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Review

Central regulation of bone mass

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ABSTRACT

The traditional view of skeletal homeostasis as a primarily endocrine activity has been expanded in recent years following the identification of direct neural pathways controlling bone homeostasis via central relays. Powerful control over both anabolic and catabolic activities have been isolated to neurons of the hypothalamus, enabling large changes in bone mass to be achieved by minute changes in the levels of these central neural signals. Initiated by studies of leptin and expanding rapidly, the breadth and complexity of this regulatory axis to bone is sure to increase. Critically though, the translation of these findings into therapeutic interventions is likely to present a greater challenge. However, the contribution to our understanding that these initial studies are making indicates an exciting potential to help to alleviate the growing challenge presented by musculoskeletal disease.

Contents

1. Control of bone remodelling

The skeleton is a dynamic set of tissues, constantly remodelling itself by the coordinated removal and replacement of minute quanta of bone by dedicated cell types, the osteoclast and osteoblast, respectively. A delicate balance between these processes is essential for maintaining both the mechanical and mineral homeostatic functions of the skeleton. Imbalance can lead to weakening which, unfortunately, is all too common, with fragility fractures due to osteoporosis extremely prevalent in the aging population, and associated with marked morbidity and mortality [1].

Historically, the prevailing view has been that bone remodelling is controlled in a predominantly endocrine manner, whilst simultaneously responding to local mechanical stimuli. Recently however, there is increasing evidence that the central nervous system contributes direct regulatory influence upon bone homeostasis via efferent neural connections. Immunocytochemistry studies initially revealed the presence of innervation and receptors for neuropeptides in bone cells, which strongly support a direct role of the nervous system in these cells ([2] – for review). More recently, retrograde trans-synaptic tracing has identified neural tracts from the

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femoral bone marrow linked direct to the central nervous system [3]. Together, these observations indicate the existence of a neuronal pathway between the brain and the bone. However, it was murine mutant models that provided the most powerful evidence for direct central control of bone mass and the importance of these murine studies to the increase in our knowledge is without question. The relevance of these models to human health, however, is yet to be fully realized, as such, where possible, human studies will be included to highlight the potential of this emerging and exciting field.

2. Dichotomous skeletal effect of leptin in mice

Interestingly, the initial identification of a direct neural output from the hypothalamus to bone came about from study of a circulating factor, leptin. Leptin, a 16 kDa protein, is a hormone secreted primarily by white adipose tissue, circulating in proportion to adipose stores and acting as an adipostat [4]. In 2000, several studies describing the skeletal effects of leptin using mutant mouse models were published. Histomorphometric analyses showed that mice lacking leptin (*ob*/*ob*) or its receptor (*db*/*db*) had higher cancellous bone volume associated with increased bone turnover [5]. This occurred despite concurrent hypogonadism and hypercortisolism, which favour bone resorption, establishing leptin's powerful anti-osteogenic effect on cancellous bone. Conversely, later that year, another group reported leptin as a potent stimulator of bone growth in *ob*/*ob* mice. Leptin treatment in young *ob*/*ob* mice increased both cancellous and cortical mineral content of the femora [6]. Leptin treatment also increased femur length, total body bone area, bone mineral content (BMC) and density (BMD) when compared to vehicle-treated controls. Similar results were observed in 15-week-old *ob*/*ob* mice treated with subcutaneous leptin infusion [11].

These apparently conflicting findings suggest differing effects of cortical and cancellous bone in *ob*/*ob* mice. Indeed, subsequent analysis has demonstrated envelope specific effects in leptin-deficient mice. Consistent with both initial studies, cancellous bone volume was greater in *ob*/*ob*, associated with elevated turnover [7], while total body bone mineral content, cortical area and mineralizing surface of the femur were reduced compared to normal mice [6,8–10]. While leptin treatment increased whole body BMC and endosteal bone formation in the *ob*/*ob* mice, no differences were seen in wild-type mice [11]. These data indicate that the primary effect of leptin deficiency on the skeleton is a reduction in cortical bone formation, which comprises 80% of the murine skeleton. In addition, to envelope specific effects, *ob*/*ob* mice also display altered responses between the axial and appendicular regions. Leptin-deficient mice were found to have increased vertebral length, lumbar BMD and cancellous bone volume but shorter femur length, femoral BMD, cortical thickness, compared to lean wild-type mice [12].

