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Histone Deacetylases in Control of Skeletogenesis

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Abstract Skeletogenesis occurs continuously during the lifespan of vertebrate organisms. In development, the skeleton is patterned and modeled until each bone achieves its optimal shape and full size. During adults, the skeleton is remodeled to maintain strength and release calcium. The bone-resorbing and bone-forming activities of osteoclasts and osteoblasts, respectively, are tightly coupled to maintain optimal skeletal health; however, during aging and disease, these cells can become uncoupled, adversely affecting skeletal health and strength. Histone deacetylases have emerged as important regulators of endochondral bone formation, osteoblast maturation and osteoclast survival. Histone deacetylases are inhibited by small molecules that are approved and/or in clinical trials as cancer therapeutic drugs or anti-epileptic agents. In this article, the roles of histone deacetylases and effects of histone deacetylase inhibitors on bone and cartilage cells are reviewed. J. Cell. Biochem. 102: 332–340, 2007. © 2007 Wiley-Liss, Inc.

Key words: osteoblast; osteoclast; chondrocyte; Runx2; epigenetics

The skeleton is a dynamic tissue throughout development and adulthood. Its major functions are to protect vital organs, support bone marrow hematopoiesis, store calcium and other ions, and provide structural support for muscles, tendons, and ligaments. Two processes, endochondral and intramembranous bone formation, are active during skeletal development. Endochondral bone formation produces the long bones of the skeleton. It begins with a cartilaginous template, which is invaded by bone resorbing osteoclasts that carve out the bone marrow cavity and subsequently by mesenchymal-derived osteoblasts that produce a collagenous matrix and proteins important for mineralization. Intramembranous bone formation begins with a condensation of mesenchymal cells, derived either from the mesoderm or neural crest, that develop into the clavicles and flat bones. Bones undergo a modeling process until the skeleton reaches it proper size and shape. Bones are also remodeled at an average rate of approximately 10% per year throughout life to release calcium needed for physiological processes and to repair microfractures. The modeling and remodeling events are mediated by the tight coupling of bone-resorbing osteoclasts and bone-forming osteoblasts.

Bone cells are responsive to external stimuli, including autocrine and paracrine modifiers, hormones, diet, and biomechanical strains. Tremendous progress has been made in understanding the molecular mechanisms responsible for osteoblast, osteoclast, and cartilage maturation in response to these factors. Essential genes, lineage-defining proteins, indispensable and modulating transcription factors, and gene signatures have been described [Cohen, 2006]. With the completion of the human genome project, an emerging area of interest in skeletal biology and other fields is epigenetic control of gene expression.

EPIGENETIC CONTROL OF GENE EXPRESSION

Epigenetics is classically defined as heritable changes in gene structure that do not affect DNA sequence and can be influenced by the environment. Modern definitions of epigenetics include the effects of DNA methylation, reversible chromatin modifications and small non-coding RNAs on gene expression [Bernstein et al., 2007; Kouzarides, 2007]. The most widely studied epigenetic modifications to chromatin are DNA methylation and post-transcriptional

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modifications of histones, including acetylation, methylation, phosphorylation, and ubiquitination. DNA methylation causes gene repression and globally decreases during aging; however, local hypermethylation of genes containing CpG islands also occurs. Histone modifications are interdependent and their roles in regulating gene expression are complex. In general, histone phosphorylation and acetylation are linked to activation of gene expression, while histone methylation can be either activating or repressing depending on context. A histone code has been proposed and is evolving as more specific and sensitive reagents and techniques are developed to monitor post-transcriptional histone modifications [Bernstein et al., 2007].

How epigenetic marks dictate chromatin structure, gene expression and phenotype in bone cells is just beginning to be understood. DNA methylation of genes encoding osteocalcin, estrogen receptor alpha, osterix, Dlx5, and receptor activator of NFκB ligand (RANKL) decreases transcription of these important molecules and may influence bone accrual [Villagra et al., 2002; Penolazzi et al., 2004; Lee et al., 2006b; Kitazawa and Kitazawa, 2007]. With respect to chromatin structure, it is known that important transcription factors such as Runx2, Twist, pRb, and SMADs, interact with histone acetyltransferases (HATs) and HDACs [Luo et al., 1998; Westendorf et al., 2002; Schroeder et al., 2004; Kang et al., 2005; Jeon et al., 2006; Lee, 2006a; Westendorf, 2006; Hayashi et al., 2007]. Recently, the osteoblast lineage-determining transcription factor, Runx2, was found associated with its target genes throughout mitosis and these genes had a "transcriptionally-poised" chromatin signature as measured by a histone 3 (H3) acetylation and H4 di-methylation pattern [Young et al., 2007]. These findings set the groundwork for understanding how epigenetic events contribute to lineage commitment and cell function.

