential in regulating skeletal development in the limb, acting through a BMP-mediated signaling pathway.

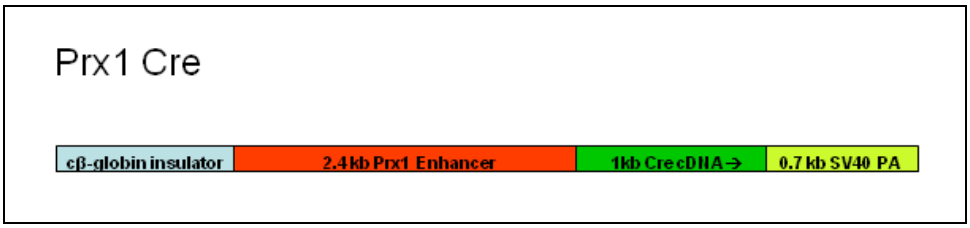

Figure 2. Schematic of *Prx1*-driven Cre transgenic construct, adapted from Logan *et al.* (12).

The designed target gene was integrated into the mouse DNA through transgenic incorporation. Verification of the specificity of the Prx1 promoter was achieved with the Z/AP reporter. The Z/AP reporter line activates the histochemical marker human placental alkaline phosphatase in cells in which Cre is expressed (Figure 3) (13).

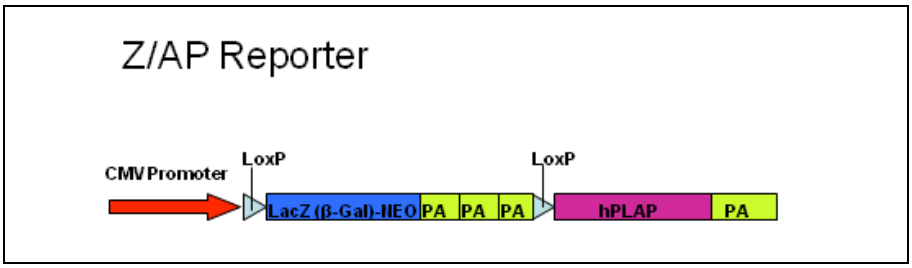

Figure 3. Representation of Z/AP reporter transgene. β-galactosidase is constitutively expressed under the strong CMV promoter. Upon exposure to Cre, β-galactosidase is removed and alkaline phosphatase is expressed (13).

Phosphatase staining indicated that Cre is strongly expressed in the budding limbs of the developing embryo. Beginning on embryonic day (ED) 10.5, Cre expression is clearly visualized in the forelimb and hind limb mesenchyme, but not in the ectoderm. By ED 16.5, Cre expression was observed throughout the developing mesoderm, including the latissimus dorsi muscle and a subset of cells deriving from periocular

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mesenchyme, indicating expression early in mesenchymal cell differentiation. However, Cre is not expressed in any internal organs or in the extra-embryonic tissues. One additional note to consider when working with this strain is the observed Cre expression in the germ line at a variable level (12). Additional lineage tracing experiments were recently reported using this strain (14). Some examples where this strain has been used to successfully delete floxed alleles include those targeting *Bmp4* (15;16), *Presenilins* (17;18), *Notch1* and *Notch2* (18), *Hif1α* (19), *β-catenin* (20), and *Tgfbr2* (21). It has also been used to activate a constitutively active allele of *β-catenin* (22).

Dermo1 (Twist2) Cre

Dermo1-Cre (sometimes referred to as *Twist2-Cre*) is also expressed in mesenchymal osteochondral progenitor cells. The *Dermo1-Cre* transgenic strain was generated by Kai Yu *et al.* in the Department of Molecular Biology and Pharmacology at Washington University Medical School (23). Unlike most Cre recombinase strains, which are generated as transgenic insertions via pronuclear injection, the *Dermo1-Cre* strain was created by a homologous knock-in of Cre into the *Dermo1* gene locus, specifically replacing exon 1 (Figure 4). This allows for more precise expression of Cre in locations where *Dermo1* is normally expressed, as it is under the control of the endogenous promoter in the normal chromosome context. *Dermo1* is a basic helix-loop-helix transcription factor that is highly expressed in the condensed mesenchyme during skeletal development, as well as other mesoderm tissues during embryogenesis (24).

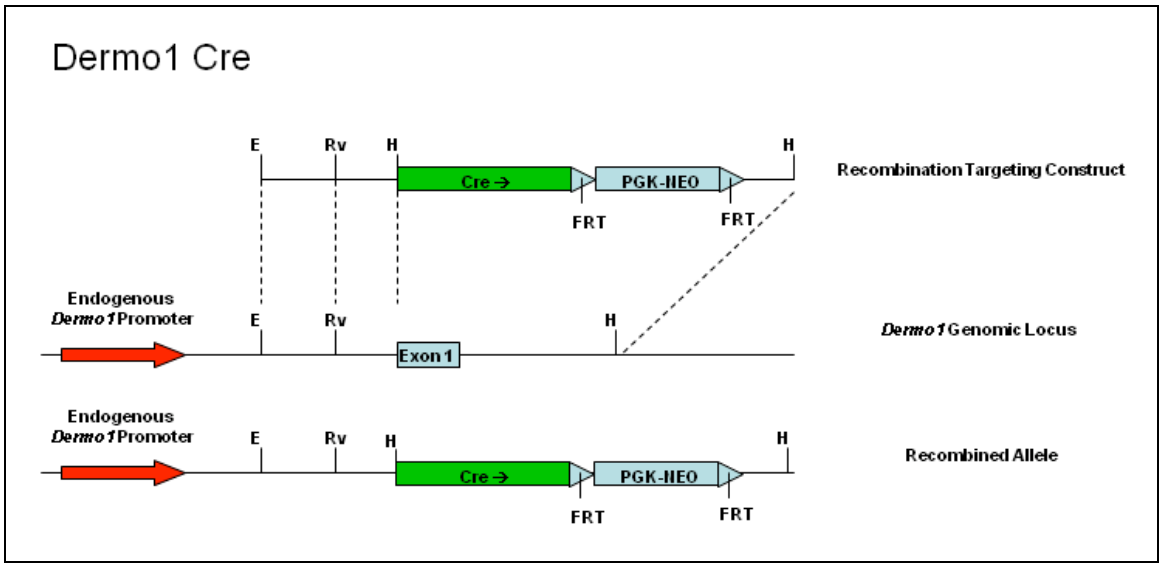

Figure 4. Diagram of the targeting *Dermo1-Cre* gene construct and representation of desired homologous integration deleting exon 1 in the *Dermo1* locus. Figure adapted from Yu *et al.* (23).

