

Suppression of HIF-1 α by Valproic Acid Sustains Self-Renewal of Mouse Embryonic Stem Cells under Hypoxia *In Vitro*

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Abstract

The developing embryo naturally experiences relatively low oxygen conditions *in vivo*. Under *in vitro* hypoxia, mouse embryonic stem cells (mESCs) lose their self-renewal activity and display an early differentiated morphology mediated by the hypoxia-inducible factor-1 α (HIF-1 α). Previously, we demonstrated that histone deacetylase (HDAC) is activated by hypoxia and increases the protein stability and transcriptional activity of HIF-1 α in many human cancer cells. Furthermore HDAC1 and 3 mediate the differentiation of mESCs and hematopoietic stem cells. However, the role of HDACs and their inhibitors in hypoxia-induced early differentiation of mESCs remains largely unknown. Here, we examined the effects of several histone deacetylase inhibitors (HDACIs) on the self-renewal properties of mESCs under hypoxia. Inhibition of HDAC under hypoxia effectively decreased the HIF-1 α protein levels and substantially improved the expression of the LIF-specific receptor (LIFR) and phosphorylated-STAT3 in mESCs. In particular, valproic acid (VPA), a pan HDACI, showed dramatic changes in HIF-1 α protein levels and LIFR protein expression levels compared to other HDACIs, including sodium butyrate (SB), trichostatin A (TSA), and apicidin (AP). Importantly, our RT-PCR data and alkaline phosphatase assays indicate that VPA helps to maintain the self-renewal activity of mESCs under hypoxia. Taken together, these results suggest that VPA may block the early differentiation of mESCs under hypoxia via the destabilization of HIF-1 α .

Key Words: Embryonic stem cell, HDAC, Valproic acid, Hypoxia, Self-renewal, HIF-1 α

INTRODUCTION

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of blastocysts during development. Because they can differentiate into any type of cell, mESCs have been used to study the molecular mechanisms in early mammalian organogenesis (Evans and Kaufman, 1981). In the presence of leukemia inhibitory factor (LIF), mESCs can be maintained indefinitely in a self-renewing state that closely resembles the pluripotent cells of the ICM. LIF signaling is initiated through the dimerization of the cytokine receptors, the LIF receptor (LIFR) and glycoprotein130, after their engagement by cytokine (Niwa *et al.*, 1998). This complex activates the Janus kinase (JAK) and the signal transducer and activator of transcription 3 (STAT3) signaling pathways, resulting in the subsequent dimerization, nuclear translocation, and target gene activation of STAT3, which are crucial for the maintenance of the self-renewal of mESCs (Ernst *et al.*, 1999; Tighe and Gudas, 2004). STAT3 has been reported to be not only

an important substrate for activated JAKs, but also a crucial regulator of the LIF-induced self-renewal of mESCs (Matsuda *et al.*, 1999). Therefore, when LIF is removed or differentiation signals are added, mESCs begin to differentiate into many types of cells, including endothelial cells (Li *et al.*, 2007), adipocytes (Bost *et al.*, 2002), neurons (Schrenk-Siemens *et al.*, 2008), and smooth muscle cells (Du *et al.*, 2004; Yu and Thomson, 2008).

During normal organogenesis, the early embryo undergoes a transient, low pO₂ concentration (~5% O₂, or hypoxia) until vascularization begins, since which may cause an increase in the oxygen requirements (Gassmann *et al.*, 1996; Land, 2004; Powers *et al.*, 2008; Simon and Keith, 2008). Hypoxia is detectable in many regions during normal embryo development (Lee *et al.*, 2001) including reproductive tract (Masuda *et al.*, 2000), the microvasculature (Intaglietta *et al.*, 1996), and the ocular vitreous (Shui *et al.*, 2006), even after well-developed vascularization. and even after a well-developed vascularization (Powers *et al.*, 2008). Hypoxia inducible factor-1 α (HIF-

