Endocrine role of bone: recent and emerging perspectives beyond osteocalcin

K J Oldknow, V E MacRae and C Farquharson
Developmental Biology, The Roslin Institute, Edinburgh, UK

Abstract
Recent developments in endocrinology, made possible by the combination of mouse genetics, integrative physiology and clinical observations have resulted in rapid and unanticipated advances in the field of skeletal biology. Indeed, the skeleton, classically viewed as a structural scaffold necessary for mobility, and regulator of calcium–phosphorus homeostasis and maintenance of the haematopoietic niche has now been identified as an important regulator of male fertility and whole-body glucose metabolism, in addition to the classical insulin target tissues. These seminal findings confirm bone to be a true endocrine organ. This review is intended to detail the key events commencing from the elucidation of osteocalcin (OC) in bone metabolism to identification of new and emerging candidates that may regulate energy metabolism independently of OC.

Key Words
- osteoblast
- osteocalcin
- metabolism
- fertility
- osteoclast

Evolution and bone
The vertebrate skeleton is one of the largest mammalian organs, providing the framework of the body, supporting the softer tissues and creating points of attachment for most skeletal muscles. In addition, the skeleton provides protection for vital organs and blood cells, assists in movement and acts as a storage system for minerals, namely calcium and phosphorus, in order to repair, micromanage and participate in fracture healing, thus maintaining a high bone quality adequate to fulfil its major functions. Uniquely, bone has the ability to renew itself through a process of remodelling. Bone remodelling is a biphasic process occurring throughout life in a constant and balanced manner, responsible for linear growth and bone maintenance during adulthood, thus demonstrating true homoeostatic functions. These processes are fully dependent upon two antagonistic cell populations: the osteoblasts and osteoclasts. The primary function of mesenchyme-derived osteoblasts is the deposition of bone matrix that is subsequently mineralised. Conversely, the haematopoietic-tissue-derived osteoclasts are a unique cell type possessing the capability to destroy the host tissue by reabsorbing mineralised bone matrix (Rodan & Martin 2000, Teitelbaum 2000, Harada & Rodan 2003, Teitelbaum & Ross 2003, Karsenty 2006). The misregulation of bone remodelling inevitably results in bone loss and disease and the most common, by far, is osteoporosis.

Considering the sheer size and dynamic homoeostatic nature of the skeleton, it is not implausible to postulate that the skeleton has a high energetic cost. Simple clinical observations add credence to the possible relationship between energy and bone, exemplified by patients with anorexia nervosa, who display decreased or arrested bone growth and low bone mass in adults (Legroux-Gerot et al. 2005). Converesly, obesity has traditionally been observed...
to have a positive effect on mechanical loading, thus providing protection from osteoporosis. Nevertheless, results from recent clinical studies have indicated that increased adiposity is associated with low total bone mineral density (BMD) and total bone mineral content (reviewed in Cau (2011)).

Insulin and the insulin receptor

For the survival of all species, the ability to precisely regulate energy production and expenditure is critical. In a once unstable environment, mammals evolved intricate paracrine, autocrine and endocrine signalling pathways that coordinate energy expenditure and storage in metabolically active tissues. Metabolic imbalance between energy intake and expenditure is detrimental, with a positive imbalance resulting in obesity and diabetes (diseases encompassed by the metabolic syndrome) or a negative energy imbalance resulting in anorexia nervosa (Fulzele et al. 2010, Fulzele & Clemens 2012). Insulin is a peptide hormone synthesised in the β-cells of the pancreatic islets of Langerhans as its precursor proinsulin and has pleiotropic roles within the body, regulating glucose homeostasis, carbohydrate, lipid and protein metabolism, and promoting cell division and growth through its mitogenic effects (reviewed in Wilcox (2005)); thus, insulin regulates whole-body energy utilisation, mediating its downstream effects by binding to the insulin receptor (IR). First identified in 1971, the IR is a heterotetrameric membrane glycoprotein situated in the plasma membrane of target cells. The IR is composed of two α and two β subunits linked by disulphide bonds. Upon binding of insulin to the extracellular α subunit of the IR, a conformational change in the intracellular β subunit is elicited, thus allowing for the binding of ATP, triggering phosphorylation of the β subunit. Accordingly, it also confers tyrosine kinase activity, leading to phosphorylation of various effector molecules including IR substrate 1 (IRS1). IRS1 can subsequently bind to further signalling molecules, mediating the cellular effects of insulin (Hubbard et al. 1994, Hubbard 1997, Kido et al. 2001).

It is well established that bone possesses a functional IR (Pun et al. 1989). Results from in vitro studies, utilising osteoblast cultures, and in vivo studies have indicated that insulin increases bone anabolic markers, modulating collagen synthesis (Rosen & Luben 1983), alkaline phosphatase production (Canalis 1983, Kream et al. 1985, Yamaguchi et al. 1993), parathyroid hormone (PTH) responsiveness (Thomas et al. 1995) and glucose uptake (Ituarte et al. 1989). Importantly, the heterogeneous distribution of IR in neonatal rat calvaria was reported subsequently (Thomas et al. 1996). Results from complementary studies indicated that insulin-challenged primary and cultured osteoclast-like cells dose-dependently suppressed osteoclast function via inhibiting resorptive pit formation, supporting the anabolic role of insulin in bone (Thomas et al. 1998). Indeed, insulin deficiency in humans exemplified by patients with type 1 diabetes mellitus (T1DM) has in some, but not all, subjects been associated with decreased bone mass (Kemink et al. 2000) coupled with poor bone regeneration following injury (Loder 1988).

DM is a group of metabolic diseases resulting from defects in insulin secretion, insulin action or both. Patients with DM have an increased risk of bone fractures; however, T1DM and T2DM result in differing osteopathy (Leidig-Bruckner & Ziegler 2001). T1DM results in low BMD, increasing fracture risk by approximately six times, whereas the fracture risk is increased by approximately only two times in T2DM compared with the general population due to bone quality deterioration (Tuominen et al. 1999, Jackuliak & Payer 2014). Specifically, T1DM patients have an absolute deficiency of insulin-like growth factor 1 (IGF1), that results in impaired bone formation and lower peak bone mass. Conversely, T2DM patients may display increased BMD due to both increased mechanical loading and hyperinsulinaemia; however, both T1DM and T2DM patients have microarchitectural bone changes, resulting in bone which has an inferior quality compared with that of the general population (Brown 2004, Yamagishi et al. 2005, Melton et al. 2008, Milczarczyk 2008, Nyman et al. 2011). As DM is beyond the remit of this review, we direct the reader to the review by Jackuliak & Payer (2014).

