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Genetic mouse models for bone studies—Strengths and limitations

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Abstract

Mice have become a preferred model system for bone research because of their genetic and pathophysiological similarities to humans: a relatively short reproductive period, leading to relatively low cost of maintenance and the availability of the entire mouse genome sequence information. The success in producing the first transgenic mouse line that expressed rabbit β-globin protein in mouse erythrocytes three decades ago marked the beginning of the use of genetically engineered mice as model system to study human diseases. Soon afterward the development of cultured pluripotent embryonic stem cells provided the possibility of gene replacement or gene deletion in mice. These technologies have been critical to identify new genes involved in bone development, growth, remodeling, repair, and diseases, but like many other approaches, they have limitations. This review will introduce the approaches that allow the generation of transgenic mice and global or conditional (tissue-specific and inducible) mutant mice. A list of the various promoters used to achieve bone-specific gene deletion or overexpression is included. The limitations of these approaches are discussed, and general guidelines related to the analysis of genetic mouse models are provided.

Keywords

Transgenic; Knock-out; Knock-in; Conditional knock-out; Gene deletion; Mouse model; Cre-lox system; Cre recombinase; Bone promoters

Transgenic approaches

Transgenic construct

Transgenesis consists in the introduction in the genome of a transgene to study the biological function of a gene of interest by overexpressing it, globally or in a tissue/cell-restricted manner. Since the insertion site of the transgene is random, two additional components, a promoter that drives transgene expression and a poly A tail that allows protein translation, must be included to ensure efficient expression of the transgene. The transgene is usually a wildtype (WT) coding DNA sequence (cDNA) or genomic DNA sequence containing exons and introns. It can also be a mutated coding DNA sequence (cDNA) that will act as a dominant negative product or that will mimic genetic mutations observed in human diseases, or an anti-sense RNA. Suicide genes, such as the diphtheria receptor (DTR), can also be expressed to eliminate the cells expressing it [1,2]. Over-expression can be targeted to specific cell type, for instance, osteoblasts, osteoclasts, or osteocytes and is achieved by placing a transgene downstream of a cell-specific promoter.

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Alternatively, global transgene overexpression can be achieved by using a house keeping gene promoter or a viral promoter. If desired, the minimal transgenic construct can be modified 1) by using inducible promoters instead of constitutively active ones to provide temporal regulation of gene expression, 2) by adding enhancers and introns to increase the level of transgene expression, or 3) by linking an epitope tag sequence to the transgene to distinguish the transgene from endogenous expression.

Production of transgenic mice—The final transgenic construct is usually microinjected into the pronuclei of fertilized mouse eggs, which are then implanted in the uterus of a surrogate mother. The transgene will be expressed by some of the offspring, named transgenic founders, which are bred to WT mice to select transgenic founders in which the transgene is transmitted through the germ line. The transgenic founders are then mated with WT mice to establish mouse lines with stable DNA integration and expression of the transgene.

Advantages and limitations—The main advantage of this technique lies in its short development time. Its main drawbacks are random integration sites and variable copy number of the transgene that lead to variable levels of expression, depending on the overall transcriptional activity of the genomic region where the transgene is integrated. The analysis of two or more independent transgenic lines, each deriving from a different founder, allows one to select the desired level of expression and to verify that the observed phenotypes are caused by the effect of the transgene rather than disruption of another gene due to random integration of the transgene. Caution must indeed be taken when breeding transgenic lines to a homozygous state since the random site of integration of the transgene may alter the gene it falls into. Homozygous transgenic lines may cause a phenotype independent of the targeted gene or lethality if the site of insertion is in a gene critical for survival. In addition, the sometime high copy number of the transgene, when brought to homozygosity, increases the chances of DNA recombination and chromosomal rearrangements, which can also generate a phenotype independent of the targeted or overexpressed gene. Importantly, promoters used to direct expression of a transgene are rarely strictly specific to any cell type, and thus careful analysis of transgene expression in multiple tissues, and at the cellular level, is essential for the validation of a founder line. Lastly, the supraphysiological levels of gene expression achievable in this approach, as well as its non-regulated nature, may not be biologically relevant, and thus interpretation of results must be done carefully.

“Targeted” transgenic mice—To overcome such drawbacks, a “knock-in” strategy based on targeted insertion of a transgene to an exact genomic locus using homologous recombination in embryonic stem (ES) cells can be used. This approach allows one to target a transgene to a transcriptionally active DNA region and to control copy number to obtain stable expression with no disruption of unknown genes whose alteration may contribute to the phenotype. The experimental design of a gene knock-in project uses the same basic principles as for a knock-out (see below), and is thus more tedious and time consuming compared to the generation of a “basic” transgenic line [3]. A new approach based on the use of engineered zinc finger nucleases, commercialized by Sigma, facilitates this approach. This technology uses an artificial zinc finger nuclease (ZFN) that consists of a designed zinc finger protein (ZFP) fused to the cleavage domain of the *Fok* I restriction enzyme. This ZFN can be redesigned to cleave new targets by developing ZFPs with new sequence specificities, thus allowing genome editing [4].

Global gene deletion

Global gene targeting consists of the introduction of specific mutations into a gene of interest by homologous recombination using ES cell technology. This approach is feasible

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thanks to the availability of the entire mouse genome sequence and to the ability of ES cells to be cultured and manipulated *in vitro* without losing their totipotency. It results in the generation of a null allele i.e., knockout (KO), allowing gene inactivation after breeding of the animals as homozygotes. The gene modification generated is constitutive and present as soon as the endogenous promoter regulating the targeted gene is turned on.

Target DNA construct

Targeting by homologous recombination requires the generation of a targeting DNA construct that requires a more complicated and time-consuming multistep strategy than generating a transgenic construct. Commercially available Bacterial Artificial Chromosome (BAC) clones are usually used to subclone the flanking upstream and downstream genomic DNA sequences (arms) of the gene of interest, which will be used to induce homologous recombination. Identical mouse backgrounds, such as 129/SV, are usually used between the BAC clone utilized for generating the construct and ES cells to avoid a decrease in recombination efficiency caused by DNA polymorphisms. Successive cloning steps are used to progressively clone the different functional entities of the construct into a single construct. The *neo* gene, which confers resistance to neomycin (G-418), is commonly used to disrupt the endogenous targeted gene and to select for ES cell clones that have integrated the targeting construct (Fig. 1). In addition, because random integration via nonhomologous recombination occurs more frequently than targeted integration via homologous recombination, the Tk^{HSV} gene is also usually placed outside of the homology region of the targeting construct. This gene, if inserted via nonhomologous recombination into the genome of ES cells, encodes a viral thymidine kinase that converts the nucleotide analogue ganciclovir into a cytotoxic product, thus allowing for negative selection of clones characterized by random insertions (cells with random insertion only are sensitive to ganciclovir).

Restriction digestions and ligations can be used for subcloning of the targeting vector, but this strategy is complicated by the length of the genomic DNA to be manipulated, and is limited by the presence and position of adapted restriction sites within the sequence to be handled. An alternative to this strategy is the recombineering technique, which exploits homologous recombination in *Escherichia coli*. In this technique, DNA is electroporated in a strain of bacteria containing an integrated defective and temperature inducible prophage carrying recombination genes that will integrate this exogenous DNA. Regardless of the cloning strategy used, the selection of a DNA probe that allows for the targeted alleles to be distinguished from the WT allele is of critical importance and should be considered very early during the cloning strategy.

