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Review

The functional implication for endothelial gap junction and cellular mechanics in vascular angiogenesis

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Abstract: Angiogenesis, the sprout and growth of new blood vessels from existing vasculature, is an important process of tumor development for the supply of oxygen and nutrition to cancer cells. Endothelial cell is a critical player in angiogenic process by modulating cell proliferation, cell motility, and cell morphology in the response to pro-angiogenic factors and environments provided by tumor and cancer cells. Recent *in vivo* and *in vitro* studies have revealed that gap junction of endothelial cells also participates in the promotion of angiogenesis. Pro-angiogenic factors modulate gap junction function and connexins expression in endothelial cells, whereas endothelial connexins involve in angiogenic tube formation and cell migration of endothelial cells via both gap junction channel function dependent or independent mechanisms. In particular, connexin might have the potential to regulate cell mechanics such as cell morphology, cell migration, and cellular stiffness that are dynamically changed during angiogenic processes. Here, we review the implication for endothelial gap junction and cellular mechanics in vascular angiogenesis.

Keywords: gap junction; connexin; angiogenesis; cell mechanics; cell migration; cellular stiffness

1. Introduction

The vascular network which supplies oxygen and nutrition is necessary for the tumor growth and cancer cell proliferation. In order to promote angiogenesis from existing blood vessels, tumor and cancer cells secret high levels of pro-angiogenic factors and provide pro-angiogenic hypoxic environments [1, 2]. In the response to these pro-angiogenic factors and environments, vascular endothelial cells (ECs) initiate angiogenic process including vascular sprouting, cell proliferation, cell migration, tube formation, and vascular stabilization [3, 4]. Notably, during these angiogenic process, ECs dynamically changes of cell mechanics that are mechanical and physiological characters determined by cytoskeletal rearrangement [5], focal adhesion formation [6], and contractile force [7], have been also observed.

Gap junctions (GJs) are consisted of connexin (Cx) family protein which has four transmembrane domains and two extracellular loop domains [8, 9]. The hexametric Cx forms a hemichannel (connexon) that docks to another connexon on the adjacent cell via extracellular domains resulting in the formation of GJ channel [8, 9]. GJ channel directly connects each cytoplasm of adjacent cells and allow the intercellular movement of small molecules and electron coupling [10]. Thus, GJ intercellular communication (GJIC) is essential for the transfer and synchronization of the intracellular environment between adjacent cells. It has been considered that GJ-mediated transfer

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and synchronization of intracellular mediators such as ions, amino acids, small metabolites, and secondary messengers are essential for orchestration of multicellular responses [10]. In addition, the C-terminal domain of Cx protein interacts with several intracellular protein such as signaling molecules [11], cytoskeletal proteins [12], and cell junctional proteins [13], indicating the possibility of GJ- and Cx-mediated regulation of cell mechanics and mechanotransduction.

EC plays a critical role in the regulation of vascular inflammation [14], blood coagulation [14] [16, 17], leukocyte adhesion and extravasation [15] [18], and angiogenesis [16] [19], thereby, the EC dysfunction is a conceivable cause of the development of cardiovascular diseases [17]. ECs predominantly express three Cxs: Cx37, Cx40, and Cx43 [18, 19] and essentially regulate GJ function and Cx expression in the response to pro-inflammatory stimuli [20, 21]. Conversely, alteration of GJ function and Cx expression in ECs is able to influence on a multiple EC functions under physiological and pathological condition [20, 22, 23]. Recent studies have indicated that abnormality of GJ and Cx expression in vascular component cells including ECs, smooth muscle cells and monocytes/macrophages contributes to atherosclerosis associated with excessive inflammation and vascular remodeling [22, 23]. In addition, more than a decade of research on GJ in ECs and angiogenesis has provided evidences of the interplay between endothelial Cxs and angiogenesis. Here, we mainly focus on GJs and Cxs in ECs and will discuss the implications of cellular mechanics for vascular angiogenesis.

