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Modeling Rare Bone Diseases in Animals

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Abstract

Purpose of review—The goal of this review is to highlight some of the considerations involved in creating animal models to study rare bone diseases and then to compare and contrast approaches to creating such models, focusing on the advantages and novel opportunities offered by the CRISPR/Cas system.

Recent findings—Gene editing after creation of double-stranded breaks in chromosomal DNA is increasingly being used to modify animal genomes. Multiple tools can be used to create such breaks, with the newest ones being based on the bacterial adaptive immune system known as CRISPR/Cas.

Summary—Advances in gene editing have increased the ease and speed, while reducing the cost, of creating novel animal models of disease. Gene editing has also expanded the number of animal species in which genetic modification can be performed. These changes have significantly increased the options for investigators seeking to model rare bone diseases in animals.

Keywords

Gene-editing; rare bone disease; animal model

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Compliance with Ethical Guidelines

Conflict of Interest

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Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

INTRODUCTION

In the past several years, the study of animal models carrying either spontaneous or induced genetic mutations has dramatically improved our understanding of gene function and basic physiological processes. The generation of animal models that closely mimic human disease, and often bear identical genetic lesions as those identified in patients, has become an essential requirement for studying underlying pathogenic processes. Today these animal models constitute an important tool for the discovery and testing of new therapeutic targets in pre-clinical research. Initially only few spontaneous animal mutations were available for study and characterization, for example fragilitas ossium mutations (1), often without knowing the underlying impacted gene. The progressive development and refinement of genetic strategies applied to the laboratory mouse, such as transgenesis, gene targeting, conditional gene inactivation and, more recently, gene editing have made it possible to generate mice (and now other animals) with a tissue-specific gain-of-function or loss-of-function of any gene of interest at an ever increasing speed. Although physiological differences exist between rodents and humans, and issues of model validity and phenotype reproducibility between species have been raised (2;3), when it comes to the musculoskeletal system, most of the signaling pathways acting on the murine skeleton are conserved in humans. Thus, the possibility to generate mouse models that both genetically and phenotypically reproduce a rare bone disease, which perhaps affects only a few patients in the world, provides the means to obtain a controlled population for study in the same place, at the same time, often in a genetically homogeneous background, and under a rigorous scientific approach. In this short review, we will discuss old and new methods of genetic manipulations and how these have been, or will be, utilized to model rare diseases of the skeleton and highlight some of the advantages and limitations of each technique.

FROM HUMANS TO MICE

Genetic diseases of the skeleton, like most genetic diseases, have been studied using a reverse or a forward genetic approach. The former studies the function of a gene, or a DNA sequence, by creating a change in that gene (usually a deletion, e.g. KO mouse) and analyzing its phenotypic effects on a model organism. The latter approach aims to identify the gene (or DNA sequence) that is responsible for an observed phenotype in a model organism or a patient/family. Occasionally, different research groups may independently take one approach or the other in the study of a related project and the published results can provide mutually informative evidence that is key to a new discovery. Such was the case, for instance, that led to the identification of *CRTAP* mutations in recessive osteogenesis imperfecta (OI) (4). Glorieux and co-workers described a recessive rhizomelic form of OI (OI type VII) in a small First Nations community in Northern Quebec (5) and mapped the disease critical region to chromosome 3p22-24.1 (6). The region contained 18 genes (but none of the collagen genes) and, at a time when next generation sequencing was not yet developed, the culprit gene was not identified. Concurrently, another group, using a reverse genetic approach, was studying the functional role of the *Crtap* gene in the skeleton and generated *Crtap*KO mice. These mice showed a severe osteo-chondrodysplasia with dramatic osteopenia and altered type I collagen synthesis, consistent with an OI phenotype. Because the human ortholog of *Crtap*, mapped to chromosome 3p22 (7) and within the

previously identified OI VII critical interval, the published results from the group in Montreal provided an essential hint that pointed to *CRTAP* as a new OI candidate gene. The sequencing of the entire *CRTAP* gene from the genomic DNA of two OI VII First Nations probands ultimately identified a homozygous single nucleotide change in intron 1 that led to abnormal splicing, frameshift, and degradation of the CRTAP transcript (4).

