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Histone acetylation regulates the expression of HoxD9 transcription factor in endothelial progenitor cells

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Abstract

The homeobox (Hox) genes encode transcription factors that are involved in the morphogenesis of body. Recent data showed that the HoxD transcription factors control the cardiovascular system development, by modulation of endothelial cell proliferation and differentiation. For our knowledge, the role of histone acetylation in expression of HoxD9 has not been studied to date; therefore, the aim of this study was to investigate the expression of HoxD9 in endothelial progenitor cells after treatment with valproic acid (VPA), a histone deacetylase inhibitor. Our results showed that VPA inhibits the histone deacetylases leaving chromatin in an acetylated state corresponding to a decondensate conformation. qRT-PCR and Western blot assays showed that the expression of HoxD9 in endothelial progenitor cells treated with VPA was increased at both gene and protein level, suggesting that acetylation regulates HoxD9 expression. Furthermore, flow cytometry analysis revealed that the expression of endothelial specific markers such as CD31, CD105, CD117 and VEGFR2 was decreased in the presence of acetylating agent, VPA. The capacity of endothelial progenitor cells to form vascular networks on Matrigel was also reduced in the presence of VPA. In conclusion, investigating the role of histone acetylation in the regulation of accessibility of transcription factors to genes involved in differentiation can contribute to understanding epigenetic mechanisms underlying the commitment of stem cells.

Keywords: differentiation, endothelial progenitor cells, histone acetylation, HoxD9, stem cells.

Introduction

The homeobox (Hox) genes encode transcription factors that are involved in the morphogenesis of body. In the mammalian genome, 39 genes were identified and classified into four groups (A–D). Each Hox gene is expressed in a subset that controls the formation of body plan of the embryo along the anterior-posterior axis, presents spatial and temporal colinearity and have specific effects in morphogenesis of tissues and organs. HoxD group form a cluster in chromosome two (2q24.1–q33.1) and comprises nine genes (HoxD 1, 3, 4, 8–13) [1, 2]. HoxD genes are involved in skeletal morphogenesis and hind limb development in particular. Targeted mutagenesis studies of HoxD genes in animal models have shown that these disturbances in gene expression alter the shape and size of skeletal elements [3]. Recent data showed that the HoxD proteins are involved also in the regulation of cardiovascular system, by modulation of endothelial cell differentiation and angiogenesis [4, 5]. Chromatin dynamics is a critical factor in achieving transcription and involve a number of mechanisms, such as methylation, histone modification, chromatin remodeling factors, varied histone incorporation and histone eviction that regulates the state of chromatin [6]. Histone acetylation is an epigenetic modification correlated with an open chromatin conformation that allows the expression of different genes such as genes involved in differentiation of stem cells to specific cell types [7]. The balance of acetylation and deacetylation is kept in control by the activity of HAT (histone acetyltransferase) and HDAC (histone deacetylase), enzymes/factors that play an important role in stem cells biology by regulating the stem cell proliferation and differentiation potential [8]. Human HDAC are grouped into four classes: Class I HDAC (1, 2, 3 and 8), with the possible exception of HDAC3, are predominantly located in the nucleus [9]. Class II HDACs (HDACs 4, 5, 6, 7, 9 and 10) are larger in size (120–150 kDa) compared to Class I HDACs, and are expressed in both the nucleus and cytoplasm [10]. HDAC11, which shares some but not sufficient homology to both Class I and II HDACs, is assigned to its own class, Class IV. All these classes have zinc containing catalytic domain, compared with Class III (sirtuins) that need NAD as a cofactor to fulfill their function [9, 10] Nonselective inhibitor of Class I and Class II HDACs, Valproic acid (VPA) promotes histone acetylation allowing the chromatin to adopt a relaxed structure facilitating the binding of ectopically expressed transcription factors or downstream secondary factors [11]. In this context, few studies have addressed the role of acetylation in function of Hox transcription factors; therefore, the aim of this study was to investigate the role of histone acetylation in expression of HoxD9 in endothelial progenitor cells (EPC), process that may be correlated with endothelial commitment of progenitor cells.

