REVIEW

AMP-activated protein kinase pathway and bone metabolism

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Abstract

There is increasing evidence that osteoporosis, similarly to obesity and diabetes, could be another disorder of energy metabolism. AMP-activated protein kinase (AMPK) has emerged over the last decade as a key sensing mechanism in the regulation of cellular energy homeostasis and is an essential mediator of the central and peripheral effects of many hormones on the metabolism of appetite, fat and glucose. Novel work demonstrates that the AMPK signaling pathway also plays a role in bone physiology. Activation of AMPK promotes bone formation *in vitro* and the deletion of α or β subunit of AMPK decreases bone mass in mice. Furthermore, AMPK activity in bone cells is regulated by the same

hormones that regulate food intake and energy expenditure through AMPK activation in the brain and peripheral tissues. AMPK is also activated by antidiabetic drugs such as metformin and thiazolidinediones (TZDs), which also impact on skeletal metabolism. Interestingly, TZDs have detrimental skeletal side effects, causing bone loss and increasing the risk of fractures, although the role of AMPK mediation is still unclear. These data are presented in this review that also discusses the potential roles of AMPK in bone as well as the possibility for AMPK to be a future therapeutic target for intervention in osteoporosis.

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Introduction

Osteoporosis is a major health problem in our aging society. One in two postmenopausal women will fracture a bone as a result of osteoporosis leading to more than 300 000 fragility fractures every year in the UK, which are important causes of morbidity and mortality (Edwards *et al.* 2007, Harvey *et al.* 2010). As the population ages, the prevalence of osteoporosis increases sharply and there is consequently a pressing need for the discovery of new molecular targets with potential drug development for prevention and treatment of this disorder. There is increasing evidence that osteoporosis, similarly to obesity and diabetes, could be another disorder of energy metabolism. A close relationship exists between obesity and osteoporosis, two diseases of body constitution that have a genetic predisposition (Rosen & Bouxsein 2006, Reid 2008). The diagnosis of postmenopausal osteoporosis is largely based on a measurement of bone mineral density (BMD), which is affected by many factors, body weight being the principal one. Positive associations between body weight and BMD have been long demonstrated; loss of body weight being often associated with bone loss and risk for osteoporosis, while gain in body weight increases bone formation due to the skeletal loading (De Laet *et al.* 2005). However, there are also data showing negative effects of obesity on BMD (Hsu *et al.* 2006,

Premaor *et al.* 2011). Body weight is made up of two components, fat mass and lean mass. The relative contribution of these two components to the variation in BMD has been controversial, although in postmenopausal women fat mass has been often shown to be important (Reid *et al.* 1992, Dytfeld *et al.* 2011). The intimate association between fat and bone has been extensively described. First, this link is based on the fact that cells which are important in obesity (adipocytes) and osteoporosis (osteoblasts) share a common cell progenitor, the mesenchymal stromal cell (MSC). This cell can differentiate into osteoblasts by expressing the transcription factor RUNX2, while expression of peroxisome proliferator-activated receptor γ 2 (PPAR γ 2) inhibits osteoblast differentiation and induces MSC differentiation into the adipogenic lineage (Gimble *et al.* 1996, Ducy 2000). The bone loss in age-related osteoporosis is associated with more adipogenesis and less bone formation (Pei & Tontonoz 2004). Second, there are direct actions on bone of hormones produced by adipocytes, such as adiponectin and leptin, as well as of hormones released by the pancreas, gut and pituitary (Cornish *et al.* 1996, 2002, Maccarinelli *et al.* 2005, Williams *et al.* 2009). In addition, the recent discovery that leptin can inhibit bone formation through hypothalamic and sympathetic nervous system relays (Ducy *et al.* 2000, Takeda *et al.* 2002, Eleftheriou *et al.* 2005) has demonstrated that bone can

not only be directly affected by hormones which have receptors on bone cells, but can also be influenced by the same hormones acting on receptors in the central nervous system (CNS). The latest demonstration of a feedback control by the skeleton of glucose and fat metabolism through the systemic release of the osteoblast-specific protein osteocalcin (Lee *et al.* 2007, Ferron *et al.* 2010, Fulzele *et al.* 2010) has reinforced the reciprocal relationship between bone and energy metabolism. Bone remodeling, occurring simultaneously in numerous parts of the skeleton, is an energy intensive process and bone constantly needs to balance energy in response to nutrient availability with growth and turnover.

AMP-activated protein kinase (AMPK) has emerged over the last decade as a key sensing mechanism in the regulation of cellular energy homeostasis and is an essential mediator of the central and peripheral effects of many hormones on the metabolism of appetite, fat and glucose (Hardie *et al.* 2006, Kola *et al.* 2006, Lage *et al.* 2008, Steinberg & Kemp 2009). AMPK senses the AMP/ATP ratio within the cell and, once activated, switches on catabolic pathways (energy generating) and switches off anabolic pathways (energy consuming). It is a key molecule in controlling metabolic diseases such as type 2 diabetes and obesity and is activated by antidiabetic drugs such as metformin and thiazolidinediones (TZDs; Zhou *et al.* 2001, Fryer *et al.* 2002, LeBrasseur *et al.* 2006). Novel findings, described in detail in this review, have shown that AMPK plays a role in bone physiology. There are *in vitro* studies demonstrating that AMPK modulators regulate bone cell differentiation and function (Kanazawa *et al.* 2007, 2008, 2009b, Molinuevo *et al.* 2010, Quinn *et al.* 2010, Shah *et al.* 2010, Jang *et al.* 2011, Mai *et al.* 2011) and two publications showing that deletions of AMPK α and β subunits in mice lead to bone loss *in vivo* (Quinn *et al.* 2010, Shah *et al.* 2010). Furthermore, AMPK activity can be regulated in bone cells by hormones, such as ghrelin and norepinephrine, that regulate food intake and energy expenditure through AMPK activation in the brain and peripheral tissues. The interest in AMPK and bone physiology comes also from the fact that metformin and TZDs have significant and opposing skeletal effects (Gao *et al.* 2010, Lecka-Czernik 2010, Mai *et al.* 2011) and the contribution of AMPK to these bone effects is still unclear. Although the elucidation of the importance of AMPK signaling in bone is still in its commencement, the revelation that AMPK activation may affect stimulation of bone formation and bone mass places AMPK signaling as a significant pathway in skeletal physiology.