Cre expression was verified using the *ROSA26* reporter (R26R). In the R26R mouse, β-galactosidase is preceded by a pair of *loxP* sites flanking a termination signal. Thus, β-galactosidase expression is activated only upon exposure to Cre (25). In doubly transgenic mice (carrying both a transgene expressing Cre and R26R), cells expressing Cre also express β-galactosidase, which is easily detected in tissues via X-gal staining (Figure 5).

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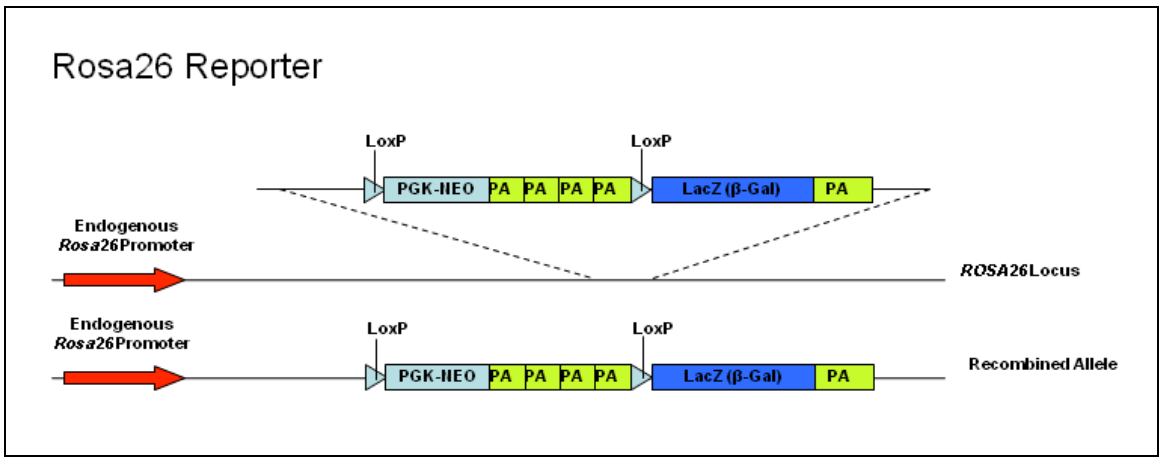

Figure 5. Schematic of ROSA26 Reporter construct. Exposure to Cre excises the end-transcription polyadenylation sequences and mediates β-Galactosidase expression (25).

This approach showed that Cre activity from the *Dermo1* promoter was detected as early as ED 9.5. Cre was found to be highly expressed in mesodermal tissues early in embryonic development, with very low expression in neural and ectodermal tissues. Later Cre activity was noted in the condensed mesenchyme, from which both osteoblasts and chondrocytes arise. At ED 16.5, β-Galactosidase activity confirmed Cre expression in both differentiated chondrocytes and differentiated osteoblasts, while bone marrow and osteoclast cells remained negative for β-Galactosidase activity. It is important to note that these studies were performed with *Dermo1-Cre* heterozygous mice, as homozygote Cre knock-in mice are not viable due to embryonic lethality. Mice homozygous for this Cre allele are functionally null for the *Dermo1* gene, as the endogenous exon 1 is replaced with the Cre recombinase cDNA (23). Examples of the use of this strain include deletion of floxed alleles of *Fgfr2* (23), *Alk3* (26), and *β-catenin* (20;27). It is also important to note that because of the high levels of Cre expression in early embryonic mesodermal tissues, this strain can also be used to conditionally delete genes in other tissues such as the lung (28;29).

Sox9-Cre

Sox9-Cre was generated by Akiyama and colleagues of the Department of Molecular Genetics at the University of Texas M. D. Anderson Cancer Center (30). The *Sox9-Cre* mice were created via homologous recombination of a targeting vector into the *Sox9* locus. The vector consisted of a 7.7-kb segment of the *Sox9* gene, with a Cre construct fused into the untranslated region of exon 3. The Cre construct was fused to an internal ribosomal entry site (IRES) and followed by an FRT-flanked PGK-NEO cassette (Figure 6).

To verify the expression of *Sox9-Cre,* homozygous Cre mice were crossed to the R26 reporter line. β-Galactosidase staining indicated that Cre was being expressed as early as ED 10.5 in the limb bud mesenchyme, and by ED 13.5, all cells in the cartilaginous primordia and perichondrium were β-Galactosidase positive. By ED 17, all chondrocytes, as well as perichondrial, periosteal, and osteoblast cells were expressing β-Galactosidase, indicating *Sox9-Cre* is expressed in the precursors of the chondrocyte and osteoblast lineages. Furthermore, β-Galactosidase staining was also noted in tendons and synoviums, indicating the mesenchymal cells expressing *Sox9-Cre* also give rise to tendon and synovial cells. In addition, these studies also showed that cell types from a variety of tissues including cells of the spinal cord, intestinal epithelium, pancreas, and mesenchymal tissue within the testis are all derived from Sox9-expressing cells (30).

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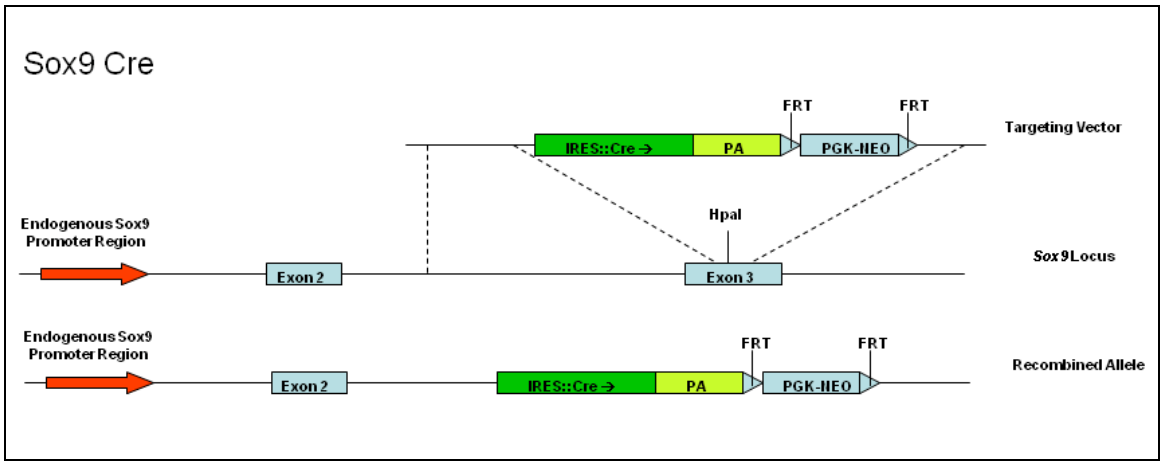

Figure 6. Homologous recombination into the *Sox9* Locus of the targeting vector. Cre expression is driven by the endogenous *Sox9* promoter region.

