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Review

In silico analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles

Judy R. van Beijnum, Arjan W. Griffioen*

Angiogenesis Laboratory, Research Institute for Growth and Development, Departments of Internal Medicine and Pathology, Maastricht University Hospital, PO Box 5800, 6202AZ Maastricht, The Netherlands

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Abstract

In vitro models have been extensively used to map gene expression in ECs but few studies have used cells from in vivo sources directly. Here, we compare different gene expression surveys on both cultured and fresh tissue derived ECs, and it emerges that gene expression profiles can be paralleled with the angiogenic stage of the cells. ECs stimulated with different growth factors in monolayer cultures exhibit gene expression profiles indicative of an active proliferative state, whereas gene expression in tube forming cells in vitro involves genes implicated in cell adhesion processes. Genes overexpressed in tumor ECs are biased towards extracellular matrix remodeling, a late event in angiogenesis. The elucidation of gene expression profiles under these different conditions will contribute to a better understanding of the molecular mechanisms during angiogenesis in both pathological and physiological circumstances and will have implications for the development of angiogenesis interfering treatment strategies.

Keywords: Angiogenesis; Gene expression profiling; Tumor endothelial marker; Extracellular matrix; Angiogenesis assay; EC heterogeneity

Contents

* Corresponding author. Tel.: +31 43 3874630; fax: +31 43 3876613.
E-mail address: AW.Griffioen@path.unimaas.nl (A.W. Griffioen).

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1. Introduction

1.1. Endothelial cell biology and angiogenesis

Endothelial cells (ECs) are among the most quiescent cells of the body since their turnover rate can be up to hundreds of days. However, during the growth of new capillaries from pre-existing blood vessels (angiogenesis), activated ECs can proliferate as rapidly as bone marrow cells [1], highlighting their capacity to readily respond to microenvironmental changes. Angiogenesis is in essence a physiological phenomenon and is a hallmark of processes such as wound healing, embryonic development, tissue remodeling and the female reproductive cycle, in which tissue growth is essential. However, angiogenesis also occurs during pathological conditions such as diabetic retinopathy, rheumatoid arthritis and cancer. The role of angiogenesis in the progression of cancer has gained particular interest once it became clear that the growth, invasion and metastasis of tumors are dependent on the formation of blood vessels [2]. In addition to providing a metastatic route, the vasculature provides the indispensable metabolic support for the tumor. Without adequate blood supply, the size of tumors is limited due to deprivation of oxygen and nutrients in the tumor cell mass. In response to these limitations, tumors start to produce angiogenic factors that activate ECs to initiate sprouting from nearby preexisting vessels [2]. The genetic change of tumor cells, leading to the formation of tumor vasculature, is referred to as the angiogenic switch [1,3]. In the tumor microenvironment, both anti-angiogenic and pro-angiogenic factors are expressed, and angiogenesis is induced once the balance tips over in favor of the latter. The expression of pro-angiogenic factors can be induced by hypoxia, and also by oncogene activation or tumor-suppressor gene mutations; the relative contribution of these events is likely to be determined by the specific type and location of the tumor [3,4]. A variety of endothelial growth factors have been described, among which vascular endothelial growth factors (VEGFs) [5], fibroblast growth factors (FGFs) [6], hepatocyte growth factor (HGF) [7] and the angiopoietins [8].

Once angiogenesis is induced, a sequence of events is set in motion. Angiogenic sprouting comprises (i) local degradation of the basement membrane and extracellular matrix surrounding the capillaries, (ii) migration of ECs in the direction of the tumor mass, (iii) proliferation of ECs, (iv) differentiation and (v) subsequently the formation of tube-like structures, in which attraction of pericytes and smooth muscle cells and deposition of new extracellular matrix occurs. During the final stages, the extracellular matrix is remodeled, and sprouts will fuse with each other to create a circulation [4,9].

Tumor progression is highly dependent on the development of a tumor vasculature by angiogenic sprouting. The precise requirements of tumors, however, may vary among different tumor types and during tumor progression. Hence, tumor phenotype heterogeneity dictates tumor vasculature heterogeneity [4]. It has been recognized that tumor vessels are irregularly shaped, tortuous and often leaky. Under normal conditions, vessel density is tightly controlled and newly formed vessels rapidly mature, but the aberrant balance of angiogenic factors produced in malignancies result in a chaotic tumor vasculature [4]. Tumor vessels fail to become quiescent; therefore, tumors have been described as ‘wounds that never heal’ [10].

1.2. Angiogenesis inhibition

Given the observation that most tumors are angiogenesis dependent, both for metabolic support and for metastatic dissemination, inhibition of angiogenesis is thought to be a promising strategy for the treatment of solid tumors [11], and likely hematological malignancies as well [12,13]. The concept of angiogenesis inhibition for cancer treatment has gained much attention over the past years and a number of different strategies have been employed, based on knowledge of EC biology and behavior during angiogenesis. To this purpose, synthetic agents have been developed that act on ECs, a number of endogenous proteins and peptides with anti-angiogenic actions have been discovered, but also the use of antibodies to interfere with the angiogenesis process has been explored.

In addition to interfering with the unwanted de novo formation of blood vessels, targeting existing tumor blood vessels for destruction resulting in tumor regression (vascular targeting), may also hold promise. ECs express genes that are not or to a lower extent expressed by other cell types [14,15] and this may offer the possibility of using gene products that are specific for a certain subset of ECs for targeting purposes. It has been shown that ECs exhibit differences in gene and protein expression, relating to their microenvironmental origin and activation status [16,17].

Tumor blood vessels express specific markers that are not present on normal blood vessels and these markers can be present on the ECs themselves, on pericytes and on the extracellular matrix [18]. By in vivo phage display selections, it was demonstrated that ECs express ‘address molecules’ that differ according to their location in the body and their specific signature [19,20]. A phage-displayed peptide that specifically bound to tumor vasculature contained an RGD sequence, known to bind integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ [19]. Another tumor vasculature specific peptide, containing the NGR sequence, was shown to bind aminopeptidase N, also known as CD13 [21]. Although this protein is also expressed in several epithelial tissues and macrophages, the cyclic NGR peptide does not bind to these cells, indicating a specific form of aminopeptidase N is present on angiogenically activated ECs [22]. Other markers that are upregulated on tumor ECs include prostate-specific membrane antigen (PSMA) [23], CD44 [17] endoglin [24] and the oncofetal ED-B domain of fibronectin [25], whereas CD34 and ICAM-1 are suppressed [16,26].

Probably, the most crucial element in both anti-angiogenic and vascular targeting approaches is the identification of specific target molecules. Since angiogenesis is not limited to pathological conditions, careful evaluation of the putative targets is warranted to prevent side effects associated with impaired physiological angiogenesis. Gene expression studies can be very helpful tools in elucidating the transcriptional differences between ECs of different sources and to identify tissue or tumor endothelial specific markers. Furthermore, gene expression analysis in this context will lead to a better understanding of EC biology during (tumor) angiogenesis. Different models have been developed to study angiogenesis, but the temporal and spatial complex actions of all factors exerting effect on ECs in vivo may not be accurately reflected in vitro.