Production of knockout mice

Subsequently, the engineered targeting construct is introduced into ES cells by electroporation and the normal gene on the chromosome is replaced with the targeting construct by homologous recombination. The ES cells that have incorporated the construct into their DNA can be selected *in vitro* by their resistance to antibiotics (G-418 for instance). ES cells containing nonhomologous recombined DNA (and thus the TK cassette) can be selected by ganciclovir. Selected totipotent selected ES cells are then injected in the blastocysts of foster mothers from a different mouse strain, such as C57BL6. The pups born from these mothers are referred to as chimeras as they are composed of cells derived from two different strains. Chimerism is visible by the presence of coat patches of different colors since cells derived from 129/SV and C57BL/6 mice give rise to an agouti and black coat colors, respectively. If a chimeric embryo possesses mutant ES-derived germ cells, the genetic alteration can be propagated to its offspring to generate heterozygote and eventually homozygote mutant mice.

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Advantages and drawbacks

Global gene targeting in the bone field greatly extended the knowledge derived from the analysis of naturally occurring mutations and has allowed for the reproduction of several human bone diseases such as osteogenesis imperfecta, various types of achondroplasiae, and others. However, this strategy also has limitations, the most important being the possible lethality of KO mice that reflects the non-redundant role of the targeted gene but precludes further analyses of its function in later stages of development. Global gene targeting also does not allow one to track gene function at specific developmental stages or cell differentiation stages. Lastly, some phenotypes caused by such genetic alterations may be difficult to interpret because they may be due to cell-autonomous, cell non-autonomous, or systemic effects.

Conditional gene targeting

The molecular "switch" enabled by conditional gene targeting allows the creation of mutations in a tissue-specific and time-specific manner and thus addresses some of the limitations of the global gene deletion strategy.

DNA construct

This strategy consists of introducing in the gene to be deleted two or more short DNA tag sequences that can be recognized by recombinases. The Cre/Lox recombinase system derived from bacteriophage P1 is the most widely used and will be discussed in detail below, but the Flp/FRT system derived from *Saccharomyces cerevisiae* can also be used. Both recombinases recognize a distinct 34-bp consensus sequence (Lox and FRT sites, respectively) and can excise the sequence between two of these sites and ligate the two generated extremities, thus "popping-out" the intervening sequence (Fig. 1) [5]. The orientation of Lox sites in a single gene and their respective positions on different chromosomes also allows one to invert sequences of DNA and to generate chromosomal rearrangements that can be useful to recapitulate, in mice, human genetic diseases. In a targeting construct, the two Lox sites are usually inserted in introns flanking exonic DNA sequence(s) crucial for gene function. The structure of this construct allows for the "floxed" gene's WT activity to be retained, whereas its cre-recombination leads to gene inactivation. It is particularly important not to interrupt endogenous gene function by the insertion of the Lox sites, and splice sites within introns as well should be avoided if possible when cloning the Lox sequences.

Production of the conditional knockout mice

This approach requires two mouse lines to be crossed, a transgenic mouse line that overexpresses the Cre recombinase in a specific tissue or cell type, and a mouse strain that contains the target gene of interest flanked by two Lox sites. Recombination and consequently inactivation of the target gene occurs only in those cells expressing the Cre recombinase. Hence, the target gene remains active in all cells and tissues that do not express the Cre recombinase. Alternatively to the use of a Cre transgenic mouse line, the Cre recombinase can be delivered to the mouse strain containing the floxed gene by viral strategies *in vitro* or *in vivo* to transmit the recombinase to "floxed" cells.

Alternative uses of conditional knockout mice

In addition to conditional gene inactivation, this approach is commonly used to determine the site of Cre recombinase activity *in vivo* [6]. This is achieved by crossing a Cre transgenic mouse line with the Cre recombinase targeted to a specific tissue or cell type, and a second mouse strain that has a reporter gene such as *LacZ*, placental alkaline phosphatase (*pALP*) or a variety of fluorescent proteins (*GFP, YFP…*), whose expression is blocked by a floxed

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stop cassette sequence (which can be polyadenylation sequences, false translation initiation codons and/or stop codons) incorporated between the 5′ regulatory elements and reporter gene. In this setting the reporter transgene is not expressed until the Cre recombinase is expressed, since the excision of the stop signal occurs only in those cells expressing the Cre recombinase, depending on the tissue specificity of the promoter used (Fig. 2). These "reporter" mouse lines are also very useful for lineage tracing studies because activation of such reporter indicates that Cre recombinase activity is present in that particular cell or was present in its progenitor [7].

Limitations and precautions

Although chromogenic or fluorescent "reporter" mice are widely used and convenient (see below), one must keep in mind that this method has limitations. Endogenous LacZ or AP activity along with non-specific background may prevent the detection of weak Cre activity that may still have significant consequences in the progeny of these Cre-positive cells if they divide, since recombination of the target floxed gene is non-reversible and permanent. Transient Cre-recombinase activity is also likely to be missed in studies focusing on a limited number of developmental stages. Therefore, complementary approaches must be utilized. These include genomic PCR from tissues or extracted cells to assess with more sensitivity recombination of the floxed alleles *per se*, or *ex vivo* measurement of the proliferation, differentiation, function or gene expression pattern of targeted cells versus non-targeted cells.

Since conditional gene deletion relies on transgenic expression of the Cre recombinase, the potency of each transgenic promoter to drive expression of the Cre recombinase is critical to achieve efficient gene recombination, and depends on several parameters mentioned in section 1 above. Transgene copy number is usually, but not always, proportional to the strength of expression. This variability can be caused by a chromosomal position effect and can relate to the site of insertion of the transgene(s), which can be in a transcriptionally more or less active chromosomal region. Comparing several founders is always useful to select for the most appropriate line with optimal level of expression.

Another important parameter to measure in conditional knockout animals is the efficiency of recombination. As mentioned above, Cre transgenic lines have differing levels of expression of the Cre recombinase, and each floxed gene has differing chromosomal accessibility to be clipped by the Cre recombinase, leading to variable recombination efficiency. Recombination efficiency can be assessed and semi-quantified by genomic PCR or Southern blot using tissues or cells extracted, and possibly purified, from the mutant mice. Enrichment of the Cre-positive cells to quantify recombination efficiency limits contamination by cre-negative cells and the artefactual decrease in recombination efficiency it generates.

Inducible gene targeting

An inducible system allows one to turn gene expression off or on by using an exogenous inducer administered to the mice. The Cre-ERT system [8,9] and the tet-ON/tet-OFF systems [10,11] have been most widely used to provide a temporal control of gene recombination.

The Cre-ERT system

In this system the Cre recombinase sequence is fused with a mutated ligand-binding domain from either the progesterone or the estrogen receptor (ER). These modified ligand-binding domains become localized to the nucleus only in the presence of synthetic steroids (RU486 and tamoxifen or 4-hydroxytamoxifen) but not in the presence of endogenous steroids.

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The CreERT recombinase system has been demonstrated to be functional *in vivo*, allowing widespread time-dependent recombination when CreERT expression is placed under the control of a strong promoter. In this system, tamoxifen can be administered systemically but also topically in studies involving skin or fracture for instance, and can be given to pregnant mothers as well to study gene inactivation in developing mouse embryos. Toxic effects of tamoxifen may be a limitation for some studies, especially studies in embryos. To alleviate this problem, a second generation of CreERT mice (Cre-ER^{T2}) that is about 10 times more sensitive to 4OH-Tamoxifen *in vivo* than the original CreERT [12] has been generated and used in several fields, including the bone field [13,14]. A drawback of this system is that it may be somewhat leaky, resulting in weak constitutive activation. In addition, recombination mediated by inducible promoters is generally mosaic and its efficiency has been dependent on tissue types, despite nearly toxic doses of inducer.