AMPK, a master sensor of cellular energy status

Structure and regulation

There has been a tremendous effort over the last two decades at trying to elucidate the structure of AMPK and its regulation and excellent reviews on the subject exist (Rutter *et al.* 2003, Carling 2004, Hardie *et al.* 2006, Viollet *et al.* 2007, Lage *et al.*

2008, Oakhill *et al.* 2009, Steinberg & Kemp 2009). Briefly, AMPK is a heterotrimeric protein, highly conserved throughout evolution, consisting of three different subunits, α catalytic and regulatory β and γ (Hardie *et al.* 1998). In mammals, there are seven genes encoding subunits (α 1, α 2, β 1, β 2, γ 1, γ 2, γ 3), that can form 12 possible AMPK heterotrimers, which exhibit differences in subcellular localization and regulation (Hardie 2007). At the molecular level, the α catalytic subunit contains the conventional serine/threonine protein kinase domain as well as the autoinhibitory sequence, the β subunit contains a glycogen-binding domain and a tethering domain for α and γ subunits, while the γ subunit contains four cystathionine- β -synthase sequence repeats, which are responsible for the binding of regulatory nucleotides (McBride & Hardie 2009, Oakhill *et al.* 2009). AMPK regulates the activities of many target proteins controlling different aspects of metabolism and therefore the regulation of its activity in the cell involves complex signaling pathways and sensing mechanisms. AMPK is activated by the binding of AMP to the regulatory γ subunit, which overall promotes the phosphorylation of Thr-172 within α catalytic subunit by upstream kinases, protects against the dephosphorylation of Thr-172 by protein phosphatases and causes allosteric activation (for reviews see Carling *et al.* (2008) and Hardie (2008)). To date, three known upstream AMPK kinases have been described, the tumor suppressor kinase LKB1, calmodulin kinase kinase (CaMKK) and transforming growth factor- β -activated kinase (TAK1), but their physiologic roles are not always very clear (Woods *et al.* 2003, Carling *et al.* 2008, Lage *et al.* 2008, Oakhill *et al.* 2009). Thus, increases in the cellular AMP/ATP ratio cause a dramatic and progressive activation of AMPK. Recent evidence demonstrates that ADP can also regulate AMPK activation by protecting AMPK from dephosphorylation (Xiao *et al.* 2011). In addition, AMPK activity can be modulated independently of AMP by activation of CaMKK β due to increasing intracellular Ca^{2+} levels (Hawley *et al.* 2005, Tamas *et al.* 2006, Sanders *et al.* 2007) and by ubiquitination and degradation (Qi *et al.* 2008). A simplified cartoon illustrating the structure and regulation of AMPK activity is shown in Fig. 1.

Function

In mammals, AMPK plays the role of an intracellular sensor that modulates the energy balance within the cell. It is activated by an increasing cellular AMP/ATP ratio, a measure that the cell experiences a decrease in energy caused by metabolic stresses that interfere with ATP production or consumption such as exercise, hypoxia, and muscle contraction. Once activated, AMPK switches on pathways that generate ATP and switches off ATP-consuming pathways that are not essential for short-term cell survival to restore the AMP/ATP ratio (Kahn *et al.* 2005, Hardie 2007). AMPK regulates many metabolic pathways in peripheral tissues by phosphorylating metabolic enzymes such as enzymes involved

Figure 1 Structure and regulation of AMPK. AMP-activated kinase (AMPK) is a heterotrimeric protein consisting of three different subunits, α catalytic and regulatory β and γ . AMPK is activated by the binding of AMP to the regulatory γ subunit that promotes phosphorylation of Thr-172 within α catalytic subunit by upstream kinases (such as the tumor suppressor kinase LKB1, calmodulin kinase kinase (CaMKK), and transforming growth factor- β -activated kinase (TAK1)) and causes allosteric activation. Increases in cellular AMP/ATP ratio caused by cellular stress or drugs (AICAR, metformin, and TZDs) activate AMPK, which maintains energy homeostasis by switching on catabolic pathways that produce ATP (energy generating) and switching off anabolic pathways (energy consuming). At the whole-body level, this is translated into changes in energy expenditure and feeding.

