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Review

Endothelial cells and pulmonary arterial hypertension: apoptosis, proliferation, interaction and transdifferentiation

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Abstract

Severe pulmonary arterial hypertension, whether idiopathic or secondary, is characterized by structural alterations of microscopically small pulmonary arterioles. The vascular lesions in this group of pulmonary hypertensive diseases show actively proliferating endothelial cells without evidence of apoptosis. In this article, we review pathogenetic concepts of severe pulmonary arterial hypertension and explain the term "complex vascular lesion ", commonly named "plexiform lesion", with endothelial cell dysfunction, i.e., apoptosis, proliferation, interaction with smooth muscle cells and transdifferentiation.

Introduction

Severe pulmonary arterial hypertension (PAH), whether idiopathic or associated with known causes (secondary forms), may have a reversible component in a minority of the patients [1,2], but most patients with severe PAH at the time of their diagnosis have persistent structural alterations of their microscopically small pulmonary arterioles, i.e., pulmonary vascular remodeling believed to be caused by angiogenic proliferation of endothelial cells (EC) [3-6]. Complex pulmonary vascular lesions at sites of bifurcations that are often glomeruloid appearing and lumen obliterating, including the so-called plexiform lesions, are frequently found in the lungs of patients with severe PAH, including the lungs from patients with Eisenmenger physiology where the lung vessels are subjected to increased (shunt) blood flow [7]. Whether these complex vascular lesions can fully explain the PAH remains controversial.

In this article, we review pathogenetic concepts of severe PAH and explain the term "complex vascular lesion," commonly named "plexiform lesion," with EC dysfunction, i.e., apoptosis, proliferation, interaction with smooth muscle cells (SMC) and transdifferentiation.

Initial EC apoptosis is followed by the emergence of apoptosis-resistant proliferating EC

Discordant stimulation of EC or an uncontrolled EC response are common events in many pathologic processes including atherosclerosis, allograft vasculopathy, hypertension, congestive heart failure, sepsis and inflammatory syndromes, and PAH [8]. These diseases have in common endothelial injury, which can result in EC apoptosis, dysfunction and activation [8].

Especially pulmonary endothelial injury caused by toxins [9], reactive oxygen species [10,11], autoimmune mecha-

nisms [5], and shear stress[12,13] likely leads to severe PAH.

A recent study showed that bone morphogenic proteins (BMP) signaling reduced apoptosis of cultured pulmonary artery EC under conditions of serum deprivation and maintained the survival of cultured circulating endothelial progenitors from normal individuals but not from IPAH patients. These results support the hypothesis that loss-of-function mutations in the bone morphogenic protein receptor II (BMPRII) could lead to increased pulmonary EC apoptosis, representing a possible initiating mechanism in the pathogenesis of PAH [14].

Taraseviciene-Stewart et al recently described that blockade of EC growth factor receptors resulted in the potentiation of PAH and marked worsening of the pathological vascular remodeling, even reproducing some of the "angioproliferative" features typical of advanced PAH and this effect was reversed by inhibitors of apoptosis, suggesting that increased apoptosis of EC in response to loss of survival signaling created conditions favoring the emergence of apoptosis-resistant cells with increased growth potential [15]. Moreover, Campbell et al and Zhao et al have shown that overexpression of EC growth and survival factors, such as vascular endothelial growth factor (VEGF) and angiopoietin-1, prevented the development of monocrotaline-induced PAH [16,17], an effect that was associated with reduced EC apoptosis. Together, the findings suggest that EC growth and the emergence of phenotypically altered vascular cells in severe PAH is the consequence of initial apoptosis and subsequent selection of apoptosis-resistant, proliferative vascular cells. This concept is consistent with recent finding describing the absence of apoptotic cells in the plexiform lesions in the lungs from patients with severe PAH [12] as well as reduction of severe PAH in the rat model [15] by treatment with simvastatin, which induced apoptosis of the EC that had obliterated the pulmonary arterioles [18].

To study the dependence of exuberant EC proliferation on initial apoptosis, we adapted the CELLMAX artificial capillary system to analyze the effects of the VEGF receptor (VEGFR) I and VEGFR II antagonist (SU5416) on human pulmonary microvascular EC (HPMVEC) under conditions of pulsatile shear stress [19].

