A histone deacetylase 7-derived 7 amino acid peptide acts as a phosphorylation carrier

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Abstract

Histone deacetylase 7 (HDAC7) belongs to the class II HDAC family and plays a pivotal role in the maintenance of endothelium integrity. There are 8 splicing variants in mouse HDAC7 mRNAs. Within the 5' terminal non-coding area of some variants, there exist some short open reading frames (sORFs). Whether these sORFs can be translated and whether the resulting peptides play roles in cellular physiology remain unclear. In this study, we demonstrated that one sORF encoding a 7-amino-acid (7-aa) peptide could be translated in vascular progenitor cells (VPCs). Importantly, this 7-aa peptide (7A) could transfer the phosphate group from the phosphorylated Ser393 site of MEKK1 to the Thr145 site of 14-3-3γ protein. The phosphorylated 7A (7Ap) could then directly phosphorylate 14-3-3γ protein in a cell-free, in-gel buffer system. The adjacent histidine and proline residues are essential for the phosphate group reception and transfer. In vitro functional analyses revealed that 7A and 7Ap increased VPC self-renewal and migration and enhanced vascular endothelial growth factor (VEGF)-induced VPC migration and differentiation toward the endothelial cell (EC) lineage, in which MEKK1 and 14-3-3γ served as the upstream kinase and the downstream effector, respectively. Knockdown of either MEKK1 or 14-3-3γ attenuated VEGF-induced VPC migration and differentiation. Exogenous 7Ap could rescue the effect of VEGF on the MEKK1 siRNA-transfected VPCs but not on the 14-3-3γ siRNA-transfected VPCs. In vivo studies revealed that 7A, especially 7Ap, induced capillary vessel formation in Matrigel plug assays, increased re-endothelialization and suppressed neointima formation in the femoral artery injury model, and promoted foot blood perfusion recovery in the hindlimb ischemia model by increasing Sca1+ cell niche formation. These results indicate that the sORFs within the non-coding area can be translated and that 7A may play an important role in cellular processes, such as proliferation, migration and differentiation, by acting as a phosphorylation carrier.

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Declaration

I, Junyao Yang, confirm that the research included within this thesis is my own work. I have been involved in the design, planning and conduct of all the experiments and the thesis writing. This includes cell functional experiments, molecular biology methods, mouse model and analysis of the results.

Expert assistance was provided in some aspects of the project by the following colleagues form the Cardiovascular Division of King's College London.

Dr.. Yanhua Hu isolated vascular progenitor cells, help to perform the mouse model and prepare the sample from the ex-vivo experiment.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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Chapter1

Introduction

1.1 Stem cells and Adventitia progenitor cells

1.1.1 Stem cell

For over 30 years, research on stem cells has been identified as a promising area for regenerative medicine. Stem cells are a class of undeveloped cells that have the notable potential to develop into many specialized cell types, and have the ability of proliferation, self-renewal and regenerating tissues (Totey, Totey, Pal, & Pal, 2009; Tuch, 2006). The widely recognized definition of a stem cell distinguished from other cell types requires two important characteristics:

1. Self-renewal: they are undeveloped cells capable of renewing themselves through many cycles of cell division while maintaining the undifferentiated state.

2. Potency: they have the capacity to be induced to differentiate to tissue- or organ-specific cells with special functions under certain environment conditions, which requires stem cells to be either totipotent or pluripotent. Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism. Pluripotency refers to a stem cell that has the potential to differentiate into any of the three germ layers. At the beginning of human development, a sperm fertilizes an egg and the resulting fertilized egg creates a single totipotent cell, a zygote. In the first hours after fertilization, this zygote divides into identical totipotent cells. Approximately four days after fertilization, these totipotent cells begin to specialize and becomes pluripotent cells (Asch, Simerly, Ord, Ord, & Schatten, 1995; Mitalipov & Wolf, 2009).

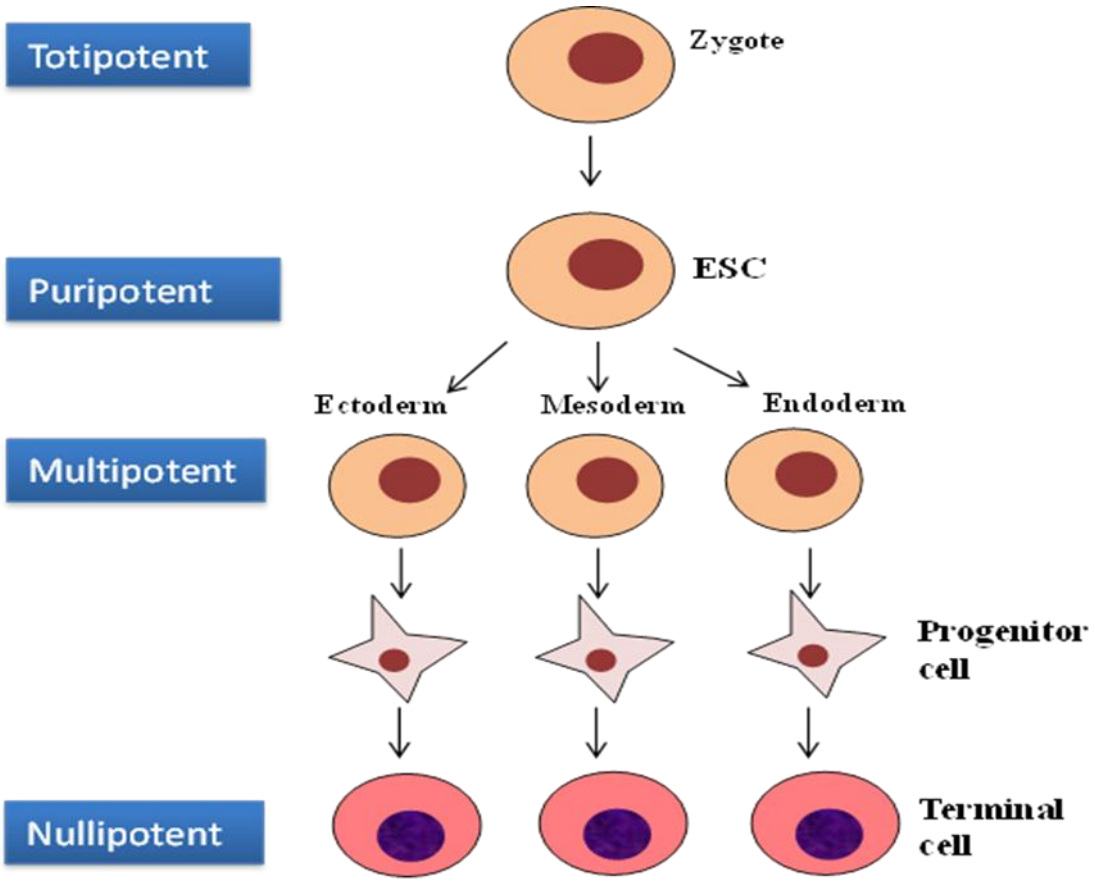


Figure 1.1 Differentiation of stem cells

Totipotent stem cells can differentiate into both embryonic and extraembryonic cell types. Such cells from an earlier stage of the embryo can construct a complete and viable organism. Pluripotent stem cells are the descendants of totipotent cells and can differentiate into nearly all cells derived from any of the three germ layers. Multipotent stem cells can differentiate into a number of cells, but only those of a closely related family of cells.

1.1.1.1 Embryonic stem cell

In mammals, parallel to former estimation, a rough classification classifies stem cells into embryonic stem cells (ESCs) and nonembryonic stem cell. ESCs derived from the inner cell mass of blastocyst stage embryos, are pluripotent stem cells with self-renewal ability and have an unlimited capacity to differentiate into all cell types. Many studies have successfully driven ESCs to differentiate into specific cell type lineages, such as endothelial cell (EC) and smooth muscle cells (SMCs) in various conditions. More established methods, including supplement ESC culture medium with VEGF for more than 21 days can induce EC-

differentiation (Xiao et al., 2006); while treatment of ESCs with PDGF-BB when cultivated on collagen-IV coated flasks will lead to SMC-differentiation (Xiao, Zeng, Zhang, Hu, & Xu, 2007). As SMCs and ECs are main cell types in the vascular wall, the ESC-derived smooth muscle cell (SMC) and endothelial cell (EC) will play crucial roles in treatment of cardiovascular diseases.

1.1.1.2 Adult stem cells

Expect embryonic development, stem cells also function as a sort of internal repairing system in many tissues, dividing essentially to replenish other cells throughout the life span of the organism. These cells are identified as adult stem cells, which have been found in different tissues and organs around the whole body. They can renew themselves and differentiate into specific lineages of cells found within the tissues they are located in. In some organs, stem cells regularly divide to repair and replace worn out or damaged tissues, such as the gut and bone marrow. In other organs, however, stem cells only divide under special conditions. Normally, when a stem cell divides, the new cell can remain a stem cell or become a differentiated cell with more specialized function, such as a heart cell. However, unlike ESC, which are known to be derived from blastocysts, the origins of some adult stem cells in specific tissues remain uncertain, though several adults stem cells have been identified such as hematopoietic stem cells and bone marrow stromal stem cells (Thomas, Lochte, Lu, & Ferrebee, 1957).

The microenvironment of the body which harbours the adult stem cells is called the "stem cell niche". A specific niche is thought to exist in many tissues and organs, such as: bone marrow, peripheral blood, heart, skin, brain and blood vessel wall (Birbrair & Frenette, 2016; Kopp, Hooper, Avecilla, & Rafii, 2009). Normally, adult stem cells are maintained in a quiescent state in the niche, but once they received stimulation signals from surrounding

injured tissues, the cells are activated to proliferate, migrate and differentiate to form new tissue (Scadden, 2006).

The advantages of adult stem cells are easy harvest and individual which will not be rejected by the immune system once transplanted back to the donor. In addition, they are possible to be stimulated in local tissue which would be more effective applying to therapy.

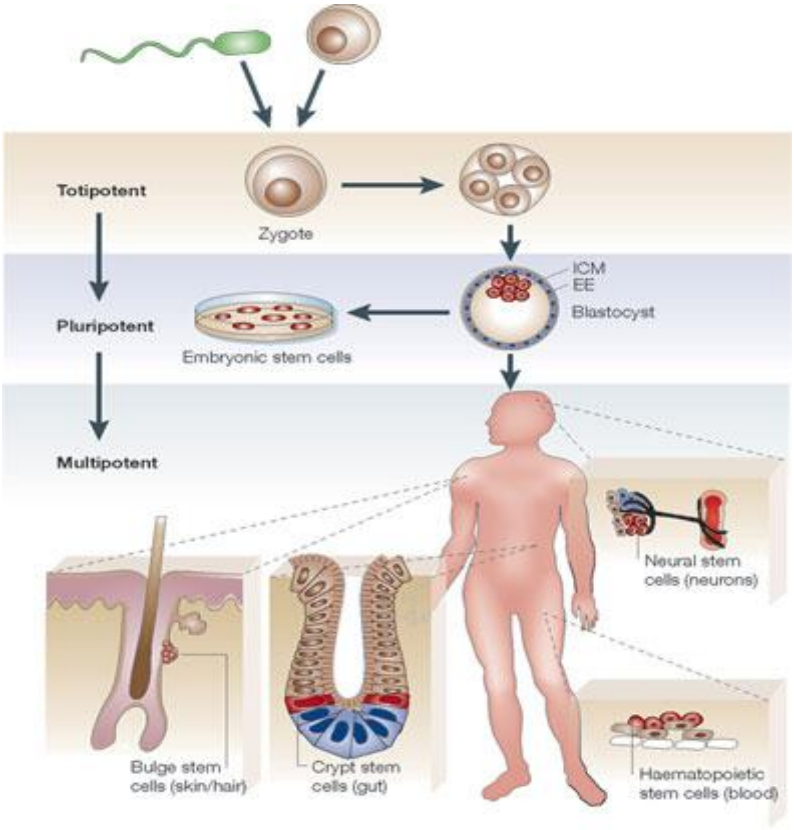


Figure 1.2 Development of human embryonic and adult stem cells.

Human ESCsare derived from the inner cells of a blastocyst which is the divided product of a zygote, a fusion of the egg and sperm. These ESCsare either self-renewing or pluripotent. Human adult stem cells reside in different adult tissues and they are also capable of self-renewal and multipotent differentiation. Whether differentiated cells are lineage restricted to the original organs of their origin or have potential to differentiate into other cell types remains uncertain (Eckfeldt, Mendenhall, & Verfaillie, 2005) .

1.1.2 Adventitia progenitor cells

1.1.2.1 Adventitia and its function

The adventitia, outermost connective tissue of most blood vessel walls, has been regarded as a network of connective tissue, including fibroblasts, perivascular nerves, and microvessels implanted in a collagen-rich extracellular matrix (ECM) (Majesky, Dong, Hoglund, Daum, & Mahoney, 2012). The vascular adventitia acts as a biological processing centre for the retrieval, integration, storage, and release of key regulators for the vessel wall function (Stenmark, Davie, Frid, Gerasimovskaya, & Das, 2006; Stenmark et al., 2013). It plays an important role in the blood vessel growth and repair and mediates cross talk between different cell types residing in the vessel wall.

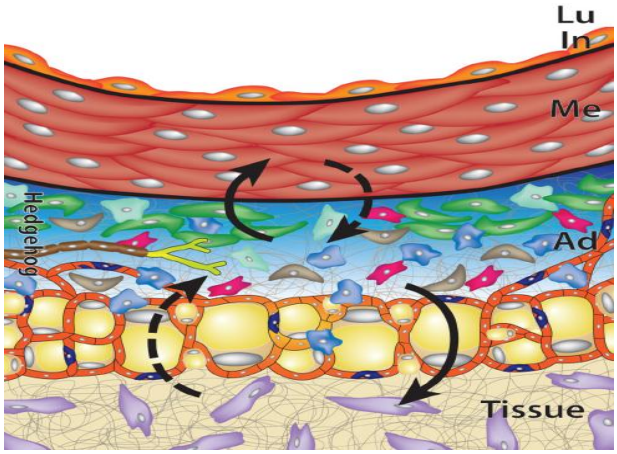


Figure 1.3 Cellular components of the adventitia of vessel wall.

The adventitia interacts with other layers of the vessel wall (top) and the surrounding tissues in which the vessel is located (bottom). Because of their specific loci, adventitial cells can participate in vessel formation, repair and disease processes. Lu, lumen; In, intima; Me, media; Ad, adventitia (Majesky, Dong, Hoglund, Mahoney, & Daum, 2011).

1.1.2.2 Adventitial progenitor cell

Adult blood vessels were believed to be quiescent, and not thought to contribute to new blood vessel formation until 1971. Based on observations of tumour tissue vascularisation, Judah Folkman and colleagues hypothesised that new vessels were formed via angiogenesis - a process in which new vessels grow from pre-existing vessels (Folkman, 1971). In the last

decade, the majority of experimental studies postulate that stem/progenitor cells reside in two main zones within the vessel wall i.e. the sub-endothelial zone (micro-vessel wall) and the adventitia (medium and large blood vessels) (Alessandri et al., 2001; E. L. Ergun, Caglar, Bozkurt, & Ergun, 2008; Pasquinelli et al., 2007; Peault et al., 2007). Though there is still some debate, it is widely believed that new vessel formation is in part dependent on stem/progenitor cells that reside within the vascular wall (Hu, Davison, et al., 2002; Hu, Mayr, et al., 2002).

Adventitial vasa vasorum constitutes a complete vascular tree-like structure, including arterioles, capillaries, and veins (Langheinrich, Kampschulte, Buch, & Bohle, 2007). Recent studies suggest a more complex and dynamic picture of the adventitia which emphasizes critical roles played by interacting adventitial cell types in growth, inflammation, repair, and disease of the artery wall (Majesky et al., 2012). It is known that normal adventitia contains resident macrophages, mast cells, T cells, B cells, and dendritic cells (Galkina et al., 2006; Mayranpaa et al., 2009; Swedenborg, Mayranpaa, & Kovanen, 2011; Zhou et al., 2010). However, recently accumulating evidence shows that the adventitia contains resident vascular stem cells/progenitor cells (Campagnolo et al., 2010; Hoshino, Chiba, Nagai, Ishii, & Ochiai, 2008; Passman et al., 2008).

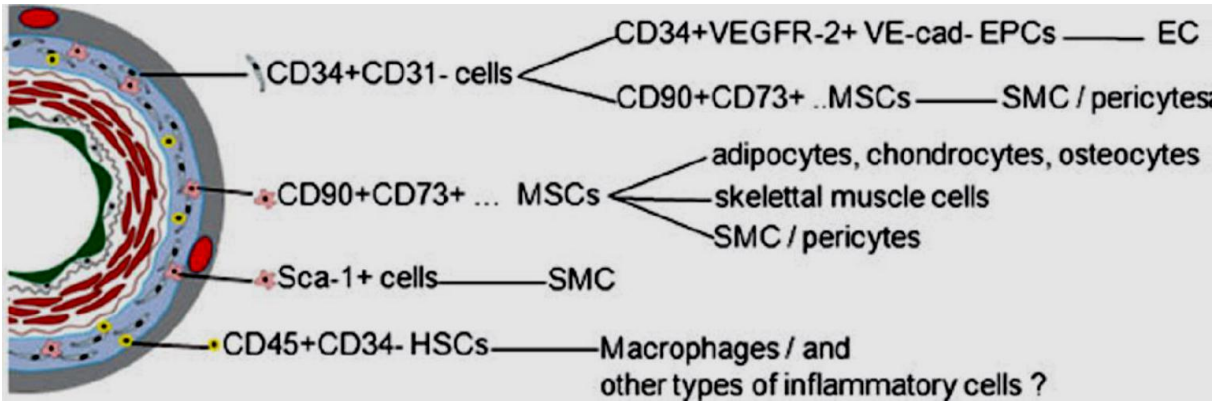


Figure 1.4 Subsets of vessel wall stem cells and their differentiation capacities.

Many studies have identified different populations of progenitor cells in the vascular adventitia using a variety of markers. These cells show a variety of differentiation capacities(S. Ergun et al., 2011).

As shown in Figure 1.4, CD34+ cells are found in the vasculogenic zone, which potentially includes two subsets: CD34+VEGFR-2+Tie+CD31- cells that differentiate into ECs and CD34-CD3-CD44+CD90+CD105+CD73 cells that differentiate into vascular SMCs. Additionally, vessel wall resident mesenchymal stem cells have also been identified within the native adventitial niche and can similarly differentiate into SMCs. Furthermore, adventitial Sca-1+ cells that reside within the vasculogenic zone from embryonic development to adulthood have the capacity to differentiate into SMCs *in vitro* under specific culture conditions. Finally, CD45+CD133+CD34-cells have been found to give rise to macrophages and other inflammatory cells.

Accumulating evidence indicates the existence of two types of circulating ECs. 5% of which are bone marrow–derived endothelial progenitor cells originated from hematopoietic stem cells(Lin, Weisdorf, Solovey, & Hebbel, 2000; Rafii, 2000), which are positive for CD34 and Flk-1 or vascular endothelial growth factor receptor 2 (VEGFR-2) (Crosby et al., 2000). The major proportion (>95%) of endothelial (progenitor) cells in blood originates from non–bone marrow tissues (Gunsilius et al., 2000; Lin et al., 2000). Hu et al reported that adventitia

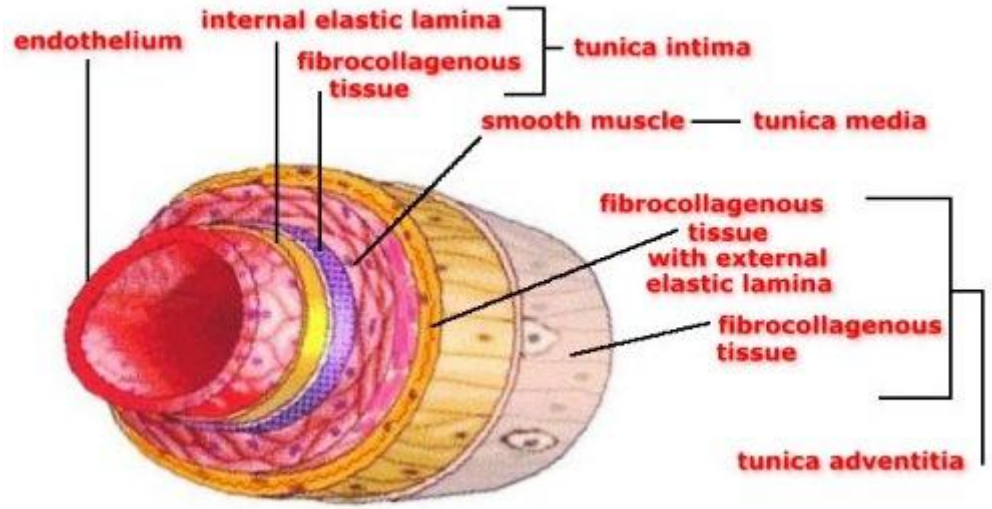
progenitors could differentiate to SMC and EC in response to VEGF stimulation, which suggest that adventitial Sca-1+ cells also have the potential to differentiate into both ECs and SMCs. They found that only the adventitia of the vessel wall contained large numbers of these marker-positive cells, and it was rare to see positive cells in the intima and media of the artery and heart tissues. Furthermore, these positive cells were more abundant in the region of the aortic root than in other areas of the adventitia. So, adventitial cells from aortic roots were cultivated using stem cell culture medium to prevent cell differentiation. Zorzi et al reported that rat thoracic aorta contained adventitial macrophage-like cells which were capable to proliferate, differentiate into macrophages and dendritic cells, and stimulate angiogenesis. A subset of these cells acquired an EC phenotype when cultured in the presence of VEGF and formed capillary-like vascular structures in a matrigel angiogenesis assay *in vitro* (Zorzi, Aplin, Smith, & Nicosia, 2010).

Under pathological conditions, the arterial adventitia undergoes remodelling in response to a variety of arterial injuries. The resident adventitial cells are often the first to be activated and reprogrammed in arterial wall remodelling, which may contain several pathological entities: the adventitial adaptive immune response predominates in chronic rejection; the adventitial sprouting of neovessels, leading to intraplaque haemorrhages, predominates in atherothrombosis; the adventitial fibrosis characterizes the response to mechanical stress the constrictive remodelling of arterial segments and initiating interstitial fibrosis in perivascular tissues (Michel et al., 2007).

In summary, these findings suggest that the adventitia maintains multiple types of progenitor cells that appear to act in concert as part of a coordinated healing response to vascular injury. The progenitor cells may also contribute to vascular disease development.

1.2 Endothelial cell

The maintenance of blood fluidity is controlled by the inner lining of the circulatory system—the endothelium, a thin layer of cells, which lines the interior surface of blood vessels and lymphatic vessels (Risau, 1997). The cells that form the endothelium are called ECs, which can be divided into lymphatic ECs contacting with lymph, and vascular ECs contacting with blood. Due to their position at the interface between blood and tissues, vascular ECs control blood fluidity and continue tissue perfusion, at the same time, they direct inflammatory cells to areas in need of defence or repair (van Hinsbergh, 2012).



(http://biology4isc.weebly.com/4-circulatory-system.html)

Figure 1.5 Structure of a blood vessel wall.

There are three layers of arteries and veins: Tunica intima (the thinnest layer), which has flat ECs lined by elastic membrane; Tunica media (the thickest layer in arteries), which formed by a thick coat of SMCs; Tunica adventitia: (the thickest layer in veins), which is made up of loosely arranged elastic and collagen fibres.

1.2.1 Endothelial cell in angiogenesis

ECs form cell-cell junctions that modulate cell signalling, functioning as selectively permeable barriers between blood and tissues. They also play an essential role in angiogenesis. In a healthy adult, quiescent ECs form a monolayer of phalanx cells, interconnected by junctional molecules such as claudins and vascular endothelial cadherin

(VE-cadherin or CD144). VE-cadherin makes adherens junction forming between ECs and regulates angiogenesis by controlling ECs adhesion, migration, proliferation, and survival via interactions with VEGF receptors. VEGF is involved in new vessel formation during embryogenesis and in proliferative diseases in adults by inducing differentiation in vascular structures and ECs proliferation.

In angiogenesis, ECs proliferate, migrate, and coalesce to form primitive vascular network, in response to angiogenic stimuli，which needs recruitment of SMCs to become mature blood vessels. Then ECs resume their quiescent phalanx state, and signals such as platelet-derived growth factor B (PDGF-B), transforming growth factor-β (TGF-β), ephrin-B2 and NOTCH cause the cells to become covered by pericytes and protease inhibitors induce the junctions re-established to ensure optimal flow distribution, which makes a mature vessel stable and functional (Herbert & Stainier, 2011). For the de novo formation of embryonic blood vessels, ECs undergo specification to either arterial or venous fate immediately following original vascular network created, which includes the differentiation, migration and coalescence of mesoderm-derived endothelial progenitors (angioblasts). During embryogenesis, ECs plant into nascent organs to give inductive signals to promote organogenesis, even in the absence of blood flow. This indicates that ECs not only form passive conduits for delivering but also establish organ-specific vascular niches, which stimulate organogenesis by releasing paracrine-tropic 'angiocrine' factors (Peter Carmeliet & Jain, 2011; Rocha & Adams, 2009).

1.2.2 Endothelial cell injury and repair

EC injury, resulting in endothelial dysfunction or the loss of proper endothelial function, is often regarded as a hallmark in the development of cardiovascular diseases. ECs injury would cause a complex cascade of events, such as endothelial denudation, platelet activation, growth factor release and further neointima formation (Tesfamariam, 2008). The factors

cause ECs injury are various under different status. *In vivo*, the major risk factor for ECs injury is shear stress that activates the endothelium and induces cell migration, proliferation and apoptosis, resulting in an increase in endothelium permeability, leukocyte and monocyte adhesion (Hahn & Schwartz, 2009). Post angioplasty, ECs are injured and activated by the implanted stent, which induces leukocytes adhesion by increasing expression of P-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1(ICAM-1), tumor necrosis factor alpha (TNF-α) and other inflammatory cytokines. Besides, injured ECs are also associated with thrombosis through inhibition of thrombomodulin (Pober & Sessa, 2007) and dysfunction in maintenance of vascular homeostasis through reducing endothelial nitric oxide synthase (eNOS) activity but increasing reactive oxygen species, which further impairs ECs and continuously induces inflammatory signalling(Cai, 2005).

Re-endothelialisation is a self-repair process after injury to maintain the protective role of endothelium in the vessel wall, which includes proliferation and migration of adjacent intact ECs, resident vascular stem cells/progenitor cells and recruited stem cells from the bone marrow. The vasculogenic zone is located between the adventitia and the media, where cells identified as CD34+CD31- are described as progenitors with the ability to differentiate into ECs, to participate in blood vessel formation and progression of atherosclerosis (Hu & Xu, 2011). Blann et al has proved that resident vascular-derived ECs contribute to endothelium repairing much more effectively in response to damaging stimulation than bone marrow originating stem cells (Blann et al., 2005), which indicates that stimulating resident stem cell to participate into endothelium repairing have therapeutic potential in angiogenesis/ vasculogenesis.

In atherosclerosis, adventitial inflammation and angiogenesis are widespread, resident stem or progenitor cells performing different structural and functional behaviours in response to injury and atherosclerotic plaques (Gutterman, 1999). In normal conditions, progenitor cells

are involved in maintaining homeostasis of the vasculature, while under pathological status, progenitor cells may also participate in neointimal lesion formation and atherosclerosis. It is believed that the media and adjacent undamaged cells, such as mature ECs and SMCs can migrate into the intima to replace dead ECs (Ross, Glomset, & Harker, 1977). Multiple research groups have demonstrated that in porcine coronary arteries and canine and rat carotid arteries, the increase in adventitial cell proliferation and migration in response to injury contributes greatly to neointimal lesion formation (Holifield et al., 1996; Mason et al., 1999; Oparil et al., 1999; Scott et al., 1996; Shi et al., 1996). In addition, SMCs and ECs in atherosclerotic plaques are reported to be derived from local resident progenitor cells in the vessel wall rather than bone marrow-derived progenitor cells (Xu, 2004). A direct evidence for the ability of adventitial cell migration was provided by monitoring the movement of transplanted cells to the adventitia of the injured artery (Stenmark et al., 2013).

Recently, it became more and more attractive that stimulation of vessel wall resident progenitor cell or recruitment of bone-marrow-derived cells could repair adult injured vessels and the expansion of pathological vessels, as the progenitor cells would become incorporated into the endothelium in a process known as postnatal vasculogenesis (Schaper, 2009). What more important is that this provides a basis for repairing and therapy for vascular injury.

1.3 Histone deacetylase

The homeostasis of histone acetyltransferase (HAT) and histone deacetylase (HDAC) is known to play a central role in the regulation of gene expression, through the modulation of chromosome assembly/disassembly and affects transcription factors access to DNA (J. H. Lee, Hart, & Skalnik, 2004). Acetylation describes a reaction that introduces an acetyl group (resulting in an acetoxy group) into a compound, namely the substitution of an acetyl group for an active hydrogen atom. The reaction involving the replacement of the hydrogen atom of a hydroxyl group with an acetyl group (CH3 CO) yields a specific ester, the acetate. The opposite chemical reaction is called deacetylation – it is the removal of the acetyl group. Histone acetylation and deacetylation involve the addition or removal of an acetyl moiety from acetyl co-enzyme A to lysine residues in the N-terminal tail on the surface of the nucelosome core of histone proteins, which is essential in gene regulation (Wu, Suka, Carlson, & Grunstein, 2001; X. J. Yang & Seto, 2003). For acetylated and deacetylated histones are considered epigenetic tags within chromatin by relaxing (euchromatin) or tightening (heterochromatin) chromatin structure, subsequently increasing or decreasing gene transcription levels. Acetylation removes the positive charge on the histones, thereby decreasing the interaction of the N termini of histones with the negatively charged phosphate groups of DNA. As a consequence, the condensed chromatin is transformed into a more relaxed structure that is associated with greater levels of gene transcription. This relaxation can be reversed by HDAC activity (Verdone, Agricola, Caserta, & Di Mauro, 2006).

HDACs are a family of enzymes that remove acetyl groups from the N-acetylated lysine residues on histones (Berndsen & Denu, 2008). Accumulating evidence indicates that HDACs are part of transcriptional co-repressor complexes and play a fundamental role in transcriptional regulation, cell-cycle progression, cell proliferation, cell differentiation and

contribute to developmental events (Kato, Tamamizu-Kato, & Shibasaki, 2004; Sterner & Berger, 2000).

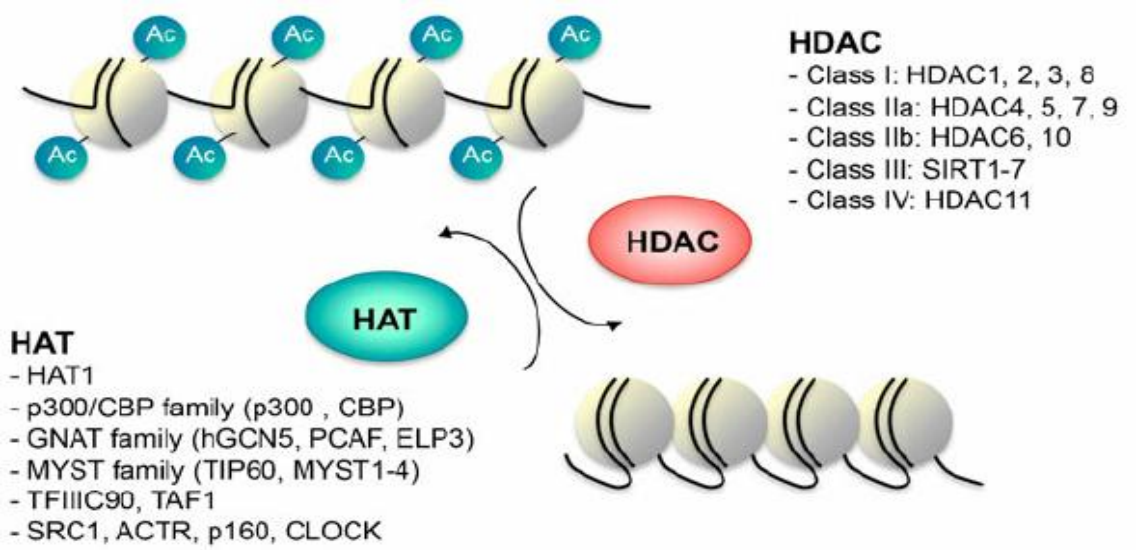


Figure 1.6 HAT and HDAC

HDACs repress transcription by deacetylation of the N-terminal tails of core-histions, which results in chromatin condensation. In contrast, HATs, such as p300 and PCAF antagonize the repressive activity of HDACs and acetylate the histone tails, nu relaxing chromatin and weakening the electrostatic interaction between positively charges histone tails and negatively charged DNA.

1.3.1 Members and characteristics of HDACS family

Mammalian genome encodes 18 HDACs, which can be grouped into four classes based on the homology with yeast HDACs (Thiagalingam et al., 2003). All members share a highly conserved deacetylase domain but differ in structure, subcellular localization and expression pattern, which results in different cellular functions (Dario Ummarino, 2013). Class I HDACs (homologous to yeast Rpd3) comprise of HDAC1, -2, -3 and -8, which are widely expressed in many human tissues and cell lines. Among them both HDAC3 and HDAC8 can shuttle between the cytosol and the nucleus, while HDAC1 and HDAC2 are exclusively located in nucleus (Longworth & Laimins, 2006; Waltregny et al., 2004). The deacetylation of histones to repress gene transcription is the main function of class I HDACs. Class I HDACs form large multi-subunit complex that associates with transcription factors and other chromatin

modifiers, except HDAC8 whose function is not clearly clarified yet (X. J. Yang & Seto, 2008). Recently, HDAC8 expression but enzyme activity was reported to mediate E-cadherin regulation(Choi et al., 2016). In mammals, HDAC1 and -2 can bind to each other forming the catalytic core of three different complexes: Sin3, nucleosome remodelling deacetylase and corepressor of RE1-silencing transcription factor. HDAC3 can form two different multi-protein complexes, nuclear receptor corepressor and silencing mediator of retinoic acid and thyroid hormone receptors. Class I HDACs are mainly found in such nuclear complexes. Furthermore, the association with several different proteins show an essential role in regulating their activity, which is highly pronounced in developmental processes that requires a global modulation of transcriptional programmes. With the exception of HDAC8, knockout of class I HDACs in mice have indeed resulted in embryonic lethality (Haberland, Montgomery, & Olson, 2009).

Class II HDACs can be divided into two sub-class groups. Class IIa members include HDAC4, -5, -7, -9, while class IIb comprises of HDAC6 and -10. Class II HDACs differ from Class I mainly for their tissue-specific expression pattern and the considerable lower deacetylase activity. Besides, their length (almost the double of Class I HDACs) and cellular localization are all different. Class II HDACs can shuttle between nucleus and cytoplasm, the activity of which is primarily regulated by its sub-cellular location in the cell, while phosphorylation by Ca^{2+} / CaMK (calmodulin-dependent kinase) at conserved serine residues promotes HDACs exporting them out of the nucleus (McKinsey, Zhang, & Olson, 2001). Mammalian class IIa HDACs (HDAC4, -5, -7, and -9) contain an N-terminal extension that interacts with other transcriptional cofactors and confers responsiveness to extracellular signals (Verdin, Dequiedt, & Kasler, 2003). They have conserved binding sites for the transcription factor myocyte enhancer factor 2 and the chaperone protein 14-3-3, which help HDACs shuttle from the nucleus to the cytoplasm. The nuclear-cytoplasm shuttling and

occurrence of post-translational modification such as phosphorylation, indicates their primary role as signal transducers in numerous tissues during development and disease (X. J. Yang & Gregoire, 2005). Class IIb family includes HDAC6 and HDAC10. HDAC10 presents a leucin-rich domain at the C-terminal. Recent reports showed HDAC10 might be up-regulated on human myeloma cell lines (Mithraprabhu, Kalff, Chow, Khong, & Spencer, 2014). HDAC6 is different from all other HDACs, as it harbours two deacetylase domains and a C-terminal zinc finger, being the important cytoplasmic deacetylase in mammalian cells (Guardiola & Yao, 2002). Cytoskeletal proteins such as α-tubulin and cortactin, transmembrane proteins such as the interferon -α receptor, and chaperones are all targets of HDAC6 (Dregan, Charlton, Wolfe, Gulliford, & Markus, 2014; Zhang et al., 2007). Class-II HDACs display distinct tissue specificity. For example, HDAC4 is abundant in skeletal muscle and brain，whereas mHDAC5 is enriched in heart and mHDAC6 is expressed highly in testis (Verdel & Khochbin, 1999). Furthermore, recent study showed that mHDAC7 is most abundant in heart and lung and low in skeletal muscle. These observations suggest that these HDACs are not redundant, but rather, have distinct physiological functions (Kao, Downes, Ordentlich, & Evans, 2000).

Class III HDACs comprise a group of protein called Sirtuins (SIRT1-7), which have been shown to promote cell survival by inhibiting apoptosis or cellular senescence in mammalian cells (Jung-Hynes & Ahmad, 2009). Although they possess a deacetylase domain, they diverge from classical HDACs as their enzymatic activity requires the cofactor NAD+. This feature suggests their involvement in metabolic functions (Longo & Kennedy, 2006). Among them, SIRT1 is the most well-characterized member and has been shown to be involved in a number of cellular processes, including gene silencing at telomere and mating loci, DNA repair, recombination, and aging (Longo & Kennedy, 2006).

HDAC11 is the latest discovered and the only member of Class IV HDACs, which has a catalytic domain at the N-terminal, indicating that HDAC11 may have a different more distinct function.(L. Gao, Cueto, Asselbergs, & Atadja, 2002). Recent study demonstrated that HDAC11, by interacting at the chromatin level with the IL-10 promoter, down-regulates IL-10 transcription in both murine and human APCs *in vitro* and *ex vivo* models (Chen et al., 2016; Villagra et al., 2009).

