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Histone Deacetylases in Skeletal Development and Bone Mass Maintenance

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Abstract

The skeleton is a multifunctional and regenerative organ. Dynamic activities within the bone microenvironment necessitate and instigate rapid and temporal changes in gene expression within the cells (osteoclasts, osteoblasts, and osteocytes) responsible for skeletal maintenance. Regulation of gene expression is controlled, in part, by histone deacetylases (Hdacs), which are intracellular enzymes that directly affect chromatin structure and transcription factor activity. Key roles for several Hdacs in bone development and biology have been elucidated though *in vitro* and *in vivo* models. Recent findings suggest that clinical usage of small molecule Hdac inhibitors for conditions like epilepsy, bipolar disorder, cancer, and a multitude of other ailments may have unintended effects on bone cell populations. Here we review the progress that has been made in the last decade in understanding how Hdacs contribute to bone development and maintenance.

1. Introduction to Bone Physiology

Despite its inert and rigid appearance, bone is a metabolically active, resilient, and regenerative tissue that serves numerous physiological roles. In addition to providing protection to vital organs and structural support for muscles, ligaments and tendons, bone is the major reservoir for essential ions (e.g., calcium and phosphorous), defends the organism against acidosis and dangerous minerals (e.g., lead), offers a supportive niche for hematopoiesis, and regulates metabolism. The vital nature of these functions provides an explanation for the evolution of a dynamic skeleton that can repair damage and adapt to external forces. Natural selection, however, has not provided adequate defense against modern challenges of aging into the seventh, eighth and ninth decades. An estimated 44 million Americans and as many as 75 million people worldwide have osteoporosis or osteopenia, placing them at an increased risk of fracture (EFFO and NOF, 1997, 2004). These numbers are expected to increase as the baby boomer generation enters their seventh decade. An osteoporotic fracture has a high economic impact and can begin a downward spiral of health and independence in the elderly, culminating in a 20% mortality rate within one year (2004). In the last several decades, knowledge of cells and molecules crucial for bone metabolism and regeneration has translated into new and effective therapies to prevent bone loss and fracture. However, some safety concerns exist regarding the long-term use of current treatments (e.g. PTH and bisphosphonates) (Vahle et al., 2002; Ruggiero et al., 2004;

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Vahle et al., 2004; Ruggiero and Drew, 2007; Lenart et al., 2008; Schneider, 2009; Solomon et al., 2009). Unraveling the complex genetic, epigenetic, and signaling components of bone biology will be essential for understanding the aging and regeneration processes of bone and for developing additional therapeutic options. Here we review the roles of histone deacetylases (Hdacs) in skeletal development and bone remodeling.

1.1 Bone Development and Modeling

During development, bones form via two processes: intramembranous ossification and endochondral bone formation. Intramembranous bones (e.g., calvaria and clavicles) arise from condensations of mesenchymal and neural crest progenitor cells. Endochondral bones (e.g., long bones, vertebrae) form when a cartilaginous anlage becomes vascularized and multipotent mesenchymal cells or pericytes are recruited to replace the cartilage extracellular matrix with tissue that eventually becomes mineralized. These precursor cells develop into osteoblasts and eventually into osteocytes, which become embedded in the mineralized matrix and serve mechanosensory roles (You et al., 2008). As the skeleton develops and grows in size, the bones undergo a “modeling” process that determines macroscopic bone structure and geometry (Martin, 2002). Another process, called “remodeling”, modifies bone microstructure, repairs damage, and contributes to the regulation of mineral homeostasis (Burr, 2002; Robling et al., 2006).

1.2 Bone Remodeling Compartments and Basic Multicellular Units

Three specialized cells (osteoblasts, osteocytes and osteoclasts) are the major participants in bone remodeling processes and are collectively called the basic multicellular unit (BMU) (Figure 1). In a resting state, the mineralized bone surfaces are lined with osteoblast-lineage cells called bone lining cells. Disruption of this lining and exposure of the bone surface as a result of structural damage, mechanical strain, or the contracture of bone lining cells in response to physiological or hormonal signals is sensed by osteocytes which initiate the formation of a bone remodeling compartment (BRC). A BRC includes a canopy of cells that are contiguous with lining cells over the BMU (Hauge et al., 2001). Cells in the canopy express typical osteoblast markers like osteocalcin and alkaline phosphatase (Hauge et al., 2001), but can also express the macrophage surface marker F4/80 (Chang et al., 2008; Pettit et al., 2008). Thus, the canopy may include both osteoblast-lineage lining cells and resident bone tissue macrophages, which have been named osteomacs. Bone marrow capillaries penetrate the BRC and provide a conduit for cells and nutrients into the otherwise isolated environment. Hematopoietic precursors are recruited to the site, perhaps by osteocytes directly, and subsequently differentiate into osteoclast precursors and fuse into large, multinucleated, polarized osteoclasts that attach to bone surfaces via integrins (Figure 2). Osteoclasts then remove the mineral and organic (e.g., collagen) components of bone tissue by secreting acids and proteolytic enzymes into a sealed resorption bay. There is an intimate relationship between the osteoblast and osteoclast cells in the BMU where osteoblast-lineage cells produce RANKL, which stimulates osteoclast differentiation (Lee and Lorenzo, 1999; Gori et al., 2000). In return, osteoclasts secrete factors and promote the release of bone matrix cytokines (e.g., TGF- β) that recruit osteoblasts to the remodeling site and stimulate their maturation, culminating in the synthesis of a new collagen-based matrix (osteoid) that forms a scaffold for nucleation and expansion of mineral crystals (Tang et al., 2009). Osteomacs in the BRC canopy may also help regulate important BMU operations such as the coupling of resorption and formation activity and mature osteoblast function and survival (Chang et al., 2008; Pettit et al., 2008).