OI provides a good example of a clinically heterogeneous disease with multiple inheritance patterns and disease mechanisms that lead to very similar clinical manifestations in affected families. In fact, while mutations in either *COL1A1* or *COL1A2* cause dominantly inherited OI, mutations in *CRTAP* and a number of recently identified genes cause recessive OI and mutations in *MBTPS2* cause an X-linked recessive form of OI (for a recent review see (8)). Moreover, dominant OI due to type I collagen mutations can cause mild to severe disease depending on the underlying genetic mutation. Because of this, several different mouse models of OI were created that mimic more or less severe forms of the disease (reviewed in (9)).

An important example in the field of rare bone diseases comes from the study of the *LRP5* gene, a member of the low density lipoprotein receptor family which functions as a co-receptor for WNT ligands (10). Biallelic loss-of-function mutations in *LRP5* cause severely decreased bone mass and eye development abnormalities in patients, a disease known as osteoporosis-pseudoglioma syndrome (10). Two independent studies identified families with genetically inherited high-bone mass and identified an identical missense mutation (Gly171 to Val) affecting a highly conserved amino acid residue in the extracellular portion of the LRP5 receptor (11;12). Interestingly, this amino acid change acts as a gain-of-function mutation and causes loss of inhibition of the WNT signaling pathway (13;14). Other families were identified with increased bone density, including autosomal dominant osteosclerosis, van Buchem disease, endosteal hyperostosis and even type I osteopetrosis and found to be carrier of *LRP5* variants, likely to be pathogenic (15). Moreover, additional mutations affecting the *LRP5* gene were also associated with familial exudative vitreoretinopathy, a disorder affecting the retinal vessel development (16;17). Therefore, the molecular genetics behind a certain clinical diagnosis is often complex and cases where mutations in different genes cause a similar phenotype (genetic heterogeneity) or different alleles cause different phenotypes (genetic pleiotropy) are rather common. These considerations must be taken into account when planning to create a new animal model of disease.

Certain diseases affecting the skeleton, whether common or rare, have an environmental component that needs to be taken into account for the generation of a faithful animal model. While the effects of secondary hyperparathyroidism on bone can be simply mimicked by administering a calcium-deficient diet to otherwise healthy mice, generating a mouse model for a disease whose etiology appears to have both a genetic and an environmental component is more challenging. Such is the case of Paget's disease of bone, where both a genetic predisposition and a viral infection were described as part of its pathogenetic process (18). The transgenic expression of the measles virus nucleocapsid gene in the osteoclast lineage causes abnormal osteoclasts and can induce Pagetic-like bone lesions in mice; however, it is likely that additional genetic predisposing factors (e.g. an *SQSTM1* mutation) are required

to faithfully reproduce all of the features observed in Paget's disease in the mouse model (19).

Additional rare diseases of the skeleton, such as Gorham-Stout disease (20), melorheostosis and others, are still currently poorly understood and occur sporadically without a clear etiology. Research studies on these rare conditions, such as a recent and interesting study on Gorham-Stout disease (21), are desperately needed to gain essential biological insight and allow the generation of useful animal models for their study.

GENETIC MANIPULATION OF ANIMALS

Transgenesis

A comparison of approaches to genetically modify mice is presented in table 1. The earliest attempts to model a human disease by modifying an animal genome utilized transgenic mice. Formally, a transgenic organism is one that harbors a gene or genetic material from another organism, regardless of the means of transfer. However, in modern mouse genetics, transgenic most often refers to mice that are produced by the method of pronuclear injection. Transgenic mice harboring a cloned gene were first created in 1980 by injecting a DNA construct into the pronuclei of fertilized mouse eggs (22). Although some modifications have been introduced for specialized situations, essentially this same approach remains the dominant method for creating transgenic mice (23). The fertilized eggs that will be injected are harvested from impregnated mice prior to fusion of the nuclei from the sperm and egg. The DNA is usually injected into the larger (male) pronucleus using a glass needle attached to a micromanipulator. Injected zygotes are then surgically implanted into the uterine horn of pseudopregnant mice leading to the birth of offspring that may or may not have undergone genomic integration of the injected DNA. In practice, roughly ten percent of offspring from such injections harbor the foreign DNA. Importantly, this approach leads to integration of the transgene into what appear to be random sites in the genome. In other words, there is no way to control the site of integration of the DNA construct using the standard approach. Moreover, the site of integration often affects the expression of the transgene due to regulatory elements near the integration site. This can lead to suppression of transgene expression or expression in cell types other than those that normally express the promoter used for the transgene. Therefore, each of the offspring harboring the transgene that is able to pass it to subsequent generations is maintained as a separate line of mice. Lines that display appropriate expression of the transgene can then be used for the desired experiments.