1.3.2 HDACs and EC differentiation

In response to vascular injury, ECs migration and proliferation contribute to the repairing of the damaged EC or denuded endothelium. Recent reports show that circulating or local resident stem/progenitor cell differentiation is also involved in this process (Rinkevich, Lindau, Ueno, Longaker, & Weissman, 2011). Through modulating chromatin structures and non-histone transcription factors, HDACs are involved in the gene expression reprograming in multiple biological processes such as cell-cycle, cell differentiation and survival (Sata et al., 2002) . Therefore, the involvement of HDACs in EC differentiation is expected.

The first evidence came from studies with HDAC inhibitors (HDACi). HDACi decreases endothelial lineage commitment of endothelial progenitor cells (Iordache, Buzila, Constantinescu, Andrei, & Maniu, 2012; Rossig et al., 2005). Rössig L et al found that the suppressive effect of HDACi on EC differentiation was mediated by the down-regulation of homeobox transcription factor HoxA9, which directs the transcription of EC markers such as eNOS, VEGFR-2 and VE-cadherin, suggesting that the HDAC-dependent activation of Hox-A9 is essential for EC differentiation(Rossig et al., 2005).

There is no direct evidence that HDAC1, 2 and 8 are involved in EC differentiation, although indirect evidence indicates that HDAC1 may suppress EC differentiation. Rajasingh J et al showed that HDACs inhibitor, trichostatin A, improved AceH3K9 and reduced HDAC1

expression in bone marrow progenitor cells, leading to differentiation into myocytes and ECs, which suggests that HDAC1 plays a suppressive role in bone marrow progenitor cell differentiation toward EC lineage (Rajasingh et al., 2011). Different from other members within class I HDACs, accumulating evidence suggests that HDAC3 possesses a pivotal function in stem cells differentiation into ECs, which is capable of repairing the damaged endothelium. VEGF is a well-known EC differentiation inducer. Xiao et al reported that VEGF up-regulated HDAC3 in ESC-derived Sca1+ cells. Over-expression of HDAC3 via adenoviral gene transfer increased, while trichostatin A or HDAC3 siRNA abolished VEGF-induced EC marker expression in Sca1+ cells, suggesting HDAC3 may function downstream of VEGF signal pathway (Xiao et al., 2006). Study from Illi B(Kajstura et al., 2005) et al demonstrated that laminar flow enhanced ESC-derived progenitor cell differentiation into EC lineage in an HDAC dependent manner. It was found that laminar flow stabilized and activated HDAC3 through the Flk-1-PI3K-Akt pathway in a ligand independent manner, which in turn deacetylated p53, leading to p21 activation, contributing to EC differentiation (L. Zeng et al., 2006). Similar mechanism is involved in VEGF-induced EC differentiation, in which HDAC3 modulates differentiation process via regulating non-histone proteins. Recent study found that unconventional splicing of HDAC3 might change HDAC3 function, inducing endothelial-to-mesenchymal transition (L. Zeng, Wang, et al., 2013). These reports suggest a critical role of HDAC3 in ECs fate determination.

Class II HDACs seem not directly involved in EC differentiation. Several groups tried to link class II HDACs with ECs differentiation, but no solid evidence has been obtained. In Spallotta F et al's reports, nitric oxide (NO) induced a cross-talk between class I HDACs (HDAC3) and class II HDACs (HDAC4 and 7), which might contribute to the neovascularization in ischemic tissue and skin repairing (Spallotta et al., 2013; Spallotta et al., 2010). However, the effect of NO on EC differentiation may be largely derived from

HDACs-mediated global hypoacetylation on pluripotency maintaining genes like Oct4, Nanog, KLF4, etc. Considering class II HDACs have only weak deacetylase activity, the histone hypoacetylation might be mainly caused by HDAC3 in the complex. Reports from other groups indicate that HDAC7 may participate in EC proliferation and cell-to-cell contact but is not involved in EC differentiation (S. Chang et al., 2006; Margariti et al., 2010). A recent report from Song Y et al (Song et al., 2013) showed that AMPK activation participated in endothelial colony forming cells differentiation. During this process, HDAC5 could be phosphorylated by AMPK. However, there is no direct evidence on the involvement of HDAC5.

1.3.3 HDAC7

HDAC7, as a member of the class II HDACs, is specifically expressed in the vascular endothelium during early embryogenesis where it maintains vascular integrity. Disruption of HDAC7 gene in mice results in embryonic lethality due to the rupture of blood vessels. In vitro study shows that HDAC7 is necessary for human umbilical vein ECs grown on Matrigel to form primitive vascular-like structures. Silencing of HDAC7 in ECs alters their morphology and motility and prevents their assembly into tube-like structures (S. Chang et al., 2006). There are still some conflicting reports concerning HDAC7 and EC proliferation (Margariti et al., 2010). Besides regulating angiogenesis, HDAC7 also play a role in apoptosis regulation. It is reported that mitochondrial and nuclear HDAC7 would redistribute to the cytoplasm upon induction of apoptotic cascade (Verdin, Dequiedt, & Kasler, 2004).

Like other members of Class II HDACs, HDAC7 also shuttles between cytoplasm and nucleus. The export of HDAC7 from nucleus seems to be regulated by phosphorylation via protein kinase D (PKD) signalling pathway. VEGF is reported to do this. The consequence is that HDAC7-mediated suppression of gene expression is removed, leading to EC

proliferation and migration (Wang et al., 2008). Though it is widely recognized that HDAC7 plays an essential role in ECs migration, the underlying mechanisms remain unclear. Some researches demonstrated that HDAC7 silencing inhibited EC migration and disturbed cell morphology via stimulating platelet-derived growth factor –B (PDGF-B) and its receptor PDGFR-β expression, partially responsible for the inhibition of EC migration (Mottet et al., 2007). Zeng's group previous studies have demonstrated that HDAC7 is involved in ß-catenin signalling and cell cycle regulation. HDAC7 forms a complex with ß-catenin and14-3-3 ε, ζ, η proteins, therefore modulating the cellular localization of ß -catenin, the expression of ß-catenin target genes and cell cycle–related genes. Overexpression of HDAC7 retains ß-catenin in the cytoplasm, whereas HDAC7 deficiency increases nuclear translocation of ß-catenin. Overexpression of HDAC7 can ablate VEGF-induced ß-catenin translocation and EC proliferation. These findings suggest that HDAC7 is crucial in the regulation of EC growth and proliferation (Haberland et al., 2009).

1.4 Alternative splicing and alternative translation

1.4.1 Alternative splicing

RNA splicing is a modification of the nascent pre-messenger RNA (pre-mRNA) transcript in which introns are removed and exons are joined. Splicing takes place within the nucleus after or concurrently with transcription in nuclear-encoded genes. All of the typical eukaryotic messenger RNA (mRNA) must undergo splicing before it can be used to produce a correct protein through translation. The mechanism of RNA splicing is complicated and is done in a series of reactions which are catalysed by the spliceosome, a complex of small nuclear ribonucleoproteins (snRNPs), but there are also self-splicing introns (Figure 1.7). In brief, the spliceosome recognizes the splicing signals on a pre-mRNA molecule, and brings the two ends of the intron together. The branch-point binding protein (BBP) and U2AF, a helper protein that recognizes the branch-point site that is displaced by the U2 snRNP, forms base pairs with the branch-point site consensus sequence, while the U1 snRNP forms base-pairs with the 5' splice junction. At this point, the U4/U6-U5 "triple" snRNP enters the spliceosome. In this complex of snRNP, the U4 and U6 snRNAs are held firmly together by base-pair interactions but the U5 snRNP is more loosely associated. Then, U6 snRNP displaces U1 at the 5' splice junction through several RNA-RNA rearrangements and break apart of the U4/U6 base pairs. Thus, there is a type of RNA-RNA rearrangement in which the formation of one RNA-RNA interaction requires the disruption of another. Finally, accuracy of splicing is increased by checking and rechecking the RNA sequences before the chemical reaction is allowed to proceed (Figure 1.7) (Rogers & Wall, 1980).

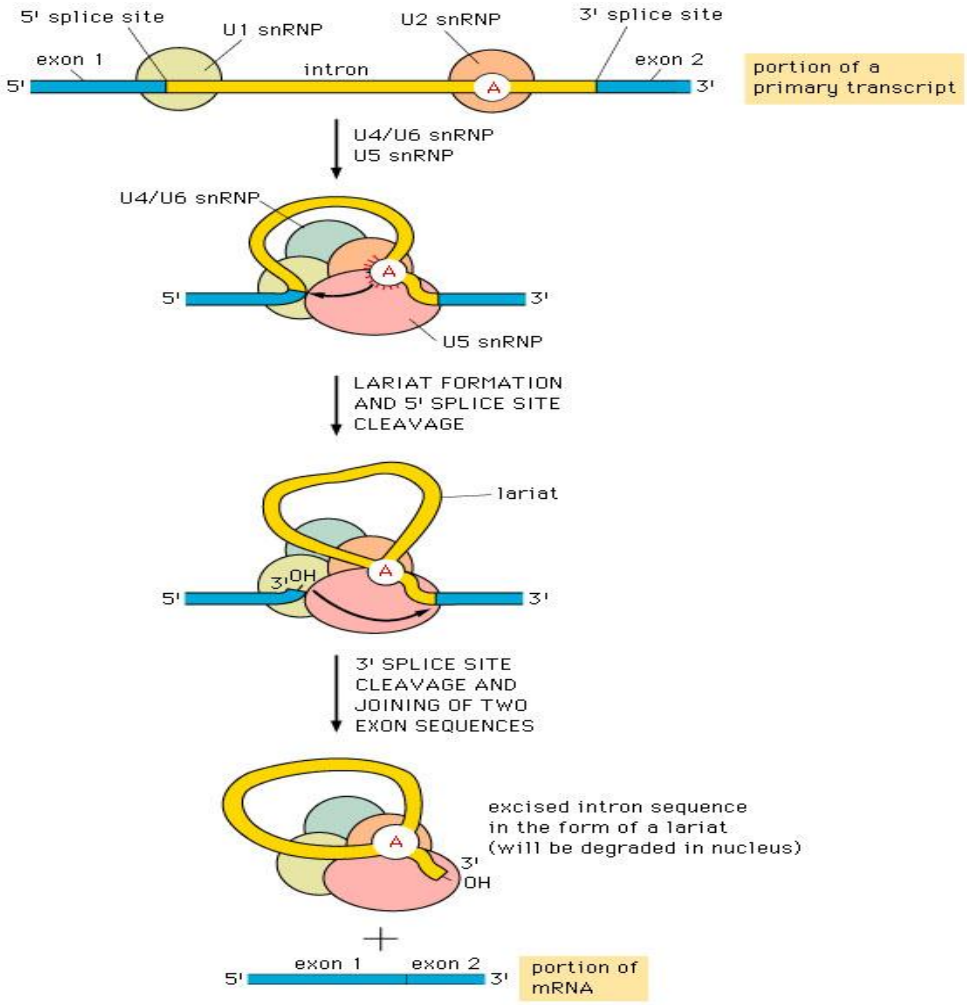


Figure 1.7 The RNA Splicing Mechanism

RNA splicing is mainly catalysed by an assembly of small nuclear ribonucleoproteins (snRNPs) which constitute the spliceosome. The spliceosome recognizes the splicing signals on a pre-mRNA molecule, and brings the two ends of the intron together. BBP and U2AF form base pairs with the branch-point site consensus sequence, and the U1 snRNP forms base-pairs with the 5' splice junction. U4/U6-U5 "triple" snRNP enters the spliceosome. U6 snRNP displaces U1 at the 5' splice junction through several RNA-RNA rearrangements and break apart of the U4/6 base pairs. In RNA-RNA rearrangement the formation of one RNA-RNA interaction requires the disruption of another.

During RNA splicing, exons are either maintained in the mRNA or targeted for removal in different combinations to produce a diverse array of mRNAs from a single pre-mRNA. This process is known as alternative RNA splicing. Alternative splicing is one of the most significant components of the functional complexity in the mammalian genome. Alternative splicing gives rise to vast diversity over gene information resulted in different protein

isoforms in different tissues, developmental states, or disease conditions (Yabas, Elliott, & Hoyne, 2015).

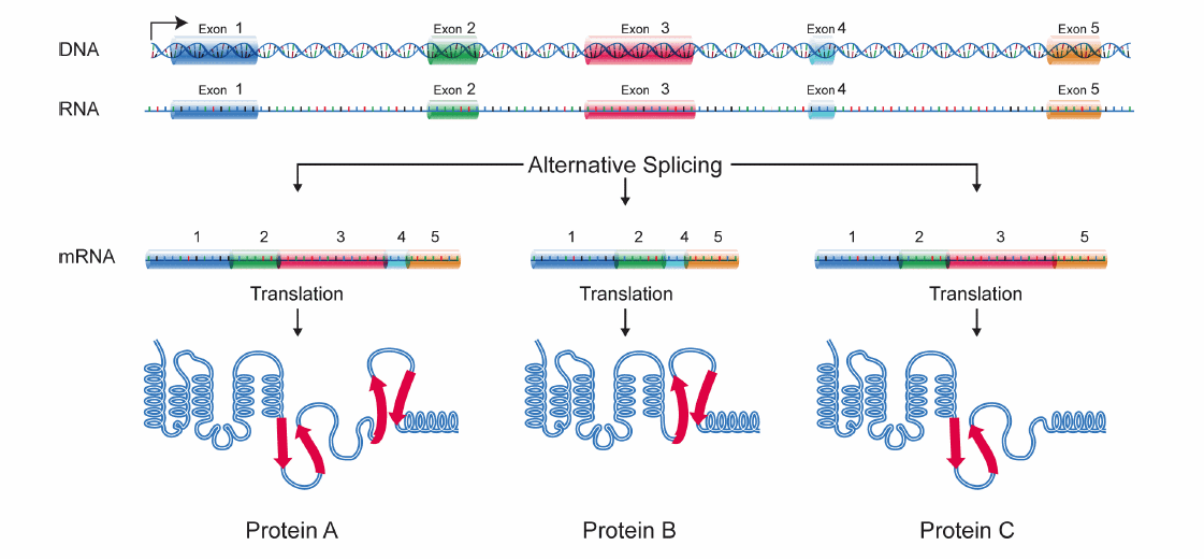


Figure 1.8 The process of alternative splicing

In DNA, the genetic information that includes the code for making a protein is located in fragments (exons, red boxes), which are interrupted by non-coding fragments (introns, green boxes). By the process of alternative splicing, the introns are removed and the exons spliced together in different combinations, generating different messenger RNAs (mRNA) that are decoded (translated) into distinct proteins.

There are 5 basic types of alternative splicing:

- Exon skipping or cassette exon: This is the most common mode in mammalian pre-mRNAs, in which case, an exon may be spliced out of the primary transcript or retained.(Sammeth, Foissac, & Guigo, 2008).
- Mutually exclusive exons: Only one of two exons is retained in mRNAs after splicing.
- Alternative donor site: An alternative 5' splice junction (donor site) is used, changing the 3' boundary of the upstream exon.
- Alternative acceptor site: An alternative 3' splice junction (acceptor site) is used, changing the 5' boundary of the downstream exon.

- Intron retention: This is the least well understood, in which case a sequence may be spliced out as an intron or simply retained. These variants are believed to be largely derived from unspliced or partially spliced pre-mRNAs, which is distinguished from exon skipping because the retained sequence is not flanked by introns(Rio, 1991). If the retained intron is in the coding region, the intron must encode amino acids in frame with the neighboring exons, or a stop codon or a shift in the reading frame will cause the protein to be non-functional (Sammeth et al., 2008).

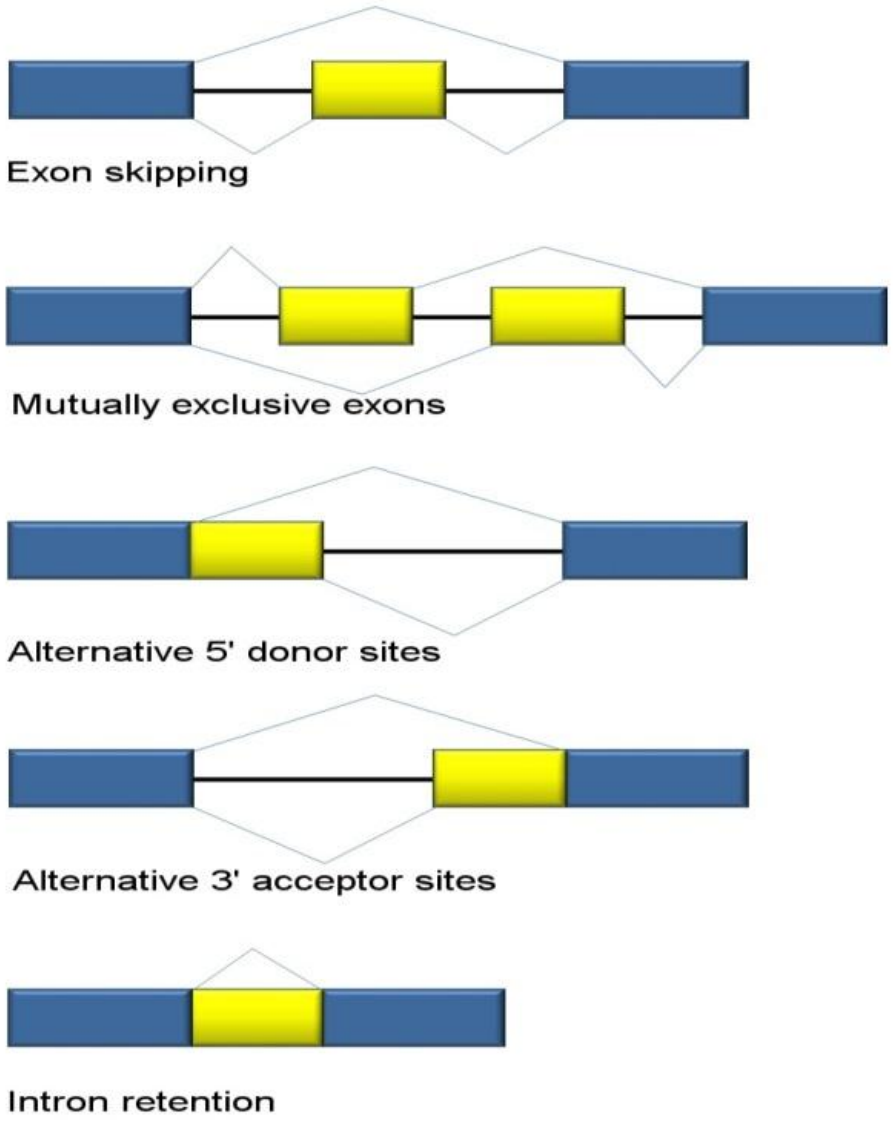


Figure 1.9 Classification of basic types of alternative RNA splicing events.

Exon skipping, mutually exclusive exons, alternative 5’ donor sites, alternative 3’ acceptor sites and intron retention.

Alternative splicing is a specific feature in eukaryotic species, which is regarded to be relative to genetic diversity within mammalian cells by increasing the number of protein isoforms that can be generated from one gene product. Through alternative splicing, different mRNA species can be derived from a single gene, giving rise to different protein variants with different even opposite functions (Pan, Shai, Lee, Frey, & Blencowe, 2008).

1.4.2 Alternative translation

Translation occurs in cytoplasm after the spliced mRNAs are exported form nucleus. ATG normally functions as the translation start codon, initiating nascent peptide synthesis by ribosome. However, recent studies have shown that translation of a peptide/protein can be also initiated from other codons within a single mRNA molecule, for example CTG initiates 15% translation, giving rise to different peptide or protein variants, which is so-called alternative translation (Bazykin & Kochetov, 2011; Jackson, 2005).

In addition to selecting different codons as start codon, there are multiple ATG codons within one mRNA molecule. These different ATG codons can serve as start codon as well, producing different peptides or proteins (Nemeth et al., 2007). Through alternative splicing, a single gene can produce different mature mRNA molecules. Through alternative translation, a single mature mRNA molecule can produce different peptides or proteins. The overall effect is to greatly increase genetic information encoded in a single gene.

1.5 VEGF and VEGF signal pathway

VEGF, originally known as vascular permeability factor (VPF), is a sub-family of growth factors, which is associated to tumor (Senger et al., 1983). Now they are normally regarded as important secreted glycoproteins, functioning in creating new blood vessels during embryonic development and after injury in which they accelerate re-endothelialisation and therefore enhance remodelling of injured arteries (Dvorak, Brown, Detmar, & Dvorak, 1995; Dvorak, Nagy, Feng, Brown, & Dvorak, 1999).

The VEGF gene contains eight exons which are highly conserved in different species (Claffey, Senger, & Spiegelman, 1995). Each exon encodes domain with specific ability in binding different molecules. The first four exons encode the signal peptide, dimerization site, VEGF receptors (VEGF-R1 and VEGF-R2) recognition site and glycosylation site. Exon 5 encodes the plasmin and matrix metalloproteinase (MMPs) cleavage sites. Eventually, exons 6, 7 and 8 respectively encode for the heparin binding site, the neuropilin binding site and the unique VEGF sequence (Keyt et al., 1996; S. Lee, Jilani, Nikolova, Carpizo, & Iruela-Arispe, 2005). The VEGF 5'- untranslated regulatory region (5'-UTR) control VEGF translation in different situations while 3'- untranslated region (3'-UTR) consists of multiple polyadenylation signals response to various stabilizing and destabilizing proteins (Figure1.10) (Konopatskaya, Churchill, Harper, Bates, & Gardiner, 2006; Seifi et al., 2012).

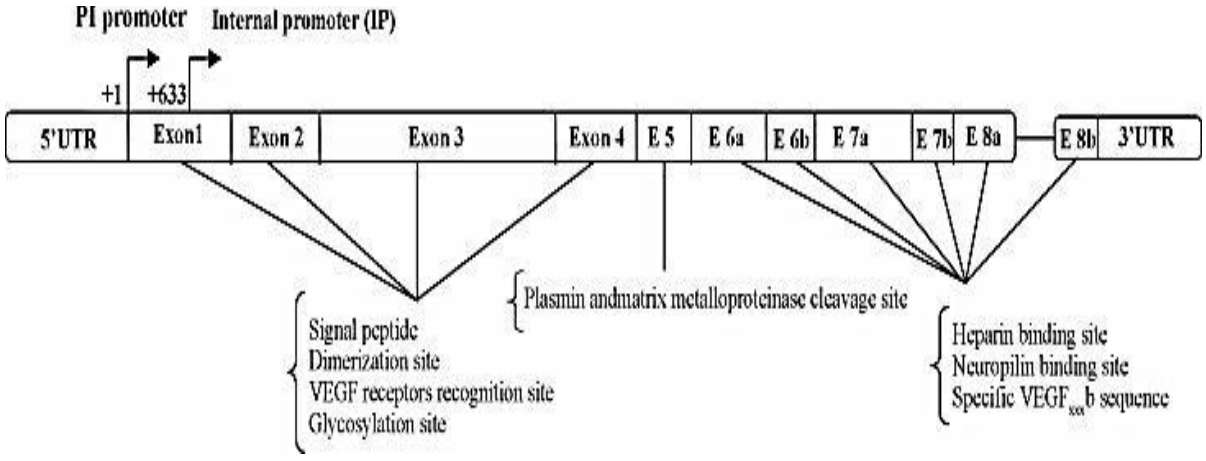


Figure 1.10 Structure of VEGF exons.

There are seven members in VEGF family: VEGF-A, VEGF-B, VEGF-C,VEGF-D, VEGF-E(or virus-encoded VEGF-like proteins), VEGF-F(a series of snake venoms) and placental growth factor (PIGF) (Jussila & Alitalo, 2002). They contain a common structure of eight specific cysteine residues in a VEGF homology domain (H. Takahashi & Shibuya, 2005). VEGF-A was first discovered and mostly studied on cells of the vascular endothelium, which has reported to stimulate EC mitogenesis, cell migration and increases microvascular permeability.

1.5.1 Alternative splicing and VEGF-A molecular diversity

As mentioned before, alternative splicing is a regulated posttranscriptional process that leads to the production of several proteins by one gene. Alternative splicing of a single pre- mRNA gives rise to at least six different isoforms of VEGF-A: 121, 145, 165, 183, 189 and 206 (numbers indicating the number of amino acid residues in each human polypeptide) (Fig 1.11) (Muller et al., 1997). These isoforms differ by the absence or presence of sequences encoded by exons 6 and 7, which alters their heparin-binding affinity and amino acid number (Cebe Suarez et al., 2006). VEGF-A121 does not bind to heparin or ECM while VEGF-A165 has moderate heparin binding ability. VEGF-A145 also contains a heparin binding domain that enable its binding to the ECM(Ferrara, Gerber, & LeCouter, 2003). VEGF-A189 and VEGF-A206 bind heparin more strongly and are sequestered in the ECM and at the cell surface. VEGF-A121, VEGF-A145, and VEGF-A165 are secreted VEGF-A splice forms while the VEGF-A183, VEGF-A189 and VEGF-A206 are the matrix bound forms. Most VEGF-producing cells express VEGF-A121, VEGF-A165, VEGF-A183 and VEGF-A189, but VEGF-A 145 and VEGF-A206 seem to be restricted to cells of placental origin (Ferrara et al., 2003; Kofler & Simons, 2015; Roy, Bhardwaj, & Yla-Herttuala, 2006; Tammela, Enholm, Alitalo, & Paavonen, 2005). Recently, many researchers believe that there are two major VEGF subfamily isoforms: one is pro-angiogenic and the other is anti-angiogenic which

differ in the sequence of exon 8 that leads to two sub-exons termed exon 8a and exon 8b (Figure 1.11). These findings contributes to VEGF angiogenesis function that is the outcome of balance between pro-angiogenic and anti-angiogenic isoform activity(Bates et al., 2002; Seifi et al., 2012).

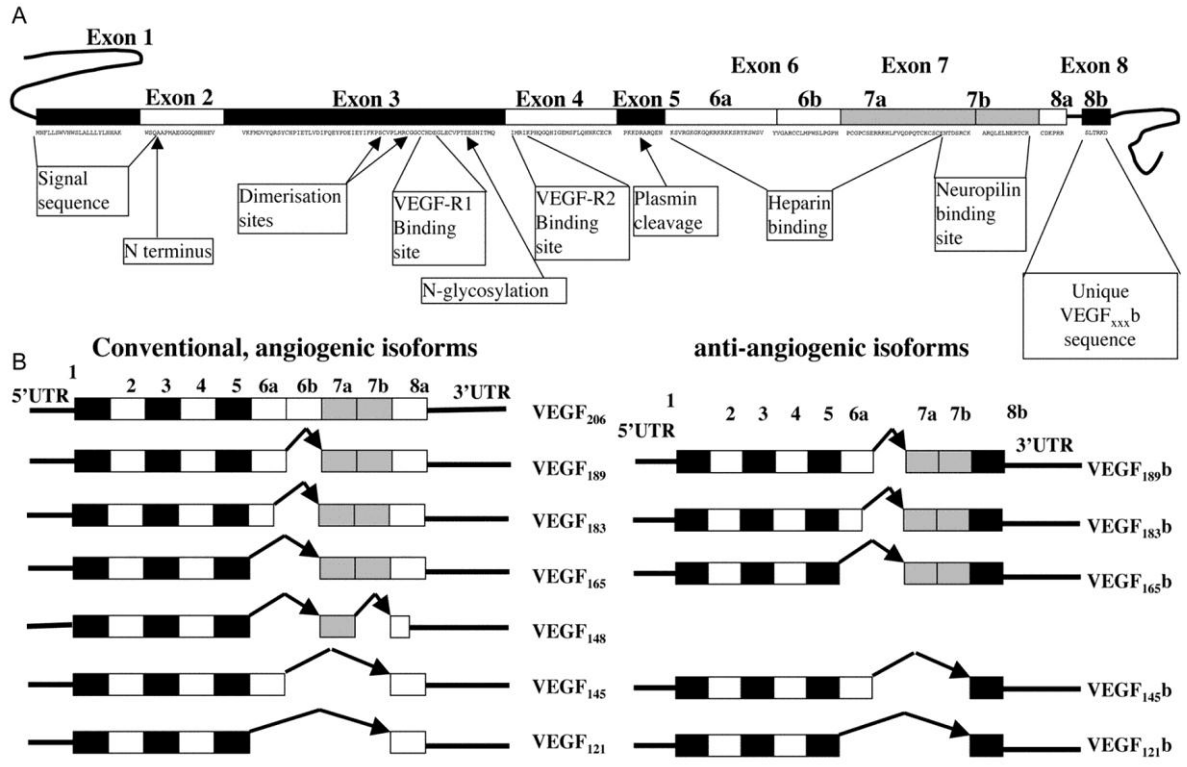


Figure 1.11 Full-length VEGF gene and two families of VEGF-A isoforms.(Nowak et al., 2008)

1.5.2 VEGF and VEGFRs

VEGF family growth factors form cysteine cross-linked dimers folded into a cysteine knot structure (Schlunegger & Grutter, 1992). Although structurally very similar, VEGF members have different physical and biological properties. They stimulate cellular responses by binding to different sites, times, and extents of specific tyrosine kinase receptors, immunoglobulin-like domains, a single transmembrane spanning region and an intracellular

portion containing a split tyrosine-kinase domain (Karkkainen & Petrova, 2000). VEGFRs can be grouped to three main subtypes, VEGFR-1 or Flt-1, VEGF-2, and VEGFR3. The first two receptors are expressed on vascular ECs, while the third one is expressed on lymphatic ECs (Holmes, Roberts, Thomas, & Cross, 2007).

VEGF/VEGF-receptor system is a significant component in the process of angiogenesis that also contains many other stimulators, inhibitors and angiogenic modulators. Different VEGF variants bind with specific VEGFRs, inducing different biologic processes. VEGF-A binds to VEGFR-1 (Flt-1) and VEGFR-2 (Holmes et al., 2007). VEGFR-2 seems to be involved in almost all of the known cellular responses to VEGF. It is expressed in almost all ECs in mouse and human embryos, while its expression is reduced towards the end of gestation (Millauer et al., 1993). VEGFR-2 is effective in transducing signals regulating cell division and inhibition of cell death. Binding of VEGF-A to VEGFR-2 leads receptor dimerization, kinase activation and autophosphorylation of specific tyrosine residues within the dimeric complex, leading to EC survival, migration and differentiation and mediation of vascular permeability. VEGF increases vascular permeability by weakening endothelial cell-cell adhesion through changing in the phosphorylation of VE-cadherin and associated catenins. Moreover, when VEGF activates ECs, VEGFR-2 interacts with the VE-cadherin/catenin complex. The VE-cadherin/catenin complex is important for the above interaction, and in cases that ECs lack β-catenin or express a truncated mutant of VE-cadherin preventing the binding with β-catenin, VEGFR-2 fails to interact with the complex (Grazia Lampugnani et al., 2003). Therefore, cytoskeletal organization is the critical determinant of life or death for ECs, and adhesive interactions of transmembrane receptors linked to the cytoskeleton through junctional plaque proteins possess a functional role. In cases of targeted inactivation of VEGFR-2 gene, it is revealed as VEGF receptors (VEGFRs) on the cell surface, causing them activated through transphosphorylation. Therefore, one of the most important effects of

VEGFR-2 is probably in maintaining the survival and apoptosis balance, which is critical for vascular haemostasis and without which vasculogenesis is disrupted, resulting in embryonic death (T. Takahashi, Yamaguchi, Chida, & Shibuya, 2001).

The role of VEGFR-1 in VEGF signalling is not clear yet, although recent studies indicate that it influences angiogenesis by recruiting monocytes and macrophages to sites of pathological angiogenesis, partially by stimulating differentiation of haematopoietic precursors (Jiang et al., 2002). The binding affinity of VEGF-A to VEGFR-2 is lower as compared with VEGFR-1, although the biological activities of VEGF-A are mainly transduced by VEGFR-2. So, VEGFR-1 is thought to act as an antagonist of VEGFR-2 which can sequester VEGF from VEGFR-2 binding, regulating vasculogenesis in the embryo (Karkkainen & Petrova, 2000). VEGFR-3 and its ligands VEGF-C and VEGF-D are important regulators of lymphangiogenesis, which mediates perpetual action and function of ligands on target cells. VEGF-C can also stimulate angiogenesis via VEGFR-2 (Ali et al., 2014).

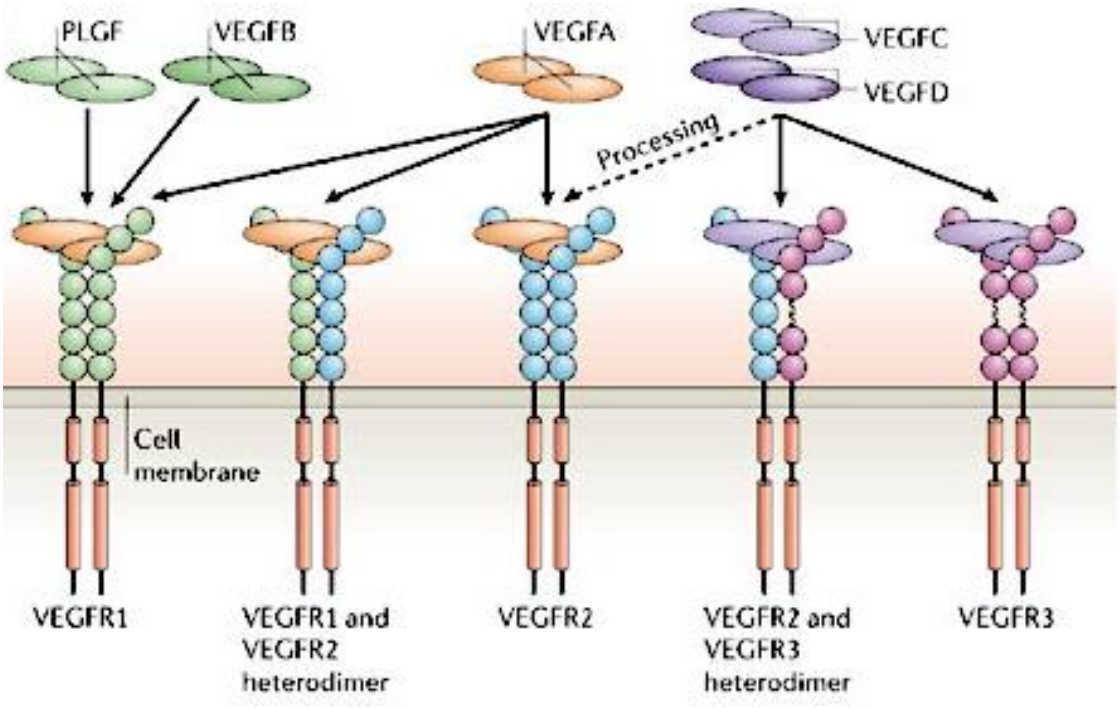


Figure 1.12 Types of VEGF and their VEGF receptors.

Interaction of VEGFs with VEGFRs. Colored arrows indicate major pathway. (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006).

1.5.3 Biologic effects of VEGFs

VEGFs are crucial in embryonic development and in other physiological and pathological conditions, including wound healing, rheumatoid arthritis, ocular neovascularization, tumor progression, endometriosis and cardiovascular diseases (Roy et al., 2006).

VEGF-A, a key molecule in induction of angiogenesis and vasculogenesis, causes proliferation, sprouting, migration and tube formation of ECs (Ferrara et al., 2003). VEGF-A was discovered as a vascular permeability factor secreted by carcinoma cell lines that stimulated the production of ascites and enhanced permeability in skin blood vessels (Senger et al., 1983). VEGF-A mediates its responses primarily by activating VEGFR-1 and VEGFR-2. Overexpression of VEGF-A resulted in large, dilated and leaky vessels due to too strong angiogenic response (Bhardwaj et al., 2003). VEGF-A also causes vasodilatation by induction of eNOS and increasing NO production(Cooke & Losordo, 2002). VEGF-A promotes EC survival by inducing the expression of anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) in the ECs, which might be related to the activation of phosphatidylinositol-3 kinase and Bcl-2 pathways (Bootle-Wilbraham, Tazzyman, Thompson, Stirk, & Lewis, 2001). Besides ECs, VEGF–A also play a role in other cell types. VEGF-A causes mitosis of retinal pigment epithelial cells and Schwann cells and promotes vascular SMC proliferation and migration (Gerber et al., 2002). VEGF-A is also reported to have hematopoietic effects on granulocyte-macrophage progenitor cells (Clauss et al., 1990). A number of signal transduction molecules are activated or modified in response to VEGF-A stimulation, such as Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and its downstream substrates, the serine / threonine kinase Akt 1, protein kinase B (PKB), PLC-y, the Scr family tyrosine kinases, the Ras GTPase activating protein, the small adaptor molecule Nck, focal adhesion

kinase C, extracellular signal-regulated kinase (ERK) and the p38 mitogen-activated protein kinase(P. Carmeliet, 2000; Claesson-Welsh, 2003; Gerber et al., 1998) (Figure1.13). The biological significance of all these interactions is not fully defined yet. However, Akt/PKB is critical in EC survival (H. Zeng, Sanyal, & Mukhopadhyay, 2001), and PLC-y-mediated activation of the ERK are crucial pathways in EC proliferation induced by VEGF.

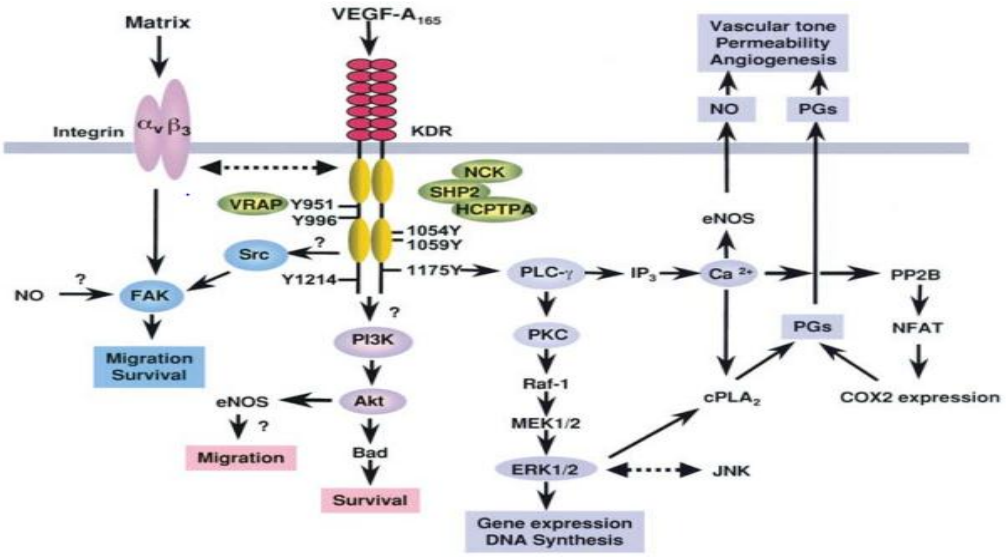


Figure 1.13 VEGF signalling pathways

Most biologically relevant VEGF signalling is mediated via KDR. VEGF-dependent EC survival is mediated, in part, via PI3K through Akt kinase. Akt also causes Ca^{2+}-independent eNOS activation through phosphorylation, an essential pathway for migration. Direct interactions between integrin avBj and KDR may also play a role in survival. PLC-y pathway is a major mitogenic signalling mechanism for VEGF resulting in PKC activation. PKC mediates activation of ERKs 112 via Raf-1 and MEK, and is involved in mitogenesis. Ca^{2+} signalling is also important for eNOS activation and NO generation.