in lipid, glucose, and glycogen metabolisms (Hardie & Pan 2002, Carling *et al.* 2003, Hardie 2003, 2004, 2008, Rutter *et al.* 2003, Hardie & Sakamoto 2006, Viollet *et al.* 2006, 2009a, Steinberg & Jorgensen 2007, Canto & Auwerx 2010). In addition to its direct acute effect via phosphorylation of metabolic enzymes, AMPK has also longer term indirect effects by regulating gene transcription, affecting expression of genes that encode those metabolic processes (McGee & Hargreaves 2008, Scott *et al.* 2009). In general, AMPK will promote energy production and upregulate the transcription of catabolic genes such as the glucose transporter 4 (GLUT4) as well as genes involved in mitochondrial biogenesis such as PPAR γ coactivator 1 α (PGC1 α ; Holmes *et al.* 1999, Zong *et al.* 2002). AMPK will also limit energy utilization to ensure cell survival, so activation of AMPK will mediate cell cycle arrest, inhibit growth and suppress protein synthesis through downregulation of mechanistic target of rapamycin (MTOR; also known as mammalian target of rapamycin) signaling (Bolster *et al.* 2002, Jones *et al.* 2005, Motoshima *et al.* 2006, Greer *et al.* 2007). AMPK activation also regulates genes involved in immunity (interleukin-2, T-cell receptor) and

vascularization (vascular endothelial growth factor, neuronal nitric oxide synthase; Levine *et al.* 2007, Reihill *et al.* 2007, Mayer *et al.* 2008). AMPK phosphorylation targets include cytoplasmic and nuclear proteins, as AMPK activation is usually confined to the cytoplasm in response to energy stress but can be observed in the nucleus in response to calcium elevation (Tsou *et al.* 2011). The exact mechanisms regulating AMPK subcellular localization are not completely understood, but a recent study has identified a nuclear export signal in the C-terminal domain of catalytic α subunit of AMPK (Kazgan *et al.* 2010). They demonstrate that AMPK α is imported into the nucleus via a Ran-GDP-dependent pathway but translocation to the nucleus can be regulated by exercise, cellular stress, and circadian rhythms. In addition to its effect on cellular energy balance in peripheral tissues, AMPK also plays a key role in the control of whole-body energy homeostasis by integrating, at the hypothalamic level, nutrient and hormonal signals that regulate food intake and energy expenditure (Andersson *et al.* 2004, Minokoshi *et al.* 2004, Dzamko & Steinberg 2009, Hardie 2011). AMPK has indeed been shown to be a major regulator of food intake by acting in hypothalamic neurons and modulating the hypothalamic fatty acid metabolic pathway (Lopez *et al.* 2007). Fasting and orexigenic signals increase AMPK activity in several hypothalamic regions, while feeding and anorectic signals inhibit it (Kola *et al.* 2005, Gao *et al.* 2007, Lopez *et al.* 2008). Nutrient- and hormone-induced alterations in AMPK hypothalamic activity have been shown to correlate with changes in neuropeptide expression in hypothalamic nuclei, indicating that AMPK activation in the hypothalamus links whole-body energy balance with neurotransmitter signaling pathways (Lee *et al.* 2005, Lage *et al.* 2008). A possible role for hypothalamic AMPK in the control of obesity is the focus of increasing interest.

AMPK is a mediator of the metabolic effects of many hormones

Many of the metabolic effects of peripheral hormones are mediated by AMPK, which plays a central role in the endocrine system (for reviews see Kahn *et al.* (2005), Kola *et al.* (2006), and Lage *et al.* (2008), Dzamko & Steinberg (2009) and Lim *et al.* (2010)). Leptin was the first hormone described to activate AMPK in the skeletal muscle, leading to inhibition of fatty acid synthesis and increase in fatty acid oxidation and glucose uptake (Minokoshi *et al.* 2002). Both phosphorylation of acetyl-CoA carboxylase (ACC) by AMPK α 2 and stimulation of PPAR α transcription are responsible for fatty acid oxidation by leptin in skeletal muscle. Leptin also stimulates fatty acid oxidation via AMPK-dependent pathways in the heart (Lee *et al.* 2004). In contrast to its stimulatory effect on AMPK activation in peripheral tissues, leptin inhibits AMPK activity in the hypothalamus, leading to an increase in appetite (Andersson *et al.* 2004, Minokoshi *et al.* 2004). Another hormone produced by the adipocytes is adiponectin, which improves glucose uptake and fatty acid oxidation in muscle, reduces hepatic glucose

production and enhances insulin sensitivity. AMPK activation has been shown to mediate all these effects of adiponectin on peripheral tissues (Yamauchi *et al.* 2002, Huypens *et al.* 2005). Adiponectin also plays an important role in the heart where it protects it from ischemia–reperfusion injury through an AMPK-dependent mechanism (Shibata *et al.* 2005). Ghrelin is a hormone produced by the gut that is also very important for the control of feeding. It has orexigenic effects mediated by AMPK activation in the hypothalamus (Kola *et al.* 2005). Ghrelin also inhibits AMPK activity in the liver and adipose tissue, upregulating gluconeogenesis and lipogenesis, but increases AMPK activity in the heart (Korbonits *et al.* 2004). In addition to mediating the actions of hormones on the CNS and peripheral tissues, AMPK also integrates the effects of several other factors, such as cytokines and neuromediators (van Thuijl *et al.* 2008).