The progesterone-binding domain of the human progesterone receptor has been mutated to one that binds the synthetic steroid RU486 (a glucocorticoid receptor antagonist) but not endogenous progesterone or glucocorticoids. This inducible Cre system is very similar to the CreERT system but has not, to our knowledge, been very much used in the bone field.

tet-ON/tet-OFF systems

The *tet* systems rely on two components, i.e., a tetracycline-controlled transactivator (tTA or rtTA) and a tTA/rtTA-dependent promoter that controls expression of a downstream cDNA in a tetracycline-dependent manner. In the *tet*-OFF system, the presence of tetracycline or its derivatives, such as doxycycline (Dox), prevents tTA to bind its target and the system is inactive. The *tet*-ON system utilizes a mutated tTA, i.e., reverse tTA (rtTA). In this case the presence of doxycycline allows the system to be turned on. *In vivo*, the system requires the breeding of two independent types of transgenic mice, i.e., transactivator mice, in which tTA or rtTA is expressed under the control of a tissue-specific promoter, and responder mice, in which expression of the gene of interest is under the control of the *tetO* minimal promoter. Addition or no addition of doxycycline to the food or drinking water of the double-transgenic mice allows *in vivo* spatiotemporal control of cDNA expression.

The *tet*-ON system requires higher doxycycline concentrations to be active compared with the concentration *tet*-OFF requires to be inactive [15]. The advantages of *tet*-ON compared with *tet*-OFF are that the transgene is not expressed until doxycycline is given to the animals and that upregulation *in vivo* is faster than downregulation. Using the *tet* systems, one can achieve a tight control of gene expression in a tissue-specific manner. It is also possible to return to a control situation by simply discontinuing doxycycline administration. The major disadvantage of the *tet* systems is that control of the expression of the acceptor construct is often leaky because of strong positional effects on the *tetO* minimal promoter.

In all of these systems, the inducible transgene can be a WT protein to be overexpressed (including the Cre-recombinase), a mutated, truncated or reporter protein, a suicide protein, or silencing RNAs.

Tissue-selective promoters used in bone cells for conditional recombination/overexpression

Skeletal morphogenesis involves sequential steps that include patterning, condensation, and organogenesis of the membranous and endochondral embryonic skeletons [16]. Three lineages give rise to the skeleton structures during early development. Neural crest cells will form the branchial arch derivatives of the craniofacial skeleton; the paraxial mesoderm will contribute to the craniofacial skeleton and form most of the axial skeleton following division of somites, and cells of the lateral plate mesoderm will give rise to limb skeleton. During

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mouse embryogenesis, between E10 and E12 days post-coitum (dpc), a group of homogenous cells condenses, acquires positional properties, and starts differentiate to form the mold of the future skeleton. Condensed mesenchymal cells can differentiate directly into osteoblasts and form flat bones such as skull bones as well as the subperiosteal region of long bones (intramembranous ossification). Condensed mesenchymal cells can also differentiate into chondrocytes to form a cartilage anlagen that is eventually replaced by bone during the process of endochondral ossification [17]. The characterization of the molecular machinery controlling these intricate processes relied heavily on the use of mutant mice, and its future refinement will depend on the optimal use of new genetic tools to target genes in specific cells and developmental time points.

Promoters driving Cre expression in specific skeletal cell types used in the bone field are described below. They are classified by the targeted cell lineage, including mesenchymal stem cell/osteochondroprogenitors, chondrocytes, osteoblasts, osteocytes and osteoclasts. Table 1 summarizes the promoter-cre lines, the reported site(s) of leakage of each promoter (if addressed), the original study describing each transgenic line, and some references describing the use of each line, when available. The Mouse Genome Informatics website (http://www.informatics.jax.org/) is a valuable resource to obtain an updated list of cre-lines for each tissues, including the skeletal system, and references of studies that have used inventoried cre lines.

Mesenchymal stem cell promoters

Floxed genes in mesenchymal or osteochondroprogenitor cells can be targeted by using the *Prx1, Dermo1* or *Sox-9 cre* transgenic mice.

Prx1 promoter

Prx1 is a paired-related homeobox gene that plays an essential role in regulating skeletal development in the limb [74]. Using transgenic mice in which a 2.4 kb *Prx1* promoter directs *Cre* expression (*Prx1-Cre* transgenic mice), Cre recombinase activity was observed in mesenchymal cells during limb bud development, in the lateral plate mesoderm, and the cranial mesenchyme, but not in the axial skeleton [18,75]. Cre recombinase activity in this line was first observed in limb buds at 9.5 dpc. By E16.5, Cre recombinase activity was detected in the entire limb bud and interlimb flank, in the craniofacial mesenchyme, and around the eyes.

As a note of caution, partially penetrant germline recombination was evident in the offspring of female (but not male) *Prx1-Cre* heterozygous mice, however, the penetrance of this apparent germline recombination event level varied depending on the particular gene flanked by *loxP* sites [18]. Therefore, males from this transgenic line should be used for targeting mesenchymal cells.

It is possible to use this promoter in an inducible fashion *in vivo* and also to purify and track cells where this promoter is active by using the *Prx1-Cre-ER^T-GFP* transgenic mice [76]. While the expression of CreER^T allows timed recombination of the floxed allele, the expression of GFP allows isolation of transgene-expressing cells from the periosteum by cell sorting as well as cell tracking. In this line, Tamoxifen injection into pregnant dams at E9 induced Cre recombinase activity and recombination at E17 in chondrocytes, osteoblasts, periosteal, and perichondrial cells, indicating that the transgene was efficiently expressed in limb bud osteochondroprogenitors. When tamoxifen was injected at E15.5 and E16.5, Cre recombinase activity persisted in perichondrial and periosteal cells, but was not detected in chondrocytes anymore. This strategy thus allows one to achieve a different level of specificity. In adult mice, daily tamoxifen injection between P19 and P23 induced Cre

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recombinase activity in the periosteum and perichondrium, but not in epiphyseal chondrocytes at P26 and up to P35. Recombined cells were predominantly found in the inner cambium layer of the periosteum in regions where the periosteum showed morphologically distinct inner and outer layers. Some of the articular chondrocytes and tendon cells also showed recombination.

Dermo1 promoter

Dermo1 (also named Twist2) is a transcription factor highly expressed in condensed mesenchyme during skeletal development and later in perichondrial cells. Because the regulatory elements of the *Dermo1* promoter were not initially characterized, *Dermo1-cre* mice were generated by inserting the *Cre-recombinase* gene within the first exon of *Dermo1*[20]. Cre recombinase activity in this line was detected as early as E9.5 in mesodermal tissues such as branchial arches and somites, but also in the skin. During embryonic endochondral ossification, Cre recombinase activity was first detected at E11.5 in the condensed mesenchyme from which both chondrocytes and osteoblasts are derived. Later in development (E16.5), Cre recombinase activity was detected in chondrocytes in growth plate cartilage and in osteoblasts and osteocytes in the perichondrium, periosteum, and endosteum;, but not bone marrow cells and osteoclasts. During intramembranous ossification in developing sutures, Cre recombinase activity was present at the osteogenic fronts and in surrounding mesenchymal tissues. Growth plate chondrocytes along with bone trabecular and cortical osteoblasts were recombined in adult mice.