The human VEGF-B gene is located on chromosome11, band q13 and contains eight exons. VEGF-B has two isoforms VEGF-B167 and VEGF-B186. VEGF-B might be related to formation of coronary collaterals. It was reported that deficient in VEGF-B resulted in development of smaller hearts and impaired recovery after induced myocardial infarction in mice (Bellomo et al., 2000). Also, VEGF-B knockout arthritis models showed reduced

synovial angiogenesis, suggesting an important role of VEGF-B in inflammatory angiogenesis (Mould et al., 2003).

The VEGF-C gene consists of seven exons and located on chromosome 4q34. VEGF-C is activated in the extracellular space by proteases to create a homodimeric protein with both VEGFR- 2 and VEGFR-3 (Enholm et al., 2001). Developmental studies, knockout models and gene transfer experiments suggest that VEGF-C with VEGFR-3 is primarily functional on lymphangiogenic effects (Y. Li, He, Zhong, Li, & Liang, 2015; Morita et al., 2015). Besides, VEGF-C plays a crucial role in tumor lymphangiogenesis and lymph node metastasis (Hisamatsu et al., 2015; Jeltsch et al., 1997).

The human VEGF-D gene is located on chromosome Xp22.31, which is a secreted glycoprotein that promotes growth of blood vessels and lymphatic vessels, and can induce remodeling of large lymphatics. The human VEGF-D binds to and activates VEGFR-2 and VEGFR-3, while the mouse VEGF-D binds only to VEGFR-3. VEGF-D is thought to have angiogenic ability both *in vitro* and *in vivo*. During embryonic development, VEGF-D deficient mice showed a lack of a profound lymphatic vessel defect (Baldwin et al., 2005; Jeltsch et al., 1997). Furthermore, VEGF-D has been proposed to have a role in tumor angiogenesis and lymphangiogenesis for its strong ability to induce angiogenesis in addition to lymphangiogenesis (Bates & Harper, 2002; Stacker et al., 2001). Thus, VEGF-D signaling is a potential target for novel anti-cancer therapeutics designed to explore anti-angiogenic approaches and to restrict metastasis (Achen & Stacker, 2012).

Human PIGF gene, encoded by seven exons spanning an 800-kb-long DNA interval, has been mapped to chromosome 14q24. Four isoforms – PIGF-1, PIGF-2, PIGF-3 and PIGF-4, have been identified (Tjwa, Luttun, Autiero, & Carmeliet, 2003). PIGF-1 and PIGF-3 are diffusible isoforms, whereas PIGF-2 and PIGF-4 have heparin binding domains (W. Yang, Ahn, Hinrichs, Torry, & Torry, 2003). PIGFs mediate their effects mainly through VEGFR-1.

PIGF-2 (Yla-Herttuala & Alitalo, 2003) can also bind to neuropilin (NRP)-1 and -2 because of an insertion of 21 basic amino acids at the carboxyl terminus (Migdal et al., 1998; Persico, Vincenti, & DiPalma, 1999). PIGF has a major role in vasculogenesis and angiogenesis in human placenta. However its functions on the angiogenic role are inconsistent. Some studies reported that PIGF binding to VEGFR-1 failed to produce EC growth and angiogenesis, while others hold the view that PIGF/VEGFR-1 signaling promotes EC viability and angiogenesis (Roy et al., 2005). PIGF has direct effects on ECs, both by inducing its own signaling and by augmenting VEGF-driven angiogenesis (Autiero et al., 2003).

VEGF-E was discovered in the genome of the parapoxvirus (Orf virus) that infects sheep, goats, and occasionally human (Lyttle, Fraser, Fleming, Mercer, & Robinson, 1994). Infection by this virus causes proliferative skin lesions in which extensive capillary proliferation. Besides, VEGF-E is reported to induce ECs proliferation, vascular permeability and angiogenesis in skin directly. And these biological functions are based on its interaction with VEGFR-2 and Nrp-1 (L. Wise, McCaughan, Tan, Mercer, & Fleming, 2007; L. M. Wise et al., 2003)

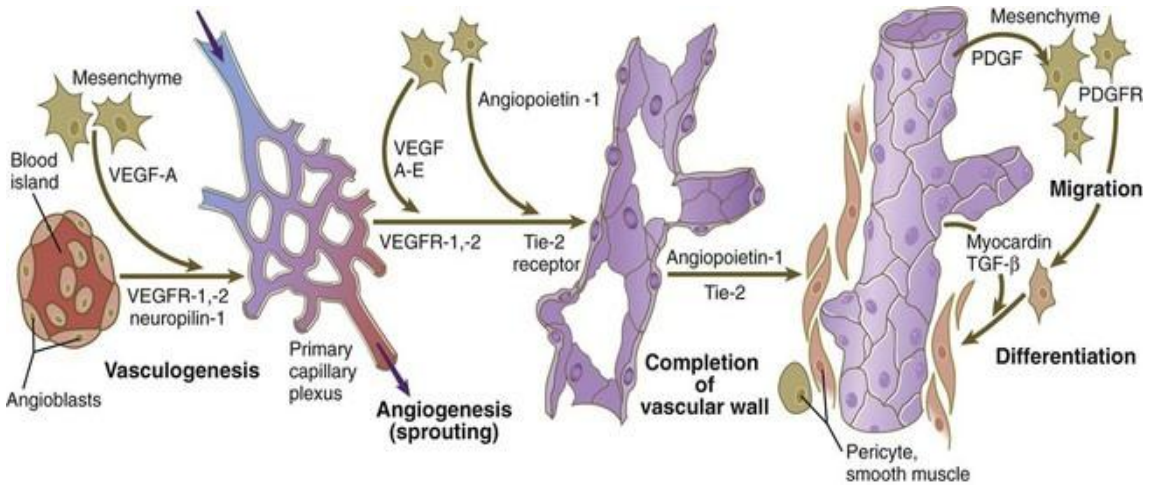
VEGF-F, identified from snake (viper) venom, consists of two VEGF-related proteins designated as vammin (110 residues) and VR-1 (109 residues), which have a 50% primary structure identity with VEGF-A165 and bind selectively to VEGFR-2 (Suto, Yamazaki, Morita, & Mizuno, 2005). As its similar structure to VEGF-A165, VEGF-F exhibits a specific blockage of VEGF-A165 activity both *in vitro* and *in vivo* (Yamazaki, Tokunaga, Takani, & Morita, 2005).

1.5.4 VEGF signaling pathways in Angiogenesis

VEGF is reported as an ECs selective mitogen, which is derived from arteries, veins and lymphatics. VEGF signalling pathways have been shown to play critical roles in development of the vascular system by regulating embryonic vasculogenesis, angiogenesis and arteriogenesis. In current study, we focus on angiogenesis. Angiogenesis is the process that new blood vessels takes shape from pre-existing post-capillary venules, where ECs respond to VEGF-A leading to sprouting, proliferating, and migrating to expand the vascular tree (P. Carmeliet & Collen, 1998). The molecular basis of angiogenesis in the embryo differs from that of pathological angiogenesis in the adult. Angiogenesis is an important mechanism of blood vessel formation in the adult (Bergers & Song, 2005).

Angiogenesis initiates with vasodilation, a process involving NO. Vascular permeability increases in response to VEGF, which is regulated by the formation of fenestrations, vesiculo–vacuolar organelles and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 and VE–cadherin, and involves Src kinases (Eliceiri et al., 1999). Therefore, the increased permeability makes it possible for extravasation of plasma proteins that lay down a provisional scaffold for ECs migrating. For ECs migration, firstly they need to loosen inter EC junction and to relieve periendothelial cell support (Maisonpierre et al., 1997). In this process, proteinases of the plasminogen activator, MMP, chymase or heparanase families influence angiogenesis by degrading matrix molecules and activating basic fibroblast growth factor (bFGF), VEGF and Insulinlike growth factor-1 (IGF-1) within the extracellular matrix (Coussens et al., 1999). Once the vessels become destabilized, proliferating ECs migrate to distant sites. During this process, VEGFs and VEGFRs have specific functions. For example, VEGF120 alone initiates angiogenesis but does not complete angiogenesis; VEGFR3 is involved in embryonic angiogenesis and pathological angiogenesis, whereas VEGF-C is angiogenic only in adult pathology; VEGF-B plays an important role in

coronary function after coronary occlusion (P. Carmeliet, Ng, et al., 1999; Dumont et al., 1998). Then, ECs assemble and fuse with pre-existing vessels, allowing vessels to increase their diameter and length with VEGF121, VEGF165 and their receptors involved in. Furthermore, ECs become quiescent and survive for years, once assembled in new vessels. In the embryo endothelial survival depended on the interaction of VEGFs between VEGFR-2, β-catenin and VE–cadherin is very important, because reduced endothelial survival causes vascular regression (P. Carmeliet, Lampugnani, et al., 1999). According to different local physiological requirements, ECs acquire specialized characteristics and perform further remodelling and 'pruning' capillary-like vessels(Risau, 1998).



（http://clinicalgate.com/cardiovascular-system-11/）

Figure 1.14 Scheme illustrating vasculogenesis, angiogenesis, and assembly of the vascular wall.

VEGFs and VEGFRs take part in all the process of vasculogenesis, angiogenesis and arteriogenesis. Angioblasts, initially expressing VEGFR-2 stimulated by VEGF-A to form the primary capillary plexus. Then competent ECs of the primary capillary plexus form vascular sprouts under additional stimulation by growth factors in the earliest stages of angiogenesis. This process followed by the recruitment of surrounding mesenchymal cells to form the cellular elements of the vascular wall.

In the developed vascular system, angiogenesis and arteriogenesis continue to play important roles in both health and disease. In response to hypoxia or injury, VEGF promotes angiogenesis to create new capillary formation. In situations such as wound healing and

injury, hypoxic tissues secrete VEGF to induce angiogenesis by inducing EC sprouting, proliferation and migration, thereby expanding the capillary network and increasing nutrient and oxygen supply (Hoeben et al., 2004). Diseases characterized by vascular occlusion, such as coronary or cerebral artery occlusions, require to promote arteriogenesis to expand pre-existing collateral anastomosis and induce new arterial growth, in which process VEGF signaling is also critical (Kofler & Simons, 2015). Understanding the VEGFs multiple function networks in angiogenesis and arteriogenesis is of great interest to develop targeted and more effective therapies for diseases characterized by inadequate blood flow.

1.5.5 MEKK1 in VEGF signalling pathway

Mitogen-activated protein kinases (MAPK), belonging to the CMGC (CDK/MAPK/GSK3/ CLK) kinase group, are protein kinases that are specific to the amino acids serine, threonine, and tyrosine. MAPKs are involved in cellular responses to mitogens, osmotic stress, heat shock and pro-inflammatory cytokines (Yujiri, Sather, Fanger, & Johnson, 1998). Mitogen-activated protein kinase kinase kinase (MEKK) is a serine/threonine kinase that occupies a pivotal role in a network of phosphorylating enzymes. The cytoplasmic serine/threonine kinases transduce extracellular signals into regulatory events that impact cellular responses (Yujiri et al., 2000). An upstream mitogen or growth factor signal initiates a module of three kinases: MEKK that phosphorylates and activates a mitogen-activated protein (MAP) kinase kinase (MEK) and finally activation of MAP kinase (MAPK; e.g., ERK). Thus, this MAP3K-MAP2K-MAPK module represents critical effectors that regulate extracellular stimuli into cellular responses, such as differentiation, proliferation and apoptosis all of which function during development(Craig, Stevens, Vaillancourt, & Camenisch, 2008).

MEKK1, a key MAP3K, appears to transduce signals for activation of c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinase 1/2 (ERK1/2). It has been reported

that MEKK1 involved in cell migration regulation. MEKK1-knockout mice exhibit altered migration of epithelial cells resulting in defective eyelid closure. Apoptosis signal-regulating kinase 1 (ASK1), a proapoptotic member of the MAP3K family, acts as a target of bFGF-mediated survival signaling in ECs (Alavi, Acevedo, Min, & Cheresh, 2007). MEKK1-MKK1/MKK2-MPK4 mitogen-activated protein (MAP) kinase cascade also reported to represses cell death and immune responses. MEKK1 translocates to the nucleus and acts as a co-repressor with p53 to down-regulate PKD1 transcriptional activity (Islam et al., 2010). During blood vessel injury, several transcription factors are activated in response to hypoxia. Among these transcription factors, activator protein 1 (AP-1) is rapidly activated by hypoxia and triggers bFGF, VEGF, and tyrosine hydroxylase gene expression. MEKK1 dominant negative mutant inhibited AP-1 activity, deleted AP-1 binding sites within the c-jun protein, resulted in hypoxia response (Minet et al., 2001).

The communication between integrin alphaVbeta3 (αvβ3) and VEGFRs acts to coordinate EC activity during angiogenesis (di Blasio, Droetto, Norman, Bussolino, & Primo, 2010). The cooperation between αvβ3 integrin and VEGF-A-mediated signals is one of the most relevant differences between VEGF-A and other angiogenic factors (Mahabeleshwar, Feng, Reddy, Plow, & Byzova, 2007). VEGF-A and integrin synergistically rescue angiogenesis in human microvascular ECs (Liu et al., 2003). It has been shown that VEGF-A regulates αvβ3 integrin function by increasing the affinity of integrins for extracellular ligands (Byzova et al., 2000). Besides, it is well documented that MAPK cascade is involved in ECs migration and immune response. Rangaswami H1 et al, reported that αvβ3 integrin mediated MEKK1 phosphorylation and MEKK1-dependent c-Jun N-terminal kinases 1 (JNK1) phosphorylation (Rangaswami, Bulbule, & Kundu, 2005; Rangaswami & Kundu, 2007). However whether VEGFs play a role to induce αvβ3 integrin mediated MEKK1 phosphorylation is still not known.

1.6 14-3-3 proteins and protein phosphorylation

1.6.1 14-3-3 proteins

14-3-3 proteins are a family of conserved regulatory molecules that are expressed in all eukaryotic cells, which are implicated in many cellular functions, including transcription, metabolism, and apoptosis (Dougherty & Morrison, 2004). They are small acidic proteins with a molecular mass ranging from 27 to 32 kDa (Aitken, Jones, Soneji, & Howell, 1995). There are seven identified 14-3-3 isoforms β, ζ, γ, η, ε, τ, and σ (stratifin/HME1) in mammals, which are highly conserved and tissue specificity(Rosenquist, Sehnke, Ferl, Sommarin, & Larsson, 2000).

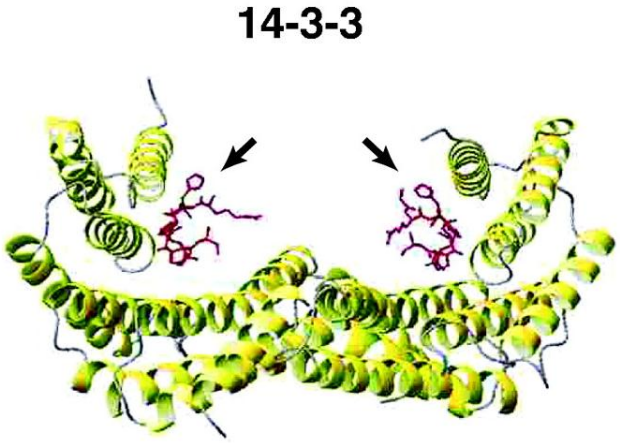


Figure 1.15 14-3-3 protein structure (Dougherty & Morrison, 2004)

14-3-3s are phosphoserine-binding proteins. The binding may be regulated by factors that bind to the consensus motifs RSXpSXP and RXY/FXpSXP (Yaffe et al., 1997). Recent studies now indicate that the 14-3-3 binding interactions may be regulated by serine/threonine protein phosphatases PP1 and PP2A and 14-3-3 play an essential role in controlling the translocation of certain proteins from the cytoplasmic and endoplasmic reticulum to the plasma membrane (Jaumot & Hancock, 2001; Ory, Zhou, Conrads, Veenstra, & Morrison, 2003). They play a central role in the phosphorylation-dependent regulation of diverse cellular processes for the ability to bind kinases, phosphatases, and transmembrane receptors.

14-3-3 protein phosphorylation is also believed to be associated with many diseases. In Parkinson's disease, increased 14-3-3 phosphorylation reduces neuroprotective potential of 14-3-3 proteins and phosphorylation of 14-3-3s at serine 232 contributes to the neurodegenerative process (Slone, Lavalley, McFerrin, Wang, & Yacoubian, 2015). During intestinal inflammation, dysfunction of 14-3-3 function induced uncontrolled Akt activation (Akt Thr308 phosphorylation), nuclear Akt accumulation resulted in intestinal epithelial cell death(Gomez-Suarez et al., 2016). Also, a lot of researches on cancer process indicate that the expression of 14-3-3 family members level is related to multiple tumor developing and suggest that it might be important to monitoring 14-3-3 levels during cancer treatment (Bergamaschi & Katzenellenbogen, 2012; Frasor et al., 2006; Z. Li, Liu, & Zhang, 2009; Matta, DeSouza, Ralhan, & Siu, 2010; Neal & Yu, 2010).

1.6.2 Cellular function of 14-3-3 protein

The 14-3-3 proteins exist in cells as homodimers and heterodimers and each molecule in the dimer contains an independent ligand-binding channel, thus a dimer can bind two pS/T sites on a single target or on separate binding partners at the same time (Gardino, Smerdon, & Yaffe, 2006). Binding of a 14-3-3 dimer can change the conformation of a target protein, mediate or prevent protein interactions and hide intrinsic localization motifs which may block the accessibility of a target protein to modifying enzymes such as kinases, phosphatases, or proteases. Therefore, 14-3-3 binding interactions can regulate the function of a wide spectrum of proteins with diverse biological activities. Multiple studies have been dedicated to understanding the role of 14-3-3 proteins in cell proliferation, migration, survival and apoptosis.

Receptor tyrosine kinases (RTKs) is cell surface receptor which initiate normal cell proliferation. In RTK pathways, Raf proteins act as direct effectors of the Ras GTPase to

promote signaling through the ERK casade. Raf proteins contain at least two phosphorylation-dependent 14-3-3 binding sites, the role that 14-3-3 binding plays in Raf regulation is complex, largely due to the different sites: Binding of 14-3-3 to the N-terminal site plays a critical role in inhibiting Raf activation. However, during the Raf activation process, 14-3-3 binding to the N-terminal site is disrupted by dephosphorylation. 14-3-3 serves a positive regulatory role in facilitating Raf dimerization via binding to the C-terminal sites to contact two Raf proteins (Garnett, Rana, Paterson, Barford, & Marais, 2005; Ritt, Monson, Specht, & Morrison, 2010).

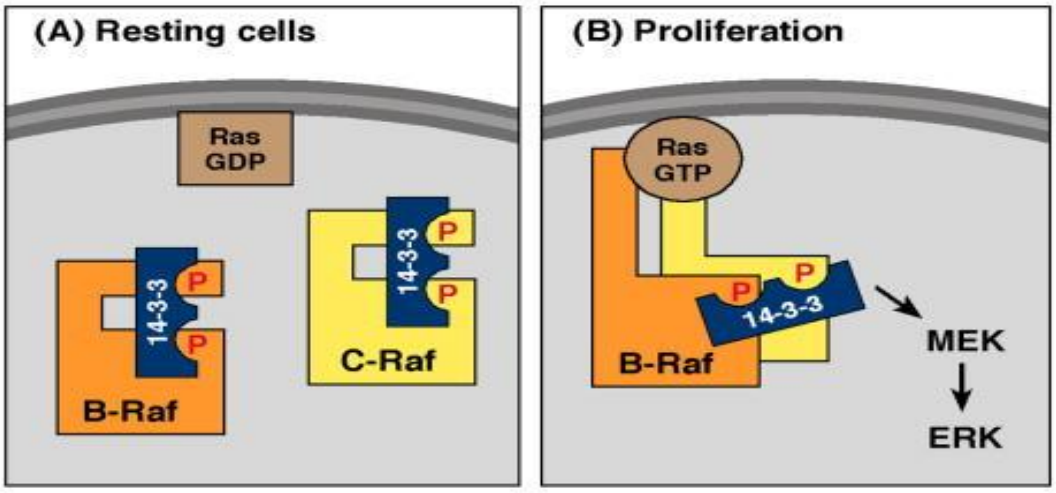
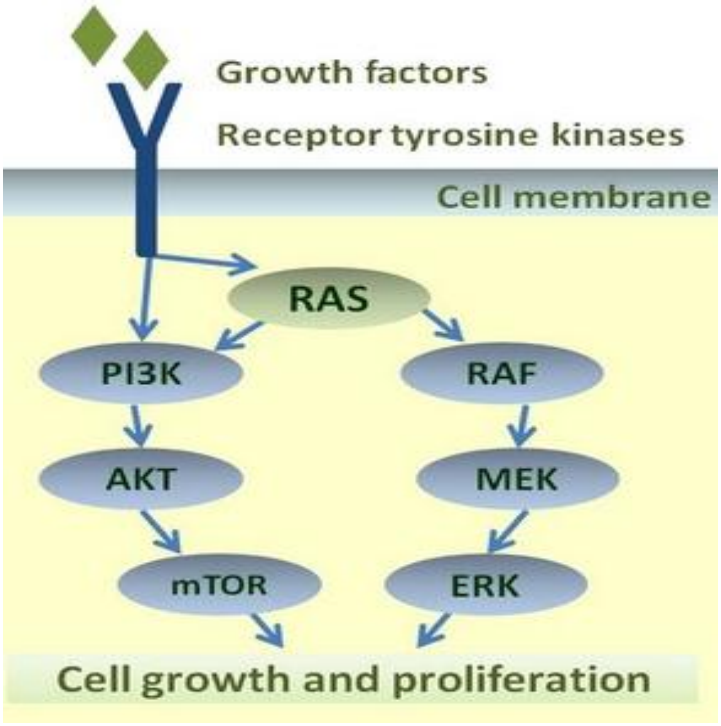


Figure 1.16 The function of 14-3-3 in Raf kinase regulation

(A) In resting cells, a 14-3-3 dimer binds to phosphorylation sites found in the Raf N-terminal regions, maintaining Raf in an inactive state. (B) In response to proliferative signals, 14-3-3 binding to the Raf C-terminal sites facilitates Raf dimerization in a Ras-dependent manner(Freeman & Morrison, 2011).

Furthermore, Raf regulation is not the only role of 14-3-3 in RTK/Ras signalling. Another critical effector of RTK/Ras signaling is the PI3K/PDK/AKT cascade, and AKT is a key kinase that generates 14-3-3 binding sites on a diverse array of target proteins. Thus 14-3-3 interact with Raf-1 in Raf-1/ERK, which control cell proliferation (McCubrey et al., 2007).



https://www.researchgate.net/figure/259347486

Figure 1.17 Signalling schematic for the Ras-Raf-MAPK and PI3K-Akt-mTOR pathways

Remodeling of the actin cytoskeletion is involved in initiation of cell migration. At the leading edge of migrating cells, this remodeling is controlled by cooperation among Rho family GTPases, protein kinases and protein phosphatases. In the last few years, 14-3-3s were identified to bind with a great number of proteins involved in actin remodeling, and PKD has been shown as a kinase that generates 14-3-3 binding sites on several of these targets. Firstly, slingshot 1-like (SSH1L) the cofilin regulator, which are activated by F-actin binding and play a significant role in actin remodeling. The ability of SSH1L binding with F-actin is inhibited by the association of SSH1L with 14-3-3 (Huang, DerMardirossian, & Bokoch, 2006; Nagata-Ohashi et al., 2004), which process is regulated by PKD (Eiseler et al., 2009; Peterburs et al., 2009). Moreover, cortactin the actin regulatory protein, is another recently identified PKD substrate that is regulated by 14-3-3 binding. Cortactin functions to enhance actin polymerization rates, which appears to be negatively regulated by 14-3-3 binding

(Eiseler, Hausser, De Kimpe, Van Lint, & Pfizenmaier, 2010). Besides, IRSp53, another molecule that involved in actin regulation, was identified as 14-3-3 binding partner. IRSp53 contains multiple protein interaction domains and acts as a signaling platform to downstream actin regulatory proteins. 14-3-3 binding was found to terminate IRSp53 signaling and disrupt actin polymerization (Cohen, Fernandez, Lazaro-Dieguez, & Musch, 2011; Mackie & Aitken, 2005; Robens, Yeow-Fong, Ng, Hall, & Manser, 2010; Scita, Confalonieri, Lappalainen, & Suetsugu, 2008). Above all, 14-3-3 binding acts to negatively regulate function in actin remodeling.

14-3-3 plays a major role in cell division cycle regulation. The association of 14-3-3 with its target proteins affects cell cycle progression through affecting protein localization or modification of their enzymatic activity. CDC25C was reported to activate the cyclin-dependent kinase CDC2 which drives the cells through mitosis. During interphase, the 14-3-3 isoforms γ and ε bind to CDC25C results in its inactivation, presumably by preventing the premature activation of CDC2 (Dalal, Yaffe, & DeCaprio, 2004). Moreover, 14-3-3γ regulates cell division mainly through interactions with cellular checkpoints proteins (Mhawech, 2005). 14-3-3γ regulates P53 by blocking its inhibitors and interacting with its regulatory proteins to regulate apoptosis (Jin et al., 2006). 14-3-3γ and ε are the only isoforms that bind to phospholipid aggregates in vesicles and membrane proteins. 14-3-3γ also interacts with organelle membrane of lysosomes, mitochondria, and Golgi. During lysosomal accumulation, 14-3-3γ is enriched at the mitochondria and plays a role in mitochondrial quality control. 14-3-3γ interacts with large pleomorphic carriers that travel from the Golgi complex to the plasma membrane and undergo fission (Miyamoto et al., 2012; Valente et al., 2012). These indicate 14-3-3γ may function as an intracellular protein trafficking.

1.6.3 14-3-3 proteins and VEGF

Clinical research found that 14-3-3 ζ, HIF-1a, and VEGF were involved in the same cascade of the malignant progression of gliomas (Cao et al., 2014). The expression levels of Mcl-1, VEGF-R2, and 14-3-3σ may be predicted to the early clinical response in head and neck tumor(Erovic et al., 2005). Our group's previous study found that HDAC7 directly binds to β-catenin and forms a complex with 14-3-3 epsilon, ζ, and eta proteins, resulting in inhibition of EC growth and leading to G1 phase elongation. VEGF treatment abolished this inhibition by increasing HDAC7 degradation leading to the translocation of β-catenin to the nucleus to induces EC growth (Margariti et al., 2010).

1.6.4 Protein phosphorylation

Phosphorylation is the most common post-translational modification that occurs in both prokaryotic and eukaryotic organisms. Phosphorylation is the attachment of a phosphate group to a molecule. The new phosphorus group can activate, deactivate or change the function of the protein, which can regulate protein function without changing the amount of protein (C. Chang & Stewart, 1998; Cozzone, Grangeasse, Doublet, & Duclos, 2004). It is estimated that 5% of the proteome contains enzymes that perform more than 200 types of post-translational modifications (Gonzalez-Montelongo, Marin, Perez, Gomez, & Diaz, 2013). These enzymes include kinases, phosphatases, transferases and ligases, which add or remove functional groups to amino acid side chains, and proteases, which cleave peptide bonds to remove specific sequences or regulatory subunits. Protein kinases play a role in a multitude of cellular processes, including division, proliferation, apoptosis, and differentiation. Phosphorylation usually results in a functional change of the target protein by changing enzyme activity, cellular location, or association with other proteins.

The catalytic subunits of protein kinases are highly conserved (Stout, Foster, & Matthews, 2004) and eukaryotic protein kinases share a conserved catalytic core common with both serine/threonine and tyrosine protein kinases (Hanks & Quinn, 1991). There are a number of conserved regions in the catalytic domain of protein kinases. In the N-terminal extremity of the catalytic domain there is a glycine-rich stretch of residues near a lysine residue, which has been shown to be involved in ATP binding. In the central part of the catalytic domain there is a conserved aspartic acid residue which is important for the catalytic activity of the enzyme (Knighton et al., 1991).

Phosphotransferase is a subclass of the transferases, comprising the enzymes that catalyze the transfer of phosphate group from a donor (usually high-energy) to an acceptor (specific substrates) (Burnett & Kennedy, 1954; C. Chang & Stewart, 1998; Cozzone, 1988; Stock, Ninfa, & Stock, 1989). Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins. However, in prokaryotic proteins phosphorylation occurs on the serine, threonine, tyrosine, histidine or arginine or lysine residues (Ciesla, Fraczyk, & Rode, 2011). Furthermore, phosphor-serine can transfer its phosphate group to serine or threonine.

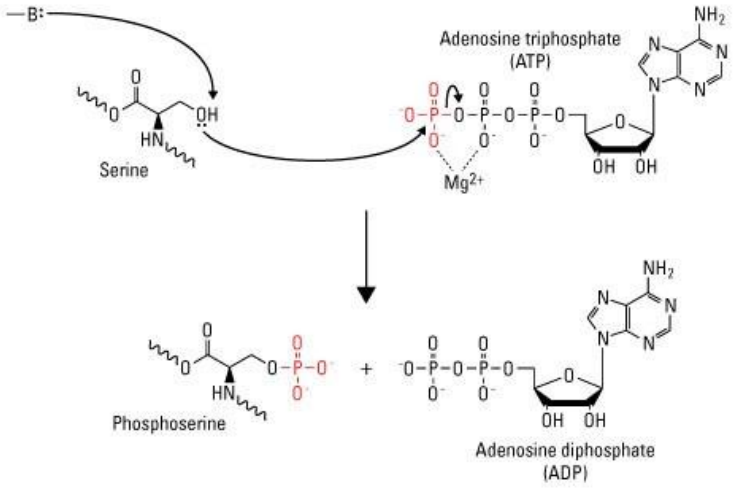


Figure 1.18 Diagram of serine phosphorylation.

Enzyme-catalysed proton transfer from the (–OH) group on serine stimulates the nucleophilic attack of the γ-phosphate group on ATP, resulting in transfer of the phosphate group to serine to form phosphoserine and ADP. (—B:) indicates the enzyme base that initiates proton transfer.

1.7 Hypothesis and aims of study

Recently, a population of progenitor cells has been identified within the adventitia of the vessel wall. These cells are found to express progenitor markers including Sca-1, c-kit, CD34, CD90, CD73 and CD45 and are able to differentiate into SMC and ECs inresponse to different stimuli (Campagnolo et al., 2015; Wong et al., 2013). They play a reparative role in angiogenesis and repairing vascular injury. HDACs are known to play a central role in the regulation of gene expression, which direct key developmental processes such as cell proliferation, migration and differentiation. HDAC7, a member of HDACs family, plays a pivotal role in the maintenance of endothelium integrity. There are 8 splicing variants in mouse HDAC7 mRNAs. Within the non-coding area of some variants, there exists a short open reading frame (ORF). Whether these ORFs can be translated and their roles in cellular physiology remain unclear

This project was originated from our previous finding supporting that one mouse HDAC7 produced a 7aa peptide from the non-coding area, which contains a serine which can be phosphorylated. Herein we intend to confirm the translation of this 7aa peptide and its role in VPCs migration. The hypothesis of the present study is that HDAC7 derived 7aa peptide function as a phosphorylation carrier and promote VPCs migration.

The aim of the present study:

1. To verify that the 7aa peptide can be translated from the first ORF in ECs.

2. To explore the role of 7aa peptide in phosphorylation transfer. Experiment Design：

Firstly, detect the phosphorylation level of 7aa peptide with VEGF stimulate. Then, try to

find the upstream kinase and downstream effector of 7aa peptide based on proteomics study. Additionally, verify the signalling pathway of upstream kinase - 7aa peptide- downstream.

3. To demonstrate the role 7aa peptide in VPCs proliferation, migration and differentiation towards EC lineage and attempt to demonstrated the underlying molecular mechanism.

4. To explore the effect of 7aa peptide in angiogenesis *in vivo*. Matrigel plug assays to detect 7aa peptide ability to induce angiogenesis in a mouse model. Injury mouse model and ischemia model to explore the 7aa peptide ability to promote angiogenesis and injury repairing.

All above not only intent to confirm the existence of the alternative translation event but also suggest that the 7aa may function as a phosphorylation carrier.

Chapter 2

Materials and Methods

2.1Materials

2.1.1 Cell culture reagents

All cell culture media and serum were purchased from Thermo Fisher Scientific (Waltham, MA, USA), while cell culture supplements and growth factors were purchased from Sigma (St Louis, MO, USA).

2.1.2 Recombinant proteins

The peptides of MEKK1 [SRRS(pSER)RIKAPSRNTC] and 14-3-3γ [KRA(pTHR)VVESSEKAYSC] were synthesized and used to raise anti-pMEKK1Ser393 and anti-14-3-3γThr145 antibodies in rabbit by GenScript (Piscataway, NJ, USA). All peptides (see list in table 2.1) and DNA fragments were synthesized by GenScript.

2.1.3 Antibodies and siRNA

The antibodies against CD31 (ab28364), Sca1 (ab51317), 14-3-3γ (ab115176) and MEKK1 (ab55653) were purchased from Abcam (Cambridge, UK). The antibodies against phosphor-Serine (P5872), phosphor-Threonine (P3555), FLAG (F1804) and HA (H6908) were purchased from Sigma. The antibody against GAPDH (sc-25778) and siRNAs [control siRNA (sc-37007), MEKK1 siRNA (sc-35899) and 14-3-3γ siRNA (sc-29584)] were purchased from Santa Cruz (Dallas, Texas, USA). All secondary antibodies were from Dakocytomation (Glostrup, Denmark). All other chemicals were purchased from Sigma. All peptides and DNA fragments were synthesized by GenScript.

Table 2.1 **A list of the 7-aa peptides**

Name	Sequence
7S	MPHASGD
7A	MHSPGAD
7Aa	MHAPGAD
7Ak	MKSPGAD
7Ar	MRSPGAD
7Al	MLSPGAD
7At	MTSPGAD
7Av	MHSVGAD
B-7S	Biotin-MPHASGD
B-7A	Biotin-MHSPGAD
B-7Aa	Biotin-MHAPGAD
B-7Ak	Biotin-MKSPGAD
B-7Ar	Biotin-MRSPGAD
B-7Al	Biotin-MLSPGAD
B-7At	Biotin-MTSPGAD
B-7Av	Biotin-MHSVGAD
7Sp	MPHA{pS}GD
7Ap	MH{pS}PGAD
7Apk	MK{pS}PGAD

7Apr	MR{pS}PGAD
7Apl	ML{pS}PGAD
7Apt	MT{pS}PGAD
7Apv	MH{pS}VGAD
B-c7Ap	MH{pS}GADK-Biotin

2.1.4 Solutions

2.1.4.1 General buffers

Phosphate buffered saline (PBS)	4.3mM Na_2HPO_4
	1.4mM KH_2CO_3
	1.4mM KCL
	pH 7.2
10X Tris buffered saline (TBS)	200mM Tris-HCl
(Used at 1X) 1	0.5M NaCl
	pH 7.6

2.1.4.2 Cell lysis buffers

WB （IP-A）	1mM EDTA
	20mM Tris-HCl pH 7.5
	120mM NaCl
	1% Triton X-100
	1x protease inhibitor Cocktail (Roche)

2.1.4.3 Immunoprecipitation buffers

IP-A	1mM EDTA
	20mM Tris-HCl pH 7.5
	120mM NaCl
	1% Triton X-100
	1x protease inhibitor Cocktail (Roche)
IP-B	1mM EDTA
	20mM Tris-HCl pH 7.5
	120mM NaCl
	1x protease inhibitor Cocktail (Roche)
IP Wash	1mM EDTA
	20mM Tris-HCl pH 7.5
	120mM NaCl
	1% Triton X-100

2.1.4.4 ELISA buffers

PBS/T	*1 x PBS*
	0.01% (v/v) Tween 20
Blocking buffer 1 x PBS	1 x PBS
	2% (w/v) BSA

Substrate solution (phosphate citrate buffer)	50mmol/L phosphate-citrate, pH5.0
	0.03% sodium perborate
	0.17mg/ml O-Dianisidine dihydrochloride
Stop solution	5M HCL

2.1.4.5 SDS-PAGE and Western blotting buffers

5x protein gel sample buffer	β-Mercaptoethanol (5%)
	Bromophenol blue (0.02%)
	Glycerol (30%)
	SDS (Sodium dodecyl sulfate) (10%)
	Tris-Cl (250 mM, pH 6.8)
Running buffer	25mM Tris/Base
	192mM Glycine
	0.1% (w/v) SDS
Transfer buffer	25mM Tris/Base
	192mM Glycine
	20% (v/v) methanol
	pH 8.5
Blocking milk solution	1 x TBS/T
	5% (w/v) skimmed milk (Tesco)
Blocking BSA solution	1 x TBS/T
	5% (w/v) BSA

Stripping buffer(Re-blot)	Purchased from Sigma

2.2 Methods

2.2.1 Cell Culture

Vascular progenitor cells (VPCs) were isolated from the outgrowth of adventitial tissues of mouse arterial vessels as described previously (Iakovou et al., 2005). Briefly, the arterial vessels were harvested from C57BL/6J mice (Charles River, Margate, Kent, UK) and cut into 2mm rings that were placed on gelatin-coated flasks and incubated at 37ºC in humidified incubator supplemented with 5% CO_2 for 6hr. Stem cell culture medium [DMEM (ATCC, Rockville, MA, USA) supplemented with 10 ng/ml recombinant human leukemia inhibitory factor (LIF; Chemicon, Temecula, CA), 10% Fetal bovine serum (FBS, ATCC), 0.1 mmol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin] were added and refreshed every other day until the cells reached 80% confluence. The cells were amplified and subjected to Sca-1+ cell purification using anti-stem cells antigen-1 (anti–Sca-1) immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated Sca-1+ cells was confirmed using flow cytometry. The Sca-1+ VPCs were maintained in stem cell culture medium and split every other day. Up to 5 passages were used in this study.