Given these insights, it has been postulated that there is a bone–energy endocrine loop. The first supportive evidence originated from the initial realisation that leptin, an adipocyte-derived hormone, inhibits both appetite (Flier & Elmquist 1997, Friedman & Halaas 1998) and bone mass accrual through a hypothalamic relay (Ducy et al. 2000). Thereafter, a rapid expansion of evidence supporting this crosstalk has occurred, further elucidating the complex roles of leptin and identifying further adipocyte- (adiponectin) and gut-derived hormones (glucagon-like peptides 1 and 2 and serotonin) that regulate bone mass, remodelling and energy homoeostasis. The revelation that bone itself regulates energy metabolism in a reciprocal manner via a secreted hormone osteocalcin (OC) was finally uncovered several years ago (Lee et al. 2007). Thus, in the last few years, an explosion of avant-garde research has explored this concept,
uncovering new and atypical roles of bone beyond its traditional functions. This aims of this review are to succinctly discuss the crosstalk between insulin and the osteoblast as well as introducing and considering new concepts beyond the current dogmas in an attempt to demonstrate the complexity of this field.

Osteocalcin

OC or bone Gla-protein was isolated from bone over three decades ago by two independent groups (Hauschka et al. 1975, Price et al. 1976) and is the most abundant osteoblast-specific non-collagenous protein (Hauschka et al. 1989). Named due to the presence of three vitamin K-dependent γ carboxyglutamic acid residues, OC is a small protein (46 and 49 amino acids long in mice and humans respectively) initially synthesised in the osteoblast as a pre–pro molecule. Vitamin K-dependent post-translational modifications occur causing three glutamic acid residues (GLU13, GLU17 and GLU20) to be γ carboxylated into Gla residues by a γ carboxylase. Final intracellular cleavages produce the mature OC, which is subsequently secreted. The presence of the three γ carboxyglutamic acid residues is critical for the structure and function of OC in the fully carboxylated state allowing the binding of OC to hydroxyapatite (HA) with a high affinity, regulating the maturation of bone mineral (Hauschka & Wians 1989, Hauschka et al. 1989). However, OC also exists in the general circulation in fully carboxylated, partially carboxylated and completely uncarboxylated forms (Plantalech et al. 1991, Cairns & Price 1994, Vergnaud et al. 1997, Schilling et al. 2005, Ferron et al. 2010a). On the basis of results from human and rodent studies, serum OC concentrations have been correlated with bone formation and osteoblast number, thus being used as a serum marker of bone formation (Brown et al. 1984; reviewed in Gundberg (2012)). To investigate the role of OC in bone health, OC-deficient mice were generated (Oc−/−); however, surprisingly no major skeletal deformities were observed in these mice (Ducy et al. 1996). In 2007, further phenotypic evaluation of these mice resulted in an unanticipated finding. Oc−/− mice were hyperglycaemic, hypoinsulinaemic and had reduced insulin secretion and sensitivity compared with WT mice. Additionally, islet size, number, β-cell mass, pancreas insulin content and insulin immunoreactivity were all markedly decreased in Oc−/− mice. Moreover, Oc−/− mice had increased fat mass and adipocyte number, being insulin-resistant in the liver, muscle and white adipose tissue (Lee et al. 2007). This study also focused on

the small number of genes encoding secreted or signalling molecules that are expressed exclusively by the osteoblast in the hope of identifying further osteoblast-enriched genes affecting energy metabolism. One gene was found to be of most interest, expressed in only two cell types: the osteoblast and Sertoli cells of the testis. This gene was Ptprv (Esp), encoding osteotesticular protein tyrosine phosphatase (OST–PTP; Mauro et al. 1994). In vitro, Ptprv coordinates the progression of the preosteoblast to a mature, mineralising cell, and in vivo it may be a critical regulator of the commitment of mesenchymal cells to the ossification of new bones during skeletogenesis (Mauro et al. 1994, Chengalvala et al. 2001, Yunker et al. 2004). It is well established that PTPs are key regulators of IR signalling in many cell types, dephosphorylating and inactivating the IR within minutes of stimulation to maintain glucose homoeostasis (Mauro et al. 1994, Hunter 1995, Schlessinger 2000, Dacquin et al. 2004, Tonks 2006, Lee et al. 2007). As a result, two mutant mice were created: a global knock out of Ptprv (Lee et al. 1996) and an osteoblast-specific knock out of the phosphatase domain of OST–PTP (Dacquin et al. 2004). Both mutants exhibited severe hypoglycaemia and hyperinsulinaemia, resulting in postnatal lethality in the first 2 weeks of life. Results from further analysis indicated that the pancreas of Ptprv−/− mice had greater islet content, number of islets, islet size and β-cell mass, resulting in increased insulin secretion. In addition, mutants were significantly more tolerant to glucose upon challenge, displaying an insulin-sensitive phenotype, thus mice were protected from induced obesity and diabetes (Lee et al. 2007, Ferron et al. 2008). In parallel, mice overexpressing full-length Ptprv cDNA selectively in osteoblasts exhibited hyperglycaemia, hypoinsulinaemia, glucose intolerance, insulin resistance, decreased β-cell proliferation, lower β-cell mass and impaired insulin secretion. Subsequently, it was noted that the phenotype of Ptprv−/−/− mice mirrored the Oc−/− mouse phenotype, while the Ptprv mice overexpressing full-length Ptprv cDNA selectively in osteoblasts were a phenocopy. Results from further genetic studies indicated that the metabolic phenotype of Ptprv−/− mice was fully corrected by removing one allele of Oc, implying that Ptprv−/− mice are a model for a gain of function of Oc, providing solid evidence that Ptprv and OC reside in the same regulatory pathway (Lee et al. 2007). Biochemical analysis revealed that Ptprv−/− mice have significantly higher serum undercarboxylated OC levels than WT controls; however, OC expression and serum levels were normal in Ptprv−/− mice, indicating that OST–PTP is involved in the decarboxylation of OC and the subsequent
release of undercarboxylated OC into the systemic circulation (Lee et al. 2007, Ferron et al. 2010a).

