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ORIGINAL ARTICLE

Blood flow and endothelial cell phenotype regulation during sprouting angiogenesis

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Abstract The role of the endothelial cell environment and shear stress induced by blood flow in phenotype determination and lumen formation has been clearly illustrated in recent studies. In the present work, a model is developed to map environmental and flow induced signals in sprouting angiogenesis to endothelial cell phenotype and lumen formation. To follow the endothelial cell lumen formation, its signaling pathway is incorporated in the present work within the phenotype determination pathway that has been recently utilized to model endothelial cell migration, proliferation, and apoptosis. Moreover, a signaling cascade for shear stress activation of endothelial cells is proposed and used for phenotype determination with activation of blood flow. A Boolean network model is employed to build a hybrid map for the relation between the endothelial cell environmental signals and the endothelial cell fate in sprouting angiogenesis with and without blood flow. This map is very useful in the development of models for sprouting angiogenesis. Moreover, this study shows that inhibition of intracellular signaling molecules, solely or in pairs, blocks angiogenic-signaling pathways and can be used to inhibit angiogenesis.

Keywords Lumen formation · Signal transduction · Blood flow · Boolean network · Shear stress

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1 Introduction

Angiogenesis, the formation of new capillaries from existing vasculature, is the main mechanism of vascularization during embryonic development and wound healing. It is also involved in processes such as tumor growth and metastasis. Blood vessels are constructed from endothelial cells (ECs) which are phenotypically inactive. In angiogenesis, external stimuli such as tumour angiogenic factors (TAF) changes EC to an active cell phenotype, which can decide to stay inert, go and/or grow. ECs of the nearby vessels start to migrate up along the TAFs gradient. Newly formed sprouts extend toward the tumour. During the extension, sprouts fuse together and form a network of capillaries. Blood flow starts in the network and successively remodels the network structure. Though dozens of cells and molecules are involved, ECs play the main role during the entire process of angiogenesis [37].

EC migration, proliferation, survival, and lumen formation are regulated by signals from extracellular matrix (ECM) and blood flow. Growth factors are the main sources of biochemical signals [23, 25]; while biomechanical signals originate from blood flow [1] and interaction of EC with ECM and with other ECs [3, 4].

Mechanosensing and mechanotransduction are performed by common proteins on the EC surface [9]. Moreover, similar signal transduction pathways are activated during vessel formation and acquirement of lumenal compartment [29]. These similarities allow studying signal transduction in angiogenesis in a unified framework. Accurate determination of EC phenotype is the main issue in this work. In the initial steps of the angiogenesis, single sprouts extend toward the tumour. EC phenotype is mainly regulated by biochemical and biomechanical signals from

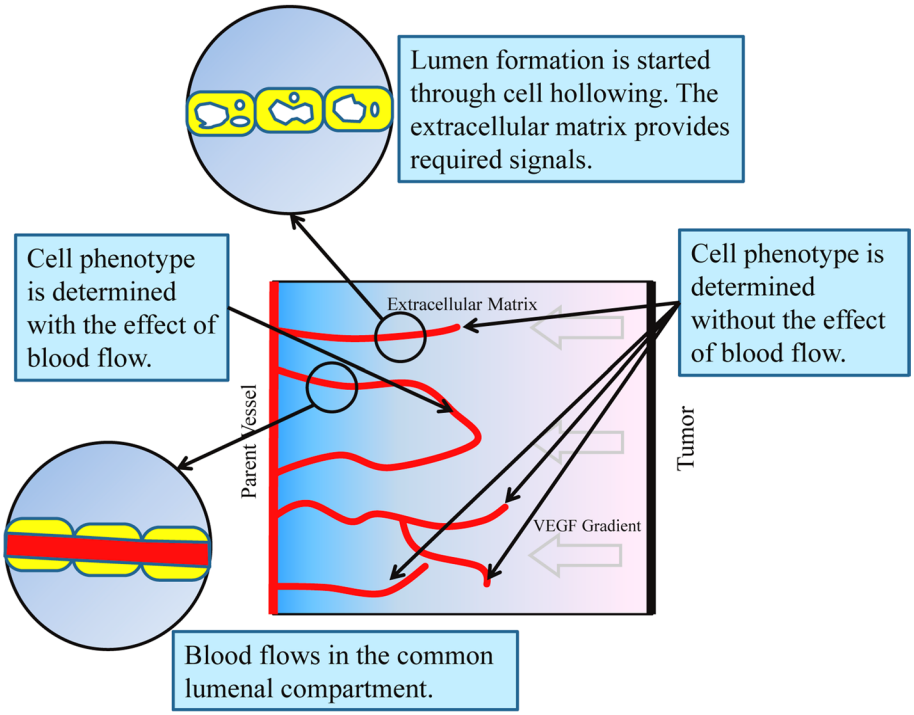


Fig. 1 Cell phenotype determination with and without blood flow

ECM; though, there are evidences from the role of interstitial flow in regulation of sprouting angiogenesis prior to anastomosis [67, 68]. After anastomosis and formation of closed loops, blood flow starts and regulates cell phenotype (Fig. 1). Different signaling pathways are activated before and after anastomosis; therefore, signaling cascades and phenotype maps are presented here in the two phases of vascular development, i.e. before anastomosis (without blood flow) and after anastomosis (with blood flow).

