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Histone deacetylase inhibition destabilizes the multi-potent state of uncommitted adipose-derived mesenchymal stromal cells

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Abstract

Human adipose-derived mesenchymal stromal cells (AMSCs) grown in platelet lysate are promising agents for therapeutic tissue regeneration. Here, we investigated whether manipulation of epigenetic events by the clinically relevant histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) alters differentiation of AMSCs. The multipotency of AMSCs was validated by their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages. High-throughput RNA sequencing and RT-qPCR established that human histone deacetylases (HDAC1 to HDAC11, and SIRT1 to SIRT7) are differentially expressed in AMSCs. SAHA induces hyper-acetylation of histone H3 and H4, stimulates protein expression of the HDAC-responsive gene SLC9A3R1/NHERF1 and modulates the AKT/FOXO1 pathway. Biologically, SAHA interferes with osteogenic, chondrogenic and adipogenic lineage commitment of multipotent AMSCs. Mechanistically, SAHA-induced loss of differentiation potential of uncommitted AMSCs correlates with multiple changes in the expression of principal transcription factors that control mesenchymal or pluripotent states. We propose that SAHA destabilizes the multi-potent epigenetic state of uncommitted human AMSCs by hyper-acetylation and perturbation of key transcription factor pathways. Furthermore, AMSCs grown in platelet lysate may provide a useful biological model for screening of new HDAC inhibitors that control the biological fate of human mesenchymal stromal cells.

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Conflict of Interest: Dr. Allan Dietz has a commercial interest in Mill Creek Life Sciences which manufactures the clinical grade commercial platelet lysate product used for maintaining adipose-tissue derived mesenchymal stem cells.

Keywords

osteoblast; bone; mesenchymal stem cell; histone deacetylase; osteogenesis; vorinostat; chondrocyte; adipocyte

Introduction

Progenitor cell populations that can differentiate into specialized tissues are critical for regenerative medicine (Rafii and Lyden, 2003). Multi-potent human mesenchymal progenitor cells have the capacity to differentiate into osteogenic, chondrogenic, cardiac, and adipogenic tissues as part of tissue regeneration strategies (Ng et al., 2008; Pittenger et al., 1999). Patient-derived human adipose-derived mesenchymal stromal cells (AMSCs) adherent to good manufacturing practice are particularly promising agents for therapeutic tissue regeneration. AMSCs are easy to manipulate, grow quickly, and have important regenerative and anti-inflammatory properties and powerful potential to improve the health of a variety of patients. Greater understanding of epigenetic events occurring within stem cell populations could help to identify novel anabolic drug targets and generally improve tissue-engineering strategies.

Epigenetic mechanisms control cellular development and maintain phenotypes in multicellular organisms by generating heritable changes in gene expression that are not linked to modifications in DNA sequence. This non-genetic cellular memory, which is sensitive to environmental cues, regulates gene expression through genomic DNA methylation, post-translational modifications of histones, as well as transcription factor interactions with mitotic chromosomes and small non-coding RNA molecules (Gibney and Nolan, 2010; Zaidi et al., 2014). Acetylation of histones relaxes chromatin and promotes recruitment of the transcriptional machinery, while chromatin condensation correlates with removal of acetyl groups (Grunstein, 1997; Peterson and Laniel, 2004). In general, histone acetylation is associated with transcriptional activation, while deacetylation of histones attenuates gene expression. This reversible process is mediated by histone acetyl transferases (HATs) that add acetyl groups on lysines and histone deacetylases (HDACs) that remove acetyl groups. Inhibiting the enzymatic activities of HDACs will alter this enzymatic equilibrium, increase histone acetylation, and globally but selectively increase gene transcription.

HDACs are expressed throughout the body and play important regulatory roles in cell growth, survival, and differentiation. Histone deacetylase inhibitors (HDIs), including suberoylanilide hydroxamic acid (SAHA, vorinostat, ZolinzaTM), are used clinically for the treatment of some cancer types, as well as non-life-threatening conditions like mood disorders and epilepsy (Kim and Bae, 2011; Peterson and Laniel, 2004). HDIs induce expression of tumor suppressor genes, DNA damage, cell cycle arrest, and apoptosis; such properties make these agents anti-tumorigenic. Currently, HDIs remain attractive targets for treatment of clinical conditions and they are considered as treatment options for major diseases including HIV and cystic fibrosis (Hutt et al., 2010; Wightman et al., 2012).