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1 α) is an important transcription factor that acts under low oxygen conditions, and the expression and activity of HIF-1 α are regulated by oxygen concentration (Semenza, 2001; Lee *et al.*, 2004; Peyssonnaud *et al.*, 2007). The stability of HIF-1 α is regulated by post-translational modifications, such as hydroxylation (Berra *et al.*, 2003), sumoylation (Bae *et al.*, 2004), acetylation (Jeong *et al.*, 2002), and phosphorylation. These modifications are mediated by the interaction of HIF-1 α with several proteins, including prolyl hydroxylases (PHDs), histone deacetylases (HDACs), ARD-1, pVHL, PKC- δ and p300/CBP (Jeong *et al.*, 2002; Kim *et al.*, 2004). In particular, class I HDACs (HDAC1 and HDAC3) enhance HIF-1 α stability and transactivation in hypoxic conditions and are considered positive regulators of HIF-1 α stability (Kim *et al.*, 2007).

Previously, we demonstrated that HIF-1 α may act as a negative regulator of the LIFR-STAT3 pathway and thus be important for the hypoxia-induced early differentiation of mESCs (Jeong *et al.*, 2007). Furthermore, we reported that PKC- δ inhibitors block the early differentiation of mESCs under hypoxia via the destabilization of HIF-1 α (Lee *et al.*, 2010). HDACs effectively inhibited HIF-1 α stability in cancer cells; however, the relevance of the HDACs as regulators of self-renewal activity in hypoxia-induced differentiation in mESCs is largely unknown. Therefore, we examined the role of HDACs in hypoxia-induced differentiation using feeder-free mESCs. Our data show that HDACs destabilized HIF-1 α protein and blocked the downregulation of LIFR-STAT3 signaling during hypoxia-induced differentiation of mESCs. Notably, valproic acid (VPA) robustly inhibited the downregulation of the LIFR-STAT3 pathway mediated by HIF1 α , which in turn sustained the self-renewal of mESCs under hypoxia. Therefore, our results suggest an inhibitory role for VPA in the hypoxia-induced early differentiation of mESCs.

MATERIALS AND METHODS

Cell culture

mESC lines, CCE cultured under feeder-free conditions were maintained in knockout-DMEM (Invitrogen, Grand Island, NY) containing 15% defined serum replacement (Knockout SRTM; Invitrogen, Grand Island, NY), 1 mM glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, 1% non-essential amino acids, 0.1 mM β -mercaptoethanol, and 1,000 U/ml LIF (ESGRO; Chemicon, Temecula, CA). For hypoxic conditions, CCE cells were incubated at 1% O₂ level with 5% CO₂ balanced with N₂ in a hypoxic chamber (Forma Scientific, Marietta, OH, USA) with an interior temperature of 37°C.

Reagents

Valproic acid, trichostatin A and sodium butyrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apicidin was purchased from Calbiochem (Darmstadt, Germany).

RT-PCR analysis and primers

Total RNA from cells was isolated using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions and quantified by spectrophotometer (NanoDrop, Nyxor Biotech). First-stranded cDNA was synthesized with 5 μ g of each DNA-free total RNA and oligo-(dT)₁₆ primer by *Moloney* murine leukemia virus reverse transcriptase (Promega,

Madison, WI, USA). One microlitre of cDNA was amplified by PCR using 1.25U of ExTaq DNA polymerase kit (Takara, Madison, WI). The primers used had the following sequences: *fgf4* forward, 5'-TACTGCAACGTGGGCATCGGA-3'; reverse, 5'-GTGGGTTACCTTCATGGTAGG-3'; *rex1* forward, 5'-CGT-GTAACATACACCATCCG-3'; reverse, 5'-GAAATCCTCTTC-CAGAAT-GG-3'; *fgf5* forward, 5'-ATGAGCCTGTCTT-GCTC-3'; reverse, 5'-GTCTGTACTTCACTGGGC-3'; *STAT5a* forward, 5'-GCTGTATCCG-TCACATTCTG-3'; reverse, 5'-CCACTGGATCAGCTCGTCGT-3'; *gapdh* forward, 5'-AAC-GGGAAGCCCATCACC-3'; reverse, 5'-CAGCCTTGGCAG-CACCAG-3'. The PCR products were separated on 1.2-1.5% agarose gels and visualized by ethidium bromide staining under a UV transilluminator.