Sox9 promoter

Sox9 is a transcription factor with a high-mobility group DNA-binding domain, expressed in all chondroprogenitors and having an essential role in chondrogenesis. The mouse *Sox9-Cre* transgenic mouse line was initially generated via a knock-in strategy to follow the fate of *Sox9* expressing cells, [7] and has not been used yet as a tool to target genes in osteochondroprogenitors [24]. Sox9-cre recombinase activity was detected in limb bud mesenchyme in E10.5 embryos and in all cells in cartilage primordia and perichondrium at E13.5. In E17.0 limb buds all chondrocytes as well as perichondrial, periosteal, and osteoblast cells, displayed Cre recombinase activity, indicating that *Sox9*-expressing limb bud mesenchymal cells give rise to both chondrogenic and osteogenic cell lineages, like Prx1-expressing cells. Comparison with the *Prx1-cre* line indicated that *Prx1*-expressing limb bud mesenchymal cells give rise to *Sox9*-expressing osteochondroprogenitors and that the *Prx-cre* line can recombine floxed genes in a broader limb bud-derived mesenchymal cell population than the *Sox9-cre* line. Cre recombinase activity was also detected in notochord, node, and endodermal linings at E8 and skin at E10.5. In E17 embryos, gonads (Sertoli cells and Leydig cells but not germ-cells), intestinal epithelial cells, neurons and glia in the spinal cord, endocrine and exocrine pancreatic cells, tendons, and synoviums also showed Cre recombinase activity. Similarly to the *Prx-1* and *Dermo1* lines, the broad spectrum of Cre recombinase expression in this non-inducible transgenic line might limit the use of this model for developmental studies.

Precautions—In all three lines, the multipotent nature of the cells where these promoters are active makes the pattern of Cre recombinase activity broad and therefore the interpretation of results relatively difficult. More specifically, because chondrocytes, osteoblasts, and osteocytes derive from common mesenchymal osteochondroprogenitor cells, Cre recombinase expression, even transient, under the control of these three promoters in osteochondroprogenitor cells will be followed by gene recombination in osteoblasts, osteocytes and chondrocytes, and also possibly in adipocytes, muscles or endothelial cells. Gene recombination in any of these descendants might indirectly and distinctly contribute to the observed bone phenotypes.

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Chondrocyte promoters

Gene targeting in chondrocytes has mostly been done by using the *Col2a1* promoter, which encodes Type II collagen, a major constituent of the growth plate made by chondrocytes. Mouse, rat, and human *Col2a1* promoter Cre lines have been generated and inducible lines are also available.

Col2α1 promoter—The mouse *Col2α1* promoter contains a small enhancer region located within the first intron of the *Col2α1* gene that in conjunction with 1 kb of the *Col2α1* promoter region efficiently drives transgenic reporter expression and recapitulates the tissue-specific patterns of *Col2α1* gene expression *in vivo* [77]. Based on this feature, the mouse *Col2α1–Cre* transgenic line has been generated and exhibited gene excision activity starting at E9 in the notochord, cranial mesenchyme, and in all cartilage primordia [26,27,30]. It is less well known in the bone field, however, that the Cre recombinase activity in the *Col2α1–Cre* line is also observed in osteoblastic cells within the primary spongiosa, as well as in trabecular osteoblasts and osteocytes in adult mice [27,29,33].

The human *Col2α1* promoter/enhancer Cre transgenic line has a very similar pattern of Cre recombinase activity and recombines floxed genes in chondrocytes but also in the developing eye, epidermis, heart myocardium and atria, cranial mesenchymal cells, the endoderm of the developing yolk sac and the spinal cord [24].

Two mouse Tamoxifen (TM)-inducible *Col2α1*–Cre lines have been created, and show similar properties to a rat TM-inducible *Col2α1*–Cre line [31,32]. In the mouse *Col2α1*–CreER^{T} line [31], weak activity could be detected in facial condensations, rostral sclerotomes, limb buds, and base of the skull as early as E10, 1 day after TM injection. Recombination time course experiments indicated that Cre recombinase activity could be first detected 8 h post-TM injection; mosaic recombination was evident 12 h post-injection and extensive recombination was detected 24 h post-injection. In this line, Cre recombinase activity was found in the perichondrium (E13.5), a major source of periosteum and osteoblast progenitors giving rise to the bone collar including both cortical and trabecular bone in the spongiosa [78]. TM injection after E13.5 avoided recombination in periosteum and bone. Cre recombinase activity could be induced post-natally until day 21, but declined thereafter to become essentially undetectable in 12-week-old mice.

In an attempt to increase sensitivity to TAM, another CreERT recombinase (CreER^{T2}) was used in association with the 1 kb mouse *Col2α1* promoter to generate the mouse *Col2α1–C*reER^{T2} transgenic line [32]. The CreER^{T2} recombinase contains a G400V/M543A/L544A triple mutation in the ER ligand binding domain (LDB) and is more sensitive to 4-OHT than is the mutant ER LBD with a single G521R substitution. In this line, upon TM injection at E15.5, Cre recombinase activity was detected 3 days later in cartilaginous areas, but not in bones formed by intramembranous formation. Upon TM injection at post-natal stages (2 and 8 week-old mice), recombination was demonstrated in cells from the resting, proliferating, and hypertrophic zones of the growth plate cartilage and in articular cartilage (1 and 6 months post-TM injection, [14]).

Cre recombinase activity in the inducible rat *Col2α1*–CreER^{T} line is localized in columnar and articular chondrocytes, as well as perichondrial cells. The inducible nature of this CreERT here again enables one to control Cre recombinase activity in specific growth plate compartments by choosing a proper developmental stage at which TM switches on Cre recombinase activity. For instance, this strategy was used to induce Cre recombinase activity in chondrocytes, but not perichondrial cells by starting TM injections at or after E12.5 [33]. TM injections in this model can also be used post-natally to recombine genes efficiently in columnar and articular chondrocytes. The tetON system is also available to induce Cre

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recombinase activity in a temporal fashion selectively in chondrocytes via the mouse *Col2α1* promoter [35]. In these *Col2α1*–DOX–Cre transgenic mice, Cre recombinase activity was observed in the cartilaginous elements of E14.5 embryos whose mother was given doxycycline since conception. P7 pups also displayed Cre recombinase activity within the entire femoral growth plate (from articular surfaces to proliferating chondrocytes) when fed milk from mothers given doxycycline. One-month-old mice also showed Cre recombinase activity throughout the growth plate when given doxycycline, however, 3 month-old mice showed Cre recombinase activity solely in articular chondrocytes upon doxycycline treatment.

Col10α1 promoter—Although not widely used yet, the mouse *Col10α1*-Cre transgenic line is another option to manipulate genes in the growth plate, based on the selective expression of Type X collagen in growth plate hypertrophic chondrocytes. Based on the observation that a 4.6 kb promoter sequence of the mouse *Col10α1* gene including a 500 bp enhancer element was sufficient to drive specific expression of a *LacZ* reporter gene in all hypertrophic cartilage zones of transgenic mice [79], a transgenic line was created by inserting the Cre recombinase into the *Col10α1* gene, using a BAC recombineering technique [36]. In this line, Cre recombinase activity was detected as early as E13.5 in long bones where hypertrophic cartilage develop and in long bone and vertebra growth plate hypertrophic chondrocytes of E16.5 and P1 mice. Cre recombinase activity was also detected in the subchondral bone marrow zone adjacent to the cartilage–bone border and in endochondral bone trabeculae. Whether this activity comes from residual LacZ activity, chondrocytes surviving in the cartilaginous core of endochondral bone trabeculae, or from leakage in osteoblasts is unclear.