In EC phenotype determination before blood flow, integrins, vascular endothelial (VE) cadherin, and transmembrane receptors (such as tyrosine kinase receptors, G-protein-coupled receptors, tyrosine-kinase-associated receptors, and serine-threonine kinase receptors) are responsible for receiving EC environmental signals [49]. Lumen formation is a key step in vascular morphogenic events that is essential for blood flow, so lumenal compartment shall be formed during sprout extension and anastomosis. An increasing number of studies try to explain acquirement of lumenal compartments in blood vessels [5, 6, 8, 20, 29, 32, 35, 56]. Using in vivo and in vitro models, signaling cascade of lumen formation has been also investigated [5, 6, 16, 20, 22, 32, 35, 48, 63]. Blood flow induces shear stress on the inner layer of the capillary wall. A number of membrane molecules and microdomains mediate mechanotransduction of shear stress and its conversion into intracellular biochemical signals. There are several candidates as shear stress sensors including ion channels, caveolae, G-protein-coupled receptors, tyrosine kinase receptors especially VEGFR2, integrins, glycocalyx, and primary cilia [1, 2, 9, 33]. Though multiple receptors are involved in signal transduction before and after anastomosis, key events in sprouting angiogenesis are regulated by VEGF specific receptor tyrosine kinases (RTKs), integrins, and VE cadherins [4].

Mapping of environmental cues to specific cell phenotypes needs a model that takes into account the intracellular molecules interactions and receptor cross talk. Regarding the intracellular signaling molecules behaviour, Boolean models can be used to simulate signaling networks. Application of Boolean network in biological and medical modelling dates back to 1960s when Kauffman [36] used a Boolean network to model the genetic regulatory networks. Li et al. [47] used a Boolean model to develop a dynamic model of guard cell abscisic acid signaling. In the context of angiogenesis, Bauer et al. [3, 4] constructed a Boolean network model of critical signaling pathways in ECs.

In the current work, comprehensive signaling cascades and phenotype maps before start of the blood flow (including lumen formation) and after it is presented. The relation between environmental signals and EC lumen formation has not been considered in the previous models. To build an integrated model for EC lumen formation prediction and phenotype determination before start of the blood flow, critical signaling pathways of ECs and lumen formation are combined and used in a Boolean network model. Moreover, a clear relation between EC phenotype and flow induced shear stress has not been presented in the previous

works, so critical signaling pathways of ECs in the presence of blood flow are proposed in this work. The signaling cascades for lumen formation and shear stress are used to develop a hybrid map to relate environmental and flow induced signals to EC phenotype and lumen formation. This map is used in tumor-induced angiogenesis simulations [7]. In addition, the model developed in this study is used to propose strategies to inhibit angiogenesis (anti-angiogenic therapy) through blockade of intracellular signaling molecules.

2 Methods

Interaction of EC surface receptors with ECM activates signaling pathways inside EC. Shear stress also activates signaling pathways inside EC. Though there are a few common signaling molecules between the pathways, different signaling pathways are activated by shear stress and interaction with ECM. Figure 2 shows a schematic from signaling pathways in EC that are activated by shear stress and interaction with ECM.

To obtain a map between environmental signals and lumen formation before anastomosis, a Boolean network model is used. When blood flow starts, the main regulator of cell phenotype is the shear stress induced by blood flow. The proposed signaling cascade for shear stress activation of EC is used to create a map between the activation signals and cell phenotype. Finally, the maps are unified to build a hybrid map for cell phenotype determination with and without flow. The reader is referred to the previous works for the descriptions and details of the method [3]. The main software that is used in this study is RBN Toolbox with some modifications on the source codes [64].

2.1 Signaling cascade of lumen formation

The signaling cascade of lumen formation is the bridge between environmental signals and EC response. Interaction of integrins and extracellular matrix activates a cascade of events in ECs. Downstream of integrin signaling, activation of Cdc42, Rac1, and Src plays a substantial role in vascular lumen formation [6, 14, 16, 38]. Cdc42, Par6, Par3, membrane type1-matrix metalloproteinase (MT1-MMP), and integrin coassociate to control EC lumen formation [60].

Downstream of Cdc42 and Rac1, other proteins are activated to transduce signals and modulate cell cytoskeleton.