HDIs also are effective modulators of the biological properties of bone. HDIs promote *in vitro* differentiation of primary osteoblasts, MC3T3 osteoblasts, and *ex vivo* calvarial cultures (McGee-Lawrence and Westendorf, 2011). HDIs induce osteogenic gene expression and accelerate matrix production in osteogenic cell lines, primary calvarial cells, and calvarial organ cultures (Iwami and Moriyama, 1993; Schroeder and Westendorf, 2005). The *in vitro* osteogenic effects of HDIs can be attributed in part to their ability to induce the activity of Runx2, the master osteogenic transcription factor that interacts with several Hdacs (Jensen et al., 2008; Lee et al., 2006; Schroeder et al., 2004; Westendorf et al., 2002). Similar to their effects on osteoblasts, HDIs enhance osteoblastic differentiation of human mesenchymal stem cells (Di Bernardo et al., 2009; Lee et al., 2009; Xu et al., 2013). However, these agents induce cell cycle arrest and alter the biological qualities of multipotent mesenchymal stromal cells (Di Bernardo et al., 2009). Thus, the biological effects and clinical utility of HDIs may depend on the cellular context.

Although HDACs have positive effects on osteogenic differentiation *in vitro*, studies examining the effects of HDIs on bone health *in vivo* have revealed negative skeletal effects of HDIs. HDIs modulate different skeletal parameters in rodents, including reduced bone mineral content, decreased trabecular volume, and decreased trabecular number (McGee-Lawrence et al., 2011; Nissen-Meyer et al., 2007; Senn et al., 2010). SAHA alleviates the tumor burden of breast and prostate cancer cells, but also reduce bone mass in an immunocompromised mouse model (Pratap et al., 2010). Despite inducing global negative effects on the skeleton, SAHA locally stimulates osteoblast activity as evidenced by increased mineral apposition and bone formation rates (McGee-Lawrence et al., 2011). Negative effects on bone homeostasis observed in mice are paralleled by results from human epidemiological studies. Valproate, which has been used clinically in the treatment of epilepsy and bipolar disease for several decades, decreases bone mineral density and increases fracture risk in children and adults (Boluk et al., 2004; Sheth et al., 1995; Vestergaard et al., 2004).

In this study, we characterized the HDACs in human AMSCs and investigated the effects of SAHA on osteogenic differentiation of these cells using protocols suitable for clinical use (Crespo-Diaz et al., 2011). We show that SAHA interferes with lineage commitment of multipotent human AMSCs into osteoblasts, chondrocytes, and adipocytes.

Materials & Methods

Cell Culture

Mesenchymal stromal cells were derived from lipo-aspirates obtained from consenting healthy donors with approval from the Mayo Clinic Institutional Review Board as previously described (Crespo-Diaz et al., 2011; Mader et al., 2013). Fat tissue was enzymatically digested using 0.075% Type I collagenase (Worthington Biochemicals) for 1.5 h at 37°C. Adipocytes were separated from the stromal vascular fraction by low speed centrifugation (400 g for 5 min). The adipose supernatant was removed and the cell pellet was rinsed with PBS and passed through 70 and 40µm cell strainers (BD Biosciences). The resulting AMSC cell fraction was maintained in Advanced MEM Medium containing 5% PLTMax (a clinical grade commercial platelet lysate product [MillCreekLifeSciences]), 2

mM Glutamax (Invitrogen), 2 U/ml heparin (hospital pharmacy), 100 U/ml penicillin, and 100 µg/ml streptomycin (Cellgro) as described previously (Crespo-Diaz et al., 2011).

Osteoblast Mineralization

AMSCs were plated in 6-well plates in maintenance medium (40,000 cells/well). The following day (day 0), maintenance medium was replaced with osteogenic medium [maintenance medium containing human osteogenic supplement (R&D Systems)]. At confluence (4 days after plating), SAHA and/or solvent (0.0005% DMSO and 0.05% ethanol) were added or removed as described in the figure captions. Media were changed every three days. On day 6, cells were fixed in 10% neutral buffered formalin and stained with 0.1% crystal violet stain (Sigma) to visualize cells independent of phenotype-specific staining procedures. On day 9, cells were fixed in 10% neutral buffered formalin and stained with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium to monitor the enzymatic activity of alkaline phosphatase (Promega). On day 21, cells were fixed in 10% neutral buffered formalin and stained with 2% Alizarin Red to visualize calcium deposition.

Adipogenic Differentiation

AMSCs were plated in 6-well plates as described above. At confluence (four days after plating), adipogenesis was induced (adipogenic supplement [R&D Systems] in maintenance medium). SAHA and/or solvent (0.0005% DMSO and 0.05% ethanol) were added or removed as indicated in the captions of the corresponding figures. Media were changed every three days. On day 14, cells were fixed in 10% neutral buffered formalin and stained with Oil Red O (Sigma) which binds to lipids and neutral triglycerides. Oil Red O stain was dissolved in isopropyl alcohol and optical density measured at 490nm using a spectrophotometer. Statistical analysis was performed with a paired t-test.

