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Perspective Article

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Genetically altered mice for bone research

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The ability to make genetic alterations in mice has had dramatic effects on the ability of bone scientists to identify the roles of specific genes in the *in vivo* setting. Particularly because the specific shapes and microarchitecture of bone closely determine the function of bone, the use of intact bones in these studies has been a particularly important advance. Here we will briefly review the progressively more sophisticated manipulations that have made these studies possible.

Injection of foreign DNA into fertilized mouse eggs, followed by the generation of mice from these eggs leads to the introduction of the foreign DNA into generally random sites in the mouse genome. By using DNA that contains transcriptional control regions, the resultant transgenic mice can express a gene of interest in tissues specified by those control regions. The number of copies of the transgene and the random sites of gene insertion lead to variable levels of expression of the transgenes, though this variation can lead to interesting results, when distinct transgenic mice are compared. The most difficult challenge in designing such mice is the choice of transcriptional control region. Often sequences required for specific and efficient gene expression in a cell type are unknown and cannot be predicted by studies in cultured cells. Consequently, trial and error is the most common approach used to design such regions. Recently, the use of very large segments of foreign DNA, carried on bacterial artificial chromosomes has made it possible to use extremely large control regions, thereby simplifying the trial and error process. Nevertheless, in all transgenic settings the most important and difficult task is the assessment of whether the transgene's control region provides the cellular specificity desired.

The use of murine embryonic stem (ES) cells for generation of genetically altered mice has been an important advance. Embryonic stem cells are useful because they can populate all the tissues of the embryo (and subsequent adult mouse) after they are injected into a host blastocyst. Germ cells from these mice can then pass along the genetic information carried in the embryonic stem cells. These cells can also be grown indefinitely in culture dishes and subjected to standard genetic manipulations. The power of embryonic stem cells results from this flexibility: cells can be manipulated *in vitro* and then converted in a series of straightforward steps into mice reflecting the *in vitro* manipulations. Long stretches of genomic DNA can be introduced into ES cells in a way that encourages the DNA to target specifically to the corresponding chromosomal region in the host ES cell. These long stretches of exogenous DNA can contain slightly altered versions of endogenous genes. If these alterations lead to deletion of crucial portions of a gene, then the resultant ES cell will be missing the normally functioning gene. Mice derived from such ES cells are called knock-out mice because the gene has been knocked out of the genome. In analogous fashion, the exogenous DNA can have limited differences from the endogenous gene that change but do not totally destroy the gene's functions. Resultant mice are called knock-in mice, after the variant gene that has been introduced into the genome.

Use of knock-out and knock-in technology allows the generation of mice that differ from littermates only in the expression of one particular gene. Studies of the bones of such mice have led to powerful conclusions about the functions of these genes in normal mice. Limitations of the methodology include the possibility that multiple genes provide the function of the studied gene. Consequently, when the gene is knocked-out, the resultant phenotype may be minimal, even though the gene contributes to an important function. Further, the analysis of phenotypes can be challenging. Effects of gene alteration may vary across developmental and post-natal time and may also vary in response to perturbations such as fracture, inflammation, hormonal changes, and so forth. Investigators often find that after they have made a genetically altered mouse, they have in fact completed the easiest and quickest part of their project!

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Another very important limitation of standard gene knock-out and knock-in technology is that all cells expressing the gene of interest express the altered gene and they do so from the time of conception. That is fine for some purposes, but, for example, if an altered gene is expressed in osteoblasts, but also in the brain, the investigator must determine what fraction of the mouse's phenotype results from direct effects on osteoblasts instead of indirect effects from brain expression. Further, if the genetic alteration affects bone development, the resulting bones may be abnormal and not be suitable for study of the effect of the genetic alteration on normal adult bone. Worst of all, the genetic alteration may lead to developmental abnormalities incompatible with life and entirely preclude study of the genetic alteration in adult bone. For these reasons, techniques have been developed to knock genes out only from certain cells and at certain times. Genes are removed from particular cells by making transgenic mice that express nucleases only in specific cell types, determined by the regulatory region used to generate the particular transgenic mouse. The nucleases, called cre-recombinase or flp-recombinase, are nucleases that cleave DNA only at specific sites designated by specific sequences not found in the normal mouse genome. These sites, called "loxP" sites for cre and "frt" sites for flp are introduced into the target gene of interest using the knock-in approach with ES cells. The resultant mouse behaves normally, because the loxP sites, for example, are introduced in "harmless" sites that flank key sequences in a gene of interest. Then this so-called floxed mouse is mated with the mouse expressing cre, for example, only in osteoblasts. The resultant progeny will express cre in osteoblasts and, in those cells only, cleave the gene of interest at the loxP sites and religate the DNA after deleting the DNA found between the loxP sites. In this way, the gene of interest will be removed only from the cells that express cre.

Further refinements of this technology depend on strategies to limit the time of expression of the cre molecule. Two strategies are commonly used. In one, the cre gene is covalently bound to the ligand-binding domain of the estrogen receptor (ER). The estrogen receptor sequence has been modified so that it doesn't bind estradiol at physiological levels, but binds tamoxifen well. The resultant cre-ER molecule is found in the cytoplasm because of the binding of the ER to cytoplasmic heat shock proteins. As a result, the cre nuclease cannot cleave DNA. When the mouse is given tamoxifen, however, the modified ER binds the tamoxifen and the cre-ER moves to the nucleus where the cre can act on its target gene.

The second strategy uses a tetracycline resistance regulatory system borrowed from bacterial plasmids and modified for use in mammalian cells in order to regulate expression of the cre recombinase gene. The tetracycline repressor is a protein that binds a DNA target sequence; binding of tetracycline derivatives by the protein blocks DNA binding. The tet repressor has been fused to a sequence from the herpes viral VP16 transcription factor to form the tetracycline-binding gene activator called tTA. In the absence of tetracycline derivatives, tTA can bind to target DNA sequences and activate transcription of adjacent genes, such as cre recombinase. When tetracycline is present, however, tTA does not bind to DNA and will not activate genes ("tet-off"). In a further modification of this system, tetracycline is required for DNA binding of a modified transactivating protein (rtTA). In this setting, ("tet-on"), a tetracycline derivative is required for activation of an adjacent gene encoding cre recombinase.Using these approaches, the expression of cre recombinase can be limited to only specific cell types and only when appropriate manipulation with tamoxifen or tetracycline occurs. In this way, a target gene flanked by loxP sites can be removed from cells of interest only when desired.

In the talk, I will illustrate the power of these methods, with a particular focus on their use in studying bone function.

Reference

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