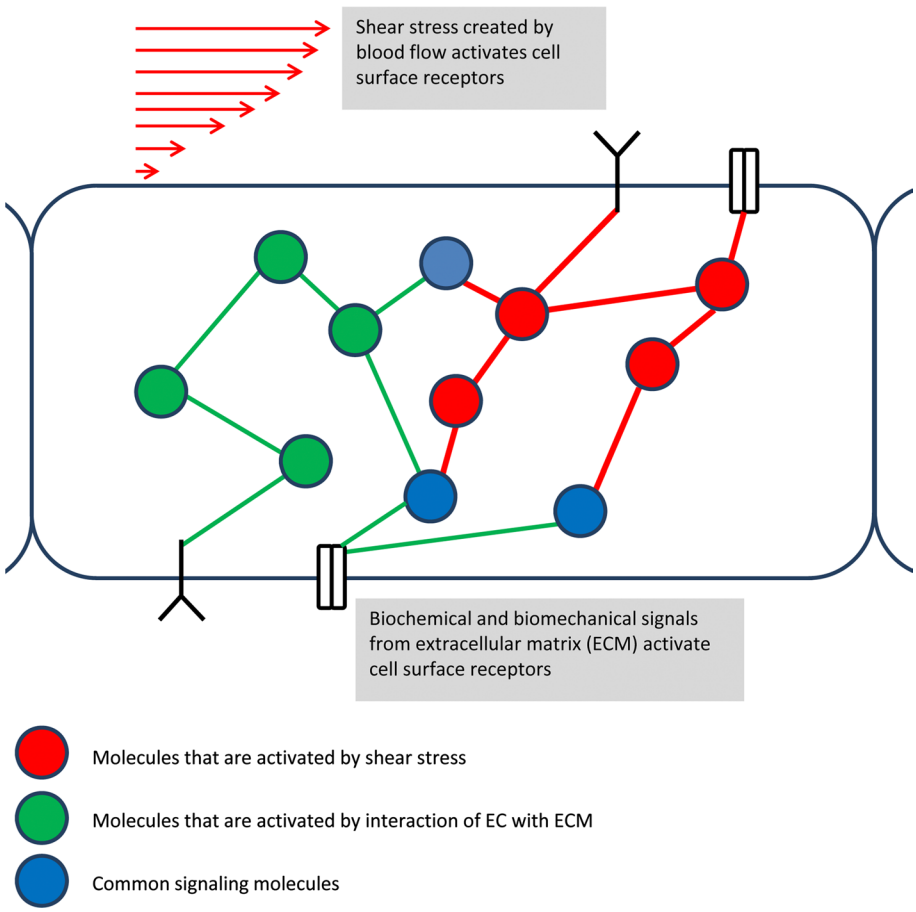


Fig. 2 Schematic from signaling pathways inside an EC

Small GTPase Rac1 activates Pak2. Cdc42 activates Pak2 and Pak4. Pak2 and Pak4 are also activated by protein kinase C (PKC), especially isoform PKCε [20, 39]. Cdc42 activates Par3, Par6, and PKCζ. Activation of Src, Pak2, Pak4, Par3, Par6, and PKCζ is required for lumen formation.

One required step in lumen and tube formation is the establishment of cell polarity [22]. An EC has two faces, one that looks the central luminal area (apical) and the other one that is connected to extracellular matrix (basal). Some proteins inside the cell control the establishment of apical-basolateral polarity. The main known proteins responsible for cell polarity in ECs are Par proteins including Par3, Par6, and atypical PKC isoform ζ. Cadherin also showed to have direct role in regulation of cell polarity [28]. Based on the available in vivo and in vitro experiments, Davis et al. [20] proposed a signal transduction pathway for EC lumen formation. A schematic of the proposed signaling pathways is shown in Fig. 3a. In the proposed signaling pathway for lumen formation in Fig. 3a, phorbol esters (that are known to enhance angiogenesis and lumen formation) used as an external activator of PKCε. It is assumed that in the process of sprouting angiogenesis, external signals exist to activate PKCε.

To incorporate the lumen formation signaling cascade model into the phenotype determination signaling pathway, a few simplifications on the signaling cascade are required. The signaling pathway in Fig. 3a is simplified through nodes information integration. Rac1 and Cdc42 are integrated in a single node without loss of information [4]. Pak2 and Pak4 are also integrated into a single node (Pak) and Par3 and Par6 into Par. The incorporation of signaling pathway of lumen formation into the signaling pathways of cell phenotype determination is performed and the result is presented in Fig. 3b.

In the schematic presented in Fig. 3b, receptors cross talk and Boolean dependence relations between intracellular molecules are shown. In this schematic, an arrow indicates an activation signal while a hammerhead indicates inhibition signal. In each box or node, the first line is the node title (signaling molecule) and the second line is its Boolean dependence relation with other nodes. The Boolean dependence relations determine activation or deactivation of nodes. For example, Grb-2/Sos activates Ras. Ras activates Raf-1 and contributes in activation of PI3K (with FAK). The Boolean operators in the dependence relation are very important in construction of the network.

The three main surface receptors of ECs are considered for this network. The first, cell–cell contact or cadherin is representative of VE cadherin in endothelial cells. The second one is RTK and represents chemical signals from VEGF in the domain. The third one, integrin, is responsible for receiving biomechanical signals from ECM molecules, which reflects the amount of attachment of ECs to ECM molecules such as matrix fibres. Activation or deactivation of any receptor and the downstream effectors, directly affects cell response. In ECs, the response is cell phenotype, i.e. proliferation, apoptosis, and/or motility, or lumen formation.