Materials and Methods

Isolation and differentiation of EPC from umbilical cord blood

The isolation and differentiation of EPC was done as previously describe [12]. Briefly, human umbilical cord blood (UCB) samples were collected at term delivery by

specialized staff at the "Gheorghe Polizu" Clinical Hospital, Bucharest, Romania with informed consent, according to EU and Romanian legislation on the collection and handling of human biological samples and personal data protection. UCB samples were serologically tested for the absence of HIV1/2, HBV, HCV and HTLV, and subjected to Histopaque (Sigma-Aldrich, Saint Louis, MO, USA) density gradient centrifugation (400 g, 30 minutes at room temperature) in order to obtain the mononuclear cells (MNC). After centrifugation, the MNC layer was harvested and washed twice in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). The MNC were plated on plastic dishes coated with Fibronectin (1 μg/cm^3, Biosciences, San Jose, CA, USA) in endothelial differentiation EBM2 medium (Lonza, Allendale, USA), supplemented with 15% FBS, 40 ng/mL VEGF, 100 μg/mL endothelial cell growth supplement, 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 50 μg/mL Neomycin (all purchased from Sigma-Aldrich). Cell cultures were maintained at 37^{0}C with 5% CO_2 and 21% O_2 in a humidified atmosphere. On day one after plating, the non-adherent cells were discarded and fresh medium was applied. To maintain optimal culture conditions, medium was changed twice a week.

Flow cytometry

Expression of endothelial progenitor cells (EPC) surface markers was assessed by flow cytometry (MoFlo FACS, Dako, Glostrup, Denmark) using 1×10^5 cells stained with fluorochrome-conjugated (Phycoerythrin, PE; Fluorescein isothiocyanate, FITC) antibodies against CD31 (PECAM-1), CD34, CD45, CD105, CD117, CD133 and VEGFR2 (Dako, Agilent Technologies, Denmark). Accutase-detached cells were washed in phosphate-buffered saline (PBS) and incubated for 30 minutes at 4^{0}C with either PE- or FITC-conjugated antibodies. For negative controls, the cells were stained with the corresponding isotype-matched IgGs (IgG1, IgG2a/b, Dako). Flow cytometry data was analyzed using the Summit 4.0 software (Dako).

Dil-Ac-LDL uptake assay

EPC were starved for 24 hours prior to 2% LPDS (Lipoprotein Deficient Serum, Millipore, Billerica, MA, USA) and then stimulated with 400 mg/mL Dil-AcLDL. The cells were washed with PBS and fixed with 2% PFA (paraformaldehyde) for 10 minutes. Subsequently, another two washes were performed with PBS and two with water then Nile Red dye (1:100) was added and incubated in the dark for six minutes. Two washes with PBS followed and one with water and fluorescence microscopy photomicrographs were obtained using Eclipse TE300 (Nikon, Tokyo, Japan).

Matrigel vascular tubes assay

To evaluate the formation of blood vessel networks in Matrigel, cells were seeded into 96-well plates at a density of 3000 cells per well. Briefly, 50 μL of Matrigel (Sigma-Aldrich, St. Louis, MO, USA) was added in 96-well plates, and left to solidify for 30 minutes at 37^{0}C. After Matrigel polymerization, cell suspension was added and incubated for 4–6 hours. Tubes formation was observed using Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Digital Net Camera DN100).

Ulex europaeus lectin binding capacity to EPC

For the assessment of *Ulex europaeus* agglutinin (UEA) binding capacity to EPC, cells were incubated with 5 mg/mL FITC – *Ulex europaeus* lectin (Sigma-Aldrich, St. Louis, MO, USA) for one hour, followed by a wash with PBS and fixed in 4% PFA for one hour. The nuclei were counterstained with DAPI (1 mg/mL). The photomicrographs were taken with a digital camera Digital Net Camera DN100 using Eclipse TE300 microscope (Nikon, Tokyo, Japan).

