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Transcriptional Analysis of Histone Deacetylase Family Members Reveal Similarities Between Differentiating and Aging Spermatogonial Stem Cells

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Abstract

The differentiation of adult stem cells involves extensive chromatin remodeling, mediated in part by the gene products of histone deacetylase (HDAC) family members. While the transcriptional downregulation of HDACs can impede stem cell self-renewal in certain contexts, it may also promote stem cell maintenance under other circumstances. In self-renewing, differentiating, and aging spermatogonial stem cells (SSCs), the gene expression dynamics of HDACs have not yet been characterized. To gain further insight with these studies, we analyzed the transcriptional profiles of six HDAC family members, previously identified to be the most highly expressed in self-renewing SSCs, during stem cell differentiation and aging. Here we discovered that in both differentiating and aging SSCs the expression of *Sirt4* increases, while the expression of *Hdac2*, *Hdac6*, and *Sirt1* decreases. When SSCs are exposed to the lifespan-enhancing drug rapamycin *in vivo*, the resultant HDAC gene expression patterns are opposite of those seen in the differentiating and aging SSCs, with increased *Hdac2*, *Hdac6*, and *Sirt1* and decreased *Hdac8*, *Hdac9*, and *Sirt4*. Our findings suggest that HDACs important for stem cell maintenance and oxidative capacity are downregulated as adult stem cells differentiate or age. These results provide important insights into the epigenetic regulation of stem cell differentiation and aging in mammals.

Keywords

Histone deacetylase; Stem cell; Spermatogonia; Differentiation; Aging; Sirtuin; Gene expression

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Introduction

Stem cell differentiation is associated with extensive chromatin remodeling, and often involves a decrease in histone deacetylase (HDAC) gene expression [1]. In adult or tissue-specific stem cells, the downregulation of HDACs has been shown to reduce self-renewal ability and a loss of ‘stemness’ by upregulating differentiation markers [2]. Other studies have shown that HDAC downregulation can also promote self-renewal in adult stem cells [3]. Mammalian HDACs are currently grouped into four classes: nuclear class I (HDAC1, 2, 3, and 8), nuclear/cytoplasmic class IIa (HDAC4, 5, 7, and 9) and cytoplasmic class IIb (HDAC6 and 10), class III (sirtuins, SIRT1-7), and class IV (HDAC11) [4, 5]. Class I, II, and IV HDACs utilize Zn^{2+} for catalytic activity; class III sirtuins rely upon NAD^+ . Some individual HDACs can compensate for the loss of other members, such as *Hdac1* demonstrating sufficiency for oligodendroglial differentiation and brain development when *Hdac2* is deleted in mice [6, 7]. However, important distinctions may exist between classes of HDACs, as recently demonstrated by the inhibition of mesodermal differentiation in embryonic stem cells (ESCs) associated with the specific knockdown of class IIa HDACs but not class I HDACs [8]. This suggests that individual HDACs within the different classes could exhibit differential regulation through distinct expression patterns.

The differentiation of adult stem cells involves chromatin reorganization that no longer favors self-renewal. In this respect, differentiating stem cells might resemble aging stem cells, in which the maintenance of ‘stemness’ and ability to repair DNA damage are compromised [9]. Recent evidence showed that downregulating HDACs induced cellular senescence in human umbilical cord blood-derived multipotent stem cells by downregulating important self-renewal factors [10, 11]. Here, using mouse spermatogonial stem cells (SSCs) as an in vivo model system for studying adult stem cell maintenance, we analyzed the gene expression profiles of *Hdac2* and *Hdac8* (class I), *Hdac9* (class IIa), *Hdac6* (class IIb), *Sirt1* and *Sirt4* (class III) during stem cell differentiation and aging. We further examined the effects of the lifespan-enhancing drug rapamycin on the transcript levels of these HDAC family members in self-renewing SSCs. Our results demonstrate that while some HDAC members are diminished upon differentiation and aging, other HDAC members are enriched, and in turn, exhibit differential responses to rapamycin. The gene expression patterns in differentiating SSCs mirror those in aging SSCs, highlighting similarities between the two processes.