Notwithstanding, it still remained unclear as to how OC carboxylation status could regulate whole-body energy metabolism. Clues came from several key studies concerning forkhead box protein O1 (Foxo1) and activating transcription factor 4 (Atf4) (Seo et al. 2009, Yoshizawa et al. 2009, Rached et al. 2010, Kode et al. 2012). Foxo1 is a transcription factor targeted by insulin and regulates glucose homeostasis in tissues involved in energy metabolism including adipocytes and hepatocytes; however, its function in osteoblasts has not been explored until recently. A Foxo1 osteoblast conditional knockout mouse was generated, that displayed decreased fasting blood glucose levels and increased insulin sensitivity. The mice also displayed a 30% increase in serum OC levels, coupled with a 75% reduction in Ptprv expression, indicative of an association between Ptprv and carboxylation status of OC. In the same study, it was demonstrated, utilising various mouse models, that heterozygous mice lacking one allele of Foxo1 in osteoblasts and one allele of Ptprv showed improved insulin sensitivity. Similarly, the metabolic phenotype was corrected in heterozygous mice lacking one allele of Foxo1 in osteoblasts by the removal of one allele of OC. Utilising these models to investigate the mechanisms underlying the phenotype, it was established that Foxo1 regulates the bioactivity of OC via OST–PTP through direct binding to its promoter, reducing serum OC (Rached et al. 2010, Kousteni 2011, 2012). In a separate study, the role of Atf4 was also investigated. Atf4 belongs to the subfamily of cAMP-response element-binding proteins/ATF basic leucine zipper proteins broadly expressed throughout the body; however, it predominantly accumulates in osteoblasts where it regulates virtually all functions of the osteoblast related to the control of bone mass including bone formation and matrix mineralisation (Yang & Karsenty 2004, Elefteriou et al. 2005, Yoshizawa et al. 2009). Atf4−/− mice primarily show phenotypic abnormalities in the skeleton; however, the global or osteoblast-specific ablation of Atf4−/− in mice results in favourable metabolic changes, including improved glucose tolerance and insulin sensitivity associated with decreased Ptprv expression. In contrast, the overexpression of Atf4 in osteoblasts reflected this phenotype, resulting in glucose intolerance associated with increased Ptprv expression. This effect was due to the direct regulation of Ptprv expression in osteoblasts by Atf4, established by a ChIP array confirming that Atf4 binds to the CRE element in the Ptprv promoter (Yoshizawa et al. 2009). Finally, it has been shown that Foxo1 co-localises with Atf4 in the osteoblast nucleus, promoting the transcriptional activity of Atf4, thus up-regulating the expression of Ptprv in osteoblasts, resulting in OC inactivation (Kode et al. 2012).

But how does Ptprv affect insulin signalling in osteoblasts? In the search for the OST–PTP substrate in osteoblasts, utilising multiple genetic and biochemical modalities, the IR was identified as a potential substrate. As a result, two studies conducted simultaneously by the laboratories of Professors Karsenty and Clemens to explore the role of insulin signalling in osteoblasts were initiated. They generated osteoblast-specific IR-deficient mice (Insr−/−) that presented with hyperglycaemia, increased peripheral adiposity, reduced insulin secretion, severe glucose intolerance and decreased levels of circulating undercarboxylated OC. These mice also displayed a skeletal phenotype with a reduction in bone acquisition due to reduced bone formation; however, the marker of bone resorption (CTx) was decreased. Upon infusion of exogenous undercarboxylated OC, the metabolic phenotype was fully corrected, indicating that insulin signalling in osteoblasts has the potential to regulate whole-body glucose homeostasis via carboxylation status of OC (Ferron et al. 2010b, Fulzele et al. 2010). It was also suggested that insulin signalling in osteoblasts might favour bone resorption, due the observation that decreased CTx levels in Insr−/− mice reflected the increase in CTx observed in Ptprv−/− mice. Utilising osteoblasts from Insr−/− and Ptprv−/− mice, Ferron and colleagues established, using a co-culture system, that WT osteoclast precursor cells cultured with osteoblasts isolated from Insr−/− mice decreased osteoclast resorption pit formation, while a 50% increase in osteoclast resorption pit formation was observed when Ptprv−/− primary osteoblasts were used in the co-culture system. Moreover, osteoprotegerin (Opg (Tnfrsf11b)), a negative regulator of osteoclast formation and function, encoding the decoy receptor for receptor activator of nuclear factor κ B ligand (RANKL), was increased by twofold in Insr−/− and decreased by 50% in Ptprv−/− osteoblasts. Further unravelling of this complex pathway revealed that insulin signalling in osteoblasts inhibited Foxo1 expression, favouring bone resorption via suppression of Opg and Twist2 (RUNX2 inhibitor; Ferron et al. 2010b, Fulzele et al. 2010, Rached et al. 2010). It appeared that osteoclasts were pivotal for the connection between bone and energy metabolism; therefore, Ferron and colleagues investigated genes associated with Opg-dependent events in the osteoclast. It was found that Tcirl1, an essential part of the plasma membrane proton pump, responsible for the acidification of the bone before bone resorption by
osteoclasts, was decreased in co-culture osteoclast/Insr<sup>b<sup>−/−</sup></sup> osteoblast models (Teitelbaum 2000, Teitelbaum & Ross 2003, Bronckers et al. 2012). These results indicated that insulin signalling in osteoblasts induces osteoclast acidification and bone resorption via decreased Opg expression. Utilising biochemical and mass spectrometry analysis, it was established that an acidic environment generated by osteoclasts situated in the resorption lacuna can decarboxylate OC present in the extracellular matrix (Engelke et al. 1991).

In addition to the classical osteoblast-specific PTP, Ptprv, which is defined by its specificity for phosphotyrosine (Alonso et al. 2004, Barr et al. 2009), 37 other mammalian classical PTPs exist. Of these, the only other identified PTP able to bind to the osteoblast IR and respond to isoproterenol treatment similarly to OST–PTP (Hinoi et al. 2008) is T-cell PTP. This finding further supports the notion that bone is involved in the regulation of glucose metabolism, increasing our understanding of the complex regulation of OC-mediated glucose homoeostasis (Zee et al. 2012) (for comprehensive and recent reviews, see Karsenty & Ferron (2012) and Ferron & Lacombe (2014)).