Fluorometric quantification of histone deacetylases

The level of histone deacetylases in endothelial progenitor cells was evaluated after treatment with deacetylating agent VPA. To establish the dose that significantly inhibits HDAC enzyme was used "Histone Deacetylase Assay Kit, Fluorimetric" (Sigma-Aldrich, St. Louis, MO, USA). Histone deacetylase quantification was performed following manufacturer's protocol: 50 μL "HDAC substrate solution" was added on 15 μL of EPC protein extract after treating the cells with different concentrations of VPA, and incubated for 30 minutes. Finally, 10 μL of developing solution was added and incubated another 10 minutes. Fluorescence was measured using TECAN reader (Infinite 200 PRO TECAN, Männedorf, Switzerland) at 350–380 nm excitations and 440–480 nm emissions wavelength.

Real time RT-PCR

To assess the gene expression level of HoxD9 in EPC stimulated with 3 mM VPA, total cellular RNA was isolated from cultured EPC using GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and reverse-transcription reaction was performed using M-MLV polymerase (Invitrogen, Carlsbad, CA, USA). The mRNA levels were quantified by amplification of cDNA using a real-time thermocycler (StepOne Plus, Applied Biosystem, USA) and SYBR Green I chemistry. Oligonucleotide primers were as follows: HoxD9 forward: 5'-CAGCAGCAACTTGACCCAAAC-3' and reverse 5'-TCCAGCTCTAGCGTCTGGTAT-3'; GAPDH forward 5'-TTGGTATCGTGGAAGGAC TCA-3' and reverse 5'-TGTCATCATATTTGGCAGGTTT-3'. PCR reaction had the following schedule: denaturation step at 95^{0}C for two minutes, 40 cycles of denaturation at 95^{0}C, 15 seconds and annealing at 60^{0}C, 40 seconds. The gene expression level of HoxD9 was normalized to GAPDH. The relative quantification was done using the comparative CT method and expressed as arbitrary units.

Immunoblot

Cell lysate preparation and Western blot analysis were done as described previously by Lupu *et al.* (2011)

[12]. Briefly, cultured cells were washed twice in PBS before lysis in 2× Laemmli's electrophoresis sample buffer (Sigma-Aldrich, St. Louis, MO, USA) and boiled for 20 minutes. Protein concentration was determined by the Amido Black method. Equal amounts of protein (50 µg) were run on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes using semi-dry system (Bioard, CA, USA). The membranes were exposed to blocking reagent TBS Blotto A (Santa Cruz, Dallas, Texas, USA), and then incubated overnight at 4^0C with the primary antibodies against HoxD9 (rabbit polyclonal, 2 µg/mL, Sigma-Aldrich, USA) followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were detected using chemiluminescence substrate solution (Pierce, Thermo Scientifc, Rockford, USA) and images were taken with a digital detection system (ImageQuant LAS 4000, Fujifilm). Quantification (TotalLabTM) of HoxD9 protein was done by normalization to β-Actin protein and expressed as arbitrary units.

Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA test; $p<0.05$ was considered statistically significant.

Results

Isolation and characterization of endothelial progenitor cells

The cells isolated from umbilical cord blood started developing after approximately two weeks culture in endothelial differentiation medium supplemented with 40 ng/mL VEGF, and 100 µg/mL endothelial cell growth supplement. These cells exhibited an epithelial-like phenotype, forming colonies of adherent cells with a cobblestone shape (Figure 1a). Flow cytometry analysis showed an endothelial phenotype, umbilical cord blood derived cells being positive for CD31, CD34, CD105, CD117 and VEGFR2 surface markers (Figure 2). Furthermore, these cells showed the ability to uptake the Dil-AcLDL as demonstrated by the presence of LDL droplets in the cytoplasm of the cells (Figure 1b). Other characteristic of EPC is the formation of vascular tubes on Matrigel basement membrane matrix and binding to *Ulex europaeus* lectin (Figure 1, c and d).