Main drugs that are AMPK activators

Besides, the physiological activators (muscle contraction) and pathological stresses (heat, glucose deprivation, and hypoxia), a variety of drugs and xenobiotics activate AMPK (Oakhill *et al.* 2009, Zhou *et al.* 2009, Hawley *et al.* 2010, Mantovani & Roy 2011). The first drug shown to activate AMPK was the nucleoside 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; Corton *et al.* 1995). It is a synthetic AMP mimetic compound that is phosphorylated in cells to AICA riboside monophosphate (ZMP). AICAR was shown to reverse many metabolic defects in animal models of insulin resistance and is still the most widely used agent to activate AMPK (Zhou *et al.* 2009). However, due to the fact that ZMP is an AMP mimetic, it can have AMPK-independent effects. It was shown, for example, that AICAR binds and inhibits the heat shock protein (Hsp90; Tomaselli *et al.* 2010) and ZMP has been hypothesized to be a toxic metabolite in the neurological disorder Lesh–Nyhan disease (Lopez 2008). In 2001, it was reported that the drug metformin, which belongs to the class of biguanides and is the most commonly prescribed oral antidiabetic drug in the USA, activates AMPK (Zhou *et al.* 2001, Hawley *et al.* 2010). Similarly, although AMPK activation is responsible for many of its cellular actions, metformin can also have AMPK-independent effects (Foretz *et al.* 2010). Interestingly, it was later shown that another class of antidiabetic drugs, the TZDs, also stimulates AMPK (Fryer *et al.* 2002), although the main therapeutic target of TZDs is the nuclear receptor PPAR γ . Metformin and TZDs were shown to indirectly activate AMPK by increasing the intracellular AMP/ATP ratio and subsequently making AMPK a better substrate for LKB1, one of the AMPK upstream kinase (Carling *et al.* 2008). Many natural products derived from traditional medicine that were claimed to have beneficial effects in diabetes and cancer can also activate AMPK. They include resveratrol, epigallocatechin gallate, berberine, and quercitine (Mooney *et al.* 2008, Turner *et al.* 2008, Zhou *et al.* 2009). Recent years have shown the development of novel pharmacological agents that

can modulate AMPK directly with the hope that these compounds will be promising in the treatment of metabolic disorders. Two new small molecules were shown to directly activate AMPK *in vivo* and *in vitro*. The first one is the compound A-79662 developed by Abbott Laboratories that belongs to the thienopyridone family. It seems a more promising selective activator of AMPK (Zhao *et al.* 2007, Scott *et al.* 2008), although it was shown to inhibit the function of the 26S proteasome by an AMPK-independent mechanism (Moreno *et al.* 2008). The second compound is the thiazolidinone PT1 from the Shanghai Institute of Materia Medica (Pang *et al.* 2008). The mechanisms of action of these two compounds are described in these reviews (Zhang *et al.* 2009, Fogarty & Hardie 2010).

Role of AMPK in bone

Subunit expression and AMPK regulation in bone cells

The different AMPK subunits have been shown to have differential tissue-specific expression and activation (Steinberg & Kemp 2009). Most AMPK subunits isoforms are expressed in bone tissue and bone cells, indicating possible combinations to form different AMPK complexes. In contrast with skeletal muscle, heart, and liver, and similar to adipose tissue, α 1 subunit is the dominant catalytic isoform expressed in bone, suggesting that it may play a major function in skeletal metabolism. Using both RT-PCR and western blot analysis, it was shown that α 1 subunit is highly expressed in bone tissue, primary osteoblasts and osteoclasts as well as in osteoblastic cell lines (Kim *et al.* 2008, Kasai *et al.* 2009, Quinn *et al.* 2010, Shah *et al.* 2010). In contrast, the α 2 subunit has a very low expression in osteoblasts and bone tissue and is not expressed in ROS 17/2.8 cells and osteoclasts (Quinn *et al.* 2010, Shah *et al.* 2010). While both β 1 and β 2 subunits are similarly expressed in bone, the γ subunit also shows differential expression in bone tissue and cells. The γ 1 subunit is the major γ isoform in bone tissue and bone cells, γ 2 being poorly expressed in osteoblasts and not expressed in osteoclasts while γ 3 has only been identified in ROS 17/2.8 osteoblastic cells (Quinn *et al.* 2010, Shah *et al.* 2010).

Several studies have demonstrated that the cell-permeable AMP analog AICAR can activate AMPK in primary osteoblasts and osteoblastic cell lines (Kim *et al.* 2008, Shah *et al.* 2010), as well as in osteoclasts and in the macrophage-like RAW264.7 cell line (Quinn *et al.* 2010). It causes a large and sustained increase in both AMPK α phosphorylation at Thr-172 and AMPK activation measured by an *in vitro* peptide phosphorylation assay. Results on osteoblastic MC3T3-E1 cell line are however divergent as AICAR was reported to either phosphorylate AMPK in those cells (Kanazawa *et al.* 2007) or to fail to induce sustained phosphorylation of AMPK α (Kasai *et al.* 2009). Another AMPK agonist, metformin, has uniformly been shown to be a potent stimulator of AMPK activation in bone marrow

progenitor cells (BMPC; Molinuevo *et al.* 2010), primary osteoblasts (Shah *et al.* 2010), MC3T3-E1 cells (Kanazawa *et al.* 2008, Kasai *et al.* 2009), ROS 17/2.8 cells (Shah *et al.* 2010), and primary bone marrow macrophages (Lee *et al.* 2010). The downstream target of AMPK, ACC, is also phosphorylated in primary osteoblasts by AICAR and metformin. There are not many AMPK inhibitors. One of them is compound C that has been shown to decrease AMPK α phosphorylation in primary osteoblasts and ROS 17/2.8 cells (Shah *et al.* 2010). However, although compound C is described as an AMPK inhibitor, it can inhibit the AKT/MTOR pathway independently of AMPK (Vucicevic *et al.* 2011) and is also known as dorsomorphin that inhibits bone morphogenetic protein (BMP) signaling (Yu *et al.* 2008). Both the BMP and the AKT/MTOR pathways are very important for osteoblastic differentiation and bone formation (Peng *et al.* 2003, Cao & Chen 2005, Sugatani & Hruska 2005, Wan & Cao 2005, Mukherjee & Rotwein 2009).