Chondrocytes

There are two principle pathways for bone formation: intramembranous and endochondral ossification. While the process of intramembranous ossification, where bone is formed without a preexisting cartilaginous template, underlies the formation of many cranial and facial bones (31), most bone is formed via the process of endochondral ossification (8). Osteochondral progenitor cells typically condense around newly forming skeletal structures in the embryo. As the structures develop, the first cells to differentiate from these progenitor cells are the chondrocytes. These chondrocytes establish the preliminary structure through extracellular secretions to form cartilage. It is along this cartilaginous scaffold that the remaining osteochondral progenitors migrate via the formation of a bone collar and differentiate into osteoblasts, which lay down the bony matrix. The process of endochondral ossification has been extensively studied and is exemplified by the description of the growth plate (8). Furthermore, in regions of the skeleton where flexibility is required, osteoblast differentiation is never achieved, and cartilage serves as the primary structural element (9).

Numerous studies have utilized Cre-mediated gene deletion within the chondrocyte lineage to gain important insights into the molecular mechanisms of chondrocyte signaling with a great degree of emphasis on understanding regulation of the growth plate. One well-established marker of chondrocyte differentiation is the expression of type II collagen. Type II collagen is an essential protein secreted into the extracellular matrix for the formation of cartilage.

Collagen II α1-Cre

To target chondrocytes, *Col2α1-Cre* (Cre expression under type II collagen transcriptional regulation) was first generated by Ovchinnikov and colleagues of the Department of Molecular Genetics at the University of Texas M. D. Anderson Cancer Center (32). *Col2α1-Cre* was generated via pronuclear microinjection. The targeting construct consisted of 3 kb of the mouse *Col2α1* promoter region, a modified first exon with a mutated initiation codon, followed by a 3.02-kb segment of the first intron ligated to a splice acceptor and an internal ribosome entry site (IRES), and finally the Cre recombinase coding region with SV40 large T antigen polyadenylation signal (Figure 7). Strains created by similar means were reported by Long and colleagues at Harvard (33).

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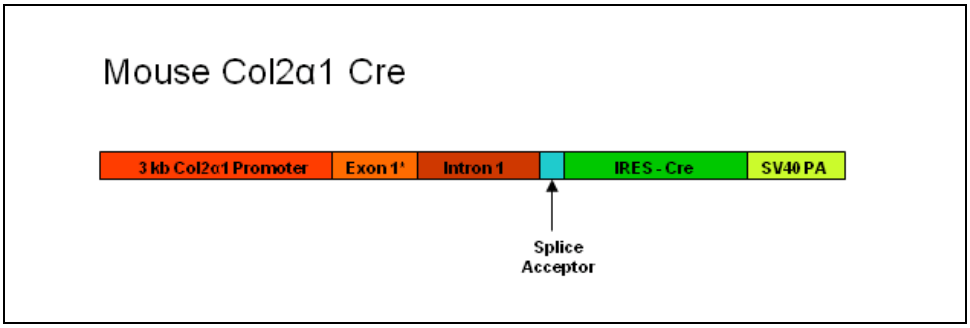

Figure 7. *Col2α1-Cre* transgene consisting of the mouse *Col2α1* promoter region, exon1, and intron 1 fused to Cre. Figure adapted from Ovchinnikov *et al.* (32).

Transgenic mice were generated with the *Col2α1-Cre* construct, and the R26 reporter was again used to characterize Cre expression. β-Galactosidase activity was first noted in the notochord and cranial mesenchyme just prior to ED 9. By ED 9.5, Cre activity was also noted in the somites and otic vesicle. At ED 11.5, very strong activity in the perinotochordal condensations and cranial mesenchyme was observed. At ED 14.5, β-Galactosidase activity was noted in all cartilaginous elements. Interestingly, some nonspecific activity was seen, particularly in the submandibular glands, along with some mosaicism, as approximately 5% of the chondrocytes stained β-Galactosidase-negative (32). *Col2α1-Cre* mice have been used to selectively delete several genes within chondrocytes including *Igf-1* (34), *Pten* (35), *Kif3a* (36), *Ihh* (37), *Gsα* (38), *Smoothened* (33), *Ilk* (39), and *Smad4* (40). In addition, "second-generation" versions have been made in which the type II Collagen α1 promoter drives the expression of a tamoxifen-inducible Cre-ER fusion protein (41;42) or the collagen II promoter is combined with a tetracycline responsive element (43) to allow for more precise temporal and spatial control of Cre expression.

A second transgenic line was created utilizing the rat α1 promoter of type II collagen to drive Cre expression in chondrocytes. This rat *Col2α1-Cre* was generated by Ernestina Schipani and colleagues of the Endocrine Unit at Massachusetts General Hospital. Unlike the original mouse-derived *Col2α1-Cre*, the rat *Col2α1-Cre* uses only 1.1 kb of the promoter region for type II collagen (44). The developed transgene also includes a splice sequence consisting of a segment from the rabbit β-Globin intron, followed by a nuclear localization signal (NLS) and the Cre cDNA and a polyadenylation signal. Specificity for Cre expression in chondrocytes was further improved by including a chondrocyte-specific enhancer element after the polyadenylation signal (Figure 8). The transgene was incorporated into the mouse genome via microinjection into fertilized eggs.