Western blot analysis

Cells were harvested and the pellets were immediately frozen in liquid Nitrogen. After thawing, the cell pellet lysed in lysis buffer (20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 1 mM Na₂EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM beta-glycerophosphate; 1 mM Na₃VO₄; 1 μ g/ml leupeptin) followed by centrifugation for 30 min at 15,000 rpm and protein concentration was determined by the BCA assay (Sigma, St. Louis, MO) and protein extracts were resolved in SDS-PAGE gels and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The protein-bearing membrane was blocked with 5% skim milk and probed with specific primary antibodies to HIF-1 α (Cayman Chemical), LIFR (Santa Cruz Biotechnology, Santa Cruz, CA), tyr-705-phosphorylated Stat3, lys-9-acetylated histone 3 (Cell Signaling Technology, Beverly, MA) and α -tubulin (InnoGenex, San Ramon, CA), followed by incubation with secondary HRP-conjugated antibodies to mouse or rabbit IgG (Pierce, Rockford, IL). Antibody detection was performed by standard procedures using ECL Plus reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence

Cells were fixated with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4) for 15 min and directly permeabilized with 0.1% Triton X-100 in PBS for 20 min. Then, cells were blocked with 5% BSA in PBS-T for 1 h at room temperature (RT). Next, anti-LIFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied overnight at 4°C and washed three times with PBS-T. Then, cells were incubated with secondary Alexa546-conjugated IgG (Molecular Probes, Inc.). Nuclear counterstaining was performed using DAPI (Molecular Probes, Inc.). Fluorescence staining was evaluated using a fluorescence microscope (Carl Zeiss, Germany).

Alkaline Phosphatase (AP) assay

The cells were fixed with 4% paraformaldehyde for 2 minutes at room temperature. Staining for alkaline phosphatase (AP) was performed using a diagnostic kit (Sigma) following protocols provided by the manufacturer.

Statistical analysis

The data are expressed as mean \pm S.E.M., and were analyzed using analysis of variance or Student's t test as appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

HDAC inhibitors downregulate the protein expression of HIF-1 α in mESCs under hypoxia

HDACs stabilize the HIF-1 α protein and their inhibitors destabilize HIF-1 α under hypoxic conditions in human cancer cells (Kim *et al.*, 2007; Lee *et al.*, 2007). However, because the effect of HDACs and their specific inhibitors varies depending on the dose, the duration of treatment, and the types of cells treated, we examined the role of several HDAC inhibitors such as sodium butyrate (SB), trichostatin A (TSA), apicidin (AP), and valproic acid (VPA) on the hypoxia-induced differentiation of CCE mESCs. Thus, we cultured mESCs with the indicated inhibitors under both normoxic (20% O₂) and hypoxic (1% O₂) culture conditions in the presence of LIF. We evaluated the effect of these inhibitors on HIF-1 α expression by Western blot analysis. HIF-1 α protein levels were higher