The experiments with human pulmonary microvascular EC (HPMVEC) seeded in the artificial capillary system demonstrated that a combined VEGF I and II receptor blocker (SU5416) induces EC apoptosis [19]. When this VEGF receptor blockade-induced apoptosis was followed by high fluid shear stress a hyperproliferative state was generated, and within 7 days phenotypically altered EC emerged [19]. These altered EC expressed the tumor marker survivin and the antiapoptotic protein Bcl-$_{XL}$ and were resistant to induction of apoptosis after challenge with TNF-α plus cycloheximide or hydrogen peroxide; in addition, the cells demonstrated survival in serum-free culture medium (Figure 1) [19].

Taken together our data reflect the paradox that growth factor-inhibition fosters the emergence of apoptosis-resistant and hyperproliferative cells [19]. This paradox has recently been described by Golpon et al [20] in experiments which resulted in the conclusion that there is "life after corpse engulfment". In these experiments it was shown that cells with apoptosis induced by UV irradiation, after they had been phagocytosed by other cells, released growth factors into the culture medium and that this conditioned medium made naïve epithelial- or endothelial cells apoptosis-resistant [20].

Whether in our shear stress experiments the SU5416 treated apoptotic cells were phagocytosed by neighboring cells of the CELLMAX system was not examined. In principle most cell types (not only professional phagocytes like macrophages) have the ability to phagocytose apoptosed cells [21-24] and we consider this possibility. It is unclear why the VEGF receptor blockade does not induce apoptosis in all of the EC and whether the surviving cells do so because they respond to survival signals which may be released by the dying cells. Alternatively or additionally

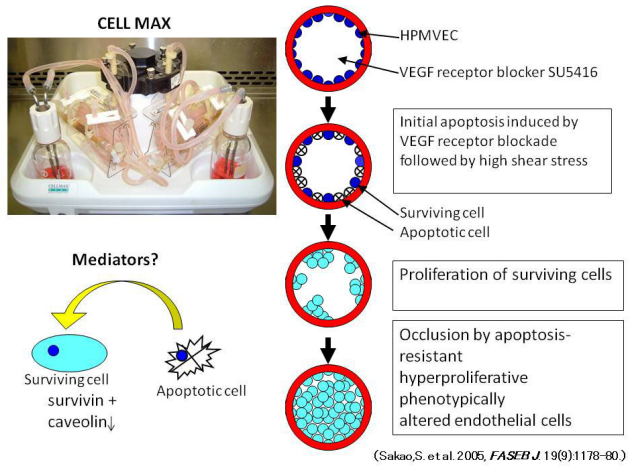

Figure 1
The CELLMAX artificial capillary modules and sequence of events that leads from initial apoptosis to proliferation of apoptosis-resistant endothelial cells. The combination of initial apoptosis induced by VEGF receptor blockade and high fluid shear stress generates apoptosis-resistant proliferative endothelial cells. *Definition of abbreviations*: HPMVEC = human pulmonary microvascular endothelial cell; VEGF = vascular endothelial growth factor; SU5416 = a combined VEGF I and II receptor blocker.

it is conceivable that the EC contain some apoptosis-resistant precursor cells which expand under the conditions of our experiments [19]. Because VEGF receptor *inhibition* allows apoptosis-resistant EC growth and because Partovian et al showed that adenovirus-mediated VEGF over-expression reduced pulmonary hypertension [25] it is not clear that VEGF causes the angiogenic growth of the lumen-obliterating EC. It is possible that over-expression of the VEGF and VEGFR II proteins in the human pulmonary vascular lesions is a reflection of a vascular repair attempt. Again, the presence of VEGF and VEGFR II in the vascular lesions does not necessarily mean that VEGF actually causes the growth of the phenotypically altered and apoptosis-resistant cells.

Consistent with the result in this *in vitro* experiment, Masri and colleagues have reported *ex vivo* that pulmonary artery EC (PAECs) isolated from patients with idiopathic PAH (IPAH) exhibit an unusual hyperproliferative potential, with decreased susceptibility to apoptosis [26]. Together with accumulating evidence from previous studies [15,19,27], this study again provides support for the concept of an apoptosis-resistant and hyperproliferative EC in IPAH.