HEK293 cells were purchased from ATCC and maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). HEK293 cells were used to amplify adenoviruses.

2.2.2 Sca-1 Positive cell sorting

As described in previous studies (X. Yang et al., 2006)，heterogeneous VPCs explanted from vein grafts were sorted using magnetic cell sorting kits (MACS) with anti-Sca-1 immunomagnetic microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). According to the manufacturer's protocol for the Anti-Sca-1 MicroBead Kit, the cultured VPCs were trypsinised and centrifuged at 300g for 10 minutes. Supernatant was removed completely and cell pellets were resuspended in 90 μl of cold buffer (phosphate-buffered saline (pH 7.2), 0.5% FBS and 2 mM EDTA) and 10 μl of Anti-Sca-1-FITC for at most 10^7 total cells. The cells were then incubated in the refrigerator (4 °C) for 10 minutes. After adding 1−2 mL of cold buffer, the cells were washed and centrifuged at 300g for 10 minutes. The cell pellet was then resuspended in 80 μl of cold buffer with 20 μl Anti-FITC MicroBeads and incubated for 15 minutes at 4°C, followed by the same washing step as above. The cells were then resuspended in 500 μl of cold buffer and ready for magnetic separation. Briefly, a column filled with magnetic beads was placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The column was rinsed with 500 μl cold buffer before applying the cell suspension. Unlabelled cells were passed through and collected for further analysis. After the column reservoir was empty, fresh 500μl cold buffer was added to the column and this washing step was performed 3 times. Finally, the column was placed in a 15 ml collection tube and flushed with 1 ml cold buffer, by firmly applying the plunger, to harvest the magnetically labelled Sca-1+ cells. Sca-1+ VPC populations were expanded for up to 5 population doublings.

2.2.3 VPC differentiation

For VPC spontaneous differentiation, VPCs were seeded in fibronectin (Sigma, 5μg/ml)-coated flasks in differentiation medium (alpha-MEM supplemented 10% FBS, 0.05mmol/L 2-mercapotoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin) for three days with medium refreshment at 48hr. For EC differentiation, the 3-day spontaneously differentiated VPCs were subjected to serum free medium in the presence of 0.1ng/ml peptides with or without 5ng/ml mouse VEGF for 4 days. The medium was refreshed every other day.

2.2.4 Adenoviral gene transfer

A 1248 base pair DNA fragment corresponding to the first 1188 nucleotides of mouse HDAC7 transcript variant 2 (NM_001204276.1) with a FLAG and an HA tag inserted into the sORF and main ORF (225aa) respectively (Figure S1A) was synthesized and cloned into pShuttle2 vector, designated as pShuttle2-HD7FH. The resulting Ad-HD7FH vector was created accordingly by using the Adeno-X expression system 1 with the protocol provided (Clontech). The viral particles were amplified in HEK293 cells. For virus infection, the undifferentiated or 3-day spontaneously differentiated VPCs were incubated with Ad-null or Ad-mHD7FH virus at 10 multiplicity of induction in serum free medium containing 0.05mmol/L 2-mercaptoethanol for 6hr, the virus containing medium was removed and fresh complete growth medium was added and incubated for 24hr, followed by further treatment and/or assessments.

2.2.5 Immunofluorescence and histology

2.2.5.1 Immunofluorescence staining of cells

Cells were seeded at a density of $1x10^4$/well in 500μl culture medium in individual chambers of a 8-well chamber slide or cry-slide coated with 0.04% gelatine. After 24 hours, culture

medium was replaced with serum free medium. Following an overnight serum starvation, the cells were treated with relevant stimuli (details will be described in corresponding experiments) and then washed twice with PBS before staining. First the cells were fixed with 4% paraformaldehyde (PFA) for 15 mins at room temperature and washed with PBS, before permeabilising with 0.1% Triton X-100 (Sigma-Aldrich) for 5 mins at room temperature. After washing with TBS 3 times, the cells were blocked with 5% normal serum (Dako) in PBS for 1 hour at room temperature. Incubation of cells with primary antibodies or blocking solution only (negative control) was performed at 4℃ overnight. The primary antibodies used and the dilutions in blocking solution for 1 hour. Then cells were washed with PBS 3 times, each time for 5 minutes. Secondary antibodies (Corresponding fluorescent-conjugated IgG antibodies were used as secondary antibodies (Invitrogen)) were applied on cells for 45 mins at 37℃ and protected from light. Following three 5 minute washes in the dark, the cells were counterstained with DAPI (1:1000 in PBS) for 2 minutes at room temperature. Images were taken by using SP5 confocal microscope (Leica, Germany), and were processed by Adobe Photoshop software. Magnification was indicated in figure legends as scale bars.

2.2.5.2 Harvesting femoral arteries for histology

Euthanasia was performed on each mouse using cervical dislocation. After death, a small incision was made in the right atrium to create an outlet for blood. The mouse was perfused slowly by injecting 20 ml of saline from left ventricle using a syringe with a 25 gauge needle. The clearing of blood in the liver served as an indicator of a good perfusion. Fixation was then performed by replacing saline with 10ml of 4% formaldehyde. The fixation tremors of the whole body were observed within seconds. An incision into the skin was made over the femoral artery, and the distal end of the injured artery was located by identification of the silk

suture for ligation. The artery was excised from surrounding tissues from the distal part, near the suture, to the proximal part, beside the abdominal aorta. The artery was then transferred into 4% formaldehyde solution at 4℃ for the further process of histology. For paraffin section preparation the fixed artery was transferred to 70% ethanol at 4℃ 24 hours before embedding in paraffin blocks. For preparation of frozen sections, dehydration of the fixed arteries was performed in 30% sucrose solution at 4℃ overnight followed by embedding them within Bright Cryo-m-Bed (Bright Instruments). The samples were then placed in liquid nitrogen for fast and adequate cooling before being cut into 5 mm thick sections. Frozen sections were stored at -20℃ before performing histological and immunofluorescence staining to assess the extent of injury and neointima formation.

2.2.5.3 Immunofluorescent staining of frozen sections

The slides containing frozen sections were thawed at room temperature for 30 minutes before staining. Rehydration of frozen sections was performed in PBS for 5 mins to remove tissue freezing matrix. To fix the tissue sections, the slides were immersed in pre-cooled acetone (-20℃) for 10 min at room temperature. After briefly washing twice with PBS, the tissue sections were blocked with 5% normal donkey serum (Dako) in PBS for 1 hour at room temperature. Following removal of the block buffer, incubation of cells with primary antibodies or blocking buffer (negative control) was performed at 4℃ overnight in a humidified chamber. The slides were rinsed thrice in PBS, 5 mins each time before the application of appropriate secondary antibodies for 45 mins at 37℃ protected from light. Following repeated 5mins washes, tissue sections were counterstained with DAPI for 5 mins at room temperature. The slides were mounted with fluorescent mounting media (Dako) and

images were acquired using an Olympus IX81 microscope with Volocity software (PerkinElmer) or an SP5 confocal microscope with Leica LAS AF software.

2.2.5.4 Haematoxylin and Eosin (HE) staining of frozen section

The slides containing frozen sections were thawed at room temperature for 30 minutes before staining. The slides were first immersed in xylene for 10mins and this was then repeated in fresh xylene solution. Rehydration was then performed using the following concentrations of ethanol: 100% ethanol for two 5 minute immersions; 70% ethanol for one 5 minute immersion. The slides were washed briefly in tap water before H&E staining. After blotting excess water from the slides with paper towel, the slides were stained in haematoxalin solution for 5 mins, followed by washing under running tap water for 5 mins. Differentiation of the staining was performed by repeatedly dipping the slides into 1% acid ethanol 5 times. Subsequently, the slides were washed under running tap water for 5-10 mins and it was confirmed that the nuclei had turned blue under the microscope. Next, counterstaining with eosin solution was performed for 5 mins before dehydration. Following brief washing in water, the slides were immersed in first 70% ethanol and then 100% ethanol twice, 2 minutes for each step. The excess ethanol was blotted and dried before the slides were immersed in xylene twice, each time for 5 mins. Finally, the slides were mounted with xylene based DPX mounting medium (Fisher Scientific) and dried overnight. All steps took place in a flow hood.

2.2.6 ELISA

For ELISA performed on cells, the undifferentiated or 3-day differentiated VPCs were seeded in 96-well plated and treated as described in figure legends. The cells were fixed with 4% paraformaldehyde at room temperature for 10min, permeabilized with 0.1% Triton X-100 in PBS for 15min, treated with 3% H_2O_2 for 20min to quench endogenous horse radish peroxidase (HRP), blocked with 10% FBS/PBS for 1hr, followed by incubation with primary antibodies at 37ºC for 1hr and HRP-conjugated secondary antibodies at 37ºC for 45min. The associated HRP was revealed by incubation with phosphate citrate buffer (50mmol/L phosphate-citrate, pH5.0, 0.03% sodium perborate) containing 0.17mg/ml O-Dianisidine dihydrochloride and measured A405nm under Genios Pro-Tecan microtiter plate reader (GeniosPro, veco, USA). For ELISA on beads, the immunoprecipitation beads or biotin-peptide pulldown assay beads were incubated with primary and HRP-conjugated secondary antibodies as described above with the A405nm measured on BioRad SmartpecTM Plus Spectrophotometer (Hercules, CA, USA).

2.2.7 Immunoprecipitation and biotin-peptide pulldown assay

The cells were lysed in lysis buffer [10mmol/L Tris-Cl pH 7.5, 120mM mmol/L NaCl, 1 mmol/L EDTA pH 8.0, 1% Triton X-100 plus protease inhibitors (Roche)] by rotation at 4°C for 1hr. The protein concentration was measured with Bio-Rad reagent with protocol provided. For immunoprecipitation assay, one mg cell lysate was incubated with 2µg primary antibody or normal IgG and three volume of Triton X-100-free lysis buffer on a rotator at 4°C for 2hr, and then 10µl Protein G-agarose beads (Sigma) was added and incubated for another 2hr, followed by PBS washing and ELISA assays. For biotin-peptide pulldown assay, 5µg Biotin-labelled peptides were incubated with 50µg cell lysates and three volume of

Triton X-100-free lysis buffer at 37°C for 1hr, and then 10µl Streptavidin-agarose beads (Sigma) was added and incubated for another hour, followed by 6 times washing with lysis buffer containing 0.5% Triton X-100. The beads was incubated with 1xSDS loading buffer (10mmol/L Tris-Cl pH 7.5, 120mM mmol/L NaCl, 2% SDS, 5% glycerol, 0.025% Bromophenol blue, 1% 2-mercaptoethanol) 95℃, 5mins. The eluate was subjected to SDS-PAGE, followed by Western blot or proteomics analysis. The beads were washed with lysis buffer containing 1% SDS for 6 times, followed by ELISA assay.

2.2.8 In gel phosphorylation

The Recombinant 14-3-3γ protein (H00007532-P01) was purchased from Novus Biologicals (Littleton, CO, USA). One hundred nanogram of 14-3-3γ protein was diluted in 250µl 0f 1xSDS loading buffer and 25µl per lane of the diluted 14-3-3γ protein was applied to SDS-PAGE. One lane together with the protein marker was cut and subjected to Silver staining (Thermo Fisher Scientific) with protocol provided. The 14-3-3γ protein bands in the remaining gel were cut and incubated in Novex Zymogram Renaturing buffer (Thermo Fisher Scientific) at room temperature for 1hr. After washing with TBST buffer (25mM Tris-Cl pH 7.5, 120mM NaCl, 1 mM EDTA pH 8.0, 0.1% Tween 20) for 3 times, the gel bands were incubated in equeal volume of TBST containing 2ng/ml peptides at 37°C for 30min. The bands were re-organized and transferred to Hybond PVDF membrane (GE Health), followed by standard Western blot procedure.

2.2.9 siRNA knockdown assay

The VPCs were sub-cultured 1:3 on fibronectin-coated 25ml-flasks in differentiation medium. Twenty four hours later, the cells were washed twice with and incubated serum free DMEM containing 0.05mmol/L 2-mercaptoethanol for 1hr. Ten µl of 10 µmol/L siRNA/flask was introduced into the cells with lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific) according to the protocol provided. The transfected cells were further cultured in stem cell medium for 72 hours, followed by VEGF treatment and migration assays. For EC differentiation assay, the transfected cells were cultured in serum free α-MEM medium in the presence of 0.1ng/ml peptides with/without 5ng/ml VEGF for 4 days, followed by EC marker analysis. During the EC differentiation, additional transfection with siRNAs was performed 48hr post the first transfection.

2.2.10 Transwell chemotaxis assay

The VPCs were harvested using trypsin/EDTA and subsequently seeded into the insert of the transwell unit with 8.0 micron pore membrane filters (Becton Dickinson Labware, USA) at 5x104 cells/200µl of serum free medium. Eight hundreds of serum-free medium containing peptides (1ng/ml) with/without VEGF (5ng/ml) were added to the holder of the unit. PBS and 1% BSA were used as control for peptides and VEGF respectively. Six hours later, the non-migrating cells inside the inner side of the insert were carefully washed with PBS and removed using a cotton tip applicator. The VPCs on the outside of the filter membrane were fixed with 4% PFA for 15 mins before staining with 0.1% crystal violet solution at room temperature for 15 mins. The cells were observed under Nikon Eclipse TS100 microscope with Nikon Ph1 ADL 20x/0.4 lense and the images were taken using Nikon DS-Fil camera and Nikon Digital Sight System and processed by Adobe Photoshop software. Data was expressed as the mean number of migrated cells in 10 fields (Figure 2.1) of each insert x 3.

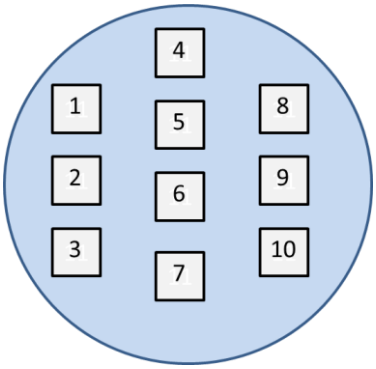


Figure 2.1 Layout of fields in each view of a transwell insert at 20x magnification

2.2.11 Scratch wound healing assay

VPCs were seeded in fibronectin-coated 12-well plates at $5x10^4$ cells per well in growth medium for 3 days with medium refreshment every other day. The cells would reach confluence after three days. Three horizontal x 2 vertical scratches were made using a 1ml pipette tip in each well. The wells were gently washed twice with PBS to remove cell debris. 1ml serum-free medium containing 1ng/ml peptide or PBS was added in triplicate. Mark 6 sites for image taking. The cells were observed under Nikon Eclipse TS100 microscope with Nikon Ph1 ADL 20x/0.4 lense and the images were taken using Nikon DS-Fil camera and Nikon Digital Sight System at 0hr, 12hr, 24hr and 36hr post-scratching, and processed by Adobe Photoshop software. Data was expressed as the mean percentage covered area by migrated cells in 6 fields of view x3.

2.2.12 Quantitative reverse transcriptase-polymerase chain reaction

Total cellular RNAs were extracted using RNeasy Mini kit (Qiagen, Manchester, UK) according to the protocol provided. One μg RNA was reverse transcribed into cDNA with random primers by MMLV reverse transcriptase (Qiagen). Using SYBR master mix (Thermo

Fisher Scientific) and Eppendorf MarsterCycler Cpgradient S (Eppendorf, Enfield, CT, USA), quantitative polymerase chain reaction was performed with 20ng of cDNA (relative to RNA amount) to amplify CD31, CD144 , SM22 and GAPDH.

Table 2.2 Primers for Real Time Polymerase Chain Reaction (PCR)

Specificity	Sequence(5'-3')
CD31 forward	5>gga gtg cct tgt gga cat cag<3'
CD31 Reverse	5'> tgc acg gtg acg tat tca ctc<3'
CD144 forward	5'>gtg cct gaa gac atc cga gtg<3'
CD144 Reverse	5'>gac ctc tgt cac tgg tct tgc<3'
SM22 forward	5'>agt gga ttg tag tgc agt gtg<3'
SM22 Reverse	5'> cag ttg gct gtc tgt gaa gtc<3'
GAPDH forward	5'>cat gtt tgt gat ggg tgt ga<3'
GAPDH Reverse	5'>aat gcc aaa ctt ctc atg ga<3'

2.2.13 Western Blotting (WB)

2.2.13.1 Whole cell protein extract preparation

Whole cell extracts were prepared from VPC in order to investigate events involved in their intracellular signaling pathways using WB. Generally, 1-2x10^5 VPC were washed with dPBS and lysed in 100µl IP-A buffer on ice for 10mins. Subsequently, lysates were collected into 1.5ml eppendorf tubes (Appleton Woods, Birmingham UK), spun, and lysates without cell debris were transferred into new 1.5ml eppendorf tubes. Cell lysates were either stored at -80°C or analysed.

2.2.13.2 Protein concentration measurement

Protein concentration of cell lysates were measured using a BCA™ protein assay kit according to the manufacturer's instructures. Firstly, 2µl of cell lysates were diluted with 8µl of dH_2O before being transferred into a 96-well Maxisorb plate (Nunc, Denmark) in duplicates, alongside a series of BSA concentrations as standards. BCA solutions were mixed at a volume ratio of 50:1 (reagent A:B) before adding 200µl into each well and incubated for 1 hr at 37°C. Absorbance was read at 710nm on a Multiskan Biochromatic™ plate reader using Ascent™.

2.2.13.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

Prior to SDS-PAGE samples were denatured by the addition of 5x gel sample buffer and subsequently boiled at 95°C for 5 mins. The denatured samples were loaded into a Nu-PAGE Bis-Tris gel prior to electrophoresis for 2 hrs at a voltage of 120V for separation of proteins. Following separation with SDS-PAGE, the proteins can be transferred onto a membrane for identification with specific antibodies. The gel was placed on two layers of wet filter paper, followed by a polyvinylidene difluoride (PVDF) membrane that had previously been hydrated with 100% methanol and equilibrated in transfer buffer (Refer Section 2.1.5.5). The membrane was in turn overlaid with additional two layers of wet filter paper, and the assembly was subsequently placed into a transfer cassette (Invitrogin, U.K.). The transfer was carried out at a constant voltage of 35V for 1.5 hrs, in a tank (Biorad, U.K.) filled with pre-cooled transfer buffer (4ºC).

2.2.13.4 Detection of proteins

Following transfer, the membrane was blocked in 5% (w/v) instant skimmed milk powder in TBS/T for 1hr and washed 3x with TBS/T. Subsequently, the membrane was incubated with primary antibody in 5% (w/v) BSA or milk for 1hr at RT before washing again. The membrane was then incubated with a secondary HRP-conjugated antibody in 5% (w/v) milk for 1hr at RT prior to detection using chemiluminescent substrate solutions, ECL™ or ECL™ plus. The protein bands were then visualised using Hyperfilm™. Membranes were usually re-probed with new antibodies after removal of the previous one by treatment with 10mls of stripping buffer for 15 mins and re-blocking.

2.2.14 Cell proliferation assays

2.2.14.1BrdU incorporation assay

The 3-day differentiated VPCs were treated different concentration of 7S or 7A peptides in the presence of 10ng/ml VEGF in serum free α-MEM medium for 24hr. The VPC proliferation assay was performed using a Cell Proliferation ELISA, BrdU (colourimetric) (Roche). BrdU labelling reagents (final concentration of 10 μM) were added 6hr prior to harvesting the cells for BrdU incorporation assays. After removing the labelling medium, 200μl of Fix Denat was added in each well for 30 mins at room temperature. The solution was then removed thoroughly before incubating with BrdU conjugated antibody for another 90 mins. After 3 washes with PBS, 100μl of substrate solution was added and incubated at room temperature. Once a change in colour was detected, 25μl of 1M H_2SO_4 was added to stop the reaction. The absorbance was measured at 450nm with correction at 690nm within 5mins of adding the stop solution. A mixture of BrdU labelling solution and its antibody in culture medium was used as blank to identify the unspecific binding of BrdU and antibody to

the plate. The wells in which cells had not been incubated with BrdU but were incubated with antibody were used as background controls to evaluate the unspecific binding the antibody to VPCs.

2.2.14.2 Limited dilution assays

The VPCs were detached with trypsin and prepared as single cell suspension. The cell suspension was diluted to 10 cells/ml in normal culture medium and aliquoted into 96-well plates with 100μl/well cell suspension containing 1ng/ml peptides. The cells were cultured for 7 days with medium refreshment every other day. The peptides were maintained at 1ng/ml for the whole process. The cells were then fixed with methanol (40%)/acetic acid (10%) and stained with Giemsa solution (Sigma). Colony formation was observed and calculated under microscope. Colonies with more than two cells (including two cells) were calculated. The area of each colony was calculated with Adobe Photoshop software and presented as the average area occupied by each single cell.

2.2.14.3 Clonogenic assay

The VPCs were detached with trypsin and prepared as single cell suspension. The cell suspension was diluted to 50 cells/ml in normal culture medium and aliquoted into 6-well plates with 2 ml/well cell suspension containing 1ng/ml peptides. The cells were cultured for 3 days. The staining and calculation protocal is the same as Limited Dilution Assays.

2.2.14.4 MTT assay

The 3-day differentiated VPCs were seeded into fibronectin-coated 96-well plates in differentiation medium at $2x10^3$cells/well. Twenty four hours later, the cells were treated with serum-free medium containing 50μm and 100μm H_2O_2 in the presence of 1ng/ml peptides for 24hr, followed by Methyl thiazolyl tetrazolium (MTT) assay with CellTiter 96 Queous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol. The absorbance at A490nm was measured on BioRad SmartpecTM Plus Spectrophotometer. PBS was included as control for H_2O_2 and peptides. Data was presented as relative A490nm with that of PBS control group set as 1.0.

2.2.15 *In vitro* tube formation assay

The procedure used was similar to that described previously (Kaufman et al., 2004). One hundred of cell suspension containing $4x10^4$ (total cell number) the 7-day differentiated VPCs was placed on top of the 50μl/well Matrigel (10 mg/ml; Becton Dickinson Labware, Bedford, MA, USA) in 8-well chamber slides (Nalge Nunc, Naperville, IL, USA). Rearrangement of cells and the formation of capillary-like structures were observed under Nikon Eclipse TS100 microscope with Nikon Ph1 ADL 20x/0.4 lense and the images were taken using Nikon DS-Fil camera and Nikon Digital Sight System at 8-18 hours and processed by Adobe Photoshop software. Data was presented as mean of tube length of each 20x view from 6x3views.

2.2.16 *In vivo* experiment

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals.

2.2.16.1 *In vivo* Matrigel plug assay

300µl of Matrigel containing 10ng/ml of 7A or 7S peptides or PBS were injected subcutaneously into the different sites in the back of each of the 12 C57BL/6J mice. The Matrigel plugs were harvested from the six mice sacrificed at day 7 and day 14 respectively post-inoculation and subjected to cry-sectioning, followed by immunofluorescence staining with anti-CD31 antibody.

2.2.16.2 Femoral artery injury model

The femoral artery injury model was introduced into ApoE-/- mice (Charles River) as described previously (Hu, Davison, Zhang, & Xu, 2003). The 100µl of 25% Pluronic-127 gel containing 10ng/ml peptide or PBS was applied surrounding the injured vessels. The injured vessels were harvested 4 weeks post-surgery and cryo-sectioned, followed by H&E and immunofluorescence staining.

Mice were weighed and anaesthetised by intraperitoneal injection of ketamine (75 mg/kg; VetalarTMV, Pfizer) combined with medetomidine hydrochloride (1 mg/kg; Domitor®, Orion). A pinch test was performed on the tail of mouse to confirm that it was fully anaesthetised. Lubricating ointment was applied to the eyes of mice to prevent corneal desiccation. Hair was removed by application of a depilatory cream from both legs to lower abdomen. The mouse was then put on an aseptic operation board and covered with an aseptic drape. The depilated area of skin was sterilized with 70% ethanol and dried with sterile cotton

swabs just before surgery. An incision into the skin over the femoral artery was made before locating femoral artery by blunt dissection of surrounding tissues. The tissues were moistened periodically using saline for irrigation. The femoral artery was isolated by gently separating the femoral nerve and vein from the femoral artery, in sequence using micro-forceps. The anterior branch of the femoral artery was looped with a 10-0 silk suture before performing an arteriotomy using a 30 gauge needle at the distal part of the bifurcation. The opening of the arteriotomy was lifted with micro-forceps before a 0.25 mm guide wire (CROSS-IT 100XT, HI-TORQUE) was introduced into the artery until it could be inserted no further. The wire was inserted and retracted 5 times before it was allowed to remain in the femoral artery for 3 mins. After gently and slowly removing the wire, the artery was immediately ligated. The same procedure was applied on the other femoral artery of each mouse. Then, 100 µl of 25% Pluronic-127 gel containing 1 ng/ml peptide or PBS was applied surrounding the injured vessels. The wound was closed after the matrigel had solidified. Anaesthesia was reversed with atipamezole hydrochloride (5 mg/kg; Antisedan, Orion) after surgery. Mice were allowed to recover in a warm container. In all experiments, mice were monitored continuously during recovery and periodically for 2 days. The injured vessels were harvested 4 weeks post-surgery and cryo-sectioned followed by HE and immunofluorescence staining.

2.2.16.3 Hindlimb ischemia model:

The hindlimb ischemia model was performed in C57BL/6J mice as described previously (L. Zeng, Xiao, et al., 2013). The right femoral artery was ligated permanently, i.e. the femoral artery was ligated at two adjacent sites with the middle part cut. The 100µl of 25% Pluronic-127 gel containing 10ng/ml peptide or PBS was applied surrounding the injured vessels. The foot blood flow was measured with LDI Doppler laser scanner (Moor Instruments, Devon,

UK) at 30min, 1 week and 2 weeks post-surgery. The reperfusion percentage was defined as the ratio of mean measurement in the foot area of ligated hindlimb to that of contralateral unligated hindlimb. The data was processed with Excel software. The flow image was processed with Adobe Photoshop software. The mice were sacrificed humanely after flow measurement at 2 weeks post-surgery. Adductor muscle tissues of ligated side were harvested and cryosectioned, followed by immunofluorescent staining with anti-CD31 and Sca1 antibodies.

2.2.17 Statistical analysis

Data expressed as the mean±SEM were analyzed using GraphPad Prism 5 software with t-test for pair-wise comparisons or analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests and significance was depicted by asterisks, *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$. A value of $p<0.05$ was considered to be significant.

Chapter 3

Results

Part A:

3.1 HDAC7 transcript variant 2 undergoes alternative transcription and translation

3.1.1 Introduction

Different from prokaryotic species, the genetic information stored in a gene is discontinuous in eukaryotic species. The genetic information containing sequence is called exon, while the interrupting sequence is called intron. For most of the genes, the expression of a gene is derived the transcription from a unique promoter. However, some genes like HDAC7 have more than one promoter region, leading to alternative transcription selection. After transcription, the nascent RNA molecules are called precursor RNAs from which the introns have to be excised, a so-called splicing event. Multiple different excision form may occur, which is called alternative splicing. Alternative splicing is a specific feature in eukaryotic species. Through alternative transcription and/or splicing, different mRNA species can be derived from a single gene, giving rise to different protein variants with different even opposite functions.

In general concept, a single mRNA molecule give rise to one peptide or protein via translation initiated from ATG codon. Actually, there are multiple open reading frames (ORF) with different size within a mRNA molecule. Whether only the main one can be translated remains unclear. Recent studies have shown that translation of a peptide/protein can be initiated from codons other than ATG. For example, CTG takes up to 15% translation. The selection of different ORFs or start codons is called alternative translation, which further increases diversity over gene information.

Due to alternative tanscribed and splicing, HDAC7 mRNA has 4 alternative splicing variants in human and 8 in mouse. Mouse HDAC7 mRNA transcript variant 2 (HDAC7-tv2) has a short open reading frame(sORF) before the main ORF that gives rise a functional HDAC7 protein within the first 350 nucleotides in 5' terminal area. These two ATG codons are

separated by about 120 nucleotides. The sORF gives rise to a 7-amino acids peptide, a very short open reading frame, which is followed by three sequential stop codons.

3.1.2 Hypothesis and Aims

Alternative translation could occur from the short open reading frame (ORF) which produces a 7-amino acids peptide (7A), a very short open reading frame in HDAC7-tv2.

The aim of this chapter is to verify that the 7aa peptide can be translated from the first ORF in ECs.

3.1.3 Results

3.1.3.1 VEGF induces HDAC7 variants 2 transcription.

In genetics, a promoter is a region of DNA that initiates transcription of a particular gene. Promoters are located near the transcription start sites of genes, on the same strand and upstream on the DNA(Gagniuc & Ionescu-Tirgoviste, 2013). HDAC7 mRNA can be transcribed from different promoters and undergo alternative splicing, giving rise to 4 transcript variants in human and 8 in mouse. In mouse, these 8 variants are derived from different promoters. HDAC7 - tv1, tv5, tv6 and tv7 are derived from promoter 1(P1), while HDAC7 – tv2, tv3, tv4 are from promoter 2(P2). In addition, the promoter of HDAC7– tv8 is different from the others, defined as promoter 3(P3)(Figure 3.1).

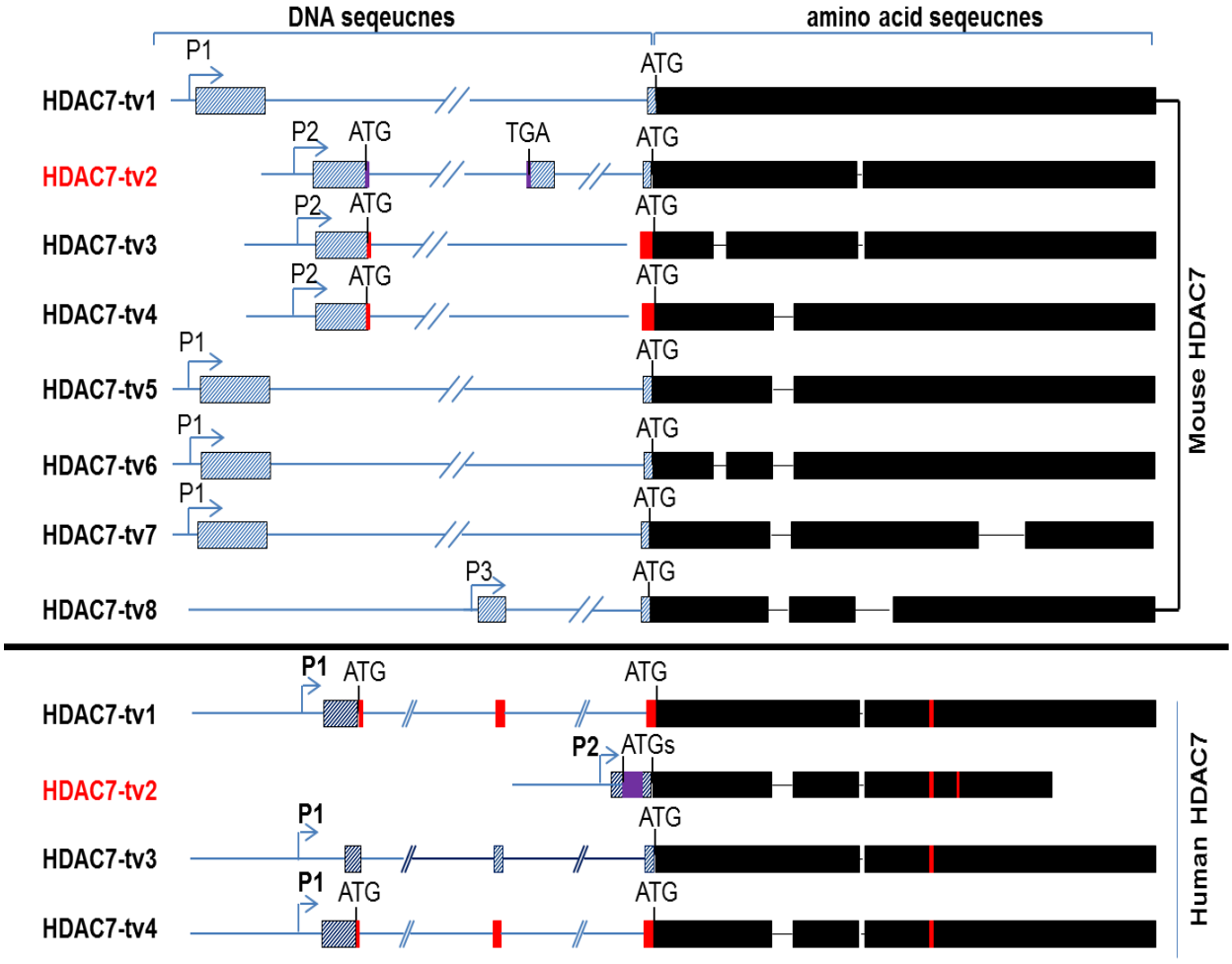


Figure 3.1 Structure of HDAC7 mRNA variants.

There are 8 transcript variants(tv) in mouse and 4 in human, which contain different promoter respectively. Mouse HDAC7 - tv1, tv5,tv6 and tv7 contain promoter 1(P1), while HDAC7 – tv2, tv3, tv4 initiate transcription from promoter 2(P2). In addition, the promoter of HDAC7–tv8 is different from the others, so we define it as promoter 3(P3). Human HDAC7-tv1, tv-3 and tv-4 contain promoter 1(P1), and only HDAC7-tv2 initiate transcription from promoter 2(P2).

To identify the transcription source, we designed specific primers from exon 1s that are derived from different promoters and performed RT-qPCR. As shown in Figure 3.2, VEGF treatment increased transcription from promoter P2 but decreased from P1 in mouse differentiated VPCs, indicating that the transcription of HDAC7 undergoes a shift from P1 to P2 during the differentiation.

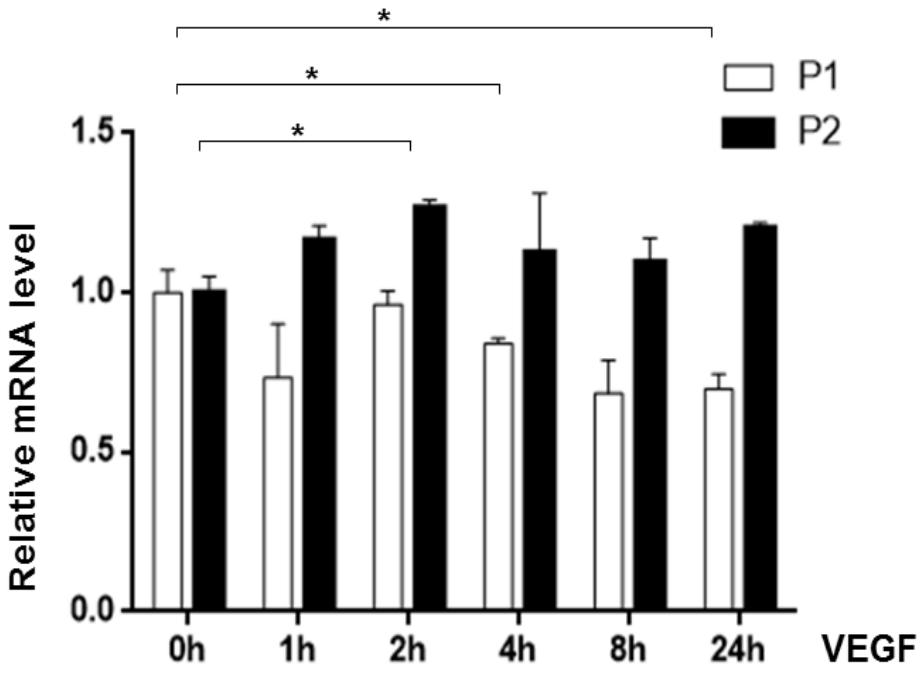


Figure 3.2 VEGF increase Mouse HDAC7 promoter 2 transcription.

The 3-day differentiated VPCs were treated with 5ng/ml VEGF for 0,1,2,4,8,24 h separately, followed by RT-qPCR analysis of transcripts level from P1 and P2. Data presented are mean of three independent experiments. *: $p<0.05$.

3.1.3.2 Alternative translation occurs from the first and second ORF within HDAC7 mRNA

Normally, translation of an mRNA initiates from the first ATG start codon. However, the translation is also initiated from the second or third start codon. As described in introduction, in the 5' untranslated region (UTR) of mouse HDAC7-tv2 (HDAC7U), there is a short ORF (sORF) encoding a 7aa peptide with three cascade in-frame stop codons. This HDAC7 mRNA exists in mouse ESCs and ECs. When the ESCs are induced to differentiate toward smooth muscle cell lineage, a 57 nucleotide sequences will be excised from the mRNA, within which the three stop codons are removed. The two open reading frames are joined together, giving rise to a bigger HDAC7 with 22 amino acids more (HDAC7F, F for fully spliced). The 7 amino acid peptide becomes the far N-terminal end of the bigger HDAC7 protein (Figure 3.3A). During the previous study on HDAC7 and SMC differentiation (Margariti et al., 2009), we compared the effect of different HDAC7 variants on EC marker expression. Theoretically, HDAC7U and HDAC7S should have a similar effect, as they both express the same HDAC7 protein. However, Over-expression of HDAC7U had less suppressive effect on EC marker expression as compared to HDAC7S although both variants expressed the same amount of HDAC7 proteins, suggesting that the non-translated area may give rise to a 7-amino acid (7aa) peptide from the first open reading frame (ORF). To test this, synthesized 7aa peptide (7A) was added to HDAC7S transfected cells and a scrabbled 7aa peptide (7S) was included as control. As shown in Figure 3.3B, addition of the peptide 7A reversed the inhibitory effect of HDAC7S, even increased EC marker expression. These results suggest that 7A peptide may be translated from the first ORF. However, the difference of EC marker expression might be from the plasmid itself, so HDAC7 protein

level should be measured in HD7U and HD7S transfected cells, which will explore in future work.