Chondrogenic Micromass Cultures

Micromass cultures were performed as described previously (Bradley et al., 2013; Bradley and Drissi, 2010; Soung do et al., 2007). Briefly, AMSCs were pelleted and re-suspended at a density of 2.5×10^7 cells/ml in maintenance medium and three 10 µl drops of cells (250,000 cells in each drop) were plated into 6-well plates. The outside of each well was rimmed with 300 µl of medium to maintain humidity while cells were incubated at 37°C for 90 minutes. Wells were then flooded with medium and incubated for three days, after which chondrogenic differentiation was induced with insulin-selenium-transferrin solution (Gibco), 50 ng/ml ascorbic acid (Sigma), and 10 mM beta-glycerol phosphate (Sigma). SAHA and/or solvent (0.0005% DMSO and 0.05% ethanol) were added/removed as described in figure captions. Media were changed every three days. On day 21, cells were fixed in 10% neutral buffered formalin and stained with alcian blue (0.5% Alcian blue (Sigma), 3% acetic acid) to detect presence of glycosaminoglycans.

Western Blotting

AMSCs were plated in 6-well plates as described above. Cells were treated with SAHA and/or solvent (0.0005% DMSO and 0.05% ethanol) as described in figure legends. After 24 hours, cells were lysed in radio-immunoprecipitation buffer (150 mM NaCl, 50 mM Tris pH

7.4, 1 % sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma) and phenylmethylsulphonyl fluoride (Sigma). Lysates were cleared by centrifugation. Protein concentrations were determined by the DC Protein Assay (Bio-Rad). Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking in 5% non-fat dry milk for 45 minutes at room temperature, primary antibodies were added overnight at 4°C, followed by secondary antibodies for 1 hour at room temperature. Proteins were visualized using an ECL + detection kit. Primary antibodies used were: Actin (1:10,000; sc-1616; Santa Cruz), Ac-H3 (K9/14) (1:10,000; 06-599; Millipore), H4 (1:10,000; 04-858; Millipore), Ac-H4 (K4/7/11/15) (1:20,000; 06-866; Millipore), pS473-AKT (1:4,000; AF887; R&D), pT24-FOXO1 (1:2,000; 2599; Cell Signaling), and NHERF1 (1:1,000; Ab3452, Abcam).

High throughput RNA-Sequencing

Total RNA was isolated from confluent AMSCs in the absence of differentiation inducers to characterize the basal state of expression by high throughput RNA sequencing using the TrueSeq method ('poly A RNA Seq'). RNA libraries were prepared according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina). Briefly, poly-A mRNA was purified from total RNA using oligo dT magnetic beads. Purified mRNA was fragmented at 95°C for 8 minutes, eluted from the beads and primed for first strand cDNA synthesis. RNA fragments were copied into first strand cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen). Next, second strand cDNA synthesis was performed using DNA polymerase I and RNase H. The double-stranded cDNA was purified using a single AMPure XP bead (Agencourt) clean-up step. The cDNA ends were repaired and phosphorylated using Klenow, T4 polymerase, and T4 polynucleotide kinase followed by a single AMPure XP bead clean-up. Blunt-ended cDNAs were modified to include a single 3' adenylate (A) residue using Klenow exo- (3' to 5' exo minus). Paired-end DNA adaptors (Illumina) with a single "T" base overhang at the 3' end were immediately ligated to the 'A tailed' cDNA population. Unique indexes, included in the standard TruSeq Kits (12-Set A and 12-Set B) were incorporated at the adaptor ligation step for multiplex sample loading on the flow cells. The resulting constructs were purified by two consecutive AMPure XP bead clean-up steps. The adapter-modified DNA fragments were enriched by 12 cycles of PCR using primers included in the Illumina Sample Prep Kit. The concentration and size distribution of the libraries was determined on an Agilent Bioanalyzer DNA 1000 chip. A final quantification, using Qubit fluorometry (Invitrogen), confirmed sample concentrations. Libraries were loaded onto paired end flow cells at concentrations of 8–10 pM to generate cluster densities of 700,000/mm² following the standard protocol for the Illumina cBot and cBot Paired end cluster kit 3. Flow cells were sequenced as 51 × 2 paired end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit 3 and HCS v2.0.12 data collection software. Base-calling was performed using Illumina's RTA 1.17.21.3. The RNA-Seq data were analyzed using MAPRSeq v.1.2.1, the Bioinformatics Core standard tool, which includes alignment with TopHat 2.0.6 (Kim et al., 2013) and gene counts with the HTSeq software. Normalization and differential expression analysis were performed using edgeR 2.6.2 (Robinson et al., 2010).