HISTONE DEACETYLASES

The human genome contains only 18 HDAC genes. By comparison, more than 1,800 genes are predicted to encode transcription factors [Venter et al., 2001]. Thus, DNA binding proteins dictate specificity, while HDACs and other co-factors serve as non-specific, broad acting modulators of gene expression. HDACs are divided into four classes on the basis of structural similarity (Fig. 1). Class I HDACs (HDACs 1, 2, 3, and 8) are widely expressed in cell nuclei. Class II HDACs are subdivided into class IIa (HDACs 4, 5, 7, 9) and class IIb (HDACs 6 and 10). They are expressed in a tissue-specific fashion and shuttle between nuclear and cytosolic compartments. Class III HDACs includes the NAD+-dependent sirtuin deacetylases, SIRTs 1–7, which are present in nuclei, the cytoplasm and mitochondria and have been associated with molecular processes during aging. Class IV contains HDAC11, but is structurally similar to class I HDACs.

HDACs are named for their deacetylase activity toward lysine residues in histones; however, it is important to recognize that they also deacetylate other proteins and they predate histones in evolution [Gregoretti et al., 2004]. Within the nucleus, transcription factors are acetylated as a means of post-transcriptional regulation. As an example, the essential osteoblast transcription factor, Runx2, is acetylated and can be deacetylated by HDAC4 and HDAC5 [Jeon et al., 2006]. Another well-defined substrate is tubulin [Hubbert et al., 2002]. In fact some HDACs are also referred to as tubulin deacetylases (TDACs) because of their crucial role in regulating microtubule structure in the cytoplasm. Thus far, most studies examining the effects of HDACs and their inhibitors have focused on their roles in gene transcription and assumed an effect on histone modification. An important area of future research will be to examine non-histone and non-transcription factor targets of HDACs in these cells more carefully.

HDACs IN CHONDROCYTES AND ENDOCHONDRAL BONE FORMATION

The crucial roles of several HDAC genes in vertebrate development were revealed in several mouse genetic knockout experiments. Deletion of class I HDACs (i.e., *HDAC1* and *HDAC2*) causes embryonic lethality, while deletion of some class IIa HDACs (i.e., *HDAC5, 7*, and *9*) leads to premature death because of cardiovascular defects with no obvious effects on skeletal development [Lagger et al., 2002; Chang et al., 2004, 2006]. Altering expression levels of another class IIa member, HDAC4, has marked effects on endochondral development [Vega et al., 2004]. *HDAC4*-depletion causes