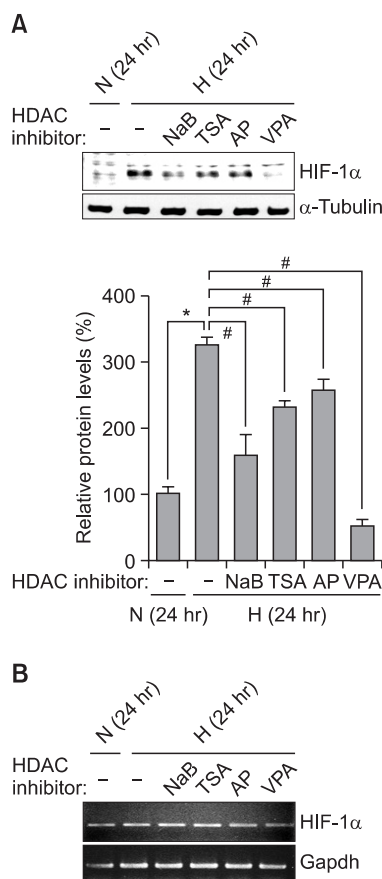


Fig. 1. HDAC inhibitors (HDACIs) decreased HIF-1 α protein levels under hypoxic conditions in mouse embryonic stem cells (mESCs). (A, B) CCE cells were treated with HDAC inhibitors, 1 mM sodium butyrate (SB), 5 ng/ml trichostatin A (TSA), 100 nM apicidin (AP), and 1 mM valproic acid (VPA), and were then immediately exposed to hypoxia for 24 h. (A) HIF-1 α and α -tubulin were examined using Western blot analysis. The graph represents the mean values \pm SD (n=3). * p <0.01; # p <0.01. (B) The expression level of HIF-1 α mRNA was examined using RT-PCR. GAPDH and α -tubulin were used as internal controls. N: normoxia, H: hypoxia; Results are representative of three independent experiments.

under hypoxia than under normoxia and markedly decreased after treatment with the HDAC inhibitors, including SB, TSA, AP, and VPA (Fig. 1A). Notably, VPA was more effective than the other inhibitors in downregulating HIF-1 α . In contrast, HIF-1 α mRNA expression did not change in CCEs treated with inhibitors under hypoxia (Fig. 1B). These results suggest that HDAC inhibitors destabilize HIF-1 α protein levels under hypoxia in mESCs.

HDAC inhibitors block the downregulation of the LIFR-STAT3 pathway under hypoxia

Previously, we reported that HIF-1 α binds to reverse HREs in the LIFR promoter, leading to the downregulation of LIFR-STAT3 signaling in mESCs under hypoxia (Jeong *et al.*, 2007). To examine the effect of HDAC inhibitors on LIFR-STAT3 signaling, we cultivated mESCs in the presence of LIF with four kinds of HDAC inhibitors, SB, TSA, AP, and VPA under hypoxic conditions. Expression of LIFR and phosphorylation of STAT3 decreased under hypoxia, whereas treatment with HDAC inhibitors effectively blocked the hypoxia-induced reduction of LIFR and phosphorylated-STAT3 (Fig. 2). Moreover, VPA markedly increased the phosphorylation of STAT3 under hypoxia to levels comparable to that of the control (normoxia). Therefore, these results suggest that HDAC inhibitors may maintain LIFR-STAT3 signaling under hypoxia.

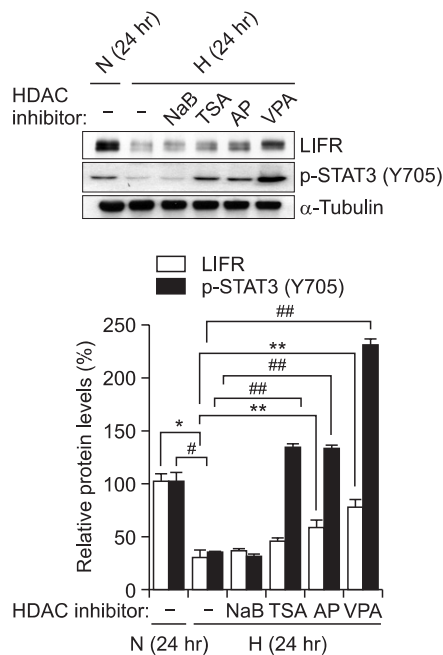


Fig. 2. HDAC inhibitors blocked the down-regulation of LIFR-STAT3 pathway under hypoxia in mESCs. CCE cells were treated with HDAC inhibitors, 1 mM sodium butyrate (SB), 5 ng/ml trichostatin A (TSA), 100 nM apicidin (AP), and 1 mM valproic acid (VPA), and were then immediately exposed to hypoxia for 24 h. Western blot analysis of LIF receptor (LIFR), phosphorylated-STAT3 (p-STAT3, at tyrosine 705 residue), and α -tubulin in CCE cells treated with HDAC inhibitors. α -Tubulin was used as internal control. Graph represents mean values \pm S.D. (n = 3). * p <0.05; ** p <0.01; # p <0.05; ## p <0.01.