A number of factors may influence the skeletal response to leptin deficiency, not the least being, the many endocrine changes associated with the loss of this pleiotropic hormone. One suggestion is that the sparing of cancellous bone during periods of diminished serum leptin may act to preserve mineral stores during periods of food restriction [13]. Consistent with such a notion, caloric restriction in mice reduces total bone mass but increases bone mass in the spine [14]. In addition, to caloric influences, the differential innervation of axial and appendicular bones may contribute to the regional differences observed. Differences in bone marrow composition are also suggested, which are illustrated by the high adiposity of *ob*/*ob* femurs compared to vertebrae [12]. A recent article suggested a bimodal threshold response to serum leptin levels, with low-dose leptin preventing bone loss, while high-dose leptin inhibiting bone growth [15]. However, despite the complexity of leptin's actions on bone, the mode of action proved a paradigm shifting discovery.

3. Central effects of leptin on bone

One of the critical developments in the area of bone metabolism in recent years has been the discovery of a central locus for the regulation of bone mass. The long form of the leptin receptor (*Ob-Rb*), which performs signal transduction, is found within the hypothalamus [16]. A shorter form of the leptin receptor (*Ob-Ra*) is co-expressed with *Ob-Rb* in the hypothalamus where it is thought to facilitate transport of leptin into the brain [17]. Critical to the notion of a central axis of leptin action, intracerebroventricular (icv) infusion of leptin, without detectable leakage in the blood stream, was able to correct the cancellous bone phenotype of ovariectomised *ob*/*ob* mice [5]. Moreover, in wild-type mice, the selective destruction of *Ob-Rb* positive hypothalamic neurons by gold thio-glucose increased cancellous bone mass, with icv leptin treatment no longer able to correct the cancellous phenotype [18]. Together, these results indicated, for the first time, that the central hypothalamic pathway is sufficient to exert leptin's effect on cancellous bone. Recently, the central leptin pathway was studied using a more targeted approach. A single hypothalamic icv injection of leptin producing adeno-associated virus in *ob*/*ob* mice resulted in normalization of body weight and recapitulation of wild-type skeletal phenotype [19]. Femoral and vertebral cancellous bone volumes were reduced to wild-type levels, while femur length and total femur bone volumes were increased to wild-type level. This reinforces the anti-osteogenic effect of leptin on cancellous and pro-anabolic actions on cortical bone as described previously, more importantly, originating from the same region of the brain. The study of leptin has firmly established the hypothalamus, as not only the seat of endocrine control of bone, but also critical in the direct neural control of this tissue.

4. Association of leptin and bone in humans

Epidemiological evidence shows that obesity is correlated with increased bone mass, and that a reduction in body weight may cause bone loss [20,21], while low body weight is a key risk factor for fracture and mortality post-fracture [22]. Taken together, these data suggest a protective effect of fat mass on bone [23] and as such a positive relationship between leptin and bone mass. However, correcting for the mechanical loading effect of body weight on bone mass has revealed both positive [24] and negative associations [25], suggesting further complexity in this relationship. One of the most likely sources of complexity in the relationship between leptin and bone mass in humans involves the development of leptin resistance, which increases markedly with increasing fat mass and age, involving the down regulation of leptin receptor expression [26,27]. A large meta-analysis of leptin and BMD in nearly 6000 postmenopausal women found that less than 1% of the variation in BMD was explained by leptin with no significant correlation with femoral neck or lumbar spine BMD [28]. Similarly, exogenous leptin supplementation has no significant effect on weight loss in humans [29]. In addition to leptin resistance, hormonal abnormalities associated with obesity, such as insulin resistance, hypogonadism and hypercortisolism further complicate the characterization of leptin's action on bone in obese patients.