2. Endothelial Cx expression and its role in vascular diseases

Cx expression pattern in ECs is dependent upon vessel type, be it arteries, veins, or lymphatic vessels. Cx37 and Cx40 are co-expressed in arterial ECs of the healthy vessels [24], whereas Cx43 has been observed characteristically in ECs of the microvasculature and at branch points of arteries that experience turbulent blood flow [24]. Cx32, Cx37 and Cx40 are present in venous ECs [25, 26]. *In vitro* studies have demonstrated Cx32, Cx37, Cx40, and Cx43 expression in both cultured human vein and artery ECs [27-29]. It has been known that alteration of each Cx expression and GJ function in ECs upon pro-inflammatory stimuli is closely correlated with EC activation. Indeed, pro-inflammatory tumor necrosis factor-α (TNF-α) reduces GJ function in EC at early phase (4hours) and then decreases the expression of Cx32, Cx37 and Cx40, but not Cx43 at late phase (24 hours) [21, 30]. LPS, is an important activator of inflammation in ECs via toll-like receptor 4, also induces serine-dephosphorylated Cx40 [31] and reduced GJ function between microvascular ECs [31, 32]. Pro-coagulant factor thrombin, which is a major trigger of thrombus formation and increased vascular endothelial permeabilization, induces rapid and acute internalization of Cx43-mediated GJ in primary pulmonary artery ECs [33]. On the other, opposite effect by which thrombin induces Cx43 expression and GJ function associating with the disruption of the endothelial barrier has been reported [34]. In this way, although some different phenotypes have been observed, these results have indicated the dynamic regulation of GJ function and Cxs expression in ECs upon pro-inflammatory stimuli at both post-translational modifications and transcriptional level.

Several studies have revealed the contribution of aberrant GJs function and Cxs expression in ECs to the promotion of endothelial dysfunction and vascular inflammatory diseases such as atherosclerosis. For example, Cx37 and Cx40 are decreased in early stage of atherosclerosis [20], while deletion of Cx40 from ECs in mice, as well as the dysfunction of Cx37, can promote the development of atherosclerosis by enhancing both monocyte adhesion and transmigration [22, 35]. Moreover, Cx37-deficient mice enhance the expression of a number of pro-inflammatory genes involved in advanced atherosclerosis [36]. Cx43 is increased in early stage of atherosclerosis [20], whereas reduced expression of Cx43 by smooth muscle cells inhibits the formation of atherosclerotic lesions [37]. Furthermore, endothelium specific deletion of Cx43 modulates renin secretion, thereby inducing hypertension [38]. A Cx43 mutation in patients with cardiac infarction has been identified as a risk factor [39]. We have previously shown not only that reduced Cx32 expression in HUVECs facilitates pro-inflammatory cytokines expression upon inflammation [30], but also that Cx32-deficient mice enhances activation of vascular inflammation and blood coagulation in septic model [30, 40]. Taken together, these studies have suggested that abnormality of GJs function and Cxs

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expression may be a trigger of various endothelial dysfunction leading to the development of atherosclerosis and vascular inflammatory diseases.

3. Alteration of GJ function and Cxs expression in ECs under pro-angiogenic stimuli

Pro-angiogenic factors have also been likely to modulate GJ function and Cx expression of ECs [41] (Fig.1). Vascular endothelial growth factor (VEGF), which plays a central role in vasculogenesis and angiogenesis [42], implicates in diverse physiologic processes including tumor angiogenesis [43, 44], diabetic retinopathy [45], wound healing [46], and tissue repair following ischemic injury [47]. VEGF-induced VEGF-receptor 2 (VEGF-R2) activation of ECs in existing vasculature is primarily an initiation step of angiogenesis and then induces sprouting, cell proliferation, and cell migration of EC [48]. *In vitro* model experiments, VEGF-induced c-Src tyrosine kinase and MAP kinases activation results in the rapid disruption of GJ function of ECs [41], and increases paracellular endothelial permeability associating with reduction of cell-cell junction [49]. Furthermore, it has been reported that the VEGF-induced disruption of GJ function correlates with the rapid internalization of Cx43 and Cx43 tyrosine phosphorylation in rat coronary capillary endothelium [50, 51]. Therefore, pro-angiogenic VEGF stimulation negatively modulate GJ function and Cxs expression in ECs in a consequence of angiogenesis-related signaling.