The above described *in vitro* experimental model appears to support the concept that apoptosis-resistant hyperproliferative EC can emerge at shear stress sensitive sites in the lung circulation in severe PAH. Although we do not address experimentally the factor or factors which confer apoptosis-resistance and phenotypical alterations of a subpopulation of endothelial stem-like cells, we suggest that blockade of the signal transduction of the obligatory EC survival factor, VEGF, in combination with high shear provide a selection pressure. The nature of the surviving and proliferating cells remains unclear. It is possible, as stated above, that the surviving and proliferating cells are precursor cells [28,29].

Cross talk between endothelial and smooth muscle cells

The interactions of EC and SMC, which exist in the close contact of a functional syncytium, are involved in a process of new vessels formation that occurs during development, as part of wound repair, and during the reproductive cycle. One basic component of this interaction is the endothelial-induced recruitment, proliferation and subsequent differentiation of SMC [30-32].

Moreover, it was shown in *in vitro* studies that several growth factors or cytokines, such as activated transforming growth factor-β_1 (TGF-β_1) and IL-1β, had been produced by the EC and SMC in coculture and they might be involved in some of the effects exerted by the coculture on these cells [31,33,34]. TGF-β_1 is a growth factor which is a potent stimulant of extracellular matrix synthesis and inhibits matrix degradation [35]. TGF-β_1 has been shown to potentiate the development of intimal hyperplasia in animal models following arterial injury [36]. Thus, TGF-β_1 appears to be an important mediator of the increased extracellular matrix deposition which occurs during vascular wall remodeling. IL-1β is one of inflammatory cytokines and its elevated serum levels in PAH have been reported [37].

Theories concerning the detailed pathobiology of PAH have focused on factors produced by EC and SMC and their response to different mediators. Prostacyclin (PGI_2), a protein produced by EC and whose known target is SMC, could be one of the vasodilators. In patients with PAH, the levels of PGI_2 are reduced [38]. Prostacyclin modulates the vasodilator response of SMC in the case of acute hypoxia [39].

We have previously hypothesized that the development of severe angioproliferative PAH is associated with initial EC apoptosis followed by the emergence of apoptosis-resistant proliferating EC [19]. However, the precise control of the balance between pulmonary arterial SMC (PASMC) proliferation and apoptosis is important in maintaining the structural and functional integrity of the pulmonary vasculature. In severe angioproliferative PAH, this balance seems to be disturbed such that there is increased PASMC proliferation and decreased apoptosis, leading to vessel wall thickening and vascular remodeling, i.e., hyperplasia of PASMC [40-43]. Indeed, severe angioproliferative PAH is characterized by complex precapillary arteriolar lesions [7,44-46], which contain phenotypically altered endothelial and smooth muscle cells [7]. Interestingly acquisition of resistance to apoptosis and increased rates of proliferation of PASMC appear to be necessary for neointima formation [47-52]. This phenotype plasticity, the dedifferentiation of mature, nonproliferative PASMC into proliferative PASMC, is a process central to vascular remodeling [53,54].

We have previously demonstrated that EC death results in the selection of an apoptosis-resistant, proliferating and phenotypically altered EC phenotype [19]. Therefore we postulated that the initial apoptosis of EC induced the release of mediators which caused VSMC proliferation. To study this hypothesis, apoptosis of microvascular EC was induced by VEGF receptor blockade using the combined VEGFR-I and II blocker SU5416 and it was shown that serum-free medium conditioned by apoptosed EC caused proliferation of vascular SMC compared with serum-free medium conditioned by non-apoptosed EC [55]. It was also shown that serum-free medium conditioned by apoptosed EC is characterized by increased concentrations of TGF-β_1 and VEGF compared with serum-free medium conditioned by non-apoptosed EC, and that

TGF-β_1 blockade prevented the proliferation of cultured vascular SMC [55]. In conclusion, EC death induced by VEGF receptor blockade leads to the production of factors, in particular TGF-β_1, which activates vascular SMC proliferation, i.e., that EC apoptosis may stimulate vascular SMC growth (Figure 2) [55].

Moreover, several recent studies showed that EC seeding of injured arterial wall segments appears to limit the SMC response to injury. It was shown that EC seeding of endarterectomized canine arteries decreased the intimal hyperplastic response [56] and that EC seeding of injured hypercholesterolemic rabbit femoral arteries also limits the intimal hyperplastic response [57]. It is, therefore, reasonable to hypothesize that apoptosed EC may lose their control over SMC allowing SMC growth.