While hypogonadism and hypercortisolism are also an issue, patients with anorexia nervosa have lower circulating leptin and thus lack leptin resistance. Anorexic patients have markedly reduced BMD, coincident with elevated (up to sevenfold) inci-

dence of spontaneous fractures [30], again supporting a positive association of leptin with bone. However, even in the absence of endocrine confounders, reports on the correlation between leptin and BMD in anorectic patients have been inconsistent. Misra *et al.* found that the direction of association between overnight fasting leptin and bone mass differed in the regression model (negative) compared with simple correlation (positive association) [31]. Interestingly, correlation studies in healthy subjects seem to suggest a gender difference, with women showing a positive correlation between leptin and BMD [32,33], while a negative correlation was evident in men [34–36]. However, some studies found no association [32,37,38]. This difference between genders may indicate an interaction between sex hormone and leptin on bone regulation. This is further strengthened by the findings in a longitudinal evaluation of early postmenopausal women, in which the significant correlation between leptin, body mass index and total body BMD was lost over time during the progression of the postmenopausal period [39].

The biology of leptin is clearly complex, with multiple axes of action. The summation of these effects on the bone mass of an individual must take into account many factors. Leptin action appears to be modulated by age, weight, gender (the most fundamental regulators of BMD) as well as more complex issues involving the competing influences of central and peripheral signalling, envelope and regional regulation with more complexity sure to come to light as the study into this fascinating molecule continues.

5. Central effects of leptin act via sympathetic nervous system

The existence of hypothalamic-mediated effects on bone homeostasis has long been appreciated, with actions via the pituitary to regulate endocrine hormone release. Indeed, leptin deficiency is characterized by numerous endocrine changes. However, the notion that leptin-responsive hypothalamic neurons may regulate bone homeostasis purely via a humoral pathway was ruled out by parabiosis studies which showed significant reduced cancellous bone mass in the icv leptin-treated *ob*/*ob* mouse but not the contralateral, non-injected *ob*/*ob* control [18].

In the absence of humoral mediation, neuronal processes were implicated, with indications that sympathetic activity was important downstream of central leptin. Sympathetic tone is decreased in *ob*/*ob* mice [40] and injection of leptin directly into the ventromedial hypothalamus activated sympathetic outflow and increased in plasma noradrenalin and adrenalin [41]. Subsequent studies using various genetic mouse models and pharmacological approaches support the involvement of sympathetic nervous system in central leptin signalling on bone regulation. Critically, bone cells were found to express functional β2-adrenergic receptors (β2AR) [18]. Blockage of the β2AR with non-selective antagonist propanolol increased cancellous bone mass in wild-type mice and protected *ob*/*ob* mice against cancellous bone loss following icv leptin treatment [18]. This was complemented by β-adrenergic receptor agonist studies that demonstrated a negative effect of treatment on cancellous bone in *ob*/*ob* and wild-type mice, without altering body weight [18,42]. Consistent with the pharmacological studies, a number of genetic studies have reinforced these findings. Disruption of dopamine β-hydroxylase, an enzyme generating adrenaline and noradrenaline, exhibited greater cancellous bone mass in mice, which remained unchanged after icv leptin infusion [18]. β2-Adrenergic receptor-deficient mice showed increased cancellous bone volume [43] while deletion of adenylyl cyclase 5, a downstream mediator of β2AR signalling, protected against age-related bone loss [44]. Together, these results strongly suggest that leptin-responsive hypothalamic neurons may inhibit cancellous bone formation via β2AR signalling.

This novel role of beta-adrenergic signalling in bone raises the important question regarding the effect of hypertensive medications and bone mass. In particular, the widespread usage of beta-blockers raises the issue of their use as a skeletal therapeutic. Despite the large sample sizes possible with such studies, the results of retrospective studies investigating beta-blocker use and fracture risk have been inconsistent. Studies have revealed both protective [45–47] and neutral findings [48,49]. A recent meta-analysis of 54 studies revealed a significant reduction in fracture risk (RR 0.86) for beta-blocker users, however, the same analysis revealed a similar effect for thiazide diuretics (RR 0.86), indicating that the anti-fracture effect may involve other components [47]. Clearly issues, such as parallel treatment and β1-selective versus β2 selective effects make such studies difficult to interpret. However, prospective studies also report a reduced fracture risk and increased BMD in beta-blocker users [50,51] or no effect [52]. Thus further effort is required before beta-blockers can be considered as a therapeutic for bone.