In addition to VEGF, basic fibroblast growth factor (bFGF) and hypoxia are well-known as the pro-angiogenic factor and environment. It has been reported that microvascular ECs facilitate GJ function and Cx43 expression in the response to bFGF stimulation [52]. The stimulation with bFGF not only increases Cx43 mRNA expression but also facilitates Cx43 localization at cell-cell interface [52]. Hypoxia condition observed in tumor tissue activates HIF pathways and induces the expression of a number of pro-angiogenic genes in cancer cells [1]. In the case of ECs, hypoxia upregulates the Notch ligand Dll4 expression and promotes activation of Notch signaling which is an essential pathway for vascular development and stabilization [53, 54]. The upregulation of Cx40 expression has been reported under hypoxia-mediated Notch signaling in ECs [54]. Recent study has shown that a Notch-Cx37-p27 axis promotes EC cycle arrest leading to vascular regeneration under shear stress [13]. These suggest that endothelial Cx and Notch might coordinate the appropriate EC proliferation and angiogenesis.

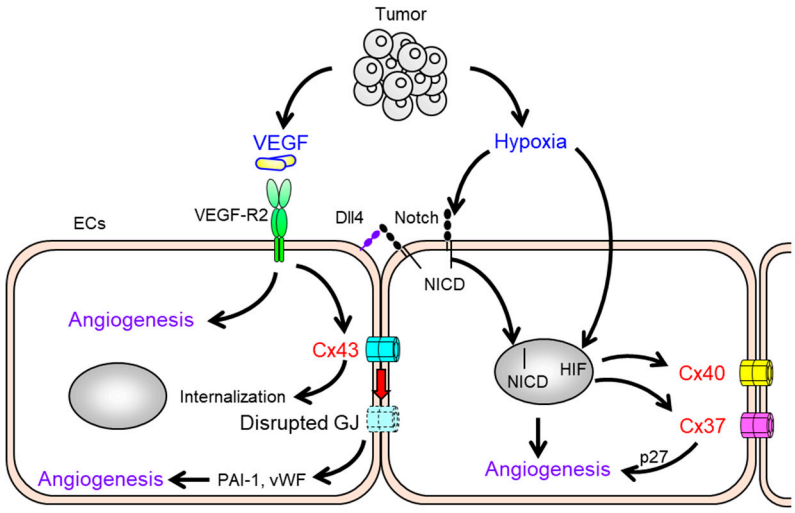

Figure 1. Alteration of GJ function and Cxs expression in ECs under pro-angiogenic stimuli. VEGF is an essential initiator of angiogenesis. ECs induce internalization and disruption of GJ formed by Cx43 under VEGF-VEGF-R2 signaling. Hypoxic condition in tumor tissue activates Notch and HIFs in EC. Notch signal including the nuclear translocation of the intracellular domain of the notch protein (NICD) induces EC function and cell mechanics that involved in angiogenesis. HIF pathways are angiogenic-related genes expression in ECs. Both signaling pathways results in angiogenesis associating with upregulation of Cx37 and Cx40 in ECs.

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Although endothelial GJ function and Cx expression are assuredly regulated by pro-angiogenic factors and environments, and further, it has been reported that Cxs expression and GJ function in tumor cell [55], myocadiac cell [56], and mesenchymal stem cell [57], tightly link to VEGF expression from these cells. For example, Cx43 knock-down in tumor cell lines increases VEGF expression and enhanced the proliferation of ECs [55]. Thus, in order to understanding the role of GJ and Cx in angiogenesis, it is necessary to elucidate the basic biology of GJ and Cx in these type cells at the interplay of angiogenesis and tumor development.