Materials and Methods

Isolation of Mouse Testicular Cells

Male FVB/NJ mice aged 1-wk-old, 3-wk-old, and 1-yr-old were euthanized and their testes were isolated for germ cell enrichment. Additionally, FVB/NJ males aged 12-days-old through 26-days-old were administered daily intraperitoneal (IP) injections of rapamycin (4 mg/kg body weight) or control vehicle (5 % Tween-80, 5 % PEG-400), beginning at postnatal day (P)12. Mice were euthanized at P26 and their testes were isolated for germ cell enrichment. All procedures and care of animals were carried out according to the Children’s Memorial Research Center Animal Care and Use Committee. Testes were decapsulated and briefly minced in ice-cold 1:1 Dulbecco’s Modified Eagle Medium–Ham’s F-12 Medium. An initial enzymatic digestion using collagenase IV (1 mg/ml) and DNase I (2 mg/ml) at 37 °C for 30 min was administered to remove interstitial Leydig cells and peritubular myoid cells from the seminiferous tubules. A second enzymatic digestion using collagenase IV (1 mg/ml), DNase I (2 mg/ml), hyaluronidase (1.5 mg/ml), and trypsin (1 mg/ml) at 37 °C for 30 min was administered to isolate germ cells and Sertoli cells from the remaining tissue. Final suspensions of single cells were prepared in ice-cold PBS containing 0.5 % BSA and 2

mM EDTA for subsequent germ cell enrichment by magnetic-activated cell sorting (MACS). PBS containing 0.5 % BSA and 2 mM EDTA is referred to as MACS Buffer.

MACS Enrichment of Distinct Germ Cell Populations

The use of MACS in this study is based upon previously established protocols [12–14]. Briefly, single cell suspensions containing germ cells in 80 μ l MACS Buffer were first incubated with 20 μ l rabbit anti-GFRA1 antibodies (Santa Cruz Biotechnology, CA) at 4 °C for 20 min with rotation. After washes, a second incubation of cells in 80 μ l MACS Buffer with 10 μ l goat anti-rabbit antibody-conjugated MicroBeads and 10 μ l anti-THY1 antibody-conjugated MicroBeads (Miltenyi Biotec, Auburn, CA) was administered at 4 °C for 20 min with rotation. The labeled cells were filtered through 30- μ m pore size mesh to remove cell aggregates, and then sorted through a separation LS column attached to a MidiMACS separator (Miltenyi Biotec). THY1+ and GFRA1+ cells were retained inside the column within the magnetic field, while unlabeled cells passed through the column and were collected as the column-depleted THY1-/GFRA1- cell fraction (CD fraction). After washes with MACS Buffer, the LS column was removed from the magnetic field and the THY1+ and GFRA1+ cells representing the undifferentiated SSC fraction were flushed out. For the enrichment of differentiating spermatogonia, CD fraction cells were subsequently reconstituted in 90 μ l MACS Buffer and incubated with 10 μ l anti-CD117 (KIT) antibody-conjugated MicroBeads (Miltenyi Biotec) at 4 °C for 20 min with rotation. These samples were then sorted through a MidiMACS LS column to collect the KIT+ cells.

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from MACS-separated cells and unsorted germ cells using the RNeasy Micro Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA samples were treated with RNase-free DNase I (Qiagen) on-column to remove genomic DNA. Yield and quality of RNA samples were determined using the NanoDrop 2000 Spectrophotometer (ThermoScientific, Wilmington, DE). Total RNA was reverse transcribed into cDNA using random hexamer primers (Life Technologies, Grand Island, NY). For quantitative RT-PCR, cDNA was added to 2 $\times$ Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) with specific oligonucleotide primer sets for the genes of interest (listed in Supplemental Table 1). Samples from three biological replicates were run in triplicate on an Applied Biosystems 7500 Real-Time PCR System using SYBR® Green dye for read-out and ROX™ dye as an internal reference. Each PCR reaction contained approximately 5–10 ng of cDNA, 1 $\times$ Power SYBR® Green PCR Master Mix, and 500 nM of each forward and reverse primer for the desired gene. *Gapdh* was used as an endogenous control. The threshold cycle (C_T), indicating the relative abundance of a particular transcript, was calculated for each reaction by the system software. Quantification of the fold change in gene expression was determined by using the formula $2^{-\Delta\Delta C_T}$, in which $\Delta\Delta C_T = [(C_T \text{ of gene of interest} - C_T \text{ of } Gapdh)_A - (C_T \text{ of gene of interest} - C_T \text{ of } Gapdh)_B]$. Fold change in transcript levels was plotted using Prism 5 software (GraphPad, La Jolla, CA). Values plotted are mean $\pm$ SEM from three biological replicates. Statistical analysis was performed using Prism 5, employing Student's *t*-test; * $p < 0.05$; ** $p < 0.01$.