Quantitative real-time reverse transcriptase PCR (RT-qPCR)

RNA was isolated using the RNeasy Kit (Qiagen) with on column DNA digestion kit (Qiagen). Isolated RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Gene expression was quantified using real-time PCR whereby each reaction was performed with 10 ng cDNA per 10 μ l, QuantiTect SYBR Green PCR Kit (Qiagen), and the CFX384 Real-Time System machine (BioRad). Transcript levels were quantified using the 2^{-C_t} method and normalized to the housekeeping gene GAPDH. The following gene specific primer sequences were used: GAPDH (Forward: ATGTTCTGTCATGGGTGTGAA, Rev: TGTGGTCATGAGTCCTTCCA), BGLAP (Forward: GGCGCTACCTGTATCAATGG, Reverse: GTGGTCAGCCAACTCGTCA), ALPL (Forward: ACTGGTACTCAGACAACGAGAT, Reverse: ACGTCAATGTCCCTGATGTTATG), OPG (Forward: GTGTGCGAATGCAAGGAAGG, Reverse: CCACTCCAAATCCAGGAGGG), and RUNX2 (Forward: TGGTTACTGTCTATGGCGGGTA, Reverse: TCTCAGATCGTTGAACCTTGCTA). Gene IDs and other primer sequences are available upon request.

Results

Multipotency of clinical-grade AMSCs

The ability of human AMSCs (prepared using our clinical grade method) to differentiate into distinct mesenchymal lineages was validated in vitro (Fig. 1A). Cells maintained for 21 days in an osteogenic cocktail produced a matrix that stained positively with Alizarin Red, a stain for calcium deposits, while cells grown in base medium did not produce a calcified matrix (Fig. 1B). AMSCs placed into micromasses and grown in a chondrogenic cocktail for 18 days incorporated Alcian Blue, a dye that detects sulfated proteoglycans deposits characteristic of functional chondrocytes (Fig. 1C). Very little Alcian Blue staining was detected in micromasses grown in base medium. AMSCs grown in base medium stained with Oil Red O, but staining was enhanced with the addition of adipogenic supplement (Fig 1D). Thus, AMSCs grown in human platelet based media supplementation have at least tri-lineage potential, with the ability to differentiate into osteoblasts, chondrocytes, and adipocytes.

Expression of HDACs and SIRT in AMSCs grown in platelet lysate

The human genome contains 11 HDACs (HDAC1-11) and 7 closely related sirtuins (SIRT1-7) that, except for the mitochondrial SIRT4 gene, encode for histone deacetylases. We utilized RNA-Seq for poly-A containing mRNAs and RT-qPCR to determine the expression levels of the full complement of HDAC and SIRT genes in AMSCs grown in 5% platelet lysate. RNA-Seq analysis revealed that HDACs 1, 2, 3 and 7 are expressed at highest expression. Medium expression is observed for HDACs 4, 5, 6 and 10, while HDAC8 and HDAC11 are present at yet lower levels, and HDAC9 expression is barely detectable in AMSCs (Fig. 2A, *top*). SIRT1 and SIRT2 are the two highest expressing sirtuins, SIRTs 3, 5, 6 and 7 are expressed at intermediate levels, while SIRT4 is barely detectable by RNA-Seq in AMSCs (Fig. 2B, *top*). Parallel expression analyses of HDACs and SIRTs by real time RT-qPCR in general corroborate the results obtained by RNASeq analysis (Fig. 2A & B). The robust expression of HDAC1, HDAC2, and HDAC3, which

together with HDAC8 are the primary targets of pan HDIs (McGee-Lawrence and Westendorf, 2011), indicate that multipotent mesenchymal cells are likely to respond to the histone deacetylase inhibitor SAHA.

SAHA induces hyper-acetylation and stimulates the AKT-FOXO1 axis in AMSCs

Considering the expression of the class I HDACs 1, 2, 3 and 8, we investigated whether AMSCs respond to SAHA, a pan-HDI. Function. Although SAHA is already very effective in generating histone hyper-acetylation and changes in gene expression within 2 hours, we selected a 24 hour treatment to ensure that cells would have time to equilibrate and alter regulatory signaling pathways (e.g., AKT signaling) (Dudakovic et al., 2013). As anticipated, treatment of confluent AMSCs with 1 μ M SAHA for 24 hour results in hyper-acetylation of histone 4 (H4) (Fig. 3A). Similarly, when sub-confluent AMSCs were assayed for histone 3 (H3) acetylation, we observed that SAHA induces hyper-acetylation of H3 (Fig. 3B). SAHA also greatly increases the protein levels of NHERF1/SLC9A3R1 (Fig. 3B), a solute-carrier protein that is activated upon inhibition or complete loss of HDAC function (Dudakovic et al., 2013). Thus, SAHA effectively blocks deacetylation of histones H3 and H4, while inducing a SAHA-responsive protein.