4. The impact of endothelial Cxs in vascular endothelial angiogenesis

Several groups have investigated the impact of Cxs for development of cardiovascular system which is closely related to angiogenesis. Mutations in the gene for Cx43 (GJA1) were found to cause a hypoplastic left heart syndrome [58]. Cx43-deficient mice, which die at birth from connatural heart malformations, have shown a reduction in the distal branching complexity and length of coronary arteries [59]. In Cx40-deficient mice, cardiac malformations have been observed [60]. Additionally, both endothelial Cx40- and Cx37-knockout mice develop severe abnormalities of the vascular function and structure [61]. Recently, loss of endothelial Cx40 leads to a reduction in vascular growth and capillary density in the neovascularization of the mouse neonatal retina [62]. We have also demonstrated that aortic vascular tissue from Cx32-deficient mice exhibit suppressed vascular sprouting of ECs [28]. Cx37 knock-out mice enhance vasculogenesis and remodeling resulting in improvement from an ischemic hindlimb injury [63]. These studies have indicated the contribution of endothelial Cxs to angiogenesis in the physiological or pathological condition.

Some reports have shown the relevance of endothelial Cxs expression and vascular angiogenic potential in ECs *in vitro* angiogenesis assay. Knockdown of Cx43 using specific siRNAs reduces tube formation and cell proliferation of human aortic ECs [64]. The downregulation of Cx43 increases angiogenesis-related factors [64], such as plasminogen activator inhibitor-1 [65] and von Willebrand factor [66], suggesting that Cx43 might directly and/or indirectly contributes to angiogenesis. Knockdown of Cx37, Cx40, or Cx43 using siRNAs has shown suppressed endothelial angiogenesis including the branching of HUVECs, elongation of cell length, and tube formation by an *in vitro* matrigel assay [67]. In gain-of-function experiments employed stable Cx-transfectants, we have demonstrated that increased expression of Cx32 markedly enhances tube length and the number of branching of EA. hy926 cells which is ECs line derived from HUVECs in matrigel tube formation [28]. On the other hand, Cx37- or Cx43-transfected EA. hy926 cells impairs tube length and the number of branching [28].

These studies have provided many evidences that endothelial Cxs expression modulate angiogenesis, however the specific role of each Cxs on angiogenesis remains unclear. Notably, it has been considered that any endothelial Cx expression may modifies other Cxs expression [28, 67, 68]. Indeed, Cx43 siRNA induces increased both Cx37 and Cx40 expression in aortic ECs. In HUVECs, Cx43 siRNA does not alter the expression of other Cxs, whereas Cx40 siRNA and Cx37 siRNA reduce Cx43 and Cx40 expression, respectively [67]. In addition, Cx32-transfected EA. hy926 cells reduce Cx43 expression and has exhibited highly angiogenic potential such as tube formation and branching [28]. Gain-of function and loss-of function assay remain to be experimentally tested, however these have indicated that alteration Cx expression patterns and their relative network of Cx expression may elicit different ECs phenotypes during angiogenic processes. This interrelated Cx regulatory network have make difficult to understand specific role of each endothelial Cxs in angiogenesis.