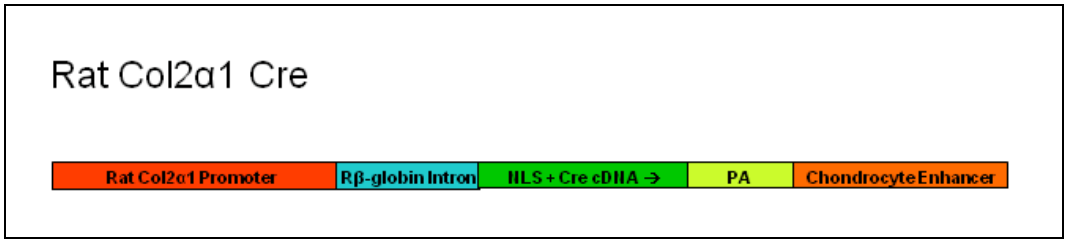

Figure 8: Diagram of the rat *Col2α1-Cre* transgene. Note the Cre cDNA is fused to a nuclear localization signal, and the enhancer element at the end of the construct increases specificity for chondrocyte expression. Figure adapted from Schipani *et al.* (44).

Confirmation of Cre expression was achieved by crossing the Cre transgenic mice to the R26 reporter line, as well as antisense Cre riboprobes on sections of hind limb. Whole mount staining of the embryos clearly indicated Cre expression across the skeleton. Additional interrogation showed Cre expression in growth plate chondrocytes by ED 15.5. Furthermore, *in situ* hybridization analysis with riboprobes identified Cre expression only in the cartilage and not in the connective tissues of the hind limb. This transgenic Cre line was initially used to create a chondrocyte-specific deletion of *HIF-1α* (44). The results from these studies also served as additional verification that the rat *Col2α1-Cre* is highly expressed in the chondrocytes of the growth plate with no detectable non-specific expression (44). This strain has also been used to selectively delete *Ppr* (45), *Vegfa* (46), and *Vhl* (47) in chondrocytes.

Collagen 10α1-Cre

Another well-established marker of chondrocyte differentiation and cartilage production is the expression of type X collagen, another matrix collagen protein. Guan Yang and colleagues of the Genetic Laboratory of Development and Diseases in Beijing, China, targeted Cre expression utilizing the α1 promoter for type X collagen (48). The *Col10α1-Cre* transgene was generated by fusing a 1.0-kb fragment of the Col10α1 promoter to a 1.2-kb Cre cDNA, followed by the 2.1-kb *hGH* polyadenylation signal (Figure 9).

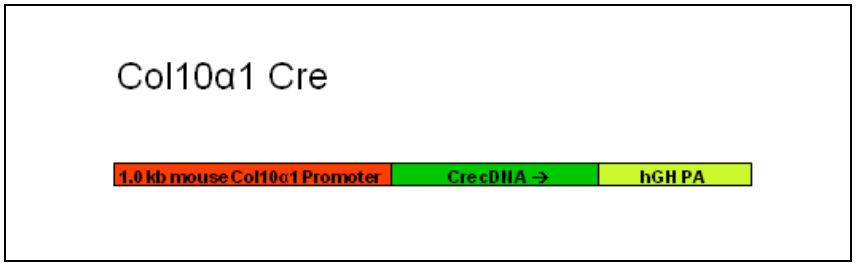

Figure 9. Schematic of *Col10α1-Cre* transgene. Total fragment length is about 4.3 kb.

To confirm the expression pattern of *Col10α1-Cre*, transgenic mice were crossed to the R26 reporter line. Cre activity was detected in cartilage primordia on ED 14.5. Furthermore, Cre mRNA expression was analyzed in varying tissue types. Cre expression was detected in the skeleton and also in the skin, but not in the lungs, liver or other soft tissues. Detailed analysis of femur sections showed β-Galactosidase staining was only visible in hypertrophic chondrocytes, and not in resting or proliferating chondrocytes. *Col10α1-Cre* is specifically expressed in lower hypertrophic chondrocytes of the cartilage lineage, with a small amount of expression in the skin.

Osteoblasts

Perhaps the most widely studied and targeted cell type in the skeletal system is the osteoblast. As the sole cell type responsible for mineralized bone deposition, studies of osteoblast activity have obvious importance in understanding the network of signaling pathways responsible for initiating mineralization and ossification of bone. While a number of different Cre lines have been established to target osteoblast specific recombination, today, the three most common are *Osterix1*-driven (*Osx1*) Cre, α1 type I collagen (*Col1α1*) Cre, and *Osteocalcin* (*OC*) Cre, each having a slightly different expression pattern.

Osterix1-Cre

During the differentiation process, an osteoblast precursor begins to noticeably change its protein expression pattern as it differentiates into a mature osteoblast (49). Two specific markers along this differentiation pathway that have been well-characterized are *Runx2* and *Osterix1*. Stephen J. Rodda and Andrew P. McMahon of the Department of Molecular and Cellular Biology at Harvard utilized the *Osterix1* marker for osteoblast differentiation by

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creating *Osx1-Cre*, a Cre recombinase under the transcriptional regulation of the *Osterix1* promoter (9). The gene construct was inserted via homologous recombination into exon 1 of the *Osterix1* locus. In addition to *Cre* expression under *Osterix1* promotional control, the gene construct also included a GFP construct fused to the Cre recombinase for easy reporter detection. Additionally, a TET-off regulatory cassette was placed in front of the Cre-GFP fusion, allowing for further regulatory capacity of Cre expression (Figure 10).

Characterization of the expression pattern of *Osx1-Cre* was performed by detection of the reporter GFP. Further verification of Cre activity was achieved by crossing the *Osx1-Cre* mice to the R26 reporter line, allowing for β-Galactosidase expression upon Cre recombination. Both reporter methods confirmed Cre activity in both endochondral and membranous bony elements, consistent with expected Osterix1 expression. Tibial sectioning at ED 14.5 illustrated Cre expression in the inner bone-forming perichondrium and sporadically in