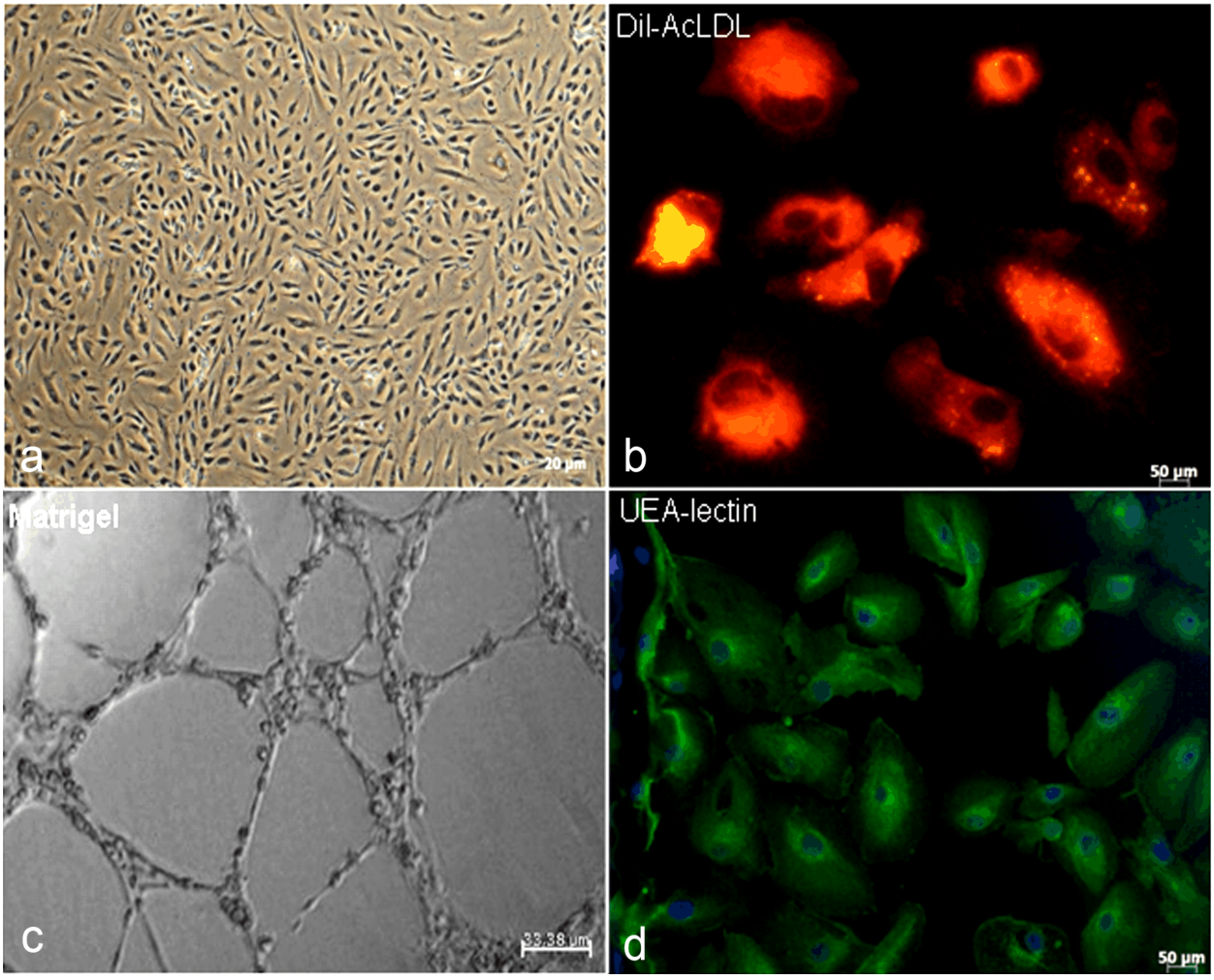

Figure 1 – ***Functional analysis of endothelial progenitors cells isolated from umbilical cord blood: (a) Epithelial-like morphology of adherent cells derived from mononuclear cells under endothelial growth factors stimulation; (b) Uptake of Dil-AcLDL observed by the presence of lipid droplets stained with Nile Red; (c) Capacity of EPC to form capillary-like tubes in Matrigel basement membrane matrix; (d) EPC ability to bind FITC-Ulex europaeus lectin.***