2. Transcriptional Regulation in Bone Cells

The dynamic and responsive nature of bone during times of development, repair, and remodeling requires rapid and temporal changes in gene expression within both the osteoclast and osteoblast lineages. Combinations of transcription factors binding to DNA sequences determine the timing of gene expression in osteoclasts and osteoblasts (Figure 2). Essential transcription factors within the osteoclast lineage include PU.1, c-Fos, NFATc1, NFkB, and MITF. In osteoblasts, Runx2 and Osterix are required; however, AP-1, Msx2, Twist, several Hox factors, Zfp521, Lef1/Tcf7, and many other transcription factors cooperatively regulate gene expression patterns. The presence of tissue-relevant transcription factors alone is insufficient to control temporal gene expression and lineage-specific differentiation processes; co-factors are required for chromatin remodeling and the recruitment of RNA polymerase II. Gene activation is associated with the recruitment of lysine/histone acetyltransferases (p300, CBP and P/CAF) by transcription factors. Conversely, lysine/histone deacetylases (Kdacs or Hdacs) bind to the same transcription factors and promote transcriptional repression. The molecular switches controlling co-factor recruitment include the epigenetic landscape, cell signaling pathways, and combinatorial transcription factor availability. These processes have become an important mechanism to consider in regards to understanding bone physiology and disease. In this review, we summarize the progress made in the last decade in understanding how Hdacs contribute to bone development and metabolism.

3. Histone deacetylases (Hdacs)

The human genome contains approximately 1800 genes for transcription factors, but just 18 genes for Hdacs. Thus, the combinatorial association of transcription factors to gene regulatory sequences dictates specificity of gene expression, whereas Hdacs serve as adaptor molecules that directly affect chromatin structure and transcription factor activity and/or may facilitate cell responsiveness to environmental cues. Hdacs exert epigenetic control of transcriptional activity by removing negatively charged acetyl groups from lysine residues in histones, which condenses chromatin and limits the accessibility of transcription factors to the DNA. Hdacs can also deacetylate non-histone proteins (Glozak et al., 2005; Choudhary et al., 2009) such as the transcription factors Runx2 (Jeon et al., 2006), p53 (Juan et al., 2000; Luo et al., 2000), and Stat3 (Yuan et al., 2005), making them more stable and/or increasing their nuclear localization. The 18 Hdacs are classified into four groups on the basis of structural and functional similarities (Figure 3). Class I Hdacs (Hdac 1, 2, 3, 8) are broadly expressed and usually found in cell nuclei. Many lines of evidence suggest that class I Hdacs are the enzymatically active subunits of multi-protein complexes that deacetylate histones (Fischle et al., 2002; Lahm et al., 2007). In contrast, class II Hdacs (Hdacs 4–7, 9 and 10) demonstrate a more tissue restricted expression pattern, shuttle between cytoplasmic and nuclear compartments in response to signaling pathway stimulation, affect cytoskeletal and tubulin structure (e.g., Hdac6), but do not appear to contribute enzymatic activity to histone deacetylation. Sirtuins (Sirt 1–7) constitute class III and require NADH for enzymatic activity. Hdac11 is the sole member of class IV and is poorly understood (Figure 3).

Several of the 18 Hdacs contribute to skeletal development and bone mass maintenance. Many of their effects in bone occur at least in part through cooperation with or inhibition of Runx2, a regulator of osteoblast function required for osteoblast differentiation and bone formation (Westendorf et al., 2002; Schroeder et al., 2004; Schroeder et al., 2005; Jensen et al., 2008). By comparison, there is a paucity of data on the roles of Hdacs in osteoclast formation and function. In the next sections and in Table 1, data from *in vitro* and *in vivo* studies for the roles of specific Hdacs in bone physiology and disease are systematically

summarized. Subsequently, the effects of broad inhibition of Hdacs with small molecules on bone density, bone cell function and fracture risk are reviewed. A summary of the general effects of germline and tissue-specific Hdac deletion was published elsewhere (Haberland et al., 2009b).

3.1 Class I Hdacs and Bone Formation

3.1.1. Hdacs 1/2—Hdacs 1 and 2 are structurally similar and typically found together in a multi-subunit protein complex (Yang and Seto, 2003). Protein and mRNA levels of Hdac1 and Hdac2 decrease during osteoblast differentiation (Lee et al., 2006; Choo et al., 2009), and accordingly, Hdac1 presence on the promoters of osteoblastic genes (e.g. osterix and osteocalcin) is lower in differentiated osteoblasts (Lee et al., 2006). Hdac1 physically associates with Runx2, decreases Runx2's transcriptional activity, and represses the stimulatory effects of p300 on Runx2 transcriptional activity (Lee et al., 2006). Moreover, Hdac1 suppression with RNAi stimulates osteoblastic differentiation (Lee et al., 2006). Taken together, these data suggest that Hdac1 plays a role in suppressing osteoblast differentiation. Germline deletion of *Hdac1* causes embryonic lethality (Lagger et al., 2002; Montgomery et al., 2007). *Hdac2* germline knockouts are viable but have a smaller body size, suggesting potential disruptions in endochondral bone formation (Zimmermann et al., 2007). Bone cell-directed knockouts for either Hdac1 and/or Hdac2 have not been described.

