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REVIEW

Contributions of neutrophils to the adaptive immune response in autoimmune disease

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Abstract

Neutrophils are granulocytic cytotoxic leukocytes of the innate immune system that activate during acute inflammation. Neutrophils can also persist beyond the acute phase of inflammation to impact the adaptive immune response during chronic inflammation. In the context of the autoimmune disease, neutrophils modulating T and B cell functions by producing cytokines and chemokines, forming neutrophil extracellular traps, and acting as or priming antigen presentation cells. Thus, neutrophils are actively involved in chronic inflammation and tissue damage in autoimmune disease. Using rheumatoid arthritis as an example, this review focuses on functions of neutrophils in adaptive immunity and the therapeutic potential of these cells in the treatment of autoimmune disease and chronic inflammation.

Key words: Neutrophils; Chronic inflammation; Autoimmune disease; Rheumatoid arthritis; Collagen-induced arthritis

Core tip: Neutrophils guide the adaptive immune response in persistent inflammation by directly and indirectly interacting with T and B cells. Understanding and manipulating these roles of neutrophils will lead to novel therapeutic approaches to prevent and treat autoimmune diseases, such as rheumatoid arthritis.

Pietrosimone KM, Liu P. Contributions of neutrophils to the adaptive immune response in autoimmune disease. *World J Transl Med* 2015; 4(3): 60-68 Available from: URL: http://www.wjgnet.com/2220-6132/full/v4/i3/60.htm DOI: http://dx.doi.org/10.5528/wjtm.v4.i3.60

INTRODUCTION

Neutrophils are polymorphonuclear granulocytes comprised of enzyme-containing granules. Neutrophils generate from the bone marrow and account for

50%-70% of circulating leukocytes in humans and 10%-25% in mice[1,2]. Under acute inflammation, particularly as a result of bacterial infection, neutrophils are the first leukocytes to respond, migrate to the site of inflammation, and kill microorganisms through phagocytosis, degranulation and generation of neutrophil extracellular traps (NETs)[3-5]. These cells have long been thought of as short-lived cells of the innate immune response.

However, recent research evidence has demonstrated that neutrophils persist beyond acute inflammation to initiate and perpetuate chronic inflammation. The onset of inflammation increases the lifespan of neutrophils in circulation, anywhere from 12 h to several days[6]. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ, inhibit neutrophil apoptosis[7]. Neutrophils also change phenotypes as inflammation persists. For instance, Neutrophils isolated from various inflammatory states show altered cell densities in gradient centrifugation[8,9]. The expression of granulocyte marker CD66b in neutrophils increases in patients with rheumatoid arthritis (RA), and decreases with the treatment by anti-inflammatory glucocorticoids, suggesting neutrophils may alter functions during chronic inflammation[10]. Glucocorticoids, a class of drugs often prescribed to patients with autoimmune disease, also inhibit apoptosis of neutrophils and increase the neutrophilic production of reactive oxygen species (ROS)[11].

Tissue-specific autoimmune disease such as RA, multiple sclerosis, and type Ⅰ diabetes are generated from unknown etiology and impact the quality of life of patients through sustained chronic inflammation driven by innate and adaptive immune responses. Neutrophils bridge the innate and adaptive immune response in autoimmune disease. This review focuses on the functional heterogeneity of neutrophils in autoimmune disease and the contribution of these roles to chronic inflammation, in the context of RA.

RA

RA is an autoimmune disease, in which the body generates antibodies against its own tissues. RA is characterized by tissue-specific autoimmune-mediated chronic inflammation that affects multiple joints and results in destruction of cartilage and bone loss[12]. Risk factors of RA are multi-dimensional and include genetic defects, infections, and environmental influences[12].

Population studies of RA estimate that genetics account for about 50% of RA disease susceptibility[13-15]. The most consistent predictor of susceptibility to RA is the link between specific major histocompatibility (MHC) Ⅱ-associated alleles of the *DRB1* gene that encode human leukocyte antigen (HLA)-DR4 and the onset of RA[16]. All of the susceptible alleles encode a conserved amino acid sequences on MHC Ⅱ[17]. RA patients with these susceptible HLA-DR4 alleles and subsequent expression of the conserved amino acid sequence on MHC Ⅱ, develop more autoantibodies associated with RA-related joint breakdown than patients without these alleles[18]. Activated antigen-presentation cells (APCs), including dendritic cells and macrophages, upregulate MHC Ⅱ surface molecule expression, which activates antigen-specific T cells and B cells and initiates the adaptive immune responses in autoimmune disease.

Bacterial infections may trigger the onset of RA. A population-based study in Sweden determined that 45% of inflammatory arthritis patients had an infection prior to the onset of early arthritis[19]. In Chlamydia-induced reactive arthritis, the microbe primes neutrophils through toll-like receptor signaling, which activates the cell to clear the infection. However, activated neutrophils can also infiltrate the joint and cause chronic inflammation[20]. Neutrophils may employ the same mechanisms in the onset of RA after an infection. Although many microbial factors have been found in synovial fluid of RA patients, there is no clear agreement that these microbial factors are the causative agents of RA[21-23].

Environmental factors, such as cigarette smoke, make provoke the development of RA in genetically susceptible populations[24,25]. Smokers with the HLA-DR shared epitope allele were 3 times more likely to test positive for rheumatoid factor (RF) than non-smokers with HLA-DR shared epitope allele[25]. Smoking increases the activation and migration of neutrophils[26,27]. Dysregulated neutrophils as a result of cigarette smoking generate a systemic inflammatory environment that is associated with autoimmune disease, such as RA and systemic lupus erythematosus[28,29].

Neutrophils account for the majority of inflammatory cells in the synovia of human RA patients, and the joints of collagen-induced arthritis (CIA) mice, a murine model of RA[30,31]. In both humans and mice, neutrophils accumulate at the pannus-cartilage interface, where much the destruction to both bone and cartilage takes places[32-34]. Thus, an in-depth understanding of neutrophil function in arthritis is vital to the prevention and treatment of this disease.

Neutrophil identification

The surface marker granulocyte receptor-1 (Gr-1) was previously used to identify neutrophil populations in murine models[35], as it was thought only mature granulocytes express Gr-1. Later researchers found that Gr-1 antibodies cross-react and bind two Ly-6 family member proteins, Ly6G and Ly6C[36,37]. While neutrophils express Ly6G, monocytes, memory T cells, and some dendritic cell subsets express Ly6C[36,38-40]. Studies utilize anti-Gr-1 antibodies to deplete neutrophils and attribute the presence of neutrophils to the ability of cancers cells to acquire metastatic phenotypes[41,42]. Although neutrophils make up the majority of Gr-1$^+$ cells in these studies, the increased metastasis after administration of anti-GR-1 antibodies cannot be attributed solely to neutrophil depletion, as Ly6C$^+$ monocyte are also depleted. In CIA, the depletion of neutrophils with anti-GR-1 antibody concluded that neutrophils were necessary for the onset and maintenance of disease,

as this treatment prevented the onset of disease and ameliorated established disease[31]. Macrophages express Gr-1 and also infiltrate the CIA joint, so this method fails to delineate the roles of neutrophils from macrophages in CIA. Currently, studies utilize antibody clone IA8 to specifically bind Ly6G and deplete neutrophils without an effect on other leukocyte populations[43].

In general, current murine studies identify neutrophils as $CD11b^{+}Ly6G^{+}Ly6C^{+}$ cells, and monocytes as $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells. Human neutrophils are defined as $CD14^{lo/neg}CD15^{+}CD16^{hi}CD33^{+}CD11b^{+}CD15^{+}CD66b^{+}$, and human monocytes are defined as $CD14^{+}CD16^{+}HLA\text{-}DR^{+}CD66^{-}$ cells[44].

Neutrophil effect on T cells

Neutrophils, unlike dendritic cells and macrophages, are not defined as APCs. APCs express antigen to T cells *via* MHC Ⅱ molecules and stimulates T cell activation with the aid of co-stimulatory molecules CD80 and CD86. In autoimmunity, autoantigen is presented and antigen-activated T cells quickly expand and migrate to the site of inflammation and induce tissue inflammation through the production of pro-inflammatory cytokines[45]. In genetically susceptible individuals, the conserved amino acid sequence on MHC Ⅱ may manipulate the antigen presentation process and cause activation of autoreactive T cells[46]. In the absence of autoimmune disease, neutrophils isolated from the peripheral blood of healthy controls produce MHC Ⅱ mRNA, but do not express the cell surface molecule[47]. Exposure of these healthy neutrophils to synovial fluid from an RA patient elicits surface expression of both MHC Ⅱ and co-stimulatory molecules[48]. Contact with T cells induces neutrophilic expression of MHC Ⅱ and costimulatory molecules[48]. Since T cells are abundant in the RA joint, neutrophils continually express MHC Ⅱ and co-stimulatory molecules and act as APCs. This further activates T cells and forms a vicious feedback loop that promotes chronic inflammation and tissue damage in the joints[48-50]. The continuous activation of T cells in the joint advances joint destruction through the production of pro-inflammatory cytokines and activation of autoreactive B cells[51,52].

Recently, studies have linked neutrophil functions to Th17 cells. Th17 cells, which produce IL-17, are potent proinflammatory mediators and have been implicated in the pathogenesis of autoimmune disease[53-56]. IL-17 can induce tissue inflammation by stimulating the recruitment of neutrophils. In the RA joint, IL-17 activates fibroblast-like synoviocytes (FLS), macrophages, and osteoblasts[53-58]. FLS activation produces the potent neutrophil chemoattractant IL-8[59,60]. Activation of macrophages produces TNF-α[61], and the combination of Il-17 and TNF-α in the joint stimulates synovial endothelial cells to produce more neutrophil chemoattractants[62,63].

Reciprocally, neutrophils help sustain Th17 cells in the joint through the secretion of Th17 chemokines CCL20 and CCL2[60]. Human neutrophils purified from the synovial fluid of RA patients express high levels of these chemokines[60]. Despite the presence of other chemokines in the joint, Th17 cells preferentially migrate toward CCL20[64]. Mice that lack the CCL2 receptor ($CCR2^{-/-}$) develop exacerbated CIA. Furthermore, Th17 and neutrophil populations expand in the lymph nodes and joints of these mice[65]. Therefore, CCL20 likely plays a more prominent role in Th17 migration than CCL2. The expansion of both neutrophils and Th17 cells in mice with exacerbated arthritis demonstrates the importance of both these cell types to the onset and maintenance of autoimmune arthritis. The reciprocal signaling between Th17 cells and neutrophils causes accumulation and activation of these cells, cultivating an inflammatory microenvironment in the joint[58,60,66].

Although reducing neutrophils in the joint is beneficial to limit sustained T cell activation, systemic neutropenia can cause infection. Some therapeutics intervene with proinflammatory events that upregulate neutrophils, instead of depleting neutrophil function. A phase Ⅱ trial that utilizes the anti-IL-17 monoclonal antibody drug, secukinumab, improves symptoms in 46% of RA patients after 16 wk of treatment, and can safely maintain these improvements through week 52[67,68]. Infection rates of patients on these drugs were 31.9% through week 52. Most infections were mild, but, interestingly, were not associated with neutropenia[68]. Simultaneously targeting neutrophils may improve IL-17 treatments, as activation of Th17 cells would decrease in addition to just decreasing the IL-17 effector molecule. However, as with many autoimmune therapies, this may leave the patient susceptible to infection.

Neutrophil effect on B cells

One of the major features of autoimmune disease is the presence of autoantibodies in circulation. In RA patients, various autoantibodies against cartilage components, chaperones, enzymes, nuclear proteins, and citrullinated proteins have been identified. However, the clinical significances and pathogenic roles of these antibodies are largely unknown, except for RF, anti-citrullinated protein antibodies (ACPA), and anti-collagen antibodies, which are all associated with joint inflammation[69-72]. Neutrophils stimulate the activation, proliferation, differentiation, and antibody-production of B cells through the production of the B cell stimulating factor BAFF (also known as BLyS)[73-75]. Peripheral blood neutrophils from both RA patients and healthy control patients express BAFF as a membrane bound molecule[76]. However, TNF-α in the RA joint releases surface-bound BAFF from neutrophils and increases the concentration of soluble BAFF[76]. High levels of soluble BAFF in the serum of RA patients correlates with high concentrations of autoantibodies[77].

Neutrophils in the joint act on B cells similarly to splenic marginal zone neutrophils, which induce antibody production and immunoglobulin class switching through the production of the B cell stimulants BAFF, APRIL, and IL-21[78]. These stimulants drive the formation of splenic germinal centers that support the proliferation and

differentiation of B cells. The blockage of BAFF decreases the size and disorganizes splenic germinal centers[79,80]. Structures similar to splenic germinal centers develop in the synovia of some RA patients[81]. A therapy that reduces soluble BAFF released from neutrophils may prevent synovial germinal center formation and reduce autoantibody production[82]. Thus, soluble BAFF from neutrophils plays a critical role in facilitating an environment, both systemically and locally, that activates B cells and perpetuates autoantibody formation.

The release of ROS by neutrophils[83,84] generates advanced glycation end-products (AGE) through oxidant-induced alteration of the structures of lipids, DNA, and proteins[83-86]. High levels of AGE in the sera of RA patients correlates with a high disease severity and high levels of inflammation markers[87]. B cells recognize ROS-modified structures as foreign molecules and produce autoantibodies against these structures[88,89]. ROS modifies type Ⅱ collagen, the main structural component in human articular cartilage and induces the production of autoantibodies to ROS-modified collagen[90-94]. In an *in vitro* study, serum from RA patients could only bind type Ⅱ collagen after exposure to ROS produced by neutrophils[95].

Autoantibodies form immune complexes in the joint, which induce neutrophil infiltration and activation through complement-mediated pathways[96,97]. B cells and neutrophils, therefore, work in concert to maintain inflammation in the RA joint.

Treatment with rituximab, a monoclonal anti-CD20 antibody that depletes B cells, decreases the severity of RA most efficiently in RA patients with high levels of autoantibodies[98,99]. Some patients experience late-onset neutropenia up to 12 mo following rituximab treatment[99]. The exact mechanism that causes late-onset neutropenia is not known. One popular hypothesis suggests B cells compete with neutrophils for resources in the developmental niche of the bone marrow as the B cells repopulate after rituximab treatment[98].

Neutrophils and NET formation

Neutrophils form NETs through a unique model of cell death known as NETosis[100]. The formation of NETs requires activated neutrophils to lose integrity of intracellular membranes prior to that of the plasma membrane[101]. In the first steps of NET formation, granules containing cytotoxic antimicrobial proteins decay, and chromatin condenses as the nuclear membrane collapses. The plasma membrane then invaginates, ruptures, and releases NETs comprised of intracellular antimicrobial contents into the extracellular space[102]. In the context of infection, the antimicrobial proteins trap and kill infiltrating microbes in the extracellular space[100,103].

NETs release citrullinated histones and proteins into the extracellular space[104,105]. Citrullination of a protein or histone is a post-translational modification (PTM) that converts arginine residues to citrulline[104,105]. This process changes the structure and antigenicity of proteins and histones, as the adaptive immune response can recognize PTM as non-self[106]. In RA, ACPA are of particular interest as an increase in ACPA correlate with an increased disease severity[107,108]. An increased propensity for neutrophils to die *via* NETosis correlates with increased levels of ACPA in the serum of RA patients[105], which suggests NETs are a major source of autoantigen in RA. In fact, proteins extracted NET-induced peripheral blood neutrophils react with sera from RA patients. Sera from RA patients react specifically with citrullinated histone H4[109].

The discovery of a conserved citrullinated antigen associated with the onset of RA opens up a novel avenue of therapeutic intervention. The enzyme PAD4 controls the citrullination of histone H4[109]. Interruption of PAD4 function could decrease citrullination of histone H4 and subsequent autoantibody production that is crucial to the development of RA.

NETosis also stimulates FLS to produce the proinflammatory cytokines IL-6, IL-8, and the Th17-associated chemokine CCL20[105]. Production of IL-6 and IL-8 aid in the polarization of CD4+ T cells to Th17 phenotypes, while CCL20 traffics Th17 cells to the site of inflammation. Thus, NET stimulation of FLS shapes a microenvironment favorable to sustained inflammation associated with Th17 cells.

Neutrophils as myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells derived from the bone marrow under pathologic conditions that suppress T cell functions[110]. Murine MDSCs are divided into two subsets based on surface expression of Ly6G and Ly6C; the $Ly6G^+Ly6C^+CD11b^+$ granulocytic-MDSC (G-MDSC) subset and the $Ly6G^-Ly6C^+CD11b^+$ monocytic-MDSC (M-MDSC) subset[111]. The counterparts of M-MDSC subset in humans have been identified as $CD11b^+CD14^+CD15^-HLA^-DR^-Lin^-$, while human G-MDSC subset are identified as $CD11b^+CD15^+CD14^{lo}HLA^-DR^-Lin^-$[111] (Table 1). M-MDSCs are immature monocytes that suppress T cell functions through the secretion of inducible nitric oxide synthase (iNOS)[112-114]. G-MDSCs are neutrophil-like cells that inhibit T cell function through the production of arginase-1[114].

First described in murine tumor models, MDSCs accumulate in the lymphoid tissues of tumor-bearing mice[115] and significantly increase in the circulation of cancer patients compared to healthy controls[116-118]. MDSCs promote tumor growth by inhibiting T-cell mediated immune surveillance and cytotoxic effects on tumor cells[119]. Recently, evidence indicates MDSCs also contribute to the regulation of autoimmune disease by inhibiting $CD4^+$ T cell proliferation and differentiation[120,121].

Neutrophil-like G-MDSCs are believed to be immunosuppressive in autoimmunity. In the experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis, G-MDSCs express high levels of programmed cell death 1 ligand 1 (PD-L1), a costi-

Table 1 Surface markers used in identification of myeloid-derived suppressor cells

	Human	Mouse
MDSC	$CD11b^+$ $CD33^+$	$CD11b^+$ $Gr\text{-}1^+$
M-MDSC	$CD11b^+CD14^+CD15^-$ $HLA\text{-}DR^-Lin^-$	$CD11b^+$ $Ly6C^+$ $Ly6G^-$
G-MDSC	$Cd11b^+CD15^+CD14^{lo}$ $HLA\text{-}DR^-Lin^-$	$CD11b^+$ $Ly6G^+$ $Ly6C^+$
Progenitor neutrophil	$CD14^{lo/neg}$ $CD15^+$ $CD16^{lo/neg}$	
Mature neutrophil	$CD14^{lo/neg}$ $CD15^+$ $CD16^{hi}$ $CD33^+$ $CD11b^+$ $CD15^+$ $CD66b^+$	$CD11b^+$ $Ly6G^+$

MDSC: Myeloid-derived suppressor cell; M-MDSC: Monocytic-MDSC; G-MDSC: Granulocytic-MDSC.

mulatory molecule that negatively regulates T cell proliferation. G-MDSCs inhibit autoantigen-priming of Th1 and Th17 cells in a PD-L1-dependent manner[122]. In CIA, MDSCs that include mostly neutrophil-like G-MDSCs suppress both T cell proliferation and $CD4^+$ T cell differentiation into Th17 cells, mainly through the production of arginase-1[123]. The depletion of MDSCs increases inflammation and disease severity, while the subsequent adoptive transfer of MDSC ameliorates arthritis[123]. Furthermore, based on our published[124] and unpublished data, M-MDSCs as well as G-MDSCs not only suppress T cell functions, but also inhibit B cell proliferation in the context of CIA.

The use of $CD11b^+Gr\text{-}1^+$ cells in most functional MDSC studies makes it difficult to confirm G-MDSCs immunosuppressive effects in autoimmune arthritis Additionally, utilization of Ly6G to recognize G-MDSCs cannot differentiate G-MDSCs from neutrophils. Similar issues exist in human MDSCs, as mature human neutrophils express many of the same surface receptors as G-MDSCs and cannot be distinguished without functional analysis[125].

Further studies comparing G-MDSC phenotypes *vs* neutrophil phenotypes are needed to determine the effect of these cells on the adaptive immune response. Current evidence indicates G-MDSCs and neutrophils act in opposing manners on the adaptive immune response[125]. Uncovering mechanisms that lead to the plasticity of G-MDSCs and neutrophils in autoimmune disease could lead to cell-based therapies that convert pro-inflammatory cells to immunosuppressive cells.

Neutrophils and potential therapeutics

As one of major contributing factors in the establishment of chronic inflammation in RA, neutrophils serve as potential therapeutic targets. Some current therapies for RA interfere with the functions of neutrophils. For example, anti-TNF-α therapies reduce IL-33 receptor expression on neutrophils and subsequently decrease neutrophil migration. Neutrophils from RA patients treated with anti-TNF-α therapies do not respond to IL-33-mediated chemotaxis[126]. Impaired chemotaxis of neutrophils may lead to a decrease in inflammation and disease severity. The wide array of effector proteins produced by neutrophils, such as BAFF, could also become therapeutic targets for RA.

NETs may serve as a novel therapeutic target for RA and other NET-associated autoimmune diseases. In addition to targeting enzymes associated with PTM, the use of DNAse to breakdown the extracellular DNA and histones associated with NETs has been suggested as a potential therapeutic in NET-associated diseases[127]. The study of NETs in RA may also reveal currently unknown citrullinated proteins that contribute to RA pathogenesis and could serve as therapeutic targets.

In CIA, the adoptive transfer of MDSCs decrease T and B cell proliferation and decreases the severity of arthritis[123,124]. The discovery of a mechanism that induces the G-MDSC phenotype from neutrophil-like cells could be crucial for the therapy of autoimmune disease. Manipulation of this mechanism will drive neutrophil-like cells toward an immuno-suppressive MDSC phenotype that impedes the super-active adaptive immune response, and thereby reduces chronic inflammation in autoimmunity. An in-depth understanding of the contributions of the joint microenvironment to the various neutrophil phenotypes, and subsequent neutrophil functions, may aid in the development of neutrophil-based RA therapies.

Similar to many autoimmune therapies, inhibition of neutrophil functions leaves the patients susceptible to infection. For this reason, the inhibition of specific enzymes, such as PAD4, may be a promising therapeutic intervention. If PAD4 can be neutralized, this could curb autoantibody production without completely diminishing the antimicrobial function of neutrophils.

CONCLUSION

Neutrophils are involved in the onset and progression of RA in a complex capacity. Neutrophils engage in several reciprocal signaling events with both B and T cells, which promote a microenvironment conducive to sustained inflammation. The formation of NETs increases the production of ROS and ACPA, which are hallmarks of RA. Neutrophils can act as pro-inflammatory cells influencing chemotaxis and immune cells signaling, but can also have a MDSC phenotype that suppresses the immune response. Since neutrophils affect many aspects of the adaptive immune response and drive chronic inflammation, the disruption of the signals between neutrophils and the adaptive immune response can serve as therapeutic targets for RA.

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MINIREVIEWS

Targeting apoptosis is the major battle field for killing cancers

Xiao-Chun Liu, Jiang-Ming Gao, Shan Liu, Li Liu, Jing-Rui Wang, Xian-Jun Qu, Bing Cai, Shu-Lin Wang

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Abstract

Targeting apoptosis is one of the major strategies for cancer therapy. Essentially, most of the conventional cancer therapeutic drugs that are in the clinical use induce apoptosis and in part necrosis of malignant cells and therefore prevent cancer progression and metastasis. Although these cytotoxic anticancer drugs are important weapons for killing cancers, their toxic side effects limited their application. The molecularly targeted therapeutics that are based on the deeper understanding of the defects in the apoptotic signaling in cancers are emerging and have shown promising anticancer activity in selectively killing cancers but not normal cells. The examples of molecular targets that are under exploration for cancer therapy include the cell surface receptors such as TNFR family death receptors, the intrinsic Bcl-2 family members and some other intracellular molecules like p53, MDM2, IAP, and Smac. The advance in the high-throughput bio-technologies has greatly accelerated the progress of cancer drug discovery.

Key words: Apoptosis; Chemotherapy; Drug targets; Drug resistance; Cancer; Translational medicine

Core tip: Chemotherapy and radiotherapy are important approaches for cancer therapy and have prolonged the lifespan and reduced the mortality of cancer patients. But chemotherapy and radiotherapy induce apoptosis in both cancer and normal cells, therefore possessing severe toxic side effects. It appears quite important to develop the biological mechanism-based drugs that that can selectively kill tumor cells but not normal

cells. Molecular targets in the apoptotic signaling pathways such as p53, TRAIL, and Bcl-2 have been identified, and molecularly targeting drugs for a variety of tumors based on these pathways are currently under development. Dissecting the genetic alterations in a particular tumor type and designing the rational drug combinations targeting different pathways can help achieve synergy in eradicating cancer cells and reversing drug resistance, and this holds great promise for the personalized treatment of cancer patients.

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INTRODUCTION

The most important concern facing current cancer therapy is the lack of tumor selective agents that kill cancer cells effectively but do not harm normal cells. In the past decade, many of the efforts have been contributed to dissecting the mechanisms underlying the pathogenesis of cancer, particularly the altered signal transduction pathways in different cancers[1-5]. Recent advances in our understanding of how a normal cell is mutated to become cancerous and the accumulation of mutations in cancerous cells leads to malignancy, progression and metastasis have greatly promoted the identification of new molecular targets for developing tumor selective therapeutics[6-10].

Apoptosis is a programmed physiological process to eradicate unwanted cells for maintaining homeostasis[11-14]. The signal transduction pathways mediating apoptosis are frequently deregulated in cancers, enabling cancer cells to evade from apoptosis and become hyper-proliferated. Most of the genetically altered signaling components in the intrinsic and extrinsic apoptotic pathways in cancers are usually the central regulators of apoptosis, which play a key role in arbitrating the fate of a cell[15,16]. The key components in the apoptotic machinery are the Bcl-2 family members including Bcl-2, Bax and Bcl-xl in the mitochondrial pathway, the extrinsic cell surface receptors such as death receptor KILLER/DR5, and some other intracellular molecules such as p53[17-25]. These signaling molecules are frequently mutated or deleted in cancers and therefore are the ideal targets for developing novel cancer targeting drugs[24-28]. Targeting of Bcl-2 with RNAi and BH3 mimetics are currently underway in the clinical trial studies and have shown significant clinical activity in selectively killing cancer cells[29-32]. High-throughput screening of chemical libraries have identified some small molecule compounds that can target p53 to activate or restore p53 functions, which already showed success in killing some of the cancer cells[33,34]. Loss of p53 in cancers confers resistance to some chemotherapeutics and tumors bearing p53 mutations are less sensitive to radiotherapy[35-37]. Optimal combination of agents targeting different targets in the signal transduction pathways in cancers leads to a synergistic effect on killing tumors and overcoming resistance to single agent treatment[25,38].

In this review, we summarize the basic understanding of the apoptotic signal transduction cascades in cancers and the crosstalk between different pathways in which the signaling components are mutated in cancers. We also introduce some of the knowledge about the mechanisms of tumor targeted drugs and chemotherapeutic agents in killing cancers. Moreover, we highlight recent advances in the technologies that are applied for identification of cancer drug targets and the strategies for cancer drug screening. The translational designs of drug combination for achieving synergy in eradicating cancer cells and reversing drug resistance will also be discussed.

DYSREGULATED CELL DEATH PATHWAYS IN CANCERS

The complex apoptotic signaling networks play a key regulatory role in maintaining homeostatic cellular progresses in living organisms[39]. There are two major signaling pathways that mediate apoptosis in mammalian cells: The extrinsic apoptotic signaling pathway and the intrinsic apoptotic signaling pathway (Figure 1). Some intracellular stimuli, such as DNA damaging agents, irradiation or oncogene activation, trigger apoptosis primarily through the intrinsic pathway which requires the activation of apoptotic regulators in the mitochondrion. Extracellular apoptotic pathway is initiated through apoptotic signaling cascades mediated by members of the tumor necrosis factor (TNF) superfamily, usually generated by the cytotoxic cells of the immune system, for example TNF, TRAIL, and FasL. Both pathways require the activation of proapoptotic cysteine proteases, caspases, to execute the cell death process[40-43]. Most of the genetically mutated intracellular signaling molecules in the intrinsic and extrinsic apoptotic pathways in cancers are the central regulators of apoptosis which arbitrate life-or-death decision of a cell. Mutations in these signaling components allow cancer cells to escape from eradicating by the host immune system and accelerates the proliferation of transformed tumor cells[44,45].