The majority of transgenic mice are designed to express a gene of interest in a specific cell type. In the context of rare bone diseases, this might involve expression of a mutant protein, previously identified in a patient population, in bone cells to explore the function of the mutant protein. An early example of this involved expression of a constitutively active mutant of the parathyroid hormone receptor in chondrocytes to mimic the human disease Jansen metaphyseal chondrodysplasia (24). Transgenesis is not usually suitable to study loss-of-function mutations. The cell type specificity of all transgenic mice depends on the activity of the gene regulatory elements included in the transgene construct. In many cases, such constructs include a DNA fragment that harbors the promoter region of a gene that is active in bone cells, such as osteoblasts. For example, different fragments of both the murine

and rat *Col1a1* gene have been used to drive the expression of numerous cDNAs in osteoblasts and osteocytes (25–32). Such constructs often do not accurately mimic expression of the endogenous gene from which the promoter was derived. If more accurate expression is desired, transgenes can be derived from large DNA fragments contained within bacterial artificial chromosomes (BACs). Such DNA fragments often contain entire genes as well as the necessary regulatory regions to faithfully mimic endogenous gene expression. One of the best known examples of this in the field of skeletal biology is the *Osx1-Cre* transgene, which expresses the Cre recombinase beginning at the earliest stages of commitment to the osteoblast lineage (33).

One of the major advantages of producing transgenic mice has been the speed with which such models can be produced, typically about six months. In addition, the cost of producing a new transgenic model has been considerably lower than for approaches involving gene targeting (discussed below). However, the costs and time saved can be offset by the randomness of transgene integration, which often requires screening of numerous lines to obtain a useful model. In addition, a major disadvantage of transgenesis is that the endogenous wild type version of any mutant gene remains intact, precluding the use of this approach to study recessive mutations. Despite these limitations, the long history of transgenesis, together with the relative simplicity of the approach, has set the stage for the rapid development of gene editing approaches utilizing pronuclear injection of mouse zygotes.

Gene targeting

The first approach that allowed modification of a specific genetic locus required the use of mouse ES cells and was termed gene targeting (34;35). Two properties of ES cells are essential for this approach. First, homologous recombination occurs efficiently in mouse ES cells, relative to other cell types. Second, mouse ES cells can contribute to organ formation, including gonads, when injected into blastocysts isolated from pregnant mice and after implantation of the injected blastocysts into foster mothers. To change a specific locus, a DNA construct is created consisting of the novel sequence to be introduced that is flanked by homology arms that guide recombination at the target locus. The desired change can range from deletion of a sequence essential for the function of the gene (knockout) to insertion of a sequence encoding a reporter gene, such as a fluorescent protein (knockin). In addition, a sequence encoding a selectable marker is required to allow selection of cells that have undergone recombination. After introduction of the construct into ES cells by electroporation, appropriately targeted clones are identified by PCR or Southern blot, followed by blastocyst injection. This approach has been used to target thousands of genes and has provided a wealth of information regarding their function.

Early after the development of gene targeting, it was realized that homozygous inactivation of some genes resulted in embryonic lethality. To overcome this limitation, the Cre site-specific recombinase, and later the FLP recombinase, was developed as a tool to delete DNA sequences in a cell type-specific manner (36;37). The Cre recombinase is an enzyme derived from the P1 bacteriophage and recognizes a 34 bp DNA sequence termed the loxP site. When a DNA sequence is flanked by loxP sites that are in the same orientation, Cre-

mediated recombination deletes the intervening sequence (38). When the loxP sites are in opposite orientations, the intervening sequence is inverted by recombination. Cell type specific gene deletion is achieved by crossing mice expressing the Cre recombinase in specific somatic cell types with mice harboring a gene of interest in which one or more essential exons are flanked by loxP sites in the same orientation. Importantly, mice harboring such loxP-flanked genes (known as floxed or conditional null alleles) can be crossed with a variety of Cre-driver strains to examine the phenotype resulting from loss of gene function in different cell types.