5. Endothelial Cxs-mediated regulation of cell migration in angiogenesis

ECs dynamically change cell mechanics such as cell morphology, cell proliferation, and cell migration during angiogenesis process [69, 70]. EC activation by pro-angiogenic factors allows tip cells to extend filamentous actin (F-actin)-rich filopodial protrusions migrating toward the required site [3, 71]. Tip cells are the leading cells of the sprouts and guide following stalk cell which proliferates to elongate the sprout [4]. A proper tuning of continued migration of tip cells and

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proliferation of stalk cells is crucial for angiogenesis [4]. Notably, the implication of endothelial Cxs in the control of EC migration has been progressively known. We have shown impaired cell migration of ECs both *in vitro* wound healing assay by using Cx32 blocking ECs and *in vivo* matrigel plaque implant assay in Cx32-deficient mice [28]. Other groups have reported that GJIC and Cx43 expression are increased in the region of cell migration and at localized to cells at the wound edge by using wounded monolayer repair assay [72]. Cx43 specific siRNA markedly suppresses cell migration of endothelial progenitor cells by transwell chamber migration assay that allowed cells to migrate through the filter membrane upon pro-angiogenic factors [73]. In addition to ECs, several type of cell such as leukocyte, epithelial cell, and tumor cell also regulate their migration via GJ channel dependent and independent function (reviewed by Matsuuchi [74] and Kameritsch [75]).

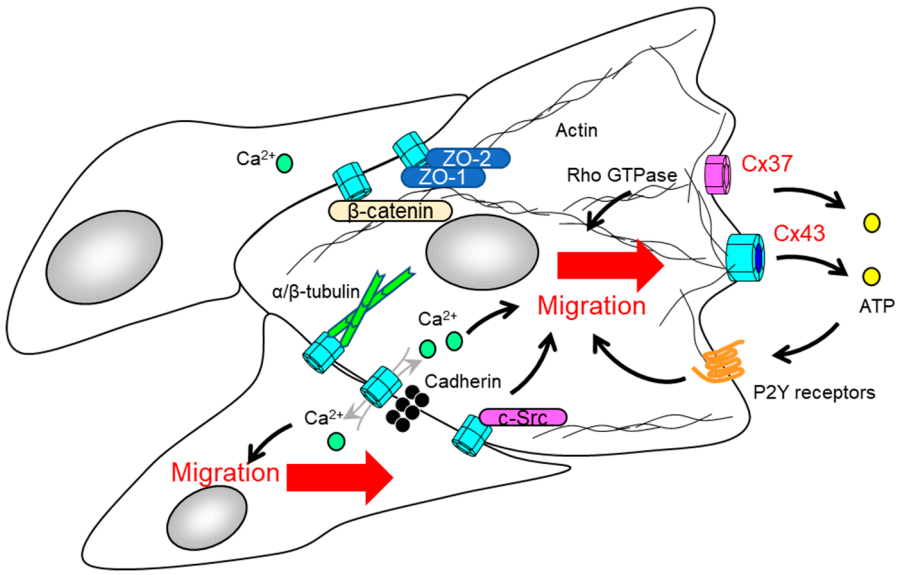

Figure 2. Endothelial Cxs-mediated regulation of cell migration. Extracellular ATP released by Cx-hemicannels activates P2Y receptors which trigger cell migration. GJ-mediated propagation of calcium waves has been required for collective cell migration. The interaction of Cx and GJ with cytoskeletal proteins or intracellular proteins orchestrate cytoskeletal rearrangement and cell migration.

Both GJ mediated cell-cell interaction and hemichannel function have involved in the regulation of cell migration in a number of cell type (Fig.2). Cultured adrenocortical cells have shown to exert intact GJIC between cells during collective cell migration [76]. In a wound assay, Cx43 expression in immortalized ECs is positively associated with cell migration and wound closure [77]. Moreover, GJ-mediated propagation of calcium waves has been required for smooth muscle cell polarization and migration [78], therefore, it is conceivable that GJIC in a cell cluster could play an important role in coordination of the migration [79]. Extracellular ATP-induced calcium signaling has been shown to modulate neuronal proliferation and migration of neuronal cells [80]. Cx hemichannels have been known as a pathway of ATP release from intracellular space to extracellular space. ATP release has been observed in glioma, glioblastoma and HeLa cells being transfected with Cx26, Cx32, or Cx43 [81]. Macrophage also releases ATP via Cx37 resulting in cell adhesion to endothelium [22]. It has been considered that ATP release via Cx hemichannels from cells may induce cell migration through calcium signaling following P2Y receptors activation in neighboring cells [81].