Valproic acid (VPA) suppresses the downregulation of the LIFR-STAT3 pathway under hypoxia in mESCs in a dose-dependent manner

VPA was more effective than other HDAC inhibitors in the attenuation of the HIF-1 α and LIFR-STAT3 pathways (Fig. 1A and Fig. 2); in addition, VPA improves the reprogramming ef-

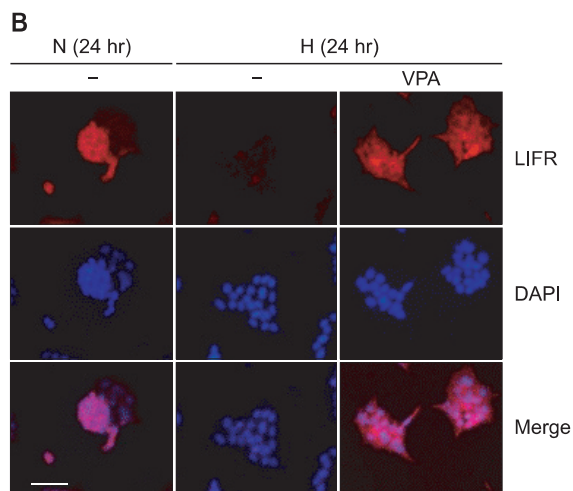
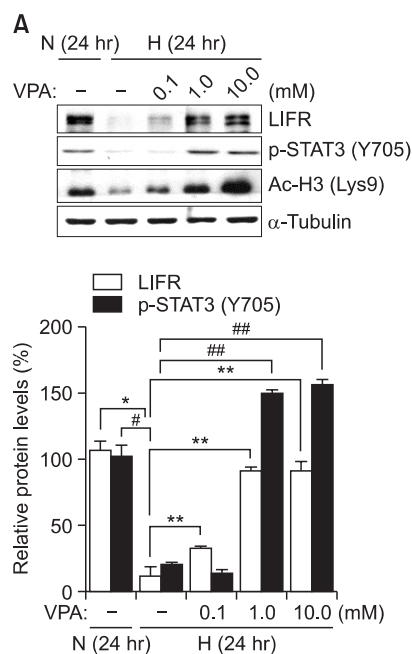


Fig. 3. Valproic acid (VPA), a HDAC inhibitor, suppressed the downregulation of the LIFR-STAT3 pathway under hypoxia in mouse embryonic stem cells in a dose-dependent manner. (A) CCE cells were treated with 0.1, 1.0, and 10.0 mM valproic acid (VPA), and were then exposed immediately to hypoxia for 24 h. Western blot analysis of LIF receptor (LIFR), phosphorylated-STAT3 (p-STAT3, at tyrosine 705 residue), lysine-9-acetylated histone 3 (Ac-H3), and α -tubulin in CCE cells treated with VPA. α -Tubulin was used as internal control. Graph represents mean values \pm S.D. ($n = 3$). * $p < 0.05$; ** $p < 0.01$; # $p < 0.05$; ## $p < 0.01$. (B) Immunofluorescent staining with LIFR (red) of cells treated for 24 h with 1 mM valproic acid (VPA) and grown under normoxic conditions (N), hypoxic conditions (H) in the presence of LIF. Nuclei are stained with DAPI (blue). Scale bar is 50 μ m.

iciency of somatic cells into induced pluripotent stem cells in both mouse and human cells (Huangfu *et al.*, 2008a; 2008b). Therefore, the role of VPA as a negative regulator of stem cell differentiation under hypoxia warrants further investigation. We treated mESCs cultivated in the presence of LIF under hypoxic conditions with 0.1, 1.0, and 10.0 mM of VPA. Expression of LIFR and phosphorylation of STAT3 was downregulated under hypoxia, whereas treatment with VPA effectively blocked the hypoxia-induced reduction of LIFR and phosphorylated-STAT3 in a dose-dependent manner (Fig. 3A). We further confirmed the effect of VPA on the hypoxia-induced differentiation of mESCs using immunofluorescence with LIFR. Undifferentiated mESCs cultured under normoxia showed abundant expression of LIFR in the plasma membrane, whereas the expression of LIFR decreased under hypoxia (Fig. 3B). Taken together, these results demonstrate that VPA inhibits the downregulation of the LIFR-STAT3 pathway under hypoxia in mESCs in a dose-dependent manner.