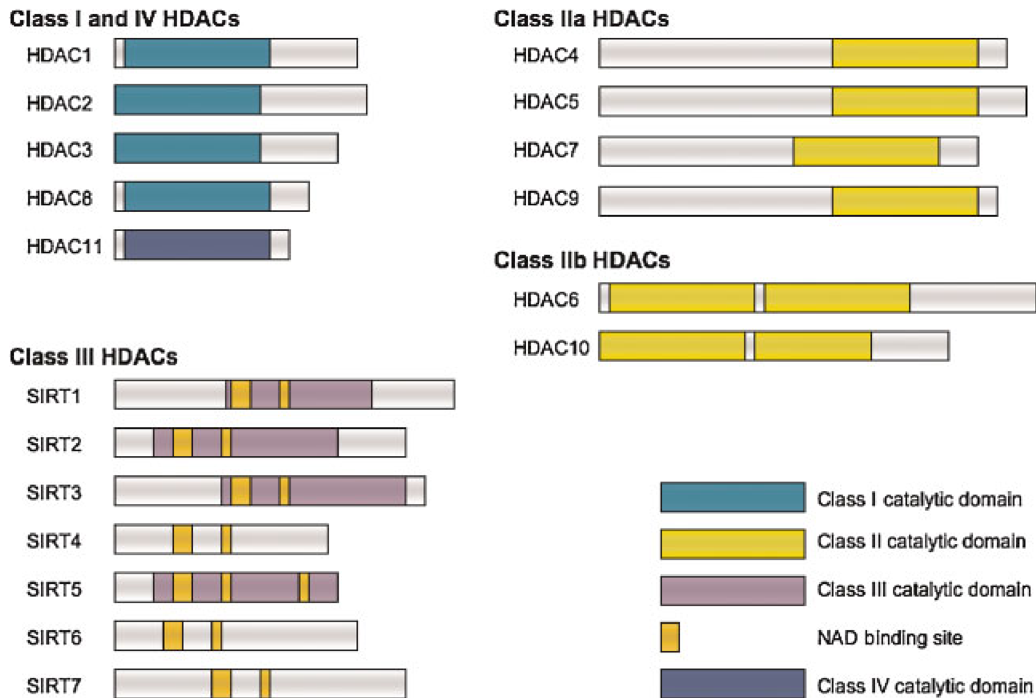

Fig. 1. *Schematic representation of HDAC structure and classification.* Mammalian cells express 18 HDACs that are divided into four classes on the basis of sequence conservation and functional similarities. HDACs in classes I, II, and IV contain at least one Zn^{+2}-dependent deacetylase domain, while the catalytic domain of class III HDACs requires NAD+. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

post-natal lethality within 2 weeks because chondrocyte hypertrophy is accelerated, leading to ectopic and premature ossification. Only bones formed by endochondral ossification are affected. HDAC4 is temporally expressed in developing chondrocytes. It is absent in proliferating cells, increases in prehypertrophic chondrocytes and then diminishes in hypertrophic cells (Fig. 2). In converse to *HDAC4* knockout mice, transgenic mice overexpressing HDAC4 in proliferating chondrocytes under the control of the α1(II) collagen promoter have no mineralized bone. This study demonstrated that HDAC4 has a crucial role in endochondral bone formation.

There are several known mechanisms whereby HDAC4 regulates skeletogenesis. One mechanism is through controlling Runx2 activity. *HDAC4*-deficient mice resemble *Runx2* transgenic mice and conversely *HDAC4* transgenic