Here, we will overview different studies that used large-scale gene expression profiling of ECs in different stages of angiogenic activation in vitro and in vivo. Gene expression changes associated with the different stages of angiogenesis that emerge from these studies are discussed and related to their potential use in the development of angiogenesis inhibitors or vascular targeting agents.

2. Gene expression profiling in angiogenesis research

2.1. Expression profiling techniques

Large-scale gene expression profiling techniques are widely used to detect changes in transcript expression levels and provide the tools to study molecular events in biological processes. For the comparison of two or more cell types, a number of techniques have been developed and employed. Serial analysis of gene expression (SAGE) provides a quantitative analysis of large numbers of transcripts, based on sequence analysis of short specific, concatenated tags. Multiple direct comparisons are possible by assessing the frequencies at which transcripts are present in the libraries [27]. Another technique, suppression subtractive hybridization (SSH) combines an effective cDNA subtraction between two samples with the requirement of only limited amounts of starting material and is, like SAGE, independent of previously cloned genes and existing sequence information [28]. SSH can be used in combination with cDNA arrays to screen for differentially expressed genes [29]. cDNA can be spotted on nylon filters for hybridization with radioactively labeled probes, usually referred to as macroarrays due to their relatively low spot density and their large size. Sequential hybridization of different samples to identical arrays allows for comparison of gene expression in the different probes.

Differential display provides a systematic and unbiased procedure to detect differences in mRNA species [30] and GeneCalling™ is a modification of this technique. Double-stranded cDNA is digested with pairs of restriction enzymes, amplified with specific linkers and resolved on polyacrylamide gel. Based on fragment length and the combination of restriction enzymes used, transcripts are identified using sophisticated database searches [31].

The advent of microarray technology has boosted gene expression profiling research [32]. High-density robotic printing of thousands of cDNAs on glass slides and subsequent two-color fluorescent hybridization and detection allows for a quantitative comparison of genes in complex probe mixtures [32]. Oligonucleotide arrays are a variation on the theme of microarrays. Complex probes of one experimental sample at the time are hybridized and signal intensities are measured by scanning [33]. A limitation of the latter techniques is that they rely fully on known sequences, but ongoing efforts to characterize and annotate the human genome are likely to enable screening the majority of expressed transcripts in the near future. Furthermore, data management and analysis are key to success when using these large-scale gene expression profiling techniques [34]. Clustering analysis can help identify coordinately regulated genes that are likely to be functionally related in the applied experimental conditions [35]. All of these techniques have their own advantages and disadvantages related to scale, effort, cost and availability of sample material. Furthermore, techniques can be combined to enhance sensitivity or throughput.

2.2. In silico profiling of ECs

The availability of public gene expression data sets has opened additional doors for gene expression profiling studies. By using different bioinformatics tools to query existing databases, it is possible to identify genes that are expressed in a particular cell type [14,15] or to overlap data sets to identify genes that are co-regulated under different circumstances [36]. In silico comparison of existing data sets may also be helpful in the elucidation of gene expression on tumor endothelium. Available databases of gene expression profiles in ECs can be overlapped with expression profiles of tumors to identify commonly regulated genes [36,37]. A minor drawback of this approach might be that only limited gene expression data of ECs from in vivo sources is available to date. Also, formats need to be interchangeable for easy data transfer and data analysis.

In this review, we analyze published data sets of genes induced in growth-factor stimulated and tube forming ECs in vitro, as well as from ECs isolated from tumor tissues. Gene expression profiles of ECs treated with bFGF, VEGF, HGF, epidermal growth factor (EGF) and different combinations thereof are reviewed and common pathways identified that play key roles in the initial activation of ECs. Differentiation of ECs in tube-like structures can be accomplished by providing three-dimensional support, leading to the induction of genes associated with this process. Finally, genes identified to be overexpressed on tumor ECs of different types of tumors are discussed. This method allows filtering out interesting candidate genes

amenable for interference in angiogenic events in different stages of progression, and can provide tools to define therapeutic agents to perform these tasks.

2.3. Gene expression associated with cytokine and growth factor stimulation of ECs in vitro

Gene expression profiling has been applied to ECs treated with different pro-angiogenic cytokines in various models to elucidate genes involved in angiogenesis. SSH in combination with differential screening was used to study transcriptional changes in human umbilical vein ECs (HUVEC) cultured for 4 h in the presence of tumor conditioned medium (CM) from the melanoma cell line C8161 and the breast cancer cell line MDA-MB231 that contain high levels of angiogenic stimulators [38]. Twenty genes were confirmed to be upregulated, some of which had previously been associated with angiogenesis, e.g., E-selectin [39] and fibronectin [40]. Cytokines associated with angiogenesis that were identified in this study included monocyte-chemoattractant protein-1 (MCP-1) [41] and interleukin-8 [42]. One of the most interesting candidate genes for follow-up was endomucin. Endomucin is a highly glycosylated transmembrane protein expressed predominantly on vascular ECs [43] and high endothelial venules, where it can function as a ligand for L-selectin [44]. Its expression seems to correlate with EC proliferation as it is increased upon stimulation with bFGF, TNFα and under the influence of tumor conditioned medium [43]. Due to its elevated expression in angiogenically activated ECs and its cell surface localization, endomucin might be a putative tumor endothelium targeting candidate. However, it remains to be determined whether a sufficiently large therapeutic window can be created for therapies not to interfere with endomucin expressed on non-tumor ECs. Furthermore, although the authors state that this in vitro model provides an accurate stimulation of the tumor environment, one can question whether 4 h of stimulation with tumor-derived factors is sufficient for ECs to adopt a tumor endothelial phenotype since tumor initiation and progression is a relatively slow process.

By using HUVEC cultured for 3 days in the presence of bFGF, VEGF and supernatant of two colorectal tumor cell lines, Caco-2 and LS174T, we created SSH repertoires that were used for macroarray screening. Twenty different genes were identified that showed upregulation in both tumor derived ECs when compared to normal ECs (isolated from fresh tissues) and in HUVEC cultured in the above mentioned conditions compared to quiescent HUVEC (van Beijnum et al., manuscript in preparation). Functional annotation of the identified transcripts showed that most genes were indicative of a high proliferative status of the ECs, since more than one third of these genes are involved in protein turnover and metabolism such as ribosomal proteins L21 and S27, and HSP90. Furthermore, a number of genes have previously been associated with angiogenesis, such as thymosin β4, MMP-10 and integrin β1. Thymosin β4 is implicated in EC migration, proliferation and differentiation by interfering with actin polymerization [45]. Exogenously applied, it acts as a chemoattractant for ECs, stimulates migration, increases MMP production [46] and induces tube formation and sprouting [45,47]. The most interesting aspect of thymosin β4 is therefore that it affects EC biology both as endogenous and exogenous peptide, creating ample opportunities for modulating EC function.