In addition to allowing the creation of conditional null alleles, Cre-loxP technology allowed the development of mice harboring specific mutations to test the impact of such mutations on protein function. While gene targeting in general allows the insertion of a desired mutation into an endogenous gene, prior to the development of the Cre-loxP system, the DNA encoding the selectable marker required for gene targeting could not be removed and often negatively affected expression of the targeted gene. Thus, any phenotype that might be observed could be due to the mutation or to the altered expression caused by the selection cassette. By flanking the selectable marker with loxP sites, it can be removed leaving only a single 34 bp loxP site, which is much less likely to influence expression of the gene into which it has been inserted. Mice harboring the Amish *COL1A2* variant, which serve as a model of osteogenesis imperfecta, were generated using this approach (39). It is also possible to create novel alleles that express the wild type protein until acted upon by the Cre recombinase. This strategy was used recently to modify the endogenous murine *Gnas* gene to express a mutation that causes fibrous dysplasia only in Cre-targeted cell types (40). These studies revealed that expression of this activating mutation in the osteoblast lineage recapitulated many features of fibrous dysplasia.

A major advantage of gene targeting is obviously the ability to make specific changes at a desired locus. One disadvantage is the longer timeframe for development of gene-targeted models, which typically require at least one year. Because of this, and the requirement for mouse ES cells, the costs of creating gene-targeted mice are also significantly greater than those for creating transgenic mice. In addition, gene targeting is not currently possible in many species, due primarily to the lack of suitable ES cells. In such species, genetic modification of somatic cells followed by somatic cell nuclear transfer is possible, but this requires such a high level of expertise that it is not a common approach for academic laboratories.

Gene editing

Although transgenesis and gene targeting have been robust approaches to generate disease models in mice and other organisms, the advent of gene editing, also known as genome editing, is revolutionizing the way in which such models are produced (41). Gene editing occurs when a double-stranded break (DSB) is introduced in DNA at a specific locus using an engineered nuclease (Figure 1). The break is then repaired by endogenous cellular machinery using one of two different pathways. Non-homologous end joining (NHEJ), as implied by the name, ligates broken DNA without regard to the sequences near the break. Repairs made by this pathway often result in small insertions or deletions (indels), which

can affect gene function if the break occurs in a protein-coding exon. Alternatively, if a DNA fragment is provided that is homologous to the DNA flanking the DSB, this fragment can be used as a homology donor to repair the break via a process known as homology directed repair (HDR). Although less efficient than NHEJ, HDR can be used to insert new sequences into the region of the DSB, allowing the creation of specific disease-causing mutations, as well as reporter genes and conditional alleles. Similar to transgenesis, gene editing can be performed in primary cells, cell lines, and in developing or adult organisms. [Figure 1]The first types of engineered nucleases that allowed creation of a DSB at a desired site were designer zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (42). In both cases, synthetic proteins are designed to recognize a specific DNA sequence. These domains are then fused to the FokI nuclease domain, which only functions as a dimer, so that two separate engineered nucleases must be created to cut a desired site. Both of these approaches have proven to be reliable tools for gene editing. However, both have the major limitation that a new pair of nucleases must be designed for each new DNA sequence that one would like to cut. Because the design process is complex and requires specialized expertise, the majority of laboratories seeking to utilize these tools must rely on collaborators or commercial entities for the design and production steps.