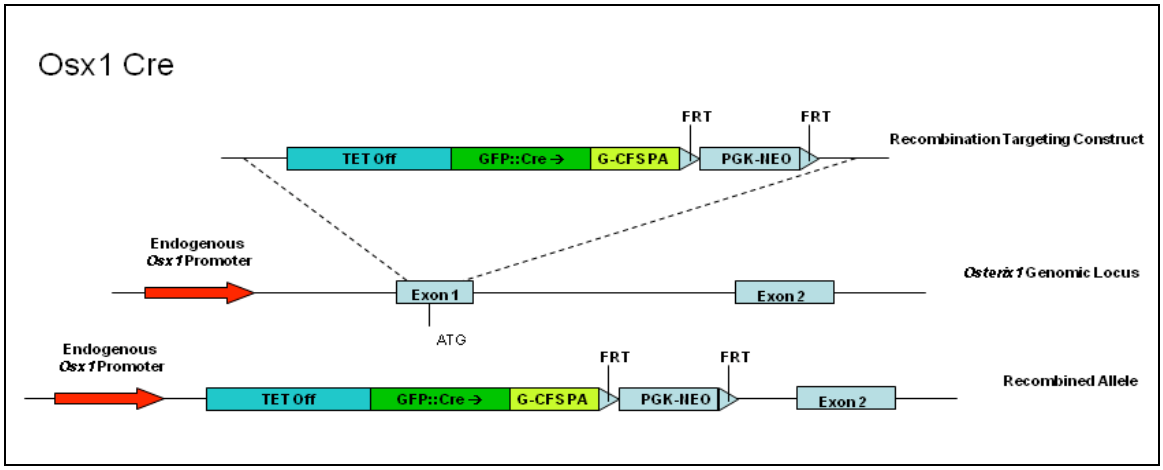

Figure 10. Targeting construct of GFP-fused Cre and homologous integration into exon 1 of the *Osterix1* gene. Homologous recombination deletes the initiation ATG signal in exon 1. Figure adapted from Rodda *et al.* (9).

hypertrophic chondrocytes by both LacZ staining and fluorescence microscopy. Further characterization illustrated that *Osx1-Cre* activity was largely restricted to the osteoblast lineage throughout embryonic and early postnatal development. This proved particularly interesting, because *Osterix1* is typically expressed in low levels in chondrocytes, but the absence of Cre activity in most chondrocytes (except hypertrophic chondrocytes) indicates that low-level Cre expression was insufficient for recombination, or that the gene construct was deficient in the chondrocyte-specific regulatory elements for *Osterix1* (9).

Col1α1-Cre

The expression patterns of type I collagen, a major protein in osteoid, were largely characterized by Rossert, Eberspaecher, and de Crombrugghe by transgenically incorporating different length fragments of the type I collagen α1 promoter fused to the β-Galactosidase reporter. Their studies showed that with a 0.9-kb promoter fragment, β-Galactosidase expression was low and restricted exclusively to the skin. However, with a 2.3-kb fragment of the type I collagen α1 promoter, high expression levels were also detected in osteoblasts and odontoblasts. Finally, the 3.2-kb promoter fragment yielded β-Galactosidase expression in tendon and fascia fibroblasts of the mesenchyme as well as osteoblasts and odontoblasts, with low levels in the skin (50). Based upon this characterization, a couple of groups have developed Cre lines under different Col1α1 promoter fragments. Romain Dacquin *et al.* of the Department of Molecular and Human Genetics at Baylor College of Medicine generated a Cre strain

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under transcriptional control of the mouse 2.3-kb promoter fragment (51). Furthermore, Liu and colleagues of the Department of Medicine at the University of Connecticut Health Center generated two *Cre* transgenes under different length segments of the rat *Col1α1* promoter: 2.3-kb and 3.6-kb (52).

The 2.3-kb mouse *Col1α1-Cre* was generated by fusing the promoter fragment to Cre recombinase cDNA, followed by an MT-1 polyadenylation sequence (Figure 11). The gene construct was then incorporated to the mouse genome through pronuclear injection (51).

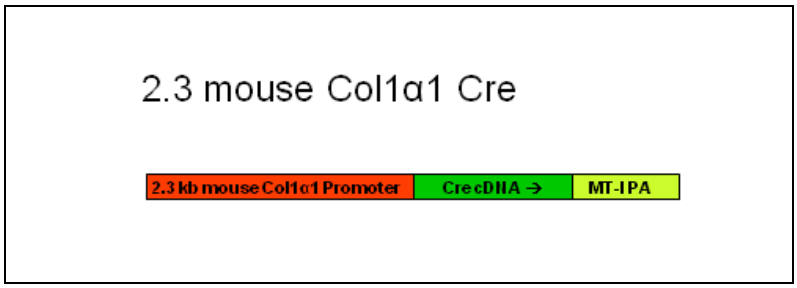

Figure 11. 2.3-kb mouse type I collagen α1 promoter *Cre* construct. Figure adapted from Dacquin *et al.* (51).

The 2.3-kb mouse *Col1α1-Cre* was characterized by crossing the Cre mice to the R26 reporter line. LacZ staining at ED 14.5 showed Cre activity in the skull and all long bone ossification centers, with very light staining in the skin of the face and hands. By ED 16.5 and at 5 days after birth, LacZ staining was found in all bones of the skeleton, while no other staining was detected in any other tissue. Furthermore, histological studies revealed the staining was unique to osteoblast cells and was not found in chondrocytes or osteoclasts (51).

Each of the rat *Col1α1* promoter Cre lines was generated via similar methods. An initial 2.3-kb promoter vector served as the starting vector. Cre cDNA was isolated and cloned into the vector after the promoter. For the 3.6-kb segment, the next 1.2 kb of the rat promoter were isolated and cloned into the vector immediately following the original 2.3-kb segment. A bGH polyadenylation signal was placed at the end of the construct (Figure 12). Integration was performed through transgenic insertion (52).

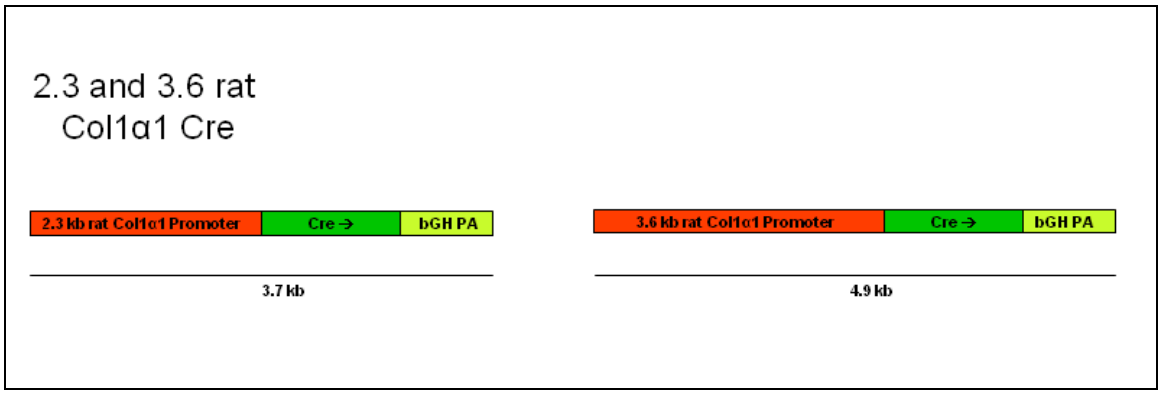

Figure 12. Representation of the 2.3-kb rat *Col1α1* promoter *Cre* construct (left) and the 3.6-kb rat Col1α1 promoter *Cre* construct (right). Figure adapted from Liu *et al.* (52).