Previous studies demonstrated that SAHA treatment and conditional knock-down of Hdac3 reduces Akt signaling in murine osteoblasts and chondrocytes (Bradley et al., 2013; Dudakovic et al., 2013). Furthermore, the SAHA-responsive gene NHERF1/SLC9A3R1 has a scaffolding function in AKT signaling (Molina et al., 2012). We therefore tested whether or not SAHA changes AKT signaling in AMSCs grown in platelet lysate under non-differentiating conditions. Western blotting revealed that treatment of sub-confluent AMSCs with SAHA enhances the levels of phosphorylated AKT and its downstream target FOXO1 (Fig. 3B). Our results do not discriminate whether increased detection of the phospho-epitopes in AKT and FOXO1 is due to changes in total AKT or FOXO1 levels or increased phosphorylation of the epitope. However, mRNA levels of AKT1 and FOXO1 do not change (Fig. 3C) compared to the ACTB and HPRT housekeeping genes. Increased detection of these AKT and FOXO1 phospho-epitopes suggests that the AKT/FOXO1 axis is stimulated by SAHA in AMSCs, perhaps by inhibition of a novel HDAC dependent pathway (Fig. 3D). Because increased AKT signaling is linked to cell survival, SAHA could be cyto-protective for uncommitted human AMSCs maintained in platelet lysate.

SAHA inhibits osteogenic differentiation of AMSCs

To assess the effects of SAHA on AMSC lineage-commitment and to avoid known inhibitory effects of HDIs on proliferating cells, SAHA was added to confluent AMSCs during osteogenic differentiation (Figs. 4 & 5). SAHA treatments with 1 μ M SAHA for six days (i.e., SAHA added at day three and six of osteogenic differentiation) reduced osteogenic AMSC differentiation as evidenced by decreased staining for alkaline phosphatase (Fig. 4A) and Alizarin Red (Fig. 4B) after 6 and 21 days, respectively, when compared to vehicle treatment. The viability of differentiating AMSCs was not significantly compromised by SAHA as verified by crystal violet staining (Fig. 4C). SAHA also reduced the expression of alkaline phosphatase (ALPL), osteocalcin (BGLAP), osteoprotegerin (OPG), and runt-related transcription factor 2 (RUNX2), which are key osteogenic markers

(Fig. 4D). Interestingly, expression of osteogenic markers increased to different degrees after drug removal. This apparent rebounding effect suggests that SAHA delays but does not irreversibly block osteogenic differentiation of AMSCs. Together, our results indicate that HDIs interfere with osteogenic lineage-commitment of AMSCs.

Previous work with mouse models and primary murine bone marrow stromal cells indicated that immature cell populations are more sensitive to HDIs treatment than committed osteoblasts (McGee-Lawrence et al., 2011). To understand how AMSCs respond to SAHA, we performed interval treatments with SAHA in which AMSCs were exposed to SAHA for one, three, or six days at different stages of osteogenic differentiation (Fig. 5A). Similar to AMSCs treated for 3 to 6 days with SAHA (Figs. 4B and 5A), AMSCs cultured in 1 μ M SAHA during early stages of osteogenic lineage-commitment between days 6 and 12 exhibited reduced Alizarin Red staining compared to vehicle treated AMSCs (Fig. 5A & B). However, SAHA administration during late stages of osteogenic differentiation (days 15–21) did not reduce Alizarin Red staining (Fig. 5C). These data together demonstrate that SAHA interferes with early but not late stages of osteogenic lineage-commitment in clinical-grade AMSCs grown in platelet lysate.

SAHA inhibits adipogenic and chondrogenic lineage-commitment of AMSCs

Previous studies showed that HDIs inhibit adipogenic and chondrogenic differentiation of mesenchymal stromal cells (Haberland et al., 2010; Lee et al., 2009). To determine the effects of SAHA on the differentiation of human AMSCs into adipocytes grown in platelet lysate, 1 μ M SAHA and vehicle were added twice (day 0 and 3) to differentiating cells. Similar to osteogenic differentiation, SAHA significantly reduced Oil Red O staining (Figs. 6A, 6B and 6C). The effects of SAHA on chondrogenesis were also assessed by micromass cultures grown in platelet lysate. The addition of 1 μ M SAHA (day 0 and 3) during chondrogenic differentiation reduced Alcian Blue staining (Figs. 6D, 6E and 6F). Our data indicate that SAHA not only interferes with osteogenic differentiation, but also suppresses adipogenic and chondrogenic differentiation of AMSCs.

SAHA mechanistically enhances transcription factor gene expression in AMSCs

AMSCs express for distinct cell surface markers that reflect their biological properties as multi-potent mesenchymal cells (Crespo-Diaz et al., 2011). Confluent post-proliferative, but un-induced AMSCs were treated with vehicle and SAHA (1 μ M) for one day. We examined expression of mRNAs for cell surface markers at the end of treatment (1 day) and three days after drug removal (4 days) in maintenance medium to understand ‘short-term’ (day 1) and ‘long-term’ (day 4) changes in gene expression. There were neither short nor long-term effects of SAHA on mRNA expression of cell surface proteins (Fig. 7A), suggesting that the cells externally retain the same cell surface markers.