The hormonal regulation of AMPK activation in bone has not been extensively studied. Adiponectin, which increases osteoblast proliferation and differentiation (Oshima *et al.* 2005) but has controversial effect on bone mass *in vivo* (Williams *et al.* 2009), can stimulate AMPK phosphorylation in MC3T3-E1 osteoblastic cells (Kanazawa *et al.* 2007). We showed that AMPK phosphorylation and activity in ROS17/2.8 cells was stimulated by ghrelin (Shah *et al.* 2010), a hormone known to stimulate osteoblast differentiation and function (Maccarinelli *et al.* 2005, van der Velde *et al.* 2008). Dexamethasone, a synthetic glucocorticoid that has been shown to induce osteoblastic differentiation and bone formation in several culture systems (Delany *et al.* 1994), stimulates AMPK phosphorylation in primary osteoblasts (unpublished results, M Shah, J Jeyabalan, B Viollet & C Chenu). We also examined the effect of propranolol, a β -adrenergic receptor antagonist that stimulates bone formation *in vivo* (Eleftheriou *et al.* 2005), on AMPK activity in ROS 17/2.8 cells and our results demonstrate that propranolol enhances AMPK phosphorylation and activity in those cells (Shah *et al.* 2010). Cytokines may also modulate AMPK activation (Steinberg *et al.* 2009) and RANKL was shown to activate AMPK phosphorylation in bone marrow-derived osteoclastic cells (Lee *et al.* 2010). In addition, other non-hormonal factors can modulate AMPK activation in bone cells and, for example, ultrasound induces AMPK phosphorylation in human fetal osteoblastic cells (Hou *et al.* 2008). This neuroendocrine regulation of AMPK in osteoblasts clearly suggests a link between AMPK activation and osteoblast function.

Effect of AMPK activation on bone cell activities in vitro and in vivo

Several studies have examined the effects of AMPK activators on osteoblast differentiation and function *in vitro* and most of them have used MC3T3-E1 mouse calvaria-derived cells. It was shown that AICAR and metformin both cause a direct osteogenic effect in MC3T3-E1 cells (Cortizo *et al.* 2006,

Kanazawa *et al.* 2007, 2008, 2009b, Jang *et al.* 2011). These actions include a dose-dependent increase in cell proliferation, type I collagen production, alkaline phosphatase (ALP) activity, and mineral deposition. These osteogenic effects could be mediated by an increase in COX-2 and endothelial nitric oxide synthase, in the activity of extracellular signal-regulated kinases 1/2 as well as in BMP2 production (Hou *et al.* 2008). Metformin was also found to regulate small heterodimer partner in MC3T3-E1 cells, an orphan nuclear receptor that stimulates osteoblastic bone formation by interacting with the transcription factor RUNX2 (Jang *et al.* 2011). The action of metformin on bone marrow mesenchymal cell progenitors has also been investigated and metformin similarly causes an increase in ALP activity, collagen synthesis, osteocalcin production, and extracellular calcium deposition *in vitro*, possibly by increasing the expression of RUNX2 (Molinuevo *et al.* 2010). We and others (Shah *et al.* 2010, Zhen *et al.* 2010) have used primary osteoblasts derived from rat calvaria to study the effect of AMPK activation in an *in vitro* model of bone formation in which the production and the mineralization of a bone matrix, can be assessed quantitatively (Utting *et al.* 2006). We confirmed in this model that both AICAR and metformin, at doses ranging between 0.5 and 100 μ M, dose-dependently stimulated bone nodule formation, while AMPK inhibitor compound C dose-dependently reduced bone formation (Shah *et al.* 2010). In this model, metformin also markedly increased cell proliferation and ALP activity (Zhen *et al.* 2010), while AICAR preferentially stimulated ALP activity after 14 days of culture, during the mineralizing phase (Shah *et al.* 2010). When primary osteoblasts were co-treated with AICAR and compound C, compound C suppressed the stimulatory effect of AICAR on bone nodule formation, supporting a role for AMPK activation in bone formation. However, these results are in contrast to the study showing that osteoblast differentiation is functionally associated with decreased AMPK activity, measured by phosphorylation levels of AMPK α subunit (Kasai *et al.* 2009). We found a similar decrease in baseline AMPK activity with time during osteoblast differentiation. This may be due to the high energy requirements and therefore elevated ATP contents observed in mature osteoblasts during bone matrix production and mineralization. Osteoblast differentiation was previously shown to coincide with changes in cellular metabolism and mitochondrial activity (Komarova *et al.* 2000). In agreement with this, our results demonstrate that when AICAR and metformin were added to primary osteoblasts at specific stages of their differentiation, although they always induced AMPK phosphorylation, their effects were more potent at later stages of osteoblastic differentiation (unpublished results, M Shah, J Jeyabalan, B Viollet & C Chenu).

Fewer studies have investigated the relationship between AMPK activation and bone resorption. Compound C has been shown to potentiate bone resorption, via inhibition of AMPK α 1 in bone marrow-derived osteoclast precursors, AMPK acting as a negative regulator of RANKL (Lee *et al.* 2010). In agreement with those results, adiponectin was

demonstrated to inhibit the stimulation by tumour necrosis factor α and RANKL of the master transcriptional factor of RANKL-induced osteoclastogenesis, the nuclear factor of activated T cells c1, through the AMPK signaling pathway (Yamaguchi *et al.* 2008). Metformin was very recently shown to reduce osteoclast differentiation and activity by stimulating osteoprotegerin (OPG) and inhibiting RANKL mRNA and protein expression in osteoblasts (Mai *et al.* 2011). Conflicting results were however shown by Quinn *et al.* (2010) who demonstrated that AICAR treatment in male mice stimulates bone loss and bone turnover with elevated rates of both bone formation and bone resorption (Quinn *et al.* 2010). The high bone resorption was due to an increase in osteoclast number. While the authors of this study confirmed that AICAR stimulates osteoclast formation *in vitro*, a result in contradiction with the previously mentioned *in vitro* studies demonstrating that AMPK activation suppresses osteoclast activity, they however showed that AICAR's effect was independent of AMPK signaling (Quinn *et al.* 2010). The effect of metformin on bone mass *in vivo* was also poorly studied, but two recent studies indicate that metformin reduces the bone loss induced by ovariectomy (OVX), in part through increasing bone formation via induction of osteoblast genes such as *Runx2* and *Lrp5* (Gao *et al.* 2010) and by reducing RANKL and stimulating OPG expression in osteoblasts (Mai *et al.* 2011).