Another less characterized *Col10α1*-Cre transgenic line was generated using the 1 kb proximal promoter of the mouse *Col10α1* gene. This line displayed Cre recombinase activity in E14.5 ribs cartilage primordiae and in some, but not all, hypertrohic chondrocytes [37].

Aggrecan promoter—Aggrecan is a major extracellular matrix (ECM) protein of both growth plate and articular cartilage. Inducible Acg-CreER^{T2} transgenic mice were generated by introducing a tamoxifen-inducible CreER^{T2} cassette in the 3′-untranslated region of the endogenous *aggrecan* gene [38]. The use of an IRES sequence and insertion downstream of the *aggrecan* gene stop codon left the endogenous gene intact in this line. The offspring of crosses between Acg-CreER^{T2} and Rosa26 reporter mice injected with tamoxifen displayed efficient Cre reporter expression in the hyaline cartilage of the growth plate and articular cartilage as well as the fibrocartilage of the meniscus, trachea, and intervertebral disks of growing and adult mice. Positive X-gal cells were first detected in the forelimb of E13.0 Acg-CreER^{T2}; R26R embryos harvested from pregnant mothers injected with 4OH-tamoxifen at E12.0. Positive X-gal staining was observed in the proliferating and hypertrophic chondrocytes. An important feature of this line is the prolonged expression of Cre recombinase in adult mice, compared to *Col2α1*–Cre based mice.

Precautions—It is important to emphasize that in most of these “chondrocyte-specific” transgenic lines, Cre recombination is evident in a significant proportion of osteoblasts, especially in adults [29]. This irreversible gene alteration is likely caused by a transient Cre activity in a subset of perichondrial osteochondroprogenitor cells that give rise to osteoblasts. Regardless of the site and time of Cre expression, this observation indicates that any osteoblastic-like or bone-like phenotype observed in mutants using these Cre lines may be mediated by a direct effect of gene deletion in the osteoblast lineage. The induction of Cre recombinase activity after E12.5 in inducible *Col2α1*–Cre lines can limit this “leakage” to osteoblasts.

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Osteoblast promoters

There are a number of Cre lines to manipulate gene expression in either immature or mature osteoblasts that are quite widely used in the bone field.

Runx2 promoter—Runx2 is a critical transcription factor of the Runx family that is required for determination of the osteoblast lineage. Runx2-Cre transgenic mice were created by inserting the Cre recombinase in the *Runx2* locus at the translational start site of the bone-specific distal promoter (P1) [40]. A cross with ROSA26 reporter mice confirmed efficient recombination at all sites of endochondral and intramembranous bone formation in neonatal mice, particularly in periosteal cells, osteoblasts, and osteocytes, but not in osteoclasts or any other mesenchymal tissues such as fat or muscles. Although this model has not been fully characterized or used extensively yet compared to other osteoblast "specific" Cre mice such as the mouse 2.3 kb *Col1α1*-Cre mice (see below), its specificity and early pattern of expression during differentiation of the osteoblast lineage may prove very useful to study osteoprogenitor fate. Expression of *Cre* in the hypertrophic chondroctyes in cartilage of this model needs, however, to be taken into account.

Osterix promoter—Immature osteoblasts can be targeted by using the *Osx–Cre* transgenic mice as well. Osterix (Osx) is a zinc finger-containing transcription factor expressed in osteoblasts of all endochondral and membranous bones. The mouse *Osx–Cre* transgenic line expresses the Cre recombinase in committed osteoblast progenitors in both endochondral and membranous-derived bones [44] in a manner that follows that of endogenous *Osx* [80]. Cre activity was observed during embryonic development from E14.5 and post-natally (P10) in the inner bone-forming perichondrium adjacent to hypertrophic chondrocytes, sporadically in hypertrophic chondrocytes, in the periosteum, and primary spongiosa. This *Osx–Cre* line is inducible. Doxycycline administration prevents Cre recombination and stopping the treatment relieves the brake on Cre recombinase expression, leading to Cre-mediated recombination. Another characteristic of this model is that Cre is fused to eGFP, which allows direct visualization of the site of expression of the transgene *in vivo* as well as lineage tracing. *Osx–Cre* mice display a slight growth delay [41] and a high prevalence of teeth malocclusion in adults of the C57BL6 strain irrespective of the targeted floxed gene (Dr. Louise Purton, personal communication).

The *Osx–CreER*^{T2} line, generated by inserting the Cre-ER^{T2} fusion cDNA and downstream poly-A element (pA) into a BAC containing the *Osterix* (*Sp7*) (*Osx*) gene promoter, was recently reported [45]. 4OH-tamoxifen treatment in pregnant mothers at E12.5–13.5 triggers recombination in embryos' mandible, the lateral calvarial bones, the proximal part of the ribs and the proximal limb bones one day later, as assessed following a cross with ROSA26 reporter mice. Cells in the perichondrium were also recombined, and tracing experiments indicated that these *Osx*–LacZ+ perichondrial cells predominantly moved inside the bone to the trabecular regions to become trabecular and endosteal osteoblasts as well as osteocytes, whereas *Col*-LacZ+ cells predominantly remain on the cortex (see below). Mosaic labeling in the pre- and early hypertrophic chondrocyte zones was also observed in *Osx–CreE*^{T2} mice, in line with the endogenous expression of *Osx* in prehypertrophic chondrocytes.

Col1α1 promoter—Mature osteoblasts on the other hand can be targeted based on their rich expression of type I collagen, the main constituent of bones. Type I collagen is also expressed in fibroblasts and other mesenchymal cells, but an osteoblast-specific enhancer has been identified in the mouse and rat 2.3 kb proximal *Col1a1* promoters which allows efficient transgene expression in osteoblasts and odontoblasts specifically [81,82].

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The mouse 2.3 kb *Col1α1*-Cre transgenic line is quite widely used in the bone field because of its robust promoter activity and transgene expression specificity to mature osteoblasts [46]. This line displays Cre recombinase activity from E14.5 in long bones and skull, and in all bones (intramembranous and endochondral-derived) at E16.5 and P5. Weak staining in digit and face skin was observed.

The rat 2.3 kb *Col1α1*-Cre transgenic line has very similar characteristics. In calvaria and long bones, Cre recombinase activity was observed in mature osteoblasts and osteocytes (cortical and trabecular cells in long bones), but not in suture mesenchyme nor cells of the inner and outer periosteum at E18 and P5.