Intracellular domain of Cxs protein interacts with other proteins that involve in being structural stability of cell-cell junction sustained by cytoskeletal scaffolds [10]. Due to the ubiquitous distribution of Cx43, many studies have been performed focusing on Cx43 and their interacting proteins. The carboxyl tail of Cx43 is indeed interacting with several cytoskeletal proteins such as F-actin, α-/β-tubulins, cadherins, and cortactin) [82-85]. For example, the membrane expression of N-cadherin or of ZO-1 is dominantly localized in the existing site of Cx43 protein [84]. The interaction of Cx43 with the cadherin family may not only be important for the mechanics of cell migration, but also generates

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intracellular signaling. Interaction of Cx43 and cadherins coordinates activation of Rho GTPases which are promoting cell motility and invasion [86, 87]. Moreover, Rac1 in migrating cell is dominantly found in forming actin-rich structures which in conjunction with E-cadherin are considered responsible for the generation of traction forces of germ cells *in vivo* [88]. Intracellular carboxyl tail of Cx43 has a number of interaction partners, thereby, the cell expressed a Cx43 lacking carboxyl tail impairs cell migration [89]. Cx43 deficiency causes an impaired polarization caused by a non-directional alignment of the microtubule organizing center. As a consequence, a loss of directionality of cell migration and then an impaired development of coronary arteries can be observed in Cx43 deficient mice. A Cx43 mutant with lack of the tubulin binding site in the carboxyl tail has shown the similar phenotypic pattern with Cx43 deficient [89], suggesting that interaction between Cx43 and cytoskeletal protein may coordinate cell mechanics and behavior.

Interestingly, Cx43 seems indeed to be important for the stability of leading processes of the neuronal cells determining the migratory pathway along the glial fibers [90]. Interesting mechanism has been elicited that control the localization of Cx43 in the cellular extensions of migrating neurons in a way that radial migration along the glia becomes possible [90]. Additionally, Watanabe and colleagues have shown that fish GJ and Cx involves in fish morphological diversity, including skin pattern formation and body shape determination [91]. Their studies have indicated that Cxs in pigment cells, xanthophore and melanophore, dictate aggregation and separation of cells resulting in pattern formation [92]. These suggest the possibility of Cxs dependent regulation of directional cell migration.

6. Cxs-mediated regulation of cellular stiffness and cell migration

The interaction between Cx and cytoskeletal proteins correlatively contributes to the regulation of cellular stiffness which is defined as the physical property of a cell to resist deformation in the response to any applied force. A contraction force which generated by the actomyosin cytoskeleton and F-actin has been inseparably connected with the regulation of cellular stiffness [93, 94]. Activation of the Rho-actomyosin signaling pathway enhances the formation of actin bundles, stress fibers, and tensile actomyosin structures [95], all of which correlate with cellular stiffness [96, 97]. Thus, interplay between endothelial Cxs and Rho family has implicated in the regulation of cellular stiffness. We have found that proinflammatory stimulation increased endothelial cellular stiffness associating with impaired GJ function, cytoskeletal remodeling, and focal adhesion formation [98]. Moreover, blockade of GJs induces the cellular stiffening associated with focal adhesion formation and cytoskeletal rearrangement, and prolonges TNF-α-induced endothelial cellular stiffening [98]. This study has provided first evidence that endothelial GJ contributes to the regulation of endothelial cellular stiffness via interaction with cytoskeletal rearrangements.