Verification of each of the rat *Col1α1* promoter Cre transgenes was performed by crossing the Cre mice to the R26 reporter line, as well as mRNA analysis from 6-week-old Cre-positive mice. Northern blots revealed that the 2.3-kB *Col1α1-Cre* was expressed in the long bone and the calvaria, with very low expression in skin, tendon,

brain, kidney, liver, and lung only detectable by overexposure of the film. Similarly, the 3.6-kB *Col1α1-Cre* was highly expressed in the long bone and calvaria, with moderate expression levels in the tendons; very low expression was detected in the brain, kidney, liver, and lung upon overexposure of the film. Additionally, histological studies of both Cre constructs, when crossed to R26 mice, showed Cre expression sufficient for recombination in osteoblasts. Specifically, the 2.3-kB *Col1α1-Cre* was expressed in mature osteoblasts of the calvaria, but not in the less differentiated cells of the suture mesenchyme. In contrast, the 3.6-kB *Col1α1-Cre* was expressed broadly in cells of osteoblast lineage in the suture mesenchyme. It was concluded that 2.3-kB *Col1α1-Cre* was more specific for osteoblast recombination, while the 3.6-kB *Col1α1-Cre* targeted a slightly broader mesenchymal Cre expression (52).

These collagen I Cre strains have been widely used for studies of osteoblast differentiation and function. For example, 2.3-kB *Col1α1-Cre* strains (of both varieties) have been used to delete floxed alleles of *Connexin43* (53), *androgen receptor* (54), *Stat3* (55), $G_s\alpha$ (56), *β-catenin* (57), *Nf1* (58), and *Pthrp* (59); and to activate a mutant version of *β-catenin* (57). The 3.6-kB *Col1α1-Cre* strain has been used to delete floxed alleles of *Mdm2* (60). In addition, a version of the 2.3-kB *Col1α1-Cre* strain expressing a fusion of Cre recombinase to a mutated ligand-binding domain of ER has also been developed and allows for tamoxifen-inducible activation of Cre activity in this lineage (61).

Osteocalcin-Cre

One of the most notable markers of mature osteoblast differentiation is the production of osteocalcin, a secreted protein that is thought to play an important role in mineralization and bone formation (49). Production of osteocalcin does not occur in an osteoblast's development until it has differentiated into a mature, bone-forming osteoblast.

Using the osteocalcin promoter as a specific marker for osteoblast cells, a number of groups have attempted to make a wide variety of different osteocalcin Cre constructs. One of these constructs, again coming from Romain Dacquin *et al*., used a 1.3-kb segment of the osteocalcin gene 2 promoter to drive Cre expression. Furthermore, they generated an artificial OG2 promoter that consisted of six tandem repeats of osteoblast-specific-*cis*-acting element (OSE) 2 followed by six tandem repeats of OSE1, followed by the Col1α1 TATA box (Figure 13) driving a cDNA Cre construct with the MT-1 polyadenylation sequence. OSE1 and OSE2 are naturally found in the OG2 promoter, but it was found that the six tandem repeats seemed to be more efficient in driving Cre expression (51).

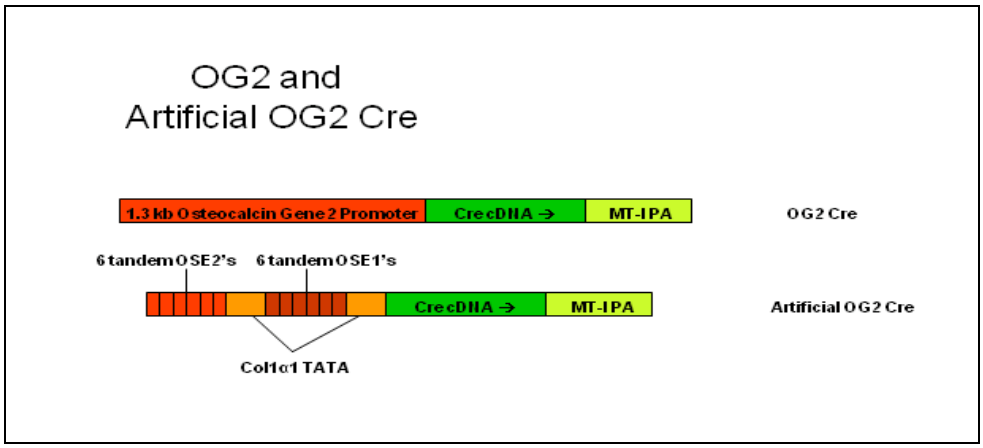

Figure 13. Schematic representations of mouse OG2 promoter-driven *Cre* (top) and an artificial OG2 promoter-driven *Cre* (bottom) with six repeats of OSE1 and OSE2. Figure adapted from Dacquin *et al.* (51).

Verification with R26 reporter mice showed that the 1.3-kb OG2 promoter-driven Cre was only expressed in bone, while the artificial *OG2-Cre* was expressed in both bone and cartilage. Their studies further indicated that Cre expression was relatively weak and/or nonspecific for osteoblasts, and they published findings that the 2.3-kB *Col1α1-Cre* was the most efficient to target osteoblasts (51).

Another form of the osteocalcin-driven Cre was generated by Zhang *et al.* from the Department of Medicine at the University of Cincinnati (62). Their *OC-Cre* construct was composed of a segment of the human osteocalcin promoter, followed by an intron for rabbit β-globin flanked by small regions of β-globin exons, followed by the cDNA sequence for Cre. Cre synthesis is terminated by the fused β-actin untranslated region (Figure 14).