The rat 3.6 *Col1α1*-Cre transgenic line is also available to target osteoblasts, but the specificity of this Cre line is different from the 2.3 kb *Col1α1*-Cre transgenic line [52]. *Ex vivo* analysis of bone marrow stromal cell and primary calvarial osteoblast cultures have shown that the 3.6 kb *Col1α1-Cre* is expressed early during osteogenic differentiation, whereas the 2.3 kb *Col1α1*-Cre is activated later and its activity is restricted to maturing osteoblasts [83,84]. Accordingly, in contrast to the 2.3 kb *Col1α1*-Cre line, the 3.6 kb *Col1α1*-Cre line displays *in vivo* Cre recombinase activity broadly in cells of the osteoblast lineage and in suture mesenchyme. During development (E18), 2.3 kb *Col1α1*-Cre activity was observed in long bones in cells of the inner layer of the perichondrium, which are destined to become osteoblasts, while 3.6 kb *Col1α1*-Cre recombinase activity was detected in the entire perichondrium. Sporadic activity was observed in growth plate chondrocytes in both 2.3 kb *Col1α1*-Cre and 3.6 kb *Col1α1*-Cre mice. Surprisingly, strong Cre activity was observed in the articular cartilage of long bones in 3.6 kb *Col1α1*-Cre mice. 3.6 kb *Col1α1*-Cre activity was also observed in tendons. These studies thus indicate that the 2.3 kb and the 3.6 kb *Col1α1-Cre* transgenic mice can be used to target different populations of osteoblasts. Importantly, Dr. Kream and collaborators have observed that *Col1α1*-Cre mediated gene rearrangement can occur in the germ line by transmission of Cre mRNA or protein in oocytes and sperm irrespective of inheritance of the Cre transgene [52]. This occurred more frequently with females than with males. They thus suggested that *Col1α1*-Cre transgenes should be transmitted through the male breeder and that genotyping should be done to identify animals that display gene rearrangement in the absence of inheritance of the Cre transgene. One study also showed that the 3.6 kb *Col1α1* promoter unexpectedly directs the expression of transgenes in the osteoclast lineage, and this effect must be considered when utilizing this promoter [85].

Temporal regulation of Cre recombinase activity can be achieved selectively in mature osteoblasts thanks to an inducible mouse 2.3 kb *Col1α1*-Cre transgenic line that was generated by using the CreER^{T2} recombinase [13]. In this line, Cre recombinase activity was detected following 4-OHT in E18.5 long bone, calvaria, rib, and vertebra osteoblasts. No Cre activity was observed in other organs such as heart, lung, liver, and kidney of embryos whose dams were treated with 4-OHT. Cre recombination was also observed post-natally in osteoblasts in 18 day-old mice upon 4-OHT treatment. The advantage of this transgenic mouse line over the non-inducible 2.3 kb *Col1α1*-Cre mouse line is to offer the possibility to study the function of a gene in osteoblasts after birth, without affecting bone development since the targeted floxed gene can be inactivated post-natally upon 4-OHT injection.

The *Col1*-CreER^{T2} transgenic line was generated by cloning the Cre-ER^{T2} fusion protein cDNA and downstream poly-A element (pA) behind a 3.2 kb fragment of the mouse type I collagen regulatory sequences [45]. The pups of *Col1*-CreER^{T2}; ROSA26 reporter mothers injected with 4OH-Tamoxifen at E13.5 displayed, at E14.5, a staining pattern similar to OSX–CreER^{T2}; ROSA26 mice, although a reduced distribution throughout the skeleton was observed, such as absence of staining in the hind limbs. Further studies confirmed that *Osx*–

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CreER^{T2} was expressed earlier in development than *Col1*-CreER^{T2}. Mosaic perichondrial recombination was observed in this model, and these cells were shown to predominantly remain in the cortex, as opposed to *Osx*–CreER^{T2} perichondrial cells that migrate to trabecular regions. No recombination was observed in cells of the growth plate.

Osteocalcin promoter—The most mature/differentiated osteoblasts can be targeted thanks to the selective expression of Osteocalcin (Ocn, also called bone γ-carboxyglutamic acid protein, or Bgp) in differentiated osteoblasts [86]. The human *OCN* promoter is first activated in fetal life just prior to birth [87,88], so it is not suitable for inactivating a gene during early development. The 1.7 kb *OCN* promoter does not drive Cre recombinase expression at a level sufficient to induce gene rearrangement in mature osteoblasts [46]. However, the 3.5 kb human *OCN* promoter has been used successfully to inactivate gene expression in mature osteoblasts using the *OCN*-cre transgenic mice that display Cre recombinase activity in calvaria osteoblasts and osteocytes starting at E17 [54] and in trabecular osteoblasts and osteocytes in 6 week-old mice [57].

Osteocyte promoters

Osteocytes represent a population of fully differentiated osteoblasts embedded within the bone matrix that is relatively difficult to study due to the absence of good cell lines, their tissue localization, and limited proliferative capacity. It is important to realize that gene deletion at any time point during differentiation of the osteoblast lineage will eventually give rise to KO osteocytes since these cells are the ultimate differentiation stage of this lineage. Osteocytes can, however, be distinguished from osteoblasts by their expression of Dentin Matrix Protein 1 (DMP1), an extracellular matrix protein that is expressed in all mineralized tissues [89,90]. Consequently, several DMP1 transgenic lines have been generated. GFP expression driven by a 7.9-kb mouse *Dmp1* promoter was found mainly in osteocytes [91]. When Cre-expression was driven by a 9.6-kb mouse *Dmp1* promoter, Cre recombinase activity was found in both dentin odontoblasts and bone osteocytes post-natally [60]. A mouse *Dmp1*-Cre-ER^{T2} is available, which allows floxed gene recombination in osteocyte post-natally only (and not during development) following tamoxifen administration [61]. The osteocyte specificity of this transgenic line seems very good, since only 1% and 9% β-gal-positive osteoblasts were detected in calvaria and femoral bones, respectively, following crossing to the ROSA26R reporter mice.

The 9.6 *Dmp1* promoter has been used to drive the expression of the diphtheria toxin receptor, a suicide gene that allowed specific ablation of osteocytes [2], as well as GPF for tracing studies [91,92]. Although the GFP moiety of the *Dmp1*-GFP mice can theoretically be used to purify osteocytes using FACS, the extraction of these cells from bone and their subsequent culture *in vitro* are technically challenging. An Immortomouse/*Dmp1*-GFP derived bone cell line (IDG-SW3) is available as an alternative to study some aspects of osteocyte biology in culture [93]. These cells express a temperature-sensitive mutant of the SV40 large tumor antigen under the control of the interferon-γ-inducible *H-2Kb* promoter (*H-2Kb*-tsA58) at 33 °C in the presence of γ-IFN, inducing continuous proliferation and immortalization. Switch to 37 °C in absence of γ-IFN allows these cells to behave as differentiated osteoblasts capable of differentiating to osteocyte-like cells under proper differentiation conditions.

Osteoclast promoters

Trap promoter—TRAP is indeed highly expressed in differentiated osteoclasts [94] and this characteristic was used to generate the mouse *Trap-Cre* transgenic line. These mice display Cre recombinase activity in long bones, vertebrae, ribs, and calvaria multinucleated osteoclasts, but also display some activity in chondrocytes. The latter is consistent with

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TRAP mRNA localization in murine chondrocytes [62,95]. Non-osseous activity was also observed in liver, spleen, stomach, heart, liver, and lung. Although the *Trap-Cre* transgenic line has not been used to create gene deletion yet, the TRAP promoter has been used successfully to drive the expression of multiple genes to osteoclasts *in vivo*, including c-fos [96], src [97], the SV40 T antigen [98], and to ablate macrophages by overexpressing the Diphteria toxin receptor [1].