A comparison of quiescent versus proliferating ECs stimulated with epidermal growth factor (EGF) by cDNA array revealed a large proportion of genes associated with cell cycle regulation to be upregulated in proliferating human dermal microvascular ECs (HDMEC) [48]. Various cyclins were induced, among which G1/S specific cyclins D1 and D2 and the mitosis specific cyclins B1 and G. Increased expression of cyclin D1 in response to VEGF and bFGF has later been reported [49]. Furthermore, cyclin D1 expression has been shown to be downregulated in ECs by the anti-angiogenic agent TNP-470 [50] and the endogenous angiogenesis inhibitor endostatin [49]. Endostatin induces cell cycle arrest in G1, associated with an inhibition of hyperphosphorylation of Rb protein and a downregulation of cyclin D1 promoter activity [49]. However, forced overexpression of cyclin D1 in ECs overrides the effect of endostatin [49]. The study by Zhang et al. [48] also confirmed the upregulation of the known angiogenesis stimulators interleukin-8, TIE-1 and TIE-2 in activated ECs. Furthermore, it stressed the involvement of different integrin subunits (αV, α5, α6 and β1) in EC adhesion and tumor angiogenesis [51,52]. As discussed earlier, integrins pose interesting candidate molecules for interference in angiogenesis [53,54]. Connective tissue growth factor (CTGF) was also upregulated in HDMEC in response to EGF and belongs to the CCN family of matricellular proteins. Several members of this family are positively involved in angiogenesis and their activities are mediated by integrins and by the induction of MMP expression [55,56]. CTGF (CCN2) and CYR61 (CCN1) are transcriptionally activated in ECs by bFGF and VEGF and are capable of promoting EC growth, migration, adhesion and survival. Furthermore, these proteins can act in a paracrine or autocrine fashion by upregulating the endothelial growth factors VEGF and bFGF [57]. CCN proteins have been recognized to be important regulators in angiogenesis during wound healing, placenta formation, fibrosis and tumor growth [57], hence attempts to interfere in the complex actions of these proteins with the aim of inhibiting tumor angiogenesis may turn out to be difficult.

Microarray analysis was used to identify genes induced by VEGF at different time points in HUVEC [58]. Over a period of 24 h, 139 genes showed induction by VEGF, of which 53 were induced within the first 2 h of VEGF stimulation. From this analysis, it was apparent that a number of genes show peak expression at early time points

and reduce their expression to basal levels within 12 h after stimulation with VEGF. Among the genes upregulated within 30 min were 3 transcription factors (early growth response (EGR) 1, EGR2 and EGR3) and 10 genes associated with cellular support, involved in metabolism, protein turnover and protein transport. Five known positive regulators of angiogenesis were identified: cyclooxygenase 2 (COX-2), heparin-binding epidermal growth factor-like growth factor (HBEGF), EGR1, CYR61 and angiopoietin-2. Induced by cytokines, cyclooxygenase-2 (COX-2) catalyzes the production of prostaglandins from arachidonic acid [59]. COX-2 metabolites promote Rac and Cdc42 activation resulting in αVβ3 integrin-mediated cell spreading and migration [60]. Furthermore, COX-2 inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs), have a protective role against the development of cancer [61], and this effect is partly due to diminished tumor angiogenesis via the inhibition of αVβ3-mediated Rac activation [51,62]. HBEGF has been associated with the induction of tube-like structures of ECs in type I collagen [63] and increased tumorigenicity associated with increased angiogenic potential [64]. EGR1 is a key transcription factor in ECs and mediates the VEGF induced upregulation of tissue factor, platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), as well as the transcription of membrane type 1 MMP (MMP-14), facilitating the invasion of ECs into the surrounding matrix [65]. Growth factor induced EGR1 transcriptional activity is inhibited by the NAB2 corepressor as well as by NSAIDs, the latter further explaining the observed protective role of these drugs [66].

A survey of genes regulated by VEGF, bFGF and the combination of both revealed only a partial overlap in expression profiles of HUVEC [67]. Interestingly, the combination of bFGF and VEGF induced a number of VEGF receptors, whereas the administration of VEGF alone failed to do so. Various cyclins and cyclin-dependent kinases were induced by the different treatments [67] Furthermore, EGR1 was identified to be induced by the combination of bFGF and VEGF, in contrast to the finding of Abe et al. who identified this transcription factor to be upregulated by treatment with VEGF alone [58]. It is interesting to note that bFGF treatment regulated the expression of the oncogenic transcription factors myc, jun and fos [67]. Induction of oncogenes was also observed in bFGF transformed ECs [68], suggesting a specific trait of bFGF action.

Relatively little overlap was also observed when the expression profiles of HUVEC treated with HGF, with VEGF, and with a combination of both were compared [69]. In addition, few genes were coordinately regulated after 4 h and 24 h of VEGF treatment. The importance of the above-mentioned key angiogenesis transcription factor EGR1 is underscored by the fact that both combination treatments (bFGF+VEGF and HGF+VEGF) induced its expression, evident already 1 h and 4 h following growth factor treatment, respectively [67,69].

The above described studies used cultured ECs stimulated with different growth factors as models for angiogenesis. It appears, however, that only a few genes are commonly regulated during growth factor stimulation of ECs (Table 1). Components of the MAPK signaling pathway (Jun, myc, MAP2K3) and cytokine signaling components (Flt1, IL-8, IL6ST, CCL2, TEK) were distinctly present, as were different cell cycle mediators (MCM2, CDC20, CCND2). Different effects on gene expression profiles by different growth factors and their combination was also evident in the studies performed by Gerritsen et al. [69] and Jih et al. [67], indicating largely separate signaling pathways are mediating the responses. Furthermore, different time points were analyzed which can have considerable effect on the identification of early acting genes and transcription factors that activate secondary genes. The gene expression platforms and data analysis in the above discussed studies differ considerably, creating additional bias. Further contributing to the observed differences can be the diversity of the ECs used in the above described studies. ECs of macrovascular origin, such as HUVEC [38,58,67,69], have recently been shown to differ substantially from microvascular ECs, such as HDMEC [48], by large scale gene expression profiling [70]. These differences are supposed to support local specialization of the vascular beds and consequently contribute to inherent differences in expression patterns.

When reviewing the functional classification rather than the identity of the genes reported to be upregulated in the six studies discussed, it emerges that cell cycle related genes make up the larger part of regulated genes, followed by genes associated with metabolism (Fig. 1, top panel). Such a 'cell cycle signature' of upregulated genes can be related to the transition from quiescent to proliferative ECs under the influence of these growth factors, an early event in angiogenesis.

