4

Creating Transgenic Mice to Study Skeletal Function

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4.1 Introduction

Rapid advances in the field of genetic engineering have led to a parallel "boom" in the number of transgenic mice generated. By combining new technologies in recombinant DNA with advances in cell and developmental biology, a host of novel animal models were developed that have specific skeletal defects. These models are useful for two majors reasons. First, many new models closely mimicking human diseases such as osteopetrosis or osteoporosis have been developed. Second, transgenic mice provide a critical test for the function of an individual gene *in vivo*. The results from these investigations have often been surprising, yet, have led to fascinating new discoveries about the role of individual proteins in the development and aging of bone tissue.

The first part of this chapter outlines methods that are typically used to generate transgenic mice. In describing these procedures, commentary is provided about the advantages and disadvantages of the two major types of transgenic mice that are currently produced, namely, "conventional" (in general results in a gain of function) or "knockout" (in general a loss of function). Variation and combinations of the two types of transgenics are also described (Section 4.6). Numerous examples of animal models generated using the procedures that have skeletal defects are listed in two tables according to the transgenic type

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they are. In some cases biomechanical defects have been documented for these mouse models either due to alterations in mineral content or composition (see Chapter 5) or from other imbalances the matrix or growth factor milieu. As transgenic methodology becomes more refined, it is reasonable to expect that many more mouse lines will be produced. They will, in turn, be important resources for the engineer interested in testing the biomechanical properties of bones with defined genetic and skeletal defects.

4.2 Construction of Conventional Transgenic Mice

Transgenic mice are designed to "overexpress" a specific gene over and above the normal patterns of genes expressed by an organism. This is done by genetic manipulation and insertion of transgenes into a host genome. Subsequently, the gene can be transmitted to its offspring. The first transgenic mouse line of this type was prepared by Brinster et al.[1] almost 20 years ago. In these early experiments foreign Xenopus (frog) ribosomal DNA was injected into the pronucleus of a fertilized mouse egg; transcripts of the foreign gene were detected indicating it was active. This work followed with a report of the successful insertion and expression of a gene coding for an enzyme[2] under the control of the regulatory apparatus (promoter) of a separate gene. The procedures described in these early studies are still currently used and are outlined in Fig. 4.1. It should be noted that several good technical manuals are available that describe in great detail the specifics of each step and are excellent guides for the novice embarking on creation of their own transgenics.[3] The material presented in this chapter is intended to provide the biomechanical engineer with a general guideline about how transgenics are produced and, further, how various mouse models differ genetically and functionally.

The first step to construct a transgenic mouse line is to isolate and characterize the gene of interest. The gene (either cDNA, which is the coding region of the gene, or total genomic DNA) is then connected (ligated) to a regulatory DNA that controls the transcription and final expression pattern of the transgene. Often, additional DNA elements are added to the transgene including intron sequences placed between the promoter and cDNA to enhance expression, and a poly-A attachment site to enhance stability of the transcribed gene (Fig. 4.1 and Ref. 4). In some cases "enhancer" sequences derived from introns or DNA distal from the promoter are added to facilitate tissue-specific gene expression.[5] The sum of these DNA parts is often referred to as a "construct." When attached to a vector or plasmid, it can be amplified in bacteria and then large quantities are purified for DNA sequence to confirm its integrity. Promoter attached to the transgene is then purified, and injected into the pronucleus of a fertilized egg. Several injected eggs are surgically transferred to a pseudopregnant mouse and the offspring born referred to as the F_0 generation. A few weeks after birth, a small piece of the tail is removed from the mice, DNA extracted, and then subject to either Southern blotting or polymerase chain reaction (PCR) for genotyping (see Chapter 3 for details). This is done to test for the presence of the transgene. Positive transgenic mice are then bred to wild-type animals and the next generation (F_1) is tested to assure that the transgene is passed on in the germline (see Fig. 4.1).

There are several categories of conventional transgenics that differ in the type (location) of trangene patterns they direct and the functional outcome (gain or loss). A description of each type and examples are listed below and in Table 4.1.

4.2.1 Ectopic Expression

In this category transgenes are placed behind a promoter that will direct their expression in tissues where they are not normally produced. This is done by specifically choosing a promoter that is active in tissues where the transgene is not made. One example in this category is a transgenic line where the myogenic (muscle)-determining gene was produced under the control of a ubiquitously expressed mouse sarcoma virus promoter. In this study, multinucleated striated myotubes (cells characteristic of muscle) were found in the brains of transgenic mice, indicating this transgene was operational *in vivo* at a site distant from where muscle is normally made.[6] In many cases, dramatic effects have been noted; overexpression of the homeobox HOXb-8 under the control of the retinoic acid β promoter resulted in animals that had mirror image duplications of their forelimbs.[7]

TABLE 4.1 Comparing Features of Various Transgenic Types

Category	Method	Result
"Conventional"		
a. Ectopic	Transgene driven by	a. Makes gene in ectopic site
b. Tissue specific	promoter that directs	b. Makes gene in specific tissues
c. Dominant-negative	expression above normal gene	c. Inactivates normal gene
Knockout	Gene targeting in embryonic stem cells	No expression from either allele; null throughout life-span
Knockin or subtle mutation	Gene targeting in embyonic stem cells	Exchange normal gene for a different or mutated gene
Cre-recombinase	Combines gene targeting of loxP with tissue-specific cre-recombinase	Tissue-specific knockout or mutation