Some of the mutated molecules in the apoptotic machinery have been identified as important markers for the distinction between normal and cancer cells, and therefore are the targets for developing tumor therapeutic drugs. The alterations in the apoptotic pathways in cancers include the Bcl-2 superfamily members in the mitochondrion. All the Bcl-2 pro-survival family members like Bcl-2 and Bcl-XL are likely to be oncogenic [15,17,18]. Bcl-2 was found to be overexpressed and implicated in the pathogenesis of myeloid and T-cell leukemia[21]. Conversely, members of the pro-apoptotic

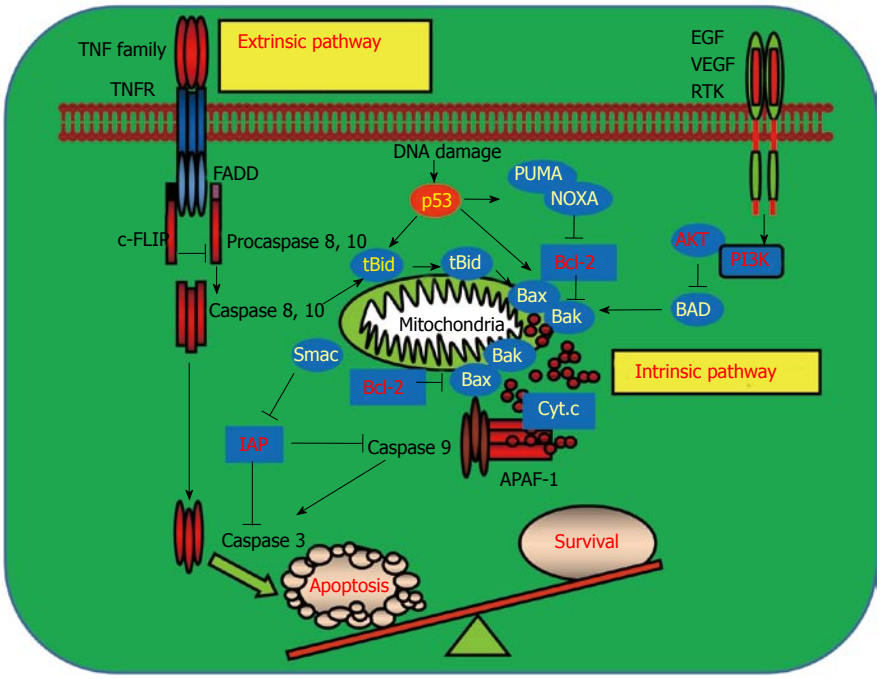

Figure 1 Signaling pathways mediating programmed cell death and cell survival. Apoptosis can be induced by extra- and intra-cellular stimuli. Activation of the cell surface receptors such as tumor necrosis factor receptor (TNFR) upon binding to their ligands leads to activation of the receptor-mediated extrinsic apoptotic pathway. Activation of death receptors triggers the cleavage of pro-caspases 8 and 10 to their active forms by recruiting adaptor protein, Fas associated *via* death domain (FADD) to form a death inducing signaling complex (DISC). The activated caspases 8 and 10 then activate the downstream effector caspase 9 and result in apoptosis. DISC formation is negatively regulated by cellular Fas-associated death domain-like interleulin-1β converting enzyme inhibitory protein (c-FLIP) which inhibits apoptosis. Intracellular stresses such as DNA damage, oncogene activation and virus infection activate the intrinsic apoptotic pathway which usually requires p53 function. p53 triggers the intrinsic apoptotic pathway by upregulating its downstream proapoptotic target genes including the p53 up-regulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), Bcl-2 associated X protein (Bax) and Bcl-2 antagonist killer1 (Bak), which in turn lead to the release of cytochrome c from the mitochondrion. In the cytosol, the released cytochrome c binds to apoptotic protease activating factor 1 (APAF-1) and caspase 9 to form the apoptosomes. Activated caspase 9 then cleaves and activates the effector caspases 3, 6, and 7 to execute apoptosis. The mitochondrial protein Smac augments apoptosis by interacting and inhibiting inhibitor of apoptosis proteins (IAPs) which block the activation of caspase 9. Akt, an important downstream target in response to the activation of receptor tyrosine kinases, inhibits apoptosis by phosphorylating and inhibiting the proapoptotic Bcl-2 associated antagonist of cell death (BAD) which prevents the activation of Bax and Bak. Bid crosslinks the extrinsic and intrinsic pathways in response to extrinsic stimuli. Activation of caspases 8 and 10 causes the cleavage of BH3 interacting domain death agonist (BID) and the truncated BID is then translocated to the mitochondrion to engage the activation of the intrinsic pathway by interacting with Bax and Bak. EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; RTK: Receptor tyrosine kinase.

subfamily such as Bax and Bak are probably tumor suppressors. Bax or Bak is frequently mutated or deleted in some gastric and colorectal cancers. Cell surface death receptor KILLER/DR5 is mutated in some of the head and neck cancers and lung cancers[46,47]. P53 is mutated or deleted in more than 50% of the human cancers. P53 induces apoptosis through regulating its downstream targets including the proapoptotic Bax, KILLER/DR5 and p53 upregulated modulator of apoptosis (PUMA). Loss of p53 impairs the apoptotic signaling pathways by aberrant control of its downstream apoptotic target genes and confers resistance to chemotherapy or radiotherapy-induced apoptosis[35-37,48-50]. Oncogenic mutations impair apoptosis indirectly by prompting or repressing the expression of the signaling molecules in cell death pathways and promote tumor progression and metastasis.

TARGETING APOPTOTIC PATHWAYS FOR CANCER THERAPY

Recent advances in our understanding of how apoptosis is activated and the mechanisms how cancers evade apoptosis have paved new revenues for developing the molecularly targeted cancer drugs[10,19,26-28]. The first link between apoptosis and cancer emerged when Bcl-2, the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death[51]. Later on, many other Bcl-2 family proteins including the proapoptotic Bax, Bak and Bok, and anti-apoptotic Bcl-2, Bcl-XL and Mcl-1 were discovered. The BH3-only proteins such as the proapoptotic Bid, Bad, Bim, PUMA and phorbol-12-myristate-13-acetate induced protein 1 (NOXA) were identified as the other key regulators which control the mitochondria-mediated intrinsic apoptotic pathway[52]. These Bcl-2 family proteins are important targets for cancer therapy (Figure 2). Several strategies have been developed by targeting the Bcl-2 family members. One approach for modulating Bcl-2 function is to target Bcl-2 with antisense Bcl-2 deoxyoligonucleotides. The other approach is to mimic the binding of a BH3 peptide to its surface groove. For example, ABT-737 and ABT-263 are BH3-mimetics that inhibit Bcl-2, Bcl-XL and Bcl-w

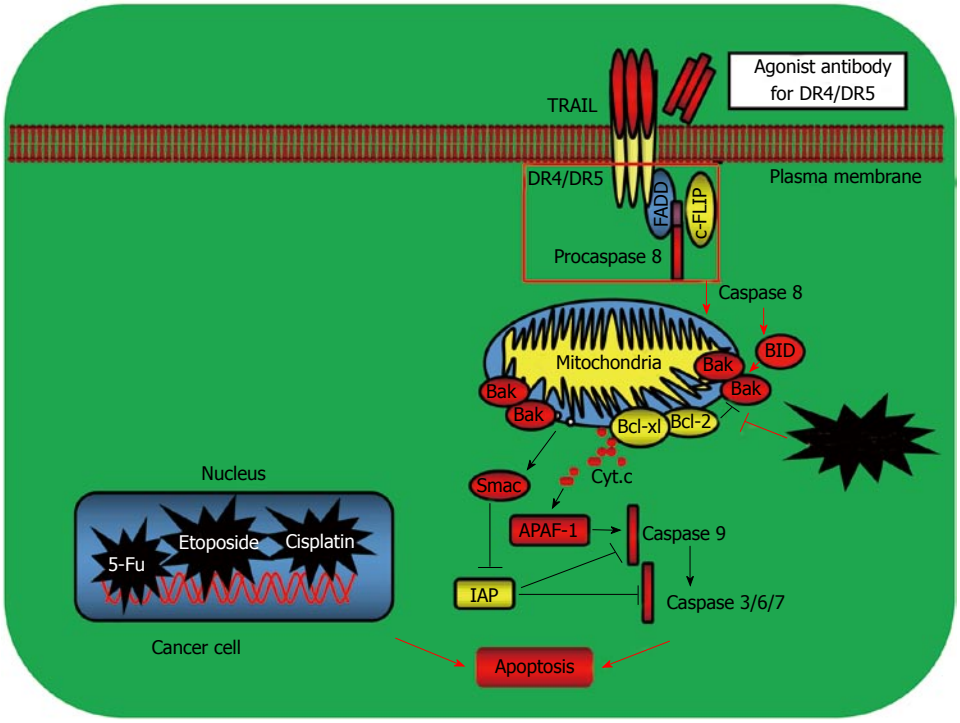

Figure 2 Examples of targeting apoptosis for cancer therapy. The prominent examples for tumor targeted therapy include the approaches by targeting the tumor necrosis factor receptor family death receptor-mediated apoptotic pathway and the mitochondrial B cell lymphoma 2 (Bcl-2) family member-mediated pathway. Activation of death receptors after binding to their ligand tumor necrosis factor related apoptosis inducing ligand (TRAIL) triggers activation of the extrinsic apoptotic pathway leading to cell death. Activation of Bax and Bak causes cytochrome c release from the mitochondrion and induces apoptosis. Bcl-2 and Bcl-XL prevent the activation of Bax and Bak and inhibit apoptosis. The mAbs targeting the TRAIL receptors have been developed and exerted significant clinical activities for some cancers such as non-small cell lung cancer (NSCLC), colon cancer, breast cancer, leukemia and prostate cancer. Small molecule BH3 mimetics activate the mitochondrial apoptotic pathway by interrupting the interaction of Bcl-2 with the proapoptotic Bax and Bak and induce apoptosis. FADD: Fas associated death domain; IAP: Inhibitor of apoptosis protein; APAF-1: Apoptotic protease activating factor-1.

Table 1 Small molecules targeting apoptosis for cancer therapy

Drug name	Mechanism of action	Drug targets
CP-31398	Restoration of wild type p53 function	Mutant p53
PRIMA-1	Restoration of wild type p53 function	Mutant p53
Nutlins	Prevent p53 degradation	p53/MDM2
RITA	Prevent p53 degradation	p53/MDM2
Oblimersen sodium	Antisense inhibitors	Bcl-2
ABT-737	BH3 mimetics	Bcl-2, Bcl-XL,Bcl-w

to liberate Bax and Bak to induce apoptosis (Table 1). Preclinical studies have shown that ABT-737 was effective as a single agent in killing small cell lung cancer (SCLC) and causing SCLC tumor regression[29-31,51-55]. ABT-737 has also been shown to synergize with other conventional chemotherapy, radiotherapy or tyrosine kinase inhibitors imatinib and gefitinib to reverse drug resistance and enhance cancer cell apoptosis[56,57]. One advantage of the BH-3-memitics as cancer therapeutics is to target specific Bcl-2 family members such as Bcl-2 or Bcl-XL which is overexpressed in certain cancer types, therefore increasing the tumor selectivity and reducing the toxicity of the anticancer drugs (Figure 2).

p53 is the most commonly mutated gene in human cancers and more than 50% of cancers carry mutations or deletions in p53, making p53 an important target for cancer therapy[58-60]. Growing evidence has shown that reactivation or restoration of p53 function in cancers will have significant therapeutic benefit. Several strategies are being explored to target tumors expressing mutant p53. Small molecules that bind to a site in mutant p53 (Y220C) increase the level of p53 with wild type conformation and activity. Other compounds (*e.g.*, PRIMA-1, CP-31398) bind to multiple mutant p53 proteins and interact with DNA binding domain, thereby promoting the proper folding of the mutant protein and restoration of p53 function[61,62]. Of all the compounds that restore wild type activity, the most progress has been made with PRIMA analogs, with the demonstration of safety in phase I clinical trial study[63] (Table 1). An alternative approach to target mutant p53 is to remove the protein by enhancing its degradation. HDAC inhibitors such as SAHA show promise in destabilizing mutant p53 by preventing HDAC6 from interacting with Hsp90[64]. Small molecule activators of SIRT1 have also been shown to lead to the deacetylation of p53 and

reduction of overall mutant p53 levels[65].

Oncogenic mutations of proteins in the intrinsic or extrinsic apoptotic pathways often lead to resistance to anticancer agents, among these molecules are p53, the Bcl-2 family members, death receptors, inhibitor of apoptosis proteins (IAPs), and the prosurvival factors such as AKT, PI3K and c-FLIP[66-69]. Mutant p53-carrying tumors showed increased resistance to commonly used chemotherapeutic agents. Overexpression of Bcl-2 confers the resistance of leukemic cells to cytotoxic chemotherapeutic agents. Similarly, other Bcl-2 family members including Bcl-XL, Bax, and BH3-only proteins such as P53 Upregulated Modulator of Apoptosis and NOXA also play regulatory roles in determining the sensitivity of cancer cells to therapeutic agents[48,49,53]. In addition to the defects in the cell apoptotic and survival machineries, there are still some other mechanisms that contribute to cancer drug resistance including the rates of drug efflux, alterations in drug metabolism and drug targets, DNA damage repair capacity, and the changes of the local tumor microenvironment. Drug resistance can also be acquired during treatment of tumors that were initially sensitive as well as the adaptive responses such as activation of other compensatory pathways. Further investigation of the molecular basis of drug resistance will help to overcome the hurdle that limits the clinical usage of cancer chemotherapeutic drugs and design rational drug combinations to restore or enhance the sensitivity of cancer drugs and improve the tumor selectivity and efficacy of cancer therapeutics[70,71].

STRATEGIES FOR CANCER DRUG SCREENING

The initial identification of a chemical compound or small molecule inhibitors to be translated into medicine generally occurred by screening molecules in the animal models and cellular assay systems with functional biomarkers or molecular targets which are specifically mutated in cancers. Therefore, the level of mechanistic understanding of the alterations in cancers and the suitable targets used determine the success of a design for cancer drug discovery. The emergence of the high-throughput and high-content technologies has driven modern cancer therapy to the molecular era that new generations of molecularly targeted cancer drugs were continuously discovered. In the past decades, new technologies have been developed for dissecting the mechanisms of cancer development and metastasis and the molecular targets have been identified for designing new cancer selective therapeutic drugs. Large scale profiling approaches such as transcriptomics and proteomics and the high-throughput sequencing techniques which comparatively quantify the expression levels of transcripts and proteins have uncovered numerous genes, proteins and biomarkers that correlate with tumorigenesis. Some of these genes and proteins have been used as good targets for developing cancer therapeutics. The most prominent examples are EGFR, BCR-ABL, VEGF, BRAF, PDGFR, p53, BCL2, MDM2, and ERBB2[24,58-65,72-78].

Target-based screening strategy focused on the rational molecular targets that are hypothesized to have a role in cancers and represents the predominant approach for cancer drug discovery. Alternatively, there is another cancer drug discovery strategy called phenotypic screening approach which is referred to as phenotypic measurement of responses upon drug treatments in the animals or cellular models and is based on the cellular phenotype or functional endpoints rather than target potency alone. Much of the early pharmaceutical and drug discovery is based on the phenotypic screening. A recent report showed that phenotypic screening achieved more success in first-in-class medicine than target-based screening. One of the successful examples of cell based phenotypic drug screening was the discovery of vorinostat, an HDAC inhibitor, for inducing differentiation of cancer cells[79].

The integration of advanced high-content imaging system into the drug screening field has aided the rapid development of cancer drug discovery[80-82]. Light microscope imaging methods evolving in the mid-90s have provided multiple cellular measurements from living cells but require continuous user intervention. The development of fluorescent proteins permitted the tracking of proteins in living cells and the use of green fluorescent protein to tag functional proteins allowed many different fluorescent analogs to be created quickly and be used as markers to study the functions, subcellular localization, trafficking and activities of particular proteins. The automation of light microscope imaging, particularly the laser-directed multicolor fluorescence of arrays of cells, formed the high content screening system. Image-based screening techniques provide invaluable readouts, for example, the changes in the morphology, proliferation, cell cycle progression, cell death, differentiation, cell migration and invasion in the cellular models before and after drug treatment. Multiplexed high content screening assays integrated measurements of multiple cellular targets in a single assay, can be applied in numerous cell types which were subjected to libraries of chemical compounds or other experimental treatments such as RNAi, and emerged as an important cancer target and drug discovery platform[83,84]. In addition, intravital imaging has gained the cellular details by tagging single cells, tissues and subcellular compartments with fluorescent proteins, through direct labelling of cells before exogenous inoculations *in vivo* or by using the specific promoters to drive the expression of genes of interest. These methods have been applied to monitor cancer drug response *in vivo* and determine the effect of cancer drugs on particular targets in animal models. Whole-body imaging techniques such as *in vivo* bioluminescence system have been used to monitor tumor progression and regression rates from multiple tissue sites during drug treatment[82,85].

Other fluorescence-based assays are also the important platforms for cancer drug screening. Examples of these dynamic technologies include the fluorescence resonance energy transfer which can be used to measure protein-protein interactions and is suitable for lead compound identification. This approach can provide a precise measurement of drug activity and insights of drug mechanism by determining the biomolecular interactions or activations[86,87]. Surface plasmon resonance imaging (SPRi) is an optical technology which allows the label-free and real-time detection of biomolecule interaction. It offers the possibility of monitoring hundreds of biological interactions simultaneously and from the binding profiles, allows the estimation of the kinetic parameters of the interactions between immobilized probes and the ligands in solution. SPRi has been applied in a variety of affinity systems, including protein/protein, protein/DNA, antibody/antigen, ligand/receptor, DNA/DNA, carbohydrate/protein, and cell/cell interactions. SPRi imaging as an affinity-based biosensor technology has been adopted for cancer drug screening, food and environment evaluation, and clinical diagnostics[88-91]. Enzymatic activation of caspases, which are a class of cysteine protease, was determined by using the peptide arrays based on the SPR imaging. This strategy used streptavidin to amplify the SPR signals of the surface-immobilized substrate peptides labeled with biotin at the C-terminus and the cleavage of the substrate peptides by caspases was detected by the increased SPRi signal. This method allowed the examination of the activities of purified caspases and caspase in cell lysate and therefore can be applicable to cell-based drug screening[92].

CONCLUSION

Enhancing or restoring apoptotic pathways in cancers are important strategies for developing cancer drugs. Some of the aberrant components in the apoptotic pathways in cancers have been identified as suitable targets for cancer therapy. These targets can be the cell surface death receptors, Bcl-2 family members, and some other intracellular molecules like p53, MDM2, IAP, and Smac. Although some of the chemical compounds or small molecule inhibitors have been discovered to modulate the activity of these targets and achieve varying degrees of success in killing cancers, drug resistance, limited patient responses and the toxic side effects still remained problematic and limited their clinic application. Further investigation of the mechanisms of apoptosis evasion in cancers and designing the rational drug combinations are still the challenges for cancer researchers in the world.

A number of high-throughput approaches including the genomic, proteomic and multiplexed imaging technologies have been applied to identify new targets in the signaling networks that are involved in cancer pathogenesis and led to discovery of new generations of target-directed chemical compounds and small molecule drugs. The natural products from plants, Chinese herbal medicine and marine bioproducts are invaluable sources for cancer drug development and many studies need to be done to discover more druggable natural products, study the mechanism of action and cure cancer patients more efficiently and less costly.

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ORIGINAL ARTICLE

Basic Study

Role of nestin in glioma invasion

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Abstract

AIM: To determine the role for the intermediate filament protein nestin in glioma invasion.

METHODS: We examined the expression and function of nestin in gliomas (Grades II-IV as defined by the World Health Organization). We determined nestin expression using Immunohistochemical methods. To elucidate nestin's biological function(s), we reduced mRNA levels by 61% and 87% in two glioblastoma-derived neurosphere lines using short hairpin RNAs and determined the effect of reduced nestin expression on glioma cell proliferation and invasion using MTS and matrigel migration assays, respectively. We also utilized quantitative real time polymerase chain reaction assays

to determine the effect of reduced nestin expression on the expression of other markers associated with glioma stem cells and their differentiated progenies.

RESULTS: We found a significant correlation between nestin immunoreactivity and astrocytoma tumor grade, with 36% of grade II, 75% of grade III, and 100% of grade IV tumors expressing significant levels of the protein when assessed using immunohistochemistry. Reduction in nestin expression had no effect on cell growth in culture, but did retard the capacity of one line to migrate *in-vitro* on matrigel. Interestingly, in the line whose migration was not affected, mRNA levels of a second intermediate filament, synemin (also knowns as desmuslin), were elevated following introduction of shRNA targeting nestin. As synemin was not induced in the line which required nestin for migration, it is a possibility that synemin may compensate for the loss of nestin in this process.

CONCLUSION: Nestin expression is prominent in high-grade astrocytomas. Nestin is not required for cell growth but it may, however, be required for cell motility.

Key words: Nestin; Stem cells; Migration; Glioma; Neurosphere

Core tip: Despite its common use as a marker of poorly differentiated stem and progenitor cells, the functional role of nestin in normal and neoplastic cells is poorly understood. Here we show that in gliomas, there is a significant positive correlation between nestin protein expression and increasing pathological grade. However, when nestin expression was inhibited, we found no significant effects on cell growth, expression of stem-cell markers, and the ability to initiate intracranial xenografts. Our data suggest that the functional role of nestin is limited, even though the migratory potential of some glioblastoma neurospheres is reduced by nestin knockdown.

Lin A, Marchionni L, Sosnowski J, Berman D, Eberhart CG, Bar EE. Role of nestin in glioma invasion. *World J Transl Med* 2015; 4(3): 78-87 Available from: URL: http://www.wjgnet.com/2220-6132/full/v4/i3/78.htm DOI: http://dx.doi.org/10.5528/wjtm.v4.i3.78

INTRODUCTION

Glioblastoma is the most common malignant primary brain tumor in adults. Despite some therapeutic advances in recent years, the prognosis for patients with glioblastoma remains dismal, and most patients die within 2 years of diagnosis. Several reports have suggested that the inability to cure glioblastoma is due to persistence of cancer stem cells, which have been shown to sustain the growth of many human tumors (reviewed in[1]). Cancer stem cells in brain tumors are thought to share many characteristics with other neural stem cells. For example, they contain proteins preferentially expressed in neural stem and progenitor cells such as CD133[2], OLIG2[3] and nestin[4,5]. The origin of stem-like cells in glioblastoma and other brain tumors is not yet clear, but they may arise from non-neoplastic adult stem cells, or from better-differentiated cells which reacquire stem-like properties *via* mutations and/or epigenetic modifications[1].

Nestin is a class VI intermediate filament protein[6], normally expressed in neuroepithelial stem and progenitor cells of the developing mammalian central nervous system (CNS). It is also expressed in adult neural stem cells lining the ventricular system of the subventricular zone and the dentate gyrus of the hippocampus, but in differentiated adult cells nestin is replaced by other intermediate filaments such as neurofilaments and glial-fibrillary-acidic-protein (GFAP)[4]. Re-expression of nestin in the adult CNS usually accompanies pathological conditions such as brain injury, ischemia, inflammation, or neoplastic transformation, and its presence in these processes may indicate a role in reactive and/or regenerative processes[7].

Nestin expression has been found in many types of brain tumors[5,8-10]. However, its functional role in tumor formation, proliferation, and migration is still not well understood. Recently, nestin has been shown to be required for prostate cancer cell migration *in-vitro* and *in-vivo*[11]. In the present study, we investigate the expression of nestin in astrocytic tumors and its functional role in glioblastoma. The latter experiments were performed in glioblastoma neurosphere lines containing stem-like cancer cells. Our results suggest that nestin is not required for proliferation *in-vitro* or engraftment *in-vivo,* but that it may be required for *in-vitro* migration in some glioblastoma cell lines.

MATERIALS AND METHODS

Clinical specimens and neurosphere lines

The tissue microarray, containing four 0.6mm cores per tumor from a number of grade II fibrillary astrocytomas, grade III anaplastic astrocytomas, and grade IV glioblastoma multiforme, was created as previously described using samples obtained from the Department of Pathology, Johns Hopkins University School of Medicine, with Institutional Review Board approval[12]. A minimum of two of the four tissue cores had to be evaluable, and contain at least 10% nestin-immunopositive tumor cells, for a case to be scored as a positive. The glioblastoma neurosphere lines HSR-GBM1 and HSR040622, were a kind gift from Dr. Angelo Vescovi and were maintained as previously described[13].

Immunostaining

Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in a hydrogen pero-

xide/methanol buffer. Unless stated otherwise, all of the following incubation steps were carried out at room temperature. First, antigen retrieval was performed by incubation of the slides in 10 mmol/L citrate buffer (pH 6.0) at 90 ℃ for 20 min. Incubation with the primary mouse anti-nestin antibody (1:5000; Chemicon, Billerica, MA) for 60 min was preceded by blocking with serum-free Protein Block (Dako Cytomation, Carpinteria, CA) for 20 min. Negative controls were performed by substitution of PBS for the primary antibody. After washing in PBS, HRP-conjugated anti-mouse IgG (1:500; Dako Cytomation) was applied to the slides for 30 min. For tyramide-based signal amplification the slides were subsequently treated using the TSA Biotin System according to the manufacturer's protocol (PerkinElmer Life Sciences, Waltham, MA). Finally, slides were incubated for 8 min with a DAB solution (Sigma, St. Louis, MO), counterstained with hematoxylin, dehydrated, cleared, and mounted.

For immunocytochemistry, neurospheres were spun onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA) using a Shandon Cytospin3 (Thermo Shandon, Waltham, MA) for 5 min at 1500 rpm. Cells were then washed once for 5 min with PBS and then fixed in freshly prepared 4% para-formaldehyde (in PBS, pH 7.4) at room temperature for 30 min. After three washes with PBS for 5 min each, cells were permeabilized using PBST (PBS + 0.2% Triton × 100) for 5 min. Blocking was performed for 45 min at room temperature with PBST containing 5% normal horse or goat serum. The primary antibodies used were rabbit anti-Ki67 1:1000 (Novocastra Laboratories, United Kingdom), and mouse anti-nestin MAB5326 1:2500 (Chemicon, Temecula, CA). Nuclei were stained with DAPI (Pierce, Rockford, IL) for 3 min in PBS. Quantitation of Ki-67 immunostaining was made by counting separately the positively and negatively stained nuclei. At least ten high-power fields containing a minimum of one hundred cells each were counted per slide using a 63X objective. Only moderate to strong staining intensity was scored as positive. Ki-67 index was expressed as the percentage of positively stained nuclei to all nuclei.

Lentivirus preparation and infection

Lentiviruses were generated essentially as previously described[11]. Briefly, 5 μg of lentiviral vector (either pSicoR or pSicoR/shNestin) and 2.5 μg of each packaging vector were cotransfected in 293T cells using the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). Oligos targeting the human nestin RNA sequence: 5′ TGCTGTTGACAGTGAGCGCGG-CTAGTCCCTGCCTGAATAATAGTGAAGCCACAGATGTA-TTATTCAGGCAGGGACTAGCCATGCCTACTGCCTCG GA-3′ (human nestin shRNA1). Twenty four hours after transfection, growth medium was replaced with DMEM containing 2% FBS. Supernatants were collected 48 h and 72 h post medium change, filtered through a 0.45-μm filter, and used directly to infect neurospheres. One round of infection was usually sufficient to infect enough cells for subsequent drug selection. Virus was allowed to infect cells for 4 h and then the cells were washed once with PBS and plated back into Neurocult medium. Seventy-two hours later, positive cells were selected in puromycin (5 μg/mL). Puromycin resistant neurospheres were expanded for 3 wk before assayed for nestin expression.

RNA extraction and quantitative real time polymerase chain reaction (RT-PCR). For RNA extraction from cultured cells, 3.75×10^5 cells were plated in 75 cm^2 tissue culture flasks each containing 10 mL of NeuroCult medium (Stem Cell Technologies, Canada) supplemented with hEGF and hFGF-b (Peprotech, Rocky Hill, NJ). Cultures were grown in the presence of 10 ng/mL puromycin (Sigma, St. Louis, MO) and incubated for 5 d in a humidified incubator. Cells were spun at 276 × g for 10 min followed by a rinse with ice-cold PBS. Cell pellets were processed for RNA extraction using the Qiagen RNeasy kits. Reverse transcription was performed as previously described[14].

mRNA levels were analyzed by RT-PCR analysis performed in triplicate with SYBR Green reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions on an I-Cycler IQ real-time detection system (BioRad, Hercules, CA). To minimize contaminating genomic DNA, a thirty minutes on-column DNase step was included during RNA extraction. The standard curve method was used to determine expression levels, and all values were normalized to actin. Oligo sequences were as follows: Nestin forward: 5′CCAGGAGCCACTGAAGACTC; nestin reverse: 5′ CCTTTCCCAGGTTCTCTTCC; actin beta forward: 5′ CCCAGCACAATGAAGATCAAT; actin beta reverse: 5′ GATCCACACGGAGTACTTG. CD133 forward: 5′ CATCCACAGATGCTCCTAAGG; CD133 reverse: 5′ AAGAGAATGCCAATGGGTCCA; OLIG2 forward: 5′ GGACAAGCTAGGAGGCAGTG; OLIG2 reverse: 5′ ATGGCGATGTTGAGGTCGTG; GFAP forward: 5′ AACTGAGGCACGAGCAAAGT; GFAP reverse: 5′ GCAGTGCCCTGAAGATTAGC; MAP2 forward: 5′ CCATCTTGGTGCCGAGTGAG; MAP2 reverse: 5′ TGGGAGTCGCAGGAGATTTTG; vimentin forward: 5′ TACCGGAGACAGGTGCAGTCCCTCA; vimentin reverse: 5′ TCACGAAGGTGACGAGCCATTTCCT; Synemin transcript variants M/H (AJ310521.1, AJ310522.1, respectively) forward: 5′ ACAGGTGCTGGAGGATGTG; Synemin transcript variants M/H reverse: 5′ CGGATC-GCCTCTACGTTACT; Synemin transcript variants M/H/L (AJ310521.1, AJ310522.1, AJ697971.1, respectively) forward: 5′ GGCCTCAGTCTGGAGGTGG; Synemin transcript variants M/H/L reverse: 5′ CCCAGATCA-CTATCTGTGGATTACT; To ensure that measurements of gene expression changes reflected a direct effect of nestin mRNA levels, we considered a significant expression level change only in cases where un-infected and scrambled control (shC) infected cells each showed significant level change as compared with shNestin (shN)

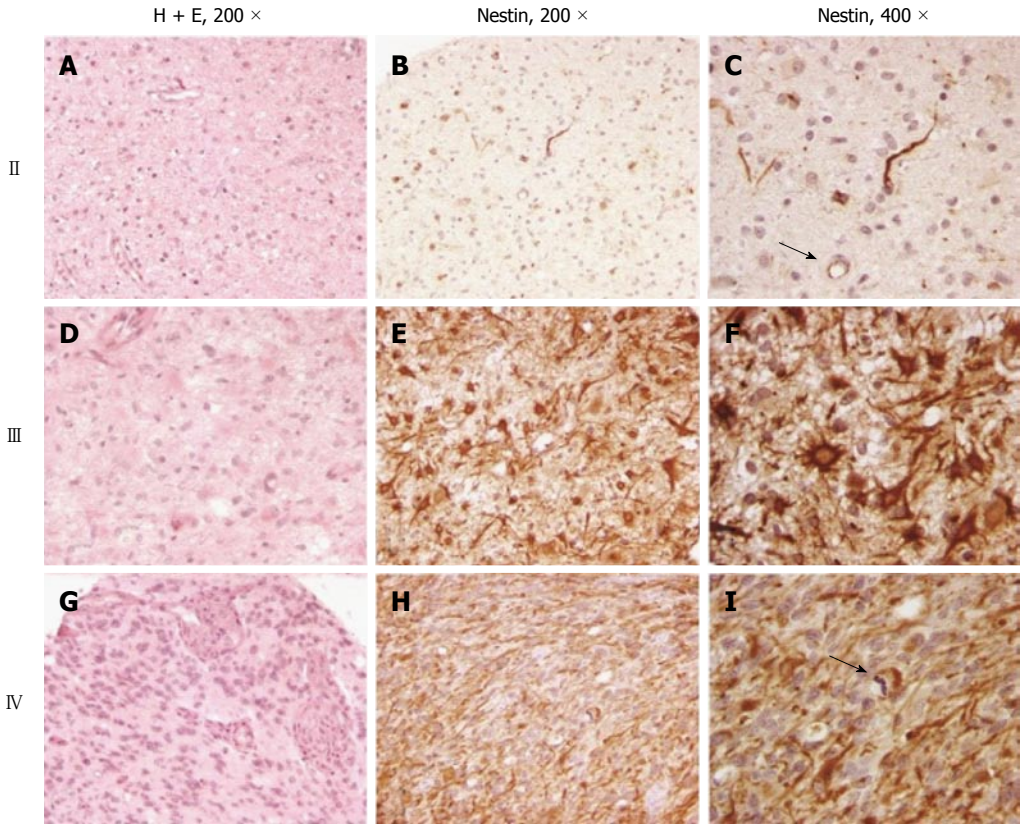

Figure 1 Nestin expression in gliomas. Diffuse astrocytomas (A) were generally either negative for nestin or contained rare positive cells (B). Endothelial cells were also often nestin positive (C, arrow). Anaplastic astrocytomas (D) generally contained moderate to frequent numbers of strongly nestin positive cells (E, F). Glioblastomas (G) were uniformly nestin positive (H, I).

infected cells.