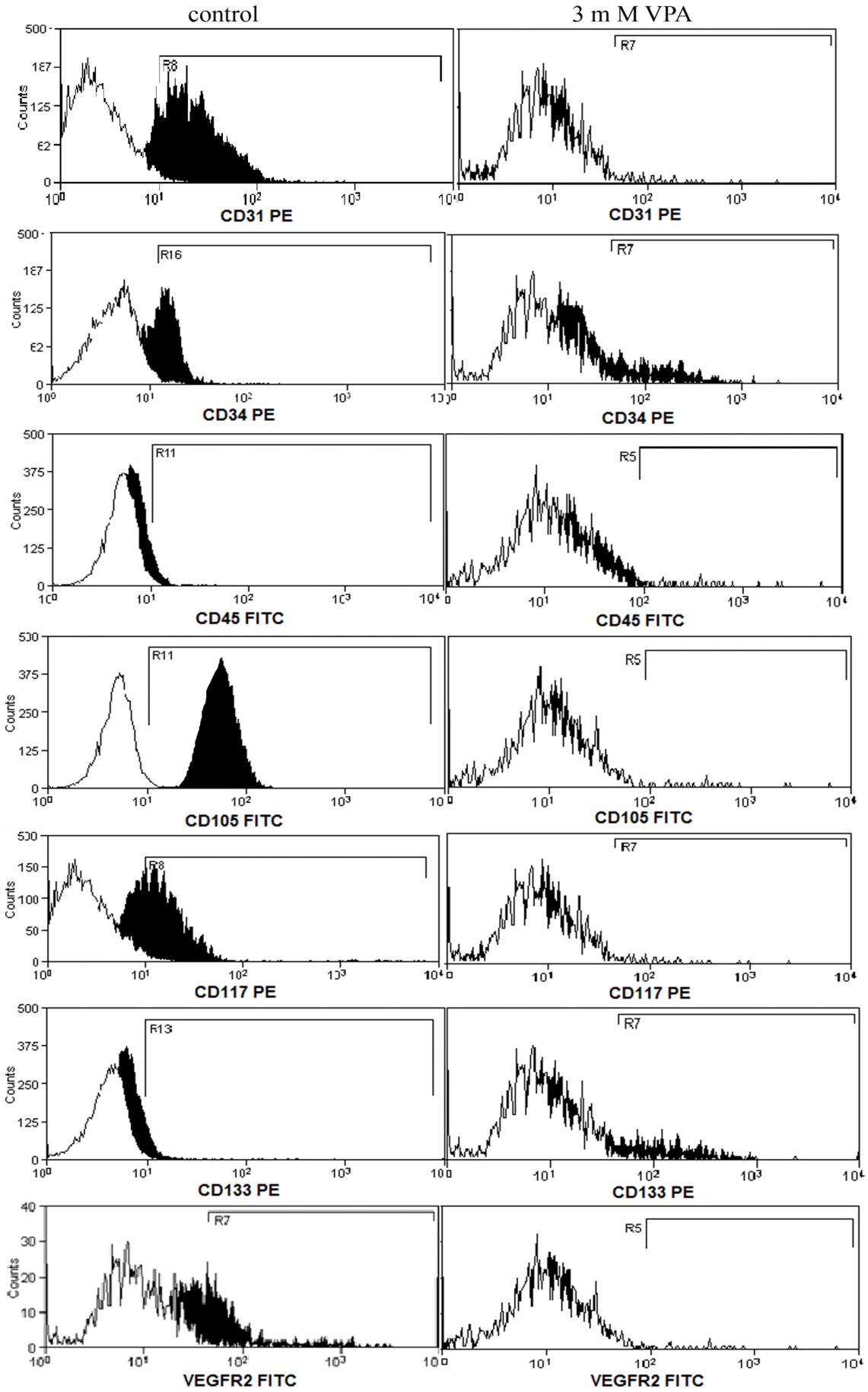

Figure 2 – ***Flow cytometry analysis of cells isolate from umbilical cord blood showed an immunophenotype positive for CD31, CD34, CD105, CD117 and VEGFR2. In the presence of VPA (3 mM), the expression of endothelial markers CD31, CD34, CD105 and CD117 is reduced.***