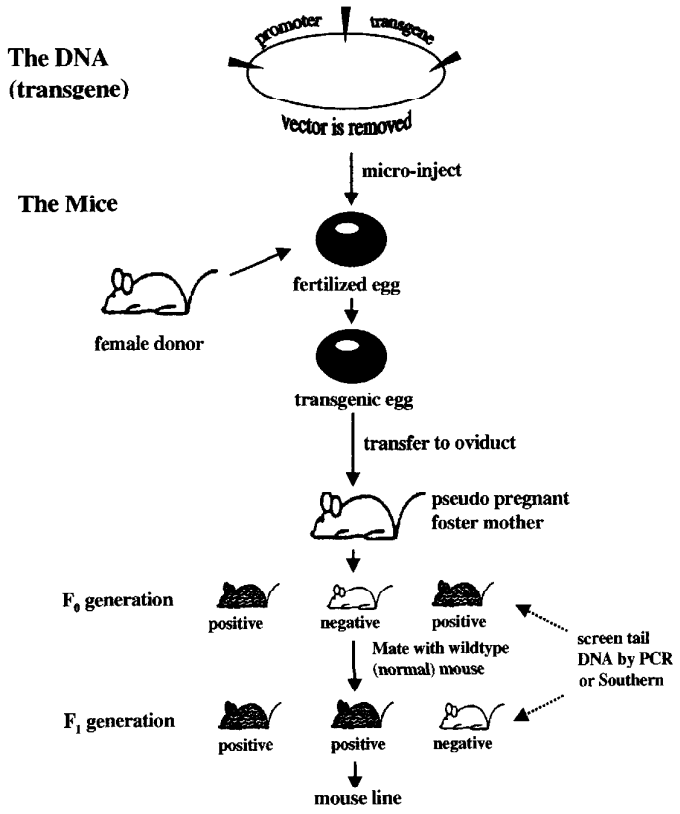

FIGURE 4.1 Construction of "conventional" gain of function mouse lines. A construct is prepared that contains a cDNA or genomic DNA attached to a promoter that regulates its tissue-specific expression. The promoter and transgene are injected into an isolated fertilized egg, which is then transferred to the oviduct of a pseudo-pregnant mouse that is the "foster mother" and host for the developing transgenic mouse. After birth, a small biopsy is taken usually from the tail and used to isolate DNA. PCR (see Chapter 3) is is then performed with the DNA to determine which offspring have the transgene. Transgenic animals are bred to each other or to other wild-type animals and their offspring analyzed for the presence of the transgene. Offspring (F_1) retaining the transgene are used for propagation of the mouse line.

4.2.2 Tissue-Specific Transgene Expression

Many proteins critical to the function of bone are, in fact, produced in numerous tissues beside bone. To study the effects of such factors specifically in skeletal tissue, promoters or DNA regulatory regions have been isolated and their temporal and spatial pattern of expression tested using markers such as β-galactosidase that can produce a blue color. By using such specific promoters, transgenic mice that overexpress transgenes in specific cells such as the osteoblast[8] or osteoclast[9] have been made. When the osteocalcin gene, which is exclusively produced in the osteoblast, was used to drive the bone-specific expression of human growth hormone, mice were generated that showed increased bone growth.[8] The osteocalcin promoter has also been used to drive growth factors; overexpression of transforming growth factor-β2 (TGF-β2) in the osteoblast led to the creation of a mouse line that had lower bone mass compared with its normal littermates.[10] Thus, by careful selection of a promoter (driving) DNA, mice can be created that express transgenes in any selected tissue. It should be noted that promoters are not available for all cell types. One promoter not yet available is for osteocyte-specific expression. Further characterization and cloning of the genes specifically made by osteocytes and other skeletal cells will be required for future advances in this area.

4.2.3 Dominant Negative Transgenics

By genetic engineering it is now possible to create genes that contain mutations that make them nonfunctional. By careful design, such mutations in addition render the normal gene nonfunctional. Due to this characteristic, they are termed "dominant." Because the gene of interest can no longer function, it is also termed "negative." A dominant negative mutation therefore overexpresses a mutant gene that makes the normal gene nonfunctional. One example in this category is a specific mutation in the TGF-β II receptor.[11] The receptor has a truncation in its cytoplasmic tail; it can bind TGF-β but cannot initiate further signaling cascades. Thus, it is possible to create mice with dominant negative mutations that are made ectopically or in a tissue-specific fashion.[12]

4.3 Construction of Gene-Targeted or Knockout Mice

The generation of mice with targeted deletions of specific genes is a more recent technology than conventional transgenics.[13] This method was developed to make cells and subsequently mouse lines that have a single gene mutation in the context or background of a normal mouse. This type of alteration differs from that discussed in Section 4.2 in that the knockout mice have no functional gene throughout their life span. To accomplish this, an additional step is added to the procedure that uses embryonic stem (ES) cells. ES cells are derived from the inner cell mass (ICM) of an early embryo and by themselves cannot easily develop into an embryo except in rare cases.[14] However, when placed into the cavity of a 3.5-day fertilized egg the ES cells can contribute to the tissues of the developing embryo. By culturing the cells *in vitro* (in a petri plate), specific gene deletions can be selected in culture using drug selections and subsequent stem cells used to generate mutated embryos. In a manner similar to that described for conventional transgenics, the chimeric mice (which are a mixture of normal and ES cells) can be bred and offspring tested for the presence of the mutated allele. The goal is to obtain transgenes that are inherited so that a mouse line can be developed with a null mutation in a single specific gene. Additional details about this procedure can be found in Ref. 15 with salient features outlined as follows.