Recent studies suggest that, in response to intimal injury, synthetic/proliferative SMC migrated to the intima can generate proinflammatory molecules to promote WBC infiltration of the artery wall [53,58,59]. EC injury caused by proinflammatory molecules may lead to EC apoptosis and SMC growth and thus a EC apoptosis-SMC growth loop could result in the progression of PAH.

It is likely that dysregulated growth factors or cytokines produced by EC and SMC exert autocrine or paracrine effects which contribute to the progression of remodeling in pulmonary artery that results in PAH.

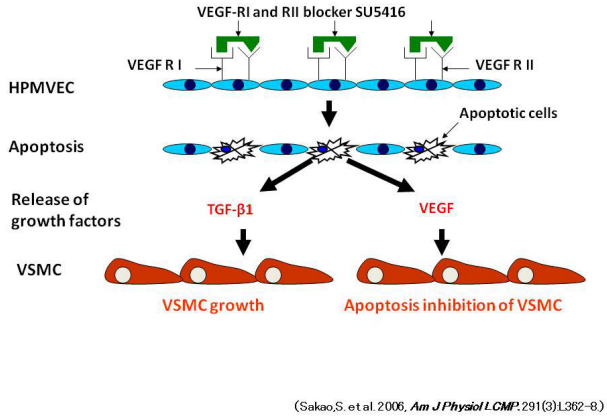

Figure 2
Sequence of events that leads from SU-5416-induced VEGF blockade to the increased growth of VSMC. VEGF receptor blockade induces apoptosis of vascular endothelial cells. Apoptotic endothelial cells release growth factors such as VEGF and TGF-β_1, and, whereas VEGF inhibits apoptosis, TGF-β_1 promotes VSMC proliferation. *Definition of abbreviations*: HPMVEC = human pulmonary microvascular endothelial cell; VSMC = vascular smooth muscle cell; TGF-β_1 = transforming growth factor-β_1; VEGF = vascular endothelial growth factor; SU5416 = a combined VEGF I and II receptor blocker.

Endothelial-Mesenchymal transdifferentiation

Transdifferentiation is a form of metaplasia and the conversion of one differentiated cell type into another, with or without intervening cell division, so this mechanism challenges the preconceived ideas that the terminal differentiated state is fixed. Indeed, it is now generally accepted that "differentiation" can sometimes be reversed or altered [60].

In the neointima formation and vascular remodeling fibroblasts in the pulmonary vascular wall play specific roles in the response to injury, including rapid migration, proliferation, synthesis of connective tissue, contraction, cytokine production, and, most importantly, transdifferentiation into other types of cells (e.g., PASMC) [61].

Hypoxia-induced changes in fibroblasts' proliferative and matrix-producing phenotypes are accompanied by the appearance of smooth muscle α-actin in tissues from pulmonary hypertensive subjects, suggesting that some of the fibroblasts transdifferentiate into myofibroblasts [62]. This transdifferentiation involves a complex network of microenvironmental factors and pathways in which extracellular matrix components as well as growth factors, cytokines, and adhesion molecules may play a role [63].

The intriguing possibility that intimal SMC may arise from the endothelium has received some attention [64,65]. In the systemic circulation, Arciniegas et al showed that mesenchymal cells that contribute to the intimal thickening may arise from the endothelium by using *in vivo* and *in vitro* methods [66].

Severe angioproliferative PAH is characterized by complex pulmonary precapillary arteriolar lesions [7,44-46], which contain phenotypically altered SMC and EC [7]. In addition to lumen-obliterating cell aggregates, which form the so-called plexiform lesions, muscularized arteries are also frequently present. Vasoconstriction as well as peptide (endothelin and angiotensin II) and nonpeptide (serotonin) growth factors have been postulated to be responsible for the muscularization of the pulmonary arteries in severe PAH [67-69]. Indeed "transitional cells" demonstrating features of both EC and vascular SMC have been identified in the plexiform lesions in the lungs from patients with severe angioproliferative PAH [70]. We hypothesize that an additional or alternative mechanism contributing to the muscularization of the pulmonary arteries may be transdifferentiation of pulmonary EC to mesenchymal cells.