To evaluate the mechanism by which SAHA inhibits cellular commitment in three different lineages, we examined the expression of selected transcription factors (TF), as well as expression of assorted biomarker genes (e.g., genes encoding housekeeping proteins, cell surface markers and cell-type specific ECM proteins). SAHA enhances both ‘short-term’ and ‘long-term’ expression of mesenchymal-lineage associated TF genes, typically by 2 to 4

fold over vehicle treated control cells (Fig. 7B). For example, the pluripotency genes NANOG, OCT4/POU5F1 and SOX2 are induced by SAHA at day 1 and remain high on day 4 (Fig. 7B, *left*). Two differentiation specific genes, including the osteogenic protein SP7/Osterix and the myogenic factor MYF5, are transiently induced at day 1 but their expression is not sustained until day 4 (Fig. 7B, *middle*). The osteoblast related gene DLX5 and the chondrogenic gene SOX6 are not detected at day 1 but clearly induced at day 4 (Fig. 7B, *right*). These data suggests that SAHA may compromise the normal multi-potent state of AMSCs by selectively enhancing the expression of TFs associated with pluripotency, or the molecular phenotypes of bone, cartilage and muscle lineages.

Discussion

Injuries and age-related degeneration of musculoskeletal tissues demand effective repair strategies. Discovery and use of stem cell populations that differentiate into specialized tissue types has provided a viable foundation for regenerative medicine. Human mesenchymal stromal cells exhibit multipotency with a capacity for differentiation into cardiac, adipose, chondrocyte or osteoblast lineages, and offer a patient-derived renewable source to support tissue restoration (Ng et al., 2008; Pittenger et al., 1999). These cells exhibit anti-inflammatory properties (Murphy et al., 2013) and possess an immuno-privileged state (Aggarwal and Pittenger, 2005; Le Blanc et al., 2004; Undale et al., 2009) that allow xenogeneic testing or use of allogeneic cells in clinical situations. The promise of these cells to provide tangible improvements to patient outcomes has made them a focus of both scientific and translational efforts.

Bone marrow is common source of human mesenchymal progenitor cells (Horwitz et al., 2002; Koc et al., 2002), despite the low yield of cells that can be obtained. Adipose tissue is an attractive alternative source for mesenchymal progenitor cells because it is readily available, and yields higher numbers of mesenchymal cells (Kern et al., 2006). In most studies, isolated human mesenchymal progenitor cells are maintained in media supplemented with fetal bovine serum. Due to the potential presence of prions and zoonotic pathogens in FBS, substitutes are necessary for ex vivo expansion of mesenchymal progenitor cells (Cobo et al., 2006; Doerr et al., 2003; Spees et al., 2004). Pooled human platelet derived media supplement that adheres to good manufacturing practice, provides the growth factors necessary for rapid expansion and chromosomal stability of mesenchymal stem cells (Crespo-Diaz et al., 2011). Our study further validates previous work that patient-derived, human adipose-tissue derived mesenchymal progenitor cells are multi-potent and have the capacity to differentiate into osteogenic, chondrogenic, cardiac, and adipogenic tissues as part of tissue regeneration strategies (Ng et al., 2008; Pittenger et al., 1999).

HDI promotes differentiation of murine osteoblasts *in vitro* (Dudakovic et al., 2013; Haberland et al., 2010; Hu et al., 2013; Iwami and Moriyama, 1993; Schroeder and Westendorf, 2005). Similarly, HDIs permit osteogenic differentiation of lineage-committed human mesenchymal progenitor cells (Di Bernardo et al., 2009; Lee et al., 2009). However, progenitor cells are sensitive to HDIs and undergo cell cycle arrest due to increased DNA damage. Evidence for these divergent effects are seen in vivo, where HDIs enhance local measurements of osteoblast activity but have an overall negative effects on bone mass in

rodents (McGee-Lawrence et al., 2011; Nissen-Meyer et al., 2007; Pratap et al., 2010; Senn et al., 2010). Furthermore, valproate usage is associated with decreases in bone mineral density and increases in fracture risk in children and adults (Boluk et al., 2004; Sheth et al., 1995; Vestergaard et al., 2004). The studies presented here demonstrate that AMSCs adherent to good manufacturing practice are also sensitive to SAHA.