3.1.2 Hdac3—Hdac3 is a transcriptional co-repressor of several transcription factors expressed in osteoblasts. Hdac3 binds Runx2, NFATc1, Zfp521 and TCF to suppress osteoblast-specific gene expression (e.g., osteocalcin and bone sialoprotein) (Schroeder et al., 2004; Lamour et al., 2007; Jensen et al., 2008; Choo et al., 2009; Wu et al., 2009; Hesse et al., 2010). Zfp521 may recruit Hdac3 to Runx2 complexes to promote repression of Runx2's transcriptional activity (Wu et al., 2009; Hesse et al., 2010). Suppression of Hdac3 in preosteoblasts by RNAi accelerates matrix mineralization and increases expression of Runx2 target genes but does not affect alkaline phosphatase expression (Schroeder et al., 2004). Taken together, these *in vitro* data suggest that Hdac3 negatively regulates the differentiation of lineage-committed osteoblasts. Germline *Hdac3* deletion is embryonically lethal (Bhaskara et al., 2008; Knutson et al., 2008; Montgomery et al., 2008), but conditional deletion of *Hdac3* in cells of the osteochondral lineage with osterix-Cre produces severe osteopenia due to reductions in trabecular number, bone formation rates and osteoblast numbers (Razidlo et al., 2010). The cyclin-dependent kinase inhibitor, p21, is upregulated in *Hdac3*-CKO calvarial bones, and bone marrow adipocyte numbers increase in these *Hdac3*-CKO animals as compared to wildtype mice (Razidlo et al., 2010). Thus, an unexplained discrepancy exists between the effects of *in vitro* Hdac3 suppression in osteoblast cell lines (increased osteoblast activity) and the *in vivo* deletion of Hdac3 in osterix-positive cells (decreased osteoblast activity and bone loss). It is possible that hypertrophic chondrocytes and/or osteoblast progenitor cells, both of which express osterix, are negatively affected by *in vivo* deletion of Hdac3, leading to the observed reduction in bone volume.

3.1.3 Hdac8—Genetic knockout studies demonstrate a crucial role for Hdac8 in intramembraneous bone formation. Germline deletion of *Hdac8* is detrimental to skull bone formation (Haberland et al., 2009a). This phenotype is recapitulated by conditionally deleting *Hdac8* in neural crest progenitor cells with Wnt1-Cre and is attributed to the upregulation of homeobox transcription factors, Otx2 and Lhx1. Interestingly, *Hdac8* depletion by Twist-Cre, Col1a1-Cre or Col2a1-Cre does not affect skull or long-bone formation (Haberland et al., 2009a). The defects in the Wnt1-Cre:*Hdac8* CKO mice share phenotypic similarities with children exposed to valproate, an Hdac inhibitor, *in utero* (Vajda et al., 2004).

3.2 Class II Hdacs and Bone Formation

3.2.1 Hdac4—Hdac4 is expressed in mature osteoblasts and prehypertrophic chondrocytes (Schroeder et al., 2004; Vega et al., 2004; Kang et al., 2005). Interaction of Hdac4 with the DNA binding domain of Runx2 may prevent Runx2 from associating with promoter elements of target genes. Hdac4 also deacetylates Runx2, and thereby represses its transcriptional activity and promotes its degradation (Kang et al., 2005; Jeon et al., 2006). Germline deletion of *Hdac4* increases bone density by promoting endochondral ossification (Vega et al., 2004). Meanwhile, transgenic mice overexpressing Hdac4 in proliferating chondrocytes demonstrate a severe deficit in endochondral ossification that leads to bone loss (Vega et al., 2004). These results mimic the phenotype of *Runx2* transgenic and knockout mice, respectively. Mice deficient in the transcription factor Mef2c also display an opposing skeletal phenotype as compared to *Hdac4*-null mice (Arnold et al., 2007). The balance between Hdac4 and Mef2c appears to regulate endochondral ossification, as *Mef2C^{+/-}; Hdac4^{-/-}* mice have a normal skeletal phenotype (Arnold et al., 2007). Hdac4 is suppressed by the microRNA miR-29b, which promotes bone formation (Li et al., 2009c). *In vitro* studies have also demonstrated that Hdac4 activity can be modulated by several different biochemical signals. For example, PTH prevents Hdac4 from repressing transcription of matrix metalloproteinase-13 (MMP-13) (Shimizu et al., 2010), a protein that facilitates breakdown of bone's extracellular collagenous matrix. PTH also decreases the interaction between Hdac4 with Runx2 in the nucleus, even though Hdac4 protein levels are increased by PTH treatment (Shimizu et al., 2007; Shimizu et al., 2010). Similarly, BMP2 treatment facilitates nuclear export of Hdac4, impeding its ability to bind and repress nuclear transcription factors like Runx2 (Jensen et al., 2008; Jensen et al., 2009).