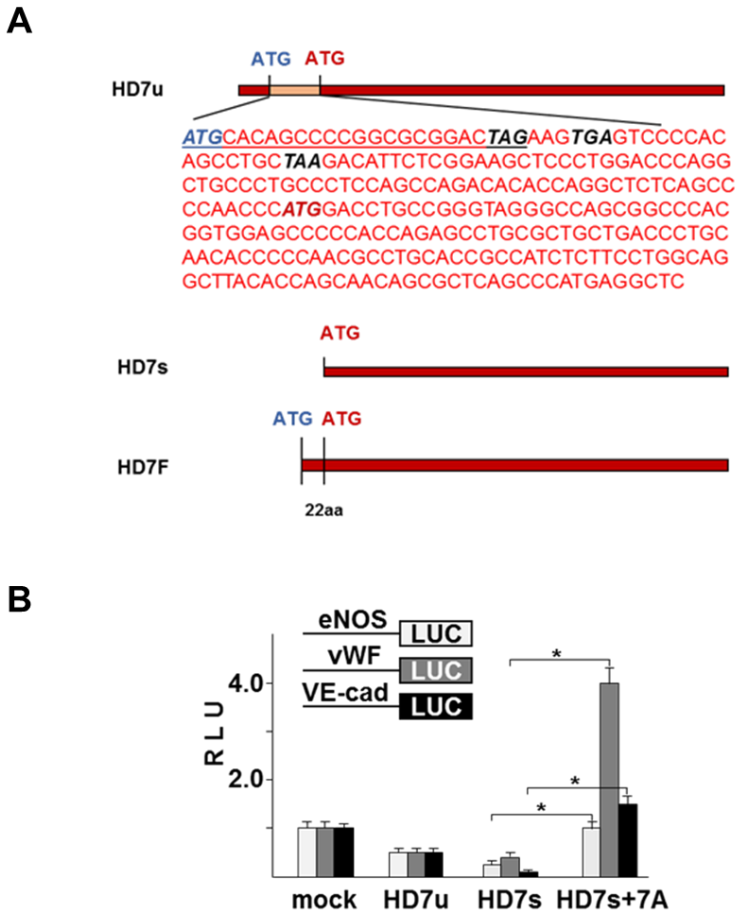


Figure 3.3 7A induces EC marker expression.

(A) A schematic illustration of the partially spliced HDAC7 (HD7u) and short HDAC7 (HD7s). The mRNA sequence of the 5' non-coding area in HD7u was shown in orange box and red characters with the short ORF underlined. The main ORF was shown in brown characters and box. ATG stands for start codon, TAG, TGA and TAA stand for stop codons. (B) Inclusion of synthetic 7aa-peptide (7A) could reverse the suppressive effect of HD7s on EC marker transcription. Embryonic stem cells were transfected with EC marker gene firefly luciferase reporter together with pShuttle-HD7u (HD7u), or pShuttle-HD7s with/without 7A. pShuttle2 empty vector was included as control (mock). Renilla-luc was included as internal control. RLU was defined as the ratio of firefly luciferase activity to that of Renilla luciferase with mock control set as 1.0. Data presented are mean of three independent experiments. *: $p<0.05$.

These findings suggest that the ATG start codon in sORF may initiate translation. Therefore, we wondered whether the sORF could be translated alone. As a peptide with only seven amino acids was not easy to be detected by routine methods, we created an artificial construct containing the whole 5'UTR and 225aa of the main ORF, designated as pShuttle2-HD7FH (Figure 3.4 A). In this plasmid, a FLAG tag was inserted upstream of the stop codon in sORF and an HA tag was fused to the 225aa of the main ORF. The expression of FLAG and HA can reflect the translation of short and main ORFs respectively. A 800 base pair DNA fragment containing Flag and HA sequences within the first and second ORF respectively was synthesized and cloned into pUC57 vector by Invitrogen. The band of interest DNA fragment released by restriction digests is shown on Figure 3.4B. The DNA fragment was then subcloned into the NheI/XbaI site of pShuttle2 vector, and then Ad-mHDAC7FH vector was created by using Adeno-X expression system 1 and viral particles were prepared with the protocol provided (Clontech). The verified bands are shown on Figure 3.4 C.

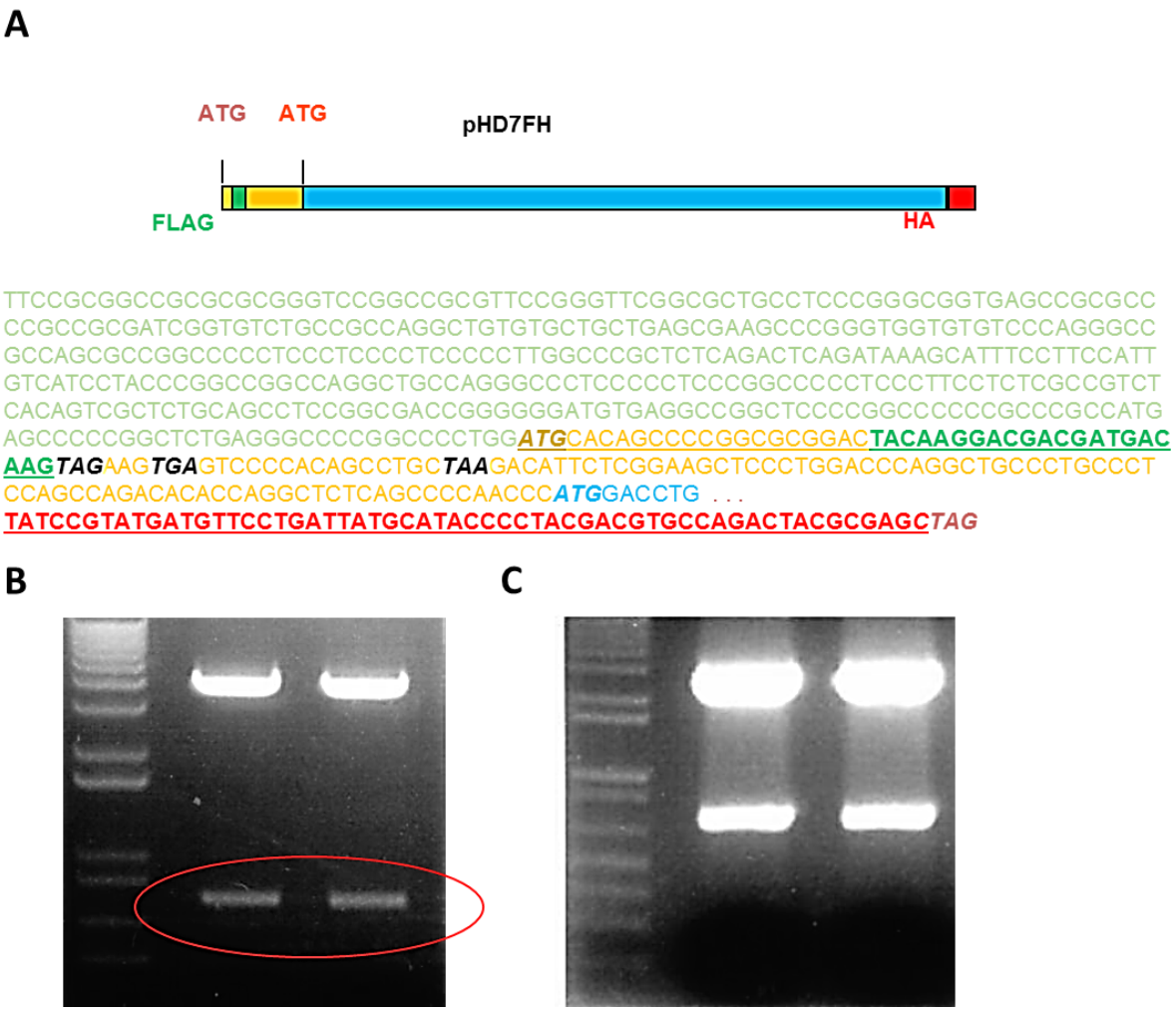


Figure 3.4 Constructure of pShuttle-HD7u-FLAG-HA vector (HD7-FH).

(A) A schematic illustration of pShuttle-HD7u-FLAG-HA vector (HD7-FH). A FLAG (bold green) and HA (bold red) sequence was inserted into the short (underlined) and main ORF (blue, 225aa downstream start codon), respectively. A TAG stop codon was included downstream of HA tag. (B) DNA fragment containing Flag and HA sequences released by restriction digests. (C) Argrose gel running verify the pShuttle-HD7u-FLAG-HA vector.

Double immunofluorescence staining detected FLAG and HA within a single cell will give us information on whether translation is initiated from the sORF or main ORF. In Ad-HD7FH-infected VPCs, both Flag and HA staining can be detected, indicating that the sORF indeed can be translated. ELISA with anti-FLAG and anti-HA antibodies revealed that VEGF might induce the translation shift from the main ORF to sORF, as the ratio of FLAG to HA tag was significantly increased in response to VEGF-treatment (Figure 3.5).

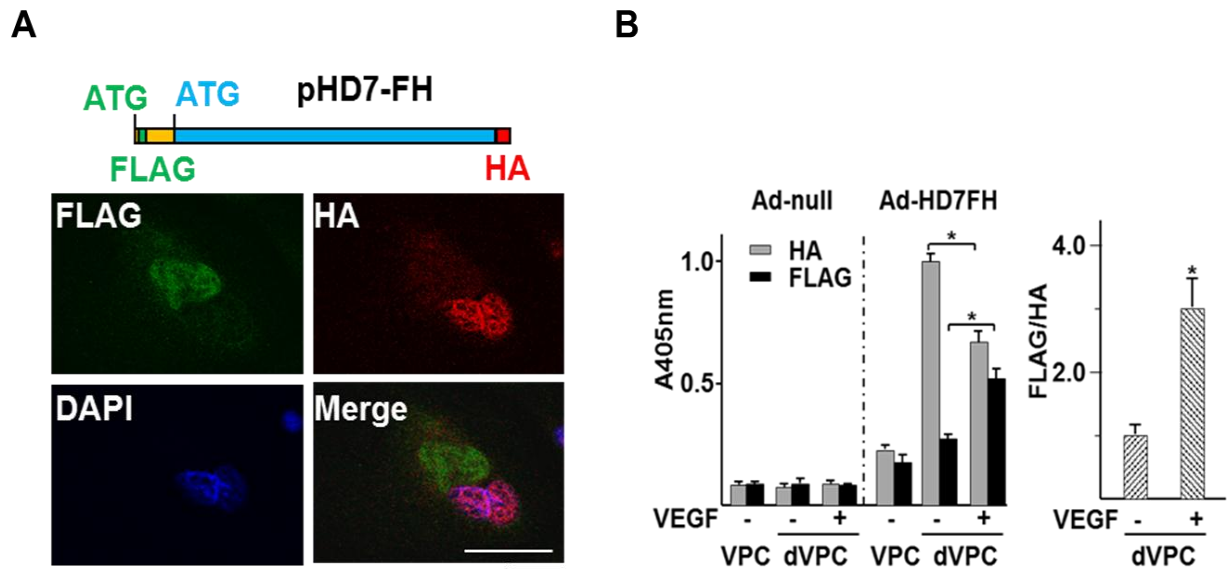


Figure 3.5 VEGF increased the 7aa-peptide translation.

(A) The short open reading frame in the 5' non-coding area could be translated in VPCs as revealed by immunofluorescence staining of 7aa-peptide-FLAG fusion peptide in pHD7-FH vector transfected VPCs. (B) VEGF increased FLAG-tagged 7aa-peptide expression. FLAG and HA expression was detected by ELISA in pHD7-FH vector infected 3-day spontaneously differentiated VPCs (dVPC). VPC: undifferentiated VPCs. 1% BSA was included as vehicle control. Data presented are representative images or mean of three independent experiments. *: $p<0.05$; **: $p<0.01$.

As descried above, 7A can be translated from the sORF and enhance EC marker expression during the differentiation of ESCs. To test whether this 7A has pro-angiogenesis effect, Matrigel plug assays were performed in C57BL/6J mice via subcutaneous implantation of peptide-containing Matrigel, followed by observation of CD31 positive cells in plug section harvested at day 7 and 14 post-implantation. At day 7 post-implantation, very few CD31 positive cells could be observed in PBS group, and only a few CD31 positive cells but no capillary structures were present in 7S group. In contrast, several capillary structures could be observed in 7A group. At day 14 post-implantation, only a few CD31 positive cells and capillary structures could be observed in both PBS and 7S groups, but multiple capillary structures were observed in 7A group (Figure 3.6). These results suggest that 7A peptide can indeed induce angiogenesis *in vivo*.

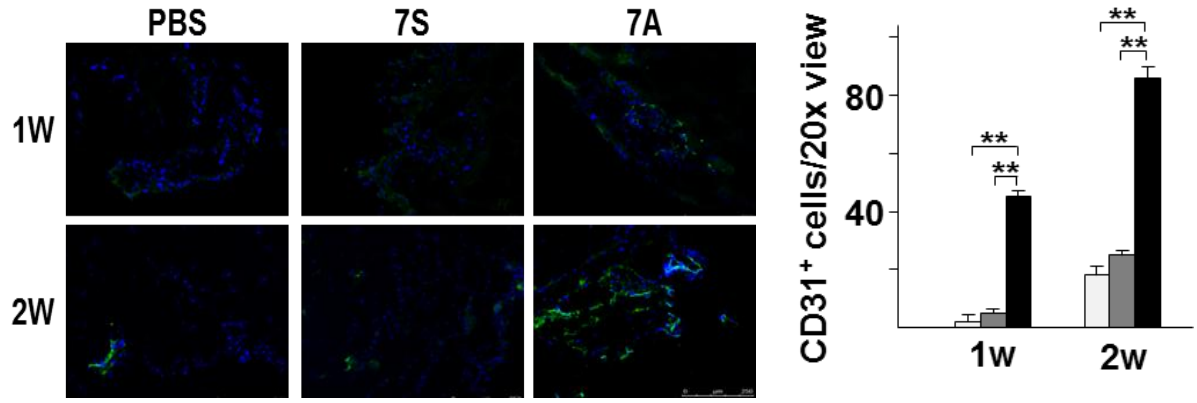


Figure 3.6 The 7aa-peptide increased angiogenesis *in vivo*.

The 7A peptide induced CD31 positive capillary vessel formation in Matrigel plug implanted subcutaneously in C57bl/6 mice. Left panel shows CD31 staining on Matrigel plug sections harvested at 1 week (1W) and 2 weeks (2W) post-inoculation. Right panel shows the average CD31 positive cells/20x view from 6 plugs for each peptide. PBS was included as control. . Data presented are representative images or mean of three independent experiments. *: $p<0.05$; **: $p<0.01$.

3.1.4 Discussion

For transcription to take place, the enzyme that synthesizes RNA, known as RNA polymerase, must attach to the DNA near a gene. Promoters contain specific DNA sequences such as response elements that provide a secure initial binding site for RNA polymerase and for proteins called transcription factors that recruit RNA polymerase. These transcription factors have specific activator or repressor sequences of corresponding nucleotides that attach to specific promoters and regulate gene expression(Gagniuc & Ionescu-Tirgoviste, 2013). In eukaryotes, transcription is more complicated than in bacteria, in part because eukaryotes have the three classes of RNA polymerase that transcribe different sets of genes. Promoters represent critical elements that can work in concert with other regulatory regions (enhancers, silencers, boundary elements/insulators) to direct the level of transcription of a given gene. In eukaryotes, the "core" promoter for a gene transcribed by pol II is most often found immediately upstream (5’) of the start site of the gene(Smale & Kadonaga, 2003). Most pol II genes have a TATA box (consensus sequence TATTAA) 25 to 35 bases upstream of the initiation site, which affects the transcription rate and determines location of the start site(Gagniuc & Ionescu-Tirgoviste, 2012).

In mouse, HDAC7 mRNA has 8 transcript variants which are derived from different promoters. HDAC7 - tv1, tv5, tv6 and tv7 use P1, while HDAC7 – tv2, tv3, tv4 are transcribed from promoter 2 (P2). VEGF induced P2 transcription in differentiated VPCs, indicating that VEGF might promote tv2 translation to produce more 7 amino acid which only exists on P2 tvs. Then the further experiment verified that VEGF not only influence the transcription of HDAC7 but also regulate its translation level with increasing the sORF translation to produce more 7aa-peptide. For the translation initiation, ribosome assembles around the target mRNA. Eukaryotic ribosomes are known to bind to transcripts in a mechanism unlike the one involving the 5' cap, at a sequence called the internal ribosome

entry site (IRES). Ribosome recruitment happens when eukaryote initiation factors elF4F and poly(A) binding protein (PABP) recognize the 5' capped mRNA and recruit the 43S ribosome complex at that location. Following recruitment of the ribosome, translation initiation happens at the start codon found within the Kozak consensus sequence ACCAUGG (Kozak, 1987). Since the Kozak sequence itself is not involved in the recruitment of the ribosome, it is not considered a ribosome binding site (Hellen & Sarnow, 2001). In HDAC7 mRNA we found the main ORF contains a Kozak sequence which might results the translation skipping. VEGF increases sORF translation may via disturbing the recognition of the Kozak sequence in main ORF or recruit specific factors in upstream of sORF, and we will explore in the further work.

Our previous study revealed that VEGF suppressed HDAC7 transcription and induced a rapid and transient degradation of HDAC7 (Margariti et al., 2010). Our group previous study demonstrated that VEGF treatment rapidly decreased endogenous HDAC7 protein via protein degradation, while U73122 and IP3 partially block VEGF-induced HDAC7 protein degradation, which indicate that VEGF induces HDAC7 degradation through the PLCγ-IP3K signal pathway. However the mechanism of VEGF suppressed HDAC7 transcription was still unknown. Herein, we found that VEGF regulates HDAC7 transcription by suppressing Promoter 1 transcription. Thus, we postulated that VEGF suppressed HDAC7 transcription might through decreasing HDAC7 promoter 1 variants transcription. Furthermore U73122 and IP3 only partially block VEGF-induced HDAC7 protein degradation, decreased HDAC7 protein might be partially due to the shift translation from HDAC7 protein to 7aa-peptide as well.

As there is no commercially available way to detect a 7aa peptide, we created a mutated HDAC7 construct mHD7-FH, in which a Flag tag was inserted into the first ORF exactly up-stream of the stop codon and an HA tag was inserted into the second ORF. We choose Flag

and HA tag not GFP and YFP which can measure directly. For 7 amino acid is very short, GFP or YFP are much longer than 7amino acid, which probably introduce artificial results. Flag and HA tag is relatively small, therefore we choose them to make the results reliable. As we transfected the plasmid to VPCs, both of the HA and the Flag signal were very low. For the progenitor cells are difficult to transfect or infect. So VPCs undergo 3 days spontaneously differentiation before the transfection. The Matrigel model staining results showed that 7A can promote angiogenesis. However, how does 7A increase angiogenesis? There are at least two hypothesizes: 7A induce progenitor cell migrate to matrigel and differentiated to EC to form capillaries; 7A directly induce EC migrate to matrigel. As far as we know, both of them occurred during the 7A induced angiogenesis.

3.1.5 Conclusions

In summary, this study confirmed that VEGF induced HDAC7 mRNA underwent alternative transcription and translation. Alternative translation occurs from the first and second ORF within HDAC7 mRNA, which will produce 7aa peptide.

Part B：

3.2 7A function as a phosphate carrier in signal transduction

3.2.1 Introduction

VEGF comprises several isoforms which bind to different receptors and promotes angiogenesis through activation of specific kinase cascade, such as MAP kinase pathway and so on. Though activation of different signal pathway regulates variety field of angiogenesis, the common feature of all the VEGF signal pathways is phosphate transfer.

The sORF encodes a 7aa-peptide (7A, MHSPGAD), which contains a serine that is one of the four amino acids that can be phosphorylated. Furthermore, the phosphate on serine can be transferred to a serine or a threonine residue. We wonder whether the serine within 7A can be phosphorylated. If so, does it play a role in VEGF signal transduction via phosphate transfer? In this chapter, we identified 7A functioned as a phosphate carrier on in the MEKK1-7A-14-3-3γ signal pathway. MEKK is a serine/threonine kinase that occupies a pivotal role in a network of phosphorylating enzymes. MEKK1, a member of MEKK, was reported to be involved in cell migration regulation. 14-3-3s, phosphoserine-binding proteins, are a family of conserved regulatory molecules that are expressed in all eukaryotic cells, which are implicated in many cellular functions, including transcription, metabolism, and apoptosis.

3.2.2 Hypothesis and Aims

We hypothesis that 7A functioned as a phosphate carrier, and receive phosphate group from upstream kinase and transfer the phosphate group to downstream target protein.

The aim of this chapter is to explore the role of 7aa peptide in phosphorylation transfer. Firstly, detect the phosphorylation level of 7aa peptide with VEGF stimulate. Then, try to find the upstream kinase and downstream effector of 7aa peptide based on proteomics study. Additionally, verify the signalling pathway of upstream kinase - 7aa peptide- downstream.

3.2.3 Results

3.2.3.1 VEGF induced 7aa-peptide phosphorylation via MEKK1

To test this, we performed immunoprecipitation assay with anti-FLAG antibody, followed by ELISA with anti-phosphoserine antibody. As shown in Figure 3.7A, serine phosphorylation was detected in Ad-HD7FH-infected VPCs as compared to control virus infected cells, which was significantly increased by VEGF treatment. Considering the phosphorylation signal could be derived from FLAG-tagged 7A or its associated proteins, we performed a special biotin-labelled peptide pull-down assay (Figure 3.7B). These experiments demonstrated that the serine residue in 7A was indeed phosphorylated and the phosphorylation could be enhanced by VEGF treatment, and that this phosphorylation was sequence specific as the serine residue in the scrabble 7aa-peptide (7S, MPHASGD) was not phosphorylated (Figure 3.7C). As expected, the substitution of serine with alanine (7Aa) totally abolished the 7A phosphorylation.

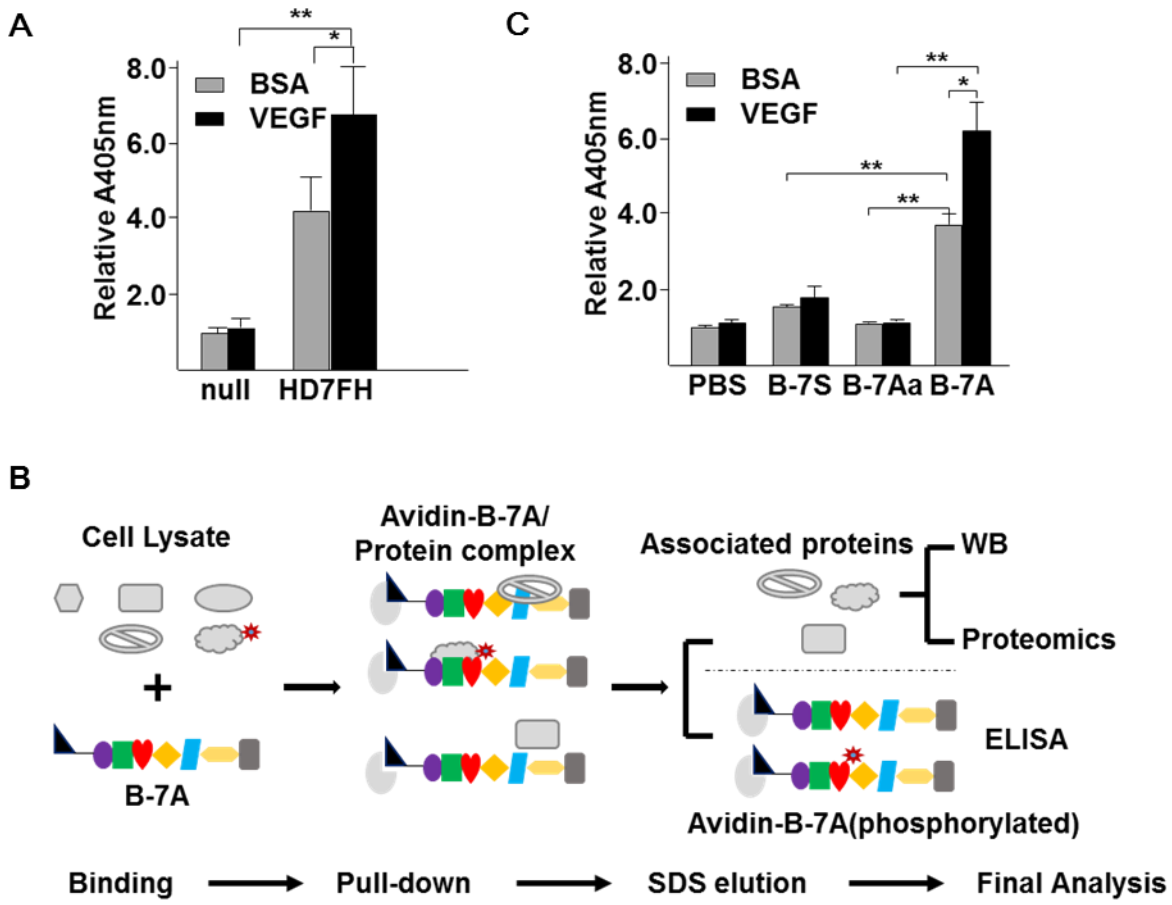


Figure 3.7 VEGF increase 7A phosphorylation

(A)VEGF increased FLAG-tagged peptide phosphorylation. The 3-day differentiated VPCs were infected with Ad-null or Ad-HD7FH virus at 100MOI and incubated in differentiation medium for 48hr. The cells were then treated with 10ng/ml VEGF for 30min, followed by immunoprecipitation with anti-FLAG plus ELISA with anti-phospho-serine antibody. 1% BSA was included as vehicle control. Relative A405nm was defined as ratio of the A405nm value with that of Ad-null/BSA group set as 1.0. (B) A schematic illustration of peptide/protein binding assay. Biotin-labelled peptides (B-7A) were incubated with cell lysate. The peptide/protein complex was pulled down by streptavidin beads. The associated proteins were eluted with SDS solution and subjected to Western blot or proteomics analysis. (C) The 7aa-peptide was phosphorylated in cell free system. Phospho-Serine in streptavidin trapped Bio-peptides was detected by ELISA. The beads were subjected to ELISA with anti-phospho-Ser antibody. Data presented are mean of three independent experiments.*: $p<0.05$.

To identify the potential upstream kinase for 7A phosphorylation, we performed a pilot proteomics analysis of the peptides-associated proteins, especially on their phosphorylation changes in response to VEGF treatment. We detailed phosphorylation changes on kinase and found that 7Ap (the phosphorylated on serine residue of 7A) and V+7A bind much more kinase than others. While 7Aa and 7Aa+VEGF bind phospho-MEKK1 (p-MEKK1) specifically (Table 3.1). Based on the proteomic results, we supposed MEKK1 as upstream kinase for 7A phosphorylation. As 7Aa cannot be phosphorylated, when 7Aa bind to p-MEKK1, it would prevent the phosphate group transfer, resulting in accumulation of p-MEKK1.

Table 3.1 **Biotin-peptide binding Phosph-kinase**

	CTL	**7S**	**7Aa**	**7A**	**P7A**	**7S+V**	**7Aa+V**	**7A+V**	**P7A+V**
5~-AMP-activated protein kinase catalytic subunit alpha-1									574
6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 2 variant 4								451	
6-phosphofructokinase type C									232
6-phosphofructokinase, liver type			13		99				211
AarF domain-containing protein kinase 4					16				
Beta-adrenergic receptor kinase 1 (Fragment)								121	
Cyclin-dependent kinase inhibitor 2A, isoform 3									173
DNA-dependent protein kinase catalytic subunit					13				
Dual-specificity testis-specific protein kinase 2								91	
Hexokinase 1, isoform CRA_f								18	
Hexokinase-2					81			103	
Hexokinase-3					32				

Homeodomain-interacting protein kinase 4				23			
Isoform 2 of ADP-dependent glucokinase						95	
Isoform 2 of Cyclin-G-associated kinase						83	
Isoform 2 of Inactive serine/ threonine-protein kinase TEX14			15				
Isoform 2 of Mitogen-activated protein kinase-binding protein 1					61		
Isoform 2 of Rho-associated protein kinase 1						31	
Isoform 2 of Traf2 and NCK-interacting protein kinase				20			70
Isoform 3 of A-kinase anchor protein 9							61
Isoform 3 of Phosphatidylinositol 4-phosphate 5-kinase-like protein 1							45
Isoform 4 of Cyclin-dependent kinase inhibitor 2A, isoform 3						37	
Isoform M1 of Pyruvate kinase PKM	312		15	716		34	
Janus kinase and microtubule-interacting protein 1				13			
Ketosamine-3-kinase							51
Mitogen-activated protein kinase kinase kinase 1		13			89		
Mitogen-activated protein kinase kinase kinase 15						39	
Mitogen-activated protein kinase kinase kinase 5						39	
Mitogen-activated protein kinase kinase kinase 6				27			42
Mitogen-activated protein kinase kinase kinase kinase 4				39		39	29
Myosin light chain kinase, smooth muscle						38	40
N-acetylgalactosamine kinase							39
Neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adapter 1							38
Nucleoside diphosphate kinase B	37						

Phosphatidylinositol 4-kinase beta (Fragment)		73				
Phosphoglycerate kinase 1		76				
Phosphoglycerate kinase 2					32	
Phosphoinositide 3-kinase regulatory subunit 5			16			
Protein kinase C alpha type			23		22	
Protein kinase C			28		22	
Protein-tyrosine kinase 2-beta			21			
Putative hexokinase HKDC1		174	54		20	19
Pyruvate kinase PKM	30		767		19	
Rho-associated protein kinase			15		19	18
Serine/threonine-protein kinase 24			14			
Serine/threonine-protein kinase 25					17	
Serine/threonine-protein kinase BRSK2					17	
Serine/threonine-protein kinase MRCK beta					17	
Serine/threonine-protein kinase SMG1	14					
Serine-protein kinase ATM					16	
Serine-threonine kinase receptor-associated protein			42			
Tyrosine-protein kinase receptor				14		

In order to verify proteomics analysis result, we performed the Bio-peptide pull down assay. We noticed that the phosphorylation of peptide RSSRIK from MEKK1 was elevated in Bio-7Aa but reduced in Bio-7A-associated samples, which was confirmed by Western blot analysis (Figure 3.8).

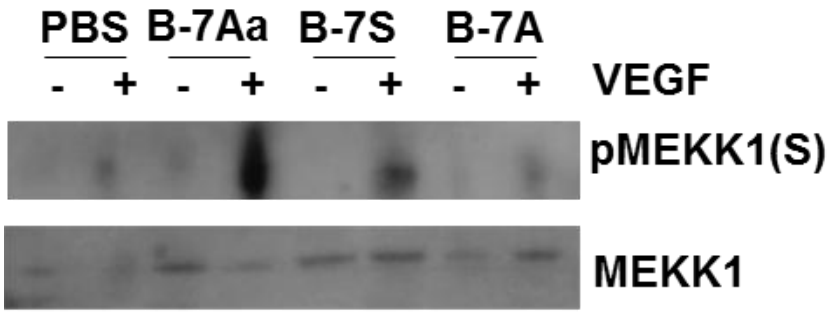


Figure 3.8 The 7aa-peptide physically interacted with MEKK1.

Bio-peptides/protein complex was trapped by streptavidin beads and subjected to Western blot analysis with anti-MEKK1 antibodies. 7S: scrabble 7aa-peptide; 7A: 7aa-peptide. 7Aa: 7aa-peptide with serine substituted by alanine. B-7S/7A/7Aa: Biotin-labelled 7aa peptides. Data presented are representative images of three independent experiments.

Then we performed time course assay and found that VEGF induced a transient increase of MEKK1 phosphorylation, peaked at 10min post-treatment (Figure 3.9A). The presence of 7A increased the de-phosphorylation of the phosphorylated MEKK1, while the addition of 7Aa could retain MEKK1 phosphorylation (Figure 3.9B, C).

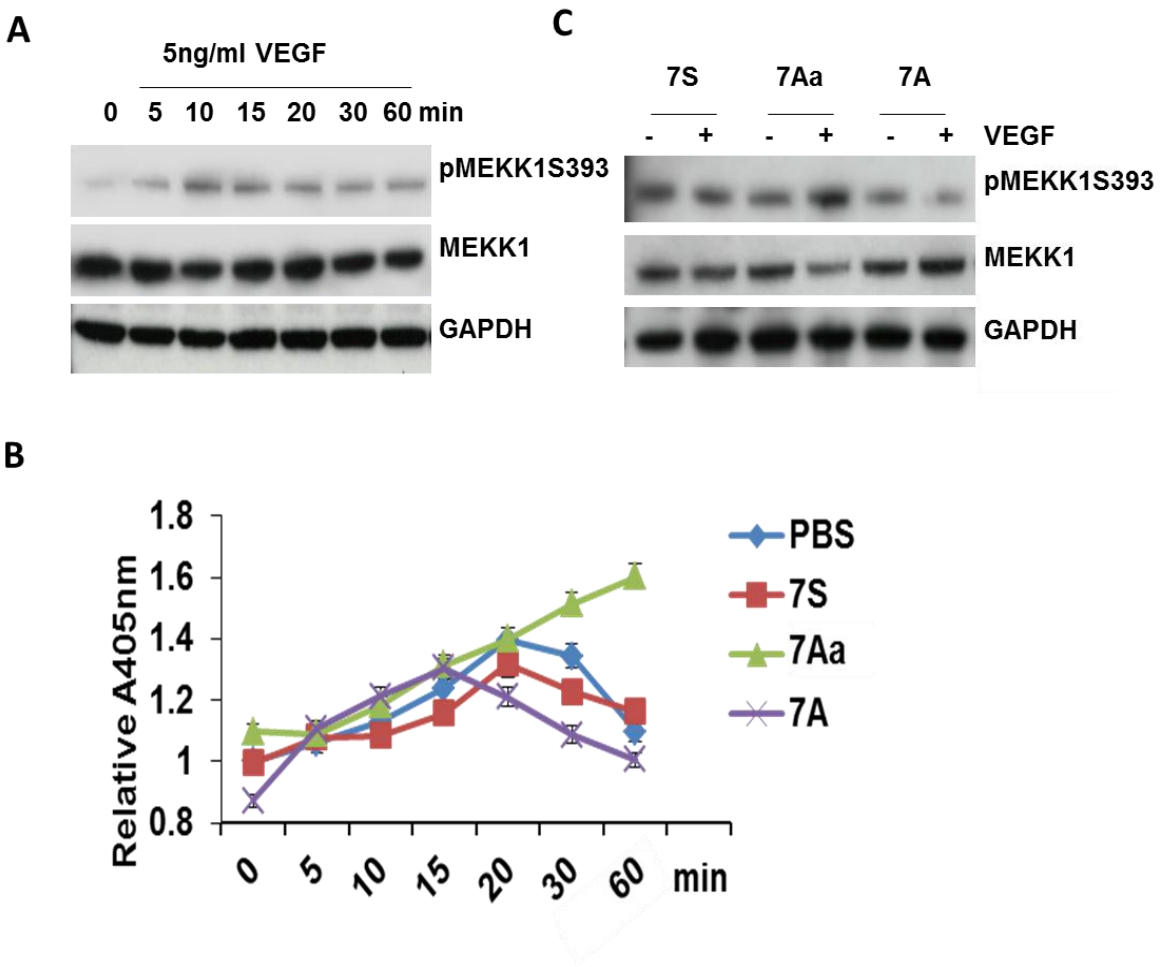


Figure 3.9 VEGF transiently activated MEKK1 phosphorylation.

(A) VPCs were treated with 5ng/ml VEGF in serum free medium for time duration indicated, followed by Western blot analysis with anti-phospho-MEKK1Ser393 (pMEKK1) and MEKK1 antibodies. GAPDH was included as loading control. (B) 7Aa retained MEKK1 phosphorylation. VPCs were seeded in 96-well plates and pre-treated with 1ng/ml peptides in triplicate for 1hr, and then treated with 5ng/ml VEGF in the presence of peptides for time duration indicated, followed by ELISA with anti-phospho-pMEKK1(Ser393) antibody. Relative A405nm was defined as A405nm value with that of PBS/0hr group set as 1.0. 7Aa retained MEKK1 phosphorylation. The VPCs were treated with 5ng/ml VEGF in the presence of 1ng/ml peptides for 30min, followed by Western blot analysis with anti-pMEKK1S393 and anti-MEKK1 antibodies. GAPDH was included as loading control. Data presented are representative images or mean of three independent experiments.

To explore 7A effect on phosphorylated MEKK1, we also performed immunofluorescence staining on VPC cell. The results show that the presence of 7A dramatically increased the de-phosphorylation of the phosphorylated MEKK1 (Figure 3.10).

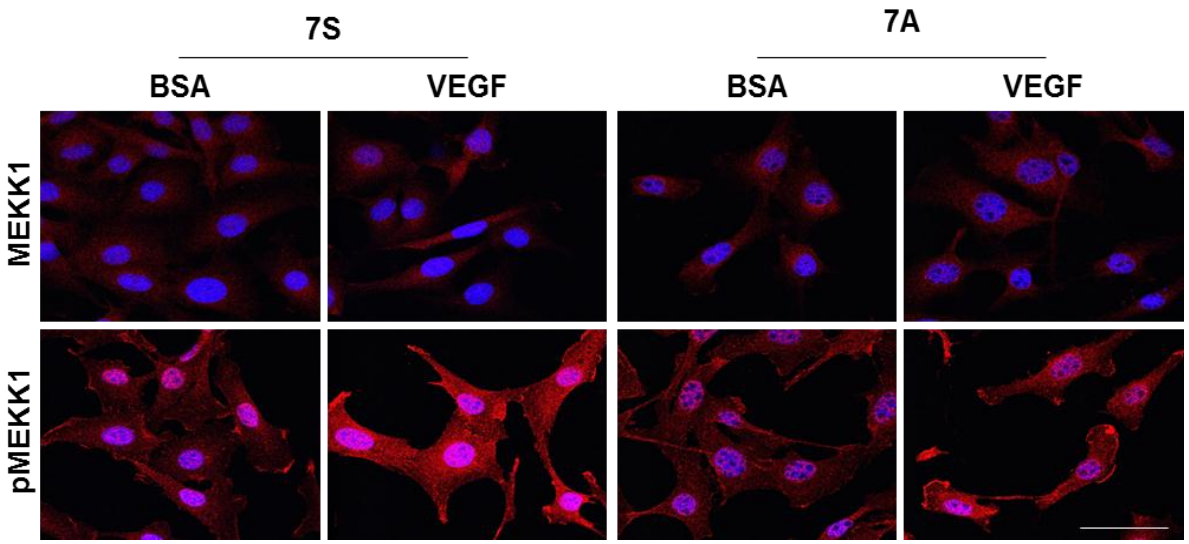


Figure 3.10 The presence of 7A attenuated VEGF-induced MEKK1 phosphorylation.