To examine this hypothesis, we incubated HPMVEC with SU5416 and analyzed these cells utilizing quantitative-PCR, immunofluorescent staining and flow cytometry analysis [71]. *In vitro* studies of HPMVEC demonstrated that SU5416 suppressed PGI_2S gene expression while potently inducing COX-2, VEGF and TGF-β_1 expression, causing transdifferentiation of mature vascular EC (defined by Dil-ac-LDL, Lectin and Factor VIII) into SM-like (as defined by expression of α-SM actin) "transitional" cells, which coexpressed both endothelial and SM markers [71]. In this experiment, the SU5416-induced transdifferentiation was independent of TGF-β_1 [71]. Although TGF-β_1 was shown to be involved in inducing endothelial-mesenchymal transdifferentiation [72] and is known to promote SM-actin expression in nonmuscle cells (EC and fibroblasts derived from various tissues) [73,74], TGF-β_1 is currently thought to be insufficient to induce expression of late SM differentiation marker SM myosin heavy chain (SM-MHC) in non-SMC lineage cells [74]. SU5416 expanded the number of CD34 and/or c-kit positive cells and caused transdifferentiation of $CD34^+$ cells, but not $CD34^-$ cells. In conclusion, this data showed that SU5416 generated a selection pressure that killed some EC and expanded resident progenitor-like cells to transdifferentiate into SM like cells (Figure 3) [71]. Further, we fully realize the limitation of our data interpretation which is based on *in vitro* studies of cultured cells. However, we believe that our data may be consistent with the concept that transdifferentiation of pulmonary EC to mesenchymal cells may contribute to the muscularization of the pulmonary arteries.

The prevailing theory of the vascular SMC contribution to vascular lesions is that in pathological states, like atherosclerosis, SMCs migrate to the intima from the media of the vessel [75]. This concept, however, has been challenged by results derived from models of vascular injury, transplant arteriosclerosis, and human allograft studies, which all indicate that a portion of the cells bearing SMC differentiation markers in intimal lesions may have originated from the hematopoietic system and/or circulating progenitor cells [76-78]. Furthermore, a recent study demonstrated that smooth muscle progenitors were present in circulating blood [79], although the origin of these cells remains unknown. Concomitantly, it was shown that ~ 60% of SMC in atherosclerotic lesions of vein grafts were derived from the donor vessel wall and 40% from the recipient, possibly from circulating blood cells [80,81]. In the aggregate these reports strongly suggest the possibility of stem or progenitor cells as a source of SMC accumulation in atherosclerotic lesions. However, not all of the SMC within intimal lesions may be derived from bone marrow cells. Recently it was shown that, in addition to circulating progenitor cells, $Sca\text{-}1^+$ progenitor cells that reside in the adventitia can transdifferentiate into SMC-like neointimal cells [82], suggesting that not only bone marrow cells but also resident vessel wall precursor cells could exist and serve as a source of SMC to form neointimal lesions.

Ingram and colleagues [29] have resolved progenitor cells within a population of EC isolated from conduit vessels in the systemic circulation. These findings suggest that EC isolated from the vessel wall are enriched with progenitor cells that rapidly proliferate and can renew the entire population. This report confirms the unexpected finding in our study [71] that there is the presence of a small number of bone marrow-derived $c\text{-}kit^+$, $CD34^+$ endothelial precursor cells among various batches of commercially available lung microvascular EC, suggesting the presence of such precursor cells in the adult lung.

The greater context of these findings, i.e., residential endothelial precursor cells and their transdifferentiation, may be a general mechanism for muscularization of vessels and, in the nondeveloping adult lung, a mechanism which participates in lung tissue homeostasis and repair of injured lung cells via utilization of resident lung tissue precursor cells.