4.3.1 DNA Preparation

To target a specific gene, it is necessary to isolate and characterize its DNA from a genomic library made from a strain of mice that is identical (isogenic) to the embryonic stem cell used. In general, this is from the 129/SVJ strain of mouse. DNA corresponding to the gene of interest is then characterized by restriction digestion and the intron (noncoding) and exon (coding) parts clearly identified. This is done so that a mutation can be designed that will inactivate the gene. Two selectable markers are then engineered into the

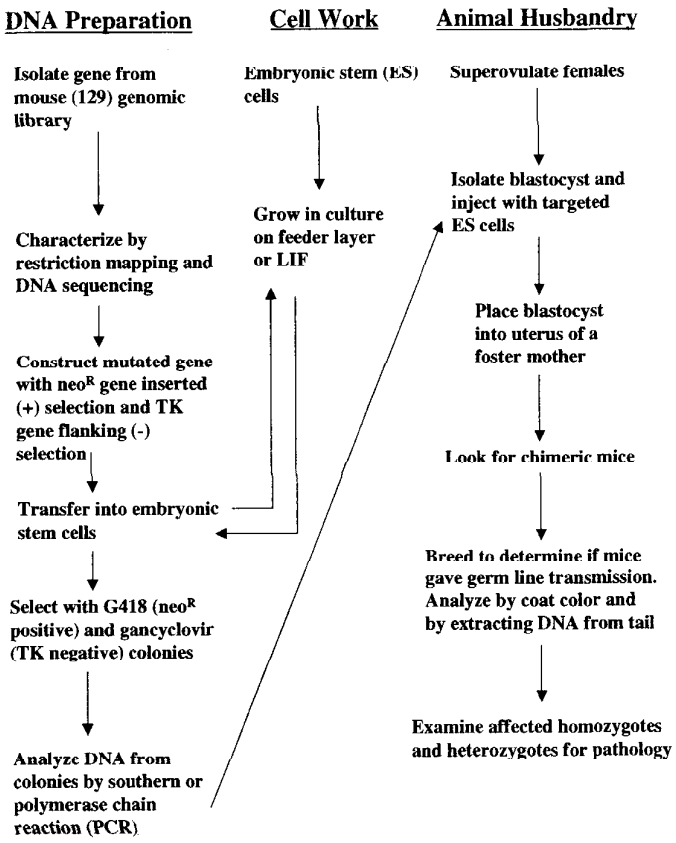

FIGURE 4.2 Outline of parallel procedures required to make a knockout mouse. Three separate lines of experimentation must be successfully performed to create a knockout mouse line. One part of the procedure involves isolation and extensive characterization of the gene that will be knocked out. A second part requires cultivation of embryonic stem cells, which are used to insert the transgene by homologous recombination (see Fig. 4.3). DNA prepared in step 1 is transferred to the ES cells, which are then treated with drugs to select for the presence of the gene at the correct gene locus. So called "targeted" ES cells are injected into a normal host blastocyst, which is then transferred to the uterus of a foster mother. ES cells incorporate into the tissues of the developing embryo and generate a "chimeric" mouse. By breeding the mouse and testing the offspring for the presence of the transgene, one determines if the targeted gene is in the "germline" and subsequently passed on to subsequent generations for the creation of a new mouse line.

isolated DNA (Figs. 4.2 and 4.3). A "positive" selection marker (i.e., neomycin or hygromycin resistance neo^R, hyg^R) is used to screen for mutant gene transfer into the ES cells. The second marker in the construct is for "negative" selection and is used to "enrich" (i.e., increase) the amount of ES cells having homologous gene transfer events (usually using thymidine kinase, TK). Genomic DNA contains repetitive elements that make up 5% of the genome, which can interfere with homologous recombination.[16] For this reason, they should be avoided when preparing a targeting construct. They can be identified by digesting the construct with restriction enzymes followed by hybridization with radiolabeled total genomic DNA.

4.3.2 Embryonic Stem Cells

While ES cells can be freshly prepared, most investigators use aliquots of cells prepared and frozen in batches for long-term storage.[17] These cells were originally isolated from the inner cell mass of very young mouse embryos (4 days old) and are characterized by rapid growth and pleuripotency (can develop into

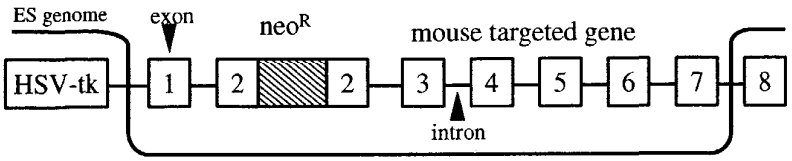

FIGURE 4.3 Details of typical targeting vector. The numbered boxes show the position of the exons, which are determined using a combination of restriction digestion (mapping) and DNA sequencing. The exons have sequences that match the mRNA while the introns do not. They are removed from the mRNA by "splicing" in the nucleus prior to exit into the cytoplasm (see Chapter 3). Using conventional techniques of genetic engineering, a "positive" selection marker is inserted within one of the exons. Shown here is one commonly used marker that codes for neomycin resistance (neo^R). It is used for selecting cells that have taken the transgene in their genome using the selection drug neomycin (G418). A negative marker is also engineered into the construct. Shown here is one such marker that codes for the TK gene. If a gene has recombined into the correct locus (see where the ES genome accepts mutant DNA by a double-crossing-over event) the TK is removed; cells that have not removed the TK are killed by treatment with gancyclovir (this is negative selection), thus enriching the population for cells that have "targeted" alleles. Cells are tested for this homologous recombination using a combination of Southern blotting and PCR (see Chapter 3). Both gene selection markers have their own promoters (usually PGK) that allow continuous (constitutive) expression if present in the cell.