2.4. Gene expression associated with tube formation of ECs in vitro

ECs grown in collagen type 1 gels form tube structures in vitro. During this differentiation process, changes in gene expression are evident. A time course of gene expression during capillary morphogenesis in 3D collagen matrices was studied using microarrays to compare expression profiles of HUVEC at 0, 8, 24 and 48 h [71]. Extracellular matrix components comprised the most abundant functional class of genes upregulated during capillary formation in this model. Most pronounced was the upregulation of collagen IVα1 and laminin γ1, both involved in basement membrane synthesis, assembly and ECM remodeling. Very interesting was the finding of a novel capillary morphogenesis gene, CMG-2, that encodes for a 45-kDa transmembrane protein and that showed binding to collagen type IV and laminin but not to fibronectin and osteopontin [71]. Integrin β1, that can dimerize with upregulated integrins α1 and α2, also shows

Table 1
Growth factor regulation of common players in in vitro models of angiogenesis

Function	Description	Gene	UniGene ID	Growth factor regulation	References
Cell cycle regulators and apoptosis related proteins	MCM2 minichromosome maintenance deficient 2, mitotin (*S. cerevisiae*)	MCM2	Hs.477481	VEGF VEGF+HGF	[58,69]
	CDC20 cell division cycle 20 homolog (*S. cerevisiae*)	CDC20	Hs.524947	VEGF VEGF+HGF	[67,69]
	Cyclin D2	CCND2	Hs.376071	EGF bFGF+VEGF	[48,67]
	DNA-damage-inducible transcript 3	DDIT3	Hs.370771	EGF bFGF+VEGF	[48,67]
	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	Hs.505777	VEGF bFGF+VEGF	[58,67]
	Caspase 3, apoptosis-related cysteine protease	CASP3	Hs.141125	VEGF EGF	[48,67]
Extracellular matrix proteins	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPINB2	Hs.514913	VEGF VEGF+HGF	[58,67]
Growth factors, cytokines and hormones	Angiopoietin 2	ANGPT2	Hs.549021	VEGF VEGF+HGF	[58,69]
	Chemokine (C–C motif) ligand 2	CCL2	Hs.303649	CM bFGF+VEGF	[38,67]
	Interleukin 8	IL8	Hs.624	VEGF EGF CM	[38,48,67]
	Diphtheria toxin receptor (heparin-binding EGF-like growth factor)	HBEGF	Hs.799	VEGF[3]	[58,67]
Metabolism	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2)	PTGS2	Hs.196384	CM VEGF	[38,58]
	Superoxide dismutase 2, mitochondrial	SOD2	Hs.487046	CM bFGF+VEGF	[38,67]
Protein turnover, modification and transport	Alpha-2-macroglobulin	A2M	Hs.212838	VEGF VEGF+HGF	[58,69]
Cell surface proteins, antigens, receptors and adhesion molecules	Low density lipoprotein receptor (*familial hypercholesterolemia*)	LDLR	Hs.213289	VEGF VEGF+HGF	[58,69]
	AXL receptor tyrosine kinase	AXL	Hs.466791	CM bFGF+VEGF	[48,67]
	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	ITGAV	Hs.436873	EGF VEGF	[48,67]
	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	Hs.218040	EGF bFGF	[48,67]
	TEK tyrosine kinase, endothelial	TEK	Hs.89640	EGF bFGF+VEGF	[48,67]
	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	TIE	Hs.788024	EGF bFGF+VEGF	[48,67]
	Thrombomodulin	THBD	Hs.2030	VEGF VEGF+HGF	[58,67,69]
	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	Hs.532082	VEGF VEGF+HGF	[67,69]
	Fms-related tyrosine kinase 1 (VEGF/vascular permeability factor receptor)	FLT1	Hs.507621	bFGF+VEGF VEGF+HGF	[67,69]
	Integrin, alpha 6	ITGA6	Hs.133397	bFGF+VEGF VEGF+HGF EGF	[48,67,69]
Signal transduction proteins	Mitogen-activated protein kinase kinase 3	MAP2K3	Hs.514012	VEGF+HGF bFGF	[67,69]
Transcription factors and chromatin associated proteins	Early growth response 1	EGR1	Hs.326035	VEGF+HGF bFGF+VEGF VEGF	[58,67,69]
	V-jun sarcoma virus 17 oncogene homolog (avian)	JUN	Hs.525704	bFGF EGF	

affinity for collagen type IV and laminin [72], stressing again the importance of extracellular matrix remodeling events during capillary formation. Also, a number of signaling pathways involved in capillary formation emerged from the data. JAK/STAT pathway signaling molecules were induced, among which gp130, leukemia inhibitory factor, JAK-1 and its inhibitor SOCS-1. Sprouty, a negative regulator of bFGF signaling, was upregulated. HUVEC transfected with a mouse sprouty4 showed a decrease in cell migration [73]. Inhibitor of differentiation genes (Id1, Id2, and Id1-3) were downregulated, as were ephrin A1 and the Eph receptors B1 and B4. This is intriguing since these proteins have been implicated in the promotion of (tumor) angiogenesis [74]. However, they might play a temporal role during the different stages of angiogenesis [75,76]. Id genes are implicated in the control of cell cycle progression and cell differentiation [77]. Heterodimers of basic helix–loop–helix (bHLH) transcription factors normally bind E-boxes in the promoters of differentiation genes, and Id proteins prevent transcription of these genes by forming non-functional heterodimers with bHLH proteins. Upregulation of Id3 can therefore prevent differentiation of ECs. Furthermore, Id proteins are upregulated in tumor ECs and induce the expression of $\alpha V\beta 3$ integrin that binds matrix metalloproteinase-2, required for EC migration, indicating that Id proteins might be involved in the switch from quiescent to angiogenic ECs. Loss of Id1 and Id3 leads to a decreased VEGF production and impaired tumor angiogenesis, suggesting that blocking Id protein expression might be an approach to block angiogenesis [77]. Indeed, knockdown of Id3 by RNA interference was able to overcome VEGF induced proliferation, migration and tube formation in HUVEC [78]. Id proteins are also required for neurogenesis [77] and it has recently been appreciated that many genes