Even in light of this new concept of bone acting as an endocrine organ, it still remains unclear as to why osteoporotic or osteopenic mice all do not display metabolic imbalances. This is indicative of a far more complex regulation of energy by bone, and indeed supportive of the notion that additional osteoblast- or osteocyte-derived factors are likely to exist.

**Male fertility and the discovery of the OC receptor**

Diet-induced obesity in rodent models leads to a decrease in sperm motility and reduced hyperactivated progression, which is associated with a trend towards a reduction in fertility potential (Ghanayem et al. 2010, Fernandez et al. 2011). In humans, obesity is associated with infertility by reducing semen quality, changing sperm proteomes and contributing to erectile dysfunction (reviewed in Cabler et al. (2010) and Palmer et al. (2012)).

The discovery of the OC receptor (GPRC6A) occurred simultaneously with the elucidation of the role of OC in fertility. Briefly, male and female patients with gonadal failure possess low bone mass; furthermore, menopause favours bone loss (Riggs et al. 1982, 1998, Wishart et al. 1995). These clinical observations led to the investigation of the possible relationship between bone and fertility. Fortuitously, it was noted that Oc<sup>−/−</sup> mice were poor breeders, as a result of from decreased testes weight with a 50% reduction in sperm count associated with impaired Leydig cell maturation and decreased circulating testosterone. Reflecting this phenotype, Ptprv<sup>−/−</sup> mice had increased male reproductive organ weights with a 30% increase in sperm count and increased circulating testosterone (Oury et al. 2011). These results indicated a link between OC and testosterone production, which was relevant to males only, as no change in circulating oestrogen or the aromatase enzyme required to convert testosterone to oestrogen (Cyp19A1) was observed in the Ptprv<sup>−/−</sup> or Oc<sup>−/−</sup> mice. In an effort to clarify the signalling mechanism underlying this pathway, several factors were taken into consideration, namely the target cells affected by OC (β-cells of the pancreas and the Leydig cells of the testis) and the sexually dimorphic aspects of OC. These clues led to the identification of GPRC6A, a G protein-coupled receptor linked to adenylate cyclase. Gprc6a is expressed in the Leydig cells, and its inactivation in mice leads to a metabolic phenotype very similar to that of Oc<sup>−/−</sup> mice characterised by glucose intolerance and decreased β-cell area and β-cell mass. In addition, these mice demonstrate defective bone mineralisation (Pi et al. 2008, 2010). Moreover, the compound heterozygous mice (Oc<sup>−/+</sup> Gprc6a<sup>−/+</sup>) had a reproductive phenotype similar in all aspects to that observed in Oc- and Gprc6a-deficient mice models (Oury et al. 2011). These results indicated GPRC6A to be an OC receptor, demonstrating that OC mediates testosterone biosynthesis. Additionally, utilising the Gprc6a<sup>−/−</sup> mouse model, it was shown that i.p. injection of OC failed to markedly stimulate ERK activity, thus having minor effects on circulating serum insulin levels, which were increased in WT mice exposed to the same treatment. GPRC6A has been shown to be integral in the promotion of β-cell proliferation during development and adulthood via OC, thus highlighting GPRC6A as an important receptor for skeletal-tissue-mediated energy regulation via the pancreas (Pi et al. 2011, Wei et al. 2014a). Most recently, Oury et al. (2013) demonstrated that OC acts via a pancreas–bone–testis axis, such that OC-stimulated testosterone synthesis is positively regulated by insulin signalling in osteoblasts and is independent of luteinising hormone (LH). No connection between Ptprv<sup>−/−</sup> and Oc<sup>−/−</sup> mice in osteoblast-stimulated oestradiol production was identified, illustrating that the regulatory mechanisms of fertility of male and female mice are vastly distinct (Oury et al. 2011).

It was noted that the reproductive phenotype of Oc<sup>−/−</sup> and Gprc6a<sup>−/−</sup> male mice was very similar to that of Lhb<sup>−/−</sup> (LH-deficient) male mice, all displaying
defective testosterone synthesis and testosterone-dependent events (Oury et al. 2011). LH is a key regulator of male fertility, favouring testosterone biosynthesis via the hypothalamo-pituitary axis (Kumar 2007). Surprisingly, further analysis of Oc<sup>−/−</sup> or Gprc6a<sup>−/−</sup> mice revealed increased circulating levels of LH, which is indicative of a dual regulation of male fertility, or of OC acting downstream, of LH (Themmen & Huhtaniemi 2000, Kumar 2007). By means of elaborate studies from Karsenty’s groups have since demonstrated that OC regulates male fertility independently of the hypothalamo-pituitary axis. Indeed, the regulation of testosterone synthesis by OC is independent of a measurable influence of Gprc6a on Lh (Lhb) expression and there is no evidence that LH regulates OC expression (Ferron et al. 2010a,b, Oury et al. 2013; reviewed in Karsenty & Oury 2014).

To emphasise the importance of the role of bone in energy metabolism, Wei et al. (2014b) evaluated the consequences of osteoblast-specific overexpression of or loss of IR in high-fat-diet (HFD)-fed mice. Results from these studies indicated that insulin resistance in bone affects whole-body glucose homeostasis in mice fed on a HFD by decreasing OC activity; moreover, it was demonstrated that SMURF1-mediated IR ubiquitination contributes to the development of insulin resistance in osteoblasts. These results support the notion that bone is a highly important site for the regulation of global energy homeostasis (Wei et al. 2014b).

What else controls OCN?

As discussed, results from a number of seminal studies have indicated that a feed-forward link exists between OC and insulin; however, leptin and glucocorticoids have been shown to negatively regulate OC activity. In brief, leptin secretion by adipocytes results in increased Ptpn1 expression via Atf4, occurring via a central pathway (Hinoi et al. 2008) and glucocorticoids decrease OC activity by suppressing osteoblast function and OC production (Brennan-Speranza et al. 2012; reviewed in Ferron & Lacombe 2014).