3.2.2 Hdac5—Like Hdac4, Hdac5 is expressed in mature osteoblasts (Kang et al., 2005); however, it may be more highly expressed in nuclei than Hdac4 (Jensen et al., 2009). Also like Hdac4, Hdac5 physically interacts with Runx2 and is capable of deacetylating Runx2 and reducing Runx2 protein levels (Jeon et al., 2006). Hdac4 and Hdac5, in conjunction with TGF- β , repress Runx2 transcriptional activity (Kang et al., 2005). *Hdac5* knockout mice are viable, fertile, and do not demonstrate gross morphological abnormalities at younger ages, but interestingly, double mutant *Hdac5:Hdac9* knockout mice are approximately one-third the size of wildtype littermates (Chang et al., 2004) (*Hdac9* knockout mice are viable and show no pathological abnormalities (Zhang et al., 2002)). The skeletal phenotype of *Hdac5* (and *Hdac9*) knockout mice has yet to be characterized. In humans, *HDAC5* was identified as a locus affecting BMD in a genome-wide association study (Rivadeneira et al., 2009), and *HDAC5* levels were elevated in two juvenile humans with primary osteoporosis (Li et al., 2009a). Elevated Hdac5 levels (via antagonism of a natural Hdac5 repressor, miR-2861) are also associated with decreased bone formation and bone loss in animal models; however, *HDAC5* is just one of a multitude of genes regulated by miR-2861 (Li et al., 2009a). Nevertheless, these genomic data identify *HDAC5* as a contributor to bone homeostasis.

3.2.3. Hdac6—Hdac6 belongs to class IIb because it contains two catalytic domains (Grozinger et al., 1999; Verdel and Khochbin, 1999) (Figure 3). Its major cellular role is as a tubulin deacetylase. Hdac6 is primarily found in the cytoplasm, but it shuttles rapidly between nuclear and cytoplasmic compartments. In osteoblast lineage cells, Hdac6 co-localizes with Runx2 in the nuclei of cells treated with a nuclear export inhibitor (Westendorf et al., 2002). Germline *Hdac6* deletion modestly increases trabecular bone mineral density via unknown mechanisms (Zhang et al., 2008).

3.2.4. Hdac7—*Hdac7* knockout mice die at embryonic day E11 due to circulatory system malformations (Chang et al., 2006). Bone-specific knockouts of *Hdac7* have yet to be described, but *in vitro* studies demonstrate the importance of Hdac7 in osteoblast

development and function. Hdac7 is abundantly expressed in both osteoblast progenitors and mature osteoblasts (Jensen et al., 2008). Runx2 promotes nuclear localization of Hdac7, and the amino terminal portion of Hdac7 is necessary and sufficient to interact with the carboxy terminus of Runx2 *in vitro* (Jensen et al., 2008). BMP2 stimulates protein kinase D, which phosphorylates Hdac7 and promotes its nuclear export (Jensen et al., 2008; Jensen et al., 2009). Specific inhibition of Hdac7 with shRNA promotes osteoblast maturation (Jensen et al., 2008).

3.3. Class III Hdacs and Bone Formation: Sirt1

Sirtuins (Sirt) 1–7 are NADH-dependent protein deacetylases that regulate transcription and aging. Sirt1 is present in mesenchymal osteoblast progenitors (Backesjo et al., 2006; Backesjo et al., 2009), but is more highly expressed in osteosarcoma cells than in normal osteoblasts (Li et al., 2009b). Activation of Sirt1 in mesenchymal stem cells promotes osteoblastic differentiation at the expense of adipocyte differentiation, and the reverse is true if Sirt1 is inhibited (Backesjo et al., 2006). Thus, Sirt1 appears to regulate the preferred differentiation pathway of mesenchymal progenitor cells. Estrogen depletion (via ovariectomy) depletes Sirt1 protein levels *in vivo*, which may contribute to the increase in marrow adiposity and bone loss observed during normal aging and in animal models of post-menopausal osteoporosis (Elbaz et al., 2009). Genetic deletion of *Sirt1* causes axial and appendicular trabecular bone loss due to increased osteoclast number and osteoclast activity and decreased osteoblast number (Edwards et al., 2007).

4. Hdac inhibitors

Class I Hdacs can be rendered enzymatically inactive by small molecules that integrate into the zinc-containing catalytic sites. Commonly used Hdac inhibitors (HDIs) belong to one of six basic structural classes: short-chain fatty acids (e.g., valproate, sodium butyrate), cyclic peptides (depsipeptide, FR901228), benzamides (MS-275), hydroxamic acids (SAHA, trichostatin A (TSA)), epoxyketones (Trapoxin), and hybrid molecules (CHAP31, CHAP50) (Drummond et al., 2005). Of these, only SAHA and valproate are clinically approved in the USA at this time, although other HDIs like MS-275 are in various stages of clinical trials (Table 2). Valproate and suberoylanilide hydroxamic acid (SAHA; vorinostat, Zolinza™) have demonstrated clinical success as treatments for epilepsy, bipolar disorder, and cancer (Phiel et al., 2001; Kumagai et al., 2007), and research into the usage of these and other HDIs for treating a wide variety of other conditions including inflammation, HIV, and cystic fibrosis is ongoing (Lehrman et al., 2005; Lin et al., 2007; Hutt et al., 2010). The reason(s) HDIs show efficacy in treating such a wide variety of disease states may be linked to the correction of deviant histone modifications triggered by the underlying conditions, thereby promoting the expression of silenced genes (e.g., tumor suppressors), or to modification of non-histone proteins. These drugs may also promote DNA damage, cell cycle arrest, terminal cellular differentiation, and cellular apoptosis, features that make them attractive therapies for treating diseases like cancer. Biochemical studies indicate that class I Hdacs (1, 2, 3, and 8) are the primary targets of existing pan Hdac inhibitors due to structural features of their enzymatic pockets. Although class II Hdacs are often found in multiprotein complexes with class I Hdacs, their deacetylase domains are dispensable for the enzymatic function of these complexes (Fischle et al., 2002; Lahm et al., 2007; Subha and Kumar, 2008). Class III Hdacs, the Sirts, are not inhibited by the above-mentioned HDIs.