Beginning in 2013, a new tool to create DSBs was developed using components of the prokaryotic adaptive immune system known as CRISPR-Cas (43). CRISPR is an acronym for Clustered Regularly Interspaced Short Palindromic Repeats and refers to sequences in prokaryotic genomes that encode small RNAs complementary to viral DNA genomes. Cas refers to CRISPR-associated proteins. In general, Cas proteins act as double-stranded DNA nucleases that are guided to target sequences by binding to small RNAs that are complementary to the target sequence. The components of the system from *Streptococcus pyogenes* have been the most widely used as molecular tools and consist of two small RNAs and the Cas9 protein. To make the system easier to use, the two small RNAs have been artificially joined to create a single guide RNA (sgRNA). To target a desired region of DNA, one simply needs to change the first 20 nucleotides of the sgRNA to a sequence complementary to the target, with the only limitation being that this 20 bp sequence must be upstream from the sequence NGG, known as the protospacer-adjacent motif (PAM). CRISPR-Cas systems from other species use different PAM sequences, vastly expanding the targeting ability of these tools. Nonetheless, since the sequence NGG occurs, on average, every 10 bp in the mouse genome (44), the *Streptococcus pyogenes* CRISPR-Cas system has been used extensively to create gene-edited mice.

Compared to either ZFN or TALENs, generating DSBs with CRISPR-Cas systems is much easier, less expensive, and faster (45). Indeed, most laboratories familiar with standard molecular biology methods can rapidly produce and use CRISPR-Cas nucleases. Moreover, the majority of core facilities involved in creating genetically modified mice have implemented CRISPR-Cas-based approaches. One reason for this is that almost any desired genomic modification can be achieved using CRISPR-Cas with efficiencies equal to or better than gene targeting with ES cells. This approach is especially amenable to altering murine genes to match rare mutations identified in orthologous human genes. The speed with which such models can be created is unprecedented. For example, a mouse model for a loss-of-function mutation can be designed and created in as little as ten weeks. Similarly,

mice with a specific missense or nonsense mutation require a similar timeframe. The latter models require a small homology donor DNA that is usually in the form of a synthetic oligonucleotide. Models requiring the insertion of loxP sites or DNA fragments of hundreds to thousands of bps usually require more time due to the low efficiency of recombination using large homology donors. However, efforts to increase the efficiency of gene editing with larger DNA fragments are being pursued aggressively.

Other than the limited recombination efficiency of HDR with large DNA fragments, the other main limitation of CRISPR-mediated gene editing is fidelity. Specifically, CRISPR-Cas nucleases can create DSBs at sites other than perfect matches to the sgRNA (46). The number of these so-called off-target cuts depends on the particular sgRNA, with some exhibiting essentially no off-target cuts while others can target numerous sites (47). As might be expected, off-target cuts result from homology with the sgRNA sequence. Thus the major approach to limit off-target activity is to identify a sgRNA target with little homology to other sites in the target genome. However, when the goal is to target a specific region to create a disease model, there may be limited choices for sgRNA candidates. Several modifications have been made to the CRISPR-Cas system to increase fidelity, such as shorting the length of sgRNAs and structural changes to the *S. pyogenes* Cas9 protein (48;49). Additional studies will be required to determine the effectiveness of these new tools in the hands of multiple laboratories. Fortunately, the limitation of low fidelity can be largely overcome by backcrossing the mutant animals to wild type breeders, thereby diluting and essentially eliminating any off-target mutations.

In addition to gene editing, the CRISPR-Cas system provides a platform for many new approaches (50). One that may be particularly relevant for models of rare bone diseases would be one that provides an alternative to the Cre-LoxP system for cell type-specific loss- or gain-of-function. A significant limitation of the Cre-LoxP system is that most Cre-driver strains display recombination activity in cells other than the desired cell type. For example, the Dmp1-Cre strains used to drive recombination to osteocytes display activity in osteoblasts and myocytes, as well as other cell types (51;52). Similarly, the Osx1-Cre transgene is active in a broad range of tissues (53). Because of this, results obtained from these models, particularly those that identify the cell type through which a particular gene mutation causes pathological changes, must acknowledge this limitation. Therefore, a CRISPR-Cas-based approach that provides better cell type-specificity would clearly be of value.