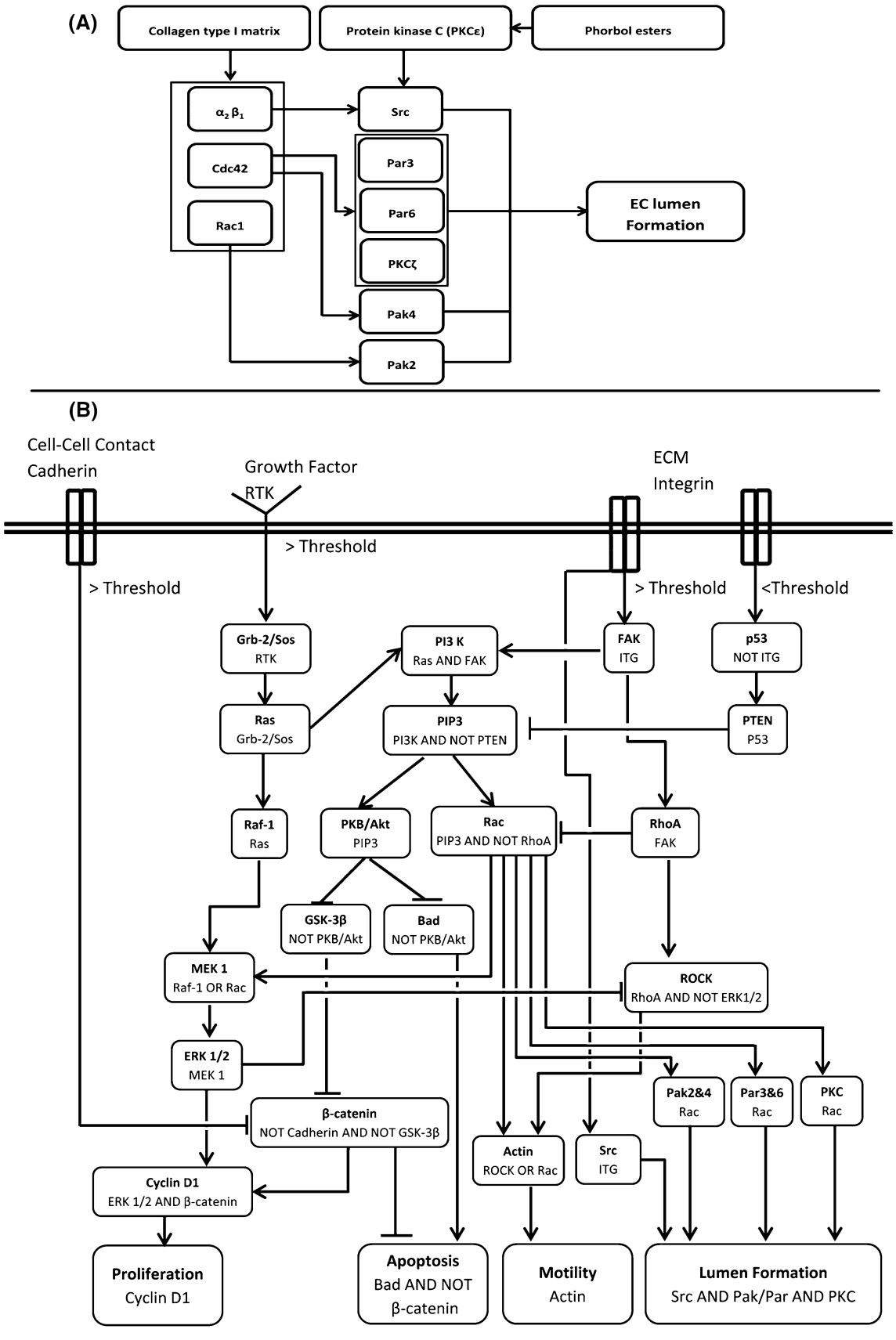
Fig. 3 **a** Schematic of general signaling network for EC lumen and tube formation. **b** Schematic of signaling network and receptor cross talk for EC phenotype and lumen formation

Rac and Rho are the main agents in cross talk between signaling pathways. Different feedback mechanisms for interplay between Rac and Rho are reported in the literature [51, 61, 62, 72]; however, no definitive mechanism for interaction of Rac with other signaling molecules especially Rho is mentioned in the literature. In the model developed here, inhibitory effect of Rho on Rac, is utilized [3].

2.2 Shear stress activation of ECs

There are considerable evidences on the effect of shear stress on EC function and phenotype. Cultured ECs reorient their longitudinal axis according to the streamlines of the flow. This will reduce the effective shear stress on ECs [70]. Several studies show that shear stress has a pivotal role on EC survival and prevention of apoptosis [21, 34]. There is also evidence that shear stress impacts EC proliferation [33, 42]. In wound healing, laminar shear stress enhances EC migration [27, 44]. In microcirculation, e.g. for a capillary network, shear stress may play a role in guidance of EC migration along the interstitial flow paths [45]. It is also reported that shear stress stimulates ECs to produce vasodilators [13, 53].

At the intracellular scale, experimental studies determine the role of cell surface receptor and intracellular signaling molecules in signaling cascade of shear stress. Integrin is involved in shear stress mechanotransduction [66] and activation of receptor tyrosine kinases (RTKs) [46]. Activation of integrin activates FAK, paxillin, c-Src, Fyn, and P130, which leads to activation of Ras-ERK pathway [30, 43]. The ERK pathway is involved in cell growth and proliferation [73]. Shear stress also activates RTKs including VEGFR2 and Tie2. The activation of RTKs is independent from VEGF [11, 31, 40, 65]. Activation of RTKs activates MAPK pathways including ERK, JNK, PI3K, and Akt through activation of Ras. These pathways are the main regulator for cell survival and inhibition of apoptosis [1, 33]. Moreover, shear stress causes rapid tyrosine phosphorylation of PECAM-1 [50]. Activation of ERK is dependent on PECAM-1 [54]. PECAM-1, VEGFR2, and VE-cadherin form a complex mechanosensory system. This system has a critical role in transduction of shear stress signals [71].