MTS growth assays

Cells were plated in triplicates at 2500 cells in 96-well culture plates and incubated in a humidified incubator for a total period of seven days. MTS (Promega, Madison, WI) reagent was added to selected wells on the first, second, and seventh days before absorbance was read at 490 nm. Fold change in cell mass was calculated as the absorbance on day seven divided by the absorbance on day one.

Spreading/migration assays on matrigel

Forty eight hours before plating neurospheres, single cells were plated at a density of 3.75×10^5 cells/mL. To prepare the matrigel substrate, 10 cm^2 culture dishes were coated with low growth factor containing matrigel (BD biosciences, San Jose, CA) at a 1:100 dilution with Neurocult medium and placed in a humidified incubator over-night. The next day, plates were rinsed once with PBS before neurosphere plating. Neurospheres were plated at a very low density in which, on average, a single neurosphere could be visualized per field. Most neurospheres attached to the matrix one hour post plating at which point the medium was replaced to minimize continuous adhesion of any remaining floating neurospheres. The position of 20-25 spheres was noted on the bottom of the plate to allow monitoring of cell spreading over time. Sphere area was calculated by multiplying the two longest perpendicular axes extending from the two furthest cells in a sphere. Each experiment was performed at least three times.

Statistical analysis

VassarStats software (http://faculty.vassar.edu/lowry/VassarStats.html) was used for statistical analyses. The significance of differences in nestin expression in astrocytomas of varying grades was analyzed using the Freeman-Halton extension of the Fisher exact probability test for contingency tables.

Statement from biostatistician

Statistical analyses in this study were reviewed by Dr. Luigi Marchionne, who is a trained biostatistician and a co-author.

In-vivo xenografts

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, at libitum access to food and water) for at least two weeks before experimentation. For xenograft studies, 1×10^5 viable cells were diluted with fresh medium and injected over 10 min into the right striatum of athymic (nu/nu) mice (Harlan, Indianapolis, IN, http://www.harlan.com). Mice were

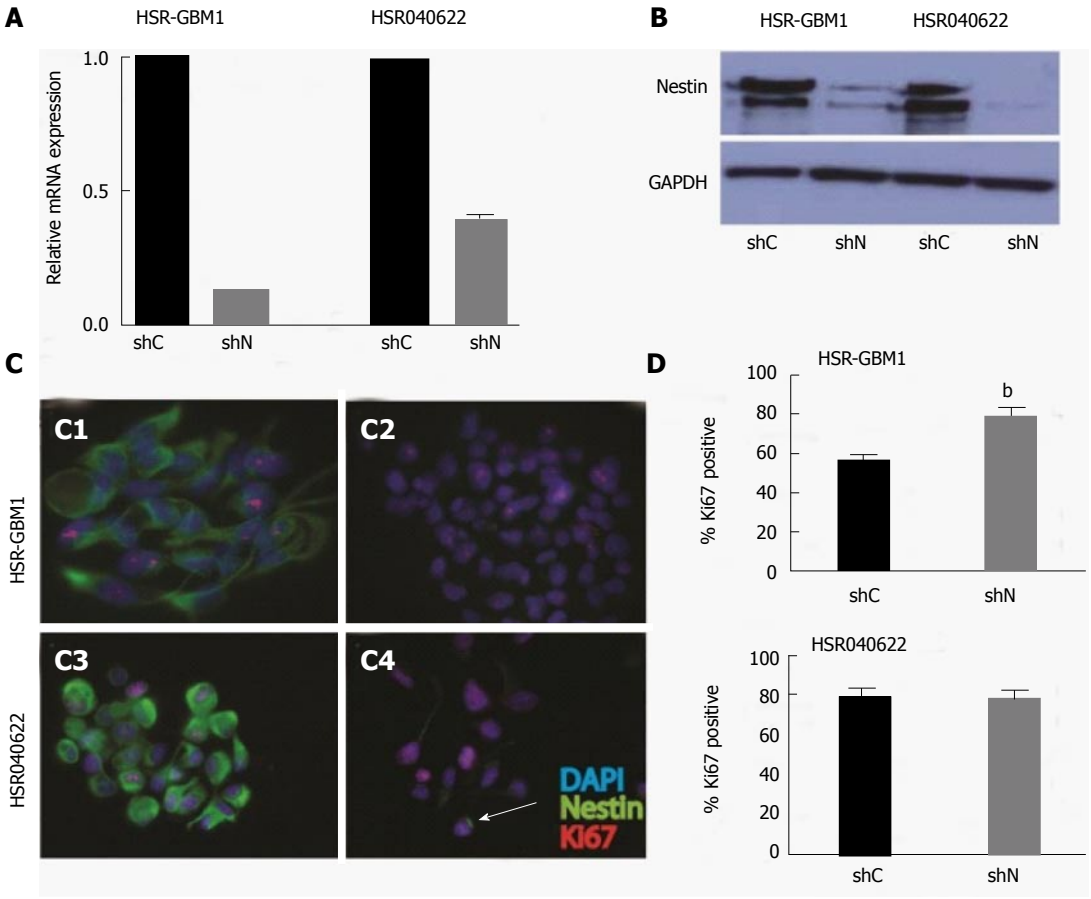

Figure 2 Nestin expression knockdown has no effect on glioblastoma cell proliferation. Quantitative real time PCR and Western Blot analyses of Nestin mRNA (A) and protein (B) expression in HSR-GBM1 and HSR040622 neurospheres confirming stable knockdown of nestin expression by short hairpin RNA. HSR-GBM1 (C1, C2) and HSR040622 (C3, C4) immuno-staining confirms nestin protein reduction in shNestin (shN) infected cells (C2, C4). Quantification of Ki67 immunoreactivity shown in D ($^{b}P < 0.01$, two-sided *t* tests). shC: Scrambled control.

monitored daily and sacrificed at the first indication of tumor development (ataxia, seizure, lethargy, or cachexia). Brains were surgically removed and fixed immediately in formalin before submission for histological analysis, as previously described[15].

Animal care and use statement: Discomfort was be minimized by the use of anesthesia during potentially painful procedures (intracranial injections). The anesthetic used was Ketamine-Xylazine in sterile saline injected intraperitoneally in accordance with the Institutional Animal Care and Use Committee.

RESULTS

Nestin expression correlates with astrocytoma grade

We assessed a total of 41 astrocytic tumors for nestin immunoreactivity in tissue arrays containing evaluable cores from 11 grade Ⅱ fibrillary astrocytomas, 16 grade Ⅲ anaplastic astrocytomas, and 14 grade Ⅳ glioblastoma (Figure 1). Immunoreactivity for nestin was identified in all tumor types, with a positive correlation between nestin immunoreactivity and increasing grade of these infiltrating astrocytic tumors. A significant level of nestin immunoreactivity, defined as 10% or more of tumor cells, was identified in 4/11 (36%) grade Ⅱ fibrillary astrocytoma (Figure 1A-C), 12/16 (75%) grade Ⅲ anaplastic astrocytoma (Figure 1D-F), and 14/14 (100%) grade Ⅳ glioblastoma, (Figure 1G-I). These differences were statistically significant ($P < 0.001$, Fisher exact test). Nestin immunoreactivity was not exclusively observed in tumor cells, and was also detected in reactive astrocytes, in microglial cells adjacent to neoplastic elements, and in endothelial cells (Figure 1C). We did not quantitate the percentage of nestin-positive cells in each tumor, but there appeared to be an increase in the extent of nestin immunoreactivity with increasing grade.

Nestin is not required for cell growth in-vitro

Proliferation and migration are important characteristics of stem and progenitor cells in the developing brain, as well as stem-like brain tumor cells[13,16]. Both processes may be influenced by cytoskeleton dynamics. To test if nestin has a role in cellular proliferation, we stably transduced two human glioblastoma neurosphere lines with lentiviruses encoding short hairpin RNAs (shRNAs) targeting either the nestin transcript (shNestin/shN) or a nonspecific scrambled sequence (shC). Utilizing this system, we achieved 87% and 61% reduction in

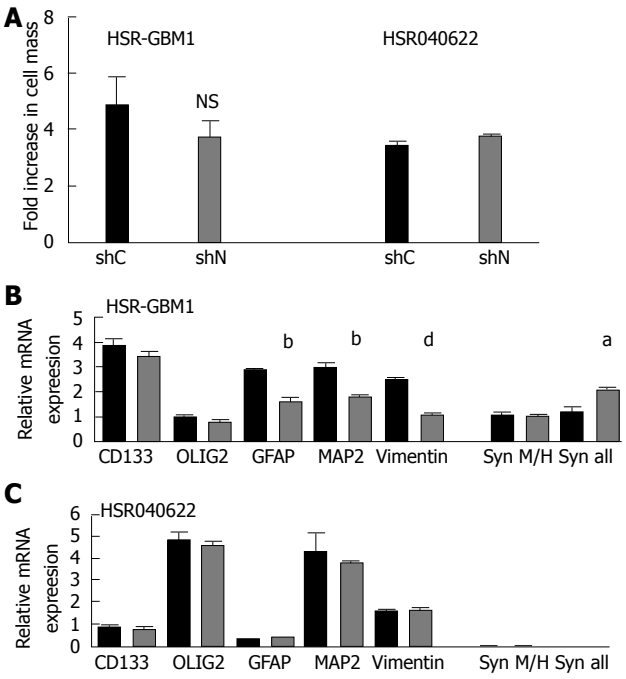

Figure 3 Effect of nestin knockdown on proliferation and the expression of stem cell and differentiation markers. A: HSR-GBM1 and HSR040622 cells stably expressing the lentivirus driven nestin shRNA show no significant difference in cellular proliferation over a period of 6 d as compared with shC (scrambled control) infected cells; B: In HSR-GBM1, lowered nestin levels has no significant effect on mRNA levels of the neural stem-cell markers CD133 and OLIG2, while statistical significant reduction of mRNA levels of GFAP (glial differentiation), MAP2 (neuronal differentiation), and the intermediate filament Vimentin was observed. The expression of the L-transcript variant of the intermediate filament synemin (syn) was significantly increased; C: In HSR040622, reduced nestin levels had no effect on expression of any of the tested genes mentioned above. It is important to note that the expression of the intermediate filament Synemin was barely detected. (NS = not significant, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$; all two sided *t* test). shC: Scrambled control; shN: ShNestin.

nestin mRNA levels in HSR-GBM1 and HSR040622, respectively, as compared to shC expressing cells (Figure 2A and B). To further evaluate the effect of reduced nestin mRNA, we examined the expression of nestin protein on a single cell level. Immunocytochemistry analysis confirmed that nestin protein was present in the majority of cells of shC infected lines (Figure 2C1, C3) and in uninfected cells (data not shown). Transduction with shN almost completely eliminated detectable nestin staining in HSR-GBM1 (Figure 2C2). In HSR040622, sporadic residual nestin positive cells could be found (arrow in Figure 2C4). Despite the dramatic reduction in nestin expression, we found no reduction in proliferation of HSR-GBM1 cells, with 56% and 80% Ki67 positive nuclei for shC and shN infected cells, respectively (Figure 2D, top panel). Similar results were observed for HSR040622 with 79% Ki67 positive nuclei for both shC and shN infected cells, respectively (Figure 2D, lower panel). To more directly test the effect of nestin reduction on proliferation, we next performed MTS assays which allow sensitive evaluation of changes in viable cell mass over time. We found no significant differences between the growth rates of HSR-GBM1 with high and low nestin levels, with 4.8 and 3.7 fold increases in cell mass for shC and shN infected cells, respectively. Similar results were observed for HSR040622, with 3.40 and 3.7 fold increases in total cell mass for shC and shNestin infected cells (Figure 3A).

Nestin knockdown does not significantly alter expression of stem cell markers

Nestin has been widely used as a marker of neural stem cells. We therefore investigated the possibility that nestin reduction may alter expression of other stem/progenitor or differentiation markers within glioblastoma derived neurospheres. We compared mRNA levels of the neural stem/progenitor cell markers CD133 and OLIG2, as well markers of glial (GFAP) and neuronal (MAP2) differentiation. Levels of the intermediate filaments vimentin and synemin were also measured, as changes in their expression could potentially compensate at least in part for nestin loss. The expression level of synemin was analyzed using two different primer pairs. The first (M/H) amplifies a product which corresponds to the M and H synemin transcript variants, while the second pair ("all") amplifies a product which represents the M, H, and L transcript variants. We found no significant differences in the mRNA level of CD133, OLIG2, MAP2, and vimentin in HSR040622 (Figure 3C). Interestingly, HSR-GBM1 cells with reduced nestin also express significantly lower levels of GFAP (56%, $P < 0.01$, two-tailed *t*-test) and MAP2 (60%, $P < 0.01$, two tailed *t*-test) (Figure 3B). In contrast, we observed an 80% increase in the level of synemin when analyzed using primers which anneal to its three transcript variants (M, H, and L; $P < 0.05$, two-tailed *t* test), but no significant change in level of the M and H transcript variants alone. We infer from these observations that the increased levels of synemin result from a significant increase in the level of synemin L in HSR-GBM1 (Figure 3B).

Nestin is required for in-vitro motility of some glioblastoma lines

Glioma cell motility plays a key role in tumor spread, and in the current inability to cure patients with these malignancies. It has been previously shown that nestin is required for metastasis of the AT6.3 prostate cancer line, suggesting it may be directly involved in cancer cell migration[11]. We therefore explored the possibility that nestin may play a role in glioma cell migration as well. Migration of HSR-GBM1 and HSR040622 was examined in a two-dimensional neurosphere outgrowth assay, which allowed migration from a small, defined number of cells to be assessed over time (Figure 4A). Glioma neurospheres were allowed to form over two days in suspension and then plated onto matrigel-coated plates. One hour after plating, neurospheres had tightly bound, forming small colonies with an average cross-sectional area of 196 μm^2 for HSR-GBM1 and 236

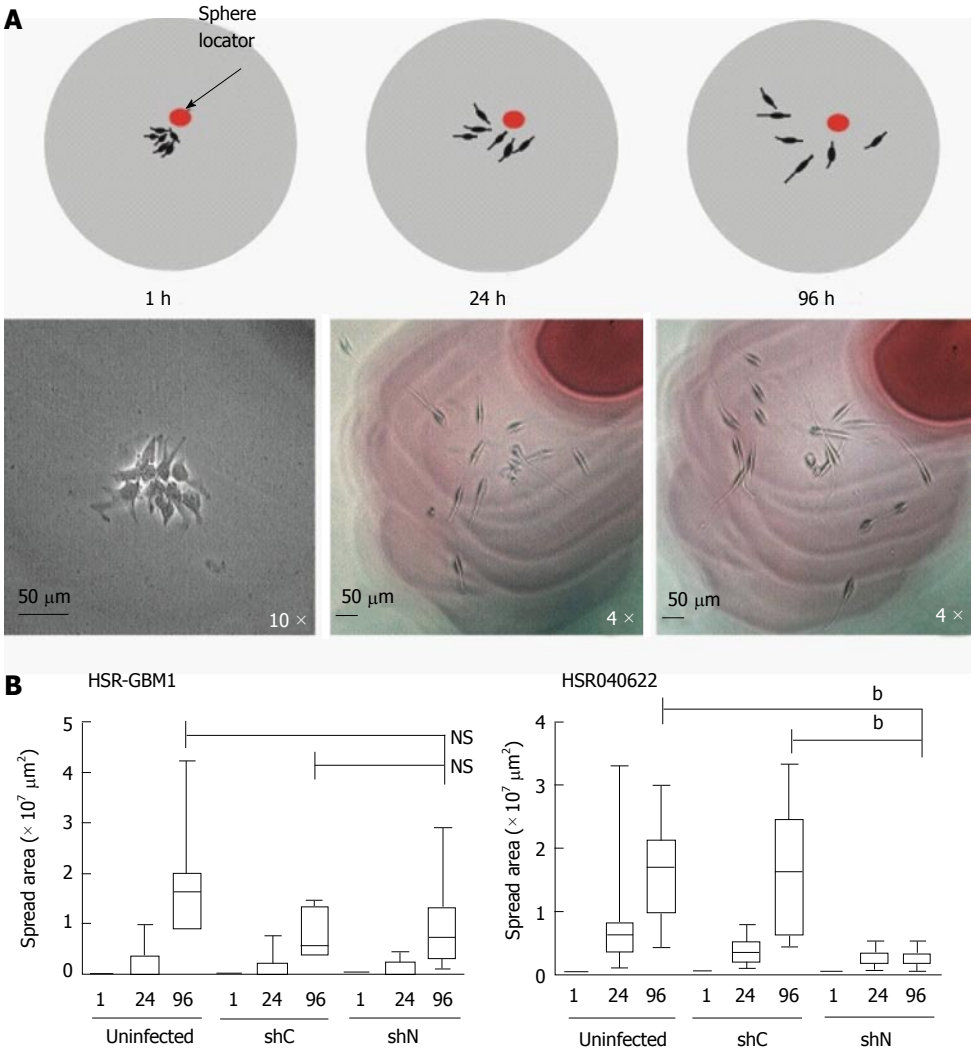

Figure 4 Effect of nestin knockdown on cell migration. A: Top, cartoon illustrating the experimental design (described in details in materials and methods). Bottom, the two-dimensional area spread of a selected HSR-GBM1 sphere is shown; B: Two dimensional area spread analysis shown no significant difference in cell migration for HSR-GBM1 (left) uninfected, shC, and shN infected cells. In contrast, a significant reduction in cell migration was observed for shN infected HSR040622 cells (right) as compared with uninfected and shC infected cells (NS: Not significant, $^{b}P < 0.001$; two sided *t* test). shC: Scrambled control.

μm^2 for HSR040622. The growth in cross-sectional area of individual colonies was then measured over time. Only limited cellular proliferation occurred over the time course of the assay, and, as we found no change in proliferation with reduced nestin levels, we believe any differences in area are due to effects on migration. Nestin knockdown dramatically reduced cell spreading in HSR040622 cells (Figure 4B, right panel) from an average area of 1.66×10^7 μm^2 for shC infected to $2.2 \times 10^6 \times 10^5$ μm^2 for shN infected cells (two tailed *t* test; $P < 0.0001$). In contrast, we found no significant decrease in migration for HSR-GBM1, with an average area of 7.24×10^6 μm^2 for shC infected and 9.12×10^6 μm^2 for shNestin infected cells (Figure 4B, left panel).

Nestin is not required for intracranial xenograft growth or in-vivo migration of tumor cells

An intracranial xenograft model was employed to compare the tumorigenicity and migratory capacity of HSR-GBM1 shC and shN infected cells. We injected 1×10^5 viable tumor cells into the right striatum of athymic nude mice and monitored tumor formation for a period of 16 wk. Xenografts formed in all animals injected with either shC or shN infected HSR-GBM1 cells, but we found no significant difference between these two lines in terms of their *in-vivo* growth. Both forming large, infiltrative tumors, resulting in death as early as 63 d following injection. Immunostaining of these xenografts confirmed that tumor cells maintained reduced nestin expression over the relatively prolonged period of *in-vivo* growth (Figure 5).

DISCUSSION

We examined nestin immunoreactivity in astrocytomas of grades II to IV, and found a significant positive correlation between protein expression and increasing pathological grade. These results are similar to those previously reported in gliomas by several other groups[4]. Dahlstrand *et al*[4], showed higher nestin expression in

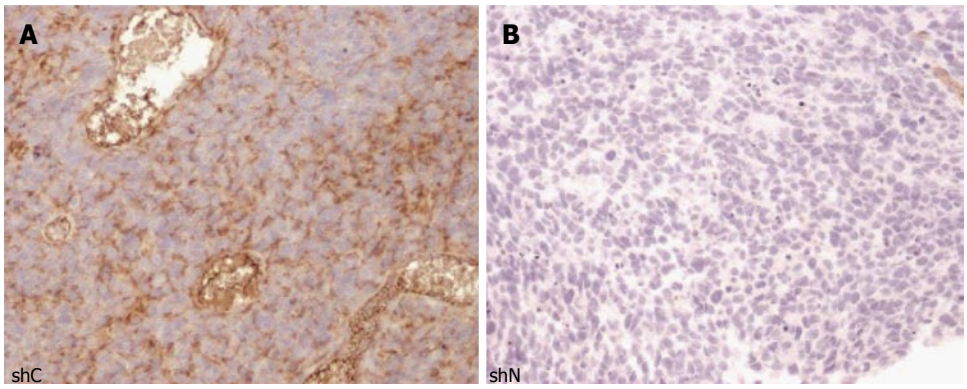

Figure 5 Nestin is not required for xenograft engraftment in HSR-GBM1. Nestin immunostaining analysis of shC (A) and shN (B) infected HSR-GBM1 cells engrafted into the striatum of athymic nude mice. No significant differences in cellular morphology or migration of tumor cells were apparent at the time the mice were sacrificed. Similar phenotype was observed when uninfected cells were injected (not shown). In all experiments, mice survived for about 63 d post injection. shC: Scrambled control.

malignant tumors such as glioblastoma when compared to lower grade glial tumors. Ehrmann *et al*[17] reported that in astrocytomas and malignant melanomas, nestin expression could be used as an auxiliary indicator of dedifferentiation and progression. Nestin expression has also been detected in non-glial malignancies, including hemangioblastomas[9], melanoma[18,19], basal epithelial breast tumors[20], prostate cancer[11], and in gastrointestinal stromal tumors[21]. In many of these, immunoreactivity correlated with increasing pathological grade as well[4,8,17,20-25]. The fact that higher grade tumors show increased nestin immunoreactivity suggests that the percentage of cells with a stem/progenitor phenotype may increase during tumor progression. In addition, the fact that nestin expression is associated with increasing grade in a very wide variety of malignancies indicates that a better functional understanding of this protein may lead to insights into how tumors progress.

Despite its common use as a marker of poorly differentiated stem and progenitor cells, the functional role of nestin in normal and neoplastic cells is poorly understood. It has recently been shown that nestin can serve as a scaffold for a number of signal transduction proteins, such as cdk5 and p35/45, thereby regulating their function[26-28]. Intermediate filaments function as cytoskeletal scaffolds in the nucleus and cytoplasm[29] and may be involved in cellular migration and metastatic potential[30-32]. In addition, it has been shown that nestin is required for migration and metastasis of prostate cancer cells[11]. Such studies highlight the need to examine potential functional roles of nestin, in addition to using it as a marker of differentiation status.

We examined the requirement for ongoing nestin expression in growth and migration of two glioblastoma-derived neurosphere lines. Growth was not significantly affected in either line when nestin levels were reduced by 60% or more using shRNA. These findings indicate that as in prostate cancer[11], glial cells appear to grow nicely with severely reduced nestin levels. It has been suggested that nestin may be only one of several intermediate filaments involved in proliferation, and therefore its loss may be compensated for by other members of this large family of proteins[11]. Indeed, we detected about a two fold-increase in synemin mRNA levels following knockdown of nestin in HSR-GBM1, although it appears that only the L transcript variant is induced (Figure 3B).

We also examined the expression of several mRNA expressed in either stem/progenitor or better differentiated cells in lines with varying nestin levels. Loss of nestin did not seem to alter expression of CD133 or Olig2, although the glial marker GFAP and the neuronal marker MAP2 were decreased somewhat following nestin knockdown. The significance of this latter observation is not clear, but the fact that CD133 and Olig2 levels were unchanged, and that cultures could be passaged for over six months with low nestin levels, strongly suggest that it is not required for the maintenance of tumor-propagating stem-like cells.

Understanding the migration of glial tumor cells is of fundamental importance if we are to eventually cure malignant brain tumors. Our results suggest that nestin may be required for such migration *in-vitro* in a subset of tumors, as the spread of HSR040622 cells was almost completely abolished *in-vitro* by nestin knockdown. In contrast, the *in-vitro* spread of the second line examined was unaffected by shRNA targeting nestin, and these cells could still form invasive intracranial xenografts despite prolonged reduction of nestin. The molecular basis for the varying requirements of glioblastoma neurosphere lines for nestin is not clear. It is possible that the increase in synemin levels observed in the HSR-GBM1 cells with nestin-targeting shRNA may compensate for the reduced nestin levels, allowing cells to migrate normally. Indeed, the intermediate filament synemin has previously been shown to contribute to the migratory properties of astrocytoma cells by influencing the dynamics of the actin cytoskeleton[33].

In summary, our studies support the concept that nestin expression is a common feature of astrocytic brain tumors, and that protein levels correlate with

tumor grade. However, the functional role of nestin appears to be limited, although the migratory potential of some glioblastoma neurospheres is reduced by nestin knockdown. Further studies will be needed to fully understand the role of nestin in migration, and the heterogeneity between different glioblastoma lines.

COMMENTS

Background

The challenges in curing glioblastoma have been partially attributed to the persistence of cancer stem cells following treatment. This subpopulation of cells have been shown to express proteins preferentially expressed in neural stem and progenitor cells such as CD133, OLIG2, and nestin. Nestin is a class VI intermediate filament protein, normally expressed in neuroepithelial stem and progenitor cells of the developing mammalian central nervous system (CNS). In this study, the authors focus on determining if nestin plays a functional role in tumor formation, proliferation, and migration as this is still not well understood.

Research frontiers

Previous work has established that nestin is required for prostate cancer cell migration *in-vitro* and *in-vivo*. In the present study, the authors investigate the expression of nestin in astrocytic tumors and its functional role in glioblastoma.

Innovations and breakthroughs

This is the first study evaluating nestin's role in tumor formation, proliferation, and migration utilizing glioma stem cells.

Applications

The findings suggest that nestin may be involved in glioblastoma cell invasion in some tumors. Inhibition of nestin expression and/or function may represent a potential therapeutic approach to reduce or inhibit glioblastoma cell spreading throughout the CNS.

Terminology

All terms used in this paper are described in the main text.

Peer-review

The relationship of nestin and glioma was well discussed in the paper.

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ORIGINAL ARTICLE

Basic Study

New categorization of human vascular endothelial cells by pro- *vs* anti-proliferative phenotypes

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Abstract

AIM: To integrally understand the effects of human vascular endothelial cells (VECs) on the proliferation of vascular smooth muscle cells (VSMCs).

METHODS: Various kinds of human VECs of different origins were co-cultured with human aortic smooth muscle cells, a representative of human VSMCs. To exclude the irrelevant effects due to growth competition between VECs and VSMCs, the proliferation of VECs had previously been arrested *via* a low-dose gamma ray

irradiation. To discriminately analyze the proliferation of VSMCs from that of VECs, the former cells were labeled with red fluorescent dye while the latter cells were labeled with green fluorescent dye before performing co-culture experiments. After 4 d, total cells were harvested and subjected to flow cytometric analyses. Decrements in red fluorescence intensities due to proliferation-mediated dilutions were measured and mathematically processed using a specific software to quantitatively evaluate the proliferation of VSMCs. The findings obtained from the flow cytometry-based analyses were further validated by microscopic observations.

RESULTS: Commercially available primary cultured human VECs exclusively promoted VSMC proliferation regardless of their tissue origins and we termed these pro-proliferative VECs as "type-I". By contrast, VECs freshly generated from human bone marrow-derived endothelial progenitors cells or human pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells suppressed VSMC proliferation and we termed these anti-proliferative VECs as "type-II". Repetitive subcultures as well as oxidative stress induced "type-II VECs to type-I" conversion along with an induction of Regulator of G-protein signaling 5 (RGS5). Compatibly, anti-oxidant treatments suppressed both the subculture-dependent "type-II to type-I" conversion and an induction of RGS5 gene. Immunostaining studies of clinical specimens indicated that RGS5 protein expressions in endothelial layers were low in normal arteries but they were up-regulated in pathological arteries including hypertension, atherosclerosis and autoimmune vasculitis in a dose-dependent manner. Overexpression and knockdown of RGS5 caused that "type-II to type-I" and "type-I to type-II" phenotype conversions of VECs, respectively.

CONCLUSION: Human VECs are categorized into two types: pro-proliferative RGS5^{high} VECs (type-I) and anti-proliferative RGS5^{low} VECs (type-II).

Key words: Vascular endothelial cells; Vascular smooth muscle cells; Human induced pluripotent stem cells; Human embryonic stem cells; Regulator of G-protein signaling 5; Oxidative stress

Core tip: There is a longstanding controversy over the effects of vascular endothelial cells (VECs) on the proliferation of vascular smooth muscle cells (VSMCs). Since the controversy came from lack of systematic studies, we performed an integrated analysis using various human VECs to quantitatively evaluate their effects on VSMC proliferation. Here we report that: (1) human VECs are classified into two groups: pro-proliferative (type-I) *vs* "anti-proliferative" (type-II); (2) oxidative stress and ageing induced "type-II to type-I" conversion; and (3) RGS5 is the responsible gene for VEC phenotype determinations. Thus, human VECs are categorized into pro-proliferative RGS5^{high} (type-I) and anti-proliferative RGS5^{low} (type-II) VECs.

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INTRODUCTION

Ischemic diseases are caused by stenosis of the artery (*i.e.*, arteriostenosis). Although cholesterol medications have produced remarkable results by reducing the rate of atherosclerosis, there still remain problems including medication-resistant patients and cases with restenosis after stent therapies. It is well known that the pathological basis of arteriostenosis is the hyperproliferation of vascular smooth muscle cell (VSMC). However, roles for vascular endothelial cells (VEC) in the development of arteriostenosis remain elusive. Controversial ideas have been raised regarding the effects of VECs on the proliferation of VSMC. From a clinical standpoint, it has been suggested that VECs prevent the proliferation of VSMC because VSMC proliferation is usually observed under circumstances where VECs are lost or dysfunctional. In accordance with this idea, VECs of hypertensive rats, but not those of normal counterparts, reportedly promote VSMC proliferation[1]. On the other hand, *in vitro* co-culture experiments using fetal human umbilical vein endothelial cells (HUVEC) and bovine aortic smooth muscle cells showed that VECs enhanced VSMC proliferation[2]. Nevertheless, this finding requires detailed validation. First, it must be re-validated by co-culture experiments using the cells of the identical species (*e.g.*, human VSMCs and human VECs). Secondly, it must be re-checked by co-culture experiments using adult samples because dynamic vasculogenesis with active VSMC proliferations occurs during fetal development. In addition, physiological events during the fetal development often resemble to pathological events in the adult life such as fetal gene expression profiles in angiosarcoma[3], coronary artery disease[4], diabetic retinopathy[5] and arteriostenosis[6] in adults. Thirdly, it must be re-validated by co-culture experiments using freshly produced VECs because characters of the cells often change during the process of *ex vivo* subcultures. For example, the quality of *ex vivo*-expanded primary cultured human cells becomes considerably lower than that of freshly differentiated human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC)[7].