VPA downregulates the expression of HoxD9 in endothelial progenitor cells

Our results showed that HDAC inhibitor, VPA induced the histone acetylation in endothelial progenitor cells. The activity of HDAC enzymes was decreased in a VPA concentration dependent manner (Figure 3), leaving chromatin in a decondensated state. The activity of HDAC enzymes was decreased with up to 52% in the presence of 3 mM VPA (Figure 3).

Gene expression analysis showed that 3 mM VPA up-regulates the expression of HoxD9, qRT-PCR data indicated that the expression of HoxD9 was increased significantly in EPC, 0.8-fold up-regulation compared to control (Figure 4a).

Furthermore, protein expression of HoxD9 was also increased, Western blot analysis showing that VPA stimulated the expression in a concentration dependent manner (Figure 4b).

After 48 hours treatment with 3 mM acetylating agent VPA, flow cytometry analysis showed that the expression of specific endothelial markers such as CD31, CD34, CD105, CD117 and VEGFR2, was decreased significantly (Figure 2).

Acetylation reduce the ability of EPC to form vascular networks *in vitro*

An essential characteristic of EPC is to form the vascular networks when are culture in collagen matrix such as Matrigel, suggesting the angiogenic potential.

Matrigel assay is most widely used to investigate the normal and pathological angiogenesis, by counting the number of capillary-like structures formed by different types of endothelial cells.

Evaluation of capillary-like structures formation on Matrigel basement membrane matrix in the presence of different concentration of VPA was performed by counting the number of tubes formation in both control and VPA treated EPC.

Were counted and analyzed six fields for each condition, and all experiments (n=6) were performed in triplicate and generated similar morphological features.

In the presence of VPA, the ability to form vascular networks was significantly reduced, 3 mM VPA decreased with up to 74% the formation of vascular networks compared with control (Figure 5) suggesting that acetylation regulates properties of EPC probably by a mechanism that involved Hox transcription factors [13].

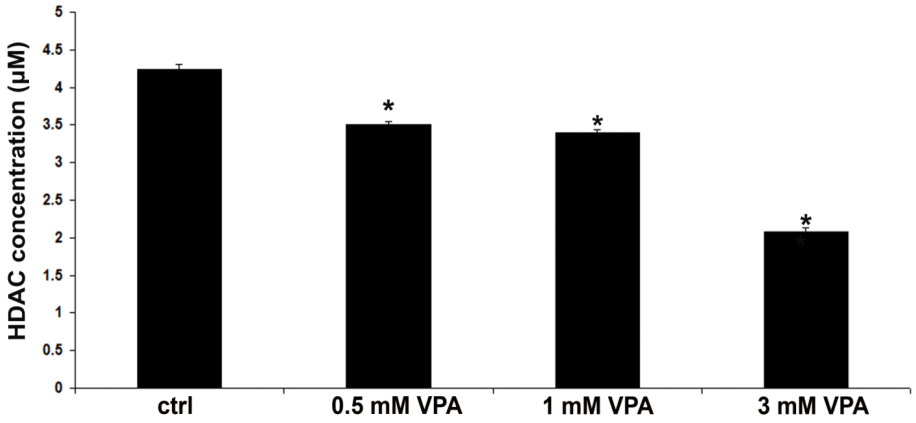

Figure 3 – ***The HDAC activity in the presence of different concentration of VPA is significantly reduced. Results are represented as mean ± standard error, n=3, *p<0.05.***