(A)
Collagen type I matrix
Protein kinase C (PKCε)
Phorbol esters
α2 β1
Cdc42
Rac1
Src
Par3
Par6
PKCζ
Pak4
Pak2
EC lumen Formation
(B)
Cell-Cell Contact
Cadherin
Growth Factor
RTK
ECM
Integrin
> Threshold
> Threshold
> Threshold
<Threshold
Grb-2/Sos
RTK
PI3 K
Ras AND FAK
FAK
ITG
p53
NOT ITG
Ras
Grb-2/Sos
PIP3
PI3K AND NOT PTEN
PTEN
P53
Raf-1
Ras
PKB/Akt
PIP3
Rac
PIP3 AND NOT RhoA
RhoA
FAK
GSK-3β
NOT PKB/Akt
Bad
NOT PKB/Akt
MEK 1
Raf-1 OR Rac
ROCK
RhoA AND NOT ERK1/2
ERK 1/2
MEK 1
Pak2&4
Rac
Par3&6
Rac
PKC
Rac
β-catenin
NOT Cadherin AND NOT GSK-3β
Actin
ROCK OR Rac
Src
ITG
Cyclin D1
ERK 1/2 AND β-catenin
Proliferation
Cyclin D1
Apoptosis
Bad AND NOT β-catenin
Motility
Actin
Lumen Formation
Src AND Pak/Par AND PKC

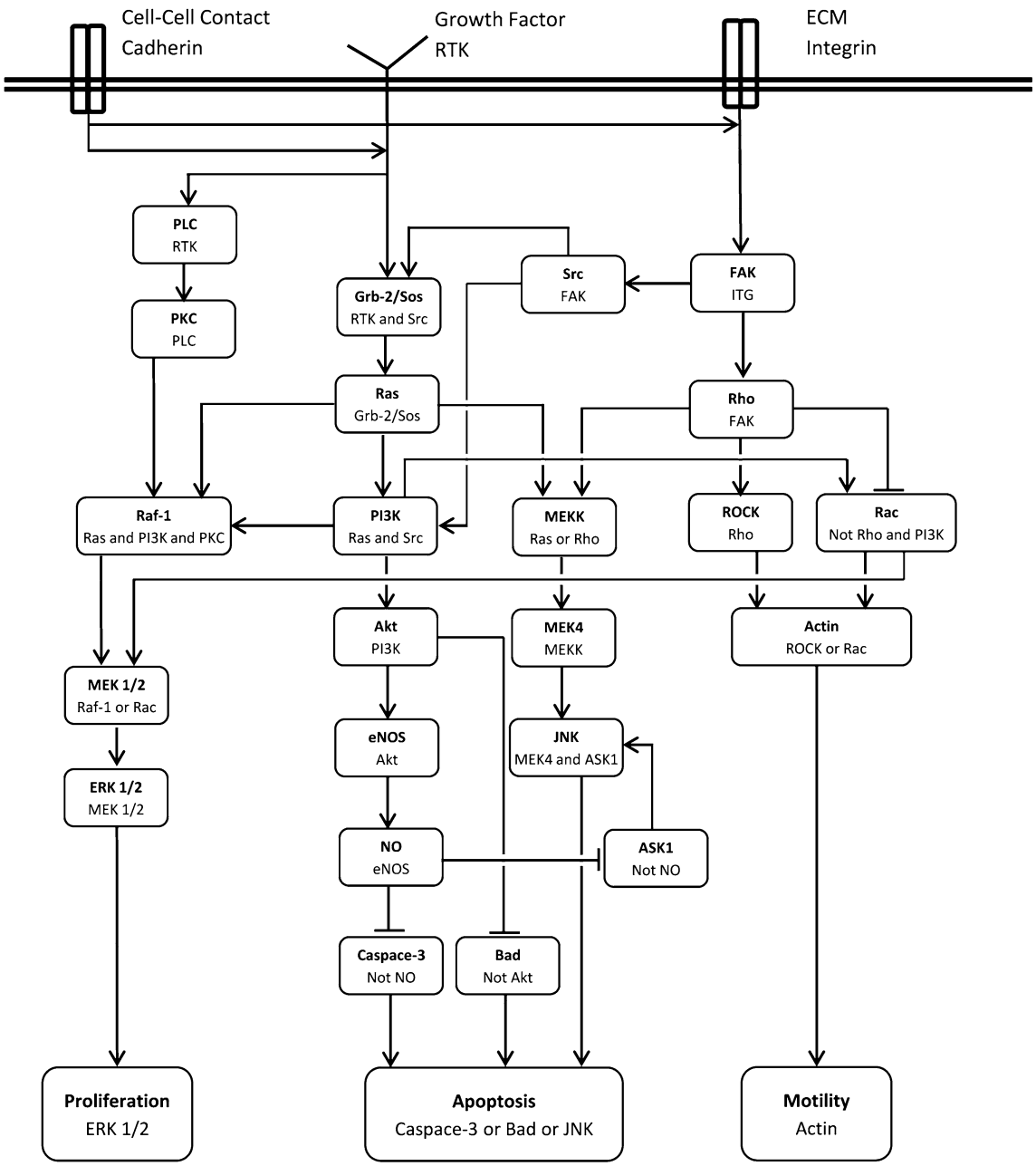


Fig. 4 Signaling cascade of shear stress activation of EC

PECAM-1 and VE-cadherin are necessary for shear stress activation of integrin [2].