Bone phenotypes of mice with deletions of AMPK subunits

The most striking evidence for a role of AMPK signaling in the regulation of bone mass came from genetic studies which analysed bone mass in mice with deletions of AMPK α and β subunits. In many mouse strains AMPK catalytic subunits are genetically redundant as single $\alpha 1$ or $\alpha 2$ knockouts (KO) are viable, yet double KOs are embryonically lethal. The metabolic exploration of AMPK $\alpha 1$ and $\alpha 2$ KO has revealed distinct physiologic roles for these two catalytic subunits (for a review of these KOs, see Viollet *et al.* (2003a)). Daval *et al.* (2005) reported no difference in body mass in AMPK $\alpha 1$ KO mice, but these mice had less fat. Energy expenditure and food intake were not modified in AMPK $\alpha 1$ KO mice (Viollet *et al.* 2009b). These mice also had normal glucose tolerance and normal response to insulin (Jorgensen *et al.* 2004). In contrast to the lack of detectable metabolic phenotype of AMPK $\alpha 1$ KO, whole-body AMPK $\alpha 2$ deletion resulted in mild insulin resistance and impaired glucose tolerance associated with insulin secretory defect (Viollet *et al.* 2003b, Jorgensen *et al.* 2004). We analyzed bone mass in both AMPK $\alpha 1$ and $\alpha 2$ KO. In agreement with the very low expression of $\alpha 2$ in bone, we found no changes in bone mass in tibia from AMPK $\alpha 2$ KO mice compared with wild-type (WT) mice (Shah *et al.* 2010). In contrast, both cortical and trabecular bone compartments were smaller in the AMPK $\alpha 1$ KO mice compared with the WT mice. AMPK $\alpha 1^{-/-}$ mice showed dramatic decrease in trabecular bone volume (-39.2%), trabecular number (-31.1%), and trabecular

thickness (-12.2%) compared with WT mice. The cortical indexes were also decreased in mice lacking AMPK $\alpha 1$, but no change in bone length was observed in those mice (Shah *et al.* 2010). Similarly, Quinn *et al.* (2010) showed that germline deletions of either AMPK $\beta 1$ or $\beta 2$ subunit result in reduced trabecular bone density and mass. Surprisingly, their bone histomorphometric analysis revealed no difference in osteoclast and osteoblast numbers between WT and AMPK $\beta 1$ or $\beta 2$ KO groups, suggesting that AMPK may influence bone cell functions other than bone cell differentiation. To investigate whether bone turnover could be stimulated in the absence of AMPK $\alpha 1$ subunit, we subjected WT and AMPK $\alpha 1^{-/-}$ mice to catabolic (OVX) and anabolic (intermittent PTH administration) hormonal challenges. AMPK $\alpha 1^{-/-}$ mice displayed a decreased bone loss after OVX in the trabecular compartment compared with WT mice. Similarly, although PTH increased cortical and trabecular bone indexes in both WT mice and AMPK $\alpha 1^{-/-}$ mice, AMPK $\alpha 1^{-/-}$ mice showed a smaller increase in bone mass in response to PTH treatment compared with WT mice (Shah *et al.* 2011). The demonstration that AMPK $\alpha 1^{-/-}$ mice are less affected by catabolic and anabolic changes in bone turnover induced by OVX and PTH, respectively, suggests that AMPK activation can influence the hormonal regulation of bone remodeling.

Possible targets and metabolic pathways regulated by AMPK in bone

The mechanisms linking AMPK activation to bone cell differentiation and bone mass remain mainly unknown. AMPK influences major metabolic pathways and it would seem intuitive that some of these pathways are also regulated by AMPK in bone cells. Since AMPK was recently shown to promote β -catenin transcription through phosphorylation of class IIa histone deacetylase 5 (Zhao *et al.* 2011), it is therefore probable that AMPK regulates osteoblast differentiation through crosstalk with the Wnt/ β -catenin pathway. Another pathway that is important for both osteoblast differentiation and osteoclastic bone resorption is the mevalonate pathway (Kanazawa *et al.* 2009a, Dunford 2010, Rogers *et al.* 2010, Yamashita *et al.* 2010). This pathway is required for the prenylation of regulatory proteins such as Ras and Rho GTPases that play a pivotal role in the regulation of numerous key cellular processes. Statins, drugs that inhibit the HMG-CoA reductase enzyme in the mevalonate pathway, stimulate osteoblast differentiation and bone formation in rodents both *in vitro* and *in vivo* (Horiuchi & Maeda 2006). Since statins may exert their vasculoprotective effects through activation of AMPK (Ewart & Kennedy 2011) and can phosphorylate AMPK *in vitro* (Rossoni *et al.* 2011), it is possible that AMPK signaling may also mediate some of the skeletal effects of statins. AMPK can indeed modulate the mevalonate pathway through repression of HMG-CoA reductase (Oliaro-Bosso *et al.* 2009) and could therefore influence this pathway in bone cells. Bisphosphonates, the widely prescribed

antiosteoporosis drugs, target the mevalonate pathway as well by inhibiting farnesylidiphosphate and geranylgeranyldiphosphate synthase (Russell *et al.* 2008, Rogers *et al.* 2010). Finally, it is also likely that AMPK activation is involved in the relationship between fat and bone in the marrow by affecting expression and/or activity of transcription factors essential for osteoblastic and adipocytic lineage commitments of MSC progenitors as discussed in the following section.