VPCs were seeded on slides and pre-treated with 1ng/ml peptides for 1hr, and then treated with 15ng/ml VEGF in the presence of peptides for 30min, followed by immunofluorescence staining with anti-MEKK1 or anti-phospho-MEKK1Ser393 (pMEKK1) antibodies. The nucleus was counterstained with DAPI. Scale bar: 50μm. Data presented are representative images of three independent experiments.

These observations imply that MEKK1 may function as an upstream kinase for 7A phosphorylation. Indeed, knockdown of MEKK1 by siRNA significantly attenuated 7A phosphorylation (Figure 3.11).

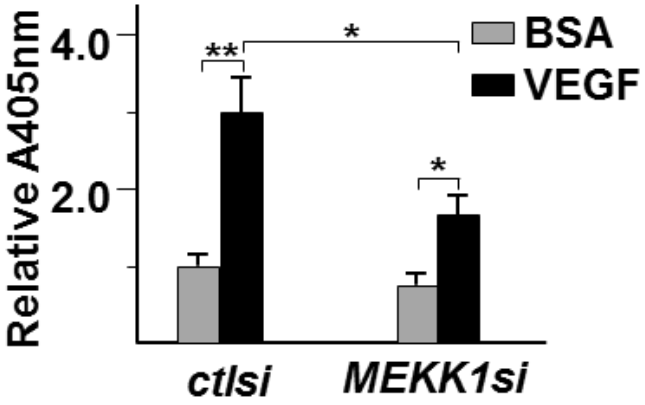


Figure 3.11 Knockdown of MEKK1 attenuated VEGF-mediated 7A phosphorylation.

The VPCs were transfected with MEKK1 siRNA (MEKK1si) and cultured for three days, followed by 5ng/ml VEGF treatment in serum free medium for 30 min. Control siRNA (ctlsi) and 1% BSA were included as siRNA and vehicle control respectively. The cell lysate was subjected to Bio-peptide/streptavidin-agarose beads pull-down arrays, followed by ELISA with anti-phospho-Ser antibody. Data presented are mean of three independent experiments. *: $p<0.05$; **: $p<0.01$.

So far, we have demonstrated that VEGF could induce HDAC7 undergo alternative transcription and translation to produce a 7-aa peptide, and this peptide could accept phosphate from MEKK1. The next question is whether 7A phosphorylation is the endpoint of VEGF-MEKK1 signalling or it can transfer out the phosphate to downstream effectors.

3.2.3.2 The phosphorylated 7aa-peptide directly phosphorylated 14-3-3γ protein

14-3-3 proteins are a family of conserved regulatory molecules ubiquitously expressed in all eukaryotic cells. Our previous study found that HDAC7 could regulate cell growth by directly binding to 14-3-3 protein (Margariti et al., 2010). From the pilot proteomics study, we noticed that the phosphorylation of a peptide (RATVVESSEK) from 14-3-3γ protein was increased in Bio-7Ap (the serine residue was synthetically phosphorylated) and VEGF-treated Bio-7A associated samples (Table 3.2).

Table 3.2 Biotin-peptide binding Phosph-14-3-3

	CTL	7S	7Aa	7A	P7A	7S +V	7Aa +V	7A +V	P7A +V
Phosph 14-3-3 protein gamma					199			35	135
Phosph 14-3-3 protein theta								15	

The physical binding of the Bio-7A and Bio-7Ap to 14-3-3γ protein was confirmed by Western blot analysis (Figure 3.12A). To test whether 7A was directly involved in 14-3-3γ phosphorylation, the phosphorylated bio-7A peptide was purified by pull-down assay and incubated with commercially available recombinant 14-3-3γ protein, followed by ELISA detection of 7Ap and Western blot analysis of phosphorylated 14-3-3γ. The phosphorylation signal in bio-7A was increased by VEGF but significantly reduced by further incubation with recombinant 14-3-3γ (Figure 3.12B, left panel). Accordingly, the 14-3-3γ phosphorylation was increased (Figure 3.12B, right panel). Direct incubation of 7Ap with recombinant 14-3-3γ protein in buffer system could increase 14-3-3γ phosphorylation (Figure 3.12C), suggesting that the phosphorylation group in the serine residue of 7Ap can be transferred to one threonine residue of 14-3-3γ protein phosphorylation.

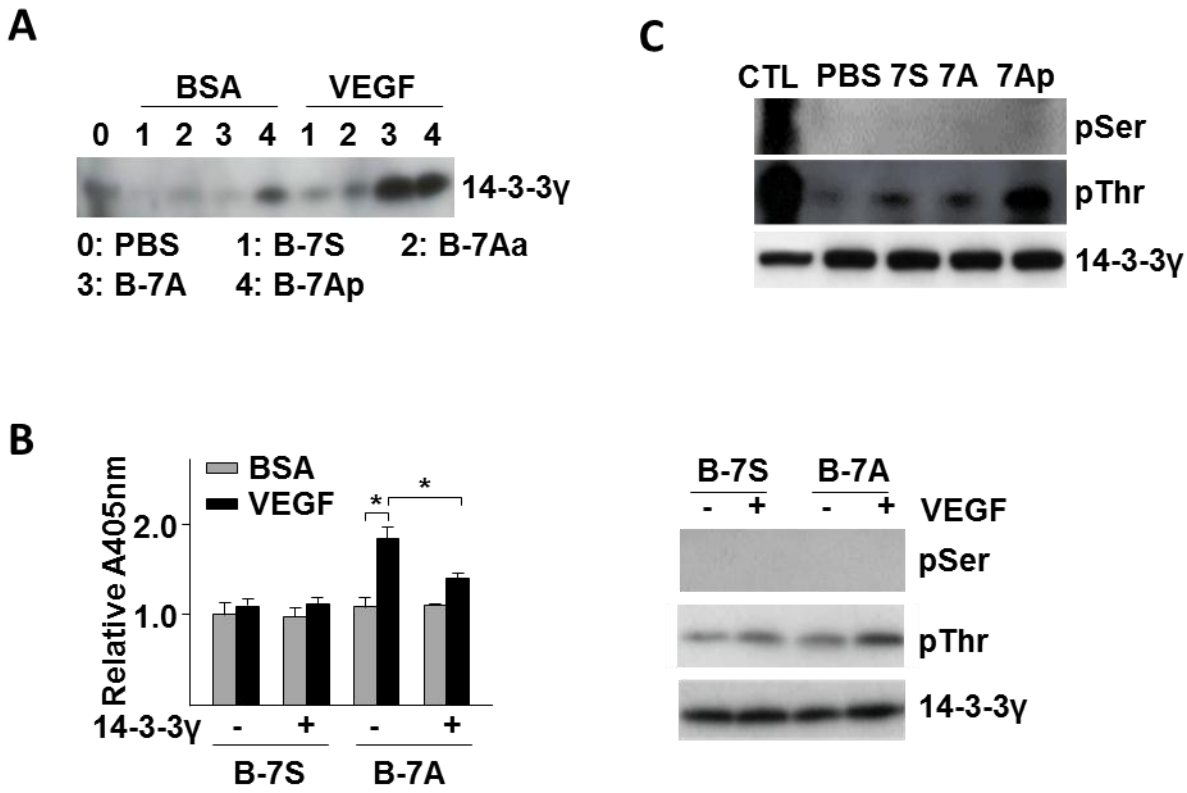


Figure 3.12 The 7aa-peptide acted as a kinase for 14-3-3γ.

(A) The phosphorylated 7aa-peptide physically interacted with 14-3-3γ. Bio-peptides/protein complex was trapped by streptavidin beads and subjected to Western blot analysis with anti-14-3-3γ antibody. (B) The 7aa-peptide mediated the 14-3-3γ phosphorylation. The cell lysate-incubated biotin-labelled peptides were trapped with streptavidin beads and washed with SDS solution to remove the associated proteins, followed by incubation with 0.1μg 14-3-3γ and elution with SDS solution. The elute was subjected to Western blot (right), while the beads were subjected to ELISA with anti-phospho-Serine antibody (left). (C) The 7Ap peptide could phosphorylate 14-3-3γ. 14-3-3γ was incubated with peptides, followed by Western blot analysis with anti-phospho-Serine (pSer) and anti-phospho-Threonine (pThr). 50μg VEGF-treated VPC lysate was included as positive control (ctl). Data presented are representative images or mean of three independent experiments. 7Ap: 7A-peptide with serine phosphorylated. *: $p<0.05$.

As shown in Figure 3.12B (right), all Bio-7S group still shows obviously band on pThr antibody. So we wonder there might be contaminated kinases in recombinant 14-3-3γ protein. To explore whether 7Ap could directly transfer the phosphorylation group to 14-3-3γ without

the facilitation of other proteins, we purified the 14-3-3γ protein band from SDS-PAGE gel and incubated with peptides in gel after being renatured. As shown in Figure 3.13, 7Ap did increase the phosphorylation of the 14-3-3γ protein, suggesting that 7Ap can act as a kinase to phosphorylate target protein.

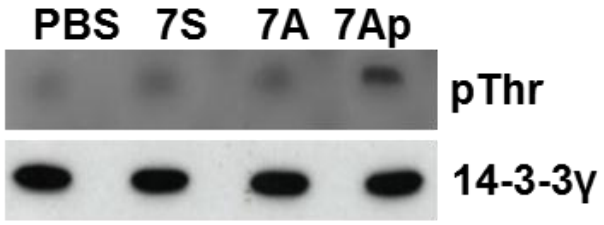


Figure 3.13 The 7Ap peptide phosphorylated 14-3-3γ in gel

The 14-3-3γ bands were cut from the SDS-PAGE gel, renatured and incubated with peptides. The gel sections were rearranged and transferred to a PVDF membrane followed by western blot analysis with anti-phospho-thr (pThr) and anti-14-3-3γ antibodies. Data presented are representative images of three independent experiments.

These results indicated that 7A could enhance VEGF-induced 14-3-3γ phosphorylation at Thr145 residue. Interestingly, the exogenous 7Ap could increase 14-3-3γ phosphorylation in the absence of VEGF treatment (Figure 3.14A), suggesting that 7A phosphorylation may be involved in 14-3-3γ phosphorylation. Immunofluorescence staining revealed that the phosphorylated 14-3-3γ was mainly located in nucleus and upregulated by VEGF treatment, which was significantly enhanced by the presence of 7A (Figure 3.14B).

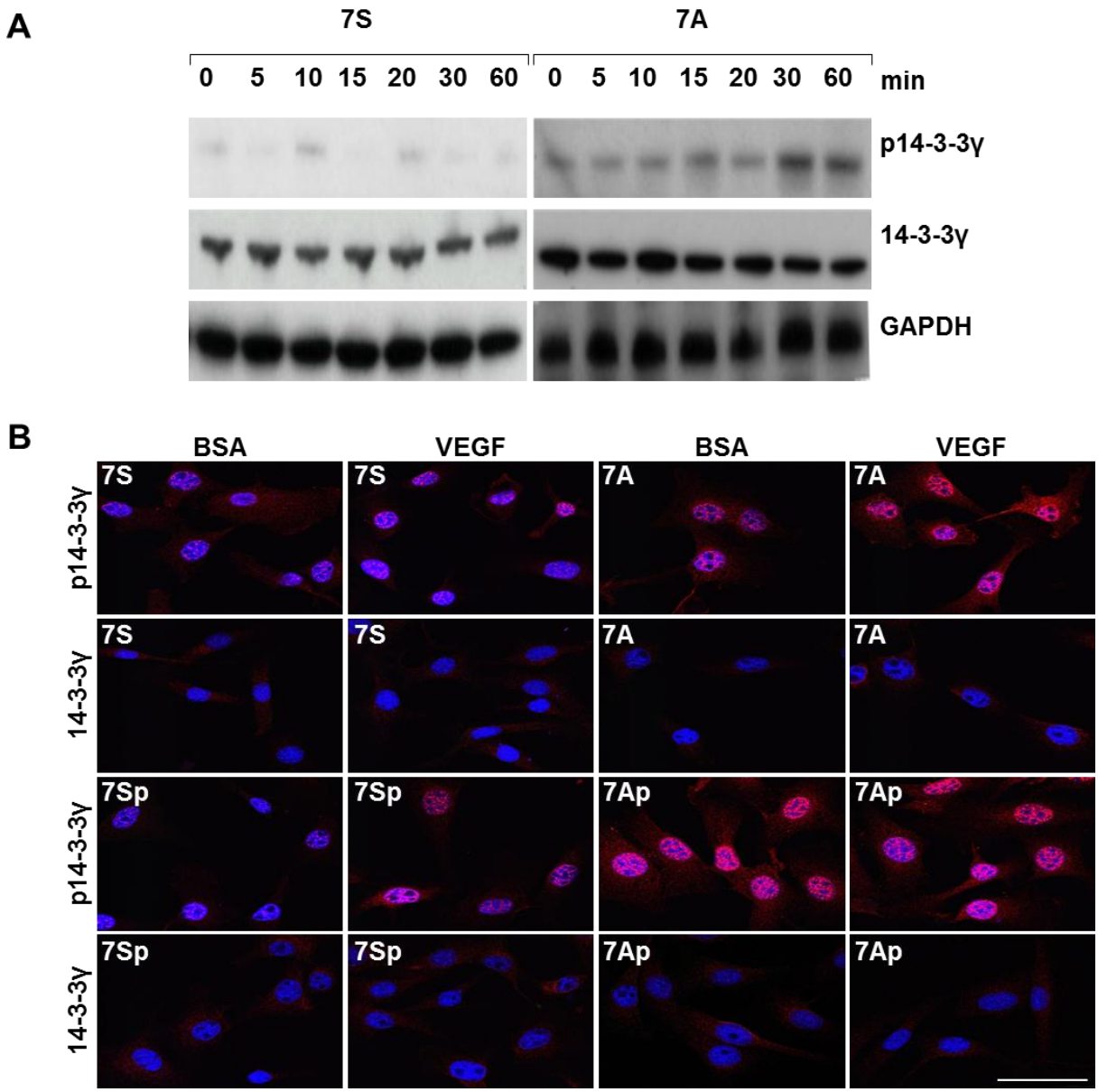


Figure 3.14 7A phosphorylation involved in 14-3-3γ phosphorylation.

(A) The 7aa-peptide enhanced VEGF-mediated 14-3-3γ phosphorylation. (B) The 7Ap induced 14-3-3γ phosphorylation and nuclear translocation. The VPCs were pre-treated with 1ng/ml 7S or 7Sp (the serine was synthetically phosphorylated) or 7A or 7Ap for 1hr and then treated with 5ng/ml VEGF in the presence of the peptides for 30min, followed by immunofluorescence staining with anti-phospho-14-3-3γThr145 (p14-3-3γ) and anti-14-3-3γ antibodies. PBS was included as control for peptides or biotin-labelled peptides. BSA was used as vehicle control for VEGF treatment. Data presented are representative images or mean of three independent experiments.

3.2.3.3 MEKK1-7A-14-3-3γ signal pathway.

As we have verified that 7A got phosphate group from activated MEKK1 and that 7Ap could transfer the phosphate group to 14-3-3γ, we wondered whether 7A bridged between MEKK1 and 14-3-3γ. Knockdown of *MEKK1* by siRNA decreased the basal and abolished VEGF-induced 14-3-3γThr145 phosphorylation (Figure 3.15A), indicating that MEKK1 is an upstream kinase for 14-3-3γ phosphorylation. In *MEKK1* knockdown VPCs, the addition of 7Ap alone could induce 14-3-3γThr145 phosphorylation (Figure 3.15B).

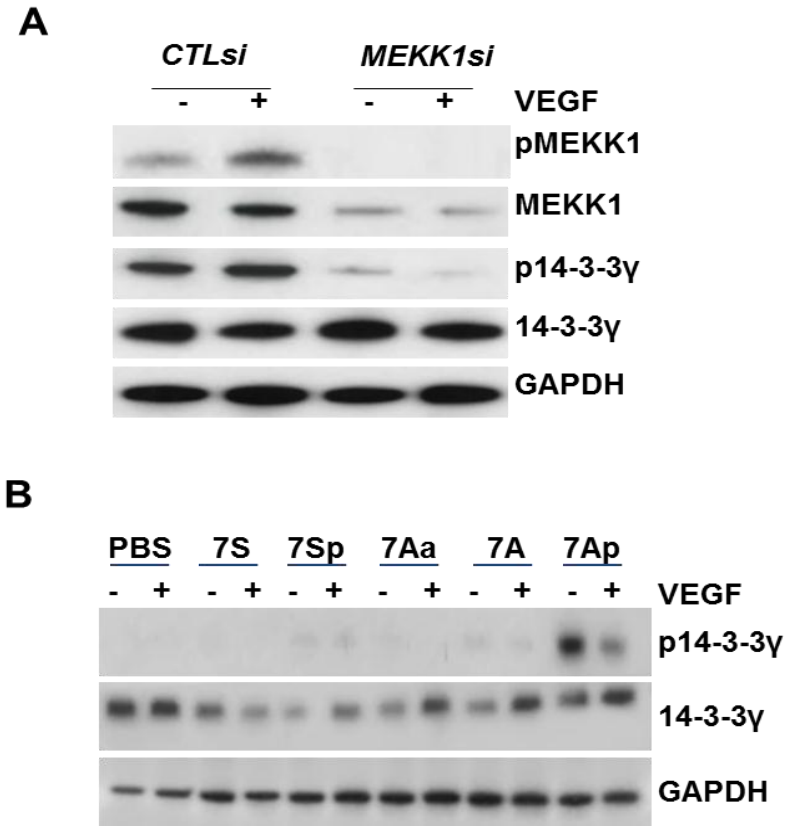


Figure 3.15 MEKK1 is an upstream kinase for 14-3-3γ phosphorylation

(A) Knockdown of MEKK1 abolished VEGF-induced 14-3-3γ phosphorylation in differentiated VPCs. (B) The 7Ap peptide induced 14-3-3γ independent of MEKK1. The MEKK1 knockdown VPCs were pre-treated with 0.1ng/ml peptides for 1hr and then treated with 10ng/ml VEGF for 30min, followed by Western blot with anti-p14-3-3γ (pThr). Data presented are representative images of three independent experiments.

3.2.3.4 The adjacent histidine and proline residues were essential for the phosphorylation transfer

To investigate whether the two adjacent amino acid residues, histidine and proline, were involved in the phosphorylation transfer, we synthesized biotin-labelled mutant 7-aa peptides, in which the histidine residue was substituted as lysine (B-7Ak), arginine(B-7Ar), leucine (B-7Al) and threonine (B-7At) respectively or proline was substituted as valine (B-7Av). The peptide pull-down assays were performed with these mutant peptides and VEGF-treated/untreated VPC cell lysates. As shown in Figure 3.16, phosphorylated serine could be detected in B-7Ak and B-7Ar under VEGF treatment although it was significantly attenuated as compared to 7A. There was no phosphor-serine signal in other three mutants. These results suggest that an alkaline residue on the left side and a proline on the right side are essential for the phosphorylation of the serine residue in 7-aa peptide.

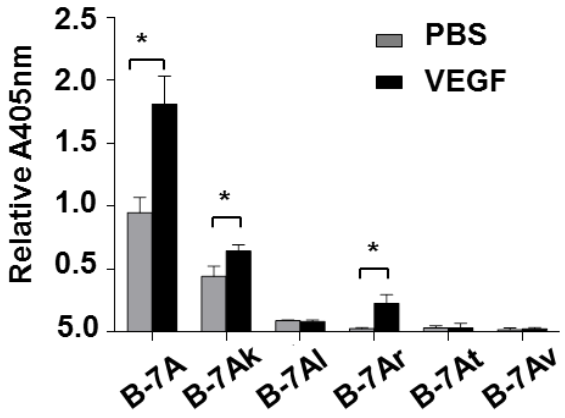


Figure 3.16 An alkaline amino acid and proline was essential for the phosphorylation reception.

An alkaline amino acid and proline flanking the serine residue was essential for the reception of phosphorylation from VEGF pathway. Biotin-labelled peptides were incubated with cell lysate from VEGF-treated/untreated VPCs (5ng/ml in serum free medium for 30min), followed by streptavidin-agarose beads pull-down and ELISA with anti-phospho-Ser antibody. Data presented are mean of three independent experiments.*: $p<0.05$.

To further assess whether these substitutions affect the phosphate group transfer to target protein, recombinant 14-3-3γ was incubated with synthetic phosphorylated mutant 7-aa peptides, followed by western blot analysis of 14-3-3γ phosphorylation. As shown in Figure 3.17, all the substitutions lost the ability to transfer the phosphate group to 14-3-3γ, suggesting that histidine and proline are essential for this phosphorylation transfer.

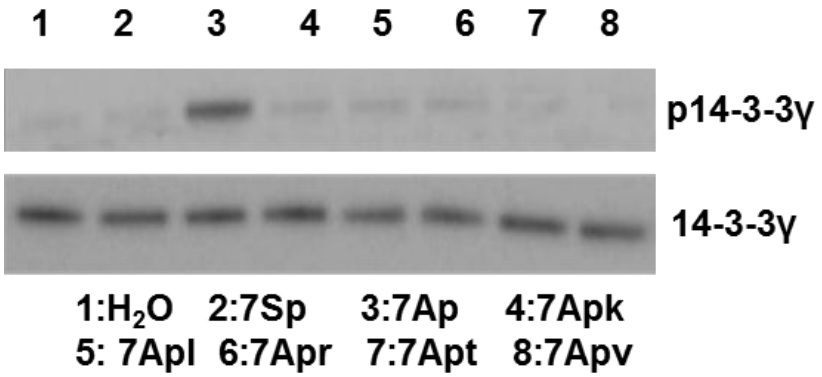


Figure 3.17 Histidine and proline are essential for this phosphorylation transfer.

Both histidine and proline were critical to phosphorylate14-3-3γ.14-3-3γ (10ng) was incubated with phosphorylated peptides (1ng) in cell free buffer system, followed by Western blot with anti-phospho-14-3-3γThr145 (p14-3-3γ) and anti-14-3-3γ antibodies.

Histidine is one of the four amino acids that can be phosphorylated in both bacterium and eucaryon (Klumpp & Krieglstein, 2005; Muimo et al., 2000). As described above, the histidine residue was not essential to receive phosphorylation at serine residue in 7-aa peptide but critical to transfer the phosphate group to 14-3-3γ, thus we wondered whether there was a phosphorylation transfer between the histidine and serine residues within 7-aa peptide. Indeed, phosphorylated histidine in N1 but not N3 position could be detected in 7Ap but not in 7Sp or 7Av (Figure 3.18A). As expected, the phosphor-serine signal was significantly reduced in 7Ap as compared to other mutants or 7Sp (Figure 3.18B).

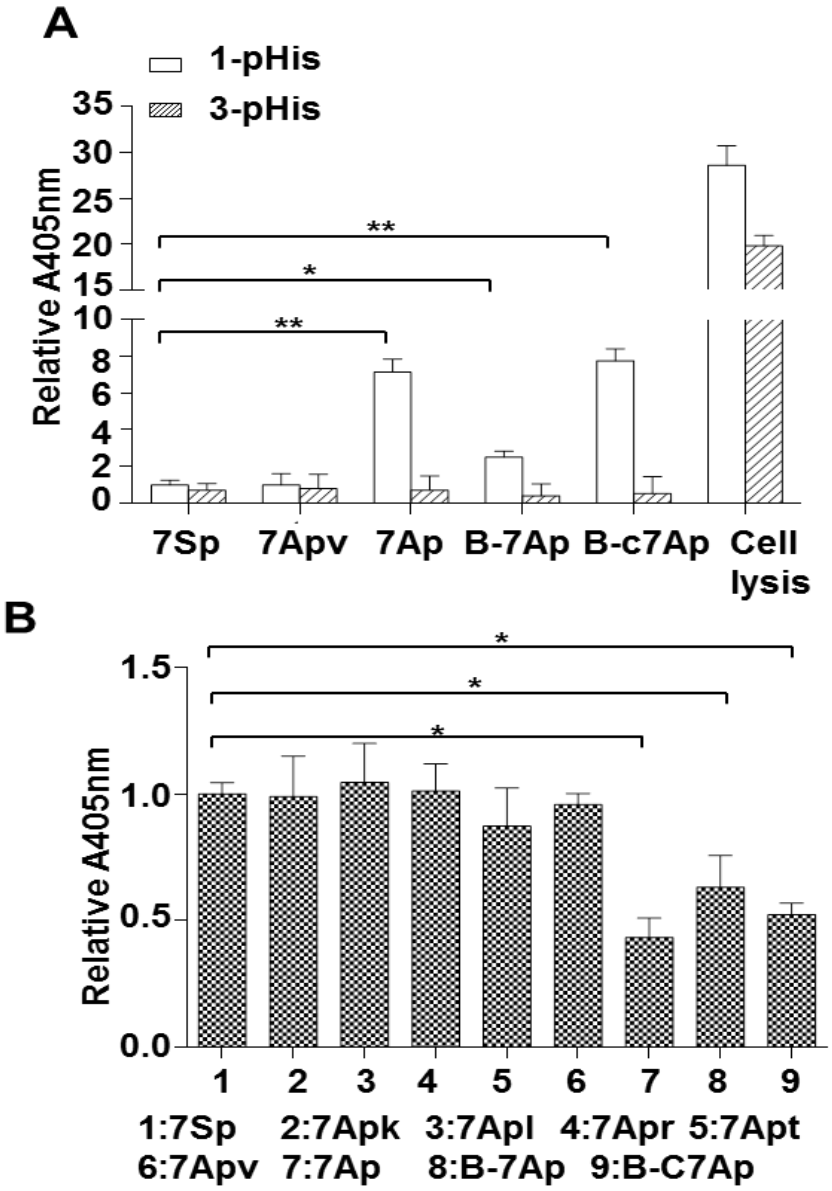


Figure 3.18 Phosphate group could be transferred from serine to histidine intra-molecularly.

The phosphorylated peptides (10μg) were coated onto microtiter plates, followed by ELISA with anti- phosphor-histidine (1-pHis and 3-pHis, A), phosphor-Ser (B) antibodies. 50μg VEGF-treated VPC cell lysate was included as positive control for phosphor-histidine. Data presented are mean of three independent experiments. *: $p<0.05$.

As histidine phosphorylation signal was low in B-7Ap and unable to be detected in peptide-pull-down assays, we synthesized a C-terminal biotin-labelled 7Ap (B-c7Ap) which kept similar level of phosphor-serine and phosphor-histidine signals. With this peptide, we assessed whether there was any change on phosphor-histidine level during phosphorylation transfer from 7Ap to 14-3-3γ. As expected, the phosphor-serine signal was significantly reduced after incubation with 14-3-3γ (Figure 3.19A). However, there was only a slight decrease of phosphor-histidine signal (Figure 3.19B).

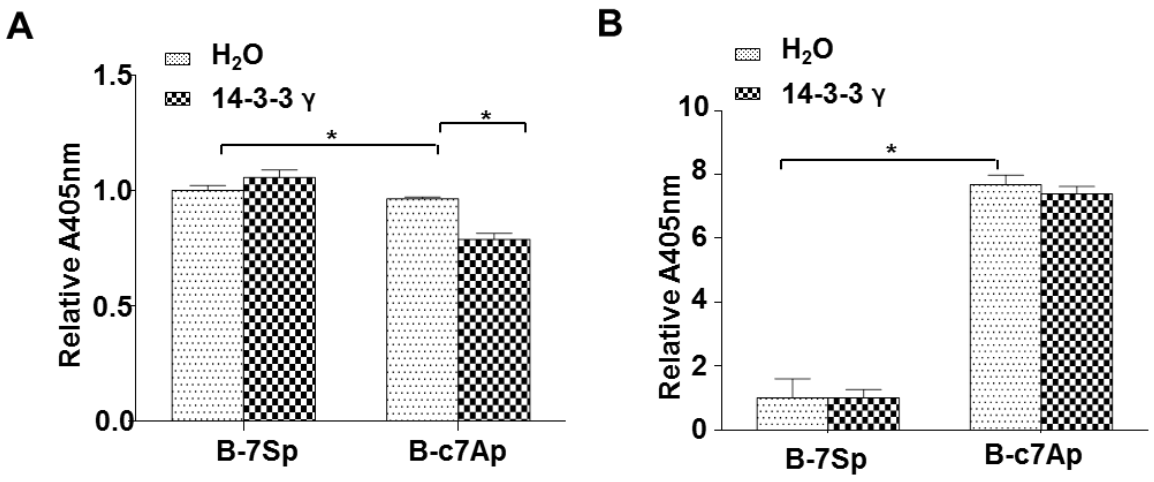


Figure 3.19 Phosphor-serine was the source of phosphate for 14-3-3γ phosphorylation.

14-3-3γ (10ng) was incubated with Biotin-labelled 7Sp (B-7Sp) or c7Ap (B-c7Ap) peptides (1ng) in cell free buffer system, followed by streptavidin-agarose beads pull-down. The beads were subjected to ELISA with anti-Phospho-Ser (A) and anti- Phospho-Histidine N1 (B) antibody. Data presented are mean of three independent experiments. *: $p<0.05$.

3.2.4 Discussion

Serine is one of three amino acid residues that are commonly phosphorylated by kinases during cell signalling pathway in eukaryotes. Phosphorylated serine residues are often referred to as phosphoserine (Gehrig, Roschitzki, Rutishauser, Reiland, & Schlapbach, 2009). As 7A contains a serine, we wonder whether it occurs phosphorylation. As shown on Figure 3.7A, more phosphoserine detected on Ad-HDAC7FH infected cell in the presence of VEGF. The phosphoserine might be from phosphorylation of phosphor-7A or 7A binding phosphoproteins. Then the 7aa-peptide phosphorylated assay was performed in cell free system, in which biotin marked 7aa-peptide was pulled down by streptavidin beads. Introduction of Biotin-Streptavidin system increase the stability of peptide-beads binding to prevent SDS elution, which can isolate peptide from binding proteins and make phosph-7A detection possible.

Normally, a kinase has multiple phosphorylation sites, each site may correspond to a set of substrates. Therefore, a kinase can participate in different cellular processes via receiving phosphate group at different sites and transferring the phosphate group to different substrates. From the proteomic data, we found that p-MEKK1 specific binding with 7Aa, especially in the presence of VEGF, indicating that VEGF induced p-MEKK1 accumulated in 7Aa group which inhibit phosphate group transfer for its similar structure with 7A. The presence of 7A accelerated the dephosphorylation of the VEGF-induced MEKK1 phosphorylation at Ser393 site, suggesting that 7A may facilitate the phosphate group transferring from this site. Thus, we demonstrated that 7A is the substrate of MEKK1 to receive phosphate group from the serine393 residue. The *MEKK1* knockdown significantly attenuated but not blocked VEGF-induced 7A phosphorylation, suggesting that MEKK1 is one of the upstream kinases for 7A phosphorylation. However, these results did not show the directly evidence for 7A binding to pMEKK1 and receiving phosphate group from pMEKK1. The phosphate group transfer

assays from recombinant pMEKK1 protein to 7A in cell-free system will be performed in future work.

7A is the substrate of MEKK1 to receive phosphate group, also transfers this phosphate group to downstream target proteins. Based on previous results and proteomic data, 14-3-3γ might be the target of 7A. In the presence of 7A, p14-3-3γ increased under VEGF treatment 30 mins. Furthermore, the 7Ap can increase P14-3-3γ without VEGF, suggesting that 7Ap might be the product of 7A with the treatment of VEGF. The binding of 7Ap to 14-3-3γ protein may cause huge conformation changes among both of them, creating enough energy to complete the phosphate group transferring from the serine residue in 7Ap to the threonine residue in 14-3-3γ. So we wonder whether the amino acid sequence in 7A is essential in the phosphorylation. Under the cell-free system, 7Ap directly phosphorylates 14-3-3γ protein. Although His/Lys or His/Arg substitution didn't affect 7-aa peptide to receive phosphorylation at serine residue, the transferring of the phosphate group to 14-3-3γ definitely needed the involvement of the histidine and proline residues. Interestingly, the N1 but not N3 site in histidine could receive the phosphate group from phosphor-serine during which the proline ring might facilitate. We may assume that upon binding to 14-3-3γ protein the phosphate group is transferred from histidine N1 site to the threonine residue in 14-3-3γ, while the empty N1 site receives phosphate from serine in which proline facilitates this intramolecular phosphorylation transfer. The significance of this striking finding is that a peptide or protein with HSP residues, such as 7A, transfers phosphorylation from a kinase to an effector independently of the direct interaction between the kinase and the effector, extending the spectrum of the effectors downstream of a kinase.

For a protein kinase, normally contains regulatory domain and kinase domain, and kinase domain contains hydrophobic motif, turn motif and activation loop, which first identified in protein kinase C. Phosphorylation at the hydrophobic motif can regulate the catalytic function,

stability or phosphatase sensitivity which shows different function on different protein kinases (Keshwani, von Daake, Newton, Harris, & Taylor, 2011). The turn motif was regarded as a phosphorylation switch that regulates the kinase binding with other proteins(Knighton et al., 1991) (T. Gao & Newton, 2002). 7aa-peptide, a small peptide, does not contain the classically kinase domains, but can transfer phosphate group and function as a kinase. Firstly, 7aa-peptide is small so it might be easier to bind with proteins, for some proteins have the groove domain such as 14-3-3γ. Then the chemic structure of 7A contains two rings (Figure 3.20), and the rings may form the net structure which is easier to share the energy and transfer the phosphate group to the target proteins. Finally, for the small molecular, 7aa might to perform more high-efficiency. However, these are speculations the mechanism and chemical basis is still unknown and we will explore in the future work.

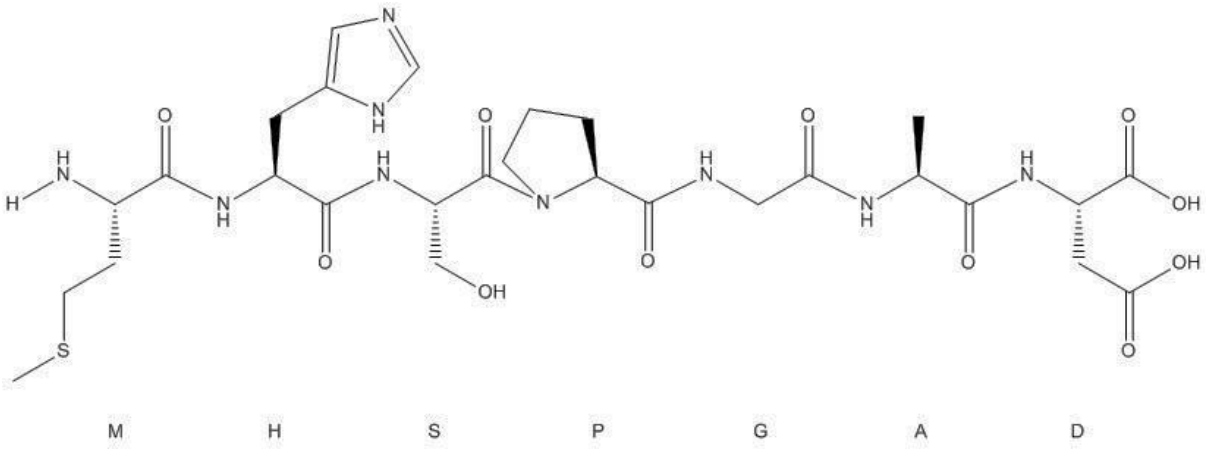


Figure 3.20 Chemical structure of 7A

3.2.5 Conclusions

7aa peptide acts as a phosphate group carrier in MEKK1-7A-14-3-3γ signal pathway. The adjacent histidine and proline residues were essential for the phosphorylation transfer.

Part C：

3.3 Function of 7A *in vitro* and *in vivo*

3.3.1 Introduction

Vascular endothelium injury is a common pathophysiological change in cardiovascular diseases. Traditional treatment aims to inhibit further injury through specific drug to protect vascular. However stimulating self-repairing to recovery the injury seems more beneficial and useful.

Adventitial vasa vasorum constitutes a complete vascular tree-like structure, including arterioles, capillaries, and veins (Langheinrich et al., 2007). Under pathological conditions, the arterial adventitia undergoes remodelling in response to a variety of arterial injuries. The resident adventitial cells are often the first to be activated and reprogrammed in arterial wall remodelling (Stenmark et al., 2011). Recent studies suggest a more complex and dynamic picture of the adventitia which emphasizes critical roles played by interacting adventitial cell types in growth, inflammation, repair, and disease of the artery wall (Majesky et al., 2012). Accumulating evidence showed that the adventitia contains resident vascular stem cells/progenitor cells (Campagnolo et al., 2010; Hoshino et al., 2008; Passman et al., 2008). These Sca-1+ progenitor cells could be cloned *in vitro*, having the migration ability and multi-lineage potential to differentiate into osteoblasts, adipocytes, pericytes, ECs and SMCs under selective differentiation-promoting stimulation in culture. Furthermore, these progenitor cells appear to act in concert as part of a coordinated healing response to vascular injury. The progenitor cells may also contribute to vascular disease development.

3.3.2 Hypothesis and Aims

We hypothesis that 7A play an role on VPCs though MEKK1-7A-14-3-3γ signal pathway and contribute to angiogenesis.

In this chapter, we aim to verify 7A effect on proliferation, migration and differentiation of VPCs, and its function in mouse ischemic and vascular injury model.