Clinical evidence: OC and metabolism/fertility

One of the earliest studies to show an association between OC and glucose metabolism was published over a decade ago. OC levels were significantly lower in diabetic patients, although OC levels increased with improved glycaemic control (Rosato et al. 1998). In many human studies only total OC levels were quantified; however, the effects on glucose metabolism via bone are attributed to undercarboxylated OC. These studies yielded mixed results with several of them indicating a positive correlation between serum undercarboxylated OC levels and enhanced β-cell function (Hwang et al. 2009, Prats-Puig et al. 2010, Pollock et al. 2011). However, results from other studies indicate no association between lower circulating undercarboxylated OC levels and higher HOMA-IR (Shea et al. 2009). Results from one recent study have indicated that there is a sex-specific action of the bone–energy homoeostasis axis with OC being associated with improved metabolic state via adiponectin in females, and via testosterone in males (Buday et al. 2013). Direct clinical evidence has been reported for the role of OC in energy metabolism, via the removal of an OC-producing osteoid osteoma, which resulted in elevated serum glucose, potentially associated with decreased levels of undercarboxylated OC (Confavreux et al. 2012). This conflicting results may be attributable to the lack of a commercially available undercarboxylated assay, or differing methodologies (Ducy 2011). Similarly, it appears that the reproductive function of OC translates to humans, with the identification of a positive association between OC and testosterone serum levels in the general population, patients with bone disorders and patients with T2DM (Hannemann et al. 2013, Kanazawa et al. 2013). Furthermore, two subjects were identified from a cohort of patients displaying testicular failure who harboured a heterozygous missense variant in one of the transmembrane domains of GPRC6A, giving credence to a role of OC function in humans (Oury et al. 2013; reviewed in Karsenty & Oury 2014).

Beyond OC

Intriguingly, recent evidence has indicated that other osteoblast-derived hormones may contribute to the emerging function of the skeleton as a regulator of energy metabolism. This was demonstrated by the partial ablation of osteoblasts in transgenic mice, which resulted in profound effects on glucose metabolism and gonadal fat mass, combined with increased energy expenditure. OC administration partially corrected the metabolic phenotype; however, it did not reverse the increased energy expenditure or decreased gonadal fat. This indicates that osteoblasts have the ability to affect glucose metabolism through both OC-dependent and -independent mechanisms (Yoshikawa et al. 2011). Herein, we will discuss novel candidates that influence energy metabolism, with a focus on emerging concepts (summarised Fig. 1).
Glucose transporter and bone

Cellular uptake of glucose is mediated by either of the two families of membrane-associated carrier proteins, namely the sodium coupled glucose transporters (SGLTs) via active transport and glucose transporter (GLUT) facilitators via facilitated diffusion (Bell et al. 1990, Carruthers 1990). The SGLT family comprises 12 members including co-transporters for sugars, anions, vitamins and short-chain fatty acids (Wright & Turk 2004). Currently, the presence of SGLT in bone has not been reported; however, SGLT2 receptor inhibitors, acting as glucose-lowering agents in the management of T2DM, have been reported to have no significant effects on bone formation and resorption or BMD in humans (Ljunggren et al. 2012). In contrast, GLUT receptors have recently been reported to be expressed in bone. To date, the GLUT family consists of 14 members subclassified into three groups, according to sequence similarities and characteristic elements (Joost &
Glut4 receptors exhibit striking tissue-specific expression, each possessing differential sensitivities to stimuli such as insulin, thus allowing for complex and specific regulation of glucose uptake according to cellular requirements (Gould & Holman 1993). It was first suggested that insulin promotes increased glucose uptake via GLUT1 in the osteoblast, independently of IGF1 signalling to increase the metabolic activity of the osteoblast (Fulzele et al. 2007). Most recently, Glut4 has been found to be expressed at similar levels to those in skeletal muscle in osteoblasts, osteocytes and chondrocytes, with the genetic ablation of Glut4 in osteoblasts/osteocytes resulting in increased peripheral adiposity associated with mild hyperinsulinemia. These mice also presented with insulin resistance. These metabolic changes were assumed to originate from osteoblasts/osteocytes as no altered gene expression was identified in the liver or adipose tissue, indicating that decreased GLUT4-mediated glucose uptake in bone is sufficient to influence whole-body metabolism (Zhu et al. 2013). Recent emerging results from two independent laboratories have indicated that, in addition to Glut4, Glut1 is necessary for bone formation and whole-body glucose homoeostasis. Moreover, Glut1 is modulated by high glucose levels (Virta et al. 2014, Wei et al. 2014a,b,c). Collectively, these results provide a deeper understanding of the role of bone in the regulation of glucose metabolism (summarised in Fig. 2).

### AMP-activated protein kinase and energy metabolism

It has recently been suggested that AMP-activated protein kinase (AMPK) is a key enzyme in the relationship between bone and fat. AMPK is a downstream component of a kinase cascade composed of differing subunits (α1, α2, β1, β2, γ1, γ2 and γ3). AMPK forms heterotrimers that exhibit differences in subcellular localisation and regulation (Hardie 2007), playing a key role in the orchestration of cellular energy homoeostasis (Hardie et al. 2006, Lage et al. 2008). In response to physiological/pathological stimuli, AMPK acts to restore cellular energy balance (AMP:ATP ratio). During cellular energy deprivation, AMPK increases the potential for ATP production via ATP-generating pathways such as fatty acid oxidation, while concurrently decreasing cellular energy-consuming anabolic processes (Corton et al. 1994, Kahn et al. 2005). Impairment of AMPK is associated with the metabolic syndrome, demonstrating its physiological requirement, reflected by the improvement of energy metabolism, namely insulin sensitivity in the presence of AMPK (Steinberg & Kemp 2009, O’Neill et al. 2011). It has recently been suggested...
that AMPK is central to the regulation of skeletal metabolism. The \( \alpha_1 \) subunit is the dominant catalytic isoform expressed in bone, and, when removed in mice, cortical and trabecular bone compartments were shown to be smaller compared with those of the WT controls (Shah et al. 2010). Moreover, the administration of metformin, a drug used widely in the control of T2DM, ameliorates hyperglycaemia and is known to activate AMPK (Stumvoll et al. 1995, Zhou et al. 2001). AMPK has been reported to enhance differentiation and mineralisation of osteoblastic MC3T3-E1 cells and dose dependently increase trabecular bone nodule formation in vitro, supporting the hypothesis of a role of AMPK in the regulation of bone formation and bone mass (Kanazawa et al. 2008, Shah et al. 2010). Recently, Jeyabalan et al. (2012) have elegantly reviewed AMPK and bone metabolism and suggested that AMPK activation may be involved in the relationship between bone and fat. Indeed, the activation of AMPK may enable the skeleton to sense energy status, initiating either adipogenesis or osteoblastogenesis depending on energy needs. This hypothesis is corroborated by the observation that AMPK reduced adipogenesis in vitro, by phosphorylating \( \beta \)-catenin, suppressing and directly phosphorylating PAR\( \gamma \) coactivators (Leff 2003, Zhao et al. 2010, Jeyabalan et al. 2012). Supporting this notion, AMPK has been shown to regulate thyroid-hormone-stimulated OC synthesis in osteoblasts, potentially indicating a direct link between AMPK and the regulation of energy metabolism via the skeleton (Kondo et al. 2013).