To address all those issues, we performed VEC/VSMC co-culture experiments using various kinds of human VECs of diverse origins. Furthermore, we performed microarray analyses to identify the key gene that determines the phenotypes of human VECs.

MATERIALS AND METHODS

Cells, tissues and reagents

HUVEC, human neonatal dermal microvascular endothelial cells (HMVEC), human adult aortic endothelial cells (HAEC) and human adult coronary arterial endothelial cells (HCAEC) were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka Japan). Human endothelial progenitors cells (EPCs) were provided as follows: Two lots of human adult bone marrow mononuclear cell-derived endothelial progenitor outgrowth cells (EPOCs) at 4th passage were purchased from BioChain Institute, Inc., Hayward, CA. Two lots of human umbilical cord blood endothelial colony forming cells (ECFC) were purchased from Lonza Group Ltd., Basel, Switzerland, and one lot of human cord blood-derived EPOCs at 4th passage, were purchased from BioChain Institute, Inc. The cells were cultured on 0.1% gelatin-coated plates using EGM®-2 BulletKit (Lonza Group Ltd. Basel, Switzerland). Human aortic smooth muscle cells of different donors were purchased from Lonza Group Ltd. (Basel, Switzerland) and cultured using SmGM™-2 BulletKit™ (Lonza Group Ltd.). Cells were re-seeded at split ratios of 1:3-1:4 twice a week. VECs within 8th passage were used in all experiments. The hESC lines (KhES-1, -3, -5) were established by the Institute for Frontier Medical Science, Kyoto University[8]. SeV-hiPSCs were established from HUVEC[9] and BJ fibroblast[9] by using iPS-Tune™ (ID Pharma Co., Ltd., Ibaraki, Japan). Ret-hiPSC lines were provided as follows: 253G1[10] and 201B7[11] were provided by CiRA at Kyoto University; #25 was provided by National Research Institute for Child Health and Development as used elsewhere[7]. Astaxanthin (A3236, Sigma-Aldrich Co. LLC. St. Louis, MO 63178, United States) was dissolved by DMSO at the concentration of 5 mmol/L. Frozen sections of clinical specimens were purchase from BioChain Institute, Inc.: normal human artery (Cat: T5595-4763 Lot: L11052523C11052523), arteries of hypertension patients (Cat: T1236013Hd-2 Lot: B502175), arteries of systemic lupus erythematosus (Cat: T1236013LUP Lot: A804253), arteries of arteriosclerosis (Cat: T1236013Hd-4 Lot: B502176).

Quantitative evaluations of VSMC proliferation

VECs were -irradiated (5 Gy) and stained with carboxyfluorescein diacetate, succinimidyl ester by using CFSE Cell Division Assay Kit (Cayman Chemical Co., Ann Arbor, MI), while VSMCs were stained with PKH26 by using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich Co. LLC., St. Louis, MO 63178) according to the manufacturer's guidance. For contact co-culture, irradiated and CFSE-stained VECs were seeded at the density of 2 × 10^5 cells/well on 0.1% gelatin-coated 24-well culture plates, and on the following day, PKH26-stained VSMCs were seeded at the density of 3.75 × 10^3 cells/well on VEC layers or gelatin layers as control. After 4 d, total cells were harvested and subjected to flow cytometry analyses by FACSCalibur™ (BD Biosciences, San Jose, CA) and FL1 and FL2 fluorescence intensities were measured by CellQuest™ Pro software (BD Biosciences). FL2 (PKH26) fluorescence intensities were further analyzed mathematically by ModFit LT™ software (Verity Software House Inc., Topsham, ME) to calculate the proliferation index. Regarding experiments on astaxanthin treatments, VECs were stained by PKH26 and VSMCs were treated with CFSE because of red colored-fluorescence interference by astaxanthin.

Microarray analyses

Total RNAs were isolated by using TRIzoL® Reagent (Life Technologies, Inc., Grand Island, NY) and subjected to GeneChip® Gene 1.0 ST array (Affymetrix, Inc., Santa Clara, CA, United States) by Pharma Frontier Co. Ltd. (Tokyo, Japan). Alterations in gene expressions were estimated as significant if > 2.0-fold increments or < 0.5-fold decrements in signal intensities were detected. Functional analyses were performed using IPA® Ingenuity Pathways Analysis software ver. 14855783 (Ingenuity Systems, Inc. Redwood City, CA) and hierarchical clustering was executed using GeneSpring GX 12.0 software (Agilent Technologies, Santa Clara, CA). All analyses were performed by Chemicals Evaluation and Research Institute (CERI, Tokyo, Japan).

RT-PCR

Total RNA was extracted from VECs using TRIzol® Reagent (Life Technologies, Inc.). First strand DNA was synthesized by using SuperScript™Ⅲ First-Strand Synthesis System kit (Life Technologies, Inc.). PCR was performed using GeneAmp® PCR system 9700 (Life Technologies, Inc) and Ex-Taq (Takara Shuzo Co. Ltd., Shiga, Japan) with a following program: the initial denaturation at 94 ℃ for 5 min, 24-28 cycles and 20-22 cycles of amplification process for *RGS5* and -actin, respectively, renature (55 ℃, 30 s), extension (72 ℃, 30 s) and denature (94 ℃, 30 s) with a final extension (72 ℃, 10 min). Primers used for RGS5 were Fw: CTGGATTGCCTGTGAGGATT and Rv: TCAGGGCATGGATTCTTTTC and those for *b*-actin were Fw: GCAGGAGATGGCCACGGCGGC Rv: TCTCCTTCTGCATCCTGTCAGC. The RT-PCR products were subjected to 1.5% agarose gel electrophoresis and the amplified DNA bands were visualized by ethidium bromide staining.

Quantitative RT-PCR

Total RNA was extracted from VECs using TRIzol® Reagent (Life Technologies, Inc.). Complementary DNA was prepared from 1 g of RNA using SuperScript™Ⅲ First-Strand Synthesis System kit (Life Technologies, Inc.), and used in quantitative PCR reactions with FAST SYBR® Green Master Mix (Applied Biosystems® from Life Technologies, Inc.). qRT-PCR was performed using the StepOnePlus™ PCR machine (Applied Biosystems® from Life Technologies, Inc.). Primers used for RGS5 were Fw: GGAGGCTCCTAAAGAGGTGA and Rv: GGGAAGGTTCCACCAGGTTC, and primers used for

GAPDH were Fw: CCACTCCTCCACCTTTGAC and Rv: ACCCTGTTGCTGTAGCCA.

Immunostaining

Cultured cells and clinical specimens were fixed by methanol/acetone (1:1) for 10 min on ice. The 1st antibody reactions were performed by using a 1:200-diluted chicken polyclonal anti-human RGS5 antibody (ab14265, Abcam plc., Cambridge, United States) and/or a 1:50-diluted rabbit polyclonal anti-human PECAM antibody (sc-8306, Santa Cruz Biotechnology Inc.) and the 2nd antibody reactions were performed by using an Alexa Fluor® 594-conjugated goat anti-chicken IgG (A11042, Life Technologies, Inc) and/or Alexa Fluor® 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies, Inc). Photomicrographs were taken by Olympus BX51 Fluorescence Phase contrast Microscope (Olympus Optical Co. Ltd.) equipped with DP-2 TWAIN digital camera system (Olympus Optical Co. Ltd.) and cellSens® standard imaging software (Olympus Optical Co. Ltd.). Regarding clinical specimens, white balance was adjusted so that autofluorescence from lamina elastic became white.

Western blotting

The 1 × 10^5 VECs were lysed by using 20 μL sample buffer solution [(2ME+) (× 2), (Cat. 196-11022) and (WAKO Pure Chemical Industries, Osaka, Japan)]. The first antibody reaction was performed by using a 1:1000-diluted anti-human RGS5 antibody (ab83230, Abcam, Cambridge, United States) or a 1:1000-diluted anti-human b-tubulin antibody (sc-9104, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) and the second antibody reaction was performed by using a 1:2000-diluted anti-rabbit IgG HRP-linked antibody (#7074S) (Cell Signaling Technology, Inc.).

Nucleofection

Expression vectors for shRNA against RGS5 were purchased from OriGene Technologies Inc. (Rockville, MD, United States). A Homo sapiens cDNA, FLJ96402, which corresponds to Homo sapiens regulator of G-protein signaling 5 (RGS5), transcript variant 1, mRNA (NM_003617.3), with two nucleotide substitutions, was purchased from National Institute of Technology and Evaluation (Tokyo, Japan), and the two substituted nucleotides were corrected by using KOD-Plus-Mutagenesis Kit (Toyobo Co. Ltd., Osaka, Japan) to become identical to the nucleotide sequences in NM_003617.3. The RGS5 cDNA was inserted into pmaxCloning™ expression vector (Lonza Group Ltd. Basel, Switzerland). Transfection was performed by using a Nucleofector™ (Lonza Group Ltd., Basel, Switzerland) according the manufacturer's guidance. The Amaxa HUVEC Nucleofector Kit (#VPB-1002, Lonza Group Ltd.) was used for HUEVC and the Amaxa Basic Nucleofector Kit Primary Endothelial Cells (#VPI-1001, Lonza Group Ltd.) was used for EPCdECs, hESdECs and hiPSdECs.

Statistical analysis

Experiments were performed independent three experiments (n = 3) and the data were analyzed according Student t test. Results were shown as averages (AV) ± SD.

RESULTS

Studies on commercially available primary cultured human VECs

The effects of VECs on the proliferation of VSMCs were quantitatively evaluated by a flow cytometry-based technique (Figure 1A and B). Briefly, "contact" or "non-contact" (*i.e.,* Boyden) co-culture experiments were performed using various kinds of human VECs, whose growths were previously arrested by a low dose gamma ray irradiation, and adult human VSMCs. After 4 d, VSMC proliferation was assessed by mathematically processing the reduction degree of the red fluorescence intensity using ModFit LT™ software.

First, the effects of commercially available primary cultured VECs were examined. As previously reported[2], the proliferation of VSMCs was up-regulated by both "contact" and "non-contact" co-cultures with HUVEC (Figure 1C and D), indicating that HUVEC enhanced VSMC proliferation *via* a soluble factor(s). These findings were confirmed by microscopic observations (Figure 1E). Similar results were obtained from HAEC, HCAEC and HMVEC (Figure 1F). Thus, commercially available primary cultured human VECs exclusively enhance VSMC proliferation *via* a soluble factor(s).

Studies on VECs generated from human endothelial progenitor cells

We next evaluated the effects of VECs that were produced from commercially available human (EPCs). Adult bone marrow mononuclear cell-derived EPCs at passage 4 were purchased. Then, EPC-derived mature VECs (EPCdECs) were prepared after additional three passages using a specialized medium (totally at passage 7) and subjected to co-culture experiments. Surprisingly, EPCdECs of the first donor (EPC1dEC) suppressed the proliferation of VSMCs under contact co-culture (Figure 2A and C). By contrast, EPC1dEC enhanced VSMC proliferation under non-contact culture (Figure 2A, right panel; Figure 2B, gray column), indicating that a potent growth-inhibitory activity, which was stronger than the growth-promoting activity of the soluble factor, was transmitted *via* cell-cell interactions. We confirmed that EPC1dEC purchased as an independent package after a half year provided similar findings (data not shown), guaranteeing the high reproducibility of our assay system. Interestingly, anti-proliferative potentials of EPC1dEC were considerably attenuated at passage 8 (Figure 2D, middle column) and finally nullified at passage 12 (Figure 2D, right column), indicating that the anti-proliferative capacity is susceptible to subculture-dependent stresses. In

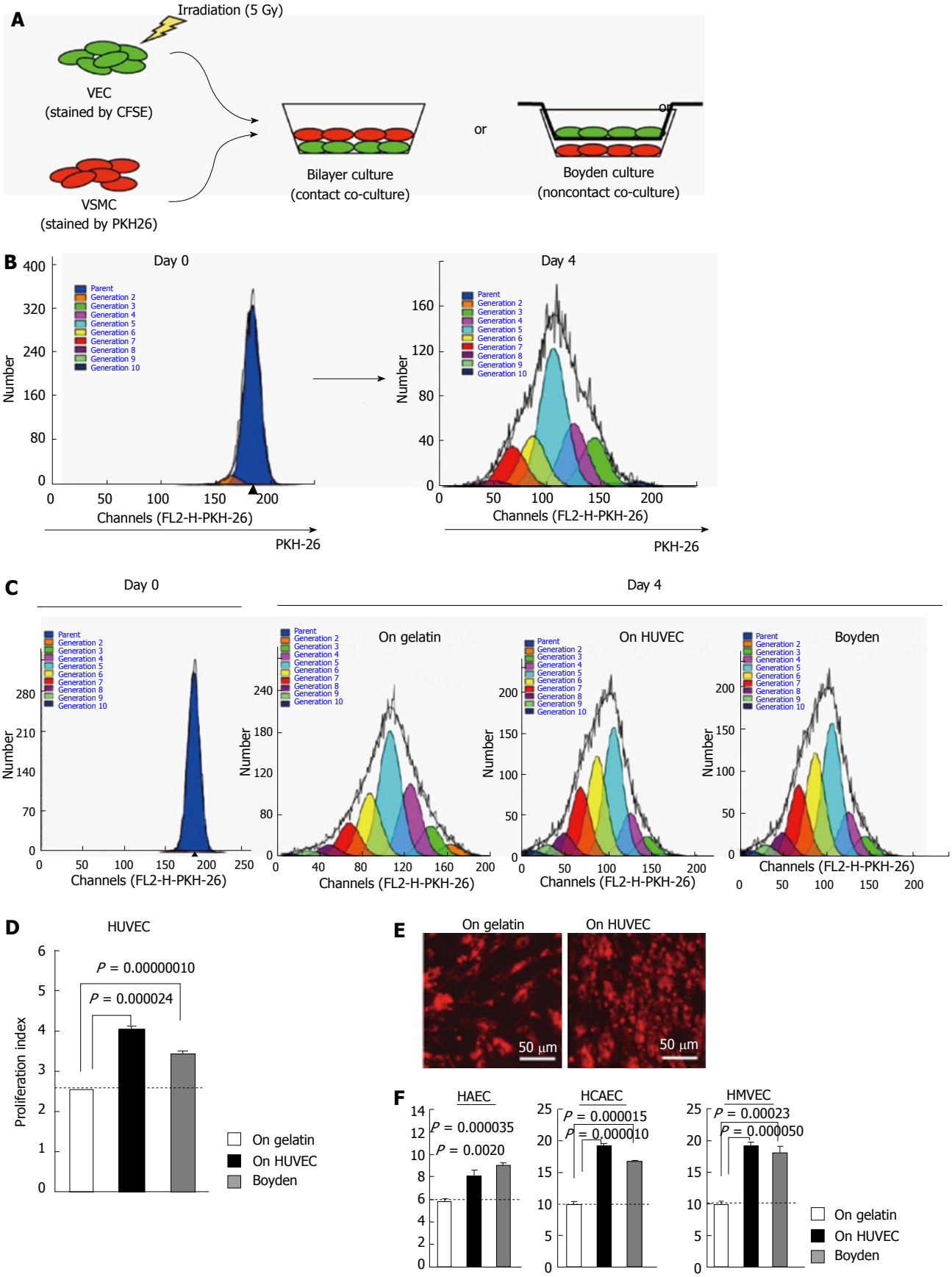

Figure 1 Phenotype determination of primary cultured human vascular endothelial cells. A and B: Illustrations of the method to calculate proliferation indexes of VEC-co-cultured VSMC *via* ModFit LT™ software-based analyses; C: Results of flow cytometric analyses of HUVEC-co-cultured VSMC with ModFit LT™ analyses; D: Results in C were statistically evaluated (n = 3); E: Fluorescent microscopy of PKH-26-stained VSMC subjected to contact co-culture with HUVEC; F: Results of VSMC-co-culture experiments using HAEC, HCAEC and HMVEC (n = 3). VEC: Vascular endothelial cells; HAEC: Human umbilical artery endothelial cells; HCAEC: Human coronary artery endothelial cells; HUVEC: Human umbilical vein endothelial cells; HMVEC: Human microvascular endothelial cell; VSMC: Vascular smooth muscle cell.

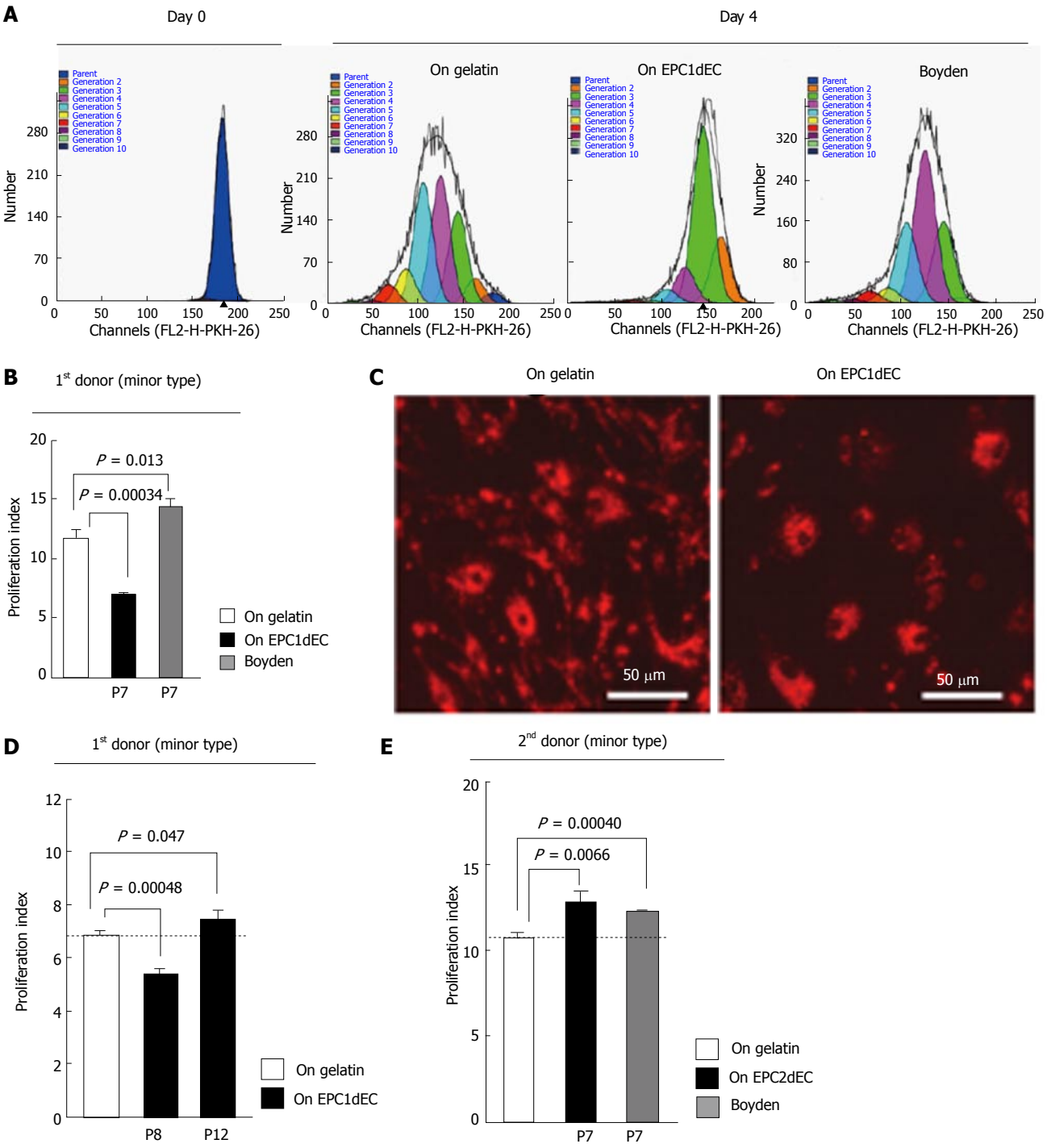

Figure 2 Phenotype determination of human embryonic stem cell-derived vascular endothelial cells. A: Results of flow cytometric analyses of hEPC1dEC[P7]-co-cultured VSMC with ModFit LT™ analyses; B: Results in (A) were statistically evaluated (n = 3); C: Fluorescent microscopy of PKH-26-stained VSMC subjected to contact co-culture with hEPC1dEC[P7]; D: Results of VSMC-co-culture experiments on the layer of hEPC1dEC at passage 8 and on that of hEPC1dEC passage 12; E: Results of VSMC-co-culture experiments using hEPC2dEC[P7] (n = 3). VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell.

addition, anti-proliferative potentials could not be detected when fixed VECs were used (data not shown), indicating that living VECs were required for transmitting the anti-proliferative capacity. We termed VECs with anti-proliferative capacities as "type-II", whereas we termed VECs with pro-proliferative capacities as "type-I".

We further examined the effects of EPCdECs of different donors. In contrast to EPC1dECs, donor 2 EPC-derived VEC (EPC2dEC) showed type-I phenotype from the earliest phase (*i.e.*, at passage 7) (Figure 2E). Moreover, all the other commercially available EPCdECs were exclusively type-I VECs (data not shown). Thus, EPCs that could produce type-II VECs belonged to a rather rare population among commercially available EPC sources. We also examined the phenotypes of VECs generated from fetal umbilical cord-derived EPCs (UCEPCdECs) and found that they were exclusively "type-I" VECs (data not shown), reflecting dynamic

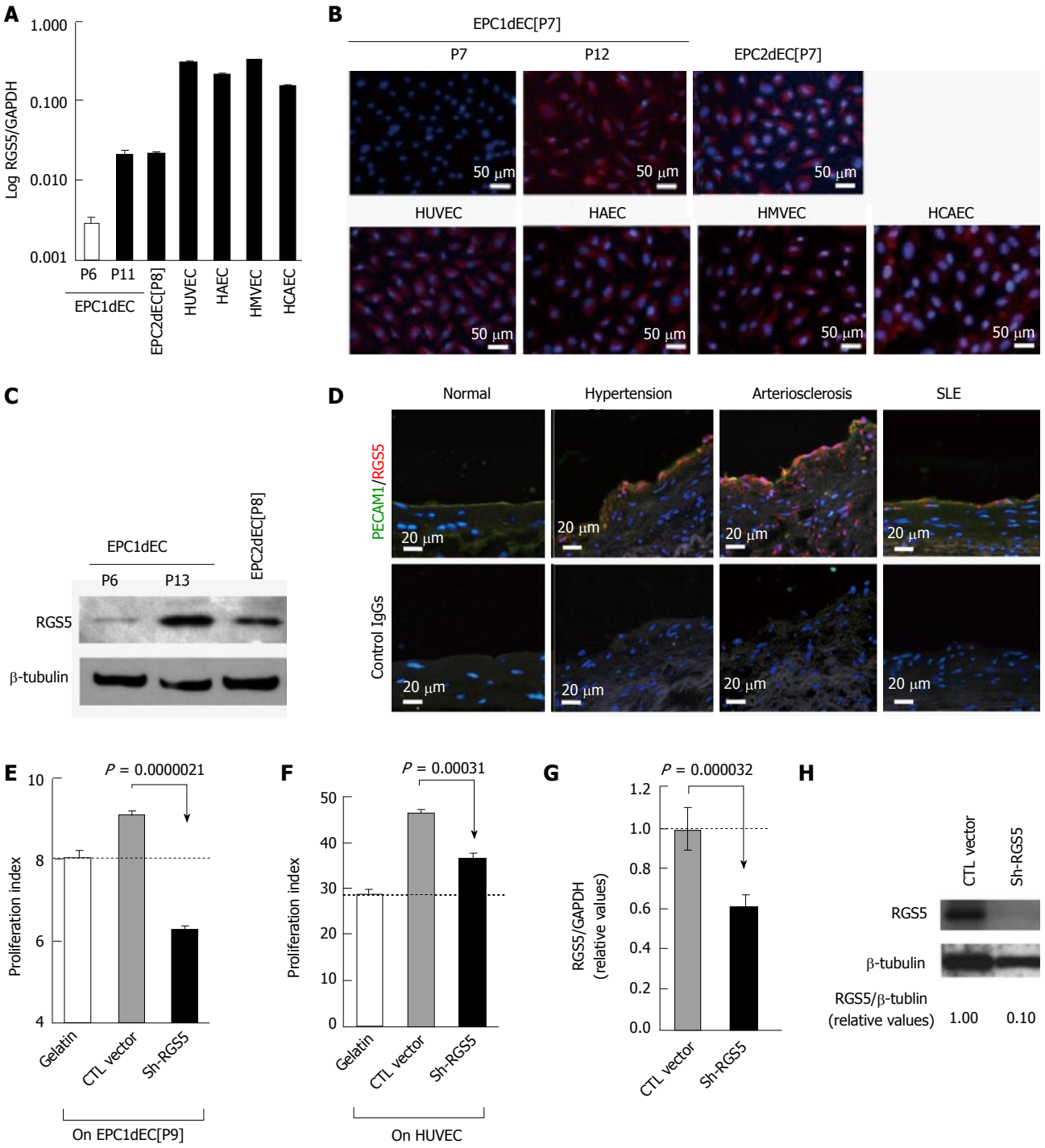

Figure 3 Regulator of G-protein signaling 5 is involved in the phenotype conversion of vascular endothelial cells. A: qRT-PCR results of RGS5 message expression were shown with normalization by GAPDH. The horizontal axis was demonstrated as logarithm; B: Immunostaining of indicated cells by using an anti-RGS5 antibody (red) with DAPI counterstaining (blue); C: Western blotting of RGS5 protein; D: Clinical specimens of the artery were subjected to immunostaining studies by using an anti-RGS5 antibody (red) and an anti-human PECAM1 antibody (green) with nuclear counterstaining with DAPI (blue); E-G: RGS5 knockdown experiments. An expression vector for shRNA against RGS5 (Sh-RGS5) or control RNA (CTL RNA) were transfected into EPC1dEC[P9] (E) or HUVEC (F). The results of VSMC co-cultured experiments (E and F) along with qRT-PCR (RGS5/GAPDH) (G) and Western blotting (H) regarding HUVEC were shown. GAPDH: Glyceraldehyde-phosphate dehydrogenase; VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell; iPSC: Induced pluripotent stem cell; PCR: Polymerase chain reaction; RGS5: Regulator of G-protein signaling 5; HUVEC: Human umbilical vein endothelial cell; HAEC: Human adult aortic endothelial cells; HMVEC: Human neonatal dermal microvascular endothelial cells.

vasculogenesis during fetal development.

RGS5 is the causative gene for phenotype conversion of human VECs

To determine the responsible gene for "type-I *vs* type-II" phenotyping of human VECs, microarray analyses were performed using "type-II" VECs, which were EPC1dEC[P7] of the two independent packages, and "type-I" VECs, which were HUVEC, HAEC, HMVEC, EPC1dEC[P12], EPC2dEC[P7] and UCEPC1dEC (GEO

Accession ID: GSE60999). We found that regulator of G-protein signaling 5 (RGS5) was the only gene that showed a discriminating expression pattern between "type-Ⅱ" and "type-Ⅰ" VECs. The results of the microarray were confirmed by quantitative RT-PCR (qRT-PCR): About one-order up-regulations of *RGS5* expressions in "type-Ⅰ" EPC1dEC[P11] and EPC2dEC[P8] and almost two-order up-regulations of *RGS5* expression in HUVEC, HAEC and HMVEC compared to EPC1-dEC[P6] (Figure 3A). Immunostaining (Figure 3B) and Western blotting (Figure 3C) further confirmed the findings. In addition, clinical relevance of RGS5 induction was evidenced by immunostaining studies using human specimens: Undetectable expression in normal subjects, mild inductions in patients suffering from hypertension or systemic lupus erythematosus and high-level inductions in patients with arteriosclerosis (Figure 3D). In the specimen of arteriosclerosis, RGS5 protein was even detected in neointima especially at perimeters of vasa vasorum, which were recognized as hollow spaces. The distribution pattern of RGS5 was highly analogous to that of oxidative stress-damaged cells[12], suggesting that oxidative stress is one of the major triggers of the "type-Ⅱ to type-Ⅰ" conversion of VECs.

The involvement of RGS5 in the phenotype conversion of VECs was verified by gene knockdown studies. As shown in Figure 3E, "type-Ⅰ" EPC1dEC[P9] was converted into "type-Ⅱ" VECs by an introduction of shRNA-RGS5 expression vector (Figure 3E). Regarding HUVEC, pro-proliferative capacities were lowered by RGS5 knockdown (Figure 3F). Although a shRNA-RGS5 introduction into HUEVC effectively lowered the levels of RGS5 message expression (Figure 3G) and protein expression (Figure 3H), its effect on phenotype alteration was rather mild probably due to particularly high RGS5 expression in HUVEC (Figure 3A). Nevertheless, the results were reproducible when distinct shRNA-RGS5 with different nucleotide sequences were introduced (data not shown). Thus, RGS5 is the causative gene for the "type-Ⅱ to type-Ⅰ" conversion of VECs.

Studies on VECs generated from human pluripotent stem cells

Although we discovered the existence of "type-Ⅱ" VECs for the first time in the world, their applications were highly limited because EPC1dEC[P7] could not be expanded without losing type-Ⅱ phenotype and because commercially available EPCdECs of other donors were exclusively type-Ⅰ VECs. To find an alternative source for the production of type-Ⅱ VECs, we examined the phenotypes of VECs that were produced from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). High-purity subculturable VECs were generated without a contamination by mural cells based on our previously reported method[13,14]. We found that human ESC-derived VECs (ESdECs) showed exclusively "type-Ⅱ" phenotypes at their early passages; however, they were converted into "type-Ⅰ" VECs after a few rounds of subcultures (Figure 4A) with up-regulated expressions of RGS5 message (Figure 4B) and protein (Figure 4C and D) although the timing of conversion differed depending on lines. Because an involvement of oxidative stress in RGS5 induction was suggested by the similarity in tissue-distributing profiles between RGS5-positive cells (Figure 3D) and oxidative stress-damaged cells[12], we examined the effect of hydrogen peroxide treatments on RGS5 gene expression. We found that hydrogen peroxide treatments induced RGS5 expressions in type-Ⅱ ESdECs (Figure 4E) and a treatment with astaxanthin, which is the most potent anti-oxidant whose singlet oxygen-quenching activity[15] and its anti-lipid peroxidation activity are reportedly superior to vitamin E by two-order[16], suppressed RGS5 inductions (Figure 4F). Moreover, astaxanthin treatment significantly delayed the timing of "type-Ⅱ to type-Ⅰ" conversion (Figure 4G), suggesting that oxidative stress is one of the major causes of RGS5 induction.