Different candidates for shear stress sensors in ECs were introduced, however, as mentioned before, main events in ECs are regulated by RTK, integrin, and VE-cadherin. Based on the available experimental data, a signaling cascade activated by shear stress is proposed here and shown in Fig. 4.

Table 1 outlines the Boolean dependence relation of the network shown in Fig. 4 and the corresponding references for each relation. The signaling cascade presented in Fig. 4 is analysed for the relation between input signals and cell phenotype.

3 Results and discussion

Results are presented in two main sections. In the first section, the model output is used to build a map for the relation between local signals and EC phenotype and lumen formation. In this section, specific strategies also are tested in the model to inhibit angiogenesis. These strategies are

Table 1 Nodes dependence relation and corresponding reference for shear stress signaling cascade

Node	Dependence relation	References
Integrin	VE-cadherin and flow	[2, 30, 33, 43]
RTK	Flow	[50]
PLC	RTK	[1, 49]
Grb-2/Sos	RTK and Src	[12]
FAK	Integrin	[2, 11, 49, 65]
Src	FAK	[49]
PKC	PLC	[49]
Ras	Grb-2/Sos	[1, 11, 12]
Rho	FAK	[49]
PI3K	Ras and Src	[3, 4]
MEKK	Ras or Rho	[1]
ROCK	Rho	[49]
Rac	Not Rho and PI3K	[3, 4]
Raf-1	Ras and PI3K and PKC	[1, 41, 49, 70]
MEK1/2	Raf-1 or Rac	[49, 58]
Akt	PI3K	[49, 50]
MEK4	MEKK	[1]
Actin	ROCK or Rac	[49]
ERK1/2	MEK1/2	[1, 49]
eNOS	Akt	[49, 50]
JNK	MEK4 and ASK1	[1, 55]
NO	eNOS	[49, 50]
ASK1	Not NO	[9]
Caspace-3	Not NO	[55]
Bad	Not Akt	[55]
Proliferation	ERK1/2	[26, 49]
Apoptosis	Caspace-3 or Bad or JNK	[55]
Migration	Actin	[49]

based on blockade of intracellular signaling molecules that may change phenotype and stop the angiogenesis process. In the second section, considering cell phenotype alteration due to blood flow, a hybrid map is obtained to explore cell phenotype determination and lumen formation with and without blood flow.

3.1 Mapping environmental signals to cell phenotype and lumen formation

The Boolean network model maps environmental cues to cell phenotypes and lumen formation. The model takes the receptors status as inputs. Then, with a random set of initial conditions for intracellular signaling molecules, the solution starts and iterates toward the final converged state. The converged state is cell phenotype and/or ability of EC to form lumen.

The state of Rac plays a central role in receptor cross talk in this model. This is verified by activating or deactivating this node, which results in different cell phenotypes [4]. For more explanation about the role of Rac and VE-cadherin in the ECs signaling cascade in angiogenesis, consult Ref. [3]. Considering two possible states for Rac (activation and deactivation) and three environmental input signals, there are sixteen possible states for the model initial conditions. For each possible state, a Boolean network model is constructed and its converged state is obtained.

In order to summarize results in a single map, the model output for all sixteen possible states of inputs and Rac are gathered in Fig. 5. A sample of the network evolution for box number 14 is also shown in the top of Fig. 5.

Each surface receptor and signaling molecule in Fig. 3 is represented by a white or black node in the leftmost column in the sample network evolution in top of Fig. 5. The nodes are numbered from 1 to 28. Nodes 1–3 correspond to the three surface receptors, i.e. cell–cell contact, RTK, and integrin, respectively. Nodes 4–24 are intracellular signaling molecules. The outputs are states of the nodes 25–28 that determine cell phenotype and lumen formation. The first column in the sample of network evolution in Fig. 5 is the initial condition of the nodes. The initial conditions for nodes 1–3 are the input signals, and for nodes 4–28 are set randomly. Iterations run from left to right and the rows show nodes evolutions during iterations. After 20 iterations, the last column shows the output signals. In the first eight iterations, column 1–8, the nodes change their states until a converged set of nodes states is obtained in iteration 9 and stays fixed until the end of solution.

It should be mentioned that the network output (state of nodes 25–28 in the last time step) is independent of initial conditions of intracellular signaling molecules (nodes 4–28). This is verified by multiple runs from several initial condition sets, which all converge to a unique set of phenotypes.

In Fig. 5, lumen formation is determined based on input signals. In the input state, the states for RTK and ECM integrin are determined. Rac activation status is on the left side of the map and contact inhibition (activation of cadherin signal) is on the right side of the map. Cadherin signal determines whether the endothelial cell has enough cell–cell contact or not. Each box in the table corresponds to a combination of input signals and Rac activation status. The resulting phenotype in the table is a combination of proliferation, apoptosis, migration, and lumen formation. The outputs are determined by letters, respectively (P: proliferation, A: apoptosis, M: migration (motility), L: lumen formation). It should be mentioned that in case of activation of apoptosis (A in the phenotype in box numbers 1–12), the cell is considered to undergo apoptosis, and other cell phenotypes are not considered. In a special case in box number 15 there is no output signal, which means that the cell is quiescent.