It may seem surprising that HDIs would be safe and tolerated agents because of the ubiquitous expression and crucial roles of Hdacs in many developmental processes. Some evidence suggests that normal cells may be resistant to toxic effects of HDIs because their cell cycle checkpoints, particularly the G2/M transition, are fully functional (Warrener et al., 2003). It also seems that resting or quiescent cells may not be affected by HDIs. Other

factors in HDI efficacy and safety are that deacetylation is a reversible process and HDIs have short-half lives. SAHA, for example, has a half-life of approximately 1.5 to 2 hours in the body following oral administration (Ramalingam et al., 2007). It is notable that SAHA's side effects (e.g. thrombocytopenia, fatigue, constipation, diarrhea) are linked to renewable tissues (e.g., hematopoietic system and GI tract). Bone is also a regenerative tissue, and thus may be susceptible to some negative consequences of Hdac inhibition. In the following section, we review the known effects of HDIs on bone cell and tissue biology.

4.1 In vitro effects of Hdac inhibitors (HDIs) on bone cells

Early *in vitro* studies suggested that HDIs could be promising skeletal therapies as they inhibited osteoclasts and stimulated osteoblasts. Recent studies, however, raise concerns about the impact of these drugs on survival of multipotent stem cells and on skeletal health *in vivo*. The *in vitro* effects of Hdac inhibitors on osteoclasts, osteoblasts, and mesenchymal progenitor cells are discussed first below and are followed by a summary of their *in vivo* effects. Table 3 summarizes all effects of HDIs on bone.

4.1.1 Osteoclasts—Several studies demonstrated that Hdac inhibition decreases osteoclast survival and activity *in vitro*. TSA promoted apoptosis in mature osteoclasts derived from bone marrow cells (Yi et al., 2007). Sodium butyrate and TSA suppressed osteoclast differentiation from hematopoietic precursors *in vitro* as well (Rahman et al., 2003; Nakamura et al., 2005). FR901228 inhibited osteoclastogenesis, prevented nuclear translocation of NFATc1, increased production of the osteoclastogenesis inhibitor, IFN- β , and decreased expression of pro-osteoclastogenesis factors c-Fos and SOCS-3 (Nakamura et al., 2005). Thus, osteoclasts are intolerant of Hdac inhibition *in vitro*.

4.1.2 Osteoblasts—The first published investigations of HDIs on osteoblasts demonstrated anabolic activity. Sodium butyrate induced alkaline phosphatase (ALP) expression in the MC3T3-E1 pre-osteoblast cell line (Iwami and Moriyama, 1993), and TSA increased osteopontin expression in C3H10T1/2 pre-osteoblast cells (Sakata et al., 2004). Schroeder et al. demonstrated that valproate, TSA, sodium butyrate, and MS-275 had stimulatory effects on several osteoblast cell lines, primary calvarial osteoblasts, and in calvarial organ cultures (Schroeder and Westendorf, 2005). In particular, TSA increased expression levels of osteoblast marker genes including type I collagen and osteopontin, and all four of these HDIs increased Runx2-dependent transcriptional activity without negatively affecting cell viability (Schroeder and Westendorf, 2005). Only high concentrations of these drugs decreased cell viability. More recently, these early observations were verified and expanded upon in a host of different osteoblast-like cell lines and calvarial models. HDIs consistently increased osteoblast gene expression, mineralized matrix deposition, alkaline phosphatase production, and Runx2 transcriptional activity *in vitro* in numerous laboratories throughout the world (Cho et al., 2005; Schroeder and Westendorf, 2005; Di Bernardo et al., 2009; Lee et al., 2009; Haberland et al., 2010). Furthermore, several HDIs (i.e. TSA, SAHA, and ScriptAid) blocked *in vitro* adipocyte differentiation and fat accumulation while simultaneously promoting osteogenic differentiation (Haberland et al., 2010). Thus, there is a large body of literature demonstrating that HDIs promote osteoblast differentiation *in vitro* or *ex vivo*.

4.1.3 Mesenchymal stem cells (MSC)—MSCs are osteoblast progenitors and promising therapies for regenerative medicine. Interestingly, valproate and sodium butyrate increased osteogenic differentiation of MSCs derived from human umbilical cord blood or adipose tissue (Lee et al., 2009), similar to their effects on osteoblast cell lines; however, these HDIs decreased proliferation and multi-lineage potential of the MSCs (Figure 4). Similarly, TSA increased calcium deposition in cultures of human bone marrow derived

MSCs (de Boer et al., 2006), but SAHA or MS-275 increased cell cycle arrest and apoptosis, and reduced stem-like qualities of bone marrow MSCs (Di Bernardo et al., 2009). Together, these *in vitro* studies provide strong evidence that HDIs promote osteoblastic differentiation of MSCs, or at least of a subset of cells in MSC populations that survive on plastic tissue culture dishes. However, the negative consequences of HDIs on the majority of cells in the culture dish raise some concerns about *in vivo* use. Like tumor cells, rapidly proliferating MSCs may be as susceptible to HDI-induced DNA damage and cell cycle inhibition.