**Bone morphogenetic proteins**

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that are members of the transforming growth factor \( \beta \) superfamily. BMPs have a critical role in embryogenesis and are important in bone and cartilage formation and function. BMPs have been the subject of other recent and extensive reviews (Chen et al. 2012). Genetic manipulation of mice has allowed a wealth of knowledge to be obtained regarding the complexity of BMPs that may have clinical relevance, such as in the treatment and clinical management of bone grafting and non-union (reviewed in Carreira et al. (2014)). Roles of BMPs in adipogenesis and energy metabolism have recently been described, including in adipocyte development, adipose cell fate determination, differentiation of committed preadipocytes and function of mature adipocyte (Tang et al. 2004, Taha et al. 2006, Huang et al. 2009). More recent results have indicated that BMPs play a role in the ‘browning’ of white adipocytes. Moreover, the genetic ablation of Bmp4 results in enlarged white adipocyte morphology and impaired insulin sensitivity, whereas overexpression of Bmp4 in white adipocytes results in reduced adipocyte tissue mass and size coupled with an increased number of white adipocyte cell types with brown adipocyte characteristics, indicating that BMP4 can regulate the induction of brown-adipocyte-like cells and insulin sensitivity by affecting white adipocyte development (Qian et al. 2013). These characteristics of BMPs appear to be conserved in human tissue, where BMP4 and BMP7 have been shown to induce the white-to-brown transition in primary human adipose stem cells (Elsen et al. 2014, Obregon 2014).

**Glycogen synthase kinase**

Glycogen synthase kinase 3 (GSK3) is composed of two mammalian isoforms, GSK3\( \alpha \) and GSK3\( \beta \), playing largely overlapping roles. Explaining simply, GSK acts mainly as a brake in many anabolic pathways including the Wnt/\( \beta \)-catenin and insulin pathways. Moreover, GSK has been implicated in a range of human pathologies including cancer, Alzheimer’s disease, non-insulin-dependent DM and bipolar disorder (reviewed in Patel et al. (2004) and Forde & Dale (2007)). Recent evidence has indicated that, in addition to the outlined pathologies, GSK3\( \beta \) functions in bone to regulate skeletal development and whole-body metabolism. It has been reported previously that germ-line loss of GSK3\( \beta \) in mice results in skeletal abnormalities; however, these abnormalities were not present in cartilage-specific GSK3\( \beta \) (GSK3B)-deficient mice, possibly due to a compensatory increase in GSK3\( \alpha \) (GSK3A) protein levels (Hoeflich et al. 2000, Kugimiyah et al. 2007, Liu et al. 2007, Gillespie et al. 2011). Subsequently, mice were created in which GSK3\( \beta \) was inactivated in early differentiating skeletal cells and osteoblasts only (Gillespie et al. 2013). These mice displayed delayed skeletal development and ossification and increased trabecular bone. However, most relevant to this review, Col1a1–Gsk3\( \beta ^{+/−} \) mice displayed decreased fat content, smaller adipocytes, pronounced hypoglycaemia and hypoinsulinaemia. Interestingly, female Col1a1–Gsk3\( \beta ^{+/−} \) mice were significantly more insulin-sensitive. These metabolic changes were independent of food consumption and undercarboxylated or total OC. The mechanisms underlying this connection still remain unclear; however, the authors suggested that these metabolic changes may be due to the hyperactivation of the insulin pathway, resulting in the uptake of glucose, or due to the presence of an unknown factor other...
than OC that contributes to increased insulin sensitivity inCol1a1–Gsk3β−/− mice (Gillespie et al. 2013).

**Osteocyte and energy**

In addition to the discussed specialised bone cells (osteoblasts and osteoclasts) osteocytes have also recently been suggested to be involved in energy metabolism. Osteocytes are the most abundant bone cells, formed from differentiated mature osteoblasts, thus becoming terminally differentiated osteocytes. Osteocytes become entrenched within the mineralised bone matrix, forming canalicular networks with other osteocytes and bone-surface osteoblasts, acting as important mediators for intracellular communication and potentially orchestrating bone remodelling. Additionally, osteocytes are able to detect gravitational forces and are thought to play a role in matrix mineralisation and phosphate homoeostasis; however, the precise functions of osteocytes still remain unclear (Karsenty & Wagner 2002, Bonewald 2007, 2011). Intriguingly, Sato and colleagues have recently suggested that osteocytes may play a role in the regulation of the control of fat mass in association with the hypothalamus. Mice were generated in which the receptor for diphtheria toxin (DT) was under the control of the dentin matrix protein 1 promoter (Dmp1). Mice then received injections of DT at 15 weeks to render them osteocyte-less mice (OL mice). Following injection, mice lost weight and white adipose tissue mass, with a drastic reduction in mesenteric and subcutaneous fat; however, these mice were not diabetic. These effects were reversed when osteocytes were replenished within the bone. The mechanism underlying this phenotype remains unknown; however, total OC was decreased in the OL mice (Sato et al. 2013). However, the DMP1 promoter also targets the osteoblast and, therefore, the assumption that the phenotype is entirely OC-driven is open to interpretation (Moverare-Skrtic et al. 2014). Moreover, Ferron & Lacombe have recently suggested the potential presence of ‘osteokines’, osteocyte-derived factors that may be implicated in the endocrine regulation of glucose metabolism; however, these factors are yet to be discovered (Sato et al. 2013, Ferron & Lacombe 2014).