Because astaxanthin treatment could not completely block RGS5 induction in type-Ⅱ ESdECs after repetitive subcultures (Figure 4F), we searched for still other candidates for type-Ⅱ VECs. Since the timing of the "type-Ⅱ to type-Ⅰ" conversion was latest in the VECs produced from KhES-5, which was established latest among the three lines of human ESCs, we hypothesized that "the more recently human iPSC lines are established, the more stably type-Ⅱ phenotype will be maintained". Since we recently established two lines of Sendai virus vector-based iPSCs (SeV-iPSCs) from HUVEC and BJ fibroblast[9], we produced VECs from these SeV-iPSCs (SeV-iPSdECs) and examined their characters. At the same time, we generated VECs from widely distributed conventional retrovirus vector-based iPSCs (Ret-iPSCs) including #25[7,17], 253G1[7,17,18] and 201B7[7,17,19,20] and examined the phenotypes of these Ret-iPSC-derived VECs (Ret-iPSdECs). Regarding #25 and 253G1, anti-proliferative potentials were undetectable even at early passages (Figure 5A, upper and middle); nevertheless, VSMC proliferations were suppressed under contact co-culture conditions compared to non-contact co-culture conditions. Therefore, weak anti-proliferative potentials were transmitted from these Ret-iPSdECs. Regarding 201B7, anti-proliferative potentials were clearly detected at early passages (Figure 5A, lower) as in the case of ESdECs (Figure 4A). In all three lines of Ret-iPSdECs, pro-proliferative capacities were augmented after repetitive subcultures (Figure 5A). Thus, comparable results were obtained from Ret-iPSdECs to ESdECs as a whole. On the other hand, SeV-iPSdECs well preserved anti-proliferative capacities until later passages (Figure 5B) without an induction of *RGS5* gene (Figure 5C and D). They also showed high resistance to oxidative stress-induced RGS5 induction (Figure 5E), supporting the idea that the most recently established human pluripotent stem cells provide the most effectual type-Ⅱ VECs.

Collectively, human pluripotent stem cells provide an excellent source for the production of anti-proliferative type-Ⅱ VECs.

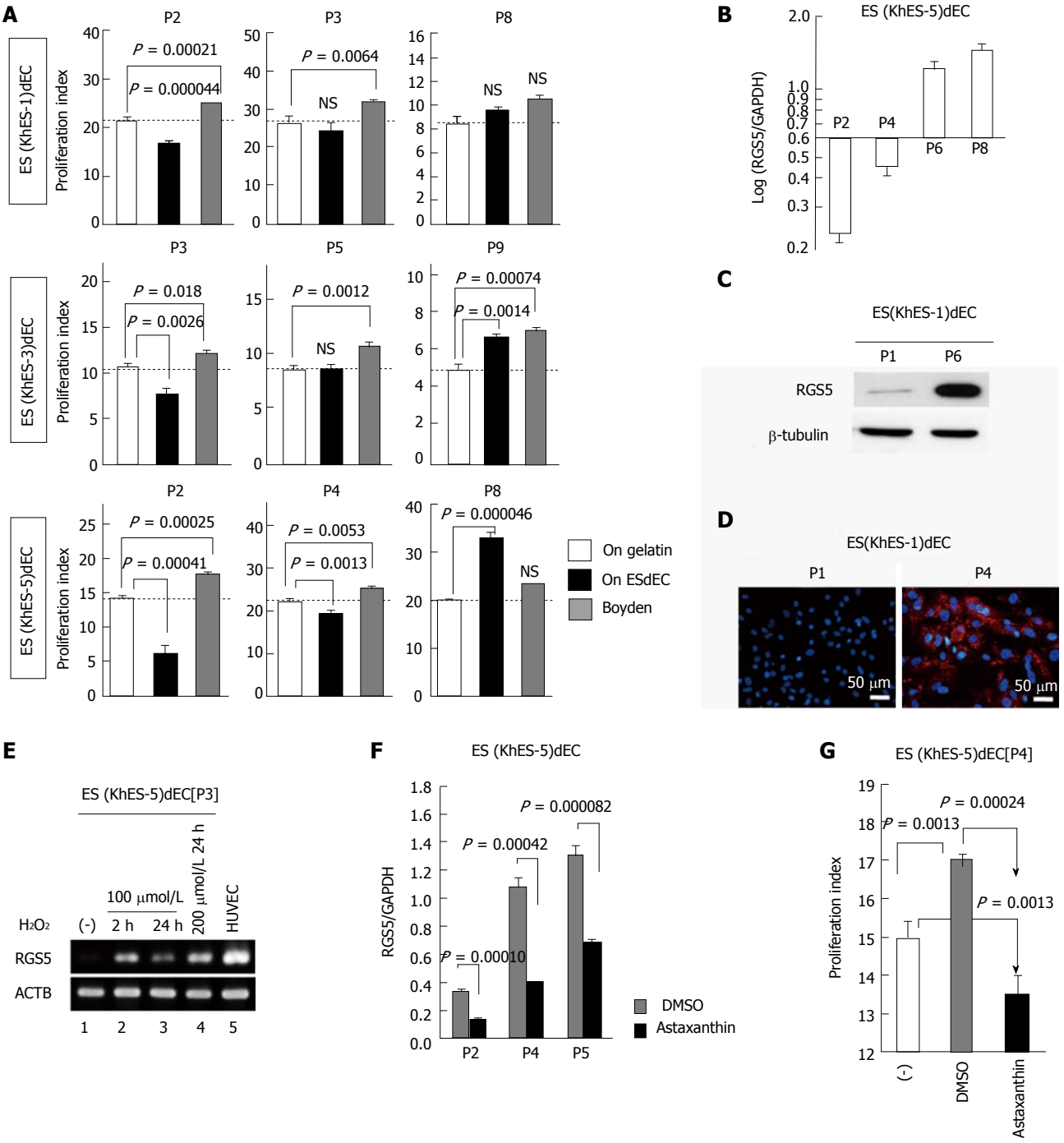

Figure 4 Phenotype evaluation of human embryonic stem cell-derived vascular endothelial cells. A: VSMC-co-culture experiments of VECs generated from three lines of hESCs (KhES-1, khES-3, khES-5) at indicated passage number (n = 3); B: qRT-PCR of RGS5 message of khES-5-derived VECs [ES (KhES-5) dEC] at indicated passage numbers; C: Western blotting of RGS5 protein in KhES-1-derived VECs [ES (KhES-1) dEC] at passage 1 and passage 6; D: Immunostaining of RGS5 protein in ES (KhES-1) dEC at passage 1 (upper) and passage 4 (lower); E: ES (KhES-5) dECs were treated with hydrogen peroxide and RGS5 message expressions were examined by RT-PCR. Lane 1: no treatment; lane 2: 100 mol/L H2O2 treatment for 2 h; lane 3: 100 μmol/L H_2O_2 treatment for 24 h; lane 4: 200 mol/L H_2O_2 treatment for 24 h; lane 5: HUVEC; F: qRT-PCR of RGS5 message of ES (KhES-5) dECs subcultured in the presence of DMSO (gray column) or 10 mol/L astaxanthin (closed columns); G: VSMC-co-culture experiments of ES (KhES-5) dECs maintained with culture medium (open column), in the presence of DMSO (gray column) or 10 mol/L astaxanthin (closed column). VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell; PCR: Polymerase chain reaction; DMSO: Dimethylsulfoxide; RGS5: Regulator of G-protein signaling 5; HUVEC: Human umbilical vein endothelial cell.

DISCUSSION

In the current study, we presented a new concept for the categorization of human VECs based on their effects on VSMC proliferation: *pro-proliferative* $RGS5^{high}$ VEC (type-Ⅰ) and anti-proliferative $RGS5^{low}$ VECs (type-Ⅱ) (Figure 6). Since oxidative stresses and subculture-dependent mechanochemical stresses induced "type-Ⅱ to type-Ⅰ" conversion along with an induction of RGS5 expression, "type-Ⅰ" $RGS5^{high}$ VECs may well be regarded

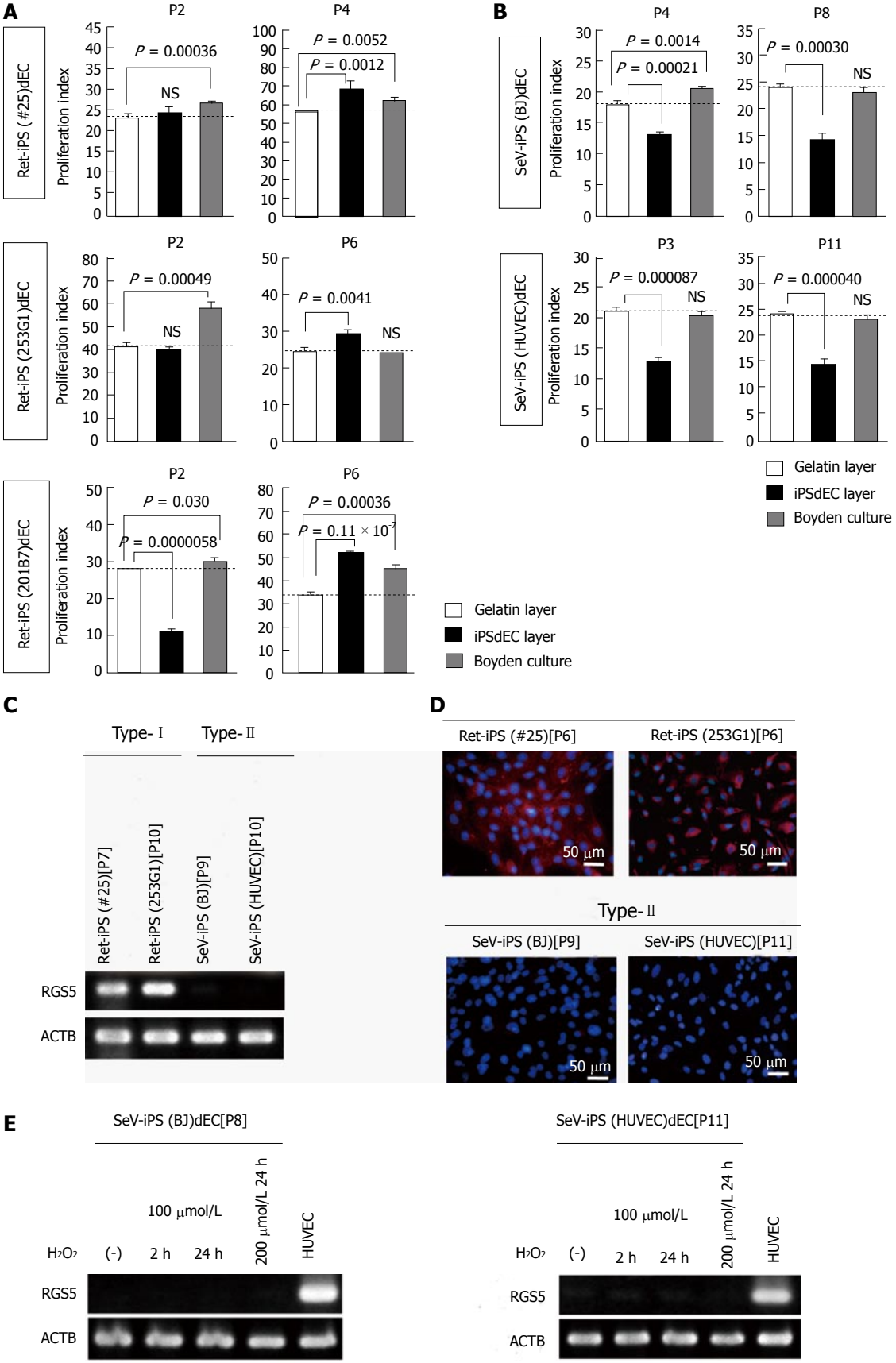

Figure 5 Phenotype evaluation of human induced pluripotent stem cells-derived vascular endothelial cells. A and B: VSMC-co-culture experiments of VECs generated from three lines of Ret-iPSCs (#25, 253G1 and 201B7); A and those from two lines of SeV-iPSCs(SeV-iPS(BJ) and [SeV-iPS(HUVEC)]; B: At indicated passage number (n = 3); C: RT-PCR of RGS5 message of Ret-iPSdECs (type-I) and SeV-iPSdECs (type-II); D: RGS5 immunostaining studies of Ret-iPSdECs (upper) and SeV-iPSdECs (lower); E: H_2O_2-treating experiments were performed in type-II SeV-iPSdECs as in Figure 4E. VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell; iPSC: Induced pluripotent stem cell; PCR: Polymerase chain reaction; RGS5: Regulator of G-protein signaling 5; HUVEC: Human umbilical vein endothelial cell.

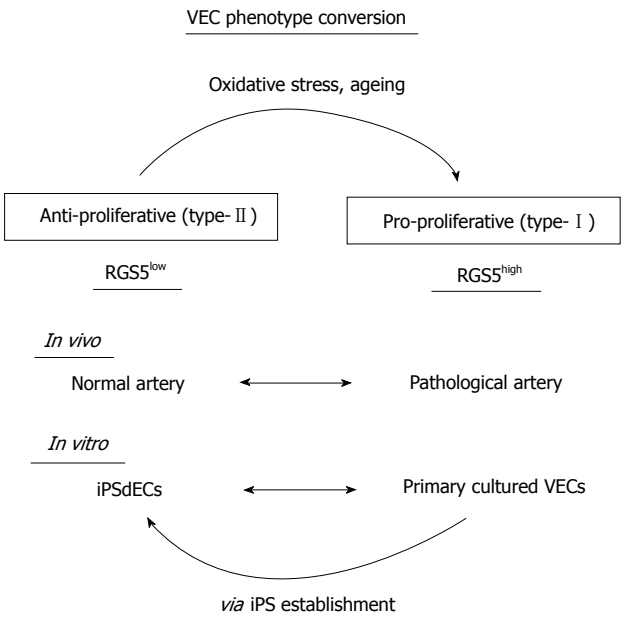

Figure 6 A model for the type-Ⅰ *vs* type-Ⅱ phenotype regulation of human vascular endothelial cells. RGS5: Regulator of G-protein signaling 5; VEC: Vascular endothelial cell; iPSC: Induced pluripotent stem cell.

as degenerative VECs. It might be shocking that *widely used* commercially available primary cultured human VECs exclusively belong to type-Ⅰ VECs. However, there is a rational reason for this idea because primary cultured cells have inevitably received multiple kinds of stresses during the process of their preparations including tissue removal, cell dissociation and cell expansion. It is almost impossible for them to preserve every character that they had in *in vivo* environments. Indeed, commercially available primary cultured human VECs preserve fundamental functions of VECs such as cord-forming activities and acetylated low density lipoprotein (Ac-LDL)-up-taking capacities; however, they may have possibly lost certain sophisticated functions before they are on the distribution routes. The type-Ⅱ anti-proliferative capacity may be one of such refined functions.

We have also shown that RGS5 is a causative gene for "type-Ⅱ to type-Ⅰ" conversion. Because EPC2dEC at passage 7 (EPC2dEC[P7]) was larger in size and showed longer doubling time than EPC1dEC passage 7 (EPC1dEC[P7]) (data not shown), EPC2dEC might be in ageing states. This idea was supported by the cluster analysis of the microarray data (GEO Accession ID: GSE61000): EPC2dEC[P7] was located closer to EPC1dEC[P12] than EPC1dEC[P7] (data not shown) and the gene function item "senescence" marked the highest value in the matching rate (data not shown). Thus, the "type-Ⅱ to type-Ⅰ" phenotype conversion may well be considered as an ageing-associated degeneration. Nevertheless, "type-Ⅱ to type-Ⅰ" conversion is not an identical concept to senescence because type-Ⅰ primary cultured human VECs show no signs of senescence. Rather, it should be considered as a sign of degeneration. A clinical study supported this idea, showing that VECs of the subcutaneous vessels of normal individuals are negative for RGS5 expression while those of scleroderma patients are positive for RGS5 expression along with an induction of interferon alpha gene[21,22]. Similar to the case of human VECs, murine VECs are reportedly negative for RGS5 expression[23]. Thus, RGS5 induction in VECs may provide a useful marker for degenerative vessels.

RGS5 plays beneficial roles depending on the kinds of cells. Lack of RGS5 expression in VECs is advantageous as reported in RGS5-deficient mice, which show an advantageous phenotype with normalization of tumor vasculatures[24]. However, up-regulated expressions of RGS5 in VSMCs reportedly bring about favorable outcomes such as angiogenesis promotion[25] and atherosclerosis improvements[26,27]. It seems that opposite effects are exerted by RGS5 between VECs and VSMCs. Thus, we have to be careful enough in performing RGS5-targetted drug discovery to avoid the side effects due to reduced RGS5 expression in VSMCs.

Although "type-Ⅱ to type-Ⅰ" conversion is usually a one-way process, "type-Ⅰ" primary cultured human VECs can be converted into "type-Ⅱ" VECs *via* "iPSC establishment and subsequent VEC differentiation" (Figure 5B). We showed that VECs generated from *freshly* established SeV-iPSCs bare high resistance to stress-induced "type-Ⅱ to type-Ⅰ" conversion (Figure 5E), and thus, they may provide an excellent tool for the transplantation therapy for the treatments of refractory arteriostenosis.

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COMMENTS

Background

Ischemia leads to the development of life-threatening diseases including ischemic heart disease and stroke. It is caused by narrowing of arteries (*i.e.*, arteriostenosis), whose pathological basis is hyperproliferation of vascular smooth muscle cells. Although roles for vascular smooth muscle cells (VSMCs) and macrophages in the development of arteriostenosis are well understood, those for vascular endothelial cells (VECs) remain controversial. Toward the development of new therapeutics, however, involvements of VECs in the progression of arteriostenosis should be elucidated.

Research frontiers

There is a longstanding controversy over the effect of VECs on the proliferation of VSMCs: Clinical observations suggest that VECs prevent the proliferation of VSMCs while *in vitro* co-culture experiments showed that human umbilical cord VECs enhanced the proliferation of bovine VSMCs.

Innovations and breakthroughs

The controversy came from lack of systematic studies, and thus, the authors performed an integrated analysis to quantitatively evaluate the effects various kinds of adult human VECs on the proliferation of adult human VSMCs. The authors have discovered for the first time that human VECs are categorized into two groups by their effects on VSMC proliferation and expression levels of Regulator of G-protein signaling 5 (RGS5): pro-proliferative RGS5^{high} VECs (type-I) and anti-proliferative RGS5^{low} VECs (type-II). Clinical relevance of our finding was supported by the fact that VECs of pathological arteries with tunica media thickening were RGS5^{high} while VECs of normal arteries were RGS5^{low}.

Applications

RGS5 expression in VECs provides a useful indicator for the drug discovery for the treatment of ischemic diseases. Furthermore, human pluripotent stem cell-derived type-II VECs will provide a useful tool for transplantation therapy of arteriostenosis.

Terminology

Regulator of RGS5 is known as an inhibitory molecules against the signaling from G protein-coupled receptor. It is reportedly involved in the regulation of VEC-VEC interaction *via* VE-cadherin and VEC-VSMC interaction *via* N-cadherin.

Peer-review

Authors tried to solve the controversy in human VECs to the proliferation of human vascular smooth muscle cells by characterize the human VECs from various sources in two groups as either pro-proliferative or antiproliferative. The studies presented herein implicate regulator of RGS5 as a modulator of VEC phenotype, where VECs expressing high RGS5 are pro-proliferative and VECs expressing low RGS5 are anti-proliferative. Oxidative stress induces RGS5 expression and shifts VECs into a pro-proliferative phenotype. The studies are novel and the manuscript is well written.

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ORIGINAL ARTICLE

Basic Study

p38 mitogen-activated protein kinase regulates type-I *vs* type-II phenotyping of human vascular endothelial cells

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Author contributions: Nakahara M and Nishio M performed the experiments and analyzed the data; Saeki K constructed viral vectors; Yuo A designed and coordinated the research; Saeki K designed the research, constructed viral vectors and wrote the paper.

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Data sharing statement: Technical appendix and dataset are available from the corresponding author (saeki@ri.ncgm.go.jp).

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Abstract

AIM: To identify kinases involved in phenotype regulation of vascular endothelial cells (VECs): Pro-proliferative G-protein signaling 5 (RGS5)^{high} (type-I) *vs* anti-proliferative RGS5^{low} (type-II) VECs.

METHODS: Proteomic kinase assays were performed to identify the crucial kinase involved in the phenotype regulation of human VECs using type-I VECs, which promotes the proliferation of human vascular smooth muscle cells (VSMCs), and type-II VECs, which suppress the proliferation of human VSMCs. The assays were performed using multiple pairs of type-I and type-II VECs to obtain the least number of candidates. The involvement of the candidate kinases was verified by evaluating the effects of their specific inhibitors on the phenotype regulation of human VECs as well as the expression levels of regulator of RGS5, which is the causative gene for the "type-II to type-I" phenotype conversion of human VECs.

RESULTS: p38α mitogen-activated protein kinase (p38α MAPK) was the only kinase that showed distinctive activities between type-I and type-II VECs: p38α MAPK activities were low and high in type-I and type-II VECs, respectively. We found that an enforced expression of RGS5 indeed lowered p38α MAPK activities

in type-II VECs. Furthermore, treatments with a p38α MAPK inhibitor nullified the anti-proliferative potential in type-II VECs. Interestingly, MAPK inhibitor treatments enhanced the induction of *RGS5* gene. Thus, there is a vicious cycle between "RGS5 induction" and "p38α MAPK inhibition", which can explain the unidirectional process in the stress-induced "type-II to type-I" conversions of human VECs. To understand the upstream signaling of RGS5, which is known as an inhibitory molecule against the G protein-coupled receptor (GPCR)-mediated signaling, we examined the effects of RGS5 overexpression on the signaling events from sphingosine-1-phosphate (S1P) to N-cadherin, because S1P receptors belong to the GPCR family gene and N-cadherin, one of their downstream effectors, is reportedly involved in the regulation of VEC-VSMC interactions. We found that RGS5 specifically bound with $S1P_1$. Moreover, N-cadherin localization at intercellular junctions in type-II VECs was abolished by "RGS5 overexpression" and "p38α MAPK inhibition".

CONCLUSION: p38α MAPK plays crucial roles in "type-I *vs* type-II" phenotype regulations of human VECs at the downstream of RGS5.

Key words: Vascular endothelial cells; Vascular smooth muscle cells; Proteomic kinase assay; p38α mitogen-activated protein kinase; Regulator of G-protein signaling 5; Sphingosine-1-phosphate; N-cadherin

Core tip: We previously reported that human vascular endothelial cells (VECs) are categorized into two types by their effects on the proliferation of vascular smooth muscle cells and the expressions of regulator of G-protein signaling 5 (RGS5): Pro-proliferative $RGS5^{high}$ (type-I) and anti-proliferative $RGS5^{low}$ (type-II) VECs. Performing proteomic kinase assays and inhibitor studies, we show here that p38 mitogen-activated protein kinase (p38 MAPK) is the crucial kinase that determines VEC phenotyping at the downstream of RGS5. Not only RGS5 overexpression suppressed p38 MAPK activities but also p38 MAPK inhibitions up-regulated RGS5 expression, indicating that "RGS5 induction" and "p38 MAPK inhibition" creates a vicious cycle in "type-II to type-I" conversions of human VECs.

Nakahara M, Nishio M, Saeki K, Yuo A, Saeki K. p38 mitogen-activated protein kinase regulates type-I *vs* type-II phenotyping of human vascular endothelial cells. *World J Transl Med* 2015; 4(3): 101-112 Available from: URL: http://www.wjgnet.com/2220-6132/full/v4/i3/101.htm DOI: http://dx.doi.org/10.5528/wjtm.v4.i3.101

INTRODUCTION

We previously reported that human vascular endothelial cells (VECs) are classified into two groups by their effects on the proliferation of vascular smooth muscle cells (VSMCs) in *in vitro* co-culture experiments along with the expression levels of regulator of G-protein signaling 5 (RGS5): Pro-proliferative $RGS5^{high}$ VEC (type-I) *vs* anti-proliferative $RGS5^{low}$ VECs (type-II)[1]. We also demonstrated that commercially available primary cultured human VECs exclusively belong to *pro-proliferative* $RGS5^{high}$ VECs (type-I). On the other hand, human bone marrow-derived endothelial progenitor cells (EPCs) and human embryonic stem cells (ESCs) produced *anti-proliferative* $RGS5^{low}$ VECs (type-II)[1]. Because RGS5 expression was hardly detectable in normal human VECs[1,2] but significantly induced under pathological conditions[1,3,4] and because oxidative stress and subculture-dependent stress induced "type-II to type-I" conversion along with *RGS5* gene induction[1], pro-proliferative $RGS5^{high}$ VECs (type-I) should be considered as degenerative VECs. The reason why widely used commercially available primary cultured human VECs belong to type-I degenerative VECs may be attributed to their histories: They have survived the drastic environmental changes from *in vivo* to *in vitro* by overcoming multiple stresses during the process of their preparations such as tissue removals, cell dissociations and cell expansions. Therefore, their characters may have changed in such a manner that they lose type-II *anti-proliferative* capacities. By contrast, EPC/ESC-derived VECs do not have such stressful histories, and thus, it is possible that they preserve type-II *anti-proliferative* phenotypes. Nevertheless, EPC/ESC-derived type-II $RGS5^{low}$ VECs were inevitably converted into type-I $RGS5^{high}$ VECs after a few rounds of subcultures[1].

In the case of induced pluripotent stem cells (iPSCs), the situations are rather complicated. Regarding VECs produced from retroviral vector-based iPSCs (Ret-iPSCs), type-II *anti-proliferative* capacities were often deteriorated from early phases and subculture-dependent "type-II to type-I" conversions were often accelerated[1]. By contrast, in the case of Sendai virus vector-based iPSC (SeV-iPSCs), "type-II to type-I" conversion was highly repressed: SeV-iPSC-derived VECs (SeV-iPSdECs) showed high resistance to subculture-dependent and oxidative stress-induced "type-II to type-I" conversions[1]. The phenotype differences between Ret-iPSC-derived VECs (Ret-iPSdECs) and SeV-iPSdECs can be explained, at least in part, by the differences in the degree of stresses. Ret-iPSCs have multiple copies of viral vectors in their chromosomes, and thus, they suffer from genome stresses. On the other hand, SeV-iPSCs completely get rid of such genome stresses because the Sendai virus vector is an RNA virus-based vector and do not integrate into the host chromosomes.

In our previous study, the signaling pathway involved in the "type-II to type-I" conversion at the downstream of RGS5 remained elusive. Because RGS5 was the only gene that showed a discriminative expression pattern between type-I and type-II VECs in microarray analyses[1], we thought that an alternative strategy

other than transcriptomic approaches was required to identify the downstream signaling events of RGS5. In the current study, we performed proteomic kinase assays to identify the crucial kinase that regulates the RGS5-mediated "type-Ⅰ *vs* type-Ⅱ" phenotyping of human VECs.

MATERIALS AND METHODS

Cells and reagents

Human umbilical vein endothelial cells (HUVEC), human neonatal dermal microvascular endothelial cells (HMVEC), human adult aortic endothelial cells (HAEC) and human adult coronary arterial endothelial cells (HCAEC) were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). The cells were cultured on 0.1% gelatin-coated plates using EGM®-2 BulletKit (Lonza Group Ltd. Basel, Switzerland). Human aortic smooth muscle cells of different donors were purchased from Lonza Group Ltd. (Basel, Switzerland) and cultured using SmGM™-2 BulletKit™ (Lonza Group Ltd.). Cells were re-seeded at split ratios of 1:3-1:4 twice a week. VECs within 8th passage were used in all experiments. The hESC lines (KhES-1, -3, -5) were established by the Institute for Frontier Medical Science, Kyoto University[2]. SeV-hiPSCs were established from HUVEC[3] and BJ fibroblast[3] by using iPS-Tune™ (ID Pharma Co., Ltd., Ibaraki, Japan). The Ret-hiPSC line of 253G1[4] was provided by CiRA at Kyoto University. Sphingosine-1-phosphate (S1P) (#62570, Cayman Chemical Co., Ann Arbor, MI, United States) was dissolved by 0.3 mol/L NaOH at the concentration of 4 mg/mL as a stock. A p38 MAP Kinase inhibitor (#506126, Calbiochem Co., La Jolla, CA, United States) was dissolved by DMSO at the concentration of 10 mmol/L as a stock. A JNK inhibitor SP600125 (#BML-EI305, Enzo Life Sciences, Inc., Farmingdale, NY, United States) was dissolved by DMSO at the concentration of 10 mmol/L as a stock. An extracellular signal-regulated kinase (ERK) inhibitor PD98059 (#513001, Calbiochem Co.,) was dissolved by DMSO at the concentration of 50 mmol/L stock. FTY720 Phosphate (#10008639, Cayman Chemical Co.,) was dissolved by chloroform at the concentration of 0.5 mg/mL as a stock. All reagents were kept at -20 ℃.

Quantitative evaluations of VSMC proliferation

VECs were g-irradiated (5 Gy) and stained with carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) by using CFSE Cell Division Assay Kit (Cayman Chemical Co., Ann Arbor, MI), while VSMCs were stained with PKH26 by using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich Co. LLC., St. Louis, MO, United States) according to the manufacturer's guidance. For contact co-culture, irradiated and CFSE-stained VECs were seeded at the density of 2×10^5 cells/well on 0.1% gelatin-coated 24-well culture plates, and on the following day, PKH26-stained VSMCs were seeded at the density of 3.75×10^3 cells/well on VEC layers or gelatin layers as control. After 4 d, total cells were harvested and subjected to flow cytometry analyses by FACSCalibur™ (BD Biosciences, San Jose, CA, United States) and FL1 and FL2 fluorescence intensities were measured by CellQuest™ Pro software (BD Biosciences). FL2 (PKH26) fluorescence intensities were further analyzed mathematically by ModFit LT™ software (Verity Software House Inc., Topsham, ME, United States) to calculate the proliferation index.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from VECs using TRIzol® Reagent (Life Technologies, Inc.). Complementary DNA was prepared from 1 μg of RNA using SuperScript™ Ⅲ First-Strand Synthesis System kit (Life Technologies, Inc.), and used in quantitative polymerase chain reaction (PCR) reactions with FAST SYBR® Green Master Mix (Applied Biosystems® from Life Technologies, Inc.). Quantitative real-time PCR was performed using the StepOnePlus™ PCR machine (Applied Biosystems® from Life Technologies, Inc.). Primers used for RGS5 were Forward: 5'-GGAGGCTCCTAAAGAGGTGA-3' and Reverse: 5'-GGGAAGGTTCCACCAGGTTC-3', and primers used for *GAPDH* were Forward: 5'-CCACTCCTCCACCTTTGAC-3' and Reverse: 5'-ACCCTGTTGCTGTAGCCA-3'.