Early after the development of CRISPR-Cas-based gene editing, mutants of Cas9 were created that lack nuclease activity but retain the ability to be guided to a specific genomic locus by a sgRNA (54). Positioning of this inactive or “dead” Cas 9 (dCas9) immediately downstream from the transcription start-site of a gene is sufficient for moderate suppression of a gene of interest (54). Fusion of an active repression domain, such as the Kruppel associated box (KRAB) domain of Kox1, with the dCas9 potentially increases the repression activity of this complex (55). Conversely, targeting of dCas9 fused to a transcriptional activation domain to the transcription start-site can be used to increase the expression of a gene of interest (55). These approaches, known as CRISPR inhibition (CRISPRi) and CRISPR activation (CRISPRa), respectively, have many potential uses, including genome-

wide loss- and gain-of-function screens. They may also prove useful as an alternative to the Cre-LoxP system for conditional loss- and gain-of-function in animal models.

For example, to suppress a desired target gene in osteocytes, one could create a single transgene expressing the dCas9::KRAB fusion protein under the control of a Dmp1 promoter fragment. This transgene could also contain a cassette for expression of the sgRNA targeting the transcription start-site of the gene of interest. Whether such a system would display better cell type-specificity than the Cre-LoxP system remains to be determined. However, unlike the all or nothing aspect of Cre-mediated recombination, small amounts of Dmp1-dCas9::KRAB expression in cells other than osteocytes may not lead to measurable target gene suppression. If such a system does display good cell type-specificity, another advantage would be that only a single genetic modification would be required for this type of model, as opposed to the two modifications required for the Cre-LoxP system. The savings in time and money for breeding would be substantial.

Perhaps one of the most exciting aspects of the CRISPR-Cas system is that it is promoting the development of non-rodent disease models. The contribution of mouse and rat models to our understanding of skeletal biology and disease is unquestionable. Nonetheless, use of these rodents has limitations with respect to the skeletal system. Paramount among these is the lack of clear Haversian remodeling. While osteon-like structures can occasionally be observed in mice and rats, these structures lack the organization seen in larger vertebrates. A recent study has shown that osteon size positively correlates with body mass in mammals and suggested that the lack of clear osteons in small mammals may be related to the need to avoid fractures (56). Be that as it may, dysregulation of Haversian remodeling, such as may occur with aging, remains difficult to study in rodents (57).

Of commonly used laboratory animal models, rabbits are the smallest and most economical species that exhibit Haversian remodeling. Importantly, the effectiveness of CRISPR-Cas in rabbits has been repeatedly demonstrated. For example, a rabbit model of X-linked hypophosphatemia (XLH) was recently created by CRISPR-Cas-mediated inactivation of the *PHEX* gene (58). Moreover, the *Rosa26* locus, which has been used extensively as a safe harbor locus for transgenes in mice, has been identified and utilized for CRISPR-Cas-mediated knockin in rabbits (59). Because of their relatively short gestation and maturation time, it is likely that the genetic modification of rabbits to study skeletal diseases will increase over the next several years.

CONCLUSIONS

Advances in multiple technologies herald a new era in the development and use of animal models of rare skeletal diseases. These include the ability to rapidly identify genetic changes in individuals with known or novel skeletal abnormalities and then to rapidly create analogous changes in animal models using technologies such as gene editing. Such models have already proven their worth by guiding the development of novel therapies. It is likely that continued advances in gene editing will eventually lead to the ability to correct disease-causing mutations at an early stage and effectively prevent or cure many of these conditions.

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Figure 1. Gene Editing with CRISPR-Cas

The Cas9 nuclease (shown in yellow) is targeted to a specific sequence in the genome by a sgRNA. Such sequences must reside upstream from the sequence NGG. The double-strand DNA cut can be repaired by Non-Homologous End Joining (NHEJ) or by Homology Directed Repair (HDR). HDR requires donor DNA with homology to the regions flanking the cut site.

Table 1

Comparison of gene modification approaches

	USEFUL FOR	ADVANTAGES	DISADVANTAGES	COST
Transgenesis	Global or cell type-specific over-expression; promoter-enhancer gene reporter studies; cell type-specific Cre expression	Well-established; low-cost; relatively rapid	Possible insertional mutation of endogenous genes; requires multiple founders; random insertion	\$
Gene targeting via homologous recombination	Gene knock-out; gene knock-in	Precise deletions or insertions	Low-efficiency; time-and effort-intensive; high-cost	\$\$\$
Gene editing using CRISPRs	Gene knock-out; gene knock-in; small, precise sequence changes	low-cost; relatively rapid; efficient; rapidly improving	Potential off-target effects	\$