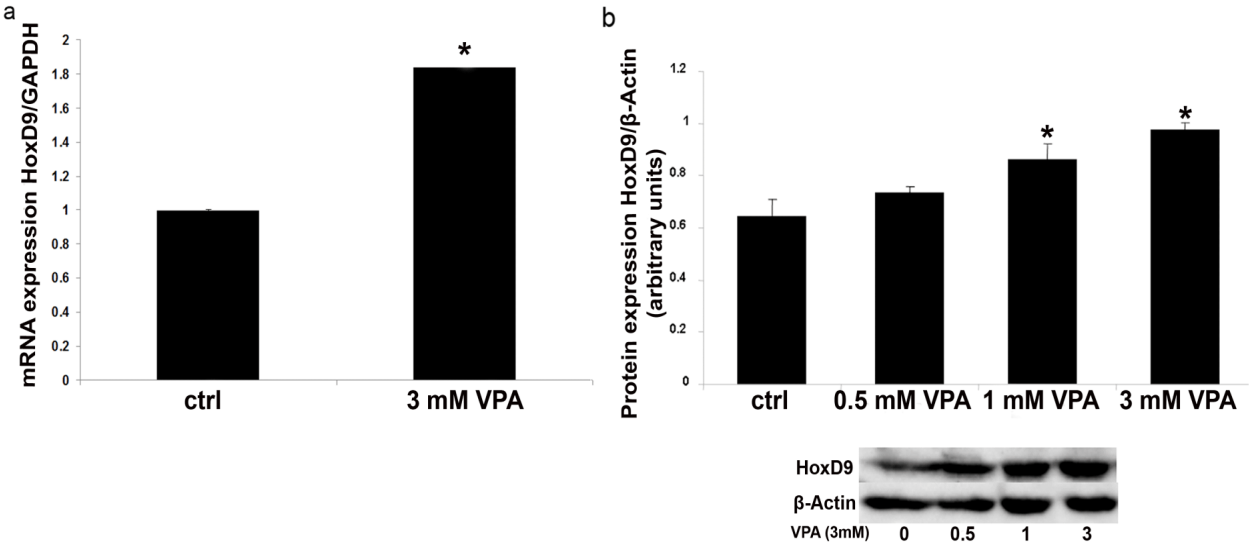

Figure 4 – ***(a) HoxD9 mRNA expression in EPC after 48 h of stimulation with 3 mM VPA. The expression of HoxD9 is significantly increased in the presence of HDAC inhibitor VPA. (b) Western-blot analysis of HoxD9 in the presence of different concentration of VPA shows an increase of protein expression dependent of VPA concentration. Results are represented as mean ± standard error, n=3, *p<0.05.***