Transgenic mice were generated through microinjection, and Cre expression was verified by crossing to the Z/AP double reporter line. Cells in which Cre is not expressed stain positive for β-Galactosidase, while cells containing Cre expression stain positive for acid phosphatase. Northern blot analysis was also used to verify Cre expression. RNA samples from different

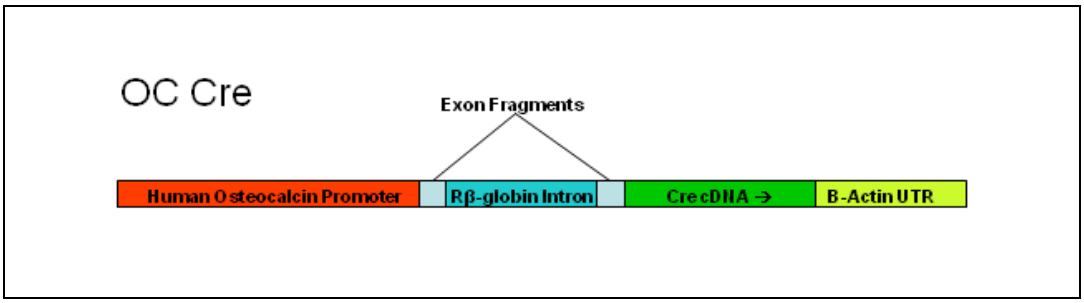

Figure 14. Diagram of the human osteocalcin promoter driving Cre expression. The promoter is separated by the rabbit β-globin intron flanked by fragments of exon. Figure adapted from Zhang *et al.* (62).

tissues indicated that the *OC-Cre* was expressed in calvaria, the femur, and the vertebrae (across the skeleton), while no detection of Cre expression was found in the brain, fat, heart, liver, kidney, skeletal muscle, or stomach. Numerical calculations between the number of β-Galactosidase-positive cells in Cre transgenic mice and control mice that were crossed to the Z/AP reporter indicated an excision index of 88.4% of osteoblasts and osteocytes, noting Cre expression in nearly 90% of the targeted cells, with extremely low β-Galactosidase-negative cells in control mice. Phosphatase staining indicated that Cre-expressing cells were not present in the calvaria on ED 16, but could easily be identified on ED 17 and 18.5 at the ossification centers. Extensive analysis verified that this human *OC-Cre* was expressed in high levels with high specificity in mature osteoblast cells (and later in the derived osteocytes) (62).

This strain has been extensively used to address gene function in differentiated osteoblasts. Floxed allele strains that have been conditionally deleted by mating to *OC-Cre* include the *IGF type 1 receptor* (62), *β-catenin* (63), *Apc* (63), *Pten* (64), *Phex* (65), *calcineurin B1* (66), *Vhl* (67;68), and *Hif1α* (67).

Osteocytes

Once completely embedded in the bone matrix, osteoblast cells change their expression patterns, stop secreting new bone, and play a more active role in regulating bone formation and resorption by other osteoblasts and osteoclasts. These changes in cell behavior are characteristic of the embedded bone cell called the osteocyte (10). Although a major role of osteocyte activity has been identified in the regulation of bone formation, the details of osteocyte function and cellular interactions are not

well-understood. Often associated with osteocyte function is the activity of odontoblasts, which are found in the teeth and exhibit characteristics similar to both osteoblasts and osteocytes (69).

DMP1-Cre

In an effort to be able to better characterize odontoblast and osteocyte activity, Lu *et al.* of the Department of Oral Biology at the University of Missouri-Kansas City School of Dentistry created a Cre line under the transcriptional regulation of the dentin matrix protein 1 (*DMP1*) promoter (69). DMP1 is a matrix protein that is highly expressed in both odontoblasts and osteocytes (70). The *DMP1-Cre* transgene is the first reported Cre gene targeted for osteocyte expression. The gene construct was created by extracting the 14-kb mouse *DMP1* promoter and fusing it to the Cre cDNA sequence. The *DMP1* promoter consisted of the 10-kb promoter region, followed by exon 1 and intron 1 and the first 17-bp initial non-coding region of exon 2 (Figure 15). The 15-kb construct was then microinjected into fertilized mouse eggs to create a transgenic line.

Analysis of Cre expression was done by crossing the *DMP1-Cre* line to the R26 reporter mice. Embryos at ED 18.5, newborns, and the bones and teeth of 1- and 4-month-old mice were taken for analysis. Femoral staining indicated strong Cre expression in the osteocytes of 6-day-old mice. Whole-mount staining indicated LacZ expression in the skull and long bones of mice at 2 weeks of age. Additionally, frozen section assays indicated Cre expression in the calvaria and long bones of newborns and 1-month-old mice in the embedded osteocytes, while very few osteoblasts stained for LacZ expression.

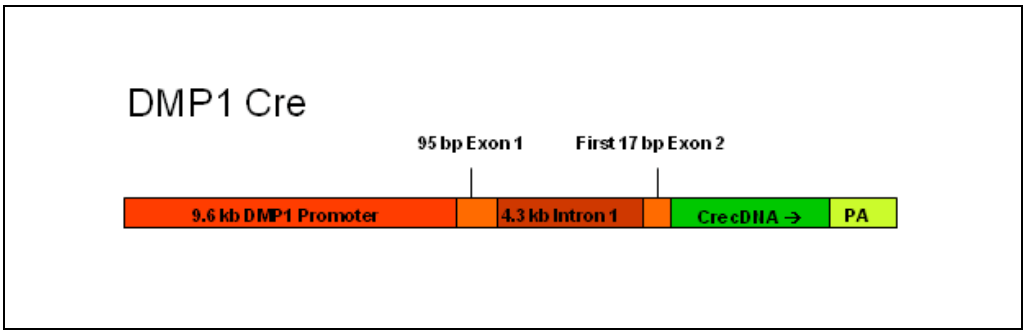

Figure 15. Schematic representation of the *DMP1-Cre* transgene. The promoter region included exon 1 and the first 17 non-coding base pairs of exon 2.

DMP1-Cre was also shown to have high expression in mature odontoblasts of the teeth through similar analysis (69). No additional studies using the *DMP1-Cre* for conditional knockout of floxed alleles have been published at this point.