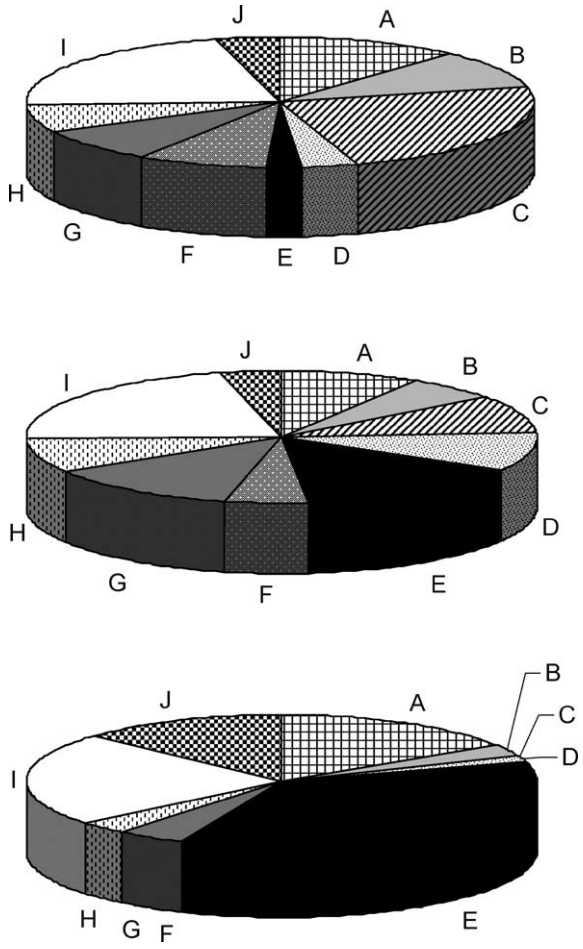

Fig. 1. Functional classification of annotated genes associated with (tumor-) angiogenesis. Upper panel: Genes upregulated by growth factor stimulation of ECs in vitro were classified according to their functions (N=464) [38,48,58,67,69] (van Beijnum et al., manuscript in preparation). Middle panel: Genes associated with capillary tube formation of ECs in vitro (N=385) [71,87,88,90,95]. Lower panel: Putative tumor endothelial markers overexpressed in ECs isolated from tumor tissue and compared to non-tumor ECs (N=69) [101–103]. Gene function was associated with signal transduction (A, squares), transcription (B, light grey), cell cycle regulation or apoptosis (C, diagonal lines), cytoskeleton (D, dotted), extracellular matrix remodeling (E, black), growth factor, hormone or cytokine action (F, dotted grey), metabolism (G, dark grey) protein turnover, modification and transport (H, dotted lines), cell surface molecules, antigens, receptors and adhesion molecules (I, white), or unknown function (J, checkerboard).

involved in the development of neuronal tissues are also implicated in angiogenesis, such as the neuropilins, ephrins and roundabout family [79–82]. Other developmental genes, among which genes involved in the Jagged/Notch pathway, have also been implicated in particular aspects of angiogenesis, such as adhesion, proliferation and migration [83–85]. The activation of Notch upon ligand binding leads to the transcription of its target genes, including the enhancer of split E(spl) bHLH transcription factors [79,86].

It is interesting to note that NRCAM, a protein involved in neuronal development, emerges as upregulated during endothelial tube formation, further extending the analogies observed between neurogenesis and angiogenesis [87,88]. NRCAM acts in concert with integrin β1 in mediating neuronal adhesion and neurite outgrowth [89]. NRCAM and αV integrin were indeed heavily upregulated in tube forming capillary ECs in collagen matrices when compared to proliferating ECs in vitro, hence NRCAM is likely to play a role in cell–cell communication and migration during angiogenesis [87,88]. Furthermore, NRCAM was extensively expressed in brain but also in placenta, a highly vascularized and angiogenic tissue [87].

In a similar study, HUVEC were cultured in 3D type I collagen gel, in the presence or absence of VEGF [90]. SSH in combination with differential screening identified 31 genes that were upregulated under the influence of VEGF in this culture model, including 4 different matrix metalloproteinases (MMP-1, MMP-2, MMP-3 and MMP-9) and the lysosomal cysteine protease cathepsin B, all of which are associated with invasion in the collagen gel. In addition, known EC adhesion molecules, including VE-cadherin, vascular cell adhesion molecule-1 (VCAM-1) and integrin α2, were upregulated in response to VEGF. Interesting novel candidate genes involved in angiogenesis include epiregulin, a member of the EGF family of mitogens and an activator of COX-2 [91,92], and VCIP, a transmembrane phosphatase with an RGD motif that mediates cell–cell interactions [93,94].

Using GeneCalling, Kahn et al. [95] aimed to identify genes upregulated in HUVEC cultured in 3D collagen gels, stimulated with phorbol myristate acetate (PMA), VEGF and bFGF. Genes of different classes were identified, but most prominently upregulated were those associated with extracellular matrix remodeling and adhesion. Of these, integrin α2, collagen IV, ADAMTS4, MMPs and cathepsin B have already been discussed. This study was later used to identify genes both upregulated in in vitro models of EC differentiation and associated with tumor vasculature by an in silico comparison [36]. In total, 128 genes were identified to be upregulated in in vitro models of angiogenesis as well as in tumor samples compared to normal tissue controls. From these, all genes that showed expression in epithelial tumor cell lines were subtracted yielding a list of 24 genes. The most prominently regulated genes included stanniocalcin-1 and OX40 ligand, previously unrecognized for a role in angiogenesis. Stanniocalcin-1 (STC1) was identified to be upregulated during tube formation of ECs [71,90,95] and during stimulation of ECs with a combination of HGF and VEGF [69]. STC1 is a secreted glycoprotein whose fish homolog regulates calcium and phosphate uptake and excretion. An angio-inhibitory role of STC1 was demonstrated by the inhibition of migration of ECs in response to HGF in the presence of recombinant STC1. Furthermore, STC1 expression might act as a stabilizing factor contributing to the maturation of newly formed vessels [96]. OX40 ligand or gp34 is a membrane-bound TNF superfamily member thought to play a role in T-cell costimulatory responses and T-cell interactions with endothelium [97,98].

OX40 binding to gp34 leads to the induction of c-*fos* and c-*jun* expression and the subsequent downstream activation of CCL5/RANTES [99].

A comprehensive overview of gene expression in endothelial tube formation in vitro in different experimental models investigated by means of oligonucleotide arrays has recently been published [88] and discusses relevant genes in light of their functional classification. Here, it also emerges that genes related to metabolism, extracellular matrix remodeling and cell surface molecules including adhesion molecules comprise an important group of regulated genes in endothelial tube formation.

A number of genes have been identified (Table 2), mainly adhesion molecules (NRCAM, VCAM, ICAM1), integrins (integrin αV, and α2) and matrix remodeling mediators (MMP-1, MMP-9, laminins α4 and γ1) that show induction in response to different combinations of growth factors in the reported tube formation models. However, when all genes reported in the above-described studies [71,87,88,90,95] are subject to functional classification, it appears that cell–cell contact, cell–matrix interactions and matrix turnover related genes represent the larger part of genes regulated in these models of tube formation in three dimensional culture (Fig. 1, middle panel). Furthermore, a clear shift in global gene expression is observed in these models representing the intermediate stages of angiogenesis (Fig. 1, middle panel) when compared to the early stages of angiogenesis (Fig. 1, top panel).