Results

Comparison of HDAC Gene Expression Profiles Between Undifferentiated and Differentiating Stem Cells

To examine the dynamics of HDAC gene expression upon SSC differentiation, we used magnetic-activated cell sorting (MACS) to facilitate the enrichment of undifferentiated primary spermatogonia expressing surface markers thymus cell antigen 1, theta (THY1) and glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1), representing the

SSC population, and the enrichment of differentiating primary spermatogonia expressing surface marker kit oncogene (KIT) [12–14]. We chose to investigate HDAC gene family members previously identified to be the most highly expressed in undifferentiated male germ cells, representing the SSC population: *Hdac2* and *Hdac8* (class I), *Hdac9* (class IIA), *Hdac6* (class IIB), *Sirt1* and *Sirt4* (class III) [15]. Utilization of MACS as an enrichment strategy allowed us to directly compare the following: undifferentiated SSCs ($2.12 \pm 0.17 \times 10^6$ cells per experiment; $N = 3$) versus unsorted germ cells ($2.05 \pm 0.13 \times 10^6$ cells per experiment; $N = 3$); differentiating spermatogonia ($2.21 \pm 0.25 \times 10^6$ cells per experiment; $N = 3$) versus unsorted germ cells (see above); and differentiating spermatogonia versus undifferentiated SSCs (Fig. 1). As expected, SSC markers *Gfra1* and POU domain, class 5, transcription factor 1 (*Pou5f1*; referred to here as *Oct4*) exhibited significant enrichment in THY1/GFRA1⁺ cells when compared to unsorted germ cells (15.5-fold and 7.3-fold, respectively), and were down-regulated in KIT⁺ cells when compared to THY1/GFRA1⁺ cells (3.9-fold and 2-fold, respectively) (Fig. 1). In contrast, differentiating spermatogonia markers *Kit* and *Sohlh2* were significantly upregulated in KIT⁺ cells when compared to unsorted germ cells (54.2-fold and 9.3-fold, respectively), as well as in KIT⁺ cells when compared to THY1/GFRA1⁺ cells (22.0-fold and 2.7-fold, respectively) (Fig. 1). For the HDAC genes we examined, *Hdac2*, *Hdac6*, and *Sirt1* transcript levels were significantly diminished in KIT⁺ cells when compared to THY1/GFRA1⁺ cells (5.4-fold, 9.4-fold, and 4.1-fold, respectively), while *Sirt4* showed significant enrichment in the KIT⁺ cells (2.8-fold) (Fig. 1). Thus, in differentiating SSCs the expression of *Sirt4* increases, while the expression of *Hdac2*, *Hdac6*, and *Sirt1* decreases.

Similarities in HDAC Gene Expression Are Observed Between Differentiating and Aging Stem Cells

Because cell differentiation exhibits many of the same molecular properties as cell aging/senescence [16], we next wondered whether SSCs from 1-yr-old mouse testes would harbor HDAC gene expression that was distinct from SSCs in 3-wk-old testes. MACS-enriched THY1/GFRA1⁺ cells were prepared from the two ages of mouse testes ($1.78 \pm 0.11 \times 10^6$ cells from 1-yr-old testes versus $1.89 \pm 0.16 \times 10^6$ cells from 3-wk-old testes per experiment; $N = 3$) and the transcriptomes were analyzed for HDAC family members. While neither *Gfra1* nor *Oct4* showed a change in expression between old and young SSCs, *Hdac2*, *Hdac6*, and *Sirt1* were significantly downregulated in the aging SSCs (5.2-fold, 6.9-fold, and 2.6-fold, respectively) (Fig. 2). In contrast, *Sirt4* was significantly upregulated in the aging SSCs (2.1-fold) (Fig. 2). These data reveal that the two sets of HDAC genes we examined show similar expression patterns in differentiating SSCs and in aging SSCs.