The acetylation levels of Histone H3 at lysine 9 (H3K9ac) are higher in mouse and human ESCs than in differentiated cells (Bártová *et al.*, 2008; Efroni *et al.*, 2008) and globally decrease during the endoderm-like differentiation of human ESCs (Krejci *et al.*, 2009). Thus, we examined the changes in H3K9ac during VPA treatment under hypoxia. The levels of H3K9ac were downregulated under hypoxia, whereas treatment with VPA effectively blocked the hypoxia-induced reduction of H3K9ac in a dose-dependent manner (Fig. 3A). These results suggest that VPA may maintain the acetylated state of histone H3, which is important for the self-renewal of mESCs under hypoxia.

Valproic acid (VPA) maintains the self-renewal and blocks the early differentiation of mESCs under hypoxia

Based on the effect of VPA on LIFR-STAT3, RT-PCR was conducted to assess the state of mESCs. Rex1 and Fgf4 are representative markers for mESC stemness and self-renewal activity, whereas Fgf5 and Stat5a are markers for early differentiation (Lee *et al.*, 2010). Expression levels of Rex1 and Fgf4 decreased under hypoxia; treatment with VPA blocked the suppression of the Rex1 and Fgf4 in a dose-dependent manner (Fig. 4A). In contrast, the expression levels of Fgf5 and Stat5a increased under hypoxia, and treatment with VPA blocked this increase (Fig. 4A). These results suggest that VPA maintains the self-renewal state and blocks the early differentiation of mESCs under hypoxia.

We confirmed the state of mESCs with alkaline phosphatase (AP), a widely used stem cell marker (Lee *et al.*, 2010). When fixed mESCs are stained with AP, undifferentiated cells appear brown or purple in compact colonies, and differentiated cells are colorless. AP staining showed many purple colonies under normoxic conditions and many colorless cells under hypoxia (Fig. 4B). However, AP-positive violet colonies markedly increased after treatment with VPA. As shown in Fig. 4B, when the data are normalized to the normoxic conditions, hypoxia decreased the AP activity ($\sim 25\%$ compared with normoxia) (Fig. 4B). But, undifferentiated colonies distinctly increased after treatment with VPA (about twofold compared with hypoxia). Thus, these results suggest that the inhibition of HIF-1 α by VPA may maintain AP activity under hypoxia.

Taken together, these results suggest that VPA may sustain the stemness of mESCs and suppress the early differentiation

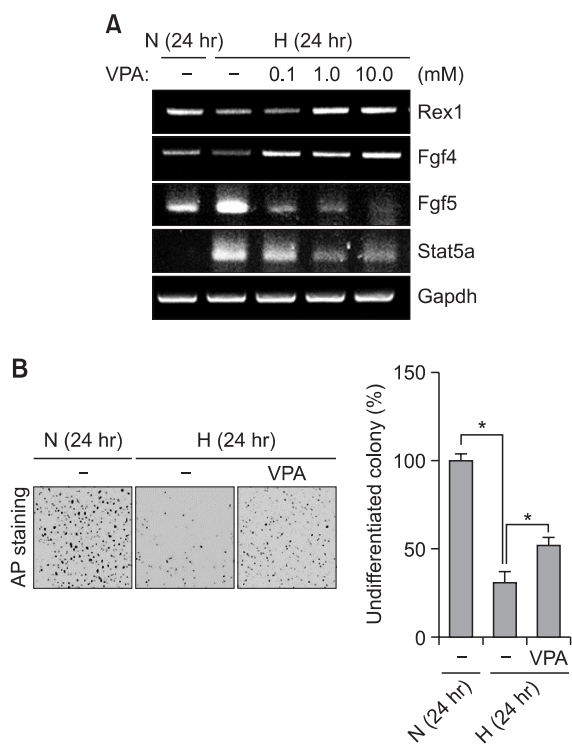


Fig. 4. Valproic acid (VPA), a HDAC inhibitor, maintained the self-renewal and blocked the early differentiation of mESCs under hypoxia. (A, B) CCE cells were grown under normoxic conditions (N), hypoxic conditions (H), and hypoxia and were treated for 24 h with 0.1, 1.0, and 10.0 mM valproic acid (VPA) in the presence of LIF. (A) The fate of mESCs was determined using self-renewal markers (rex1 and fgf4) and early differentiation markers (fgf5 and stat5a) expression levels using RT-PCR. Gapdh was used as an internal control. Results are representative of three independent experiments. (B) Undifferentiated mESCs were identified by alkaline phosphatase (AP) activity assay staining (blue). Undifferentiated colonies (%) were quantified by calculating the AP-positive colonies as described in 'Materials and Methods'. Graph represents mean values \pm S.D. (n = 4). * $p < 0.01$.

of mESCs under hypoxia.