Immunostaining of N-cadherin

Human ESC/iPSC-derived cells were fixed by 4% paraformaldehyde for 15 min at room temperature. The 1st antibody reaction was performed by using a 1:100-diluted rabbit polyclonal anti-human N-cadherin antibody (ab12221, Abcam plc, Cambridge, MA, United States). The 2nd antibody reaction was performed by using an Alexa Fluor® 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies, Inc). Photomicrographs were taken by Olympus BX51 Fluorescence Phase contrast Microscope (Olympus Optical Co. Ltd.) equipped with DP-2 TWAIN digital camera system (Olympus Optical Co. Ltd.) and cellSens® standard imaging software (Olympus Optical Co. Ltd.).

Western blotting

The 1×10^5 VECs were lysed by using 20 μL sample buffer solution (2ME+) (× 2) (Cat. 196-11022) (WAKO Pure Chemical Industries, Osaka, Japan). The first antibody reaction was performed by using a 1:1000-diluted anti-human RGS5 antibody (ab83230, Abcam, Cambridge, MA, United States), a rabbit polyclonal anti-p38α antibody (C-20) (sc-535, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States), a rabbit polyclonal anti-p38 (phospho T180) antibody (ab51050, Abcam plc.), a rabbit monoclonal anti-c-Jun (60A8) (#9165, Cell Signaling Technology, Inc., Beverly, MA, United States), a rabbit monoclonal anti-Phospho-c-Jun (Ser73) (D47G9) (#3270, Cell Signaling Technology, Inc.), a rabbit monoclonal anti-Phospho-c-Jun (Ser63) (54B3) (#2361, Cell Signaling Technology, Inc.), a rabbit polyclonal anti-Phospho-c-Jun (Ser243) Antibody (#2994, Cell Signaling Technology, Inc.), a rabbit polyclonal anti-c-

Jun (phospho T93) antibody (ab28854, Abcam plc.) or a 1:1000-diluted anti-human β-tubulin antibody (sc-9104, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) and the second antibody reaction was performed by using a 1:2000-diluted anti-rabbit IgG HRP-linked antibody (#7074S) (Cell Signaling Technology, Inc.).

Immunoprecipitation

The 3 × 10^6 HUVECs were lysed by 500 μL of RIPA buffer. Immunoprecipitation was performed using a mouse monoclonal anti-human RGS5 antibody (sc-390245, Santa Cruz Biotechnology Inc.) or a goat polyclonal anti-human S1P1 antibody (sc-16070、Santa Cruz Biotechnology Inc.), anti-human S1P2 antibody (sc-31577, Santa Cruz Biotechnology Inc.), anti-human S1P3 antibody (sc-16076, Santa Cruz Biotechnology Inc.). For control, normal mouse IgG (sc-2025, Santa Cruz Biotechnology Inc.) or normal goat IgG (sc-2028, Santa Cruz Biotechnology Inc.). Precipitated samples were subjected to western blotting using a goat polyclonal anti-human S1P1 antibody (sc-16070, Santa Cruz Biotechnology Inc.) and a rabbit polyclonal anti-human RGS5 antibody (ab-83230, Abcam plc.).

Proteomic kinase assays

Proteome Profiler Antibody Arrays were performed using Human Phospho-Kinase Antibody Kit (#ARY003B, R&D Systems Inc., Minneapolis, MN, United States) according manufacturer's guidance. In brief, 5 × 10^6 cells were lysed by using 500 μL lysis buffer and, after centrifugation, 334 μL of the lysate was diluted by 2 mL blocking buffer and used for the assay. The intensity of each spot was measured by using a free Image J software and its percentage to that of reference spot was plotted in a bar chat.

Gene transfer

A Homo sapiens cDNA, FLJ96402, which corresponds to *Homo sapiens* RGS5, transcript variant 1, mRNA (NM_003617.3), with two nucleotide substitutions, was purchased from National Institute of Technology and Evaluation (Tokyo, Japan), and the two substituted nucleotides were corrected by using KOD-Plus-Mutagenesis Kit (Toyobo Co. Ltd., Osaka, Japan) to become identical to the nucleotide sequences in NM_003617.3. The cDNA was inserted into pmax-Cloning™ expression vector (Lonza Group Ltd. Basel, Switzerland). The 2 × 10^5 human ESC-derived VECs (ESdECs) were transfected with 3 μg vectors using a Nucleofector™ (Lonza Group Ltd.) as reported elsewhere[1] and subjected to Western blotting. Alternatively, RGS5 cDNA was inserted into the simian immunodeficiency virus (SIV) vector (ID Pharma Co., Ltd., Ibaraki, Japan) in either forward (SIV-RGS5) or reverse (SIV-control) direction. The 2 × 10^5 ESdECs were infected with SIV vectors at MOI = 80. After 6 d, during which the cells were subcultured twice, immunostaining studies were performed.

Statistical analysis

Experiments were performed independent three or four experiments (n = 3 or 4) and the data were analyzed according Student t-test. Results were shown as averages ± standard deviations (AV ± SD).

RESULTS

Proteomic kinase assays using multiple pairs of type-Ⅰ and type-Ⅱ VECs

During our trials to identify the downstream signaling target of RGS5, we found by coincidence that there was a clear difference in the phosphorylation state of c-JUN protein between type-Ⅰ and type-Ⅱ VECs. c-Jun protein was hyper-phosphorylation at multiples sites such as Ser63, Ser73, Ser243, Thr93 and Thr170 in type-Ⅱ human VECs including ES(KhES-5)-derived VECs [ESdEC(KhES-5)] at early passages (Figure 1, blue rectangles). By contrast, c-Jun protein was hypo-phosphorylated in type-Ⅰ VECs including ESdEC (KhES-5) at late passages and commercially available primary cultured human VECs (Figure 1, red rectangles). Therefore, we hypothesized that certain kinases would be working at the downstream of RGS5 for the "type-Ⅰ *vs* type-Ⅱ" phenotyping of human VECs. It is known that c-Jun protein is phosphorylated by multiple kinases including c-Jun N-terminal kinase (JNK), p38α MAPK, ERK, glycogen synthase kinase 3 (GSK) and casein kinase 2. To identify the pivotal kinase involved in the determination of VEC phenotypes, a systemic analysis to quantitatively evaluate the activation states of various kinases using multiple pairs of type-Ⅰ and type-Ⅱ VECs is of great use. For this aim, we applied a commercially available proteomic kinase assay, where cell lysates were reacted with an array of site-specific phosphorylation antibodies of 43 kinases on a nitrocellulose membrane to simultaneously detect the activation state of each kinase *via* an ordinary immunoblotting procedure.

First, we compared the profiles of protein kinase activities between ESdEC(KhES-5) at passage 2 [ESdEC(KhES-5)[P2]], which showed type-Ⅱ phenotype[1], and those at passages 10 [ESdEC(KhES-5)[P10]], which showed type-Ⅰ phenotype[1]. As shown in Figure 2A, protein kinase activities were generally higher in ESdEC(KhES-5)[P2] (type-Ⅱ) than type-Ⅰ ESdEC(KhES-5)[P10] (type-Ⅰ) in accordance with the result in Figure 1. To narrow the list of candidate kinases involved in type-Ⅰ/type-Ⅱ phenotyping, we performed proteomic kinase assays using VECs generated from KhES-3 line of ESC at passage 2 [ESdEC(KhES-3)[P2]], which showed type-Ⅱ phenotype[1], and those at passages 8 [ESdEC(KhES-3)[P8]], which showed type-Ⅰ phenotype[1]. ESdEC(KhES-3) provided similar results to ESdEC(KhES-5) regarding several kinases including p38α MAPK, GSK3, AMPK and AKT (Figure 2B, Panel A) and kinase that phosphorylated p53 and HSP60 (Figure 2B, Panel B). To further narrow the list of candidate kinases, we performed proteomic kinase assays

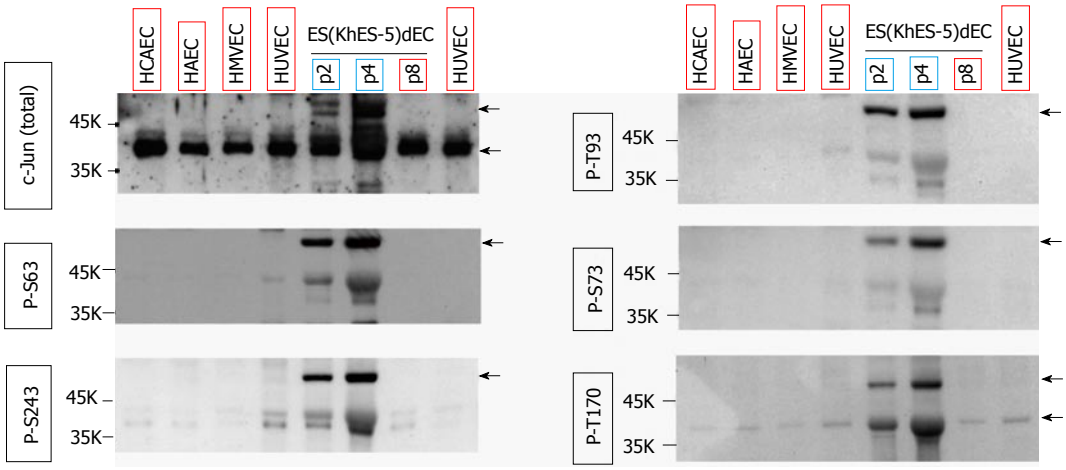


Figure 1 Western blotting assays. HCAEC, HAEC, HMVEC, HUVEC and ESdECs were subjected to western blotting using indicated antibodies. Arrows indicate the specific bands recognized by the antibodies. HCAEC: Human adult coronary arterial endothelial cells; HAEC: Human adult aortic endothelial cells; HMVEC: Human neonatal dermal microvascular endothelial cells; HUVEC: Human umbilical vein endothelial cells; ESdECs: Embryonic stem cells-derived vascular endothelial cells.

using VECs generated from KhES-1 line of ESC at passage 2 [ESdEC(KhES-1)[P2]], which showed type-Ⅱ phenotype[1], and those at passages 9 [ESdEC(KhES-1)[P9]], which showed type-Ⅰ phenotype[1]. Regarding several cases including p38α MAPK, GSK3, AMPK and AKT, consistent results were obtained among the three lines of ESdECs (Figure 2C, Panel A). We still performed proteomic kinase assays using SeV-iPS-derived VECs (SeV-iPS(HUVEC)dEC), which showed type-Ⅱ phenotype[1], and HUVEC, which showed type-Ⅰ phenotype[1]. As shown in Figure 2D, only p38α MAPK provided consistent results: Higher p38α MAPK activities in type-Ⅱ VECs and lower p38α MAPK activities in type-Ⅰ VECs. To confirm this finding, we further performed proteomic kinase assays using Ret-iPS-derived VECs [Ret-iPS(253G1)dEC] with type-Ⅰ phenotype[1] and SeV-iPS(HUVEC)dEC with type-Ⅱ phenotype[1]. Again, p38α MAPK activities were higher in type-Ⅱ VECs than type-Ⅰ (Figure 2E). We finally performed proteomic kinase assays using additional commercially available primary cultured human VECs including HAEC, HCAEC and HMVEC, all of which showed type-Ⅰ phenotype[1], and obtained compatible results (data not shown).

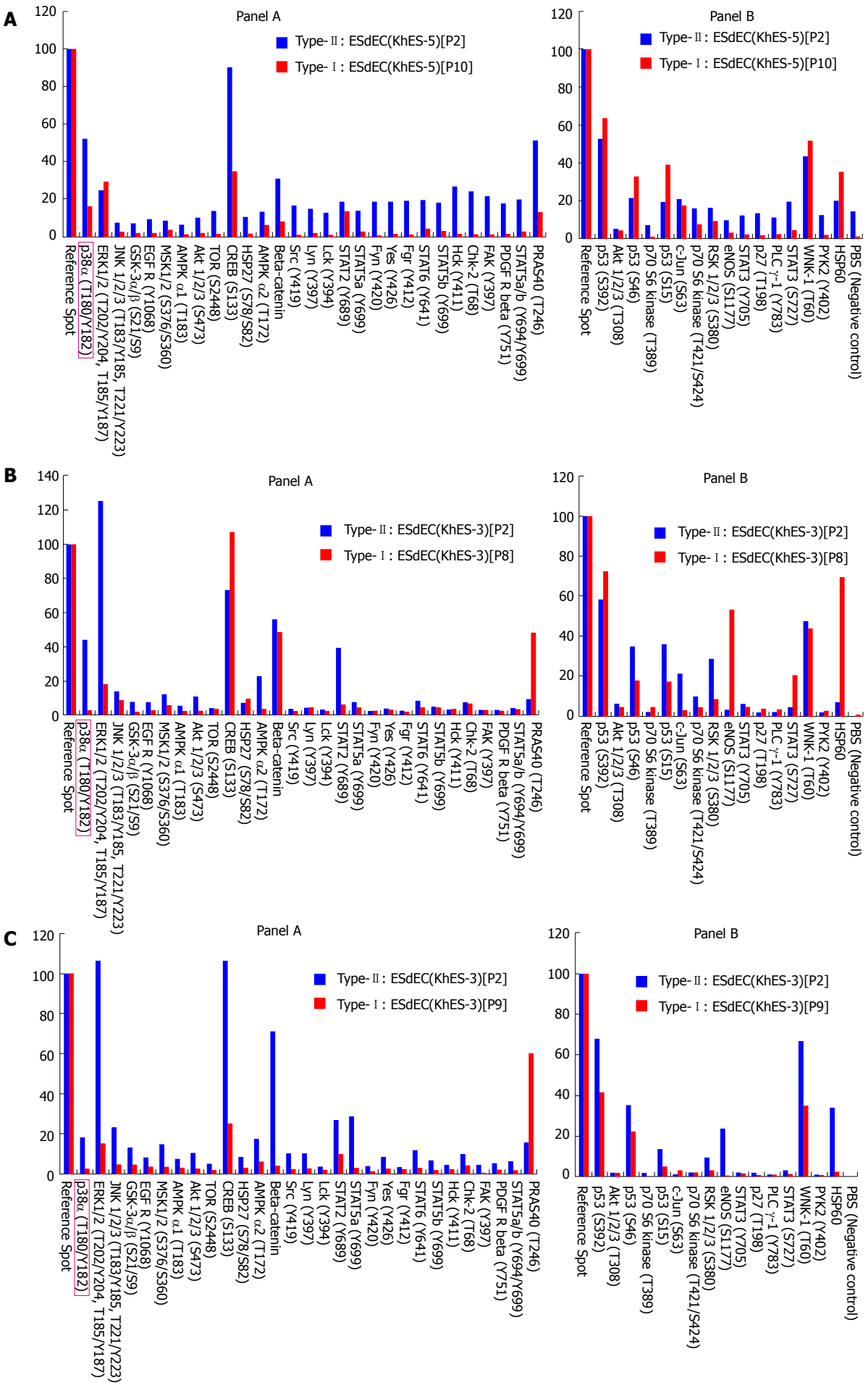
Thus, we obtained p38α MAPK as the strongest candidate for the kinase that is involved in the "type-Ⅰ *vs* type-Ⅱ" phenotyping of human VECs.

Verification of the involvement of p38 MAPK in phenotype regulation of human VECs

To verify the involvement of p38α MAPK in RGS5-mediated phenotype regulation of human VECs, we first examined whether an enforced expression of RGS5 would affect p38α MAPK activities. As shown in Figure 3A, transfection of the RGS5-expression vector into type-Ⅱ ESdECs lowered Thr180 phosphorylation of p38α MAPK, and thus, depressing the activity of p38α MAPK. Next, we examined the effects of the p38α MAPK inhibitor on the phenotype regulation of VECs. Treatment with a p38α MAPK inhibitor (Figure 3B, gray column), but not with a JNK inhibitor (Figure 3B, hatched column) or an ERK inhibitor (data not shown), abrogated type-Ⅱ *anti-proliferative* capacities of ESdECs (Figure 3B), confirming the involvement of p38α MAPK in RGS5-mediated "type-Ⅱ *vs* type-Ⅰ" phenotyping. Interestingly, we found that p38α MAPK inhibitor treatments up-regulated RGS5 expression (Figure 3C). Thus, there is a positive feedback loop between "RGS5 induction" and "p38α MAPK down-regulation", running a vicious cycle for the unidirectional conversion from type-Ⅱ VECs to type-Ⅰ VECs.

RGS5 disturbed sphingosine-1-phosphate-dependent signaling events

It is known that RGS family proteins function as inhibitory molecules against G protein-coupled receptor (GPCR)-dependent signaling. It is also known that sphingosine-1-phosphate (S1P), whose receptors belong to the GPCR family, activates various kinases including p38α MAPK, ERK and JNK and plays crucial roles in the regulation of VEC-VSMC interactions *via* N-cadherin[5]. Therefore, we examined the effects of RGS5 expression on the signaling from S1P to N-cadherin. First, we examined the possible interactions between RGS5 and S1P receptors. As shown Figure 4, RGS5 co-precipitated with S1P receptor 1 ($S1P_1$) (Figure 4A), but not with $S1P_2$ or $S1P_3$ (Figure 4B), in type-Ⅰ $RGS5^{high}$ VECs. In addition, treatments with FTY720-P, an inhibitor of S1P-dependent signaling, induced "type-Ⅱ to type-Ⅰ" conversions (Figure 4C). Thus, RGS5 induced "type-Ⅱ to type-Ⅰ" conversion, at least in part, by interfering the S1P/$S1P_1$-dependent signaling. To further confirm the disturbance of S1P-dependent signaling by RGS5, we examined the effects of RGS5 on the subcellular localization of N-cadherin because $S1P_1$ receptor activation in VECs is reportedly required for N-cadherin-dependent VEC-VSMC adhesion[5]. We found that the localization of N-cadherin at intercellular junctions was abrogated by an enforced expression of


A
Panel A
Type-Ⅱ: ESdEC(KhES-5)[P2]
Type-Ⅰ: ESdEC(KhES-5)[P10]
Panel B
Type-Ⅱ: ESdEC(KhES-5)[P2]
Type-Ⅰ: ESdEC(KhES-5)[P10]
B
Panel A
Type-Ⅱ: ESdEC(KhES-3)[P2]
Type-Ⅰ: ESdEC(KhES-3)[P8]
Panel B
Type-Ⅱ: ESdEC(KhES-3)[P2]
Type-Ⅰ: ESdEC(KhES-3)[P8]
C
Panel A
Type-Ⅱ: ESdEC(KhES-3)[P2]
Type-Ⅰ: ESdEC(KhES-3)[P9]
Panel B
Type-Ⅱ: ESdEC(KhES-3)[P2]
Type-Ⅰ: ESdEC(KhES-3)[P9]
Reference Spot
p38α (T180/Y182)
ERK1/2 (T202/Y204, T185/Y187)
JNK 1/2/3 (T183/Y185, T221/Y223)
GSK-3α/β (S21/S9)
EGF R (Y1068)
MSK1/2 (S376/S360)
AMPK α1 (T183)
Akt 1/2/3 (S473)
TOR (S2448)
CREB (S133)
HSP27 (S78/S82)
AMPK α2 (T172)
Beta-catenin
Src (Y419)
Lyn (Y397)
Lck (Y394)
STAT2 (Y689)
STAT5a (Y699)
Fyn (Y420)
Yes (Y426)
Fgr (Y412)
STAT6 (Y641)
STAT5b (Y699)
Hck (Y411)
Chk-2 (T68)
FAK (Y397)
PDGF R beta (Y751)
STAT5a/b (Y694/Y699)
PRAS40 (T246)
p53 (S392)
Akt 1/2/3 (T308)
p53 (S46)
p70 S6 kinase (T389)
p53 (S15)
c-Jun (S63)
p70 S6 kinase (T421/S424)
RSK 1/2/3 (S380)
eNOS (S1177)
STAT3 (Y705)
p27 (T198)
PLC γ-1 (Y783)
STAT3 (S727)
WNK-1 (T60)
PYK2 (Y402)
HSP60
PBS (Negative control)

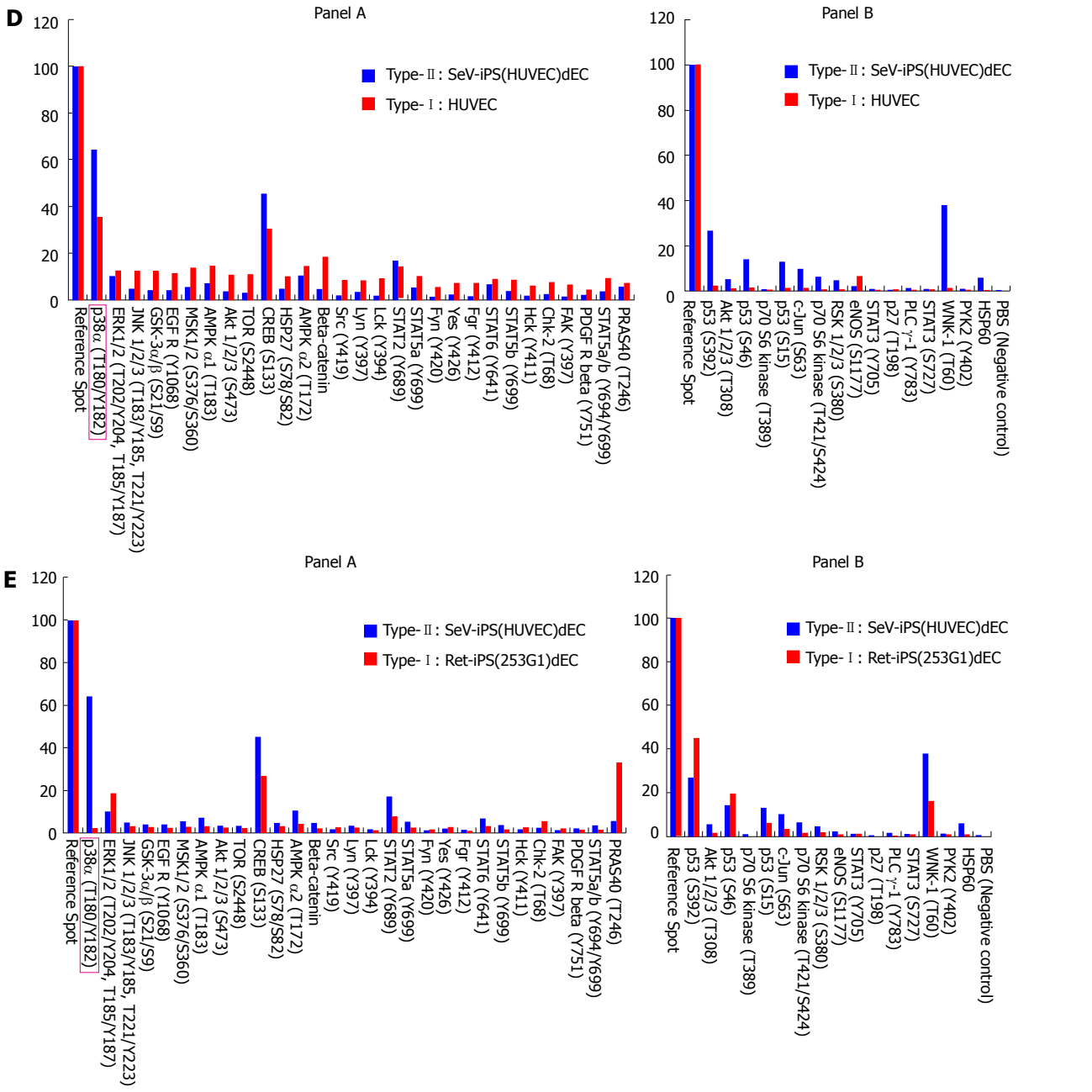


Figure 2 Proteomic kinase assays. Lysates of various pairs of type-Ⅰ and type-Ⅱ vascular endothelial cells were prepared as indicated and used for proteomic kinase assays. ESdECs: Embryonic stem cells-derived vascular endothelial cells; iPS: Induced pluripotent stem cells.

RGS5 in type-Ⅱ VECs (Figure 5A). We also examined the intracellular localization of N-cadherin in various human VECs. In accordance with a previous report[6], N-cadherin localization at intercellular junctions was hardly detectable in type-Ⅰ commercially available primary cultured human VECs (Figure 5B). In addition, repetitive subcultures abrogated the localization of N-cadherin at intercellular junctions in type-Ⅱ ESdECs (Figure 5C). We also examined the effects of an inhibitor of p38α MAPK, which is the pivotal downstream kinase of RGS5 in the phenotype regulation of human VECs (Figures 2 and 3) and found that p38α MAPK inhibition abrogated the localization of N-cadherin at intercellular junctions (Figure 5D). Finally, we examined N-cadherin expressions in clinical specimens of the patients with hypertension, arteriosclerosis and systemic lupus erythematodes (SLE)-associated vasculitis. Our previously report showed that RGS5 expressions in VECs were up-regulated in pathological situations in a severity-dependent manner[1] in accordance with an earlier report on the study of scleroderma patients, which showed endothelial RGS5 overexpression in subcutaneous vessels[3,4]. Compatible with those previous findings, N-cadherin expressions were abrogated in pathological arteries (Figure 5E). Thus, RGS5 overexpression disturbs S1P/S1P$_1$–dependent signaling events in human VECs.

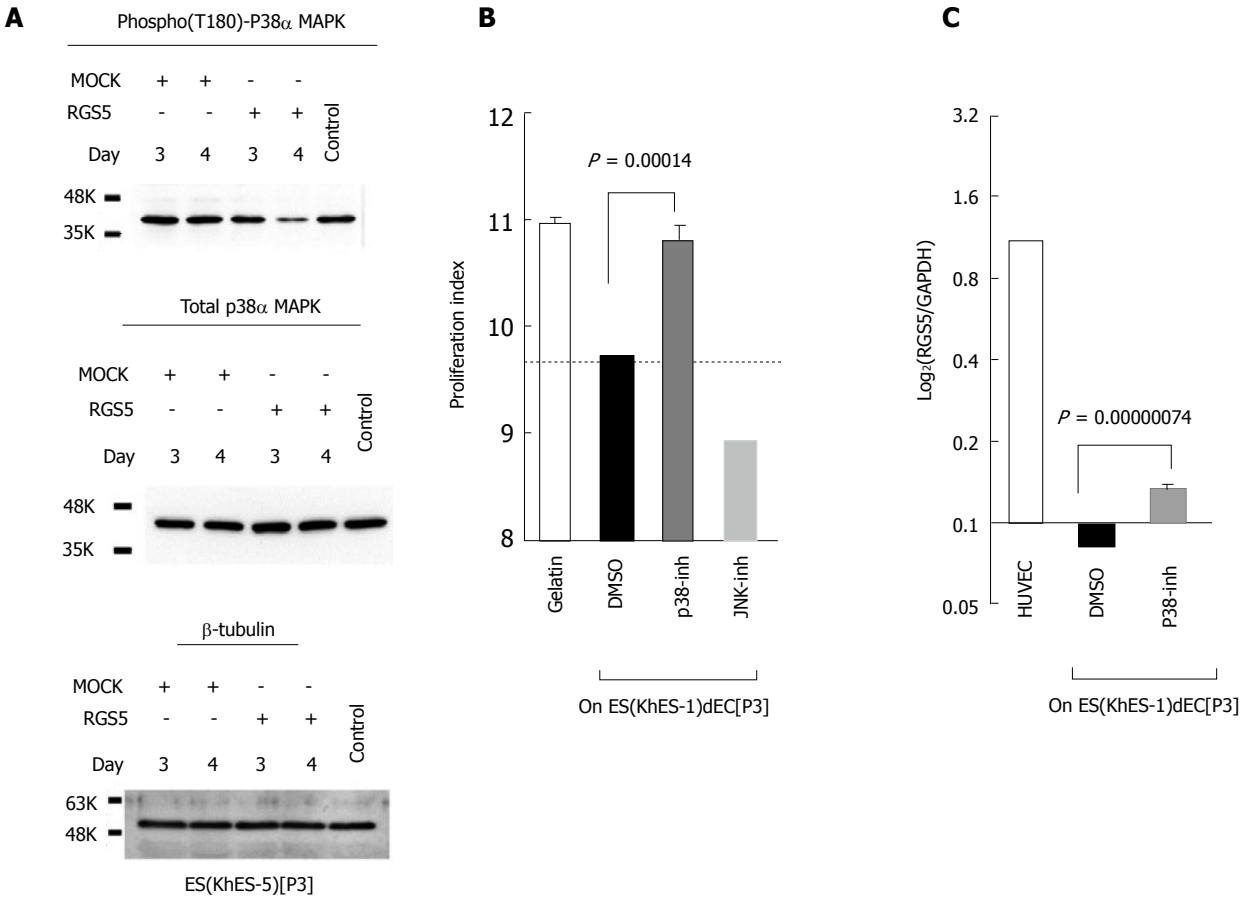


Figure 3 Involvement of p38 mitogen-activated protein kinase in the phenotype regulation of human vascular endothelial cells. A: Type-Ⅱ ESdECs were transfected with either an RGS5 expression vector or an empty vector by nucleofection and subjected to Western blotting using indicated antibodies after 3 or 4 d; B: Type-Ⅱ ESdECs were treated with either 0.1% DMSO, a p38 MAPK inhibitor (10 μmol/L) or a JNK inhibitor (10 μmol/L) for three days and subjected to co-cultures with human aortic smooth muscle cells (n = 3, AV ± SD); C: Type-II ESdECs were treated by a p38 MAPK inhibitor (10 μmol/L). Next day, RGS5 expressions were examined by qRT-PCR (n = 4, AV ± SD). RGS5: Regulator of G-protein signaling 5; ESdECs: Embryonic stem cells-derived vascular endothelial cells; DMSO: Dimethylsulfoxide; JNK: Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; qRT-PCR: Quantitative real-time polymerase chain reaction; HUVEC: Human umbilical vein endothelial cells.

DISCUSSION

In the current study, we identified p38α MAPK as a pivotal kinase that is involved in phenotype regulations of human VECs at the downstream of RGS5. Therefore, human VECs are finally categorized as follows: Pro-proliferative RGS5^{high} p38α MAPK^{low} VECs (type-Ⅰ) and *anti-proliferative* RGS5^{low} p38α MAPK^{high} VECs (type-Ⅱ) (Figure 6).