Stem Cells Exposed to Rapamycin Exhibit HDAC Gene Expression Profiles that Reflect a More Undifferentiated State

Mouse SSCs display a loss of regenerative ability during the aging process [17–20], but undergo active expansion in vivo when exposed to the lifespan-enhancing drug rapamycin [21]. Rapamycin inhibits mammalian target of rapamycin (mTOR) signaling and can increase the lifespan of worms, flies, and mice [22–24]. Furthermore, rapamycin blocks SSC differentiation by inhibiting phosphatidylinositol 3-kinase/Akt signaling pathways [25]. In light of these findings, we decided to test whether rapamycin-exposed SSCs would exhibit a reversal in the HDAC gene expression patterns we saw in the aging and differentiating SSCs. We implemented an established regimen in which male mice were administered intraperitoneal injections of rapamycin or control vehicle daily for two weeks [21, 26–28]. Following these treatments, single cell suspensions of germ cells were prepared from isolated testes and subjected to MACS-enrichment of THY1/GFRA1⁺ cells ($1.96 \pm 0.08 \times 10^6$ cells from rapamycin-exposed mice versus $1.80 \pm 0.13 \times 10^6$ cells from vehicle-exposed mice per experiment; $N = 3$). Upon analysis of the rapamycin-exposed and vehicle-exposed SSC

transcriptomes, we observed an enrichment of *Gfra1* and *Oct4* expression in the rapamycin-exposed SSCs (3.2-fold and 2.7-fold, respectively) (Fig. 3). These data reflect enhanced SSC self-renewal that had previously been noted [21, 28]. Interestingly, *Hdac2*, *Hdac6*, and *Sirt1* exhibited a significant upregulation in the rapamycin-exposed SSCs (4.5-fold, 3.1-fold, and 2.6-fold, respectively), while *Sirt4*, along with *Hdac8* and *Hdac9*, was significantly downregulated in these cells (2.2-fold, 5.3-fold, and 2.7-fold, respectively) (Fig. 3). We conclude that exposure of SSCs to rapamycin, which inhibits cell differentiation and promotes stem cell self-renewal, results in HDAC gene expression patterns that are opposite of those seen in differentiating and aging SSCs: specifically, increased *Hdac2*, *Hdac6*, and *Sirt1* and decreased *Sirt4*.

Discussion

For these studies, we have demonstrated that individual HDAC members within three distinct classes exhibit differential gene expression in self-renewing, differentiating, and aging SSCs. The expression of class I–III members *Hdac2*, *Hdac6*, and *Sirt1* correlates with adult stem cell maintenance, while the expression of class III member *Sirt4* associates with differentiation and aging in these stem cells. A recent report from Luzzani et al. (2011) examined the transcript levels of HDAC family members in mouse and human ESCs that were in vitro differentiated [29]. The authors found that class I *Hdac2* and *Hdac3* were differentially regulated in differentiating mouse ESCs (downregulated and upregulated, respectively), and that both class IIa *HDAC5* and class IIb *HDAC10* were upregulated in differentiating human ESCs [29]. We observed a similar down-regulation of *Hdac2* in our differentiating mouse SSCs. As HDAC2 associates with HDAC1 to form repressive complexes, and HDAC1 has been shown to control ESC differentiation [30], the altered transcript levels of *Hdac2* might be coordinated with *Hdac1* in SSC differentiation. Our analysis of HDAC family gene expression levels in these studies is important, however, we acknowledge that their potential functional significance remains speculative in the absence of protein assessment and view such examination as integral to future studies.

Our finding that *Sirt1* and *Sirt4* are differentially regulated in self-renewing, differentiating, and aging SSCs supports recent evidence that *Sirt4* negatively regulates *Sirt1* and oxidative metabolism in adult tissue [30]. Interestingly, SIRT4 catalytic output appears limited to NAD⁺-dependent ADP-ribosyl transferase activities with no deacetylase capabilities [31], suggesting that its function might be distinct from other sirtuins within differentiating and aging cells. SIRT1, the family member most closely related to yeast Sir2, is required for lifespan extension mediated by caloric restriction; modulation of *Sirt1* expression through elevation or reduction results in delayed or premature aging, respectively [32]. Thus, decreased *Sirt1* expression, together with reduced *Hdac2* levels in differentiating and aging SSCs, might reflect an alteration of both chromatin organization and oxidative metabolism that would negatively impact stem cell maintenance.