AMPK and the relationship between bone and fat

Osteoblasts and adipocytes arise from a common precursor, the pluripotent MSC. Both cell types are found in the bone marrow and a number of lineage-specific transcription factors and multiple extracellular and intracellular signaling pathways regulate differentiation into osteoblast or adipocyte lineages. Wnt/ β -catenin signaling pathway and RUNX2 induce osteoblastogenesis while inhibiting adipogenesis, whereas PPAR γ plays a pivotal role in adipogenesis while inhibiting osteoblastogenesis (Lecka-Czernik *et al.* 1999, Ahdjoudj *et al.* 2004, Kang *et al.* 2007, Takada *et al.* 2007). This reciprocal regulation of PPAR γ and RUNX2 underlies the lineage commitment of MSC progenitors to adipocytes or osteoblasts and consequently the age-related increase in bone marrow fat and bone loss (Pei & Tontonoz 2004). The activation of PPAR γ 2 using rosiglitazone was shown to cause differentiation of bone marrow-derived MSC into adipocytes while blocking osteoblast differentiation via suppression of RUNX2 (Gimble *et al.* 1996). In addition, activation of PPAR γ 2 in cells of the osteoblast lineage has been shown to convert them to terminally differentiated adipocytes. In contrast, PPAR γ 1 isoform induces osteoclastogenesis from hematopoietic stem cell pool and bone resorption by regulating c-FOS expression and increasing the expression of RANKL (Lazarenko *et al.* 2007, Wan *et al.* 2007). Interestingly, AMPK has been shown to phosphorylate β -catenin, suppress PPAR γ expression, and reduce adipogenesis *in vitro* (Zhao *et al.* 2010). Moreover, it can modify PPAR γ activity by directly phosphorylating PPAR γ coactivators (Leff 2003, Jager *et al.* 2007). Since AMPK was also shown to affect RUNX2 expression in BMPC (Molinuevo *et al.* 2010), AMPK activation could therefore be a signal for the skeleton to sense energy status and initiate adipogenesis or osteoblastogenesis depending on energy needs. It could also be an indication for the skeleton to instigate the appropriate bone remodeling response associated either with augmentation of energy-producing processes or decreased energy-costing processes. Changes in metabolic pathways indeed occur during bone cell differentiation and function to allow high ATP generation for bone matrix production and mineralization, as well as for bone resorption (Komarova *et al.* 2000, Kim *et al.* 2007). The potential role of AMPK in the regulation of bone homeostasis is shown in Fig. 2.

Figure 2 AMPK and the regulation of bone homeostasis. Bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption. Activation of the AMPK pathway stimulates osteoblast differentiation and bone formation via enhancing the osteoblast-specific transcription factor RUNX2. Activated AMPK can also lead to the inhibition of osteoclast formation and bone resorption possibly via suppression of the nuclear factor of activated T cells c1 (NFATc1). Activation of AMPK inhibits adipogenesis by reducing the expression of major genes involved in adipogenesis, including PPAR γ . The AMPK signaling pathway may also determine adipocyte and osteoblast differentiation from mesenchymal progenitors by regulating the expressions of Runx2 and PPAR γ .

AMPK and the skeletal effects of antidiabetic drugs

AMPK is activated by antidiabetic drugs such as metformin and TZDs. Both drugs have complementary mechanisms of action to control glycemia, TZDs directly target insulin resistance while metformin reduces hepatic glucose production. TZDs, such as rosiglitazone and pioglitazone, significantly improve insulin sensitivity but at the cost of increased marrow adiposity and bone loss (de Paula *et al.* 2010). Although TZDs function mainly by activating PPAR γ , they can also rapidly activate AMPK in mammalian tissues and cells independently of their effect on PPAR γ -mediated gene transcription (LeBrasseur *et al.* 2006). Furthermore, TZDs possibly affect AMPK indirectly by causing release of adiponectin (Maeda *et al.* 2001) leading to activation of AMPK and reduction in glucose production (Lee & Kim 2010). While type 2 diabetes itself imposes a higher risk for fractures, there is also growing clinical evidence that TZD treatment decreases BMD and increases incidence of fractures (Grey *et al.* 2007, Grey 2008, 2009, Lecka-Czernik 2010). As mentioned previously, rosiglitazone has been shown to increase adipogenesis in BMPC and to decrease osteoblastogenesis (Benvenuti *et al.* 2007, Molinuevo *et al.* 2010). In addition, several *in vivo* studies have demonstrated that administration of TZDs to rodents induces adverse skeletal effects, including a decrease in total body BMD, trabecular bone volume and osteoblast number, while it increases the number of marrow adipocytes (Rzonca *et al.* 2004, Soroceanu *et al.* 2004, Ali *et al.* 2005, Li *et al.* 2006,