We find that clinical grade AMSCs express relatively high levels of the class I HDACs 1, 2, 3 and 8 that are known to be inhibited by SAHA. SAHA treatment of AMSCs induces hyper-acetylation of histones H3 and H4 as expected. However, this HDI also enhances the levels of phospho-AKT, phospho-FOXO1 and the scaffolding protein NHERF1, which has been linked to AKT signaling. The latter suggests that, even though SAHA can have cytotoxic effects on highly proliferative cell types, SAHA may promote AKT dependent cell survival of AMSCs. The stimulatory effects of SAHA on AKT signaling in human AMSCs are in contrast to earlier findings obtained with mouse MC3T3 osteoblasts and primary chondrocytes, where SAHA inhibits AKT phosphorylation (Bradley et al., 2013; Dudakovic et al., 2013). We propose that the effects of HDAC inhibitors like SAHA are distinct for different mesenchymal cell types.

Previous studies indicated that SAHA can promote osteoblast differentiation in MC3T3 cells (Dudakovic et al., 2013) prompting the current study to examine its effects on osteogenic differentiation of clinical grade human AMSCs to support potential bone tissue engineering applications. However, when we tested the biological effects of SAHA on tri-lineage differentiation of AMSCs into osteogenic, chondrogenic and adipogenic cell fates, we observed that SAHA blocks cell fate commitment into each of these mesenchymal lineages. These findings parallel those made with mouse bone marrow-derived stromal cells (BMSCs) in which SAHA inhibits osteogenic lineage commitment (McGee-Lawrence et al., 2011). Our present results with human AMSCs and these previous studies with mouse BMSCs collectively suggest that the effect of SAHA depends on the state of lineage-commitment. Experiments with interval treatments revealed that both in human AMSCs (this work) and mouse BMSCs (McGee-Lawrence et al., 2011), the inhibitory interval for SAHA is within the first two weeks of osteogenic differentiation after which the cells become refractory to SAHA.

To understand the mechanism by which SAHA alters lineage-commitment of AMSCs, we screened a panel of mesenchymal transcription factor genes for modulations upon SAHA treatment. This analysis revealed that SAHA can simultaneously up-regulate both pluripotency genes (NANOG, OCT4/POU5F1 and SOX2) and principal gene regulators that control osteogenic (e.g., SP7/Osterix, DLX5), chondrogenic (e.g., SOX6, NKX3-2) and myogenic (e.g., MYOD, MYF5) cell fates. Thus, SAHA simultaneously facilitates adoption of a more immature pluripotent state, while provoking differentiation into very distinct mesenchymal lineages. The concurrent up-regulation of these diverse transcription factors may destabilize the multi-potent state of adipose-tissue derived mesenchymal stromal cells. Consequently, SAHA blocks directed commitment of immature cells into a single mesenchymal lineage. In conclusion, the clinical efficacy of SAHA as a bone-anabolic epigenetic inhibitor may be realized in local or ex vivo applications that accelerate the maturation of phenotype-committed osteoblasts.

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Figure 1. Validation of the multipotency of clinical grade AMSCs grown in platelet lysate
 Diagram depicting the experimental set up for validating the tri-lineage potential of clinical-grade AMSCs grown in platelet lysate (PL) using different differentiation supplements and measuring lineage-specific histochemical outcomes (**A**). AMSCs were induced into osteogenic, chondrogenic or adipogenic cell fates using standard differentiation cocktails followed by histochemical staining. AMSCs cultured in base/osteogenic medium ('Osteo') for 21 days exhibit Alizarin Red staining reflecting osteogenic differentiation (**B**). Alcian Blue staining is shown for chondrogenic differentiation upon culture in base/chondrogenic medium ('Chon') for 18 days (**C**). Oil Red O staining for adipogenic differentiation is observed for AMSCs induced in base/adipogenic medium ('Adipo') for 14 days (**D**). Representative microscopy images of duplicate cultures that reveal extracellular matrix staining are shown for osteogenic and chondrogenic differentiation cultures. The right column in Panel D shows a 5X enlargement of the image to show intracellular Oil Red droplets.

Figure 2. Expression of HDACs and SIRT6 in AMSCs

AMSCs were grown in maintenance medium and RNA was isolated from un-induced confluent cells. RNASeq for polyadenylated mRNAs (**top**) or real-time RT-qPCR quantitative (**bottom**) was used to measure expression levels of HDACs (**A**) or SIRT6 (**B**). For each bar, the average normalized gene counts (reads per kilobasepair per million reads, RPKMs) are shown for AMSCs from three different patients ($n = 3$, mean $\pm$ STD). RNASeq and RT-qPCR values generally correspond well among AMSCs from distinct patients, but there are differences in relative detection of distinct histone deacetylases (e.g., HDAC3).