M lysozyme promoter—M lysozyme, whose function is to cleave peptidoglycan off intruding bacteriae, is expressed in myeloid cells and monocytes/macrophages giving rise to osteoclasts. Mouse *LysM*-Cre transgenic mice were generated by a knock-in strategy inserting the *Cre* cDNA into the endogenous mouse *M lysozyme* gene [67]. Strong Cre recombinase activity in this line was detected by genomic Southern blot analyses in purified $F4/80^+$ peritoneal macrophages and Gr1+ peritoneal neurophils. Weak Cre activity was detected in sorted $CD11c^+$ dendritic cells.

Cathepsin K (Ctsk) promoter—*Ctsk* is more selectively expressed in osteoclasts [99–101]. The mouse *Ctsk-Cre* transgenic lines showed Cre recombinase activity in long bones, calvaria, and ribs [62,70]. Non-osseous activity was detected in stomach, salivary gland and liver [62]. Another mouse *Ctsk-cre* line was created by a knock-in strategy [70]. In this line, Cre activity was detected in skull and long bones at E16.5 and P7, consistent with the appearance and skeletal localization of functionally mature osteoclasts, and in vertebra multinuclear osteoclasts in adult mice.

CD11b promoter—*CD11b* encodes the α-subunit of the Mac-1 leukocyte integrin heterodimer, a major adhesion molecule and a commonly used hematopoietic surface marker. This gene is upregulated during myeloid differentiation, with the highest levels in mature monocytes, macrophages, and neutrophils [102]. CD11b is also expressed along the osteoclast differentiation pathway from mononucleated early progenitor cells until mature polynucleated osteoclasts [71,103,104]. Two human *CD11b-Cre* transgenic mouse lines have been generated. Both lines display Cre recombinase activity in hematopoietic organs including bone marrow, spleen and thymus. Cre recombinase activity was detected in peritoneal macrophages and bone marrow granulocytes [71]. B220-positive lymphocytes subpopulations and macrophages showed low level of Cre recombinase activity in the spleen. Strong Cre recombinase activity was detected in *ex vivo* generated TRAP-positive multinucleated osteoclasts. Very low level of recombination was detectable in testis, liver, kidney, brain, heart, and lung, possibly consistent with peripheral blood contamination. Cre recombinase activity was observed in the brain in several microglia-like cells, which derive from common precursor with macrophages.

Phenotypic analyses and interpretations

The availability of mouse models that allow gain or loss-of-function of specific genes *in vivo* has profoundly transformed the field of bone biology for the last 10 years. This “genetic” approach is now widely used to demonstrate the contribution of a given gene in a variety of biological processes such as bone development, bone remodeling, bone cancer metastasis, or bone repair. Although it is usually considered as the “gold standard” to ascribe a function to a specific gene *in vivo*, it is not devoid of limitations, especially if one loses sight of what actually happens in these mutant models, and thus can lead to serious misinterpretations and erroneous conclusions. Things become even more complex when a gene is more widely expressed or is involved in the regulation of multiple physiological processes. The literature is nowadays very much loaded with sometime erroneous conclusions and interpretations from global mutant mice. Science is an evolving business and every scientist will likely be proven wrong about something in the future, but the more

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general use of conditional KO models and their comparison to global and other Cre models should bring substantial information in the near future, if one keeps in mind the limitations of genetic models.

The cases of mutations or loss of function of a specific gene whose expression is restricted to bone cells is *a priori* the least prone to ambiguity, although the gene of interest might have distinct role in immature versus fully differentiated cells. Therefore, although a global KO would seems sufficient to address the role of this gene in bone homeostasis, the conditional approach brings a critical advantage to dissect putative distinct roles at various time of differentiation at the cell level or at various times of development. Examples of this would be the cases of *NF1*, a gene that is mutated in patients with neurofibromatosis type I, or β-catenin. Knock out of *Nf1* in mature osteoblasts, using the 2.3 kb *Col1α1-Cre* mice revealed the contribution of this gene to collagen synthesis, *Rankl* expression and bone mineralization, but did not affect osteoblast differentiation, leading to a high bone mass associated with high bone turn over [50]. In contrast, deletion of *Nf1* in immature osteoprogenitors revealed its role in promoting early differentiation, and led to a low bone mass phenotype [19,29].

β-catenin is a key component of the canonical Wnt signaling pathway and plays a crucial role in multiple steps during chondrogenesis and chondrocyte maturation. Global β-catenin deficiency resulted in embryonic lethality prior to formation of the skeletal elements [105]. Conditional deletion of *Catnby*, the gene encoding β-catenin, in mesenchymal precursors promoted chondrogenesis [22,106], suggesting that β-catenin inhibits early mesenchymal cell differentiation into cartilage. However, conditional deletion of *Catnby* in chondrocytes using the *Col2α1–Cre* mice decreased chondrocyte proliferation and delayed chondrocyte maturation [107]. Thus, β-catenin has opposite roles depending on the stage of development. It inhibits chondrogenesis, but once cartilage has formed, it promotes the maturation of growth plate chondrocytes.

Another consideration to keep in mind is that although a gene may seem cell specific, various physiological or pathological conditions may switch on or off the expression of a gene in adults. A typical example would be stress-responsive genes like *Atf4* for instance. The use of promoter specific for mature or differentiated bone cells is thus advantageous for its cell selectivity, but data from these models should be interpreted with caution since the function of the targeted gene in earlier differentiation stages is not taken into account. On the other hand, promoters active in progenitor cells that give rise to multiple sublineages will likely give a more biologically relevant picture of the phenotype, but the low specificity of such promoters makes the analysis of the results more complex. Interpretation of results in studies limited to a single Cre transgenic line to delete a gene of interest must thus be done with caution and comparison of the results obtained following deletion the same floxed gene with different Cre lines is the best approach to understand the gene function in a given lineage.

Importantly, even a restricted and transient expression of the Cre recombinase during development or later will irreversibly recombine a floxed gene in cells that may proliferate and later constitute a significant part of the cell population in the targeted tissue of mutant mice. The *Col2α1–Cre* lines represent a good example where Cre recombinase activity is expressed, likely transiently, in a pool of progenitor cells giving rise to chondrocytes but also osteoblasts, and possibly other mesenchymal cells. Crosses with reporter mice may not detect this event due to its transient nature or because of the sensitivity of the reporter system (most often time β-gal staining). In addition, authors interested in growth plate development may seldom report leaky expression in the marrow space or in adults. Interpretation of the results obtained from such conditional KO mice must thus be done with

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caution, keeping in mind that the system is never perfect and that other cell types than the one thought to be targeted may contribute to the observed phenotypes or mechanisms.

Another important issue is the genetic background of the mutant mice under study. Because bone parameters, including bone mass, differ significantly between mouse strains, it is crucial to use WT controls that originate from the same mutant colony, and if possible to have a colony backcrossed to a pure background. When using conditional knockouts, “WT” control animals can be Cre-negative; flox/flox or Cre-positive; +/+. The first control takes into account any possible interference from the introduction of the *Lox* sequences, while the second takes into account the possible effects of Cre recombinase activity on the genome.

Lastly, gender and age are important factors that should be considered when interpreting results. Studies too often report absence of phenotype using a single age time point and gender, which can lead to erroneous conclusions if the gene under study regulates processes that are slow, rapid, compensated by redundant mechanism(s), or gender-specific.