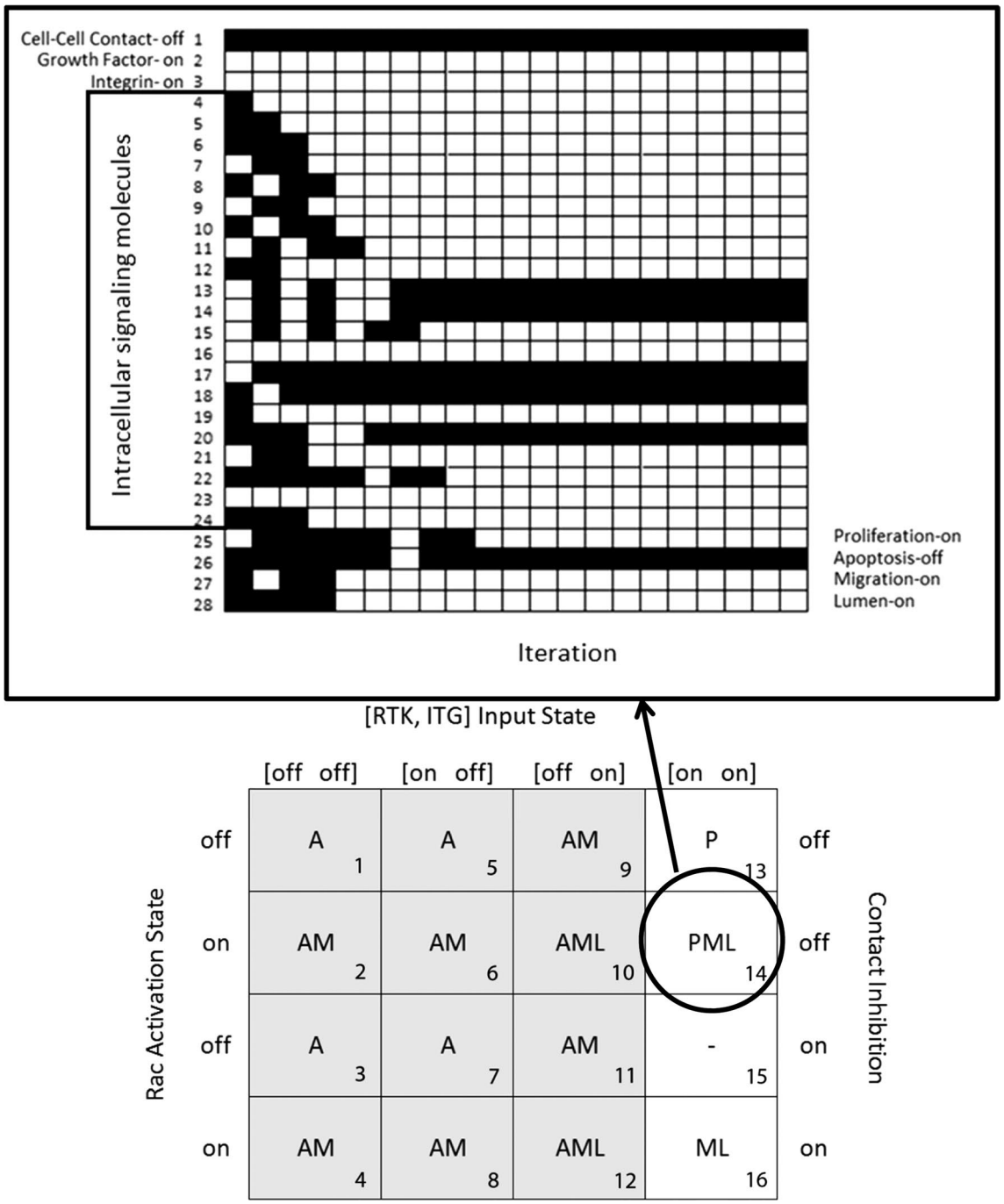


Fig. 5 Prediction of cell phenotype and lumen formation based on external signals

This model is developed based on the results of multiple in vivo and in vitro experimental models. Fundamental role of integrin in EC lumenogenesis has been shown by multiple in vitro and in vivo models [5, 17, 18, 24, 59]. Receptor blocking antibodies of integrin, completely inhibits Lumenogenesis in ECs [17, 39]. The Map developed in this study agrees completely with the experiments on the role of integrin in lumenogenesis. Moreover, it is shown that blocking antibodies of integrin can cause collapse of an existing capillary network [10, 19]. This is due to the apoptotic signal that is produced when integrin signal is off.

The key role of Cdc42 and Rac1, collectively named as Rac in this study, in vascular lumenogenesis is also shown by multiple experimental studies [6, 52, 57, 74]. Davis et al. [20] show that siRNA suppression of Cdc42 and Rac1 significantly inhibits lumenogenesis in ECs. Accordingly,

in the map developed in Fig. 5, ECs form lumen only when Rac (Cdc42 and Rac1) activation state is on.