Excitingly, results from other recent studies have indicated that osteocyte-derived fibroblast growth factor 23 (FGF23) functions in an endocrine manner. Since its identification in 2000, FGF23 has been shown to be most highly expressed in bone (osteocyte), acting as an important hormone in regulating serum phosphate levels primarily via actions on the kidney (Shimada et al. 2004) (reviewed in Bonewald & Wacker (2013)). In addition to the role of FGF23 in phosphate homoeostasis and bone mineralisation, the PHEX, DMP1, FGF23, KLOTHO and the MEPE/ASARM peptide axis has been demonstrated to be involved in the regulation of energy metabolism via the bone (David et al. 2009a,b). Briefly, mouse models either overexpressing MEPE, ASARM peptides or infused ASARM peptides display increased adiposity, are hyperglycaemic and have increased OC, whereas FGF23-null mice are hypoglycaemic (ASARM peptide modulates PHEX–DMP1-mediated FGF23 expression; Rowe et al. 1996, David et al. 2009a,b, 2011). Intriguingly, patients subjected to a 4-h euglycaemic–hyperinsulinaemic clamp show increased FGF23 that correlates positively with insulin infusion (Winther et al. 2011). These combined data are indicative of key roles for FGF23 in energy metabolism (reviewed in Rowe (2012)).

**Fracture burden and global energy metabolism**

It seems plausible that fracture may be associated with a large metabolic expense, thus directly affecting global energy metabolism. Reviewing the literature, we found no clear link between fracture burden and energy metabolism. However, Hamann and colleagues have recently assessed the effects of intermittent PTH on metabolic function in both diabetic and non-diabetic rats, with internally stabilised induced subcritical femoral defects. PTH had no effect on body weight, glucose tolerance or pancreatic islet morphology in both groups, despite PTH therapy resulting in bone anabolic effects and bone defect repair. Unfortunately, the authors were unable to detect undercarboxylated OC; however, they reported no change in carboxylated OC between vehicle and PTH-treated non-diabetic and diabetic rats (Hamann et al. 2014). These results are surprising as intermittent therapy is known to increase serum levels of OC (Neet et al. 2001, Greenspan et al. 2007). These insights indicate that improved fracture repair may not have a global effect on energy metabolism. Paradoxically, it has been shown that vitamin K-dependent γ carboxylation of OC positively enhances the efficacy of PTH following a closed fracture osteotomy. After osteotomy, carboxylated OC increased by 18% from baseline and uncarboxylated OC was increased by 100% after surgery; however, insulin sensitivity was not assessed (Shimizu et al. 2014).
**Sphingolipids and PHOSPHO1**

Sphingolipids are a large class of lipid molecules containing a sphingoid backbone, derived from the condensation of an amino acid and fatty acid; modifications of this basic structure result in a large sphingolipid family (Hannun & Obeid 2011, Mullen et al. 2012). Sphingolipids are primarily synthesised *de novo* in the endoplasmic reticulum and Golgi apparatus, before transportation to the plasma membrane and endosomes; however, sphingomyelinases also play vital roles in sphingolipid biosynthesis. Categorised as acidic, alkaline or neutral, sphingomyelinases cleave sphingomyelin, thus generating ceramide and phosphocholine (Merrill et al. 1997, Marchesini & Hannun 2004, Futerman & Riezman 2005). Until recently, sphingomyelins were considered structurally inert; however, they are now accepted to be fundamental signalling molecules, responsible for eliciting a wide range of signalling properties and cellular functions, encompassing roles in the regulation of cell growth, proliferation, differentiation, programmed death, death, senescence, adhesion, migration, inflammation, angiogenesis and intracellular trafficking. Current efforts are focused on deciphering the mechanisms underlying these varied roles, enabling a greater understanding of sphingolipid metabolism and lipid generation and action (Hannun & Obeid 2008, Merrill 2011, Airola & Hannun 2013; reviewed in Gault et al. (2010)).

Recent *in vitro* results have indicated that sphingolipids are implicated in osteoblast and chondrocyte apoptosis and in the regulation of osteoclastogenesis (Takeda et al. 1998, MacRae et al. 2006; reviewed by Khavandgar & Murshed (2014)). *In vivo*, sphingolipid metabolism plays a critical role in skeletogenesis; mouse models lacking the ceramide-generating neutral sphingomyelinase 2 enzyme (nSMase2/SMPD3 – gene-targeted Smpd3<sup>−/−</sup> and *fro/fro* mice) display gross skeletal abnormalities, including deformed long bones, short-limb dwarfism, hypomineralisation, delayed dentin mineralisation and enamel formation (Aubin et al. 2005, Stoffel et al. 2005, Alebrahim et al. 2014). Conversely, the overexpression of SMPD3 in osteoblasts only (*fro/fro*;Col1a1–Smpd3 mice) corrects embryonic bone abnormalities, demonstrating a direct role of SMPD3 in skeletal mineralisation (Khavandgar et al. 2011, 2013). However, the mechanisms underlying this role, while remaining unclear, are now becoming a little more evident.

As highlighted, SMPD3 hydrolyses sphingomyelin to phosphocholine (Stoffel et al. 2005), which is subsequently hydrolysed into choline and phosphate by the bone-specific phosphatase PHOSPHO1 (Houston et al. 2004, Stewart et al. 2006, Roberts et al. 2007). Complete ablation of Phospho1 in mice results in a similar phenotype to that of *fro/fro* mice, with Phospho1<sup>−/−</sup> mice having significant skeletal pathology, spontaneous fractures, bowed long bones, osteomalacia and scoliosis in early life (Huesa et al. 2011, Yadav et al. 2011, 2014, Rodriguez-Florez et al. 2014). These results indicate that PHOSPHO1 and SMPD3 are within the same metabolic pathway required for skeletal mineralisation in the mouse (Khavandgar Z, Oldknow KJ, Murshed M & Farquharson C, unpublished observations).