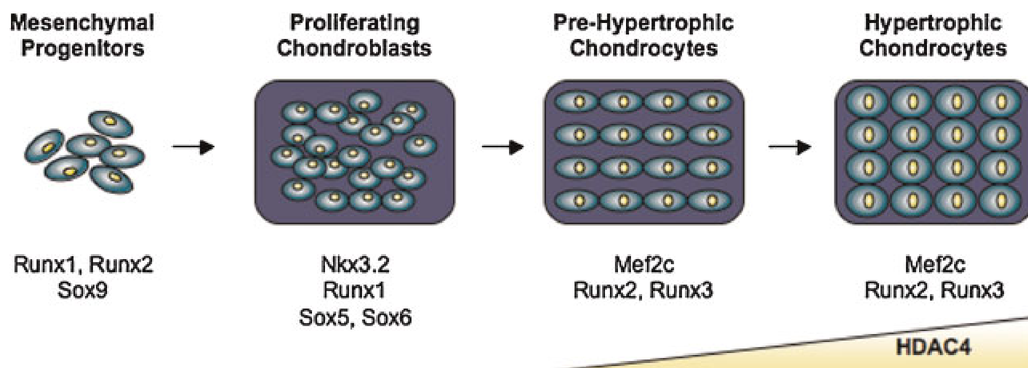

Fig. 2. *HDACs and transcription factors in chondrocyte maturation during endochondral bone formation.* HDAC4 is a negative regulator of Runx2 and Mef2 in pre-hypertrophic chondrocytes and promotes differentiation of hypertrophic chondrocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mice are phenotypically similar to *Runx2*-deficient mice [Vega et al., 2004]. HDAC4 physically interacts with Runx2 and can block Runx2-dependent transcription. Accordingly, *HDAC4*-null mice prematurely express the Runx2-target gene, Indian hedgehog (Ihh), which is secreted by prehypertrophic chondrocytes to control chondrocyte hypertrophy and activate osteoblast differentiation in the perichondrium leading to osteoblast invasion. Ectopic ossification in *HDAC4*-deficient mice is partially reversed by deleting one allele of another transcription factor gene, myocyte enhancer factor-2c (MEF2c) [Arnold et al., 2007]. Conversely, impairments in endochondral chondrocyte hypertrophy, ossification, and longitudinal bone growth in $MEF2c^{+/-}$ mice are partially rescued by $HDAC4^{+/-}$ mice. MEF2 transcription factors are expressed in endochondral cartilage as early as E12.5 and bind HDAC4. HDAC4 blocks MEF2c-induced transcription of the collagen 10a promoter. Thus, HDAC4 interacts with and regulates two transcription factors, Runx2 and MEF2c, essential for proper chondrocyte maturation.

HDACs also bind and regulate other transcription factors that contribute to chondrocyte maturation. In cooperation with Runx2, Runx3 controls chondrocyte hypertrophy [Yoshida et al., 2004]. HDAC4 binds Runx3 weakly but efficiently deacetylates Runx3 in non-chondrocytic 293T cells [Jin et al., 2004]. In contrast, Runx1, which is downregulated during chondrocyte maturation and hypertrophy [Wang et al., 2005], does not interact strongly with HDAC4 [Durst et al., 2003]. Sox6 is expressed in prechondrocytes and contributes to proper skeletal development. It interacts with HDAC1 and recruits it to the cyclin D1 promoter in 293T cells [Iguchi et al., 2007]. Nkx3.1 and Nkx3.2 are transcriptional repressors and also capable of interacting with HDAC1 repressor complexes in non-chondrocytes [Kim and Lassar, 2003; Lei et al., 2006]. These interactions between important chondrocyte transcription factors and HDACs need to be functionally verified in chondrocytes because the interactions might be influenced by tissue-specific co-factors. In addition, more experiments are needed to understand the roles of all HDACs in chondrocytes. Tissue-specific knockout mice will be essential because HDAC depletion is usually lethal; however, experiments examining HDAC expression patterns during chondrocyte maturation and differentiation would provide useful data to guide RNA interference (RNAi), overexpression and knockout studies.

HDACs IN OSTEOBLASTS

Several HDACs have been identified as regulators of osteoblast maturation. Using a panel of osteoblast-precursor and osteosarcoma cell lines, as well as primary osteoblasts, we found that some HDACs (e.g., HDAC3 and HDAC7) are expressed in all osseous cells, others are predominantly expressed in progenitors (e.g., HDAC1) or mature osteoblasts (e.g., HDAC4 and HDAC6), and some were barely detectable with available antibodies (e.g., HDAC2, HDAC5, HDAC8) [Westendorf et al., 2002; Schroeder et al., 2004]. Thus, at any one time, osteoblasts express multiple HDACs to control gene expression (Fig. 3A). These HDACs bind many crucial osteoblast transcription factors, notably, Runx2, Smads, Twist, and pRb [Luo et al., 1998; Westendorf et al., 2002; Schroeder et al., 2004; Kang et al., 2005; Jeon et al., 2006; Lee, 2006a; Westendorf, 2006; Hayashi et al., 2007]. Individual inhibition of several HDACs stimulates osteoblast maturation in vitro. Suppression of HDAC1 by RNAi accelerated osteoblast maturation, with an increase in alkaline phosphatase production [Lee et al., 2006a]. Reduction of HDAC3 levels by RNAi also accelerated osteocalcin gene expression and matrix mineralization, but did not affect alkaline phosphatase [Schroeder et al., 2004]. Suppression of HDAC4 or HDAC5 by RNAi or dominant negative proteins relieved TGFβ-mediated and Smad3-dependent repression of the osteocalcin promoter and enhanced matrix mineralization of caIB 2T3 cells [Kang et al., 2005]. Suppression of HDAC4 or HDAC5 by RNAi also increased total and acetylated levels of Runx2 [Jeon et al., 2006]. Together, these data indicate that inhibition of individual HDACs is sufficient to promote osteoblast differentiation but different HDAC complexes might have distinct roles during the process.