3.3.3 Results

3.3.3.1 The 7aa-peptide induces VPC colonies formation and proliferation.

As described above, the 7A peptide could be alternatively translated in VPCs in response to VEGF treatment and might be involved in signal transduction via acting as a phosphorylation carrier. The next question is what role it may play in the cellular behaviour. BrdU incorporation and MTT assays revealed that 7A had no effect on cell proliferation (Figure 3.21A) on serum free medium or cell survival under oxidative stress (Figure 3.21B).

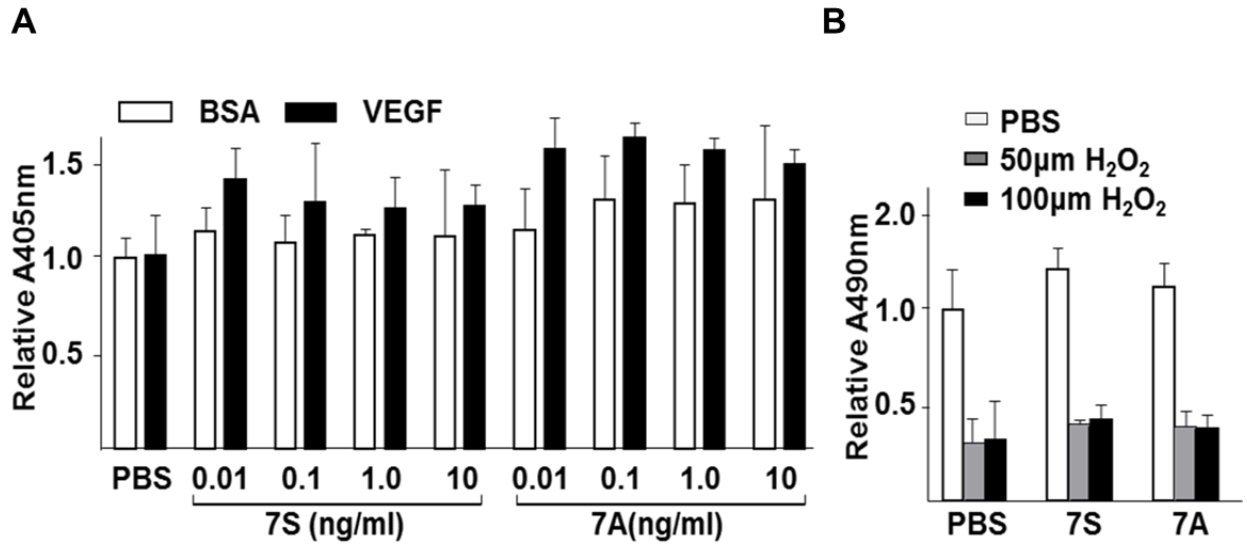


Figure 3.21 7A had no significantly effect on VPC proliferation and survival

(A) The 7aa-peptide slightly increased VPC proliferation. The 3-day differentiated VPCs were treated different concentration of 7S or 7A peptides in the presence of 10ng/ml VEGF in serum free α-MEM medium for 24hr. BrdU labelling reagents were added 6hr prior to harvesting the cells for BrdU incorporation assays. PBS and 1%BSA were used as control for peptide and VEGF respectively. The relative A405nm was defined as that of PBS/BSA group set as 1.0. (B) The 7aa-peptide had no effect on cell survival under oxidative stress. The 3-day differentiated VPCs were treated 50μm and 100μm H_2O_2 for 24hr in the presence of 1ng/ml 7S or 7A peptide, followed by MTT assays. The relative A490nm was defined as that of PBS group set as 1.0.

As progenitor cell have the self- renew function, we wonder whether 7A has effect on VPC self–renew. Therefore, in a limited dilution assay was performed in 96-well plates with normal growth medium, the presence of 7A especially 7Ap significantly increased the colony numbers and cell numbers within a colony (Table 3.3). Besides, in clonogenic assay performed in 6-well plates, 7A especially 7Ap dramatically increased the cells numbers per colony (Table 3.4) and the average area occupied by per cell (Figure 3.22). These results suggest that 7A especially 7Ap increase VPC self-renewal and may also increase VPC migration.

Table 3.3 Distribution of VPC Colonies (7days)

	2～10	11～50	51～100	＞100	Total Colonies	Cells/ Colony
PBS	13	20	5	0	38	24
7S	7	18	8	0	33	31.2
7Sp	3	17	6	0	26	32.1
7A	7	15	12	7	41	55.7
7Ap	0	32	13	11	56	66.9
7Aa	12	20	6	0	38	30.2

Table 3.3 Distribution of VPC Colonies (7days)

VPCs were seeded in 96-well plates at density of one cell/100μl/well in the presence of 1ng/ml peptides in stem cell medium for 7 days with medium refreshment every other day. PBS was included as vehicle control. Cells and colonies were observed under microscope after staining with Giemsa. The Table shows the distribution of colonies with different cell numbers, total colony number and average cell number per colony. Data presented were mean of three independent experiments.

Table 3.4 Distribution of Cell Numbers per Colony (3days)

%	2～5	5～10	11～15	16～20	＞20
PBS	24.18	35.34	23.25	14.42	2.79
7S	33.23	37.27	19.56	5.9	4.03
7Sp	32.14	38.09	23.81	2.38	3.57
7A	17.36	25.19	27.67	17.74	12.02
7Ap	10.22	20.12	29.41	21.52	18.73
7Aa	30.54	42.36	19.35	4.73	3.01

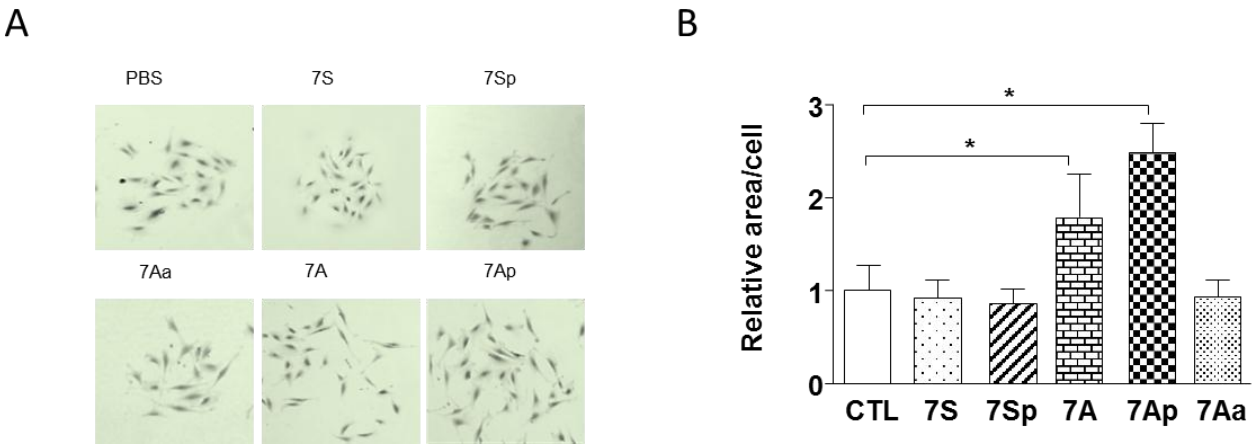


Figure 3.22 7A increased VPC migration.

VPCs were seeded in 6-well plates at density of 100 cells/well in the presence of 1ng/ml peptides in stem cell medium for 3 days. PBS was included as vehicle control. Cells and colonies were observed under microscope after staining with Giemsa. Table 5.2 shows the distribution of colonies with different cell numbers, total colony number and average cell number per colony Panel (A) shows cell distribution of typical colony in each group. Panel (B) shows the average area occupied by a single cell within a colony. Data presented were representative image or mean of three independent experiments. *: $p<0.05$.

3.3.3.2 The 7aa-peptide enhanced VEGF-induced VPC migration via MEKK1-7Ap-14-3-3γ

In order to identify the effect of 7A on VPC migration, The transwell migration assays were performed and the results showed that 7A increased and significantly enhanced VEGF-induced VPC migration. 7Ap alone dramatically stimulated VPC migration, which was comparable to that observed in VEGF plus 7A (Figure 3.23). The stimulatory effect on VPC migration was also observed using the wound healing model (Figure 3.24).

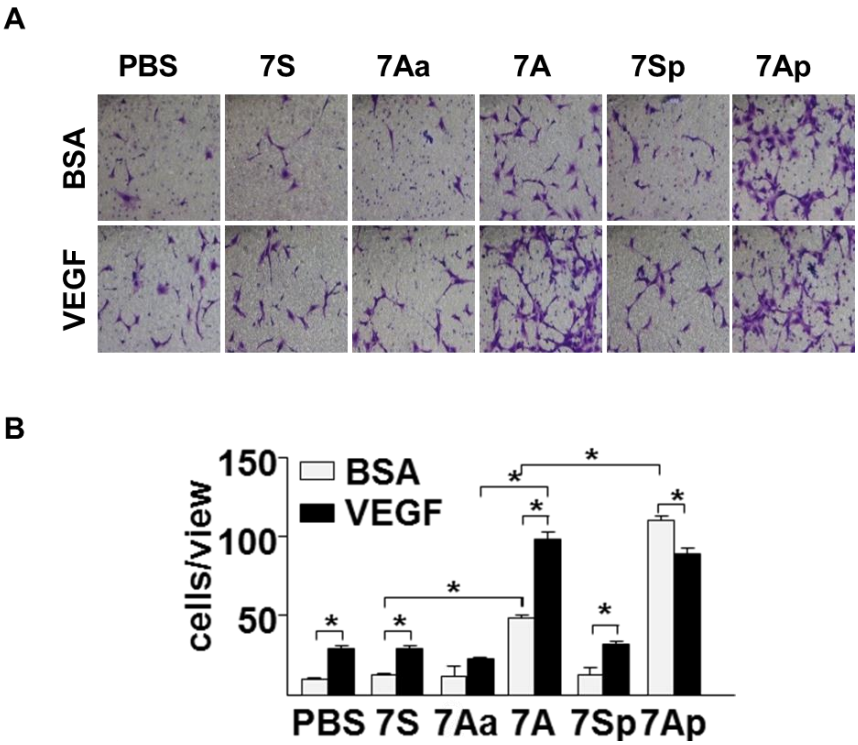


Figure 3.23 The 7aa-peptide enhanced VEGF-induced VPC migration

The 7aa-peptide enhance VEGF-induced VPC migration. The 3-day differentiated VPCs were seeded in the insert and serum free medium containing 1ng/ml peptides with/without 10ng/ml VEGF was added into the holder of transwell plates. Migrated cells were stained with crystal violet and observed under microscope 6hr later. (A) Panel shows the images of the migrated cell (B) panel shows the average cells/view. 1% BSA was included as control. Data presented were representative image or mean of three independent experiments. *: $p<0.05$.

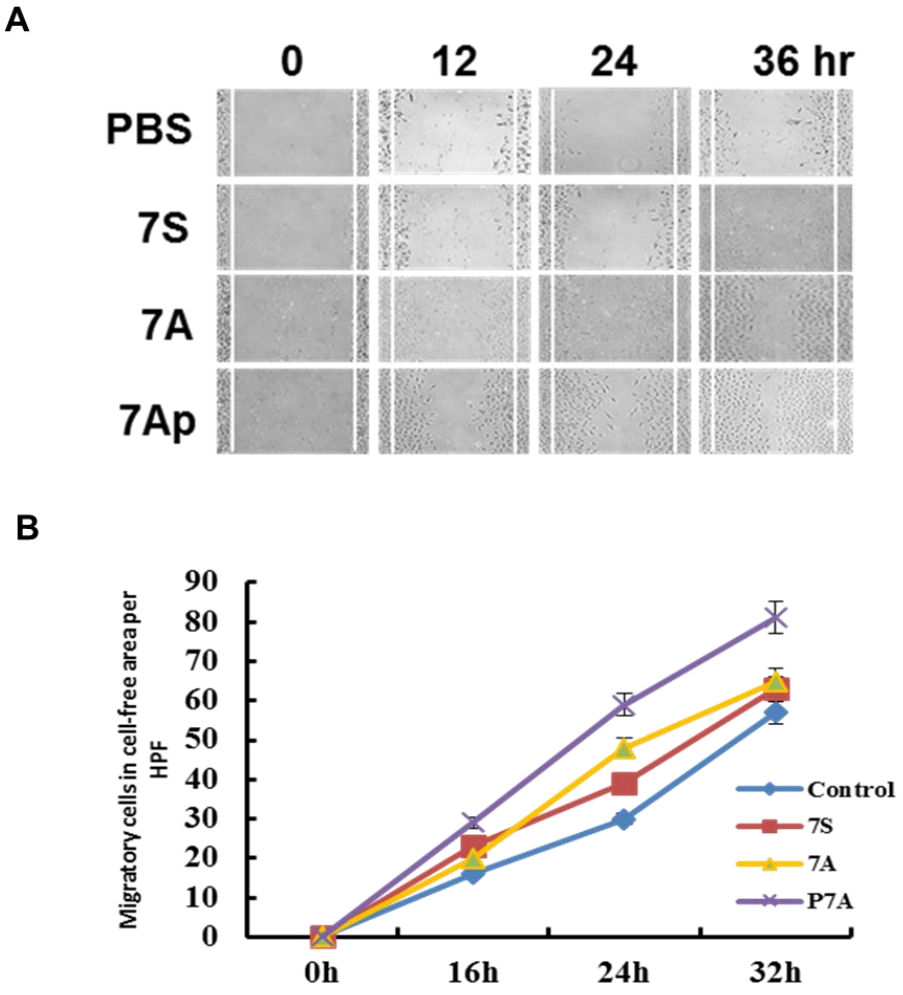


Figure 3.24 7Ap significantly increased VPC migration in a wound healing model.

Wound was introduced into confluent 3-day differentiated VPCs by tip scratching, and incubated with α-MEM medium containing 2% FBS and 1ng/ml 7S, 7A or 7Ap peptide. (A) Images were taken at 0, 12, 24 and 36hr post scratching. (D)The migrated cells in scratched area were counted from 3 views per scratching, 3 scratchings per well and 3 wells per peptide. PBS was included as control. Data presented were representative image or mean of three independent experiments.

As we have verified that MEKK1 is an upstream kinase for 14-3-3γ phosphorylation. We wonder whether MEKK1-7Ap-14-3-3γ signal pathway involved in 7A induced VPCs migration. In the transwell migration assays, knockdown of MEKK1 or 14-3-3γ decreased the basal level and abolished VEGF-induced VPC migration (Figure 3.25), suggesting that both MEKK1 and 14-3-3γ are essential for VPC migration. In MEKK1 or 14-3-3γ knockdown cells, 7A had no stimulatory effect any more, suggesting that MEKK1 and 14-3-3γ are also essential for 7A-mediated VPC migration. However, 7Ap alone could induce VPC migration in MEKK1 but not in 14-3-3γ knockdown cells, indicating that 7Ap is downstream of MEKK1 but upstream of 14-3-3γ.

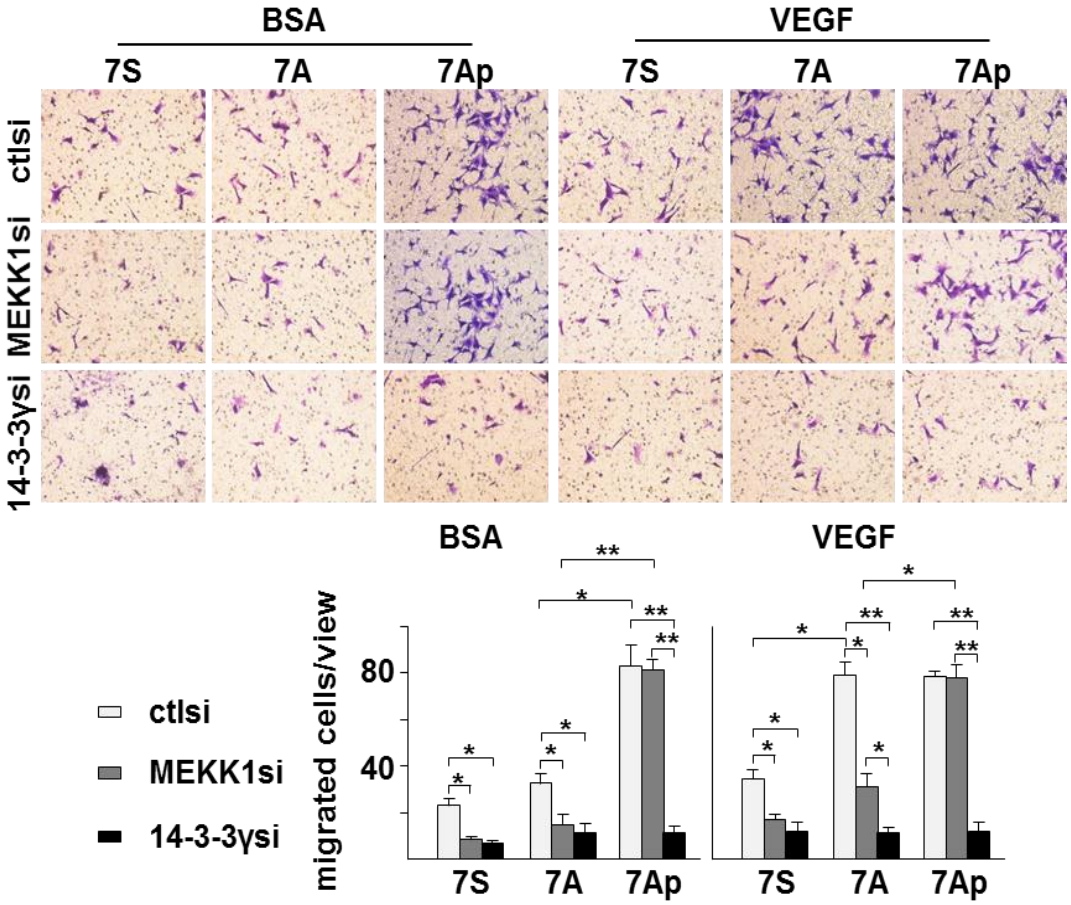


Figure 3.25 MEKK1-7Ap-14-3-3γ signal pathway mediated VEGF-induced VPC migration

Knockdown of MEKK1 or 14-3-3γ abolished VEGF-induced VPC migration. Transwell migration assays were performed on control siRNA (ctlsi), MEKK1 siRNA (MEKK1si) and 14-3-3γ siRNA (14-3-3γsi) transfected VPCs with 1ng/ml peptides and 10ng/ml VEGF. The upper panel shows the crystal violet staining images while the lower panel shows the mean value of migrated cells per view. Data presented are representative images or mean of three independent experiments. *: $p<0.05$. **: $p<0.01$.

3.3.3.3 7aa-peptide enhanced VEGF-induced VPC differentiation toward EC lineage *in vitro* via MEKK1-7A-14-3-3γ signal pathway

When ESCs differentiate into smooth muscle cells (SMCs), the HDAC7 transcript variant 2 undergoes further splicing, leading to the incorporation of 7A into the far N-terminal end of HDAC7 protein (Margariti et al., 2009). Thus, we assumed that the 7A might be involved in cell differentiation process. VPCs were cultured in differentiation medium in the presence of 7A and/or VEGF, followed by quantitative RT-PCR analysis of EC and SMC marker expression. As shown in Figure 3.26A, 7A increased CD31 and CD144 mRNA levels, which was significantly enhanced by VEGF although VEGF alone only had slight effect. The effect of 7Ap alone on CD31 and CD144 expression was comparable to the combined effect 7A and VEGF. 7A alone had no effect on SM22 expression but significantly decreased SM22 expression in the presence of VEGF, although VEGF had slight increasing effect. 7Ap alone significantly decreased SM22 expression. These results suggest that 7A especially 7Ap favours VPC differentiation toward EC lineage while suppresses SMC differentiation. The EC differentiation was further confirmed by tube formation assays (Figure 3.26B).

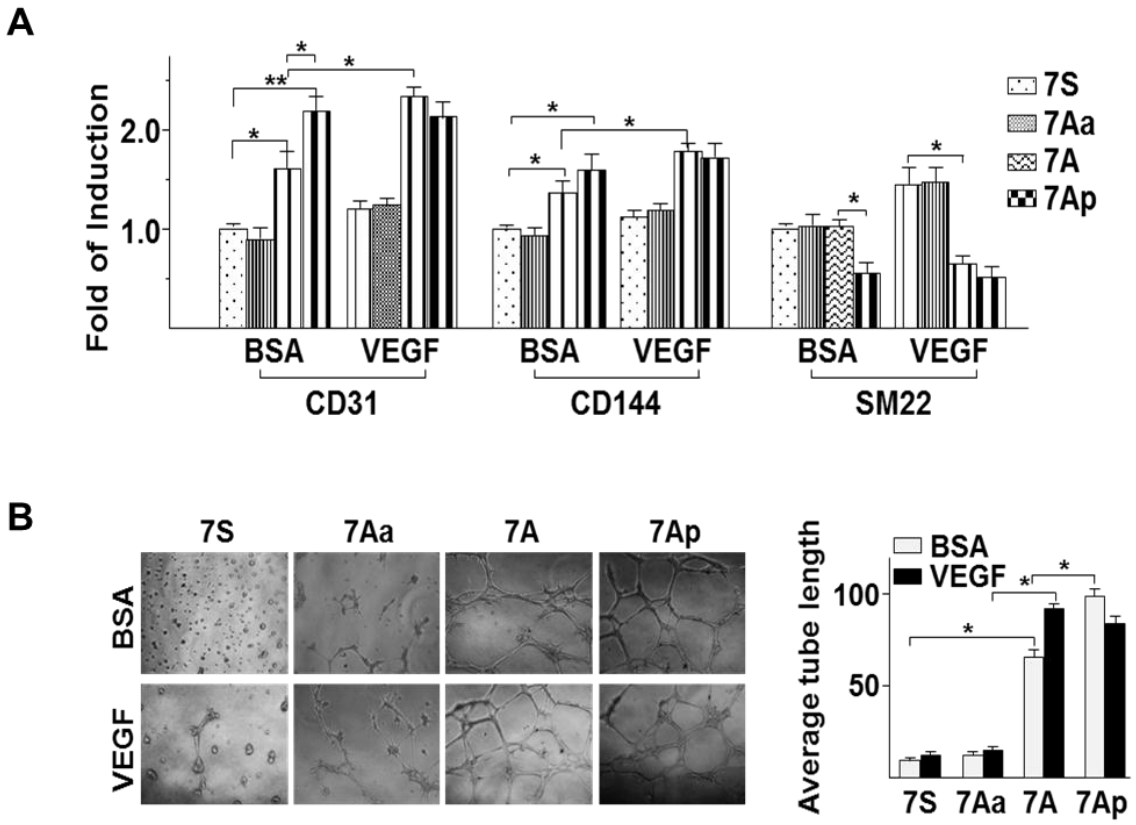


Figure 3.26 The 7aa-peptide enhance VEGF-induced VPC differentiation toward EC lineage.

The 7aa-peptide induced VPC differentiation toward EC lineage. The VPCs were cultured in differentiation medium in the presence of 0.1ng/ml peptides with/without 10ng/ml VEGF for 7 days, followed by quantitative RT-PCR analysis of CD31, CD144 and SM22 mRNA levels (A) or Matrigel tube formation assess (B). Data presented are representative images or mean of three independent experiments. *: $p<0.05$. **: $p<0.01$.

We have identified 7A is downstream of MEKK1 but upstream of 14-3-3γ, and MEKK1-7Ap-14-3-3γ involved in 7A regulated VPC migration. Similar trend was observed on their effect on CD31 and CD144 expression in differentiated VPC cells (Figure 3.27). Interestingly, knockdown of MEKK1 especially 14-3-3γ increased SM22 expression. The inhibitory effect of 7Ap on SM22 expression was decreased by 14-3-3γ knockdown. These results suggest MEKK1, 7A and 14-3-3γ form a signal pathway, contributing to VPC differentiation toward EC lineage.

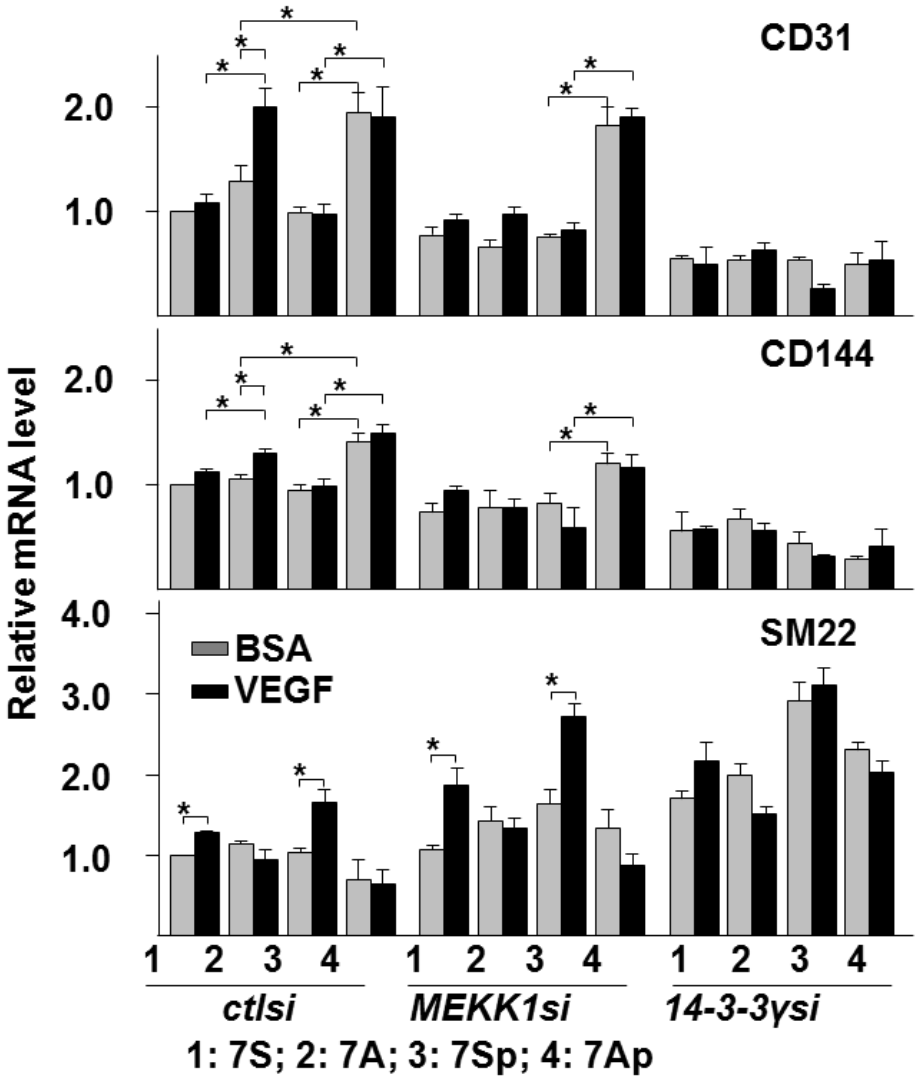


Figure 3.27 MEKK1-7A-14-3-3γ mediated 7aa-peptide induced VPC differentiation toward EC lineage.

The VPCs were cultured in differentiation medium and transfected with siRNA in the presence of 0.1ng/ml peptides with/without 10ng/ml VEGF for 7 days, followed by quantitative RT-PCR analysis of CD31, CD144 and SM22 mRNA levels. Data presented were representative image or mean of three independent experiments. *: $p<0.05$.

3.3.3.4 The 7A increased vascular injury repair and angiogenesis in ischemic tissues *in vivo*.

As 7A is able to enhance EC differentiation from VPCs *in vitro*, several animal models have been performed to check whether this peptide could also induce VPC differentiation *in vivo*. A wire-guided femoral artery injury model was then introduced in ApoE$^{-/-}$ mice (Xiao et al., 2006). Because 7A can enhance EC differentiation from VPCs *in vitro*, several animal models have been performed to assess whether this peptide could also induce VPC differentiation *in vivo*. Pluronic-127 gel containing 10ng/ml of peptides or PBS was applied surrounding the adventitia of the injured vessels, which were harvested 4 weeks post-surgery. The HE staining of cryo-sections revealed that 7A or 7Ap administration significantly reduced the neointima formation (Figure 3.28).

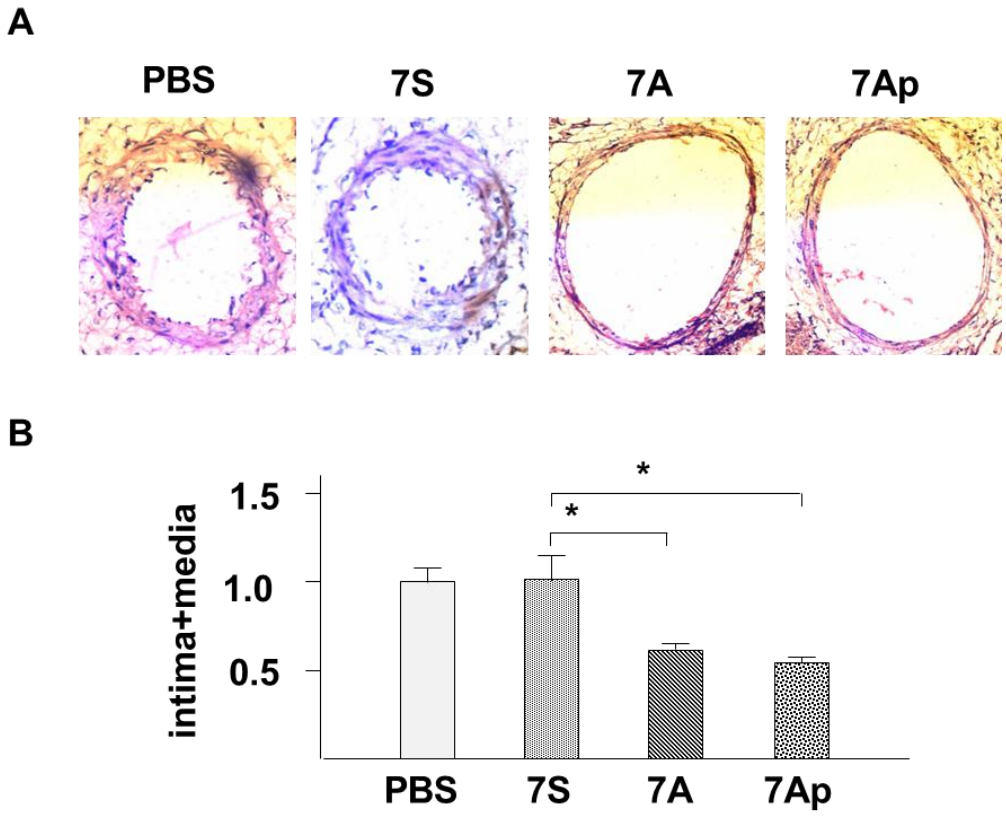


Figure 3.28 The 7A and 7Ap peptides attenuated neointima formation.

The 7A and 7Ap peptides attenuated neointima formation in mouse femoral artery wire-guided injury model. A panel shows the H.E. staining images of injured vessel sections 4 weeks post-surgery. B panel shows the average intima plus media area from 6 mice with that of PBS group set as 1.0. Data presented were representative image or mean of three independent experiments. *: $p<0.05$.

Besides, A hindlimb ischemia model was introduced in C57BL/6J mice, in which Pluronic-127 gel containing 10ng/ml peptides was applied surrounding the injured vessels (L. Zeng, Xiao, et al., 2013). Foot blood perfusion was measured by a Doppler Scanner at day 7 and day 14 post-surgery. These experiments were performed in 8-month (Figure 3.29A) and 10-weeek (Figure 3.29B) old mice. Although different age mice showed different recovery rate (PBS groups in both figures), 7Ap had a similar trend in promoting the foot blood perfusion recovery.

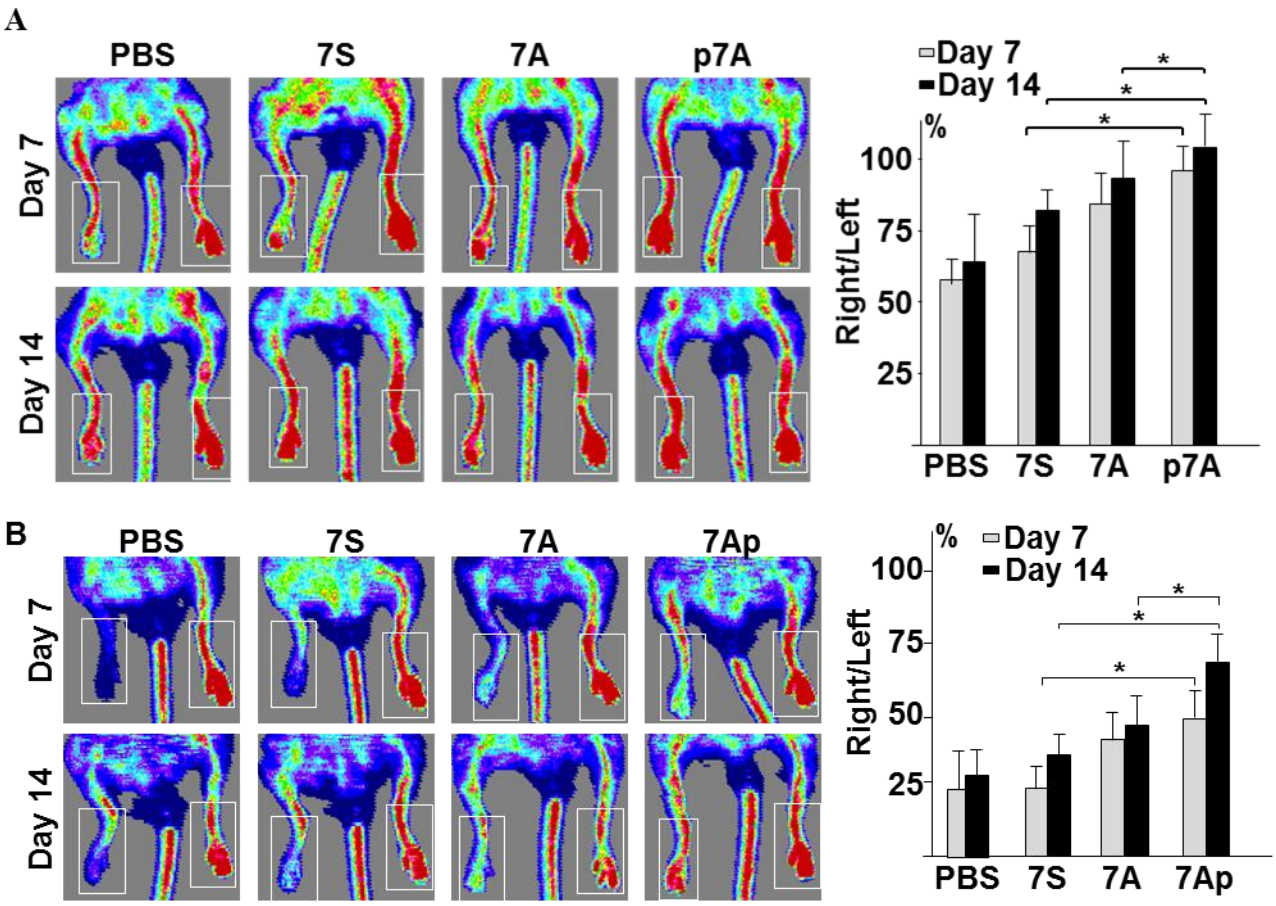


Figure 3.29 The 7A especially 7Ap peptide increased foot blood perfusion in mouse hindlimb ischemia model.

The hindlimb ischemia model was introduced into 8-month (A) / 10-week (B) old C57bl/6 mice by ligating and cutting the femoral artery in the right side. 200µl of F-127 pluronic gel containing 10ng/ml peptides was applied to the injured area. Foot blood flow was measured by Doppler Scanner at day 7 and day 14 post-surgery. Left panel shows the representative images of 6 mice for each group. The right panel shows the ratio of foot blood flow in the right injured side to that of the left uninjured side. *: $p<0.05$.

Immunofluorescence staining on the skeletal muscle sections with anti-CD31 and anti-Sca1 antibodies revealed more double positive cells in the 7Ap group (Figure 3.30). Importantly, many Sca1+ cell clusters were noted in the 7Ap group (Figure 3.30). These results suggest that 7A, especially 7Ap, stimulates VPC migration, proliferation and differentiation toward the EC lineage *in vivo*, contributing to vascular injury repair and angiogenesis in ischemic tissues.

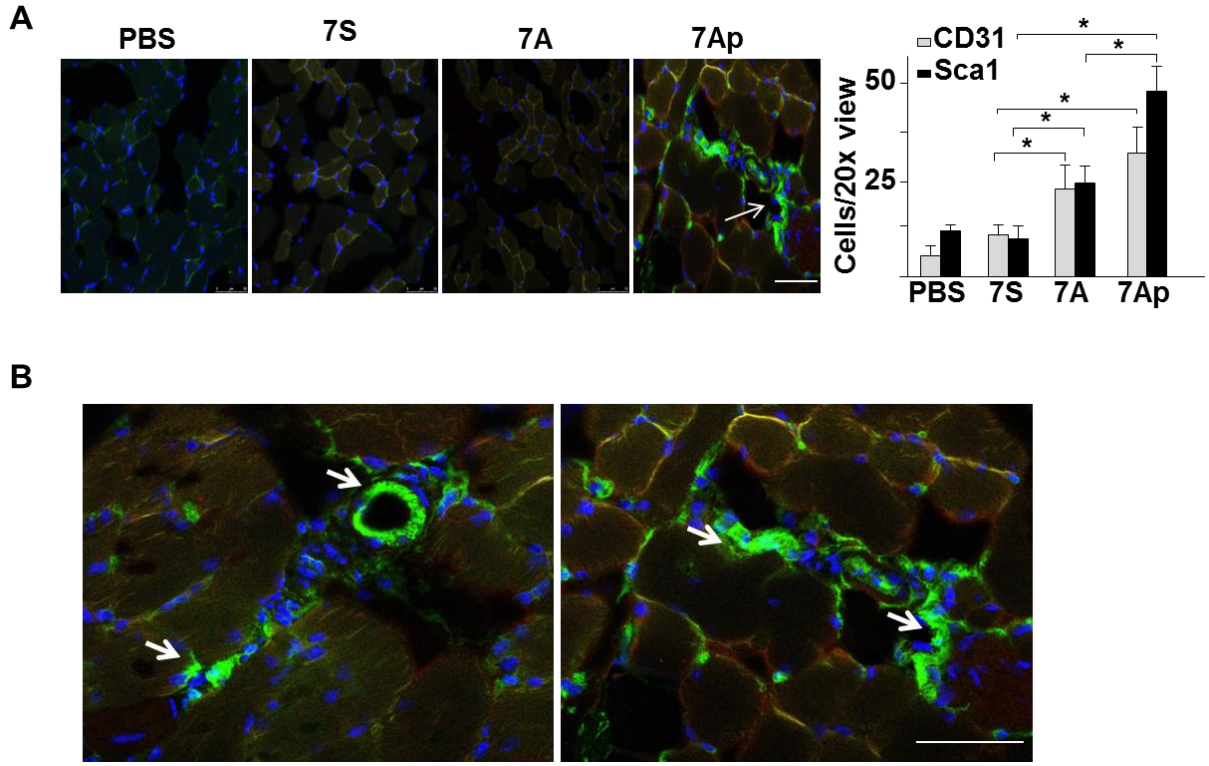


Figure 3.30 7A, especially 7Ap, stimulates VPC migration, proliferation and differentiation toward the EC lineage *in vivo*.