Lifespan in model organisms can be extended by inhibiting mTOR signaling with rapamycin [22–24]. Since rapamycin also expands mouse SSCs in vivo [21], the observed increase in *Hdac2*, *Hdac6*, and *Sirt1* transcript levels in rapamycin-exposed SSCs correlates with their relative abundance in self-renewing, but not differentiating or aging stem cells. Class II HDAC gene products have been shown to associate with mTOR-activated hypoxia inducible factor 1 alpha (HIF1α) to ensure HIF1α stability in cells undergoing active growth and metabolism [33]. Downregulation of *Hdac9* in rapamycin-exposed SSCs likely reflects this inhibition of mTOR and its downstream regulatory network. The decrease in *Hdac8* and *Sirt4* in SSCs upon rapamycin treatment suggests that their regulation is modulated by mTOR signaling, and that stem cell differentiation and aging events likely utilize this

signaling pathway, in turn, to affect HDACs and subsequent epigenetic modifications that influence cell fate.

The observed changes in gene expression upon aging and following rapamycin treatment are likely not attributable to potential changes in the SSC populations enriched by MACS, as the numbers of THY1/GFRA1+ cells isolated under each condition are roughly equivalent. Rapamycin blocks differentiating spermatogonia and subsequent spermatogenesis by inhibiting the mTOR complex 1/phosphatidylinositol 3-kinase/Akt signaling and progression through the cell cycle, but conversely promotes SSC maintenance through the upregulation of GFRA1 signaling [21, 25]. Thus, we conclude that our enrichment for THY1/GFRA1+ cells from young, old, and rapamycin-exposed testes yields similar cells for each condition.

In summary, members of class I–III HDACs maintain distinct gene expression profiles in undifferentiated adult stem cells, and undergo similar dynamics with differentiation and aging. Our findings suggest that HDAC genes important for stem cell maintenance and oxidative capacity, *Hdac2*, *Hdac6*, and *Sirt1*, are downregulated as adult stem cells differentiate or age, while an HDAC gene associated with cells in a differentiated or oxidative state, *Sirt4*, is upregulated. These results provide important insights into the epigenetic regulation of stem cell differentiation and aging in mammals.

Supplementary Material

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Acknowledgments

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Fig. 1. HDAC class I-III genes exhibit distinct expression levels in differentiating SSCs. MACS-enriched germ cells representing undifferentiated and differentiating spermatogonia (THY1/GFRA1+ and KIT+, respectively) were compared to unsorted testicular cells or to each other. Fold changes in transcript levels were set relative to endogenous control *Gapdh* (baseline value of 1). Student's *t*-test was performed to assess significance between each SSC marker and the endogenous control; * $p < 0.05$; ** $p < 0.01$

Fig. 2.

HDAC gene expression profiles in aging SSCs are similar to those in differentiating stem cells. MACS-enriched THY1/GFRA1+ spermatogonia from 1-yr-old male mice ($1.78 \pm 0.11 \times 10^6$ cells per experiment; $N = 3$) were compared to samples from 3-wk-old males ($1.89 \pm 0.16 \times 10^6$ cells per experiment; $N = 3$). Fold changes in transcript levels were set relative to endogenous control *Gapdh* (baseline value of 1). Student's *t*-test was performed to assess significance between each SSC marker and the endogenous control; * $p < 0.05$; ** $p < 0.01$

Fig. 3.

Rapamycin-exposed SSCs exhibit a reversal in the HDAC gene expression observed in aging and differentiating stem cells. MACS-enriched THY1/GFRA1+ spermatogonia from rapamycin-treated male mice ($1.96 \pm 0.08 \times 10^6$ cells per experiment; $N = 3$) were compared to samples from vehicle-treated males ($1.80 \pm 0.13 \times 10^6$ cells per experiment; $N = 3$). Fold changes in transcript levels were set relative to endogenous control *Gapdh* (baseline value of 1). Student's *t*-test was performed to assess significance between each SSC marker and the endogenous control; * $p < 0.05$; ** $p < 0.01$.