6. The neuropeptide Y system

Subsequent to the identification of leptin's actions in the hypothalamus, a number of central pathways to bone have been identified. One of the most generalized and powerful involves the neuropeptide Y (NPY) system. This system comprises three ligands: NPY, peptide YY (PYY), and pancreatic polypeptide (PP) mediating its actions through five Y receptor subtypes: Y1, Y2, Y4, Y5 and y6 [53,54]. NPY is produced by neurons of the central and peripheral nervous systems, and is present in both sympathetic and parasympathetic nerve fibres, often co-secreted with noradrenaline [55]. NPY-ergic neurons are abundant in the hypothalamus of the brain, with high levels in the arcuate nucleus and ventromedial hypothalamus [56–58], as well as some non-neuronal sites [59,60].

7. The NPY system in bone – early evidence

Early studies identified NPY-immunoreactive fibres in bone, most commonly associated with blood vessels [61–64], but also cells in the periosteum and bone lining cells [61,62]. Central NPY treatment was associated with a reduction in bone mass [5]. Importantly, NPY treatment in osteoblastic cell lines inhibited the cyclic AMP response to parathyroid hormone and norepinephrine [65,66], suggesting the presence of functional Y receptors on bone cells and a possible regulatory role for NPY. However, reports of Y receptors in bone were contradictory, however, some studies reported the presence of a Y receptor on human osteoblastic and osteosarcoma-derived cell lines and bone marrow cells [67,68]. Thus while suggestive, a role for NPY in the direct regulation of bone cell activity via receptor interactions was still to be firmly established.

8. Hypothalamic Y2 receptor effects on bone

As in the study of leptin, confirmation of a NPY-mediated effect in bone came with the production of mutant mouse models. To date, two Y receptors have been connected with skeletal homeostasis, Y1 and Y2. Both receptors are abundant in the hypothalamus as well as in peripheral nerves [69–71]. The first Y receptor model evaluated for skeletal activity was $Y2^{-/-}$, due to the known co-localisation of Y2 and leptin receptors on neurons within the arcuate nucleus [72,73]. Initial analysis of the distal femur of germline $Y2^{-/-}$ mice revealed a twofold greater cancellous bone volume associated with

a greater rate of bone formation [8,74]. Parameters of bone resorption were unchanged except for a modest elevation in osteoclast number. Critically, the bone anabolic response seen in germline *Y2*$^{-/-}$ mice was recapitulated in adult mice following conditional deletion of Y2 receptors solely from the hypothalamus, demonstrating a pivotal role for central Y2 receptors in this pathway [74]. The skeletal changes observed in germline and conditional *Y2*$^{-/-}$ mice occurred in the absence of measurable changes in bone active endocrine factors. Thus these findings indicated that the anabolism resulting from Y2 receptor deletion was not mediated by endocrine effectors of bone turnover but rather through a neural mechanism.

9. Y1 receptor and bone

Two recent publications have identified Y1 as a second Y receptor active in the regulation of bone and have indicated that a neural-mediated link exists between the central Y2 receptors and the cells of bone. Similar to Y2-deficient mice, loss of Y1 expression resulted in a generalized anabolic phenotype, with greater bone mass and formation [75]. The bone phenotype, however, differed from *Y2*$^{-/-}$ mice in several critical aspects. Most importantly, conditional deletion of hypothalamic Y1 receptors had no effect on bone homeostasis, indicating a non-central mechanism for Y1 action in bone. The existence of a direct Y1-mediated effect on anabolism was further suggested following the identification of Y1 expression in osteoblastic cells *in vivo* [75]. While, treatment of wild-type osteoblast-like cultures with NPY resulted in a decrease in cell number, this response was completely absent in *Y1*$^{-/-}$ cultures, indicating functional osteoblastic Y1 receptors. Moreover, this osteoblastic Y1 expression may be directly involved in the *Y2*$^{-/-}$ phenotype. *Y1*$^{-/-}$*Y2*$^{-/-}$ mice do not display an additive phenotype in bone, and Y1 expression is substantially reduced in osteoblast-like cultures from *Y2*$^{-/-}$ mice [76].

The role of osteoblastic Y1 activity has yet to be fully defined, and assessments may be complicated by interactions with immune cells. The Y1 receptor has also been shown to play an important role in the regulation of the immune system [77]. *Y1*$^{-/-}$ mice showed a bimodal change in immune function. T cells from *Y1*$^{-/-}$ mice were intrinsically hyper-responsive to activation, but produced a reduced number of effector T cells, due to a defect in the antigen presenting cell (APC) population. This Y1-mediated regulation of T cell activity is of particular interest in bone biology given the emerging role of the immune system in the regulation of bone remodelling and the pathophysiology of osteoporosis. Oestrogen deficiency results in an increase in adaptive immune function leading to increased production of TNFα by activated T cells, and may significantly contribute to the greater osteoclastogenesis and resorption postmenopause [78]. Although their role in the control of anabolism is yet to be fully elucidated, these studies indicate that Y1 signalling may be a critical downstream component of the neural regulation of bone mass.