HDACs IN OSTEOCLASTS

Very little is understood about how specific HDACs control osteoclasts. HDAC1 is recruited to the promoters of important osteoclast genes, *NFATc* and *OSCAR*, by STAT3 (PIAS3) to regulate their expression and inhibit osteoclast

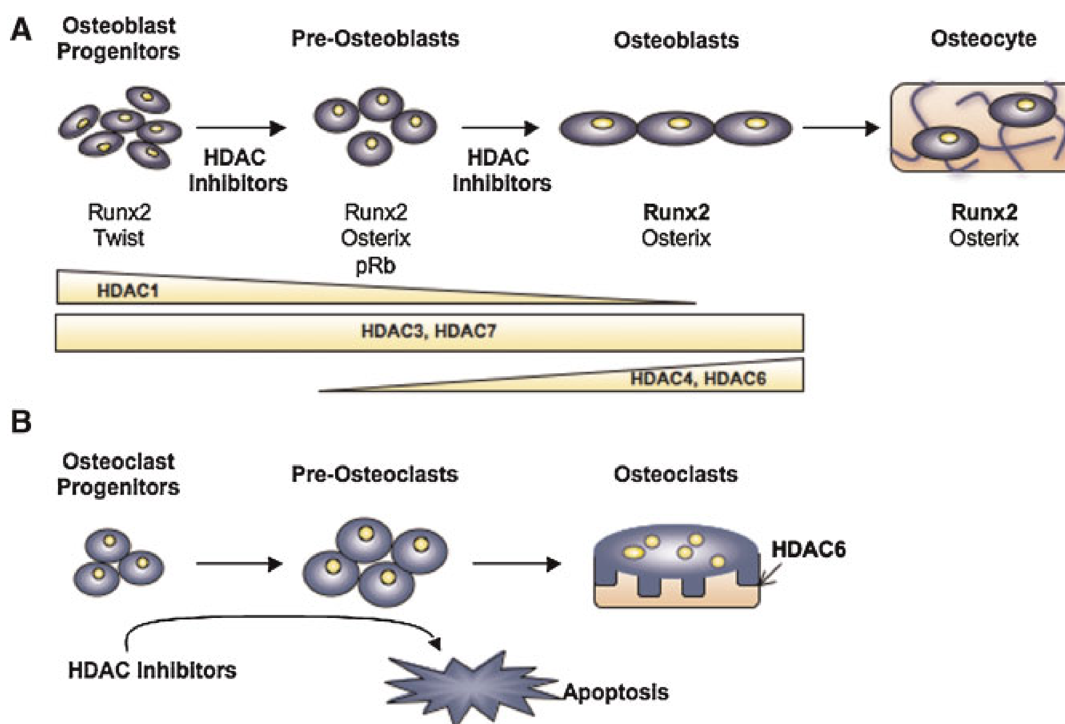

Fig. 3. *HDACs in osteoblast and osteoclast maturation.* **A**: Several HDACs are expressed at different times during osteoblast maturation. Inhibition of individual HDACs by RNAi or broad repression with HDIs promotes terminal osteoblast differentiation. **B**: HDAC6 contributes to the formation of a sealing zone and HDAC inhibitors induce osteoclast apoptosis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

maturation [Kim et al., 2007]. The osteoclast is an extremely specialized cell of hematopoietic origin. Its purpose is to resorb mineralized bone and degrade the extracellular collagenous matrix during bone remodeling. It accomplishes this by migrating to areas in need of repair and establishing a tight sealing zone to protect neighboring cells and tissues from the potent acids, lysosomal enzymes and proteases it releases to remove bone. Distinct from potential roles in gene regulation and as discussed above, HDACs have crucial roles in organizing the actin cytoskeleton and microtubule network, which is extremely important during osteoclast recruitment to remodeling sites, formation of the sealing zone, and bone resorption. HDAC6 is expressed in splenic osteoclasts and is a likely candidate to control microtubule acetylation, which increases during osteoclast maturation (Fig. 3B) [Destaing et al., 2005].

