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Analysis of temporal and spatial properties of protein interactions in the pharyngeal cancer stage development, based on GO term

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Abstract

Angiogenesis is one of the main and well-studied processes sustaining the tumoral cell growth. The development of new blood vessels by sprouting from the existing ones is a multistep process and involves numerous gene products. By networking a set of 92 genes involved in angiogenesis, we have built a putative model which could provide a theoretical framework for identifying the spatial and temporal interaction progress of the encoded proteins.

Key words: Angiogenesis, Angiogenic stages, Cancer, Networking model, Protein interaction

1. Introduction

The onset of neoplasia is characterized by a balance between the proliferative and respectively, the apoptotic processes of the transformed cells. In this stage, they are receiving oxygen and nutrients by diffusion, with the cost of limiting the growth of tumor mass to 0.4 - 2 mm [1-3].

When the proliferation rate of transformed cells overcomes the apoptotic rate and the oxygen amount becomes insufficient, the metabolism of the neoplasic cells starts to change. The required energy is obtained through anaerobic glycolysis, which drops down the pH values in the range of 5.6 to 6.8 [4], creating conditions for local acidosis. In the same time, tumors follow one of four strategies to receive the required quantity of oxygen and nutrients: inducing and initiating the angiogenic shift (the common way); expansion of preexisting blood vessels, with no need for developing their own vasculature; vasculogenesis, which means the building of new blood vessels from precursor cells derived from bone marrow cells; generating of a tube network by the tumor cells (this way is yet viewed as uncertain)[1].

As a response to the lower pH values of the local environment, the transformed cells gradually release the hypoxia-induced factor (HIF1). Meanwhile the ubiquitination and proteasomal degradation of its oxidized form, by the VHL protein (von Hippel-Landau protein) are slowed down. HIF1 is a hypoxia-induced stress released molecule with a key role in the adaptation of tumor cells to acidosis, acting as a transcription factor for over 40 molecules [5] involved in the positive regulation of angiogenesis [4] (VEGFs, PDGFs, IL8, ANGPT2[4, 6] etc.) and in other cellular processes (cell proliferation, cell survival, apoptosis, cell adhesion, motility, cytoskeletal structure, extracellular matrix synthesis, iron metabolism,

glucose metabolism etc.) [7]. On the other hand, the tumor cells start to release chemoattractants for the M2 macrophages, which infiltrate into the surrounding tissues and start to produce inflammation and angiogenic factors in the tumor microenvironment [2,8].

It is known that low oxygen and glucose levels coupled with low extracellular pH values are potent triggers for the angiogenic shift, through the activation of HIF1 and by chemoattracting and activating the immune system cells (leukocytes). The angiogenic shift is marked by tilting the balance in favor of angiogenic factors (Figure 1). In Figure 2, this series of events corresponds to the pre-angiogenic stage of tumor development.

Figure 1. The angiogenic shift. The action of angiogenic molecules overrules the action of angiostatic ones.

Under the action of angiogenic molecules, the pericytes and the endothelial cells start to proliferate, disassembly the extracellular matrix and lose any cell-cell and cell-matrix adhesion. This stage is called angiogenesis initiation.

In the stage of differentiation, stabilization, remodeling and maturation of vessels, endothelial cells continue to proliferate and start to migrate towards the increasing gradient of angiogenic chemoattractants, giving rise to new blood or lymph vessels that start to sprout from the existing ones [1,2], growing and branching into the tumor and leading to intensive growth of the neoplasia, which enter the angiogenic or vascular stage.

After the development of a vessel network, the transformed cells start to spread into neighboring tissues and into blood, which carries them to distant sites, where metastases can be developed [1,2]. If the new vessels are not perfused by blood, they start to regress due to endothelial apoptosis [6], but this is not the case of a growing tumor, which requires high amounts of glucose and nutrients, provided only by a well-developed network of blood vessels. Therefore, angiogenesis is a key process for tumor development and one of the most investigated processes [8]. In this study, we used existent knowledge of gene interaction included in public databases to look at the possible networks created by a set of genes most frequently involved in angiogenesis.

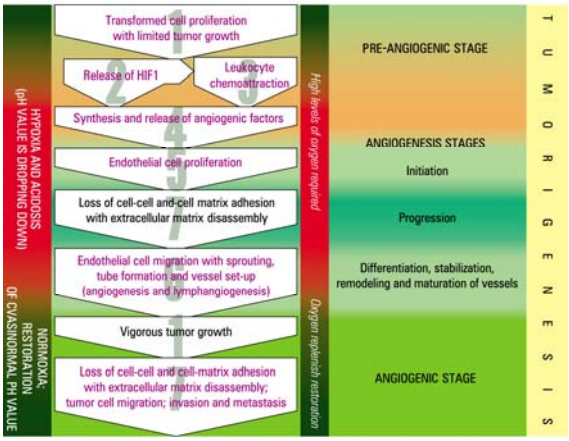


Figure 2. Main phases in neoplasias development (left), and angiogenesis stages (right), depending on oxygen pressure and pH value in the tumoral microenvironment.