Lazarenko *et al.* 2007). The published literature suggests that the effects of TZDs on bone may however vary depending on the strain/sub-strain of mice, gender, age, dose, and duration of treatment, as well as the hormonal status and the anatomical location (Ackert-Bicknell *et al.* 2009). For example, female rats treated with rosiglitazone did not exhibit any adverse effect on bone, while ovariectomy in these rats led to a significant rosiglitazone-induced bone loss, indicating that TZDs may enhance the bone loss induced by estrogen deprivation (Sottile *et al.* 2004). This is in agreement with a recent study in transgenic mice over-expressing PPAR γ in osteoblasts, in which the bone loss in female became evident only after ovariectomy, although the male mice also exhibited reduced bone mass (Cho *et al.* 2011). Clinical studies have also shown that TZDs have greater effects on bone loss (Berberoglu *et al.* 2010) and risk of fractures (Kahn *et al.* 2008) in postmenopausal women with type 2 diabetes than in males. Furthermore, bones of young, growing mice were shown to be less affected than those of adult and older mice (Lazarenko *et al.* 2007). The involvement of AMPK in the mechanism of action of TZDs in bone has not yet been investigated, but rosiglitazone has been shown to ameliorate alcoholic fatty liver via the adiponectin/SIRT1/AMPK pathway and to indirectly increase AMPK activity by inhibiting complex I of the respiratory chain to result in increased AMP/ATP ratio (Lim *et al.* 2010, Shen *et al.* 2010). It is also well known that PPAR activity can be modulated by phosphorylation (Burns & Vanden Heuvel 2007); phosphorylation of PPAR γ coactivator, p300, by AMPK was shown to reduce its interaction with PPAR γ and to repress transcriptional activation function of the receptor (Yang *et al.* 2001, Leff 2003). Therefore, we cannot exclude that PPAR γ is also a transcriptional target of AMPK as shown for PPAR α (McGee & Hargreaves 2008).

In contrast, metformin induces an osteogenic effect *in vivo* and *in vitro*, possibly mediated via RUNX2 and activation of AMPK (Cortizo *et al.* 2006, Gao *et al.* 2010, Lee *et al.* 2010, Molinuevo *et al.* 2010, Zhen *et al.* 2010). Co-treatment of BMPC with metformin partially inhibits the adipogenic action of rosiglitazone (Molinuevo *et al.* 2010). Experimental studies have shown that metformin prevents bone loss in ovariectomized rats, reducing RANKL expression in osteoblasts and therefore inhibiting osteoclast differentiation and activity (Mai *et al.* 2011). In addition, metformin treatment has been shown to stimulate bone healing in a craniotomy defect model in both control and diabetic rats (Molinuevo *et al.* 2010). The effect of metformin on bone in humans has been poorly studied, but due to the safety problems of rosiglitazone, there is a regain of interest for metformin action on bone. Borges *et al.* (2011) recently performed a randomized, parallel group, double-blind, multicenter study comparing the efficacy and safety of Avandamet (AVM; rosiglitazone/metformin) and metformin on BMD after 80 weeks of treatment in drug-naïve type 2 diabetes mellitus patients. A decrease in lumbar

spine and total hip BMD were observed in the AVM group while in the metformin group modest increases in lumbar spine and total hip BMD were detected, suggesting that metformin treatment in diabetic patients could have beneficial effects on bone.

Targeting the AMPK pathway for osteoporosis treatment?

This overview of experimental studies and clinical data clearly indicates that manipulating the AMPK pathway has an impact on skeletal metabolism. Owing to its regulation of the coordination between anabolic and catabolic metabolic pathways, AMPK could represent an attractive therapeutic target for osteoporosis. This is supported by *in vitro* studies which overall imply that AMPK pathway is beneficial for bone formation while inhibiting bone resorption and by the low bone mass phenotype of mice with germline deletions of AMPK subunits. However, there is no proof yet that AMPK activation can lead to bone formation *in vivo*. In addition, there are a few conflicting studies describing the deleterious effect on bone of two AMPK activators, AICAR and TZDs. AICAR has been shown to stimulate bone resorption both *in vitro* and *in vivo* in mice (Quinn *et al.* 2010) and TZD treatment in humans leads to an unbalanced remodeling in favor of bone resorption (Grey *et al.* 2007, Grey 2009, Berberoglu *et al.* 2010, Lecka-Czernik 2010). The involvement of AMPK activation in this negative impact on skeleton of AICAR and TZDs has not yet been proven. This pinpoints a major problem for all these studies, which is that drugs that are currently used to activate AMPK are non-specific. Furthermore, the widespread AMPK cellular actions make difficult the use of these activators *in vivo*. New pharmacological AMPK activators are in development for the treatment of diabetes that could be effective in specific target organs and achieved through isoform-specific activation of AMPK. The development of novel AMPK agonists selective for bone could be a similar option considering that AMPK isoforms expression in bone is different from other tissues. The possibility of targeting both osteoporosis and the rapid increase in body weight after the menopause would be very attractive and AMPK, if confirmed to be important for bone formation *in vivo*, could be a very appealing strategy. Obesity is also a major risk factor for osteoarthritis (OA) and the AMPK pathway could be similarly involved in OA cartilage (Kang *et al.* 2010). However, despite the rapid progress made during the last decade to clarify the physiological importance of AMPK in diabetes, obesity, insulin resistance, cancer, and cardiovascular diseases, there are still many challenges in understanding the role of AMPK activation in each tissue, the cumulative effects of AMPK activation at the whole-body level, before one can target AMPK therapeutically for disorders of energy metabolism. All the more, the studies investigating the role of AMPK in skeletal physiology are just beginning and much effort is needed to

clarify the function of AMPK in bone and its contribution to the skeletal effects of antidiabetic drugs. While mutation in the $\gamma 2$ subunit of AMPK causes cardiomyopathy associated with cardiac glycogen storage disorder (Arad *et al.* 2007), no mutation of AMPK has yet been linked to human bone disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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