3.2 Targeted inhibition of signaling molecules and anti-angiogenic strategies

The model developed in this study is used to examine the effect of targeted inhibition of specific signaling molecules on cell phenotype. In this method, using blocking antibodies or technologies to suppress the expression of individual genes, specific signaling molecules are inhibited. This means that the inhibited node cannot transduce the signals to downstream effectors. In the model, inhibition of signaling molecules is equivalent to setting the output signal of the inhibited node permanently off, regardless of the input signals. Inhibition of intracellular signaling molecules can change the cell fate. The change may make the ECs unable to form lumen, proliferate, and migrate. In ideal conditions, the ECs undergo apoptosis. Making the ECs unable to form lumen or drive them to undergo apoptosis are excellent strategies to inhibit angiogenesis. Phenotype alterations may disrupt the angiogenesis process and are also considered as anti-angiogenic strategies. To examine this concept, cell phenotypes correspond to inhibition of intracellular signaling molecules are presented in Table 2.

Table 2 Summary of the inhibited nodes with desired anti-angiogenic effect and the mechanism of production of the effect

Inhibited nodes	Anti-angiogenic effect
Grb-2/Sos	Apoptosis
Ras	Apoptosis
PI3K	Apoptosis
PIP3	Apoptosis
PKB/Akt	Apoptosis
FAK	Apoptosis
Rac1	Inhibition of migration
β-Catenin	Inhibition of proliferation
Actin	Inhibition of migration
Cyclin D1	Inhibition of proliferation
Src	Inhibition of lumen formation
Pak/Par	Inhibition of lumen formation
MEK 1 and Raf-1	Inhibition of proliferation
MEK 1 and ERK 1/2	Inhibition of proliferation
MEK 1 and GSK-3β	Inhibition of proliferation
MEK 1 and Bad	Inhibition of proliferation
MEK 1 and p53	Inhibition of proliferation
MEK 1 and PTEN	Inhibition of proliferation
MEK 1 and RhoA	Inhibition of proliferation
MEK 1 and ROCK	Inhibition of proliferation
ERK 1/2 and Raf-1	Inhibition of proliferation
ERK 1/2 and GSK-3β	Inhibition of proliferation
ERK 1/2 and Bad	Inhibition of proliferation
ERK 1/2 and p53	Inhibition of proliferation
ERK 1/2 and PTEN	Inhibition of proliferation
ERK 1/2 and RhoA	Inhibition of proliferation
ERK 1/2 and ROCK	Inhibition of proliferation
ERK 1/2 and GSK-3β	Inhibition of proliferation

3.3 Cell phenotype alteration due to flow in the loop

When blood flows in the capillaries, it acts as the main regulator of cell phenotype. ECs are activated by shear stress magnitudes as low as 0.2 dyne/cm^2 [15, 69]. Activation means that both integrin and RTK are activated and this does not depend on their specific ligands [1, 31]; however, activation of RTK and integrin depends on the activation of VE-cadherin [2].

In cell phenotype determination without blood flow, apoptotic signal is dominant except for the cases that both RTK and integrin signals are active. Similarly, flow activates both RTK and integrin; as such, a hybrid map is derived for EC phenotype with and without blood flow. In the signaling cascade presented in Fig. 4, the Boolean network model has a fixed output, which is the activation of proliferation and migration signal. The final hybrid map, which is obtained from integration of the model results with and without blood flow is shown in Fig. 6.

The outputs are shown by letters, P: proliferation, M: migration, PM: proliferation and migration, PML: proliferation, migration and lumen formation. When blood flows in the capillary and creates shear stress on capillary walls, activation of integrin and RTK depends on VE-cadherin. This means that when VE-cadherin is not activated, the results are similar to no flow conditions. By contrast, when the VE-cadherin signal is active, the EC starts to proliferate and migrate, without relation to the Rac state.

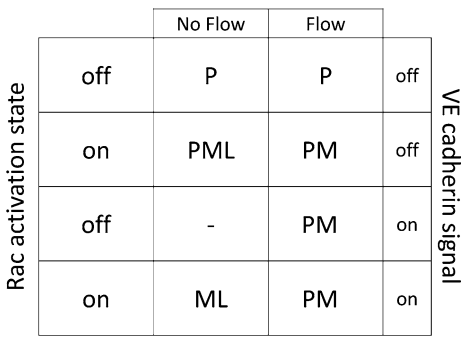


Fig. 6 Hybrid map for EC phenotype considering both flow and no flow conditions

4 Conclusions

A Boolean network model of receptor cross talk for lumen formation is presented. Moreover, signaling cascade of shear stress activation of ECs is proposed and the resulting phenotypes in angiogenesis are obtained. This is, to our knowledge, the first study that presents a comprehensive model of cell phenotype determination and lumen formation based on environmental cues and blood flow. Effect of inhibition of each intracellular signaling molecule and possible anti-angiogenic effects are also investigated in this study. The model predicts that inhibition of intracellular signaling molecules, solely or in pairs, can be used to inhibit angiogenesis, thus posing a strategy to achieve anti-angiogenic effects. Moreover, this study shows the dependency of phenotype to blood flow. ECs survive when both RTK and integrin are activated, with and without flow. The hybrid map developed in this study is a valuable map in modelling sprouting angiogenesis.

Compliance with Ethical Standards

Conflict of interest None.

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