HDAC INHIBITORS

Several natural and synthetic small molecule inhibitors of HDACs exist (Fig. 4) [Minucci and Pelicci, 2006]. They act by incorporating into the catalytic site of HDACs [Finnin et al., 1999]. Numerous studies now indicate that general inhibition of HDAC activity with small molecule inhibitors accelerates osteoblast maturation in vitro. Broad-acting HDAC inhibitors (i.e., trichostatin A, valproic acid, sodium butyrate, MS-275 or SCOP402) accelerate alkaline phosphatase production and matrix mineralization of osseous cells in vitro and calvarial explants ex vivo [Iwami and Moriyama, 1993; Schroeder and Westendorf, 2005; de Boer et al., 2006; Jeon et al., 2006]. HDAC inhibitors also increase the expression of osteopontin and RANKL in osteoblasts [Fan et al., 2004; Sakata et al., 2004; Chen et al., 2007], block glucocorticoid cell cycle arrest in osseous cells [Smith and Frenkel, 2005], activate ERKs and stimulate osteoblast differentiation of multipotent bone marrow-derived mesenchymal cells [de Boer et al., 2006; Chen et al., 2007]. These results are consistent with results from the RNAi studies described above wherein specific HDACs were suppressed in osteoblasts. They also support the notion that HDAC inhibitors facilitate terminal cellular differentiation. Moreover, they agree with data indicating that relative HDAC activity

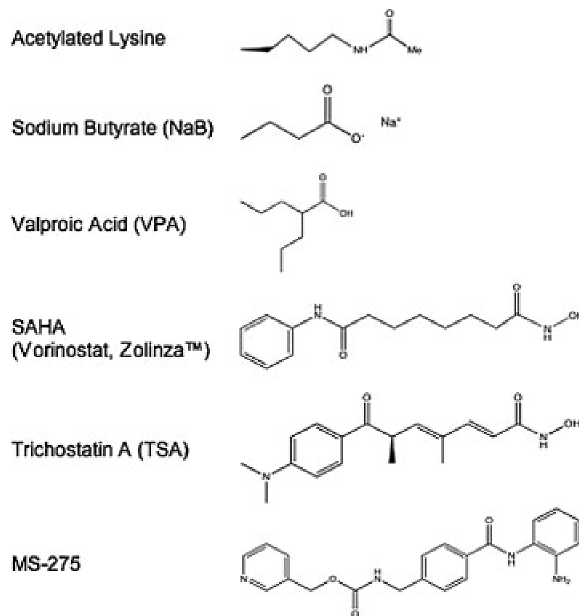

Fig. 4. *Chemical structures of common HDAC inhibitors.* HDAC inhibitor structures are aligned with the structure of an acetylated lysine side-chain.

decreases during osteoblast maturation [Lee et al., 2006a].

In contrast to their positive effects on in vitro osteoblast maturation, HDIs decrease the survival and maturation of osteoclasts. Before sodium butyrate was recognized as an HDI, it was shown to decrease the formation of tartrate resistant acid phosphatase (TRAP)-positive multinucleated cells from bone marrow cells because of its toxicity to the latter cells [Iwami and Moriyama, 1993]. Newer and more potent HDIs also prevent osteoclastogenesis. Trichostatin A (TSA) suppressed the differentiation of osteoclasts, but not macrophages, from bone marrow cultures [Rahman et al., 2003] and induced $p21^{WAF}$ expression, which contributed to osteoclast apoptosis [Yi et al., 2007]. Depsipeptide (Romidepsin) suppressed in vitro osteoclastogenesis by blocking RANKL-induced nuclear translocation of NFATc1 and by increasing production of IFN-β, an inhibitor of osteoclastogenesis [Nakamura et al., 2005]. In this study, depsipeptide also prevented bone destruction in a rat model of rheumatoid arthritis. Finally, SAHA abolished osteoclastogenesis by suppressing several events leading to NF-κB activation [Takada et al., 2006].