10. Neuropeptide Y interaction with leptin

Both leptin-deficient and NPY receptor-deficient models have been identified as involving hypothalamic-mediated control of bone homeostasis. Interestingly, these two molecules have a close association within the hypothalamus, suggesting the possibility of a commonality in mechanism. Indeed, NPY has been demonstrated as an important downstream effector of leptin signalling. In the arcuate nucleus where a proportion of NPY-ergic neurons co-express the leptin receptor [79], expression of NPY is elevated following the reduction in leptin due to starvation [80–82] and in *ob*/*ob* mice [83]. Administration of leptin to *ob*/*ob* mice reduces the elevated levels of NPY [84,85], while central injection of NPY mimics many of the characteristics of leptin deficiency, including hyperphagia, hyperinsulinemia, decreased thermogenesis, and the development of obesity [8,86–88]. Conversely, deletion of NPY partially corrects the obesity phenotype of *ob*/*ob* mice [89]. Thus the exact relationship between the *ob*/*ob* and *Y2*$^{-/-}$ models was unknown.

In terms of skeletal changes, several lines of evidence suggested that leptin and NPY-mediated pathways to bone were similar. *Y2*$^{-/-}$ *ob*/*ob* double mutant mice did not show an additive effect on cancellous bone volume or formation [8]. Male *Y2*$^{-/-}$*Y4*$^{-/-}$ double knockout mice revealed a synergistic increase in cancellous bone volume, compared with *Y2*$^{-/-}$ mice in a gender-specific manner, coincident with a marked reduction in plasma leptin in male, but not female *Y2*$^{-/-}$*Y4*$^{-/-}$ mice [90]. However, several studies also suggested distinct pathways. Continuous administration of NPY into wild-type mice, mimicking the increase in *ob*/*ob*, has been shown to reduce cancellous bone volume, suggesting that NPY and leptin may use different pathways to control bone mass [5]. Destruction of specific hypothalamic regions using chemical ablation techniques indicated that the leptin effect on cancellous bone originated from a region without NPY expression [18]. However, it was the examination of cortical bone response in *Y2*$^{-/-}$ and *ob*/*ob* mice that provided definitive evidence for separate pathways. Leptin deficiency produces a low bone mass phenotype reducing cortical mass and formation, whereas, germline and hypothalamic *Y2*$^{-/-}$ mice display an opposing phenotype, with greater cortical mass and formation activity, a relationship further enhanced following correction for the greater body weight of *ob*/*ob* [8]. In addition, exogenous elevation of central NPY levels, as evident in *ob*/*ob* did not block the *Y2*$^{-/-}$-mediated anabolic response, even in the presence of elevated serum leptin [7]. Thus it appears that although related in their expression in some regions of the hypothalamus, the afferent pathways mediating the skeletal effects of Y2 and leptin appear distinct.

11. The cannabinoid receptors

The endocannabinoid system mediates its actions via two cannabinoid receptors (CB1 and CB2) and like NPY receptors couple to inhibitory G-proteins [91]. Unlike other neurotransmitters, endocannabinoids are not released from vesicles, but are generated as needed [92]. CB1 is primarily found within the CNS [93] while CB2 is predominantly expressed in peripheral tissues [94]. Recent studies have revealed that cannabinoid receptors are abundantly expressed in osteoblasts and osteoclasts, and play a role in the control of bone homeostasis by a centrally mediated mechanism. The CB1 receptor plays a significant role in regulating BMD [95]. It has been demonstrated that mice with inactivation of CB1, have increased bone mineral density and additionally are protected against ovariectomy-induced bone loss [95]. CB1 action does appear to be direct, with the synthetic cannabinoid receptor antagonists AM251 (CB1), SR144528 (CB2) and AM630 (CB2) able to inhibit osteoclast formation and bone resorption *in vitro*, and both AM251 and SR144528 inhibit osteoclastic bone resorption, thus protecting against ovariectomy-induced bone loss *in vivo* [95]. Moreover, CB1-knockout mice were resistant to the inhibitory action of AM251 on osteoclast formation, which indicates that cannabinoid antagonism inhibits osteoclasts, which is in part mediated by the CB1 receptor [95].