Interestingly, both Phospho1- and Smpd3-deficient models exhibit decreased body size, indicating that, in addition to the *de novo* pathway, the sphingomyelinase pathway may have the potential to regulate energy metabolism (Stoffel et al. 2005, Oldknow et al. 2013). Supporting this notion, results from metabolic studies conducted in our laboratory have highlighted the finding that Phospho1 ablation confers remarkable protection against obesity and diabetes in mice, independent of serum levels of uncarboxylated and undercarboxylated OC (Oldknow et al. 2013). The mechanisms underlying this metabolic protection in both Phospho1- and Smpd3-deficient models remain unclear; therefore, it is important to determine whether concentrations of either circulating or bone-derived choline/ceramide are decreased in these models. Choline supplementation by others results in hepatic insulin resistance (Wu et al. 2013). Moreover, the impairment of *de novo* synthesis of choline via phosphatidylethanolamine N-methyltransferase, which catalyses the methylation of phosphatidylethanolamine in the liver, protects mice from diet-induced obesity (Jacobs et al. 2010). However, in contradiction to the results of these studies, it has recently been reported that choline can promote liver health by maintaining cholesterol homoeostasis (Al Rajabi et al. 2014). Furthermore, *de novo* ceramide accumulation results in an alteration in metabolism (Summers et al. 1998, Merrill 2002, Yang et al. 2009, Ussher et al. 2010). Pharmacological inhibition of dihydroceramide desaturase 1 (DES1), an enzyme involved in the *de novo* pathway of sphingolipid metabolism (responsible for the insertion of a double bond into the sphingosine backbone of prevalent sphingolipids, e.g. conversion of dihydroceramide into ceramide), improves insulin sensitivity (Bikman et al. 2012). Such DES1<sup>−/−</sup> mice have alterations in energy expenditure, and haploinsufficiency of DES1 in the mouse model protects against lipid- and glucocorticoid-induced insulin resistance. (Holland et al. 2007, Siddique et al. 2013).
Taken together, these findings strongly support a role of sphingolipids in the endocrine function of bone; however, the importance of ceramide and choline in energy regulation by the skeleton has not yet been fully investigated.

**Ectonucleotide pyrophosphatase/phosphodiesterase 1**

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) is the founding member of the NPP family. These glycoproteins have pleiotropic roles in hydrolysing phosphodiester or pyrophosphate bonds in various substrates, including nucleoside triphosphates, lysophospholipids and choline phosphate esters (Bollen et al. 2000, Stefan et al. 2005, Zimmermann et al. 2012). Specifically, NPP1 forms disulphide-bonded homodimers and is highly expressed in the plasma membrane and mineral-depositing matrix vesicles of osteoblasts (Johnson et al. 1999, 2001, Vaingankar et al. 2004, Terkeltaub 2006). Thus, NPP1 has been identified as a critical regulator of tissue mineralisation, hydrolysing nucleotides into extracellular inorganic pyrophosphate (PPi), a potent inhibitor of HA crystal formation in mineralisation-competent tissues (Terkeltaub 2001). Mice lacking NPP1 (Enpp1<sup>−/−</sup>) have severe mineralisation defects in long bones and calvariae, with pathological peris INCIDENTAL roles in mineralisation-competent tissues (Terkeltaub 2001). Mice lacking NPP1 (Enpp1<sup>−/−</sup>) have severe mineralisation defects in long bones and calvariae, with pathological peris incidental soft tissue and medial arterial mineralisation associated with abnormally low PPi levels (Sali et al. 1999, Johnson et al. 2003, Anderson et al. 2005, Mackenzie et al. 2012a,b). In addition to its recognised roles in mineralisation, increased NPP1 expression has been associated with insulin resistance in both in vitro and in vivo models by negatively modulating IR signalling. (Maddux et al. 1995, Belfiore et al. 1996, Costanzo et al. 2001, Goldfine et al. 2008, Prudente et al. 2009, Huesa et al. 2014). Additionally, insulin-resistant subjects have been found to have NPP1 overexpression in skeletal muscle, adipose tissue, fibroblasts and lymphocytes (Frittitta et al. 1997, 1998, Teno et al. 1999, Stenz & Kitabchi 2007, Goldfine et al. 2008). Combing the necessity of NPP1 for mineralisation and the known role of NPP1 in insulin resistance led ourselves and our colleagues to investigate whether NPP1 has a functional role in bone as a novel regulator of energy metabolism. Genetic ablation of Enpp1 resulted in insulin sensitisation and mildly improved glucose homeostasis. Upon challenge with a chronic HFD, Enpp1<sup>−/−</sup> mice displayed improved insulin tolerance and resistance to obesity. Unlike the Phosho1<sup>−/−</sup> mice, Enpp1<sup>−/−</sup> mice displayed increased levels of undercarboxylated OC and the bone resorption marker CTX, which is indicative of increased insulin signalling in osteoblasts favouring resorption by osteoclasts (Huesa et al. 2014). However, the results of in vitro studies did not reveal a role for NPP1 as a modulator of insulin signalling, indicating a more complex underlying pathway. Taken together, results from our laboratory indicate a far more complex story underlying the reciprocal regulation of bone and energy metabolism.

**Perspective**

The concept of the whole-body study of physiology has established the skeleton as a bona fide endocrine organ, considerably expanding the classical view of bone towards it being a more complex organ. These provocative results have challenged and fascinated researchers, resulting in an increased number of laboratories working in this field. Further exploration of the endocrine role of the skeleton is necessary in the search for additional candidates for molecules involved in the skeletal control of whole-body energy metabolism. The potential therapeutic implications of these recent findings have not yet been fully exploited. Whether the use of OC is efficacious in the treatment of DM remains to be determined. Indeed, many unanswered questions remain and some have been highlighted previously by others, including the following: does OC regulate insulin secretion over the short/long term? How does the osteoblast or osteocyte sense and use glucose or other fuels? Do bone cells utilise glucose or amino acids? Does bone fracture increase whole-body energy expenditure? Do osteocytes truly have an effect on energy metabolism? (Martin 2007, Fulzele & Clemens 2012). The answers to these challenging questions are unquestionably attainable, and should ultimately result in better diagnosis, clinical management and treatment of patients with metabolic diseases.

**Declaration of interest**

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

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