Table 2
Regulation of common players in in vitro models of 3D collagen tube formation

Function	Description	Gene	UniGene ID	Growth factor regulation	References
Cell cycle regulators and apoptosis related proteins	BCL2-related protein A1	BCL2A1	Hs.227817	PMA+bFGF+VEGF PMA+bFGF+VEGF+HGF	[88,95]
Cytoskeleton proteins	Gelsolin	GSN	Hs.522373	PMA+bFGF+VEGF+HGF PMA	[71,88]
	Myosin IC	MYO1C	Hs.289226	PMA+bFGF+VEGF PMA	[71,95]
Extracellular matrix proteins	A disintegrin and metalloproteinase domain 10	ADAM10	Hs.172028	PMA+bFGF+VEGF+HGF bFGF+VEGF	[87,88]
	Collagen, type IV, alpha 1	COL4A1	Hs.17441	PMA+bFGF+VEGF PMA	[71,95]
	Cathepsin B	CTSB	Hs.520898	PMA+bFGF+VEGF VEGF	[90,95]
	Laminin, alpha 4	LAMA4	Hs.213861	PMA+bFGF+VEGF+HGF PMA	[71,88]
	Laminin, gamma 1 (formerly LAMB2)	LAMC1	Hs.497039	PMA+bFGF+VEGF+HGF PMA	[71,88]
	Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	Hs.83169	PMA+bFGF+VEGF VEGF	[90,95]
	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase)	MMP9	Hs.297413	PMA+bFGF+VEGF VEGF	[90,95]
	Nidogen 2 (osteonidogen)	NID2	Hs.369840	PMA+bFGF+VEGF+HGF PMA+bFGF+VEGF	[88,95]
	Tissue factor pathway inhibitor 2	TFPI2	Hs.438231	PMA+bFGF+VEGF+HGF PMA+bFGF+VEGF PMA	[71,88,95]
Growth factors, cytokines and hormones	Placental growth factor, VEGF-related protein	PGF	Hs.252820	PMA+bFGF+VEGF PMA	[71,95]
	Stanniocalcin 1	STC1	Hs.25590	PMA+bFGF+VEGF PMA VEGF	[71,90,95]
	Transforming growth factor, beta 1	TGFB1	Hs.1103	PMA+bFGF+VEGF bFGF+VEGF	[87,95]
Metabolism	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	HMGCR	Hs.11899	PMA+bFGF+VEGF+HGF PMA	[71,88]
Protein turnover, modification and transport	Peptidylglycine alpha-amidating monooxygenase	PAM	Hs.369430	PMA+bFGF+VEGF+HGF PMA+bFGF+VEGF	[88,95]
Cell surface proteins, antigens, receptors and adhesion molecules	Ectonucleoside triphosphate diphosphohydrolase 1	ENTPD1	Hs.523173	PMA+bFGF+VEGF+HGF PMA	[71,88]
	Chemokine (C-X-C motif) receptor 4	CXCR4	Hs.421986	PMA+bFGF+VEGF+HGF PMA+bFGF+VEGF	[88,95]
	Dipeptidylpeptidase 4 (CD26)	DPP4	Hs.368912	PMA+bFGF+VEGF+HGF PMA	[71,88]
	Fms-related tyrosine kinase 1 (VEGF/VPF receptor)	FLT1	Hs.507621	PMA VEGF	[71,88,90]
	Intercellular adhesion molecule 1 (CD54)	ICAM1	Hs.515126	PMA+bFGF+VEGF+HGF PMA	[71,88]
	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	Hs.532082	PMA+bFGF+VEGF PMA	[71,95]
	Integrin, alpha 2 (CD49B)	ITGA2	Hs.482077	PMA+bFGF+VEGF+HGF PMA+bFGF+VEGF PMA VEGF	[71,88,90,95]
	Integrin, alpha V (CD51)	ITGAV	Hs.436873	PMA+bFGF+VEGF+HGF bFGF+VEGF PMA	[71,87,88]
	Neuronal cell adhesion molecule	NRCAM	Hs.21422	PMA+bFGF+VEGF+HGF bFGF+VEGF	[87,88]
	Podocalyxin-like	PODXL	Hs.16426	PMA+bFGF+VEGF PMA	[71,95]
	Vascular cell adhesion molecule 1	VCAM1	Hs.109225	PMA+bFGF+VEGF+HGF VEGF	[88,90]

2.5. Gene expression associated with angiogenic ECs in vivo

To date, only a very limited number of studies have attempted to characterize the gene expression profile of tumor ECs. The major challenge in doing so is to obtain a sufficiently pure population of cells. The difficulty lies in the fact that only a few percent of the tissue mass consists of ECs, and that the ECs are embedded in extracellular matrix components and surrounded by pericytes. Ideally, the isolated cells should be used in downstream applications immediately, omitting any subculturing steps or time consuming isolation procedures that can introduce changes in gene expression [100,101]. Antibody-based procedures in combination with magnetic bead separation have been used to isolate ECs from tissues [101]. In addition, high-speed fluorescence activated cell sorting can be applied to isolate ECs from tissue sources [100].

The first study published to tackle these technical challenges used SAGE technology to identify markers in ECs isolated with magnetic beads [101]. Both a negative selection using antibody-coupled beads directed against epithelial cells, leukocytes, monocytes and macrophages and a positive selection using P1H12 antibody-coupled beads ensured the selection of a highly pure population of ECs from both colon tumor and normal colon tissue. SAGE tags were categorized as pan-endothelial markers when expression was high in normal ECs, tumor ECs and in HUVEC and human microvascular ECs (HMVEC). Tumor endothelial markers (TEMs) showed high expression in tumor-derived ECs but not in normal ECs or in HUVEC, HMVEC, or cell lines. A substantial number of previously uncharacterized genes have been classified as TEMs in this study. This can be partly attributed to the technique used, SAGE, which is not dependent on previously documented or cloned genes. Also, as ECs represent only a small fraction of the total cell population in tissues, libraries constructed from these tissues will only reveal endothelial genes that are very highly expressed [101]. Interestingly, TEMs showed a strong bias towards genes functioning in extracellular matrix turnover such as collagens Iα1, Iα2, IIIα1, IVαI, VIα3 and XIIα1, and matrix metalloproteinases MMP-11 and MMP-2, stressing the importance of ECM remodeling during angiogenesis in vivo. When expressed as a percentage of tags, 82% of the TEMs coded for proteins that function in ECM remodeling and cell migration [101]. Furthermore, many TEMs were involved in adhesion and cytoskeletal remodeling, already discussed to be involved in tube formation. Genes thought to play a role in the initiation of angiogenesis, e.g., nuclear signaling molecules, and genes involved in cell-cycle regulation, metabolism and proliferation were almost completely absent [101].