Figure 3. Molecular effects of SAHA on AMSCs

Western blotting of confluent AMSCs that were treated with 1 μM SAHA or vehicle for 24 hours show that SAHA treatment increases histone H4 acetylation, but not total H4 protein as expected (**A**). Western blot analysis of sub-confluent AMSCs shows that SAHA stimulates accumulation of acetylated histone H3 and up-regulates the levels of the HDAC responsive protein SLC9A3R1/NHERF1 and enhances phosphorylation of AKT and FOXO1 (**B**). This apparent stimulation of the AKT/FOXO1 axis in AMSCs contrasts with findings with pre-osteoblastic MC3T3 cells where SAHA is inhibitory of AKT (Dudakovic et al., 2013). Gene expression analysis demonstrates that AKT and FOXO1 levels are not affected after 24 hour SAHA treatment (**C**). Gene expression data were obtained by RT-qPCR and normalized to GAPDH, which was set at 100 (n = 3, mean ± STD). The diagram summarizes the modes of action of SAHA in AMSCs based on results presented in A and B (**D**). HDACs support the deacetylation of H4 and H3 histone and suppress expression of NHERF and FOXO1, whereas SAHA opposes these changes. SLC9A3R1/NHERF1 may inhibit AKT signaling in mouse osteoblasts (Dudakovic et al., 2013) (dashed diagonal line), but our data (see panel B) indicate that SAHA dependent induction of NHERF1 does not inhibit total AKT phosphorylation in AMSCs. Instead, our results suggest that HDACs may control the AKT-FOXO1 pathway through a different mechanism that is sensitive to SAHA in AMSCs.

Figure 4. SAHA inhibits early osteogenic lineage commitment

SAHA (1 μ M) and vehicle were added on days 3 (confluent cells) and 6 of osteogenic differentiation (indicated by arrows). SAHA and vehicle were removed on day 9, cells washed with PBS, and cells were either harvested or further differentiated. Alkaline phosphatase stain after 9 days of differentiation (A). Alizarin Red staining after 21 days of differentiation (B). Crystal Violet stain after 9 days of differentiation (C). Gene expression analysis of RNA collected at various time points of differentiation (D). Gene expression data were obtained by RT-qPCR and normalized to GAPDH, which was set at 100 ($n = 3$, mean $\pm$ STD).

Figure 5. AMSCs committed to the osteogenic lineage are refractory to SAHA inhibition
Diagram showing the design of experiments described in B and C, and a summary of results obtained for Alizarin Red staining presented in Figures 4 and 5, as well as data not shown (A). SAHA (1 μ M) and vehicle were added on days 6 and 9 (treated days 6–12) of osteogenic differentiation followed by Alizarin Red staining after 21 days of differentiation (B). SAHA (1 μ M) and vehicle were added on days 15 and 18 (treated days 15–21) of osteogenic differentiation followed by Alizarin Red staining after 21 days of differentiation (C).

Figure 6. SAHA inhibits adipogenic and chondrogenic lineage-commitment of AMSCs
 Cell cultures were stained with Oil Red O after 14 days of adipogenic differentiation (A). SAHA (1 μ M) and vehicle were added on days 0 (confluent cells) and 3 (treated days 0–6) of adipogenic differentiation. SAHA and vehicle were removed on day 6, cells washed with PBS, and differentiation continued. Representative microscope images (5X zoom) of cultures stained with Oil Red O (B). Quantification of the Oil Red O stain (n = 3, optical density $\pm$ STD) (C). Alcian Blue stain after 18 days of chondrogenic differentiation (D). SAHA (1 μ M) and vehicle were added on days 0 and 3 (treated days 0–6) of chondrogenic differentiation. SAHA and vehicle were removed on day 6, cells washed with PBS, and differentiation continued. Representative images of micromass cultures (E). Quantification of Alcian Blue stain in micromass cultures (n=3, ImageJ stain intensity $\pm$ STD) (F).

FIGURE 7. SAHA enhances expression of multiple mesenchymal-related transcription factors in AMSCs

SAHA (1 μ M) and vehicle were added at confluence in maintenance medium. RNA was collected one and four days after treatment. For the four day time point, SAHA and vehicle were removed one day after treatment, cells washed with PBS, and grown in maintenance medium for three additional days. A panel of genes for cell surface markers (**A**) and tens of transcription factors (**B**) with known functions in mesenchymal lineage commitment or phenotype maintenance was selected from the literature and their expression was analyzed by RT-qPCR analysis after SAHA treatment. SAHA did not significantly change expression of cell surface genes ($n = 3$ (3 replicates each), mean $\pm$ STD) (**A**). Genes induced at both one and four days after an initial one day SAHA treatment (**B, left**). Genes with a short-term response that are induced after only one day of SAHA treatment (**B, middle**). Transcription factor genes induced with a delayed response that exhibit up-regulation only the removal of SAHA for three days (**B, right**). The graphs plot the fold change (FC) for the expression of SAHA versus vehicle (SAHA/veh) in technical triplicates of AMSCs from two distinct patients ($n = 2$ (3 replicates each), mean $\pm$ STD). Data for the panel of transcription factors was filtered for average FC > 1.8 , with a minimal FC > 1.4 in each of the two patients.