The peptide 7Ap increased Sca1+ (green) cell migration into the ischemic tissue and differentiation into CD31+ cells (red). (A) The left panel depicts representative immunofluorescence staining images on a skeletal muscle section from the injured leg. Arrow indicates the Sca1+ cell niche. The right panel shows the mean±SEM CD31+ /Sca1 + cells from six 20x views of each of the six mice per group. Scale bar: 50 μm. (B) 7Ap increased Sca1+ cell niche formation in ischemic tissues. Arrows indicate the Sca1+ niche. *: $p<0.05$.

3.3.4 Discussion

14-3-3γ protein belongs to 14-3-3 family, a group of conserved regulatory molecules, that are expressed in all eukaryotic cells. 14-3-3 family was already found to participate in the formation of the adherens junctions through interaction with catenins and plakoglobin complex, therefore controlling the neighboring cell-to-cell connections, and the intercellular and extracellular matrix-to-cell communications (Klezovitch & Vasioukhin, 2015; Morrison, 2009; Sehgal et al., 2014). In current study, 14-3-3γ protein is the effector of the MEKK1-7A-14-3-3γ signal pathway, as knockdown of 14-3-3γ abolishes VEGF or 7Ap-induced VPC migration and differentiation toward EC lineage.

Recently, *in vitro* experiments indicated that 14-3-3γ overexpression also played a potential role in cancer invasion (Raungrut et al., 2014), suggesting that 14-3-3γ protein plays an important role of in cell migration. In this study, knockdown of 14-3-3γ protein abolished VEGF or 7Ap-induced VPC migration, confirming the role of 14-3-3γ protein in VPC migration. VEGF or 7Ap increased 14-3-3γ protein phosphorylation at Thr145 site. Interestingly, increased phosphorylated 14-3-3γ was translocated to the nucleus, while the 14-3-3γ in cytoplasm shows no significantly changes. On the one hand, the nuclear translocation of 14-3-3γ protein may break down the cadherin/catenin/plakoglobin/14-3-3complex formation. Therefore, the cells lose cell-to-cell connection, contributing to cell migration. On the other hand, the phosphorylated 14-3-3γ protein in the nucleus may be involved in the regulation of gene transcription that is responsible for cell migration and differentiation. However how 14-3-3γ regulating cell migration is still not clear, and need to explore in further work.

In vivo, ischemic model were performed on 10 weeks and 8 months mouse. 7A and 7Ap shows much more effective on the 8 months mouse compared with 10 weeks mouse, though 7AP increased blood recovery significantly on both. 10 weeks mouse might be more sensitive

to injury, which will result in much stronger self-recovery and cover the 7A effect. The staining results suggested 7Ap might induce VPC migration to form a nitch like structure. A single cell colony formation assay verified that 7Ap promote VPC self-renew, proliferation and migration. As 7Ap shows such a strong function in promote angiogenesis in mouse, we wonder whether it would be also functional in human, which may have the potential to be a new therapeutic way in vascular disease. The role of 7Ap in human ECs and ES will be verified in our future work, and which may provide the basis of 7Ap to be a potential new drug.

Progenitor cell differentiation is such a complicated process that multiple signalling pathways are involved in. 7Ap promoted VPCs differentiation towards functional EC. Knockout 14-3-3γ abolished 7Ap-induced EC differentiation, indicating MEKK1-7A-14-3-3γ pathway is involved in EC differentiation. Besides, proteomic data also showed that 7A may participate in cell cycle regulation, as binding with CDK and other related proteins. Whether 7A regulates cell differentiation via cell cycle regulation would be an interesting project, and we will give a brief describe in the future work part.

3.3.5 Conclusions

The 7aa-peptide induces VPC proliferation, VEGF-induced migration and differentiation toward EC lineage in vitro via MEKK1-7A-14-3-3γ signal pathway. Furthermore, the 7A increased vascular injury repair and angiogenesis in ischemic tissues in vivo.

Chapter 4

Concluding Discussion and Future Work

4.1 Including Discussion

It is widely accepted that one mRNA molecule produces one peptide/protein. However, more and more evidence suggests that one mRNA molecule may produce several peptides through different ORFs or non-ATG-translation (Chu, Ma, & Saghatelian, 2015; Cleary & Ranum, 2014). In this study, we demonstrate that a sORF within the 5'-UTR of mouse HDAC7 transcript variant 2 can be translated to produce a biologically active 7aa-peptide, contributing to the $Sca1^+$ progenitor cell self-renewal, migration and differentiation toward EC lineage. The most important finding from this study is that this 7aa-peptide can participate in the signal transduction via acting as phosphorylation carrier, shedding new lights on the biological functions of the HDAC7 gene.

4.1.1 HDAC7 transcript variant 2 undergoes alternative transcription and translation

There are eight transcript variants in mouse HDAC7 mRNA molecules, producing different HDAC7 proteins with two types of the N-terminals. The transcript variants 3 and 4 have 22 amino acids more in the N-terminal as compared to other variants. In the 5'UTR of HDAC7 transcript variant 2, there is a sORF encoding 7aa-peptide with three cascade in-frame stop codons. The further splicing of this mRNA will join the sORF with the main ORF, producing the same N-terminal as transcript variants 3 and 4 (Margariti et al., 2009). The information indicates that the start codon of the sORF can really initiate the translation.

However, it is unknown whether this sORF can be translated alone. In this study, with the artificial construct pShuttle2-HD7FH, we demonstrate that this sORF is indeed translated in VPCs. The co-existence of FLAG and HA at a comparable level within single VPC suggests that both ORFs can be translated simultaneously. There is no overlap on FLAG and HA immunofluorescence staining, reflecting that there is no interaction between the 7aa-peptide and HADC7 protein and that there is no further splicing to join the two ORFs. Indeed, we

didn't detect HDAC7 in Bio-7A peptide pull down/proteomics studies. Our previous study showed that there was no splicing occurred in ESCs (Margariti et al., 2009). The exclusive staining of FLAG in the cytoplasm indicates that the 7A only locates in the cytoplasm. This should be true as it doesn't contain nuclear location signals.

4.1.2 7aa-peptide is essential for VPCs to maintain the pluripotency

The translation of the 7aa-peptide from the sORF may be very important for the VPCs to maintain the pluripotency. The first supporting evidence to this notion was that 7A especially 7Ap significantly increased VPC colony formation and cell numbers within a single colony. Further direct evidence came from the observation that 7Ap significantly increased $Sca1^+$ cell niche numbers in a mouse model. The indirect evidence was the observation that the ratio of FLAG to HA was significantly reduced upon the spontaneous differentiation with the removal of leukemia inhibitor factor. Further detailed investigation will be required to study whether leukemia inhibitory factor is involved in the translational regulation of the sORF and the underlying mechanisms.

In addition to VPC self-renewal, 7A also seems to be involved in VEGF-induced VPC migration and differentiation toward EC lineage. VEGF could increase sORF translation as revealed by the increase in the ratio of FLAG to HA in differentiated VPCs under VEGF treatment. Exogenous 7A could enhance VEGF function on the aspects of migration and differentiation toward EC lineage. Of course, detailed investigation will be required to study how VEGF drives the translation of the sORF.

4.1.3 7aa-peptide can function a kinase to transfer phosphate group

Besides demonstrating the translation of the sORF, the most important and striking finding of this study is that a 7aa-peptide can transfer phosphate group like a kinase. Protein phosphorylation is one type of post-translational modifications, which plays an important role in the cellular signal transduction. A phosphate group is originally transferred from a donor like ATP to a protein kinase, which in turn transfers the phosphate group to its substrate via protein-protein interaction. During this process, the kinase experiences phosphorylation-to-dephosphorylation shift. Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins (Burnett & Kennedy, 1954; C. Chang & Stewart, 1998; Cozzone, 1988; Stock et al., 1989). Normally, a kinase has multiple phosphorylation sites, each site may correspond to a set of substrates. Therefore, a kinase can participate in different cellular processes via receiving phosphate group at different sites and transferring the phosphate group to different substrates.

In this study, we demonstrate that 7A is the substrate of MEKK1 to receive phosphate group from the serine393 residue. The *MEKK1* knockdown significantly attenuated but not blocked VEGF-induced 7A phosphorylation, suggesting that MEKK1 is one of the upstream kinases for 7A phosphorylation. The presence of 7A accelerated the dephosphorylation of the VEGF-induced MEKK1 phosphorylation at Ser393 site, suggesting that 7A may facilitate the phosphate group transferring from this site. The retaining of MEKK1Ser393 phosphorylation by the presence of 7Aa confirms that 7A acts as the phosphate group recipient. The serine/alanine substitution makes 7Aa lacking the ability to receive phosphate group. To receive the phosphate group, an alkaline aa at the histidine residue seems essential as lysine or arginine substitution only slight reduced but leucine or threonine substitution totally abolished VEGF-induced 7aa phosphorylation. The proline ring seems critical for this phosphorylation process as substitution with valine (the same carbons) ablated 7-aa phosphorylation. Strikingly, 7A can directly transfer this phosphate group to the Thr145

residue of 14-3-3γ protein in a way like a kinase. The 7Ap not only increased 14-3-3γ protein phosphorylation at Thr145 site in living cells but also in a cell-free in-gel reaction system. Under the cell free system, 7Ap directly phosphorylates 14-3-3γ protein. The binding of 7Ap to 14-3-3γ protein may cause huge conformation changes among both of them, creating enough energy to complete the phosphate group transferring from the serine residue in 7Ap to the threonine residue in 14-3-3γ.

The significance of this striking finding is that a small peptide such as 7A transfers phosphorylation from a kinase to an effector independent of the direct interaction between the kinase and the effector, extending the spectrum of the effectors downstream a kinase.

4.1.4 MEKK1-7A-14-3-3γ signal pathway is involved in 7Ap-induced VPC migration and differentiation

14-3-3γ protein is the effector of the MEKK1-7A-14-3-3γ signal pathway, as knockdown of 14-3-3γ abolishes VEGF or 7Ap-induced VPC migration and differentiation toward EC lineage. 14-3-3γ protein belongs to a family of conserved regulatory molecules, participating in the formation of the adherens junctions through interaction with catenins and plakoglobin complex, therefore controlling the neighboring cell-to-cell connections, and the intercellular and extracellular matrix-to-cell communications (Klezovitch & Vasioukhin, 2015; Morrison, 2009; Sehgal et al., 2014). An intact adherens junction keeps the cells quiescence via suppressing cell migration and proliferation. It has been reported that high level of 14-3-3γ protein was related to cancer cell invasion while ablation of 14-3-3γ protein led to neural migration delay (Raungrut et al., 2014; Wachi et al., 2015), suggesting that 14-3-3γ protein plays an important role of in cell migration.

In this study, knockdown of 14-3-3γ protein abolished VEGF or 7Ap-induced VPC migration, confirming the role of 14-3-3γ protein in VPC migration. VEGF or 7Ap increased 14-3-3γ protein phosphorylation at Thr145 site, which was translocated to the nucleus. The nuclear translocation of 14-3-3γ protein may break down the cadherin/catenin/plakoglobin/14-3-3γ complex formation. Therefore, the cells lose cell-to-cell connection, facilitating cell migration. Moreover, the phosphorylated 14-3-3γ protein in the nucleus may be involved in the regulation of gene transcription that is responsible for cell migration and differentiation.

4.1.5 The translation shift between 7aa-peptide and HDAC7 protein

HDAC7 plays an important role in the maintenance of the cadherin-catenin complex (Hui, Brunt, & Husain, 2010; Margariti et al., 2010). It has been documented that PKD-mediated phosphorylation contributes to HDAC7 degradation leading to cell migration and proliferation (Sinnett-Smith et al., 2014; Wang et al., 2008). A recent report showed that VEGF-PKD1-HDAC7 pathway contributed to endothelial progenitor cell migration and differentiation toward EC lineage (Yu et al., 2014).

In this study, we demonstrated that a short ORF encoding a 7aa-peptide could be translated from the 5'-UTR together with the main ORF as revealed by the occurrence of both FLAG- and HA-tagged peptides within a single cell. Importantly, there existed a translation shift from the main ORF to the short one within VPCs in response to VEGF treatment as revealed by the increase of FLAG to HA ratio. This translation shift will decrease HDAC7 protein, providing an alternative strategy to control HDAC7 protein level. The decrease of HDAC7 protein may affect the cadherin-catenin complex, contributing to cell migration and proliferation. Taken together, we can assume that the changes on the cadherin-catenin

complex can be derived from the combined effect of the occurrence of the 7A and the decrease of HDAC7 protein.

In summary, VEGF induces HDAC7 mRNA to undergo translation shift from the main ORF to the short one in VPCs, giving rise to a 7aa-peptide (7A). On the other hand, VEGF activates MEKK1 via phosphorylation at the Ser393 residue. The activated MEKK1 transfers the phosphate group from Ser393 to the serine residue within the 7aa-peptide. The phosphorylated 7aa-peptide (7Ap) in turn transfers the phosphate group to the Thr145 residue within 14-3-3γ protein, leading to cadherin-plakoglobin-catenin-14-3-3γ complex disruption and 14-3-3γ nuclear translocation. Other signal pathways may be also activated by 7Ap. The overall effect is to induce VPC proliferation, migration and differentiation toward EC lineage, leading to angiogenesis and vascular injury repair *in vivo* (Figure 4.1). The significance of this study is to discover that a small peptide can function like a kinase.

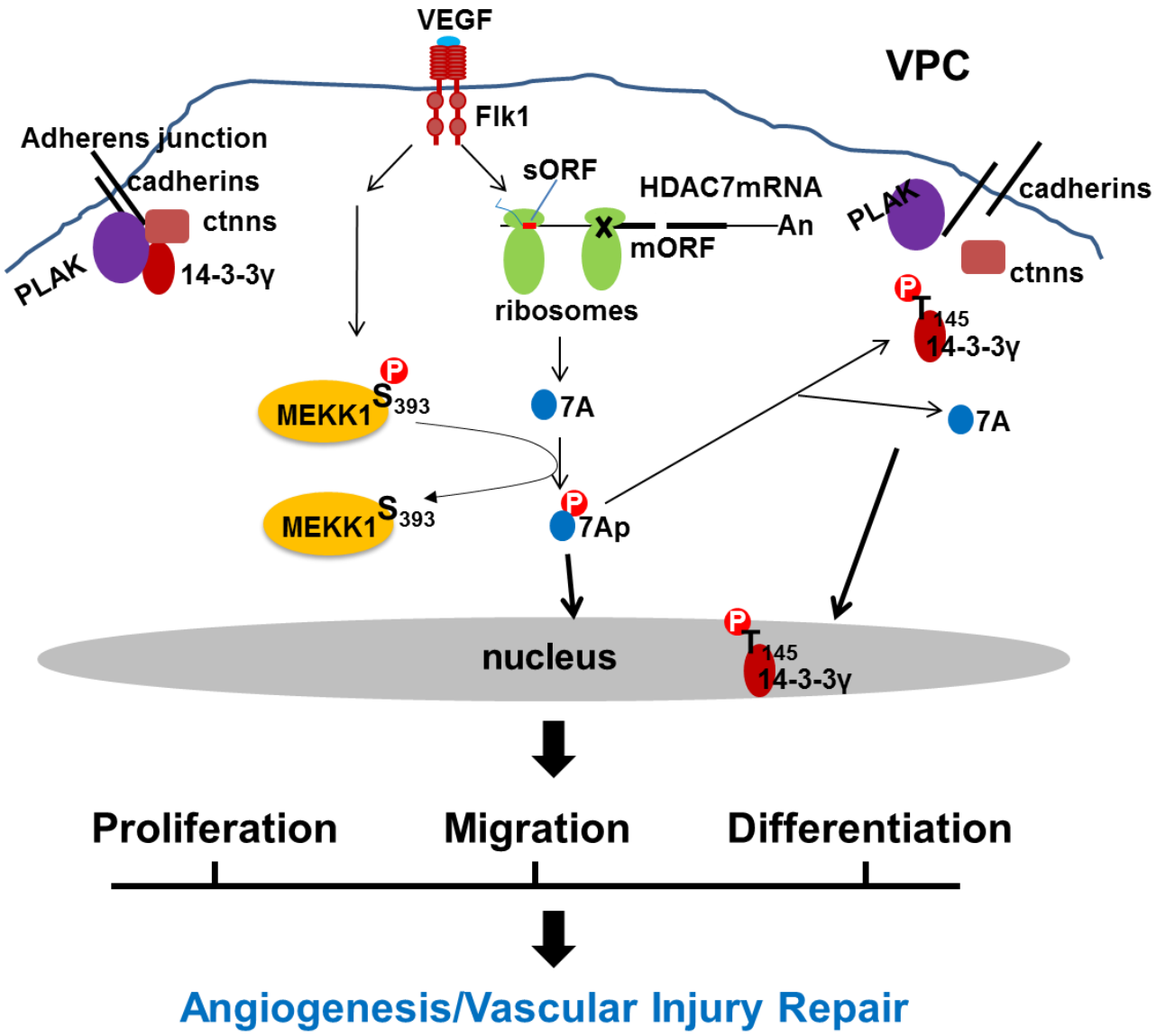


Figure 4.1: A schematic illustration of VEGF-induced HDAC7 alternative translation and its role in VPC proliferation, migration and differentiation.

VEGF binds to its receptor VEGFR-2, triggering the translational shift of HDAC7 from the main ORF to the sORF, giving rise to a 7aa-peptide (7A). VEGFR-2 activates MEKK1 phosphorylation at Ser393 site. The phosphorylated MEKK1 transfers the phosphate group from Ser393 to the serine residue of 7A, forming the 7Ap. 7Ap transfers the phosphate group to Thr145 site within 14-3-3γ, leading to 14-3-3γ nuclear translocation, therefore disrupting the cadherin/plakoglobin (PLAK)/catenins (ctnns)/14-3-3γ complex. 7Ap may also activate other pathways. On the other hand, the decrease of HDAC7 protein may also contribute to the disruption of the cadherin/plakoglobin (PLAK)/catenins (ctnns)/14-3-3γ complex. The overall effect is to increase VPC proliferation, migration and differentiation toward EC lineage, contributing to angiogenesis and vascular injury repair *in vivo*.

4.2 Future Work

4.2.1 Translational regulation of sORF and mORF of HDAC7-tv2

Much effort has been made in this project to elucidate the 7A acts as a phosphate group carrier to transfer phosphate group from MEKK1 to 14-3-3γ. The production of 7A and the translational regulation of sORF of HDAC7 is still unknown. We wonder that protein or non-coding RNA might bind to the upstream sequence of sORF or mORF to block or facilitate the access of ribosome.

Firstly, we will compare the alternative translation among different cell types and conditions. In details, Ad-HD7FH infection will performed on ES, differentiated ES, dES plus LIF or VEGF, VPCs, differentiated VPCs, dVPCs plus LIF or VEGF, mECs, mSMCs. ELISA with anti-FLAG and anti-HA antibodies will be performed to detect the translation of sORF and mORF5. These experiments will give us information whether there is alternative translation during these cellular processes and the potential role of the 7A peptide.

Then, we will construct mHD7NC reporter system, in which the 5'UTR of mouse HDAC7 mRNA was inserted in pSI-Check2 vector with the Renilla luciferase ORF fused with HDAC7 mORF. The expression level of Renilla luciferase will represent HDAC7 mORF expression. Transfection of this construct into ES, differentiated ES, dES plus LIF or VEGF, VPCs, differentiated VPCs, dVPCs plus LIF or VEGF, mECs, mSMCs, followed by Renilla/Firefly luciferase activity assay. These experiments will confirm data obtained from ELISA and provide a useful and convenient reporter system.

Moreover, DNA fragments complementary to the upstream sequence of sORF and mORF will be synthesized respectively and transfected together with the HDAC7NC reporter into those cells to confirm data obtained from above. In order to identify the potential regulatory

proteins, Biotin-labelled RNA fragments will be synthesized identical to the upstream sequence of sORF and mORF respectively. RNA/Protein pull-down assay with cell lysate will be performed and isolated from cells described above, followed by proteomics analysis. These experiments will identify the potential regulatory proteins for the alternative translation.

Finally, we will analyse the changes of the regulator proteins in different cells or different conditions. RT-PCR analysis will be performed to detect mRNA level of these proteins; Western blot will be performed to analyse the protein level changes and modification changes under different conditions; Double immunofluorescence staining or immunoprecipitation assay will be performed to detect the association of this candidate with translation initiation factors; A modified ChIP assay plus RT-PCR will be performed to amplify HDAC7 mRNA and ribosomal RNA; Mutant candidate protein, in which the modification site was substituted with amino acid that is unable to undergo the modification, will be created to compare the mutant and wild type gene on HDAC7NC reporter. These experiments will give us information whether this protein bridges HDAC7 mRNA and ribosome. They will also give us the information on the role of the modification in the regulation of alternative translation.

4.2.2 Explore the mechanism of the phosphorylated 14-3-3γ drive VPC migration and differentiation.

There is no much work on 14-3-3γ, till now only 111 papers have been recorded in total. These are mainly in neuroscience and cancer research. In this study, my data revealed that this protein could be phosphorylated by 7Ap at Thr145 site, which drove the protein nuclear translocation. Knockdown of this protein abolished 7Ap-induced VPC migration and differentiation toward EC lineage. There are two main questions to be answered.

One question is that: Is the phosphorylation of Thr145 site essential for 14-3-3γ nuclear translocation and how is this regulated? We will create the Ad-FLAG1433G and Ad-FLAG1433GT145V viral constructs. A FLAG tag will be fused to the N-terminal of 14-3-3γ protein. In the Ad-FLAG1433Gm construct, the Thr145 is substituted with valine, losing the ability to be phosphorylated. Immunofluorescence staining or cellular fractionation of virus infected VPCs that are treated with 7Ap will confirm the essentiality of Thr145 phosphorylation in 14-3-3γ nuclear translocation. Next, we will association of 14-3-3γ with kinesin. Kinesin is a motor molecule, trafficking proteins from cytoplasm into nucleus. The co-immunoprecipitation or double immunofluorescence staining assays will give us information whether there is affinity difference between the wild type and mutant 14-3-3γ to kinesin. These experiments will provide a mechanism on 14-3-3γ nuclear translocation.

The other one is that: How does the phosphorylated 14-3-3γ drive VPC migration and differentiation? First and forest, the two viruses-infected VPCs were treated with 7Ap, followed by migration and differentiation assay respectively. These experiments will give direct evidence on the role of Thr145 phosphorylation in VPC migration and differentiation regulation. It is established that Akt is involved in cell migration. Our previous study has shown that Akt can form a complex with mTOR (Martin D, JBC 2014). It has been reported that 14-3-3γ can up-regulate mTOR signalling. So, we will assess whether 14-3-3γ associates with mTOR and Akt and whether Thr145 phosphorylation facilitate this interaction. Co-immunoprecipitation and double staining will be performed using anti-FLAG and anti-mTOR or Akt. We will also detect mTOR and Akt phosphorylation in control siRNA and 14-3-3γ siRNA knockdown VPCs or viruses-infected VPCs following 7Ap treatment. Furthermore, immunoprecipitation will be performed with anti-FLAG antibody from cell lysates isolated from the two viruses-infected VPCs following 7Ap treatment. The 14-3-3γ associated proteins will be subjected to proteomics analysis. The proteins missing

in the mutant 14-3-3γ may be the potential candidates responsible for migration and differentiation. We will also pay attention to the phosphorylation changes of the associated proteins. Some of the modification changed proteins are the potential candidates, and the identification of potential candidates need to be verified using knockdown or pathway analysis. As phosphorylated 14-3-3γ is located in the nucleus, it is possible that this protein functions as a co-factor for gene transcriptional regulation. We can perform ChIP assay with anti-phospho-14-3-3γThr145 antibody. The isolated DNA fragment can be subjected to microarrays or DNA sequencing. The VPCs are paired as treated/untreated with 7Ap. These experiment might to draft the way of phosphorylated 14-3-3γ drive VPC migration and differentiation.

4.2.3 Explore the mechanism of 7Ap regulate VPC proliferation.

Firstly, we will verify whether 7A translation is essential for VPC proliferation. Synthesize a DNA fragment complementary to the upstream sequence of the sORF and perform transfection into VPCs. These cells will be infected with Ad-mHD7FH, followed by ELISA with anti- FLAG and anti-HA antibodies. These experiments will assess whether the DNA fragment can block the translation of 7A. VPCs will be transfected with the DNA fragment followed by proliferation assay. These experiments will give us information on whether 7A is essential for cell proliferation. Use CRISP/Cas technique to create an ES or VPC cell line that the sORF is deleted. Exogenous 7A or 7Ap can be added during the creation process. When the cell line was established, its self-renewal will be assessed in the absence or presence of exogenous 7A or 7Ap. If 7A is essential, there may be no colony formation in the absence of exogenous 7A.

Secondly, we will explore the effect of 7Ap on VPC cell growth and cell cycle progression. VPCs are treated with PBS, 7S, 7A or 7Ap for 7 days, followed by calculation of cell size and cell number every day to get information whether 7Ap has effect on cell growth in size and doubling time. VPCs are treated with PBS, 7S, 7A or 7Ap for 24hr, followed by FACS analysis of cell cycle to get information whether 7Ap affect each cell cycle phase.

Finally, we will identify how 7Ap drives VPC proliferation. From our pilot proteomics data, we found that 7A/7Ap was associated with some cyclin-dependent kinases. These CdKs may be good candidates on this aspect. We will go through the database again to check whether there are other proteins that are involved in cell cycle control. Then we will perform Western blot, cellular fractionation, immunofluorescence staining to check candidate modification and cellular location changes and perform microarrays on VPCs+7Sp vs VPCs+7Ap, VPCs vs mutant VPCs and VPCs+ZK770 vs VPCs+ZK771. Comparison among these three databases may find some key genes that play a role in VPC self-renewal.

Chapter 5

Publications

5.1 Journal Articles

5.1.1 Published

1. **Junyao Yang,** Andriana Margariti, Lingfang Zeng. **Analysis of Histone Deacetylase 7 (HDAC7) Alternative Splicing and Its Role in Embryonic Stem Cell Differentiation Toward Smooth Muscle Lineage.** *Methods Molecular Biology*, 1436, 978-1-4939-3665-6.

2. Zeng L, Li Y, **Yang J**, Wang G, Margariti A, Xiao Q, Zampetaki A, Yin X, Mayr M, Mori K, Wang W, Hu Y, Xu Q. **XBP 1-Deficiency Abrogates Neointimal Lesion of Injured Vessels Via Cross Talk With the PDGF Signaling.** *Arterioscler Thromb Vasc Biol*. 2015 Oct;35(10):2134-44. doi: 10.1161/ATVBAHA. 115.305420. Epub 2015 Aug 27.

3. **Yang JY**, Wang Q, Wang W, Zeng LF**. Histone deacetylases and cardiovascular cell lineage commitment.** *World J Stem Cells*. 2015 Jun 26;7(5):852-8. doi: 10.4252/wjsc.v7.i5.852.

4. **Junyao Yang**, Qian Wang and Lingfang Zeng**. Histone deacetylases in vascular remodelling.** *Chinese Journal of Hypertension* [J] May 2014, Vol. 22 No.5:403-409.

5. Hu YW[1], **Yang JY[1]** , Ma X, Chen ZP, Hu YR, Zhao JY, Li SF, Qiu YR, Lu JB, Wang YC, Gao JJ, Sha YH, Zheng L, Wang Q. **A lincRNA-DYNLRB2-2/GPR119/GLP-1R/ ABCA1- dependent Signal Transduction Pathway Is Essential for the Regulation of Cholesterol Homeostasis and Inflammatory Reactions.** *J Lipid Res*. 2014; 55(4): 681-697.

6. Martin D, Li Y, **Yang J**, Wang G, Margariti A, Jiang Z, Yu H, Zampetaki A, Hu Y, Xu Q, Zeng L. **Unspliced X-box-binding protein 1 (XBP1) protects endothelial cells from oxidative stress through interaction with histone deacetylase 3**. J Biol Chem. 2014 Oct 31;289(44):30625-34.

7. Zhao Y, Li Y, Luo P, Gao Y, **Yang J**, Lao KH, Wang G, Cockerill G, Hu Y, Xu Q, Li T, Zeng L. **XBP1 splicing triggers miR-150 transfer from smooth muscle cells to endothelial cells via extracellular vesicles.** *Sci Rep.* 2016 Jun 24;6:28627. doi: 10.1038/srep28627.

8. Yan-Wei Hu, Jia-Yi Zhao, Shu-Fen Li, Jin-Lan Huang, Yu-Rong Qiu, Xin Ma, Shao-Guo Wu，Zhi-Ping Chen, Ya-Rong Hu, **Jun-Yao Yang**, Yan-Chao Wang, Ji-Juan Gao, Yan-Hua Sha, Lei Zheng and Qian Wang. **RP5-833A20.1/miR-382-5p/NFIA-Dependent Signal Transduction Pathway Contributes to the Regulation of Cholesterol Homeostasis and Inflammatory Reaction.** *Arterioscler Thromb Vasc Biol*. published online August 14, 2014.

9. Hu YW, Zhang P and **Yang JY** *et al*. **Nur77 decreases atherosclerosis progression in apoE(-/-) mice fed a high-fat/high-cholesterol diet.** *PLoS One.* 2014 Jan 31;9(1):e87313. doi:10.1371/journal.pone. 0087313. E Collection 2014.

10. **Yang JY**, Hu YW, Zhang P, Zheng L, Wang Q. Progress of Niemann-Pick type C1 Like 1 on cholesterol metabolism. *Sheng Li Xue Bao.* 2012 Dec 25;64(6):721-8.

5.1.2 In preparation

1. **Junyao Yang**, Jing Xu, Ka Hou Lao, Andriana Margariti, Qiang Zhao, Wei Ding, Gang Wang, Lei Zheng, Zhongyi Zhang, Yanhua Hu, Wen Wang, Qingbo Xu, Qian Wang, and Lingfang Zeng. **A histone deacetylase 7-derived 7-amino-acid peptide acts as a phosphate group transporter.** (submitted manuscript)

2. **Junyao Yang**, Wen Wang and Liangfang Zeng et al. **XBP1 Splicing Promotes Wound Healing via Modulating Endothelial Nitric Oxide Synthase.**(Awaiting submission)

5.2 Meeting Abstracts

1. **Junyao Yang**, Qian Wang, Wen Wang, Lingfang Zeng. **HDAC7-derived 7aa peptide may function as a phosphorylation carrier.** Arteriosclerosis, Thrombosis, and Vascular Biology Peripheral Vascular Disease (ATVB | PVD) 2016 Scientific Sessions May 5-7, 2016, May 5 – 7, 2016. Omni Nashville. (Poster Presentation)
2. **Junyao Yang**, Kao Hou, Lao, Lingfang Zeng. **HDAC7-derived 7aa peptide may function as a phosphorylation carrier.** BCS Annual Conference, 6 – 8 June 2016 in Manchester. (Poster Presentation)
3. **Junyao Yang**, Yi Li, Lingfang Zeng**. Scratch activated XBP1 splicing promote endothelial cell migration.** Arteriosclerosis, Thrombosis, and Vascular Biology Peripheral Vascular Disease (ATVB | PVD) 2015 Scientific Sessions May 7–9, 2015, San Francisco, California. (Poster Presentation)
4. **Junyao Yang**, Lingfang Zeng. HDAC7-derived 7aa peptide may function as a phosphorylation carrier. BSCR Autumn Meeting-Cardiovascular Signalling in Health and Disease, University of Reading, Palmer Building 8-9September 2014. (Poster Presentation)

5. **Junyao Yang**, Qian Wang, Wen Wang, Lingfang Zeng. **HDAC7-derived 7aa peptide may function as a phosphorylation carrier.** 2013 SEMS Postgraduate Symposium 2013, Queen Mary, University of London. (Poster Presentation)
6. **Junyao Yang**, Wen Wang, Qian Wang, Lei Zheng, Lingfang Zeng. HDAC7-derived 7aa peptide may function as a phosphorylation carrier.2013 Yue Gui Qiong, National Clinical Laboratory Meeting.(Invited Oral Presentation and First prize)
7. **Junyao Yang**, Wen Wang, Qian Wang, Lei Zheng, Lingfang Zeng. HDAC7-derived 7aa peptide may function as a phosphorylation carrier. The 19th Annual Meeting of the Chinese Life Science Society –UK.(Oral Presentation)

Abbreviations

7A, 7aa-peptide	**7 amino acids peptide**
7Ap	**Phosph-7A**
7Sp	**Phosph-7S**
AP-1	**activator protein 1**
ASK1	**Apoptosis signal-regulating kinase 1**
bFGF	**basic Fibroblast Growth Factor**
BBP	**branch-point binding protein**
Bcl-2	**B-cell lymphoma 2**
Bio-7A	**Biotin marked 7A**
Bio-7Ap	**Biotin marked 7Ap**
Bio-7S	**Biotin marked 7S**
Bio-7Sp	**Biotin marked 7Sp**
CD3	**cluster of differentiation 3**
CD31	**cluster of differentiation 31**
CD34	**cluster of differentiation 34**
CD44	**cluster of differentiation 44**
CD45	**cluster of differentiation 45**
CD73	**cluster of differentiation 73**
CD90	**cluster of differentiation 90**
CD105	**cluster of differentiation 105**

CD133	**cluster of differentiation 133**
CD144	**cluster of differentiation 144**
cDNA	**complementary DNA**
CM	**conditioned medium**
ctrl	**control**
DAPI	**4',6-diamidino-2-phenylindole**
ddH_2O	**Double distilled water**
DMEM	**Dulbecco's Modified Eagle's Medium**
DMSO	**Dimethyl Sulfoxide**
dNTPs	**deoxyriobonucleotide triphosphates**
ECs	**Endothelial Cells**
ECM	**Extracellular matrix**
EDTA	**Ethylenediaminetetraacetic Acid**
EGF	**Epidermal Growth Factor**
ELISA	**Enzyme-linked immuno sorbent assay**
ELISA	**Enzyme-Linked Immunosorbent Assay**
eNOS	**Endothelial Nitric Oxide Synthase**
ERK	**Extracellular signal–Regulated Kinase**
ERK1/2	**Extracellular signal–Regulated Kinase 1/2**
ESCs	**Embryonic Stem Cells**
FBS	**Fetal bovine serum**

FGF	**Fibroblast Growth Factor**
GFP	**green fluorescent protein**
HDAC	**Histone acetyltransferase**
HDACi	**Histone acetyltransferase inhibitors**
HE stian	**Haematoxylin and Eosin stain**
HRP	**Horseradish Peroxidase**
ICAM-1	**Intercellular Adhesion Molecule 1**
IGF-1	**Insulinlike growth factor**
IL	**Interleukins**
JNK	**c-Jun N-terminal kinase**
JNK1	**c-Jun N-terminal kinase1**
kSDS	**Sodium Dodecyl Sulfate**
LIF	**Leukemia Inhibitory Factor**
MAPK	**Mitogen-activated protein kinase**
MEKK1	**Mitogen-activated protein kinase kinase kinase 1**
MMP	**matrix metalloproteinase**
MMPs	**Matrix Metalloproteinases**
mRNA	**Messenger RNA**
mRNA	**messenger RNA**
MTT	**Methyl thiazolyl tetrazolium**
NO	**Nitric Oxide**

NO	**Nitric Oxide**
P1	**Promoter1**
P2	**Promoter2**
PDGF	**Platelet-Derived Growth Factor**
PDGF-B	**platelet-derived growth factor B**
PDGF-BB	**platelet-derived growth factor BB**
PDGFR-β	**Beta-type platelet-derived growth factor receptor**
PKB	**protein kinaseB**
PKD	**protein kinase D**
PlGF	**Placental Growth Factor**
PI3K	**Phosphatidylinositol-4,5-bisphosphate 3-kinase**
PP1	**protein phosphatases 1**
PP1A	**protein phosphatases 2A**
pre-mRNA	**pre-messenger RNA**
qPCR	**Quantitative polymerase chain reaction**
qPCR	**quantitative real time Polymerase Chain Reaction**
RTKs	**Tyrosine kinases**
RT-PCR	**Reverse transcription polymerase chain reaction**
Sca-1	**Stem cells antigen-1**
SEM	**Standard Error of the Mean**
SiRNA	**small interfering RNA**

siRNA	**small interfering RNA**
SMCs	**Smooth Muscle Cells**
snRNPs	**small nuclear ribonucleoproteins**
sORF	**Short open reading frame**
SSH1L	**slingshot 1-like**
TGF-β	**Transforming Growth Factor beta**
TNF-α	**Tumor Necrosis Factor alpha**
VCAM-1	**Vascular Cell Adhesion Molecule-1**
VE-cadherin, CD144	**Vascular endothelial cadherin**
VEGF	**Vascular endothelial growth factor**
VEGF	**Vascular Endothelial Growth Factor**
VEGFR	**Vascular Endothelial Growth Factor Receptor**
VEGFRs	**Vascular endothelial growth factor recepters**
VPCs	**Vascular Progenitor Cells**
WB	**Western Blot**
µg	**microgram**
µg	**microgram**
µl	**microliter**
µl	**microliter**
µm	**micrometre**
mg	**minigram**

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