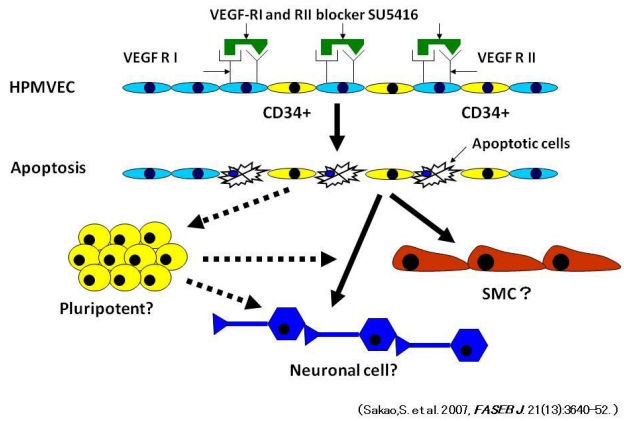

Figure 3
Sequence of events in HPMVEC that lead from VEGF blockade by SU5416 to transdifferentiation to smooth muscle-like cells. Endothelial cell death induced by VEGF receptor blockade and subsequent selection of progenitor-like cells leads to transdifferentiation to smooth muscle-like cells and neuronal cell. Dotted arrows mean hypothetical sequences of events. *Definition of abbreviations*: HPMVEC = human pulmonary microvascular endothelial cell; VSMC = vascular smooth muscle cell; SU5416 = a combined VEGF I and II receptor blocker.

Genetic and/or epigenetic factors in PAH - a perspective

Genetic mutations, like BMPRII mutations that have been found in patients with familial and nonfamilial forms of IPAH [83], may contribute to cell growth control. Indeed, there is a growing literature that associates BMP and their receptors with cell growth control, even in cancers [84-86].

Not only somatic cell mutations may contribute to the hyperproliferative, apoptosis-resistant endothelium phenotype, but the unusual EC phenotype could also arise from a normal resident or itinerant lung cell population as a result of genomic events [71,87].

Not only "genetic", but also "epigenetic factors", should be considered as factors or conditions which induce the hyperproliferative, apoptosis-resistant endothelium phenotype. Epigenetics, here understood as a bridge between genotype and phenotype, can influence gene expression without changing the underlying DNA sequence, i.e., epigenetic modifications can express themselves via DNA methylation and histone modifications [88-91]. Dietary and hormonal influence can be envisioned to affect the pulmonary vessels in patients with IPAH, initiating or amplifying changes in the EC residing along the pulmonary vessels [92,93].

It is hypothesized that apoptosis-resistant, phenotypically altered and transdifferentiated EC may arise by genetic and epigenetic mechanisms.

Conclusion

It is tempting to speculate in the context of PAH that following EC apoptosis a selection of cells characterized by a high proliferative potential, including resident progenitor cells, results in a prevalence of hyperproliferative, apoptosis-resistant pulmonary vascular lesion cells that contribute to the irreversible and progressive nature which characterizes many forms of severe PAH (Figure 4).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SS conceived of the report, contributed to its design and conception and drafted the manuscript. KT drafted the manuscript and contributed to its design and conception. NV contributed to its design and drafted the manuscript. All authors read and approved the final manuscript.

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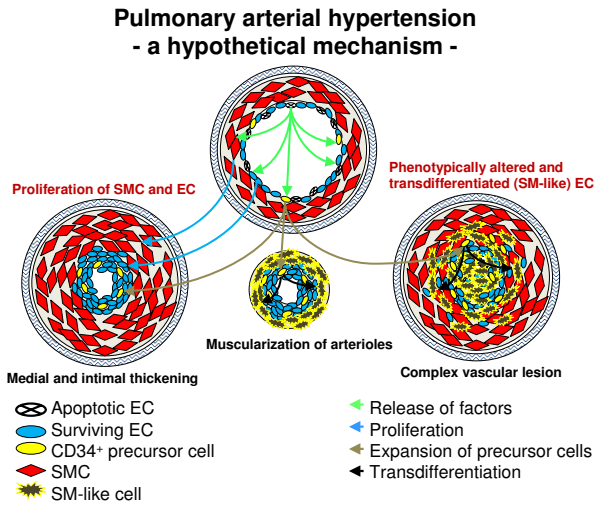

Figure 4

A hypothetical mechanism of pulmonary arterial hypertension. Sequence of events that leads from endothelial cell initial apoptosis to proliferation of apoptosis-resistant endothelial cells and vascular smooth muscle cells and endothlial-mesenchymal (SM-like) transdifferentiation. Apoptotic endothelial cells may release some kinds of factors that generate apoptosis-resistant proliferative endothelial cells, promote vascular smooth muscle cell proliferation and result in subsequent selection of progenitor-like cells leads to endothlial-mesenchymal (SM-like) transdifferentiation. These events may be a general mechanism for intimal and medial hypertrophy, muscularization of arterioles and complex vascular lesions. *Definition of abbreviations*: EC = endothelial cell; SMC = smooth muscle cell; SM-like = smooth muscle-like

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