Very recently, SAGE profiles were published representing malignant brain endothelium and invasive breast carcinoma endothelium [102,103]. Two normal brain EC samples were compared with three glioma-derived EC preparations. The majority of glioma endothelial markers (GEMs) function in extracellular matrix remodeling or are expressed as surface molecules involved in cell–cell interactions and adhesion. Collagens Iα1, IVαI, IVα2 and VIα1 were shown to be overexpressed in glioma ECs, as were a number of matrix metalloproteinases [102]. A similar profile is apparent in breast tumor ECs compared to normal breast ECs [103]. Combining the information in these three SAGE data sets, it is readily apparent that genes involved in extracellular matrix remodeling and cell–cell or cell–matrix contact represent the majority of upregulated genes during tumor angiogenesis in different types of tumors (Fig. 1, bottom panel; Table 3).

Table 3
Tumor endothelial markers in brain, breast and colon identified by SAGE

Function	Description	Gene	UniGene ID	Expression		
				Madden et al. [102]	St. Croix et al. [101]	Parker et al. [103]
Extracellular matrix proteins	Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	Hs.111779	Brain	Colon	Breast
	Collagen, type I, alpha 1	COL1A1	Hs.172928	Brain	Colon	
	Collagen, type III, alpha 1	COL3A1	Hs.443625	Brain	Colon	
	Collagen, type IV, alpha 1	COL4A1	Hs.17441	Brain	Colon	
	Collagen, type IV, alpha 2	COL4A2	Hs.508716	Brain		Breast
	Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	Hs.83169	Brain	Colon	
	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase)	MMP9	Hs.297413	Brain		Breast
	CD164 antigen, sialomucin	CD164	Hs.195727	Brain	Colon	
	Serine (or cysteine) proteinase inhibitor, clade E (nexin, PAI-1), member 1	SERPINE1	Hs.414795	Brain		Breast
Cell surface proteins, antigens, receptors and adhesion madhesion molecules	Thy-1 cell surface antigen	THY1	HS.134643	Brain	Colon	
	Plexin domain containing 1	PLXDC1	Hs.125036		Colon	Breast
Unknown	*Homo sapiens* clone FLC1492 PRO3121 mRNA	EST		Brain		Breast

From the available SAGE data sets, a number of genes can be identified that are overexpressed on the endothelium of different tumors. Colon tumor and glioma ECs overexpressed collagens Iα1, IIIα1, IVα1 and CD164/sialomucin-like [101,102]. Collagen IVα2, matrix metalloproteinase 9 (MMP-9), SerpinE, SPARC and an EST were coordinately upregulated in both glioma EC and breast tumor EC when compared to their respective normal counterparts [102,103]. Interestingly, almost no overlap in tumor endothelial genes is seen between colon and breast tumors, though the functional classes of upregulated molecules do indicate similarities in gene expression (Table 3). Most of these genes are associated with the basement membrane (BM), a specialized form of extracellular matrix. It has been recognized that BM components such as collagen IV, laminin, nidogen and SPARC are key regulators of angiogenesis. Type IV collagen provides the scaffold for BM assembly and is together with collagens type XV and type XVIII associated with vasculature. ECs are generally quiescent when bound to the BM suggesting this matrix provides growth arresting cues [104]. During angiogenesis, MMP-9 is produced that mobilizes sequestered growth factors and degrades collagen type IV, thereby disrupting BM organization and exposing cryptic domains of partially degraded collagens. These domains are proangiogenic, and further stimulate EC proliferation [105]. When BM degradation reaches completion, MMP-9 resistant collagen fragments remain that are generally anti-angiogenic, such as arrestin and tumstatin. The anti-angiogenic activity of these endogenous inhibitors generated by proteolytic cleavage of collagens is mediated by binding to integrins, mainly type β1 [104].

SPARC expression has previously been associated with ECs in various tissues [106,107]. It is involved in tissue remodeling and proteolytic peptides produced by MMPs in the extracellular matrix can have differential effects on angiogenesis [108].This interplay between several overexpressed genes in tumor ECs may hold promise for the design and application of therapeutic modalities.

One major concern that arises from the above described studies is the uncertainty that the genes that were identified are truly markers of tumor angiogenesis rather than physiological angiogenesis. Indeed, most of the TEMs described by St. Croix et al. were also expressed during angiogenesis of corpus luteum formation and wound healing [101]. Furthermore, in all published SAGE studies, a relatively small number of samples was used: 2 invasive breast tumor specimen were compared to 1 normal breast sample [103], EC isolated from 1 colon tumor was compared to EC from 1 normal colon [101] and EC from 2 normal temporal lobotomies were compared to 3 grade III or grade IV gliomas [102].

The expression of extracellular matrix remodeling related genes and those involved cytoskeleton and cell interactions seems a predominant theme in ECs in vivo (Fig. 1C, bottom panel). The identification of novel genes and genes previously not thought to be involved in angiogenesis by using unbiased gene expression profiling techniques will shed new light on the process of angiogenesis and the possible interference therein for therapeutic purposes. Since angiogenesis is not limited to pathological conditions, careful evaluation of putative markers for their tissue specificity is warranted if these are to be used in targeting undesired angiogenesis.

3. EC diversity

3.1. Microenvironmental interactions in angiogenesis

ECs in a particular microenvironment interact with various types of cells, such as fibroblasts, pericytes, immune cells, tumor cells or organ cells. In addition, ECs can interact with various ECM components and are exposed to different growth factors and cytokines released by its neighbors [109]. As a result, EC lining different vessels exhibit morphological and functional specializations. ECs derived from arteries, veins and tissues clearly differed in global gene expression profiles, related to various processes such as ECM remodeling and coagulation cascades [14,70]. A gene expression comparison of cerebral ECs (HCEC), part of the blood–brain barrier, with HUVEC revealed that HCEC are characterized by the expression of genes associated with neuroprotection and growth support. Marked differences in growth factor protein release between HCEC and HUVEC in culture were observed that supported the gene expression data [110]. It has been demonstrated that tumor ECs isolated from renal cell carcinomas (RCC) retain their persistently activated phenotype in culture. Initially it was shown that RCC tumor ECs downregulate adhesion molecules such as ICAM-1, ICAM-2 and CD34 [111]. Later, it was shown that RCC tumor ECs were resistant to serum-withdrawal induced apoptosis, a higher proliferation rate and more pronounced tube formation in comparison to HMEC [112]. Thus, it seems that the original microenvironment programs the endothelial phenotype and that these properties persist (at least to some extent) in both in vitro behavior and gene expression.

3.2. Tumor angiogenesis models

One of the advantages of using cell culture models that mimic an in vivo situation in vitro is the virtually unlimited supply of cells that can be obtained for these purposes. Furthermore, assay conditions can be carefully monitored and can be kept constant ensuring a certain degree of reproducibility. However, in cell culture systems, cells are no longer in their natural environment and might respond aberrantly to certain stimuli, giving a false representation of the in vivo situation [113]. Also, in primary cell cultures such as HUVEC, the culture process itself can induce changes in these cells [100,114].

When regarding angiogenesis in cancer, tumor ECs have generally resided in a tumor microenvironment for months to years, whereas culture systems only cover a time period of days. This discrepancy between in vitro and in vivo conditions might translate in an incomplete or hampered mimic of in vivo conditions. Thus, though in vitro approaches might provide useful information in certain settings, the results require cautious interpretation.