It is widely accepted that p38α MAPK plays important roles in the acquisition of stress resistance. It was shown that p38α MAPK mediated cell survival in response to oxidative stress by inducing antioxidant genes[7]. The involvement of p38α MAPK in the acquisition of oncogenic stress resistance was also reported[8,9]. These findings support the idea that the activity of p38α MAPK should be maintained at relatively high levels to prevent the "type-Ⅱ to type-Ⅰ" conversions by various kinds of stresses including oxidative stress and aging. It was also reported that macrophage deficiency of p38α MAPK promoted apoptosis and plaque necrosis in advanced atherosclerotic lesions[8]. Thus, p38α MAPK plays indispensable roles in the amelioration of ischemic vascular diseases in both VECs and macrophages.

We also showed that there exists a vicious cycle between "RGS5 inductions" and "p38α MAPK inhibitions". Because this cycle induces an intensifying expression of RGS5 over time, "type-Ⅱ to type-Ⅰ" conversion, which is induced by oxidative stress or aging[1], is generally an irreversible process. Our previous *in vitro* studies demonstrated that RGS5 expression in type-Ⅰ VECs cannot be nullified unless they are subjected to iPSC establishment and subsequent VEC differentiation[1]. On the other hand, the cancellation of RGS5 expression can be observed under *in vivo* conditions. It was reported that, although RGS5 expression was induced in the endothelial cells of subcutaneous vessels in scleroderma patients, it returned to normal after high dose immunosuppressive therapy followed by autologous hematopoietic cell transplant[10,11]. Thus, it seems that there are a sufficient amount of endothelial progenitor cells (EPCs) that can produce healthy RGS5^{low} VECs (type-Ⅱ) in the bone marrow of scleroderma patients and these EPCs contribute to the regeneration of healthy vessels after an intensive therapy. In this sense, not only autologous iPSC-derived VECs but also autologous

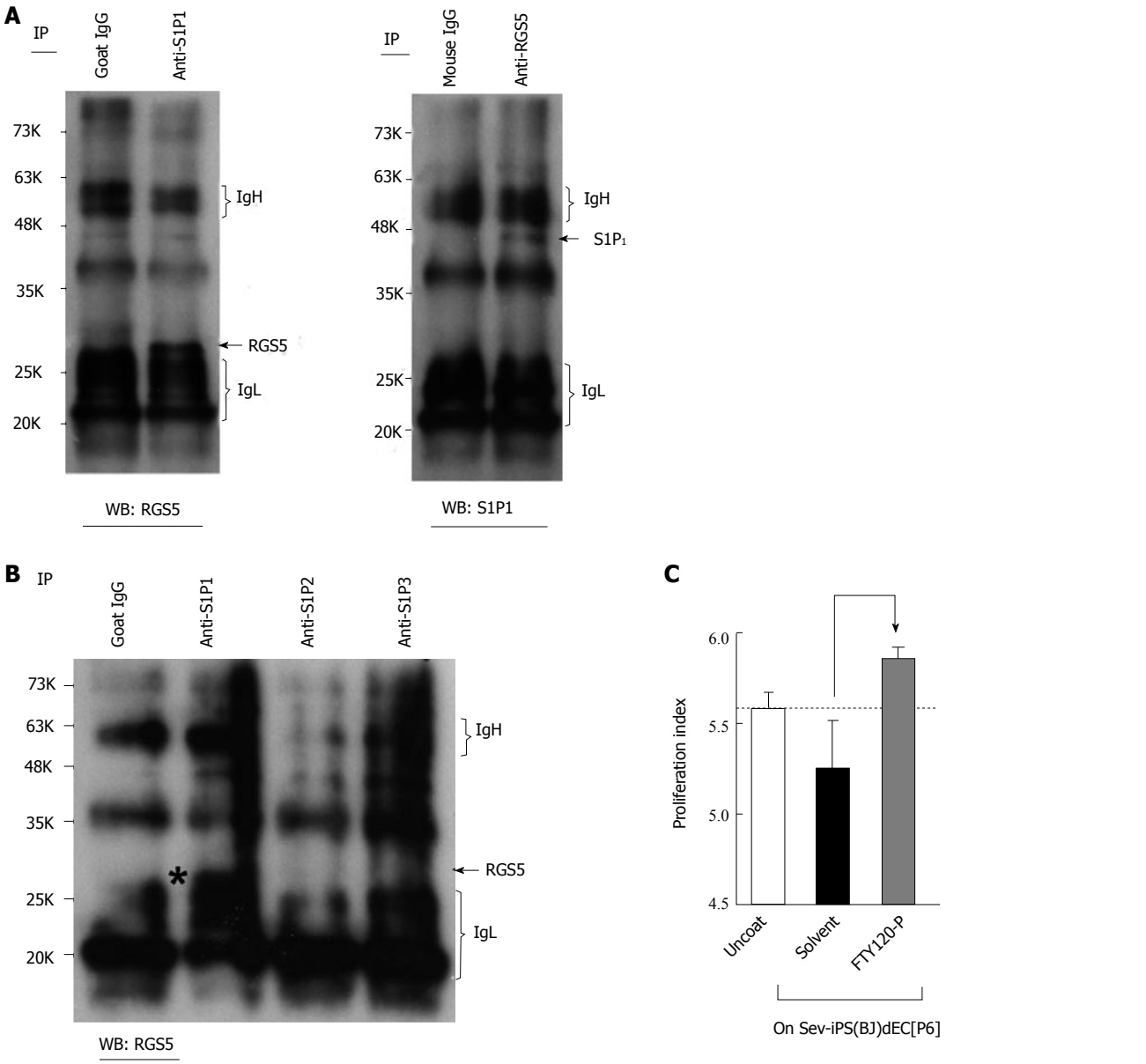
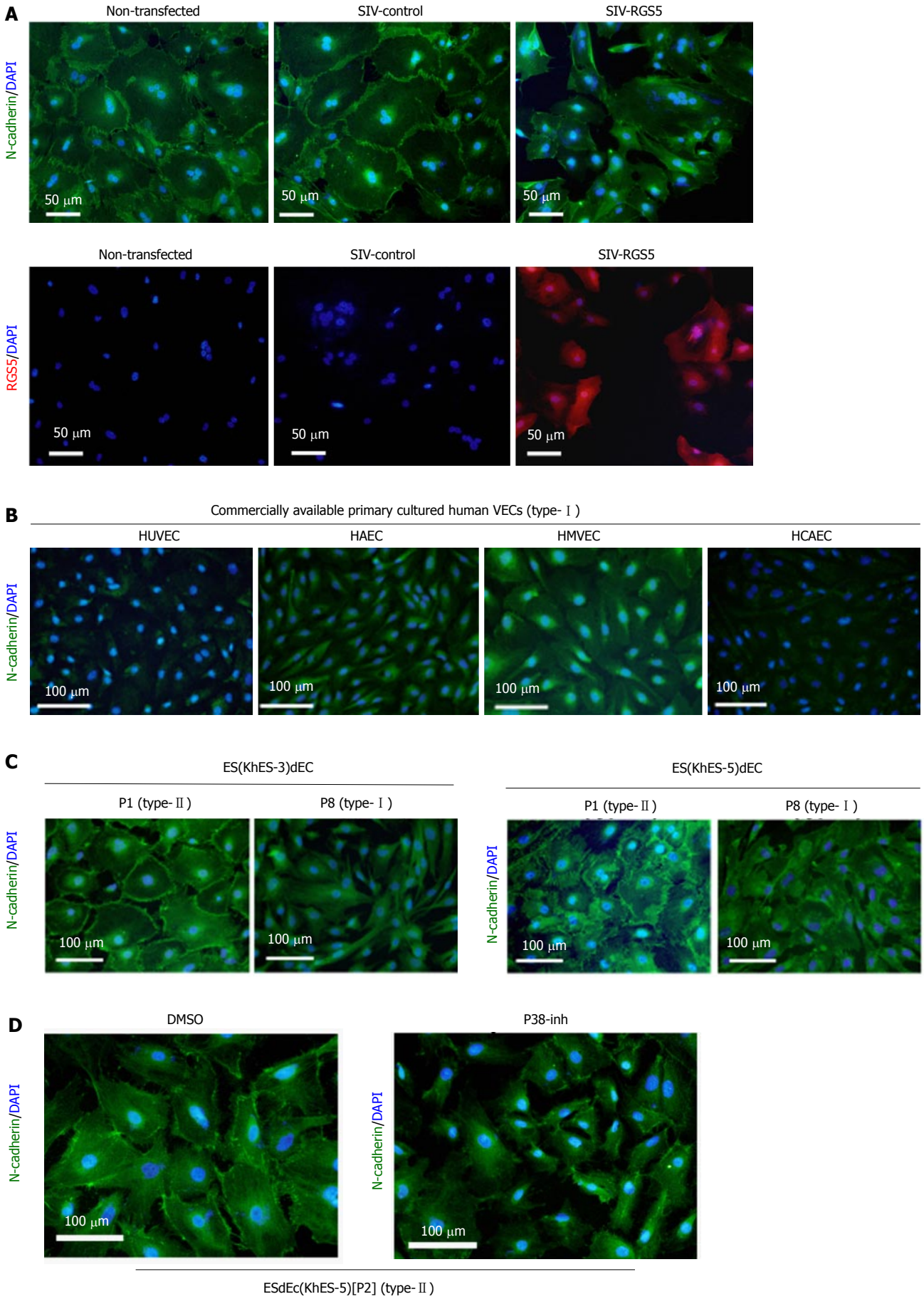


Figure 4 Interaction between sphingosine-1-phosphate receptors and regulator of G-protein signaling 5 in type-I vascular endothelial cells. A and B: Immunoprecipitation (IP) followed by Western blotting (WB) was performed using HUVEC lysates and indicated antibodies; C: Human aortic smooth muscle cells were subjected contact co-culture experiments with type-II VECs with or without 1 nmol/L FTY720-P (n = 3, AV ± SD). HUVEC: Human umbilical vein endothelial cells; VECs: Vascular endothelial cells; SeV-iPS: Sendai virus vector-based induced pluripotent stem cells; RGS5: Regulator of G-protein signaling 5.

hematopoietic stem/progenitor cells may provide a powerful tool for the transplantation therapy for the treatment of vascular diseases.

We also demonstrated that RGS5 disturbed S1P-dependent signaling events. It was reported that the $S1P_1$ activation in VECs was required for N-cadherin-dependent adhesion with mural cells and that knockdown of N-cadherin expression resulted in destabilization of vascular structures[5]. Our immunostaining studies showed that the localization of N-cadherin at intercellular junctions was abrogated in pathological arteries (Figure 5E). Thus, loss of N-cadherin localization at intercellular junctions may possibly promote the arteriostenosis *via* the destabilization of VEC-VSMC adhesions. Our results (Figure 5B), in accordance with a previous report[6] showed that commercially available primary cultured human VECs lacked the localization of N-cadherin at intercellular junctions. Although the molecular basis for the lack of N-cadherin localization at intercellular junctions has not been elucidated so far, an inappropriately enhanced expression of RGS5 in type-I VECs may possibly be involved in the progression of this process, at least in part.

There may be diverse signals that induce RGS5 expressions. In addition to our previous finding that oxidative stress and subculture stress induced RGS5 expression[1], Jin *et al*[12] reported that RGS5 worked as a hypoxia-inducible apoptotic stimulator in HUVEC. Therefore, it seems highly reasonable to think that RGS5 is a common downstream effector of various stressors in human VECs. Although the direct downstream signaling event remains elusive, RGS5 may provide a


A
Non-transfected
SIV-control
SIV-RGS5
N-cadherin/DAPI
RGS5/DAPI
50 μm
B
Commercially available primary cultured human VECs (type-Ⅰ)
HUVEC
HAEC
HMVEC
HCAEC
N-cadherin/DAPI
100 μm
C
ES(KhES-3)dEC
ES(KhES-5)dEC
P1 (type-Ⅱ)
P8 (type-Ⅰ)
N-cadherin/DAPI
100 μm
D
DMSO
P38-inh
N-cadherin/DAPI
100 μm
ESdEc(KhES-5)[P2] (type-Ⅱ)

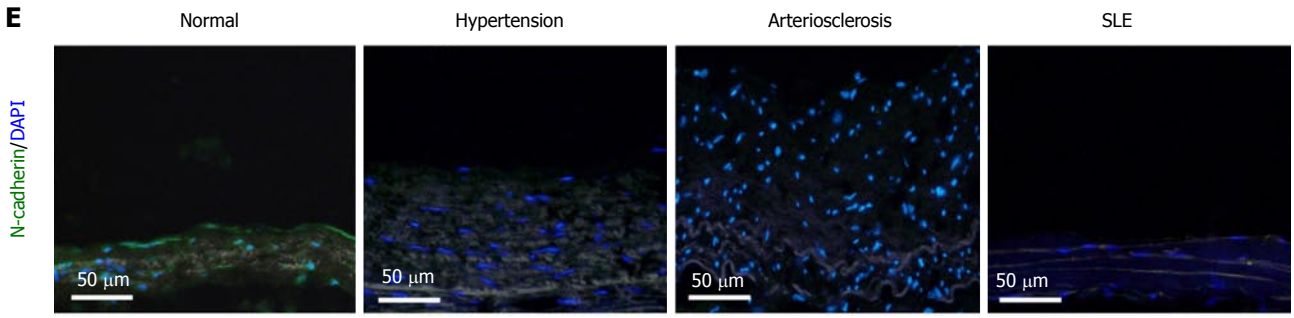


Figure 5 Effects of regulator of G-protein signaling 5 on the intracellular localization of N-cadherin. A: Type-II VECs were infected with SIV vectors carrying either human RGS5 cDNA (SIV-RGS5) or inverted human RGS5 cDNA (SIV-control) and subjected to immunostaining using an anti-N-cadherin antibody (green) or anti-RGS5 antibody (red); B-E: Immunostaining studies using an anti-N-cadherin antibody (green) with nuclear counterstaining with DAPI (blue) were performed using commercially available primary cultured human VECs (type-I) (B), ESdECs at early (type-II) and late (type-I) passages (C), ESdECs at early passages (type-II) treated with 0.1% DMSO or a p38 inhibitor (10 μmol/L) (D) and clinical specimens (E). HCAEC: Human adult coronary arterial endothelial cells; HAEC: Human adult aortic endothelial cells; HMVEC: Human neonatal dermal microvascular endothelial cells; HUVEC: Human umbilical vein endothelial cells; ESdECs: Embryonic stem cells-derived vascular endothelial cells; RGS5: Regulator of G-protein signaling 5; DMSO: Dimethylsulfoxide; VECs: Vascular endothelial cells.

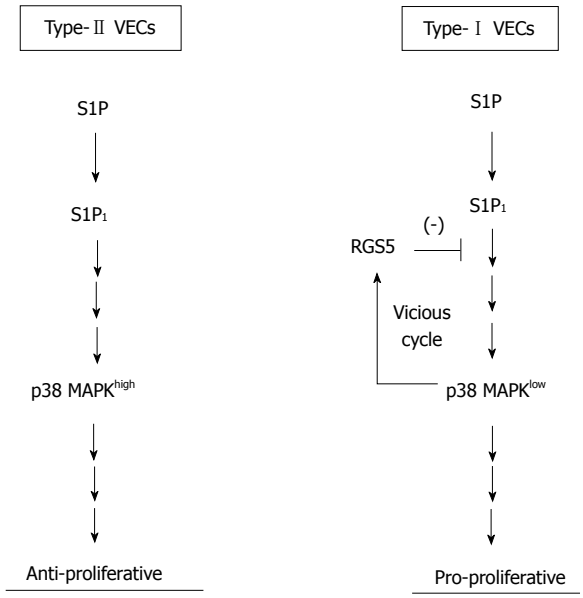


Figure 6 A model for roles of p38 mitogen-activated protein kinase in the regulation of vascular endothelial cells phenotypes. MAPK: Mmitogen-activated protein kinase; RGS5: Regulator of G-protein signaling 5; S1P: Sphingosine-1-phosphate; VECs: Vascular endothelial cells.

good candidate for drug discovery in various vascular diseases.

Collectively, the preservation of p38α MAPK activity at higher levels in VECs provides a new strategy in the drug discovery for the treatment of ischemic diseases.

ACKNOWLEDGMENTS

We thank Mr. Shinnosuke Suzuki and Mr. Yoshinori Yanagi for technical assistance.

COMMENTS

Background

The pathological basis of ischemia is hyper-proliferation of vascular smooth muscle cells (VSMCs). Although there had been a longstanding controversy over the effect of vascular endothelial cells (VECs) on the proliferation of VSMCs, it was recently clarified that human VECs are categorized into two groups by their effects on the proliferation of VSMCs: Pro-proliferative VECs (type-I) *vs* anti-proliferative VECs (type-II). Various stresses such as oxidative stress and aging induce "type-II to type-I" conversion of human VECs.

Research frontiers

Regulator of G-protein signaling 5 (RGS5), which is reportedly induced in endothelial cells of pathological vessels, is identified as a causative gene for "type-II to type-I" conversion.

Innovations and breakthroughs

Signaling events that are working up-stream and down-stream of RGS5 in "type-II to type-I" conversion remain elusive. By applying proteomic kinase assays, we have clarified that RGS5-mediated p38α mitogen-activated protein kinase (p38α MAPK) suppression is the crucial downstream signaling event for "type-II to type-I" conversion.

Applications

p38α MAPK activity in type-II VECs even under stressful conditions may provide a useful indicator in drug discovery for ischemic diseases.

Terminology

p38α MAPK is reportedly required for an acquisition of stress resistance in various cell types.

Peer-review

In this study, the authors have identified that p38α MAPK is a crucial downstream effector of RGS5 in type I-type II VECs conversion. This finding provides a new strategy in the drug discovery for the treatment of ischemic disease. In general, this is a quite interesting and nice study. Experiments were well designed with appropriate controls and executed. Conclusions are significant and justified based on the high quality data. This manuscript certainly deserves to be published.

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ORIGINAL ARTICLE

Basic Study

Pro- *vs* anti-stenotic capacities of type-I *vs* type-II human induced pluripotent-derived endothelial cells

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Abstract

AIM: To verify *in vivo* relevance of the categorization of human vascular endothelial cells (VECs) into type-I (pro-proliferative) and type-II (anti-proliferative).

METHODS: Endothelial layers of murine femoral arteries were removed by wire injury (WI) operation, a common technique to induce arteriostenosis. Type-I and type-II VECs produced from human induced pluripotent stem cells (iPSCs), whose characters were previously determined by their effects on the proliferation of vascular smooth muscle cells in *in vitro* co-culture

experiments, were mixed with Matrigel® Matrix. The mixtures were injected into subcutaneous spaces around WI-operated femoral arteries for the transplanted human iPSC-derived VECs (iPSdECs) to take a route to the luminal surface *via* vasa vasorum, a nutrient microvessel for larger arteries. Histologies of the femoral arteries were examined over time. The presence of human iPSdECs was checked by immunostaining studies using an antibody that specifically recognizes human VECs. Degrees of stenosis of the femoral arteries were calculated after three weeks. To determine the optimal experimental condition, xenotransplantation experiments were performed under various conditions using immunocompromised mice as well as immunocompetent mice with or without administration of immunosuppressants.

RESULTS: Because immunocompromised mice showed unexpected resistance to WI-induced arteriostenosis, we performed xenotransplantation experiments using immunocompetent mice along with immunosuppressant administrations. After one week, luminal surfaces of the WI-operated arteries were completely covered by human iPSdECs, showing the efficacy of our novel transplantation technique. After three weeks, type-I-iPSdECs-transplanted arteries underwent total stenosis, while type-II-iPSdECs-transplanted arteries remained intact. However, untransplanted arteries of immunosuppressant-treated mice also remained intact by unknown reasons. We found that transplanted human VECs had already been replaced by murine endothelial cells by this time, indicating that a transient existence of human type-II-iPSdECs on arterial luminal surfaces can sufficiently prevent the development of stenosis. Thus, we re-performed xenotransplantation experiments using immunocompetent mice without administrating immunosuppressants and found that arteriostenosis was accelerated or prevented by transplantation of type-I or type-II iPSdECs, respectively. Similar results were obtained from the experiments using human embryonic stem cell-derived VECs at early passages (*i.e.*, type-II) and late passages (*i.e.*, type-I).

CONCLUSION: Pro- and anti-stenosis capacities of type-I and type-II human iPSdECs were verified, respectively, promising a therapeutic application of allogenic iPSdECs.

Key words: Vascular endothelial cells; Vasa vasorum; Arteriostenosis; Wire injury; Human induced pluripotent stem cells

Core tip: We previously reported that human vascular endothelial cells (VECs) were classified into two categories by their *in vitro* effects on the proliferation of vascular smooth muscle cells: Pro-proliferative VECs (type-I) and anti-proliferative VECs (type-II). Applying our new technique to transplant human VECs onto the luminal surface of endothelial layer-removed murine arteries, the *in vivo* relevance of the concept for VEC categorization was validated. Transplantation of pro-proliferative VECs (type-I) resulted in total stenosis while that of anti-proliferative VECs (type-II) completely blocked the development of arteriostenosis. Thus, pro-stenosis (type-I) and anti-stenotic (type-II) capacities were verified *in vivo*.

Nishio M, Nakahara M, Saeki K, Fujiu K, Iwata H, Manabe I, Yuo A, Saeki K. Pro- *vs* anti-stenotic capacities of type-I *vs* type-II human induced pluripotent-derived endothelial cells. *World J Transl Med* 2015; 4(3): 113-122 Available from: URL: http://www.wjgnet.com/2220-6132/full/v4/i3/113.htm DOI: http://dx.doi.org/10.5528/wjtm.v4.i3.113

INTRODUCTION

In our previous report, we presented a new concept for the categorization of human vascular endothelial cells (VECs) based on their effects on the proliferation of human vascular smooth muscle cells (VSMCs) in *in vitro* co-culture experiments: Pro-proliferative VECs (type-I) and anti-proliferative VECs (type-II)[1]. We also showed that commercially available primary cultured human VECs were exclusively pro-proliferative VECs (type-I)[1] compatible with an earlier report[2]. Although anti-proliferative (type-II) VECs were producible from commercially available human endothelial progenitor cells depending on donors[1], they were inevitably converted into "type-I" VECs after repetitive subcultures[1]. Human embryonic stem cells (ESCs) stably produced "type-II" VECs; however, ESC-derived type-II VECs were inevitably converted into type-I after repetitive subcultures[1]. Molecular analyses identified that the causative gene for "type-II to type-I" conversion was Regulator of G-protein signaling 5 (RGS5)[1], which is reportedly expressed in the endothelia of pathological vessels but not those of normal vessels[1,3,4]. Besides repetitive subcultures, oxidative stresses also induced "type-II to type-I" conversion[1] Although anti-oxidant treatments lowered the degree of RGS5 induction and delayed the time of "type-II to type-I" conversion, they could not completely block RGS5 induction or "type-II to type-I" conversion[1]. Thus, the problem of obtaining sufficient amounts of type-II VECs remained unsolved.

This problem was resolved by utilization of exogenous gene-free human induced pluripotent stem cells (iPSCs). Although type-II VECs were producible from conventional retrovirus vector-based exogenous gene-containing human iPSCs (Ret-iPSCs) depending on lines, they were inevitably converted into type-I VECs after repetitive subcultures[1]. By contrast, type-II VECs generated from recently established Sendai virus vector-based human iPSCs (SeV-iPSCs)[5] showed particularly high resistance to "type-II to type-I" conversion[1]. This may be explained by an empirical rule that "the older the establishment time of ESC/iPSC is, the more prone

to phenotype conversion the ESC/iPSC-derived VECs are" due to larger cumulative stresses. Moreover, SeV-iPSCs get rid of genome stress attributed to vector insertions into chromosomes. Indeed, Ret-iPSCs often expressed RGS5 message in immature states and RGS5 expression levels were further augmented after VEC differentiation. Thus, not only the passage numbers of iPS-derived VECs but also the quality of iPSCs (inc. the time and the method of establishments) are crucial determinants of the stability of type-Ⅱ characters of iPSC-derived VECs (iPSdECs).

In the current study, we validated *in vivo* relevance of the new concept for the categorization of human VECs by transplantation experiments using iPSdECs: Ret-iPSCdEC (type-Ⅰ) and SeV-iPSCs-derived VECs (SeV-iPSCdEC) (type-Ⅱ). For this aim, we established a new technique to transplant human VECs onto luminal surfaces of murine arteries utilizing a route *via* vasa vasorum. By applying this unique technique, we verified the *pro-stenosis* and *anti-stenosis* capacities of type-Ⅰ and type-Ⅱ human VECs, respectively. We also show that xenotransplantation of human VECs to murine arteries was effectively performed even under immunocompetent conditions because transplanted VECs have exerted their full effects within a short period (< 1 wk) before rejected by immune systems. Our studies not only prove the *in vivo* relevance of our new concept for the categorization of human VECs but also suggest the possible application of *allogenic* human iPS-derived VECs to therapeutic purposes.

MATERIALS AND METHODS

Cells

The hESC line (KhES-5) was generously provided by the Institute for Frontier Medical Science, Kyoto University[5]. A SeV-hiPSC line [SeV(BJ)-hiPSC] were established by using iPS-Tune™ (DNAVEC Corp., Ibaraki, Japan) from BJ fibroblast[6]. A Ret-hiPSC line (#25) was established from human fetus lung cells (MRC-5) by infecting recombinant retroviruses expressing the four factors (Oct3/4, Sox2, Klf4 and c-Myc) at Department of Reproductive Biology, Center for Regenerative Medicine, National Research Institute for Child Health and Development as used elsewhere[7,8]. 253G1[9] was provided by CiRA at Kyoto University and used elsewhere[7,8,10]. The type-Ⅰ and type-Ⅱ VECs were generated from Ret-hiPSC and SeV-hiPSCs, respectively[1].

Immunostaining

The 1st antibody reactions were performed by using a 1:50-diluted rabbit polyclonal anti-human-specific PECAM antibody (sc-8306, Santa Cruz Biotechnology Inc.)[11] or a 1:420-diluted anti-smooth muscle actin antibody (A5228, Santa Cruz Biotechnology Inc.) and the 2nd antibody reactions were performed by using an Alexa Fluor® 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies, Inc.) or an Alexa Fluor® 488-conjugated goat anti-mouse IgG (A11029, Life Technologies, Inc.), respectively. Photomicrographs were taken by either Olympus FluoView™ FV1000 Confocal Microscope (Olympus Optical Co. Ltd., Tokyo, Japan) or Olympus BX51 Fluorescence Phase contrast Microscope (Olympus Optical Co. Ltd.) equipped with DP-2 TWAIN digital camera system (Olympus Optical Co. Ltd.) and cellSens® standard imaging software (Olympus Optical Co. Ltd.).

Wire injury operation

The operation was performed as described elsewhere[12]. Nine-week-old ICR or NOD/SCID mice were anesthetized by intraperitoneally administrating the mixture of hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol tartrate (5 mg/kg) using 26 gauge needles. After depilation and disinfection, a 2 cm-long skin incision was made at the inguinal area to expose the femoral artery. After ligating the profunda femoris artery, a small incision was made on its proximal site, from which a 0.014-inch diameter guidewire (Cook Medical Inc., Bloomington, IN, United States) was inserted until it reached the bottom of the aorta descendens. Then, the guidewire was moved back and forth 10 times and rotated 5 times to evenly and completely exfoliate the endothelial layer. Finally, guidewire was removed and the profunda femoris artery was ligated at its most proximal site. After one week to three weeks from the operation, mice were anesthetized by intraperitoneally administrating the mixture of hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol tartrate (5 mg/kg) using 26 gauge needles and then subjected to perfusion fixation by administrating 50 mL of PBS and subsequently 100 μL of 4% paraformaldehyde solution *via* the left ventricle. Specimens of femoral artery were prepared and embedded into paraffin blocks for histological analyses.

Pervasa vasorum transplantation

An anti-asialo-GM1 antibody (300 μg) (WAKO Pure Chemical Industries, Osaka, Japan) was injected into the tail vein of 9-wk-old ICR or NOD/SCID mice, which were subsequently subjected to WI operations. On the other hand, iPSdECs/Matrigel® mixture were prepared just before the end of the WI operation as follows: 1×10^6 iPSdECs were centrifuged and kept in ice-cold sterile 1.5 mL tube, to which 20 μL of ice-cold Matrigel® Matrix was promptly added and mixed. The iPSdECs/Matrigel® mixture was injected into the subcutaneous space around the outer membrane of the femoral artery by using 200 μL pipet tip. An anti-asialo-GM1 antibody (300 μg) was injected into tail vein twice a week to block NK-mediated rejection in some experiments.

All procedures involving animals, including WI operations and pervasa vasorum transplantation (PVVT), were reviewed and approved by the Institutional Animal Care and Use Committee of National Center for Global Health and Medicine, Tokyo, Japan (Authorization No. 15014). The animals were acclimatized to laboratory

conditions (23 ℃ ± 3 ℃, 12 h/12 h light/dark, 55% ± 15% humidity, ad libitum access to food and water) prior to experimentation. The animal protocol was designed to minimize pain or discomfort to the animals by using mixed anesthetics as described above.

Statistical analysis

Experiments were performed independent three experiments using three mice (n = 3) and the data were analyzed using student t test. Results were shown as averages ± standard deviations (AV ± SD).

RESULTS

A new method to transplant human VECs on luminal surfaces of murine arteries.

The luminal surfaces of the arteries are continuously exposed to high-pressured and pulsatile blood flows, and thus, it is difficult for the transplanted human VECs to safely land and stably reside on the luminal surfaces if they are intravenously or intra-arterially injected into the blood stream. Thus, as alternative approach, we applied a strategy to take a rout *via* vasa vasorum, which is a nutrient vessel that supply or drain the walls of the larger arteries and veins. We hypothesized that the vasa vasorum would effectively guide the transplanted human VECs at least to the middle portion of tunica media and the human VECs would then migrate to the luminal surface, which is the natural site for them to reside.

For an effective replacement of the endothelial cells of the murine femoral artery by the transplanted human VECs, the host endothelial layer was mechanically removed by wire injury (WI) operation, which is a very common technique to induce experimental arteriostenosis. Then, human iPSdECs were transplanted by our unique technique termed "PVVT", where human iPSdECs were mixed with Matrigel® Matrix and the mixtures were simply injected into subcutaneous spaces around WI-operated femoral arteries (Figure 1). First, we performed experiments using immunodeficient NOD/SCID mice, which lack functional lymphocytes and permit the engraftment of a wide range of human cells. However, these mice did not develop arteriostenosis even after WI operations by unknown reasons (data not shown). Similar results were obtained from nude mice, which suffer from deficient T cell function (data not shown). Therefore, we performed experiments using immunocompetent ICR mice along with an administration of anti-asialo GM1 monoclonal antibody (αAGM1)[13] to prevent immunorejections by natural killer (NK) cells. The reason why we used αAGM1 is as follows. It is known that endothelial cells of the venus graft are replaced by arterial endothelial cells within several days from the coronary artery bypass surgery. Thus, an inhibition of the long-term immunorejections by T/B cells, which are usually induced a couple of weeks later, is not required for our purpose. On the other hand, short-term immnoreactions by NK cells, which are usually induced after several days from transplantation, might possibly affect the efficiency of the transplantations. Therefore, we performed xenotransplantation under the condition where NK cell activities were blocked by an administration of αAGM1.