The current crop of HDIs has broad specificity for multiple, if not all, HDACs [Hu et al., 2003; Gurvich et al., 2004]. Despite their lack of specificity, many are in clinical cancer trials and appear to be relatively safe and effective in combination with other treatments [Minucci and Pelicci, 2006]. Vorinostat (SAHA or Zolinza^{TM}) received FDA approval in October 2006 for the treatment of advanced therapy-resistant cutaneous T-cell lymphoma. Another HDI, VPA, is a commonly prescribed anti-epileptic drug. Little is known about the effects of broad-acting HDIs on the skeleton, but recent reports demonstrated that long-term VPA treatment causes osteopenia or osteoporosis and increased fracture risk in epileptic patients [Guo et al., 2001; Sato et al., 2001; Boluk et al., 2004; Vestergaard et al., 2004]. The mechanism by which VPA causes bone loss is not clear but may be related to certain characteristics of epilepsy such as low physical inactivity or insufficient vitamin D or calcium intake [Guo et al., 2001]. VPA inhibition of the succinate semialdehyde dehydrogenase and succinate semialdehyde reductase enzymes might also contribute to the phenotype. Because available HDIs affect most HDACs, large-scale efforts are underway to identify small molecules that specifically block the activity of single HDACs [Yoshida et al., 2003].

FUTURE PROSPECTS AND DIRECTIONS

Current in vitro evidence indicates that inhibiting HDACs promotes osteoblast maturation and suppresses osteoclast maturation. Together with the extensive literature documenting the anti-cancer effects of HDIs, these data suggest that HDIs might be effective against metastatic tumors and associated osteolytic bone disease. The available data also suggest that targeting HDACs might be a novel strategy for treating diseases associated with abnormal bone mass and strength as well as for bone tissue engineering. Enthusiasm for HDIs as a novel class of anabolic agents is tempered however because epileptic patients treated with VPA for extended periods of time have an increased incidence of osteoporosis. Fracture risk in these patients is dose-dependent [Vestergaard et al., 2004]; therefore, adverse effects of HDIs on bone mass might be controllable. More studies with animal models are needed to understand how HDIs affect skeletal health. In addition, non-invasive bone density scans on cancer patients treated with HDIs will provide useful information on how these drugs affect the

human skeleton and how they might be combined with other drugs or biologics to prevent skeletal damage.

What might explain the contradictions between the in vitro and in vivo data? It is important to remember that coupling of osteoblasts and osteoclasts is essential for bone remodeling. RANKL is expressed on the osteoblast surface and is crucial for promoting osteoclast maturation. HDAC inhibition stimulates RANKL expression [Fan et al., 2004], which in vivo would increase the number of osteoclasts that can resorb bone. HDAC inhibition also decreases the expression of the estrogen receptor alpha in breast cancer cells [Reid et al., 2005] and in osteoblasts (Westendorf, unpublished work); therefore, HDIs might prevent bone formation by decreasing sensitivity to hormonal stimuli.

A limitation of the current HDI crop is their lack of specificity. Many HDIs will inhibit all HDACs, although a few are more selective. While we wait for specific small molecule inhibitors to be developed, much can be learned at the molecular level by using RNAi and tissue-specific animal models to alter expression levels of each HDAC. For example, we found that HDAC3 suppression and HDIs both promote bone formation in vitro, but HDAC3 suppression does not increase the expression of alkaline phosphatase as HDIs do [Schroeder et al., 2004; Schroeder and Westendorf, 2005]. Thus, suppressing single HDACs might be more favorable in certain situations.

To fully understand the mechanisms of HDAC action in skeletal cells, a greater understanding of HDAC expression levels and localization in cells of the chondrocytic, osteoblastic, and osteoclastic lineages is required. Certain HDACs might be temporally expressed in these lineages and this would limit their interactions with transcription factors and substrates. Class II HDACs are shuttled across the nuclear membrane and in some cells demonstrate predominant cytoplasmic localization. This would indicate that non-histone substrates are affected by HDAC inhibition. Finally, genome-wide epigenetic profiling of HDAC interactions with DNA, which can be accomplished by hybridizing DNA collected in chromatin immunoprecipitations with probes on tiling chip arrays (ChIP-on-Chip), will identify crucial regulatory elements and genes controlled by HDACs. This should be done in all cell types.

In conclusion, HDACs have crucial roles in promoting skeletogenesis. Only the tip-of-iceberg has been revealed with regard to how HDACs control bone formation and remodeling. The effects of HDAC inhibitors on bone health will be widely and aggressively pursued at the clinical and molecular levels in the next few years. Advances in epigenomic technologies will provide the means to understand how HDACs control skeletogenesis.

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