4.2. In vivo effects of Hdac inhibitors on bone

4.2.1 Animal models of bone loss with Hdac inhibitor therapy—A handful of studies have recently examined the effects of HDIs on bone density *in vivo*. Valproate adversely affects rodent bone mass, but there seems to be a yet-unknown genetic component, as there are strain-related differences in the skeletal effects of valproate. In particular, seven- to eight-week old AKR/J, Balb/c, CBA, and C3H/H3J mice had reduced BMC following oral administration of valproate, but A/J, DBA/2J, and 129T2 mice did not. (Senn et al., 2010). Valproate also reduced bone mineral content of the total femur in young Wistar rats (Nissen-Meyer et al., 2007), and chronic oral ingestion of valproate lowered trabecular bone volume fraction and trabecular number in the proximal tibia of C3H/HeJ mice (Senn et al., 2010). Interestingly, serum osteocalcin increased in animals following valproate therapy (Nissen-Meyer et al., 2007), but histological indices of osteoblast and osteoclast activity were unchanged (Senn et al., 2010). SAHA also decreased trabecular bone volume fraction and trabecular number in the distal femur of C57Bl/6 mice. These negative effects resulted from an overall decrease in bone formation via decreased osteoblast number, with no change in circulating or histomorphometric indices of bone resorption (McGee-Lawrence et al., 2010). Unexpectedly, SAHA-treated mice had increased indices of local osteoblast activity including mineral apposition rate and bone formation rate (paralleling increases in osteoblastic gene expression and mineralized matrix production seen in osteoblasts following *in vitro* exposure to Hdac inhibitors (Schroeder and Westendorf, 2005; Di Bernardo et al., 2009; Lee et al., 2009)). These data suggest that in normal mice, SAHA causes bone loss by reducing osteoblast number, even while increasing the activity of mature osteoblasts (McGee-Lawrence et al., 2010). This is consistent with the pro-differentiation effects of HDIs on osteoblasts *in vitro* (Iwami and Moriyama, 1993; Sakata et al., 2004; Cho et al., 2005; Schroeder and Westendorf, 2005; Di Bernardo et al., 2009; Lee et al., 2009; Haberland et al., 2010). Finally, in a preclinical animal model designed to test the effects of SAHA on tumors growing within the bone microenvironment, SAHA reduced tumor burden in long bones of immunocompromised mice, but bone mass of the contralateral limbs was reduced and associated with an increase in bone resorption (Pratap et al., 2008). Thus, the integrity of the immune system may alter the responses of bone cells to HDIs. This is an important clinical consideration as the immune system is suppressed in many cancer patients.

4.2.2 Clinical reports of bone loss following Hdac inhibitor therapy—Human epidemiological studies concur with the negative effects of HDIs on rodent bone density. Valproate has been used since the 1960s as a treatment for epilepsy, bipolar disease and other mood disorders. In several patient cohorts, prolonged exposure to valproate decreased bone mineral density in both axial and appendicular sites in children and adults (Sheth et al., 1995; Boluk et al., 2004; Elliott et al., 2007), leading to increased fracture risk (Vestergaard et al., 2004). These studies have many confounding factors, including general decreased physical activity of epileptic patients. However, children born to mothers treated with valproate have an increased chance of developing craniofacial bone defects (Vajda et al., 2004). There are conflicting reports regarding how valproate alters bone remodeling to cause bone loss in humans. Circulating osteocalcin (a marker of bone formation) was reported to

be both higher (Sato et al., 2001; Oner et al., 2004; Kim et al., 2007) and lower (Rieger-Wettengl et al., 2001; Tsukahara et al., 2002) in patients receiving long-term valproate therapy compared to controls. Serum and urinary markers of bone resorption were reported to increase, decrease, or remain unchanged (Sato et al., 2001; Tsukahara et al., 2002; Kim et al., 2007). It should be noted that valproate inhibits enzymes (e.g. succinate semialdehyde dehydrogenase and -reductase) other than Hdacs (van der Laan et al., 1979; Loscher, 1981); thus, it is unclear that its capacity to stimulate bone loss *in vivo* is specific to deacetylase inhibition.

5. Clinical Relevance and Future Directions

Clinical observations and *in vivo* animal experiments with SAHA and valproate suggest that bone mass should be closely monitored in patients on long-term HDI therapy. It is unclear whether concurrent administration of an osteogenic therapy (e.g., bisphosphonates, PTH, denosumab) can attenuate HDI induced bone loss. Further research is needed to better define the mechanism by which HDIs reduce bone mass *in vivo* (e.g., increased turnover or decreased bone formation) before effective counter-therapies can be selected for evaluation. The negative skeletal consequences of broad-acting HDIs that are currently in clinical trials may be considered an acceptable side effect for oncologists and their patients with osteosarcoma or advanced metastatic tumors because of the beneficial effects of these HDIs on tumors within bone (Pratap et al., 2008). However, next generation HDIs will ideally target individual Hdacs. While these drugs are under development, knockout mouse models can provide valuable information about the most suitable drug targets and define the roles of specific Hdacs in bone formation. At the molecular and cellular levels, much remains to be understood about the roles of Hdacs in bone biology. Class I Hdacs appear to play a crucial role in genome integrity and cell viability, while class II Hdacs may regulate the duration and intensity of cell signaling cascades; however, just a paucity of pathways have been studied so far. A better understanding of the roles of Hdacs in altering the epigenome of osteoblasts and osteoclasts, especially during the aging process, will also provide insights into bone degeneration and potentially regeneration.