many tissues of an organism). Details about the culture conditions required to keep the cells in a "undifferentiated state" are outlined in detail in Ref. 15. Briefly, the cells must be maintained either on a coating of gelatin or over a layer of embryonic fibrobasts that have been previously inactivated by mitomycin C or by irradiation so that they can no longer divide. To inhibit differentiation, the cells are passaged often and supplemented with the cytokine leukemia inhibition factor (LIF). DNA constructs are transferred (transfected) (see Chapter 3) into the ES cells and screened for the presence of the transgene using the drug G418. If the transgene is randomly integrated, the thymidine kinase gene will be present, and will kill the cells treated with gancylovir. This latter procedure is a negative selection that eliminates cells that have not undergone homologous recombination or gene targeting. Colonies resistant to the two drugs are isolated, expanded, and frozen for future growth. DNA is subject to restriction digestion and Southern blotting or PCR (see Chapter 4). DNA from transgenes with homologous recombination will have characteristic banding patterns when analyzed by electrophoresis.

4.3.3 Animal Husbandry

ES cells containing targeted transgenes are used for injection into host blastocystes. Usually 15 or more cells are used for each injection using finely drawn glass needles under a microscope. The procedure is similar to DNA injections into fertilized pronuclei (conventional transgenics), except the ES cells and blastocystes are much larger. Injected blastocystes are allowed to recover and are then transferred into a foster mother; mice that are born are chimeric. The extent of chimerism can be judged by the coat color; ES cells are from mice that are agouti, which has a light brown color (129) while the recipient host blastocyste is usually from mice with a black coat (C57). One strives to obtain a high percentage of contribution of ES cells (agouti color) to assure that the transgene will be in the germ cells that are used for inheritance. Chimeric mice are bred and offspring tested for the presence of the transgene as described above. If the F_1 generation has the transgene, then it is considered to be "germline transmission" and the line can be maintained indefinitely. In this first generation, the trangenic mice will be heterozygous with one mutated allele. By breeding heterozygous mice, new animals can eventually be generated that are normal (wild-type), heterozygous (one normal and one mutant allele), or total knockouts (two mutant alleles). Mice can be maintained on a mixed background (129/C57) or bred against different strains to change the background over time. This may be a critical feature in the manifestation of the phenotype; it may be more or less severe depending on the background of the mouse line. Recent studies showed that different strains of mice vary in bone mass as a result of intrinsic differences in rates of bone formation.[18]

One category of "loss-of-function-mice" that will not be discussed in detail relies on retrovirus insertion of DNA randomly into the genome to inactivate genes. Although some interesting models like the Mov 13 mouse have been described in this category,[19,20] the procedure is limited because it cannot control the site and subsequent type of gene that is inactivated. Similarly, several mouse models of skeletal disease have been created by spontaneous or induced mutation in unknown genes. Often, the phenotypes are very interesting but, again, they are limited because the primary gene defect is unknown. Nevertheless, by linkage analysis the causative gene in this latter case can, in theory, be identified using "reverse genetics."

4.4 Examples of Transgenic Mice with Skeletal Phenotypes

There are many transgenic mouse lines that show skeletal alterations due to either overexpression of the transgenes or expression of mutant proteins that inactivate the function(s) of the normal endogenous genes. They include growth factors, cytokines, receptors, and structural matrix genes (Table 4.2 and Ref. 21).[22–48] The expression of some transgenes was targeted to bone tissues through bone-specific promoters such as osteocalcin (osteoblast specific) or TRAP (osteoclast specific), and others were expressed in nonbone tissues. The phenotypes range from osteopetrosis (too much bone) to osteoporosis (not enough bone) resulting from impaired formation or to altered activities of the osteoblasts and osteoclasts. In other cases, skeletal abnormalities resulted from defects in cartilage and matrix structures. In most cases, the phenotypes were consistent with data drawn from *in vitro* studies; however, others were surprising or even contradictory to previously reported results. For example, interleukin-6 (IL-6) was long thought to be a bone resorption cytokine; however, overexpression of IL-6 in transgenic mice decreased osteoclast number and bone resorption.[38] Despite these sometimes paradoxical data that are learned from these mice lines, they provide many new skeletal models that can be analyzed further to delineate pertubations in their molecular, cellular, or biomechanical integrity.

4.5 Examples of Knockout Mice with Skeletal Phenotypes

By gene targeting using homologous recombination, knockout mice have been generated with expected or predicable bone defects (Table 4.3 and Refs. 49 to 84). These include vitamin D receptor, alkaline phosphatse, calcitonin, and cathepsin K. On the other hand, additional functions were discovered from knockout mice. For example, deletion of 24-hydroxylase, which is thought to play a role in the degradation of vitamin D3, resulted in impaired vitamin D catabolism as well as intramembranous bone ossification. Further, targeted disruption of the widely expressed gene *c-src* led to defects primarily in bone tissues. Deletion of two structurally related bone-enriched matrix genes osteopontin[84] and bone sialoprotein (see Chapter 5) had no discernible phenotype *in vivo* possibly due to redundancy, while lack of a transcription factor cbfa-1 implicated to regulate their expression led to dramatic changes in the skeletal formation. Consistent results, on the other hand, were obtained from the separate deletion of osteoprotegrin (OPG)[49] and its ligand.[50] The results obtained were opposite to the phenotype in osteoprotegrin transgenic mice[21] and, taken together, strongly suggest that OPG is an inhibitor of osteoclast differentiation and activity. Therefore, they may be a potential therapeutic agent for the treatment of osteoporosis.