CB2 has a more clearly peripheral expression pattern [96]. Karsak *et al*. demonstrate that the *CNR2* gene, encoding the CB2 receptor, plays a role in the regulation of bone mass in man [97]. A genetic association study returned a significant association between CB2 variants and BMD of single polymorphisms and haplotypes encompassing the *CNR2* gene on human chromosome

1p36. CB2-deficient mice have a distinctly accelerated age-related trabecular bone loss and cortical expansion, even though cortical thickness remains unaltered [96]. Despite the loss of bone, *CB2*$^{-/-}$ mice exhibit increased mineral appositional rate and bone formation rate. These alterations are reminiscent of human osteoporosis. Furthermore, Ofek *et al.* demonstrated that functional CB2 receptors are expressed in cells of both the osteoblast and osteoclast lineages [96]. These *in vitro* studies indicate that CB2 signalling contributes to the maintenance of bone mass by (i) stimulating stromal cells/osteoblasts directly; (ii) suppressing monocytes/osteoclasts, both directly and by inhibiting osteoblast/stromal cell receptor activator of NF-κB ligand (RANKL) expression. Jointly these data suggest that the cannabinoid system plays an important role in the regulation and maintenance of bone mass through the signalling of both the CB1 and CB2 receptors. However, whereas CB1 actions may be, at least in part, centrally mediated, CB2 actions are peripheral in nature.

12. The melanocortin system and CART

Melanocortins are a complex family comprising a number of endogenous ligands and several receptor subtypes in which there are five melanocortin receptors (MCRs), identified as G-protein coupled receptors MC1–5 [98,99]. The endogenous agonists are all derived from precursor proopiomelanocortin (POMC), of which α-, β- and γ-MSH (melanocyte-stimulating hormone) and adrenocorticotropic hormone (ACTH) elicit their action by interacting with the MCRs [98]. In addition to the melanocortin agonists, agouti-related protein (AgRP) has been identified as a high-affinity antagonist [100].

The regulation of bone homeostasis by this system centres around the action of melanocortin 4 receptor (MC4R) expressed in hypothalamic neurons and reveals that bone resorption is also under central, neural control. Patients deficient in MC4R are known to exhibit a high bone mineral density resulting from a decrease in bone resorption [101]. Importantly, the increased BMD is still evident following correction of the obesity that is characteristic of MC4R deficiency [101]. Mechanistic studies in mice have enabled dissection of this pathway to bone, and interestingly, have implicated another hypothalamic neuropeptide, cocaine- and amphetamine-regulated transcript (CART). Hypothalamic CART expression is increased in *MC4R*$^{-/-}$ mice, which display a high bone mass phenotype due to decreased osteoclast number and function [43,102], as evident in human studies. Additionally, MC4R mutant mice lacking one or two copies of CART exhibited a significantly lower bone mass [43,102], demonstrating increased CART signalling, is critical to the low-bone-resorption/high-bone mass phenotype observed in MC4R-deficient mice. Consistent with this interaction, *Cart*$^{-/-}$ mice display a low bone mass phenotype due to increased bone resorption [43]. These interactions, defining an osteoclastic regulatory loop are hampered by that lack of identification of a receptor for the CART neuropeptide.

13. Conclusion

The burgeoning field of skeletal neuro-regulation has highlighted a new paradigm emerging in skeletal research with broader implications for tissue homeostasis in general. The existence of these efferent neural pathways has identified a novel action for the hypothalamic nuclei in the control of peripheral tissue homeostasis. This action, in a complementary fashion with endocrine actions via the pituitary, identifies a further level of sophistication in the regulation of bone mass. Additionally, this work has stimulated the examination of bone cells for neural receptors and may enable direct intervention to harness the powerful anabolic and catabolic activities for therapeutic benefit. The powerful genetic resource of mutant mice has enabled clearly defined pathways to be identified. However, the effective translation of these findings to human studies has been less clear and remains a significant challenge facing this field at present.

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