On a more general note, obviously and despite similar bone biology between mice and human, there are a number of differences between the two species that make extrapolations of mouse results to human conditions, diseases and treatment hazardous. Those include different tissue organization, continuous growth in mice versus growth plate closure in humans, differences of posture and patterns of mechanical loading, different remodeling activities, distinct genetic evolution and others. This being said, the findings obtained in genetically modified mutant mice shed light onto important biological mechanisms that will always eventually increment our knowledge of bone physiology, regardless of whether the results in mutant mice can be directly extrapolated to humans physiopathology or not.

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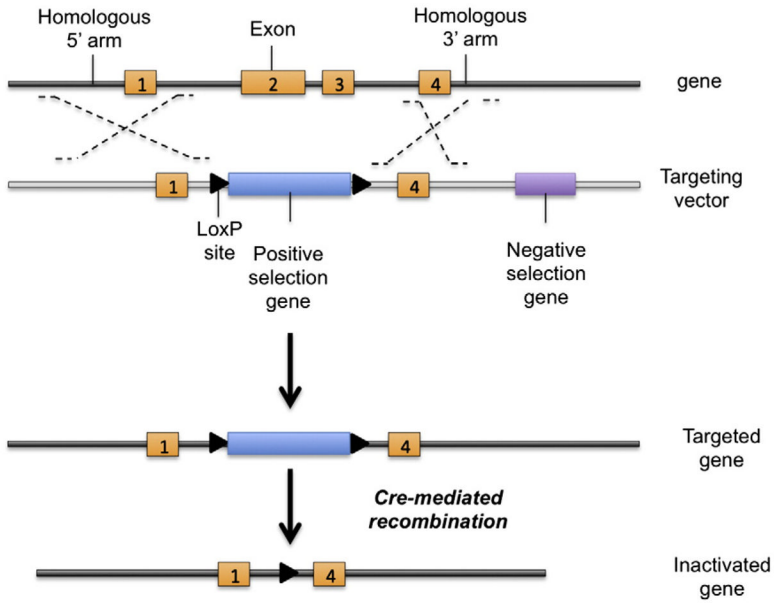

Fig. 1.

Schematic presentation of a conventional targeting construct. Several kilobases of genomic DNA on either side of the target gene are cloned around a drug-selection marker. *LoxP* sequences flank the positive drug-selection gene in the targeting construct. After the cloned DNA (targeting vector) is introduced into ES cells, positive and negative drug selection is performed in culture. Cre recombinase can delete the DNA sequence between the *IoxP* sites, thereby deleting part of a targeted gene in ES cells.

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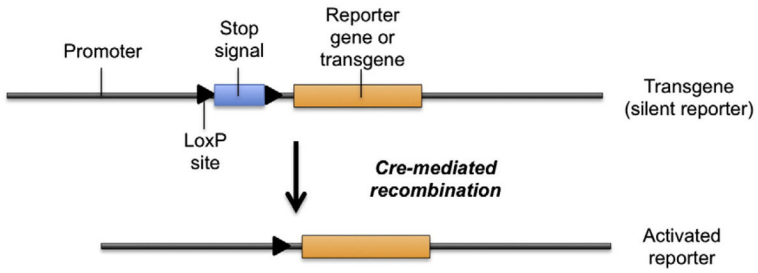

Fig. 2.

The Cre/LoxP system can be used for cell tracking purpose or to conditionally active expression of a transgene. The transgenic construct contains a stop cassette/signal flanked by two LoxP sites between a promoter and a reporter gene such as LacZ or GFP, or a transgene such as a suicide gene. The cross between Reporter and Cre-expressing mice leads to excision of the STOP cassette and transcription of the reporter gene or transgene.

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Table 1

Main Cre transgenic lines used for the analysis of bone-related genes. This list is not exhaustive.

Cell type	Promoter	Reported leakage	Examples of gene targeted	Original description
Osteochondro progenitors	Prx-Cre	Tendons, interlimb flank [18]	*Nf1* [19]	[18]
	Dermo1-Cre (mouse)		*Fgfr2* [20]	[20]
			Z/EG, *Ai9* [21]	
			Catnby [22]	
	Sox9-Cre (mouse)	Notochord, node, endodermal linings at E8, skin at E10.5, gonads, intestin, spinal cord, pancreas, tendons and synoviums at E17.0	*Tace*, Osx [7]	[23]
Chondrocytes	Col2A1-Cre (human)	Developing eye, epidermis, heart myocardium, endoderm of the developing yolk sac and cranial mesenchymal cells [24]	*V-Egf* [24]	[24]
			Fosl2 [25]	
	Col2a1-Cre (mouse)	Liver [26]	*Trsp* [26]	[30]
			Pten [27, 28]	
			Nf1 [29]	
	Col2a1CreER^{T} (mouse)			[31]
	Col2a1CreER^{T2} (mouse)	Osteochondroprogenitors		[32]
	Col2a1CreER^{T} (rat)	Osteochondroprogenitors	*Smo* [33]	[33]
	Col2a1 DOX-Cre (mouse)		*Ext1* [34]	[35]
	Col10a1-Cre (BAC mouse)	Subchondral and endochondral bone marrow cells		[36]
	Col10a1-Cre (1kb mouse)	Skin, perichondrium, some trabecular osteoblasts		[37]
	Acg-CreER^{T2} (mouse)			[38]
Osteoblasts	Runx2-CreER^{T} (mouse)	Cartilage	*Vegf*164 [39]	[40]
	Tet-OSX-Cre (mouse)		*P53/Rb* [41]	
			A-Raf/B-Raf [42]	[44]
			Cxcr4 [43]	
	OSX-CreER^{T2} (mouse)		*Vegf*164 [39]	[45]
	2.3kb Col1A1 -Cre (mouse)	Weak activity at E14.5 in skin and digits [46]	*Adrβ2* [47]	[46]
			Hdac8 [48]	
			Lrp5 [49]	
			Nf1 [50]	
	2.3kb Col1A1-Cre ER^{T2} (mouse)		*Osx* [51]	[13]
	2.3kb Col1A1 (rat)	Sporadic activity in chondrocytes before E18 [52]		[52]
	3.2kb Col1-CreER^{T2} (mouse)			[45]
	3.6kb Col1A1-Cre (rat)	Tendons	*Rb1*[53]	[52]
		Sporadic activity in chondrocytes before E18		

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Cell type	Promoter	Reported leakage	Examples of gene targeted	Original description
		Strong activity in articular chondrocytes [52]		
	OCN-Cre (human)		*Igf* [54]	[54]
			Notch1 [55]	
			Cnb1 [56]	
			Smad4 [57]	
			Pten [58]	
Osteocytes	15kb Dmp1-Cre (mouse)		*Pkd1* [59]	[60]
	10kb Dmp1-creER^{T} (mouse)	Small % of osteoblasts	*Ppr* [61]	[61]
Osteoclasts	Trap-Cre (mouse)	Chondrocytes, liver, spleen and stomach, heart, liver and lung [62]	*Ilk* [63]	[62]
	Lysozyme-Cre (mouse)		*Gr* [64]	[67]
			ER-a [65]	
			JunB [66]	
	Cat K-Cre (mouse)	Stomach, salivary gland (line 1) and liver (line 8) [62]	*Bcl-x* [68]	[62, 70]
			Dicer [69]	
	CD11b-Cre (human)	Peritoneal mcrophages, bone marrow granulocytes	*S1P$_1$* [72]	[71]
		Low level in spleen, testis, liver, kidney, brain, heart, and lung [71]	*Ikkβ* [73]	