An important point to note is that only a fraction of all the mouse lines listed in Tables 4.1 and 4.2 have been tested for biomechanical integrity. An example of two knockouts that have been examined for biomechanical properties are osteocalcin[68] and biglycan,[69] both of which are extracellular matrix proteins. Both are expressed by the osteoblast but are structurally very different; osteocalcin is a small protein with a γ carboxylation post-translational modification, while biglycan is a small proteoglycan whose mass is composed primarily of carbohydrates. Mutations in these abundant structural genes showed no profound effects on the development of the bones (i.e., gross abnormalites in shape or number) but, rather, they had age-related effects on the accumulation of bone mass (see Fig. 4.4). Specifically, a disruption of the biglycan gene led to a decrease in bone formation and less bone mass, while osteocalcin mutant mice had

TABLE 4.2 Examples of Transgenic Mice with Skeletal Phenotye

Gene	Promoter	Phenotype	Ref.
		Overexpression (Gain of Function)	
Osteoprotegrin	Human ApoE	Osteopetrosis due to decreased osteoclast formation	21
IGF-I	Osteocalcin	No change in bone formation rate Increased bone density and osteocyte number	22
TRAP (tartrate-resistant acid phosphatase)	TRAP/SV40 enhancer	Increased bone resorption Decreased trabecular bone volume	23
Soluble tumor necrosis factor receptor	α1-anti-trypsin	Protected against bone loss caused by estrogen deficiency	24
Human osteoblast stimulating factor-1	Human osteocalcin	Increased bone density	25
Human TGF-β2	Rat osteocalcin	Progressive bone loss Increased bone matrix deposition and defective bone mineralization Hypoplasia of the clavicles	10
Interleukin 4	lck	Osteoprosis due to decreased osteoblast activity	26
Stretch-activated cation channel (α-rENaC)	Osteocalcin	Increased bone formation rate and bone density	27
Vitamin D receptor	GOSCAS	Increased bone formation rate Thicker and stronger bone	28
Human G-CSF	SRα	Osteoporosis due to increased bone resorption	29
c-fos	H-2Kb class I MHC	Osteosarcoma	30
Human BMP4	BMP4	Decreased body size Failure in the development of hair and whisker follicles, high incidence of neonatal death Increased apoptosis in cartilage and hair follicles	31
Human bFGF	Phosphoglycerate kinase	Shortening and flattening of long bones, moderate macrocephaly	32
Human TNF-α	Human TNF-α	Arthritis	33
Interferon γ	Mouse immunoglobin λ chain enhancer	Osteochondrodysplasia characterized by thin cortical bones, fractures, disruption of the epiphyseal plate and degeneration of articular cartilage	34
Ets2	Mouse metallothionein	Neurocranial, viserocranial, and cervical skeletal abnormalities similar to Down's syndrome	35
Human growth hormone	Rat osteocalcin	Longer femora and greater mid-diaphyseal cross-sectional geometry associated with impaired mechanical integrity due to lower ash content, presence of cartilage and woven bone and greater porosity in the mid-diaphysis	8, 36
HTLV-I tax	LTR	High bone turnover and myelofibrosis	37
Human IL-6	H-2Ld	Decreased osteoclast number and bone resorption	38
PTHrP	Collagen type II	Chondrodysplasia characterized by short-limbed dwarfism and a delay in endochondral ossification	40
Mutant PTH/PTHrP receptor (constitutively active)	Rat α 1 (II) collagen	Delayed mineralization, decelerated conversion of proliferative chondrocytes into hypertrophic cells and prolonged presence of hypertrophic chondrocytes	39
Natriuretic peptide	Human serum amyloid	Skeletal overgrowth	41
		Dominant-Negative Expression	
Mutant human α1 (I) collagen	Human α (I) collagen	Short bones, decreased collagen and mineral content, poor mechanical properties and spontaneous fractures	42

(*Continued*)

TABLE 4.2 Examples of Transgenic Mice with Skeletal Phenotye (Continued)

Gene	Promoter	Phenotype	Ref.
Mutant α1 (II) collagen	Mouse α (II) collagen	Short limbs, hypoplastic thorax, abnormal craniofacial development, disruption of the normal organization of growth plate with decreased proliferating zone and increased hypertrophic zone, defect in endochondral ossification	43
Mutant α1(IX) collagen	Rat collagen II	Osteoarthritis and mild chondrodysplasia	44
Truncated type X collagen	Chick type X	Compression of hypertrophic growth plate cartilage, decreased bone formation, leukocyte deficiency and lymphopenia	45
Mutant β-1 integrin	Osteocalcin	Thinner bone and increased osteoclast number and bone resorption in female mice	46
Truncated TGF-β type II receptor	Metallothionein	Osteoarthritis	47
Klotho	Gene interrupted by foreign transgene	Osteoprosis, infertility, short life span, arteriosclerosis, and skin atrophy	48

an increase in bone formation resulting in "thicker" bones. Similarly, both mice lines had biomechanical pertubations that were quite distinct. Biglycan-deficient mice had spontaneously decreased yield energy and failure load at 6 months of age[69] while osteocalcin mice showed increased bone strength only after ovarectomy.[68] As can be seen by the phenotypes listed in Tables 4.2 and 4.3 many of the trangenic lines have been well characterized at the tissue and cellular level and thus offer an immense resource for the biomechanical engineer interested in testing the relationship of a specific gene to biomechanical soundness.