It has been considered that endothelial cellular stiffness may be a determinant factor of leukocyte adhesion to endothelium. In general, leukocyte senses the stiffness of extracellular substrate by integrin-ligand interaction and adheres more strongly to stiff substrate [99]. ECs materially work as a substrate during leukocyte adhesion and migration process. Leukocyte integrin assumes both selective and cohesive adhesion via the binding to distinct endothelial adhesion receptors such as the intercellular adhesion molecule 1 (ICAM1) [93]. Integrin increases the binding avidity to ligands correlated with the endothelial cellular stiffness, while integrin-focal adhesion complex generates the contractile force in cell and transduces the force into a mechanosignaling [100, 101]. These suggested the possible mechanism which regulates leukocyte adhesion and activation via physical endothelial cellular stiffness [102].

In addition to leukocyte, it has been reported that ECs themselves also modulate their migration, proliferation, and morphological changes in the response to extracellular substrate stiffness [103, 104]. Thus, it has been shown a possibility that stiffening ECs in existing vasculature is favor to recruit pro-angiogenic tip cells and stalk cells at the sprouting spots (Fig.3). Of note, VEGF-induced cytoskeletal rearrangement and impaired GJ function might be supposed to increases EC stiffness. Stiff ECs may recruit endothelial progenitor cells and support the cell proliferation and elongation of stalk cells. Taken together, a series of studies suggest the concept that GJ-mediated EC stiffening might facilitate

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angiogenic process of recruited ECs by being the activator of mechanosensing and transduction pathway.

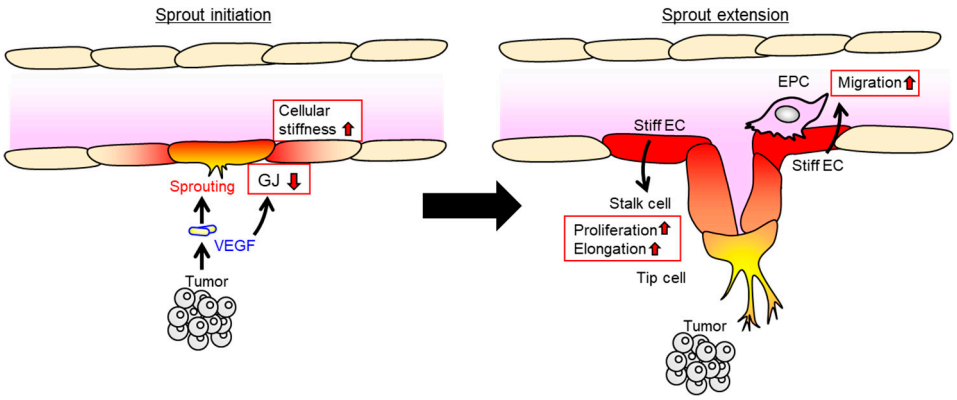

Figure 3. Potential role of endothelial cellular stiffening in angiogenesis. VEGF-induced GJ reduction increases the stiffness of ECs in sprout initiation phase. Stiff ECs provide the favorable environment for recruitment of endothelial progenitor cells, while stiff ECs support adjacent stalk cell proliferation and elongation.

7. Conclusions

We are beginning to understand that GJ and Cx in ECs might be a center for connection between biological function and cell mechanics in the context of angiogenesis. In this review, we provide an overview of the endothelial GJ function and Cxs expression found in pro-angiogenic condition and the functional role of endothelial GJ and Cxs in cell mechanics during the angiogenic process. Cell mechanics-based mechanisms hold promise the better understanding for physiological and pathological angiogenesis. Although several studies have demonstrated GJ-/Cx-dependent regulation of angiogenesis, the mechanisms are still speculative and controversial. Additionally, GJ- and Cx-mediated interactions in a number of other type cells such as vascular smooth muscle cell, pericyte, fibroblast, macrophage, and tumor cell also contribute to tumor angiogenesis through the expression of pro-angiogenic factors. Thus, further studies in the basic biology of GJ and Cx in these type cells would be required for elucidation with a particular emphasis on the interplay of angiogenesis and tumor development. We have speculated that GJ and Cx targeting approaches may be relevant to the development of the treatment of cancer patients.

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