Osteoclasts

While much about the processes surrounding bone formation and resorption is not well understood, it is speculated that osteoblasts, osteocytes, and osteoclasts work in parallel to maintain a continually changing and dynamic skeletal structure. Osteoclast activity has been associated with tight regulation of blood calcium ion concentrations. It is known that Ca^{2+} ion concentrations play crucial roles in skeletal muscle and cardiac contractions, and the process of bone resorption by the osteoclasts, liberating free Ca^{2+} ions, may be involved in this homeostasis (11). Dampening osteoclast activity has also been one of the major targets of osteoporosis therapies by the class of drugs referred to as bisphosphonates (71).

TRAP-Cre and *CtsK-Cre*

Targeting osteoclasts for Cre expression then proves to have a variety of uses in an

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http://www.bonekey-ibms.org/cgi/content/full/ibmske;5/5/151
doi: 10.1138/20080312

effort to further interrogate bone resorption pathways. Chiu and colleagues of the Department of Medicine at the University of Melbourne used the promoter regions of two individual osteoclast markers, tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CtsK) to generate Cre expression in osteoclasts (72). TRAP is responsible for catalyzing the hydrolysis of a number of esters and anhydrides in the resorption of bone (73), while CtsK is a lysosomal cysteine protease that degrades type I collagen (74).

The *TRAP-Cre* and *CtsK-Cre* lines were both generated through transgenic integration of the designed gene construct. The *TRAP-Cre* construct consisted of a segment of the promoter region of *TRAP* including exons 1B and 1C. Original TRAP and CtsK vectors already existed, so isolation of the promoter regions was relatively easy. *TRAP-Cre* was created from the pTRAP-GFP vector in which the Cre cDNA coding sequence was fused to the promoter region and modified through standard digestion and cloning techniques to generate *TRAP-Cre*. Similarly, *CtsK-Cre* was generated from the pGL3-CK5.0 plasmid, consisting of promoter nucleotides -3359 to +1660 of the *CtsK* gene, which was fused to Cre cDNA and modified as above to generate the *CtsK-Cre* construct (Figure 16) (72).

Verification of the Cre lines was initially performed via mRNA expression studies through RT-PCR of each of the transgenic mice generated. The two mice with the highest Cre expression in bone and lowest expression in non-ossified tissues were chosen for each Cre line for further studies. Further validation was conducted by crossing the chosen *TRAP-Cre* and *CtsK-Cre* mice to the R26 reporter line. In short, the *CtsK-Cre* lines showed moderate LacZ staining in the long bones, calvaria, and ribs,

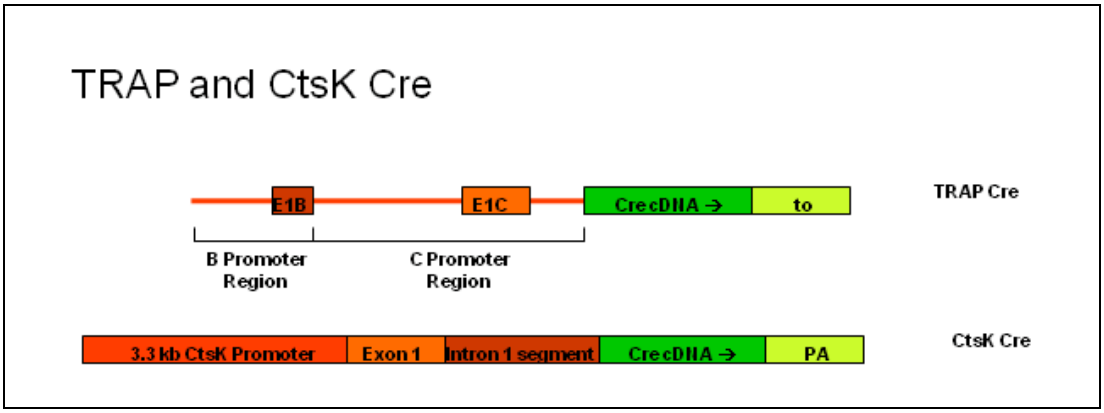

Figure 16. Diagram of *TRAP-Cre* (top) and *CtsK-Cre* (bottom). *TRAP-Cre* was designed from the pTRAP vector that contains two of the 3 exon promoters: 1B and 1C (75). *CtsK-Cre* was designed including the *CtsK* promoter and exon1, as well as a fragment of intron 1.

with low levels observed in a small number of non-mineralized tissues such as the liver. Furthermore, histological analysis of *CtsK-Cre* in the long bones showed osteoclast staining for LacZ. Very few bone marrow cells stained positive, indicating that *CtsK-Cre* is expressed at a later stage in osteoclast development.

TRAP-Cre analysis showed intense staining for β-Galactosidase in the long bones, vertebrae, ribs, and calvaria. Again, nonspecific staining was observed in a few soft tissues such as the liver and heart. Furthermore, the histological data for *TRAP-Cre* showed β-Galactosidase activity in the osteoclasts of the long bones, as well as in proliferating and hypertrophic chondrocytes. In short, expression of Cre was highly contingent upon transgenic integration site, as different donors displayed very different Cre expression. However, both *TRAP-Cre* and *CtsK-Cre* generated Cre activity in osteoclasts, though it seemed *CtsK-Cre* had

the highest specificity, while *TRAP-Cre* demonstrated the highest expression levels (72). In addition to the *TRAP-Cre* and *CtsK-Cre* lines, Ferron and Vacher have also created a transgenic strain that directs expression of Cre under the *CD11b* promoter. These mice express Cre during the differentiation of both the myeloid and osteoclast lineages (76).

More recently, Nakamura and colleagues independently made a *CtsK-Cre* line in which the coding sequence of Cre was knocked into the endogenous *CtsK* locus behind the endogenous ATG site using recombineering (77). This strain was used for studies in which *estrogen receptor α* was specifically deleted in osteoclasts (78).

Summary

Cre-mediated DNA recombination has become an extremely valuable tool throughout scientific research. The ability to modify essential genes specifically in the skeleton and at different stages in cellular differentiation has already provided an important understanding of the processes of bone development and osteoporosis. The wide range of skeletal-specific Cre genes will, no doubt, continue to expand, while studies with these important strains will further contribute to our knowledge of skeletal development, as well as uncover novel therapeutic techniques targeting osteoporosis, skeletal metastasis, and other skeletal diseases.

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