4.6 New Frontiers: Designer Mice

4.6.1 Tissue-Specific Knockout Using Cre-recombinase

Some genes when knocked out from conception are lethal. Although these observations clearly show the importance of a protein, they limit examining its function later in life. For this reason, a technology has been developed where genes can be knocked out at a specific time and location. In this procedure promoters with the desired developmental expression regime are connected to the enzyme cre-recombinase (Fig. 4.5). The enzyme by itself has no effect on normal eukaryotic cells. It was originally isolated from λ phage (virus that infect bacteria) and has the ability to remove a stretch of DNA that has loxP DNA sequences attached to both ends. The short loxP sites are placed at the ends of (or flanking) a gene being tested using genetic engineering and homologous recombination (see previous section). Thus, two mice strains are created: one with a promoter connected to cre-recombinase so that it will be made in a specific place at a specific time during development. The second mouse strain contains loxP sites outside (flanking) the gene that is to be deleted. By crossing the two mouse strains, both genetic modifications can be incorporated into a single mouse that will, subsequently, have the gene with loxP sites deleted in tissues that express the cre-recombinase. This is referred to as a "conditional allele" in contrast to a total knockout, which is termed a *null* allele. The advantage of making the two types of mice separately is that the cre-recombinase transgenic lines can be used repeatedly to make additional mice defective in any gene with previously engineered loxP sites. An example of this procedure was recently described for the enzyme glucokinase, which is essential for glucose homeostasis. Using various promoters to drive the cre-recombinase, the gene was ablated either in the β cell of the pancreas or in the liver.[85] The differential knockout of the enzyme showed that pancreatic cell expression was more critical to hyperglycemia while the liver expression was important for glycogen synthesis and glucose turnover. Further, by careful placement of the loxP sites within the targeted gene, mice have been made with only a portion of the

TABLE 4.3 Examples of Knockout Mice with Skeletal Phenotype

Gene	Phenotype	Ref.
Osteoprotegrin	Osteoporosis due to impaired osteoclast formation, arterial calcification	49
Osteoprotegrin ligand	Toothless, osteopetrosis due to lack of osteoclasts, lack of lymph nodes, defect in thymocyte differentiation	50
Transcription factor PU.1	Osteopetrosis due to lack of osteoclasts	51
c-fos	Osteoporosis and absence of tooth eruption Hematopoietic defects	52, 53
c-sac	Osteopetrosis due to impaired osteoclast function	54
Tartrate-resistant acid phosphatase	Mild osteopetrosis due to impaired bone resorption	55
Calcitonin	Decreased bone density	56
Cathepsin K	Osteopetrosis due to impaired osteoclast function	57
β-3 Integrin	Osteoscelerosis Dysfunctional osteoclasts and osteblasts	58
Cbfa-1	Lack of bone formation due to maturational arrest of osteoblasts	59
PTHrP receptor	Accelerated chondrocyte differentiation	60
Vitamin D receptor	Hypocalcemia, hyperparathyroidism, rickets and osteomalacia, alopoecia, and infertility	61
24-hydroxylase	Impaired vitamin D3 catabolism, Impaired intramembranous bone ossification	62
Thyroid hormone receptors	Decreased bone growth, disorganized growth plate and delayed ossification, decreased bone mineral content and serum IGF-I level	63
BMP-3	Increased bone density	64
Alkaline phosphatase	Spontaneous fracture, defective mineralization Epileptic seizures and death before weaning	65
Connexin 43	Small dome-shaped skull Impaired intramembraneous bone formation Decreased ALPase activity in osteoblasts	66
β-Adrenergic receptor kinase	Decreased bone formation in heterozygous mice	67
Osteocalcin	Increased bone formation	68
Biglycan	Osteoporosis due to decreased bone formation	69
Osteonectin	Osteopenia	70
IL-6	Protection from bone loss caused by estrogen deficiency	71
IL-1 receptor	Prevention of ovariectomy-induced bone loss	72
Collagen II α1	Lack of extracellular fibrils in cartilage with disorganized chondrocytes, absence of endochondrial bone and growth plate in long bone	73
BMP7	Holes in the basisphenoid bone and xyphoid cartilage, retarded ossification of bones, fused ribs and vertebrae, a kinked tail, polydactyly of the hind limbs	74
LIF receptor	Severe osteopenia of perinatal bone associated with increased osteoclast number	75
FGF receptor 3	Deafness, kyphosis, scoliosis, crooked tails, curvature and overgrowth of long bones and vertebrae	76
Gli 2 and 3	Lack of trabecular bone, presence of calcified cartilage and absence of primary ossification center in the diaphysis	77
IGF-1	Delayed bone ossification	78
PTHrP	Abnormal endochondral bone formation and lethal skeletal displasia	79
TGF-β1	Decreased bone mineral content and longitudinal growth and elasticity, dental abnormalities	80, 81
NF-KB1 and NF-KB2	Osteopetrosis due to lack of osteoclast differentiation	82
GMCSF (OP/OP)	Osteopetrosis	83
Osteopontin	Altered osteoclast function *in vitro*	84