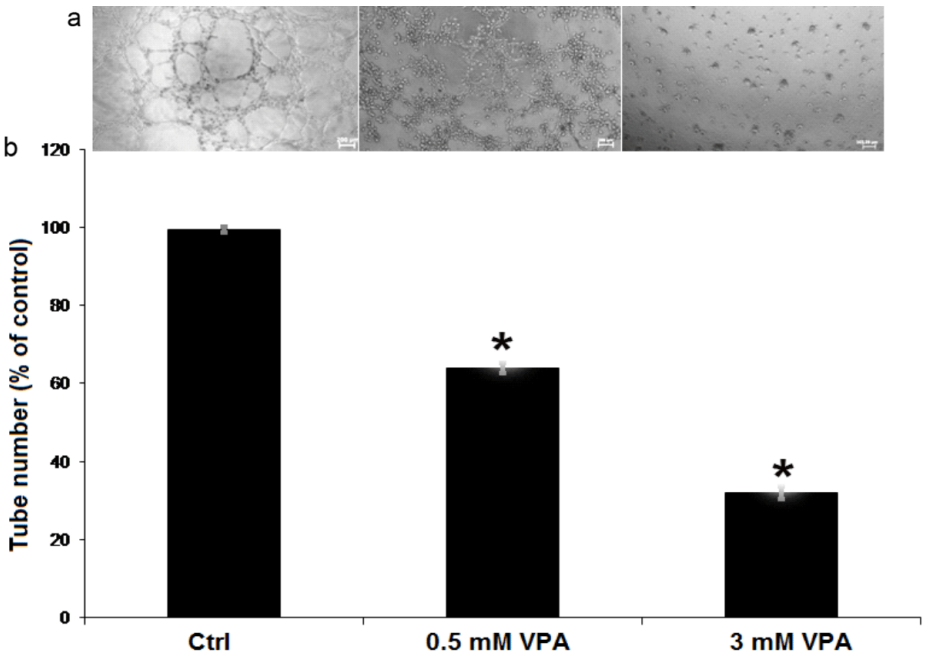

Figure 5 – ***Evaluation of capillary-like structures formation on Matrigel basement membrane matrix in the presence of VPA (a). The number of capillary-like networks was determined using Axio-Vision 4.8 software (b). Note that in the presence of 3 mM VPA EPC was not capable to form capillary-like networks. Results are represented as mean ± standard error, n=6, *p<0.05.***

Discussion

Since their initial discovery, EPC have raised great enthusiasm given their therapeutic promises in a variety of disorders including cardiovascular diseases, burn wounds, diabetes, atherosclerosis [14]. EPC were also isolated from the bone marrow, peripheral blood, contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels or by secreting factors that improve tissue function [15, 16]. Epigenetic modifications control the differentiation mechanisms by making the chromatin accessible for transcription in certain regions, allowing the necessary spatial and temporal control of genes. The histone acetylation level is regulated by the activity of HAT and HDAC enzymes during embryonic development and cellular differentiation [17]. Hox genes act at many levels within development: they are effectors genes that regulate the formation of the tissues and organs and act as executive genes regulating other genes that in turn control large networks of other genes. Hox genes promote cell division, cell adhesion, apoptosis, and cell migration, processes that are required for differentiation of precursor cells into their terminal specialized cells [18, 19]. HoxD8, HoxD9, HoxD10 and HoxD12 proteins are functionally equivalent in inducing cell cycle arrest and neuronal differentiation of BE(2)-C cells. HoxD3 is able to induce cell cycle arrest and morphological differentiation but fails to upregulate the neuronal marker NEFM and HoxD4, HoxD11 and HoxD13 [20]. Furthermore, recent data showed that HoxD9 gene regulate muscle cell growth, proliferation, differentiation and innervations, abnormalities in this gene may causes muscle denervation, resulting in muscular dystrophy. HoxD9 gene is also involved in differentiation of mesenchymal cells into cartilage and new bone *via BMP* gene family [13]. The expression of HoxD *in vivo* on the chick chorioallantoic membrane retained endothelial cells in an invasive state and prevented vessel maturation leading to vascular malformations and endotheliomas. Therefore, HoxD regulates endothelial cells gene expression associated with the invasive stage of angiogenesis [21, 22].

Another mechanism by which HoxD gene mediates conversion of endothelium from the resting to the angiogenic/invasive state is by interacting with basic fibroblast growth factor (bFGF) urokinase plasminogen activator (uPA) and integrin *ab*3. In the chick chorioallantoic membrane model, the sustained expression of HoxD3 retained endothelial cells in invasive state and prevented vessel maturation leading to vascular malformations and endotheliomas [21]. The expression of HoxD genes is temporal regulated, the expression of HoxD10 is maximal at three days after stimulation with angiogenic factors, whereas the expression of HoxD3 increased after three days, indicate that differentiation and maturation of endothelial cells is accompany by the changes in Hox gene expression [23].

Conclusions

Discovering the role of histone acetylation in the regulation of accessibility of transcription factors to genes involved in differentiation can contribute to understanding epigenetic mechanisms underlying the commitment of stem cells.

Conflict of interests

The authors declare that they have no conflict of interests or competing financial interests.

Acknowledgments

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