Several attempts have been made to overcome these limitations. Co-culture of ECs with tumor cells allows studying the interaction between the different cell types. Increased proliferation and network formation of HUVEC was apparent in co-culture with U87 glioma cells. Interestingly, global gene expression profiles of EC in co-culture concordantly shifted towards more genes involved in ECM remodeling and cytoskeletal changes [115]. Depending on the model used, direct interactions, or bidirectional cross-talk over a membrane can be analyzed.

Endothelial precursor cells (EPC), derived from CD34+/AC133+ bone marrow progenitor cells were evaluated as a model for tumor ECs in comparison to widely used microvascular ECs (HMVEC). Only partial overlap (~30%) in gene expression patterns of HMVEC and EPC was evident, pointing towards intrinsic differences between these cells. Hence, it was postulated that EPC could be a better model for tumor ECs than HMVEC or HUVEC. However, the expression of 565 EPC gene tags overlapped with tumor EC, whereas 503 HMVEC gene tags overlapped with tumor EC [116], suggesting that EPC provide only a marginally better model for tumor EC.

Although apparently similarly regulated, the exact differences, if existing, between angiogenic ECs under physiological circumstances and angiogenically activated ECs in tumors have not fully been elucidated. Different models have been developed to study angiogenesis in vitro, however, it may be difficult to accurately mimic the temporal and spatial complex actions of all factors exerting effect on ECs in vivo. Therefore, extrapolation of data generated by in vitro experiments to the in vivo situation might be limited.

3.3. EC heterogeneity and therapeutic interference

EC diversity observed in tissues, large and small vessels, arteries and veins, and tumors constitutes a challenge for the development of therapeutic strategies for the interference with angiogenesis. From the above-described SAGE analysis, it was apparent that there was only very limited overlap in genes regulated concordantly in breast and colorectal cancer EC, but that both colon tumor EC and breast tumor EC associated genes were overexpressed in glioma EC. This indicates tumor EC heterogeneity between different tumors and holds implications for the design of future targeted therapies. Also, this heterogeneity may lead to differences in responsiveness to current (anti-angiogenic) therapies. In addition, EC heterogeneity may exist within a tumor [109].

The markedly different global gene expression profiles in growth factor stimulated ECs, tube forming ECs in 3D collagen and ECs isolated from tumors (Fig. 1), stresses the influence of environmental factors on phenotype and gene expression. Therefore, for both the identification of putative therapeutic targets and for the evaluation of novel therapeutics, careful evaluation of the model systems used is warranted.

4. Implications and future directions

Different studies have attempted to find novel targets for angiogenesis interfering therapies by elucidating gene expression patterns of ECs. A number of investigators stimulated ECs in culture with different cytokines and growth factors [38,48,58,67,69], whereas others used tube formation models in vitro [71,87,88,90,95]. Only very few attempts have been made to profile ECs from in vivo sources, mainly due to cumbersome isolation techniques that are required to isolate sufficiently pure populations of ECs from tissues [101–103]. These different approaches, cell sources and cytokines applied, have led to the identification of different subsets of genes regulated under these circumstances. In general, genes involved in ECM or BM remodeling, e.g., Collagen IVα1, MMP-1 and MMP-9, showed upregulation in both endothelial tube formation in vitro and in tumor endothelium in vivo. Also, different integrins were identified in the diverse EC sources described here. Some of these molecules have been explored for therapeutic interference in cancer. Different inhibitors of matrix metalloproteinase activity have been developed for anticancer therapy [117] but they were not very successful in clinical trials [118]. Inhibiting EC adhesion to ECM components by blocking $\alpha V\beta 3$ integrin induces apoptosis in ECs and tumor regression [119]. Currently, two αV integrin inhibiting agents, monoclonal antibody Vitaxin and cyclic RGD-like peptide cilengitide, are about to enter the market (www.medimmune.com, www.merck.de). Furthermore, different proteolytic fragments of BM collagens have proven anti-angiogenic activity [120]. Gene expression profiles emerging from the different models described, although not exhaustive, are best viewed in the context of the distribution of the identified genes over a number of functional classes. When the functional classification of the reported genes associated with in vitro growth factor and cytokine stimulation of ECs growing in monolayers is regarded, it is apparent that cell cycle-related genes make up the larger part of regulated genes, followed by genes associated with metabolism (Fig. 1, top panel). Genes associated with tube formation are those involved in cell–cell and cell–matrix contact, extracellular matrix turnover and signaling (Fig. 1, middle panel). The involvement of extracellular matrix remodeling proteins is even more evident in ECs isolated from fresh tissues (Fig. 1, bottom panel).

The observation that very few genes overlap in the above mentioned types of studies might reflect the different activation of the used EC sources. Stimuli applied in vitro might be incomplete for a perfect mimic of the tumor microenvironment. It is possible that the concentrations of cytokines and growth factors used are not representative for the in vivo situation. In vitro activation of ECs with only one angiogenic growth factor may even overstimulate one pathway and silence others. Likewise, the presence of other specific types of cells such as pericytes, stromal or epithelial cells in vivo influences the phenotype and hence the gene expression profile of the endothelium by a plethora of different signals. Furthermore, one might speculate what the influence of time is; tumor ECs have generally been exposed to their tumor environment for months to years, whereas most in vitro studies cover only a limited period of time. It has been proposed that tumor ECs have further progressed into the sequence of angiogenic events, whereas cultured ECs are at the early stages of angiogenesis. This could explain the gradual increase (from confluent monolayers of cultured cells, via in vitro stimulated tube forming cells to tumor-derived ECs) of the proportion of expressed genes that are associated with extracellular matrix remodeling, a late event in angiogenesis.

Novel genes and genes not previously associated with angiogenesis identified by gene expression profiling studies will provide more insight in the molecular events that direct angiogenesis and may hold clues for the development of novel therapeutic moieties. Tumor endothelium specific markers that are expressed on the cell may be targeted for vessel destruction by, for example, antibodies conjugated to cytotoxic drugs. Gene products that play a crucial role in the active sprouting of capillaries from existing vessels are putative candidates for therapeutic interference in the angiogenesis process.

The successes in isolating pure fractions of ECs has provided the opportunity to study gene expression in ECs from in vivo sources directly, without the introduction of artifacts by culturing steps. Also, bioinformatics approaches to identify novel endothelial markers [15,36,37,121] and to functionally annotate them [122] are promising. Furthermore, public availability of gene expression data in uniform formats provides a tool for surveying gene expression profiles over a large number of experiments to identify common denominators of certain processes and signaling pathways [123–126]. Taken together, these progresses in understanding angiogenic EC biology, and the concomitant identification of molecules pivotal for this process, might eventually result in the development of novel, potent and specific angiogenesis modulators for therapeutic purposes.

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