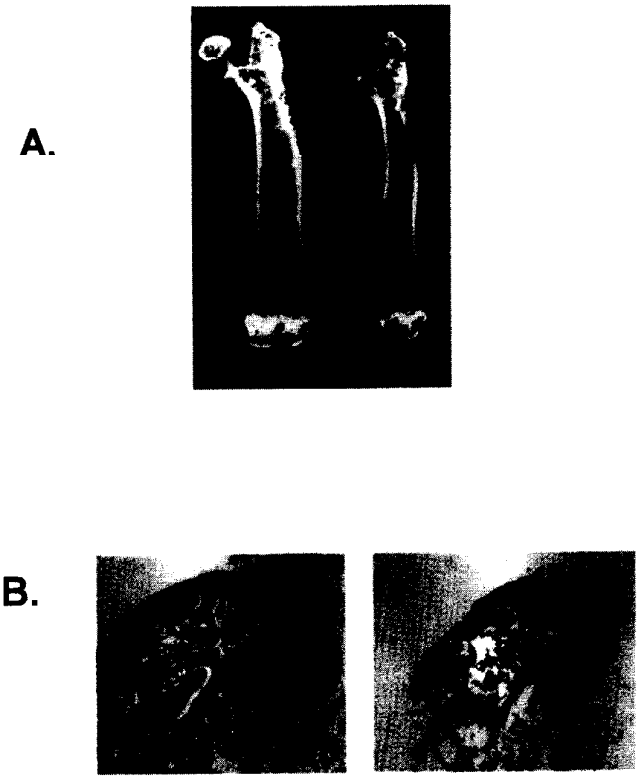

FIGURE 4.4 Femurs of transgenic mouse with knockout of biglycan gene. (A) Faxitron of femurs of 6-month-old normal (+/0) and biglycan (−/0) mice. The mice are designated with a second alelle "0" because biglycan is located on the X chromosome; male mice have no second biglycan allele. (B) Sections through the femoral head of the bones shown in panel A. Note the relative loss of trabecular volume designated by an* and thinning of the cortex in the mutant (0/) compared with normal littermates (0/+).

targeted gene deleted also in a tissue-specific manner.[86] In the future, one can expect to see mice conditionally knocked-out or mutated in specific skeletal cells such as osteoblast and osteoclast or in any place where cell-specific promoters become available.

4.6.2 Point Mutations or Subtle Gene Alterations

In an attempt to refine the knockout technology, further new methods were developed to create small (not total knockout) changes in genes. This is an ideal way to study protein structure–function relationships *in vivo*. This strategy is referred to as "hit and run,"[87] "in out,"[88] "tag and exchange,"[89] or "double replacement."[90] Essentially, the technique uses two homologous recombination steps: one to insert selectable markers (sometimes called a cassette) for targeted gene uptake (the hit) and a second step to replace the normal gene with a mutated version (the run or replacement). This strategy was successfully used to create a "site-directed" mutation in the collagenase cleavage site of type I collagen. Animals with this mutation showed skin defects and altered osteocyte activities.[91]

4.6.3 From Knockout to Knockin

Variations of the gene targeting have also been used to replace one gene for another such that the inserted gene is under the control of the replaced gene. This is done to determine whether a structurally similar