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Figure 1.

Diagram of a basic multicellular unit (BMU) inside a bone remodeling compartment (BRC). The renewal of bone tissue from the coordinated action of osteoclasts, which resorb bone tissue via production of acid and proteolytic enzymes, and osteoblasts, which synthesize new, unmineralized bone matrix (osteoid) is referred to as a BMU. Bone remodeling by osteoclasts and osteoblasts takes place inside a BRC, formed when osteocytes respond to physiological stimuli (hormones, structural damage, etc.) and signal for the formation of a canopy of lining cells over the bone tissue to be remodeled. Bone marrow capillaries, which penetrate the canopy, provide a conduit for cells and nutrients to enter the local environment.

Figure 2. Schematic of A) osteoblast and B) osteoclast differentiation from their respective precursors. Genes that are considered hallmarks of various differentiation stages or cell function are displayed above the cell diagrams in blue text. Transcription factors essential for progression of the differentiation process are shown below the cell diagrams in black text.

Figure 3.
Schematic representation of the structure and classification of the 18 mammalian Hdacs.

Figure 4.

Hdac inhibitors (HDIs) can have detrimental effects on mesenchymal stem cell proliferation, survival, and pluripotency while simultaneously stimulating osteoblast differentiation and activity. These deleterious effects on stem cells, which could ultimately deplete the osteoblast progenitor population, may help explain why HDIs have a stimulatory effect on osteoblastic cell lines *in vitro* but can cause bone loss *in vivo*.

Table 1

Skeletal consequences of genetic Hdac deletion in mouse models.

Class	Hdac	Knockout (KO)/ Conditional KO (CKO)/ Transgenic (Tg)	Cells affected	Result	Reference
I	1	KO	All (germline deletion)	• Embryonic lethal	(Lagger et al., 2002; Montgomery et al., 2007)
I	2	KO	All (germline deletion)	• Reduced body size (↓ vertebrae/pelvis size)	(Zimmermann et al., 2007)
I	3	KO	All (germline deletion)	• Embryonic lethal	(Bhaskara et al., 2008; Montgomery et al., 2008)
		CKO	Osteochondral lineage (<i>Osterix: Cre</i> deletion)	• cortical bone loss • trabecular bone loss • decreased bone formation rate • decreased Ob.N • increased marrow Ad.N	(Razidlo et al., 2010)
I	8	• KO • CKO • CKO • CKO • CKO	• All (germline deletion) • Pre-osteoblasts (<i>Twist1: Cre</i> deletion) • Osteoblasts (<i>Col1a1: Cre</i> deletion) • Chondrocytes (<i>Col2a1: Cre</i> deletion) • Neural crest cells (<i>Wnt1: Cre</i> driven deletion)	• ossification defects in frontal and interparietal bones • No effect • No effect • No effect • ossification defects in frontal and interparietal bones	(Haberland et al., 2009a)
II	4	• KO • Tg	• All (germline deletion) • Chondrocytes (<i>Col2a1</i> promoter driven)	• ectopic chondrocyte hypertrophy and enhanced endochondral ossification • no endochondral ossification	(Vega et al., 2004)
II	5	Tg	All (antagonization of Hdac5 repressor)	• trabecular bone loss • decreased cortical bone strength • decreased bone formation rate • decreased Ob.N	(Li et al., 2009a)
II	6	KO	All (germline deletion)	• increased trabecular bone mineral density	(Zhang et al., 2008)
III	SIRT I	KO	All (Germline deletion)	• trabecular bone loss	(Edwards et al., 2007)

Class	Hdac	Knockout (KO)/ Conditional KO (CKO)/ Transgenic (Tg)	Cells affected	Result	Reference
				<ul style="list-style-type: none">• Increased Oc.N• Decreased Ob.N	

Table 2

Current Hdac inhibitors in clinical trials.

Drug name	Common Name	Structural class	Conditions under evaluation	Clinical Trial IDs
4SC-201	Resminostat	Hydroxamic acid	<ul style="list-style-type: none"> Hepatocellular carcinoma Hodgkin's lymphoma 	NCT00943449 NCT01037478
AR-42	(none)	Hydroxamic acid	<ul style="list-style-type: none"> Multiple myeloma Chronic lymphocytic leukemia Lymphoma 	NCT01129193
CHR-2845	(none)	Hydroxamic acid	<ul style="list-style-type: none"> Hematological disease Lymphoid malignancies 	NCT00820508
CHR-3996	(none)	Hydroxamic acid	<ul style="list-style-type: none"> Solid tumors 	NCT00697879
FR901228; NSC630176	depsipeptide; romidepsin; Istodax	Cyclic peptide	<ul style="list-style-type: none"> Lymphoma 	NCT00383565 NCT00007345
ITF2357	Givinostat	Hydroxamic acid	<ul style="list-style-type: none"> Hodgkin's lymphoma Polycythemia vera 	NCT00792467 NCT00928707
JNJ-26481585	(none)	Hydroxamic acid	<ul style="list-style-type: none"> Leukemia Lymphoma Myelodysplastic syndromes 	NCT00676728
LBH589	Panobinostat	Hydroxamic acid	<ul style="list-style-type: none"> Lung carcinoma Head and neck cancer Prostate cancer Breast cancer Hematopoietic/Lymphoid cancer Lymphoma Neoplasms Leukemia Multiple Myeloma Graft-Versus-Host Disease Recurrent Malignant Gliomas Hepatocellular Carcinoma Renal Cell Carcinoma Advanced Solid Tumors Myelodysplastic Syndrome (MDS) Post-transplant lymphoproliferative disorder 	(>10)