DISCUSSION

The hypoxia-inducible factor-1 α (HIF-1 α) is an important transcription factor for many cellular responses to hypoxia that include angiogenesis, glycolysis, resistance to apoptosis, and differentiation (Maltepe *et al.*, 2005). Therefore, a specific regulator of HIF-1 α may be a useful agent to study many aspects of cellular regulation, particularly in cell-fate decisions (Simon and Keith, 2008). Here, we describe the negative role of VPA (a HDAC inhibitor) in hypoxia-induced differentiation of mESCs via the downregulation of HIF-1 α .

Among the various classes of HDAC enzymes, class I and II HDACs deacetylate and thus stabilize HIF-1 α protein under hypoxia (Kim *et al.*, 2007). In addition, HDAC inhibitors suppress the deacetylating activity of HDAC and induce acetylation of HIF-1 α , which leads to the destabilization of HIF-1 α (Kim *et al.*, 2007). More specifically, sodium butyrate (SB) is

an inhibitor of class I and class IIa HDACs; apicidin and valproic acid are specific inhibitors of class I HDACs; trichostatin A is potent pan-HDAC inhibitors (Kim *et al.*, 2007). In the present study, treatment of mESCs with HDAC inhibitors led to a marked reduction of HIF-1 α protein levels under hypoxia (Fig. 1A). Therefore, the inhibitory effect of HDACs on the downregulation of HIF-1 α in mESCs may be mediated by the inhibition of class I and/or II HDACs.

The decrease in early differentiation markers expression was most dramatic in cells treated with VPA. A possible explanation for the inhibitory role of VPA in mESCs is based on the notion that HDAC inhibitors may suppress the hypoxia-induced differentiation of mESCs by promoting cellular responsiveness to LIF. Previous reports showed that FR901228, an inhibitor of histone deacetylases, increases the sensitivity to IL-6-type cytokines in different cell types, including normal fibroblasts, epithelial cells, macrophages, splenocytes, and various tumor cell lines (Blanchard *et al.*, 2002). This report is consistent with our data showing the effect of HDAC inhibitors in maintaining and enhancing the LIFR-STAT3 pathway under hypoxia (Fig. 2, 3), which suggests a suppressive role for HDAC inhibitors. However, the opposite role for HDAC inhibitors in differentiation has also been reported. For example, TSA promotes myocardial differentiation when added to 7-day-old embryonic bodies (Karamboulas *et al.*, 2006) and enhances neuronal differentiation when added to embryonic neural stem cells (Balasubramanian *et al.*, 2006) via transcriptional activation of restricted and specific genes (McCool *et al.*, 2007). In these experiments, the inhibitor dose and the duration of treatment were different from those of our experiments; note that the effect of HDACis varies depending on the dose, the duration of the treatment, and the differentiation state of the treated cells. More importantly, in these experiments, the differentiation of mESCs was induced in the absence of LIF and thus no IL-6-type cytokines exist whose sensitivities are increased by HDACis. In contrast, we cultured mESCs under hypoxia in the presence of LIF. Therefore, our results suggest that HDACis may increase the responsiveness to LIF under hypoxia, which leads to the suppression of the differentiation of ESCs.

In summary, our data indicate that VPA had a negative effect on HIF-1 α stability in mESCs under hypoxia. Furthermore, VPA prevented the attenuation of the LIFR-STAT3 pathway by partially increasing the responsiveness to LIF, which led to the inhibition of differentiation under hypoxia. Taken together, these results suggest that VPA suppresses the hypoxia-induced differentiation of mESCs and may be important for understanding the molecular mechanisms underlying the physiological events during mammalian embryogenesis.

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