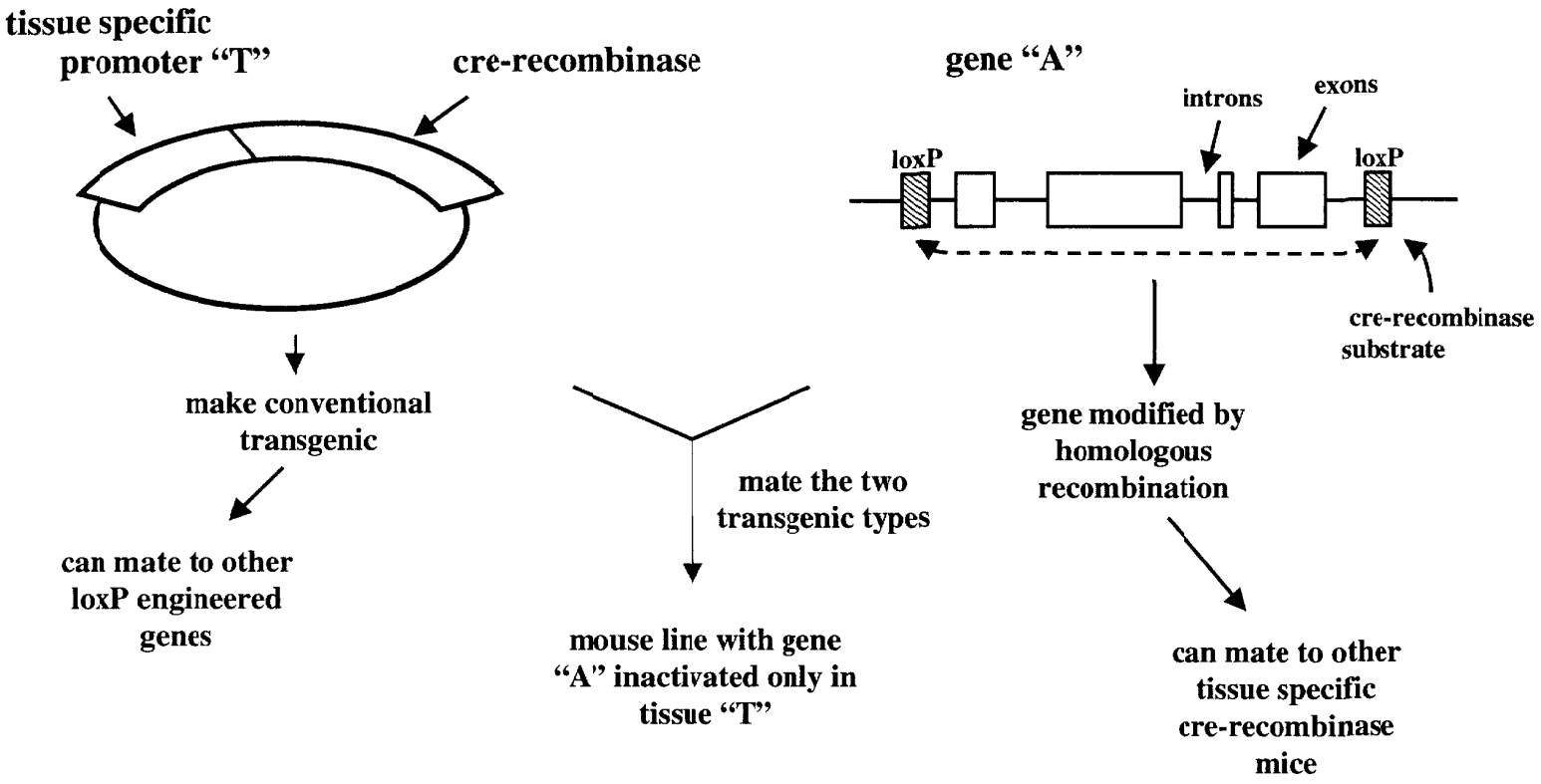

FIGURE 4.5 Construction of cre-recombinase tissue specific knockouts. This experiment uses a combination of conventional transgenic mice (described in Fig. 4.1) and targeted (homologously recombined) genes. In the first case a mouse line is made that expresses the enzyme cre-recombinase under the direction of a tissue-specific promoter. A second mouse line is made that has loxP sites engineered at either side of the gene being tested for function. LoxP sequences are removed in the presence of cre-recombinase. By breeding two mice lines, a third line is created that will inactivate the gene with the loxP sites only in a select tissue and are considered "conditionally" knocked out. The first two lines made by this approach can be bred to other mice in any number of combinations ultimately to create mice that have inactivated genes in any tissue for which specific promoters are available.

protein could "rescue" the ablated gene if expressed at the same time and place during development.[92] One could imagine testing, for example, whether the actions of the many isoforms of TGF-β are interchangeable in the context of a null background. In this case one could exchange TGF-β1 for TGF-β2 (under the control of TGF-β1 promoter) to determine if the second isoform could compensate for the action of the first. Undoubtedly, new phenotypes will continue to arise from these types of experiments creating even more models to test for bone structure and function.

4.6.4 Inducible Gain of Function: Timing Is Everything

To control the production of the transgene, new techniques were devised where genes can be induced or repressed in the live organism through the administration of a soluble agent. Two examples in this category use either ecdysone, which is a fly protein,[93] or tetracyline,[94] which represses or induces genes depending on the DNA used for construction. This latter procedure has been used in numerous studies using cultured cells and should be useful to examine the role of a gene at a specific window of time during the life span of the mouse.

4.6.5 Tissue-Specific Gene Inactivation: Combining Space and Time

By using the trangenic technologies described, it is now possible to destroy specific cell types by connecting a poison to a cell-specific promoter. This has been done using diphtheria toxin[95,96] or the TK gene. This latter approach, which relies on the subsequent application of gancylovir, has an advantage because the timing of cell destruction can be controlled. By creating trangenic mice that had the TK gene under the control of the osteocalcin[97] or type I collagen promoters,[98] the osteoblasts in the skeleton were inactivated leading to induced osteopenia. An advantage of this procedure is that it is reversible or "conditional"; withdrawal of the drug removes the toxic effects on the osteoblast and subsequently leads to a wave of new bone formation.

4.7 Summary

In summary, there are numerous ways to create trangenic mice and each method has advantages and disadvantages. With conventional transgenics the overexpressed gene must have effect over the endogenous one. Further, because the transgene is randomly integrated into the host chromosome, it is possible that it can become inactivated or expressed in an unusual pattern due to effects from nearby gene sequences. Integration of foreign DNA could also inactivate genes within the host.[48] If the overexpression of the gene is lethal, inducible methods are an alternative. Some methods differ genetically but have a similar outcome; a dominant-negative tissue-specific transgene is quite similar to a cre-recombinase tissue-specific knockout. In either case, the gene of interest would be inactive or not expressed in a specific tissue.

Due to the nature of the way knockout mice are made, they should not have DNA integration effects if screened properly. There is, however, the possibility of gene redundancy where no phenotype is detected because a second gene takes over the function of the ablated gene. Knowing the structure and abundance of related genes, one can create mice with "double knockouts." This is done by breeding two separate single knockouts with each other through two generations (the first generation is all heterozygote). Using this strategy, the authors have discovered that the small proteoglycan decorin partially compensates for biglycan in regulating bone mass.[99]

Having created a mutant mouse line, an evolving trend is to combine all aspects of basic biology in the analysis of the transgenic. For example, knowing that bone formation is affected in a particular mouse model (see Chapter 1) one might isolate bone-forming cells and study their activity in culture (see Chapter 2). Detailed molecular analysis could then be carried out using the procedures outlined in Chapter 3. Mineral character and composition would also be critical to examine either *in vivo* or *in vitro* (see Chapter 5). By mixing and matching the host of genetic features available, an "Alice's Restaurant" picture emerges: "You can get anything you want." In summary, anything is possible in creating transgenic mice; one is limited only by well-characterized genes and promoters and the proper analyses.

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