Drug name	Common Name	Structural class	Conditions under evaluation	Clinical Trial IDs
MS-275	Entinostat	Benzamide	<ul style="list-style-type: none"> Colorectal cancer Renal cell carcinoma Leukemia Solid Tumor Myelodysplastic syndromes 	NCT01105377 NCT01038778 NCT01132573 NCT01159301 NCT00101179 NCT00466115 NCT00313586
PCI-24781	(none)	Hydroxamic acid	<ul style="list-style-type: none"> Lymphoma Hodgkin's Disease Multiple Myeloma Leukemia 	NCT01149668 NCT00724984
PXD101	Belinostat	Hydroxamic acid	<ul style="list-style-type: none"> Thymoma Thymic carcinoma Lymphoma Lung carcinoma Malignant Epithelial Neoplasms Myelodysplastic Syndromes 	NCT00589290 NCT00865969 NCT00926640 NCT01090830 NCT00274651 NCT00357162
Suberoylanilide hydroxamic acid (SAHA)	Vorinostat	Hydroxamic acid	<ul style="list-style-type: none"> Acute Myeloid Leukemia Breast Cancer Lung carcinoma Lymphoma Pancreatic Cancer Solid Tumor Ovarian Cancer Gastric Cancer Multiple Myeloma Hodgkin's Disease Polycythemia Vera Adenocarcinoma of the Pancreas Fallopian Tube Cancer Peritoneal Cancer Melanoma Essential Thrombocythemia Small Intestine Cancer 	(> 10)
SB939	(none)	Hydroxamic acid	<ul style="list-style-type: none"> Solid Tumors Hematologic malignancies Prostate Cancer 	NCT00741234 NCT00504296 NCT01075308
Valproic Acid	Valproate, Depakote, Depakene, Depacon, Stavzor	Short chain fatty acid	<ul style="list-style-type: none"> HIV Infections 	(> 10)

Drug name	Common Name	Structural class	Conditions under evaluation	Clinical Trial IDs
			<ul style="list-style-type: none">• Myelodysplastic Syndromes• Sarcoma• Ovarian Cancer• Cervical Cancer• Glioma• Hypersplenism• Leukemia• Neuroectodermal Tumor• Lymphadenopathy• Lymphoma	

Table is current as of August 2010

Table 3Summary of *in vitro* and *in vivo* effects of Hdac inhibitors on bone.

Cell/animal model	Effect
Osteoclasts	<ul style="list-style-type: none"> Promote apoptosis (Yi et al., 2007) Suppress differentiation/maturation (Rahman et al., 2003; Nakamura et al., 2005)
Osteoblasts	<ul style="list-style-type: none"> Promote differentiation (Haberland et al., 2010) Increase alkaline phosphatase production (Iwami and Moriyama, 1993; de Boer et al., 2006) Increase osteoblastic gene expression (Sakata et al., 2004; Schroeder and Westendorf, 2005; Haberland et al., 2010) Increase mineralized matrix production (de Boer et al., 2006; Haberland et al., 2010) Increase Runx2 transcriptional activity (Schroeder and Westendorf, 2005)
<i>In vitro</i> Mesenchymal stem cells (MSC)	<ul style="list-style-type: none"> Promote apoptosis (Di Bernardo et al., 2009) Promote cell cycle arrest (Di Bernardo et al., 2009) Decrease proliferation (Di Bernardo et al., 2009; Lee et al., 2009) Decrease pluripotency (Di Bernardo et al., 2009; Lee et al., 2009) Increase osteoblastic lineage differentiation (Cho et al., 2005; de Boer et al., 2006; Di Bernardo et al., 2009; Lee et al., 2009) Decrease adipocytic lineage differentiation (Lee et al., 2009; Haberland et al., 2010) Decrease neural lineage differentiation (Lee et al., 2009) Decrease chondrocytic lineage differentiation (Lee et al., 2009)
<i>In vivo</i> Humans	<ul style="list-style-type: none"> Decrease bone mineral density (Sheth et al., 1995; Boluk et al., 2004; Elliott et al., 2007) Increase fracture risk (Vestergaard et al., 2004) Cause teratogenic craniofacial defects (Vajda et al., 2004)
<i>In vivo</i> Rats	<ul style="list-style-type: none"> Decrease bone mineral content (Nissen-Meyer et al., 2007)
<i>In vivo</i> Mice	<ul style="list-style-type: none"> Decrease trabecular bone mass (strain-dependent) (Pratap et al., 2008; McGee-Lawrence et al., 2010; Senn et al., 2010)