After one week from the transplantation, WI-operated arteries underwent entire loss of endothelial layers with fibrin clot-like substances on the luminal surfaces of the arteries of non-transplanted mice (Figure 2A and B, open arrows). By contrast, the luminal surfaces of the arteries of human iPSdEC-transplanted mice were thoroughly covered by the endothelial cells (Figure 2B, closed arrows). Immunostaining studies using an antibody that specifically recognizes human PECAM1 but not murine PECAM1[11] confirmed that the endothelial cells that covered the luminal surfaces of the arteries were indeed human VECs (Figure 2C and D).

Thus, our new PVVT technique guaranteed the effective transplantation of human VECs onto the luminal surfaces of murine arteries.

Type-Ⅰ and type-Ⅱ iPSdECs exacerbated and prevented the development of arteriostenosis, respectively

Next, we analyzed the histologies of the arteries after three weeks from the transplantation. We found that type-Ⅰ iPSdECs-transplanted arteries underwent almost complete stenosis (Figure 3A, lower left). By contrast, "type-Ⅱ" iPSdEC-transplanted arteries showed intact morphologies (Figure 3A, lower right). For quantitative evaluations, we calculated the ratio of the square measure of the lumen and that of the area surrounded by external elastic membranes in cross sections as an indicative of stenosis. We found that transplantation of "type-Ⅰ" iPSdECs exacerbated the development of arteriostenosis (Figure 3B). Histological analyses (Figure 3C, left panels) and immunostaining studies using anti-smooth muscle actin (Figure 3C, right panels) confirmed the presence of hyper-proliferated VSMCs within the neointima. Unexpectedly, AGM1 administration significantly inhibited the development of arteriostenosis (Figure 3A, upper right), and thus, we could not detect significant differences between "WI + αAGM1 + transplantation (-)" and "WI + αAGM1 + iPSdEC (type-Ⅱ)" groups (Figure 3B). Therefore, although we could successfully demonstrate the pro-stenosis capacity of type-Ⅱ iPSdEC, we failed in showing the anti-stenosis activity of type-Ⅱ iPSdEC in these experiments. We hypothesized that immunosuppressive states inhibited the development of arteriostenosis as observed in the case of immunodeficient NOD/SCID mice and nude mice (data not shown).

When we examined the presence of human iPSCdECs after 3 wk from transplantation, we noticed that the transplanted human VECs already became undetectable on the arterial luminal surfaces (Figure 3D), indicating that transplanted iPSCdECs had already been replaced by host's endothelial cells by this time as expected from the finding obtained from bypass surgeries. Our observation at the same time indicates that the time

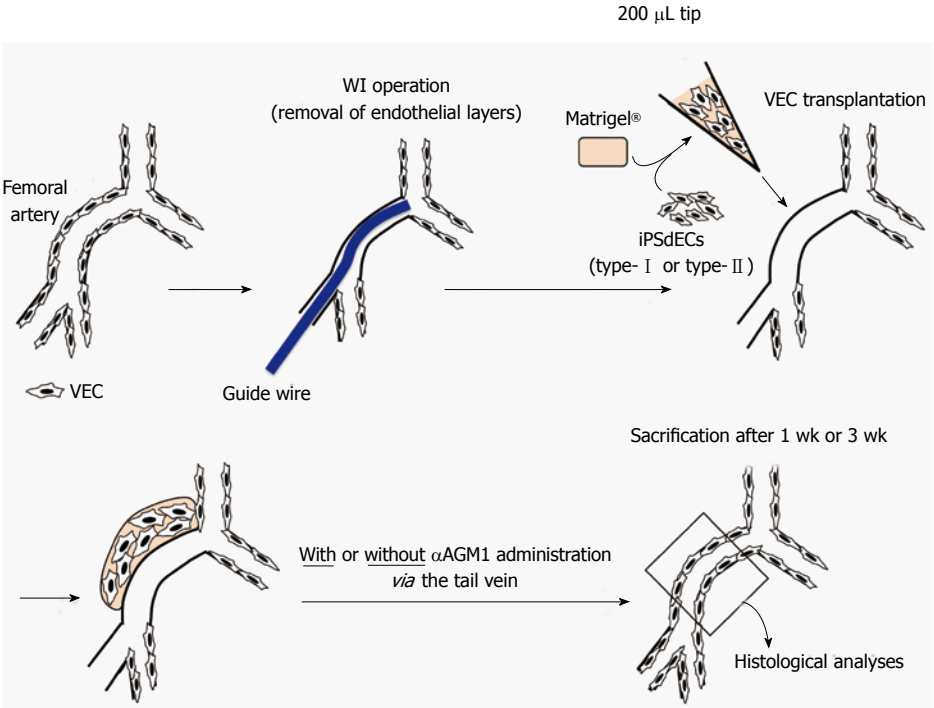
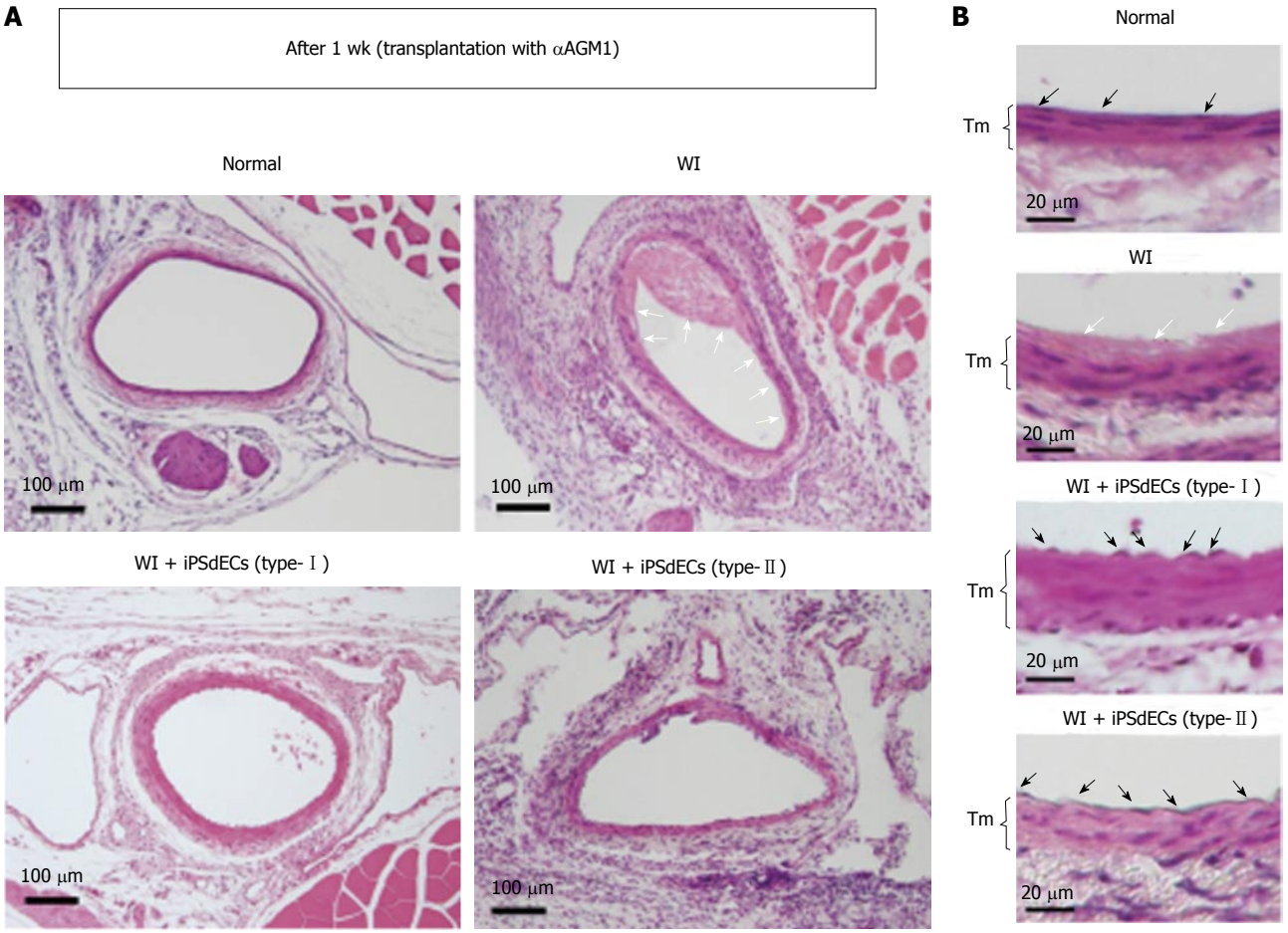


Figure 1 The procedure of pervasa vasorum transplantation. Human iPSCdECs were mixed with Matrigel® Matrix and the mixtures were put into the subcutaneous regions around the WI-injured femoral arteries. WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSdECs: iPSC-derived VECs; αAGM1: Anti-asialo GM1 monoclonal antibody.



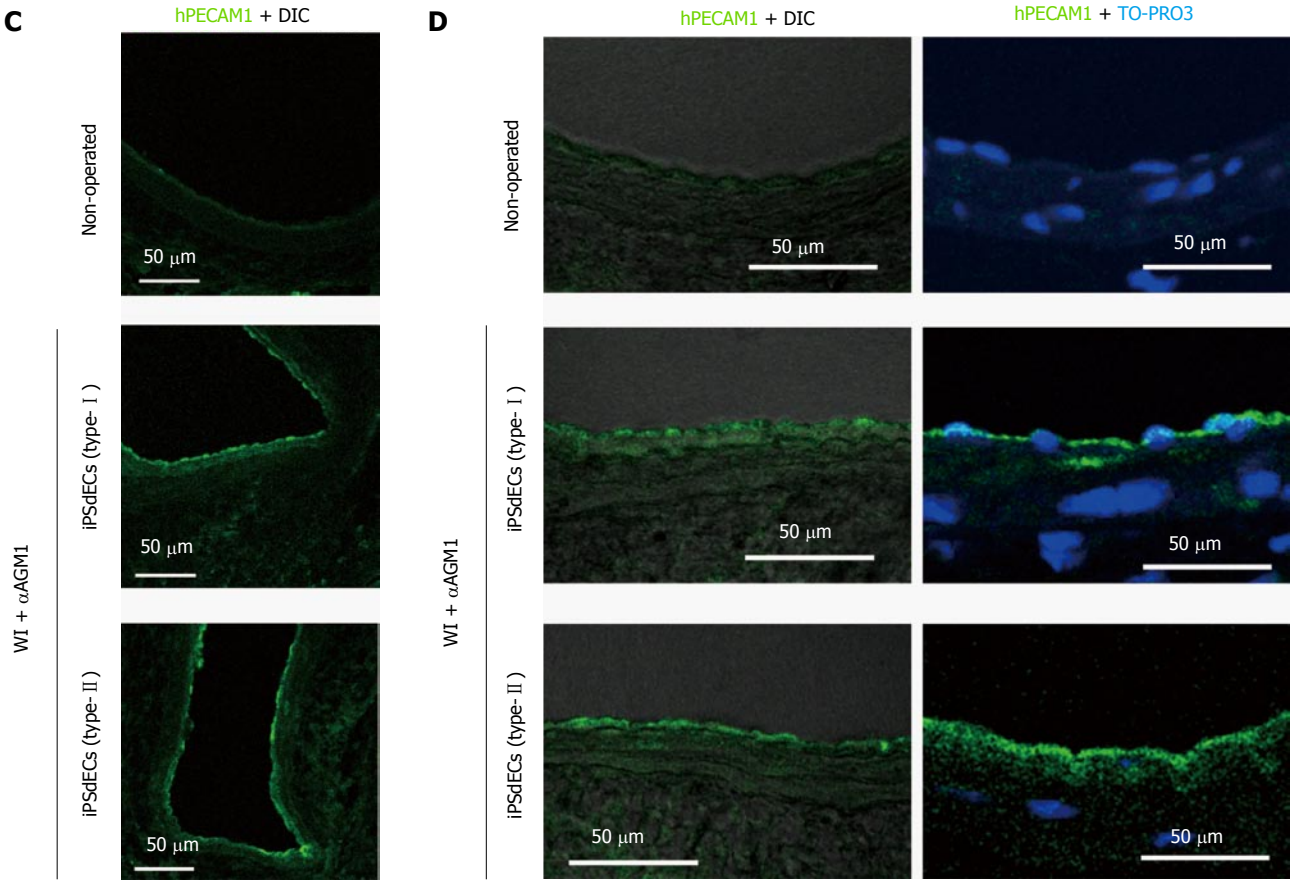
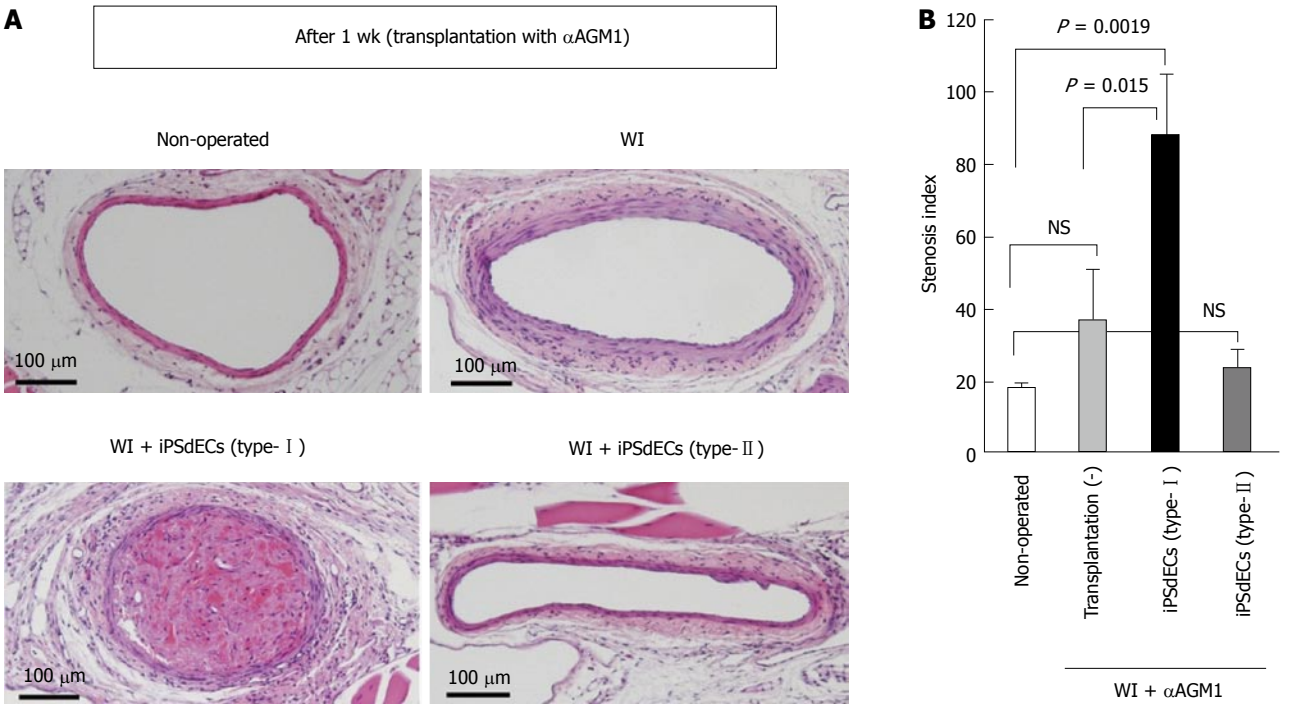


Figure 2 Histological analyses after one week. A and B: WI-operated femoral arteries that were transplanted with type-Ⅰ or type-Ⅱ iPSdECs were examined after one week from PVVT in mice regularly administrated with asialo GM1 antibody αAGM1). Open arrows indicate fibrin deposits, closed arrows indicate nuclei of endothelial cells and Tm indicate tunica media; C and D: Con-focal microscopies of immunostained samples using anti-human PECAM1 antibody with differential interference contrast (DIC) (C) or nuclear counterstaining by TO-PRO3 (D). WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSdECs: iPSC-derived VECs; αAGM1: Anti-asialo GM1 monoclonal antibody.



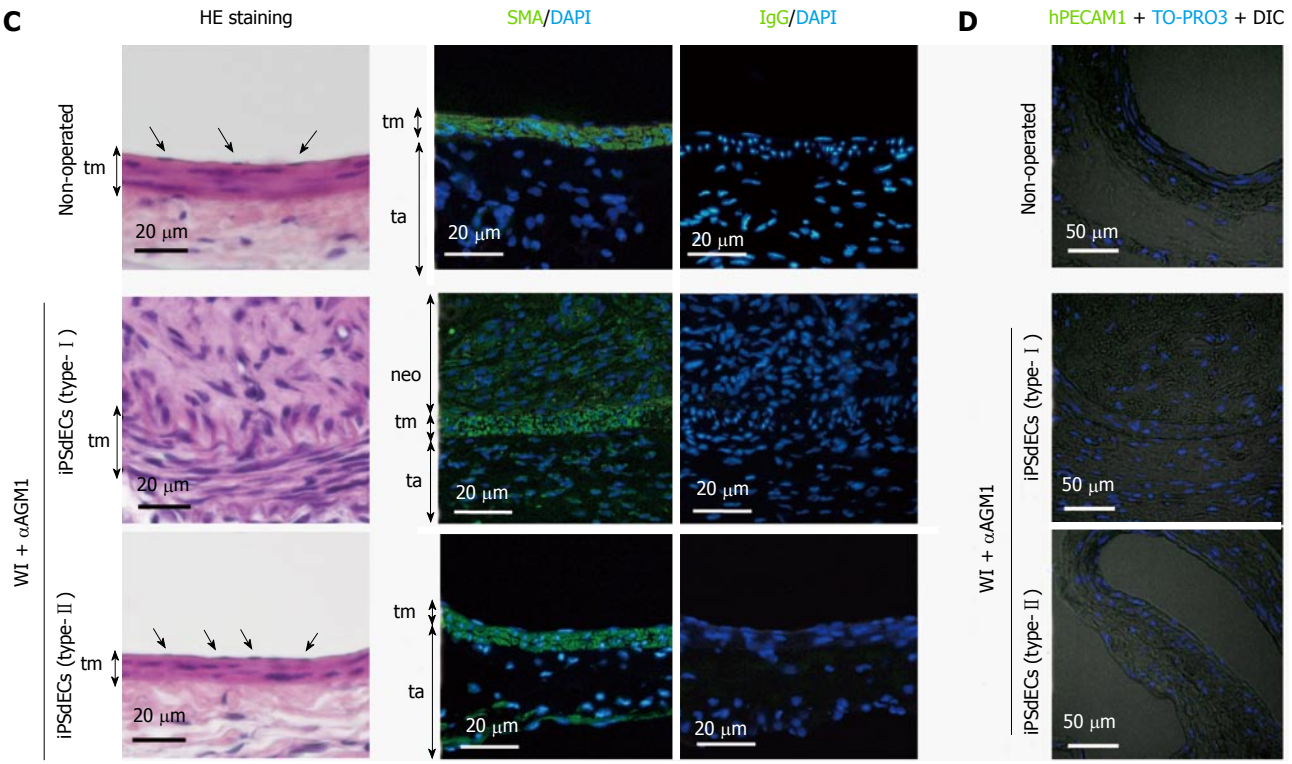


Figure 3 Histological analyses after three weeks. Histologies of WI-operated femoral arteries after three weeks from PVVT in mice regularly administrated with aAGM1 were examined. A: Photographs of HE-stained samples at low magnification; B: Stenosis indexes were calculated and statistically analyzed by student-t test. Data were presented as average (Av) ± standard deviation (SD). Experiments were performed using three mice for each condition (n = 3); C and D: High magnification photographs of the samples with HE staining (C, left), immunostaining using anti-smooth muscle actin (SMA) antibody with nuclear counterstaining by DAPI (C, right) and immunostaining using anti-hPECAM1antibody with DIC and nuclear counterstaining by DAPI (D). WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSdECs: iPSC-derived VECs; αAGM1: Anti-asialo GM1 monoclonal antibody.

period required for the transplanted iPSdECs to exert their full effects was rather shorts (about one week). Thus, we re-performed the transplantation experiments without administrating αAGM1. We confirmed that arteriostenosis was exacerbated by type-Ⅰ iPSCdECs transplantation under these conditions (Figure 4A, lower left, and 4B, lower left). By contrast, the development of arteriostenosis was completely prevented in type-Ⅱ" iPSdECs-transplanted arteries (Figure 4A, lower right, and 4B lower right). Without an administration of αAGM1, WI-operated arteries underwent arteriostenosis (Figure 4A, upper right, and 4B upper right). Thus, *anti-stenosis* capacities of type-Ⅱ iPSdECs, as well as the *pro-stenosis* capacities of type-Ⅰ iPSdECs, were verified (Figure 4C). We also examined the effects of transplantation with human ESC-derived VECs (ESdECs) at early passages (type-Ⅱ) and at late passages (type-Ⅰ)[1]. Although ESdECs at early passages (type-Ⅱ) underwent "type-Ⅱ to type-Ⅰ conversion" after a few rounds of subcultures *in vitro* and thus their type-Ⅱ characters were not as solid as type-Ⅱ SeV-iPSdECs, we could detect clear differences in the results of transplantations between ESdECs at early passages (type-Ⅰ) and those at early passages (type-Ⅱ) (Figure 4D and E).

Collectively, the *in vivo* relevance of the concept for the categorization of human VECs, type-Ⅰ (pro-proliferative) and type-Ⅱ (anti-proliferative), were verified *in vivo*.

DISCUSSION

In the current study, we verified the *in vivo* relevance of the new concept for the categorization of human VECs by showing that *pro-proliferative* VECs (type-Ⅰ) exacerbated the stenosis of injured arteries whereas *anti-proliferative* VECs (type-Ⅱ) prevented the development of arteriostenosis. Our finding highlights an unexpected importance of the endothelial cells for the maintenance of vascular structures, demonstrating that VECs serve as not only the simple cover for the luminal surface but also a crucial regulator of the proliferation of VSMCs. Therefore, an approach to the preservation of type-Ⅱ VEC phenotypes provides a new strategy for the treatment of arteriostenosis. Although the major cause of arteriostenosis is currently hypercholesterolemia-based atherosclerosis and anticholesteremic agents has been exerting high therapeutic effects, new VEC-targeted drug discoveries may further contribute to the control of ischemic diseases especially in the cases of restenosis after stent therapies or resistance to cholesterol medications.

We found by chance that arteriostenosis is strongly inhibited under immunosuppressive conditions. This

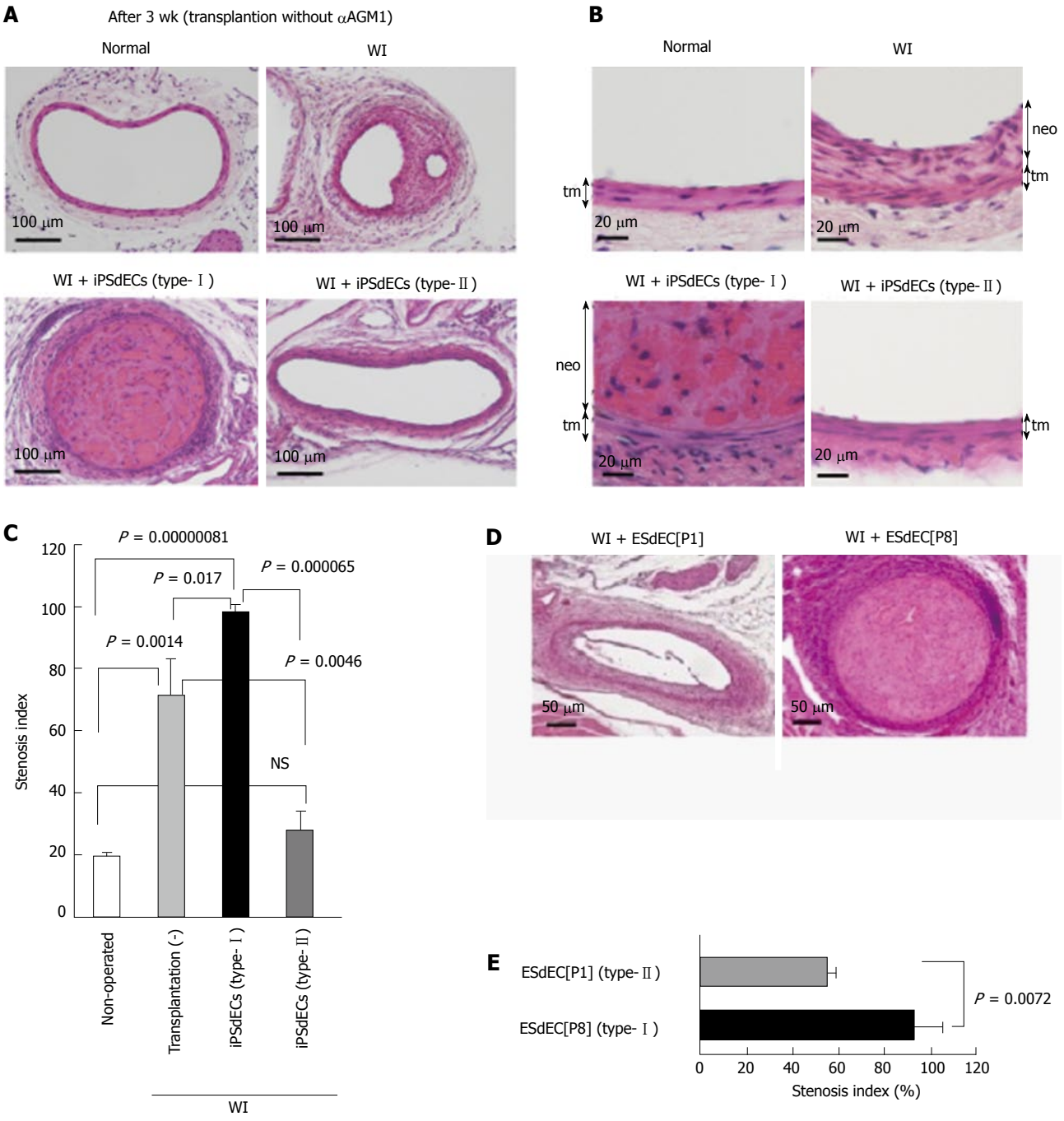


Figure 4 Transplantation without immunosuppression. A-C: WI-operated femoral arteries transplanted with type-I or type-II iPSdECS were examined after 3 wk from PVVT in mice without αAGM1 administration. Photographs of HE-stained samples at high magnification, those at low magnification and calculated stenosis indexes (Av ± SD, *n* = 3) (C) were shown; D and E: HE staining (D) and calculated stenosis indexes (*n* = 3) (E) of WI-operated femoral arteries after 3 wk from ESdECs transplantation without αAGM1 administration. WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSdECs: iPSC-derived VECs; αAGM1: Anti-asialo GM1 monoclonal antibody; ESdECs: ESC-derived VECs.

finding may explain, at least in part, the high effectiveness of immunosuppressive agents used in the drug-eluting stent. The reason why immunosuppressive states prevent the development of arteriostenosis remains elusive. In WI-injured arteries of immunodeficient NOD/SCID mice, cellular compartments of tunica media were lost by unknown reasons (data not shown). Because a similar phenomenon was observed in nude mice (data not shown), a certain component of T cells might possibly be involved in the regulation of this bizarre phenomenon. We are currently studying which components of T cells are involved in this phenomenon.

In the current study, we also established a new transplantation technique termed PVVT, which guarantees the effective transplantation of human VECs onto the luminal surface of murine arteries. PVVT is a very simple technique, which can be performed by putting human VEC-embedded gels into subcutaneous spaces

adjacent to arterial walls through a small skin incision. It can even be carried out as an ambulatory treatment if it is clinically applied in the future. Although we cannot completely exclude the possibility that there are still other paths than *vasa vasorum*, our new transplantation technique provides the easiest and safest way to transplant VECs on the luminal surfaces of injured arteries. Another merit of our PVVT technique is that it can be performed even under immunocompetent conditions, which indicates that *allogenic* iPSdECs are used as effectively as *autologous* iPSdECs when clinically applied. Moreover, a short-term requirement of iPSdECs makes the clinical application of human iPSdECs much safer. In the case of *allogenic* iPSdECs, the risk of tumor formation following transplantation will be lowered to a minimum because *allogenic* iPSdECs will be immunologically rejected by adaptive immune systems. Collectively, PVVT-based therapies will widen the applicability of iPSdECs to clinical purposes.

Thus, our finding will shed a new light to an advanced understanding of vascular biology and contribute to the therapeutics development for the control of ischemic diseases.

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COMMENTS

Background

Human vascular endothelial cells (VECs) are categorized into two groups by their effects on the proliferation of vascular smooth muscle cells (VSMCs) in *in vitro* co-culture experiments: Pro-proliferative VECs (type-I) *vs* anti-proliferative VECs (type-II).

Research frontiers

It remains elusive whether pro-proliferative and anti-proliferative human VECs indeed exert pro-stenotic and anti-stenotic potentials *in vivo*, respectively. To valuate the characters of human VECs *in vivo*, however, an innovative technique that guarantees high-efficiency transplantation of VECs onto the luminal surface of the artery, which is exposed to high-pressured blood stream, is required.

Innovations and breakthroughs

The problem to effectively transplant human VECs onto the luminal surface of the murine artery has been resolved by the novel transplantation technique, where gel-embedded VECs were injected into the substances space around arteries through a small incision opening on the skin surface. By applying this technique, *in vivo* relevance the concept for the categorization of human VECs was validated, demonstrating that transplantation of pro-proliferative VECs (type-I) and anti-proliferative VECs (type-II) resulted in deterioration and prevention of stenosis in the injured arteries, respectively.

Applications

Towards the control of ischemic diseases, transplantation of human iPSC-derived type-II VECs to the luminal surface of injured arteries *via* a vaso vasorum route may provide a new adjunct therapy with high efficacy and high safety but low risk of restenosis after revascularization.

Terminology

Vasa vasorum is a feeding microvessel that supplies the cells in tunica media of larger blood vessels including smooth muscle cells and fibroblasts. Wire injury is a technique to mechanically remove the endothelial cells from arterial lumens by the movement of a metal wire in a longitudinal as well as vertical direction, causing the development of arteriostenosis.

Peer-review

The manuscript is about two types of vascular endothelial cells, which have anti and pro proliferative effect on vascular smooth muscle cells. The study is well-design and written.

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