2. Methods

We have analyzed the relationships between some genes whose expression products are involved in angiogenic processes and which are comprised in the panel of *TaqMan® Array 96-Well Plates Gene Signature for Human Angiogenesis* (cat.no.4414071, APPLIED BIOSYSTEMS, Foster City, CA 94404, USA). The predicted relationship between the products of the analyzed genes were identified using the String® program version 9.1 and 10.0 [9], available at http://string-db.org (accessed between June 2015 and November 2016). This software allows users to customize the active prediction methods (neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases, text mining), the confidence of displayed interactions, the additional players and other parameters (e.g., different processes in which a gene, a set of genes or a protein are involved). The networks can be displayed in many fashions (by confidence score of relationships, by evidence of interactions, by actions or by interactive mode, which allows moving, adding or deleting some nodes).

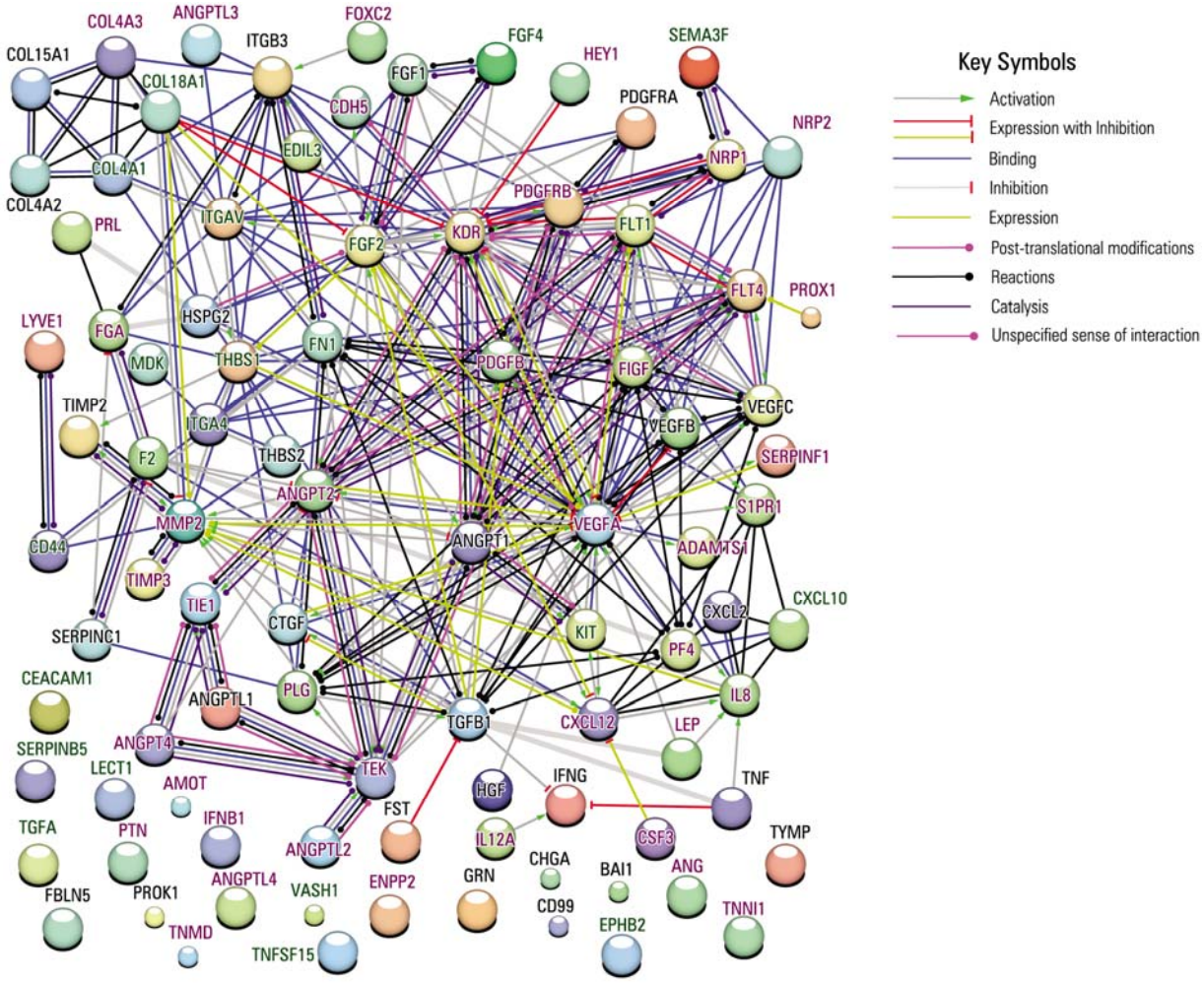
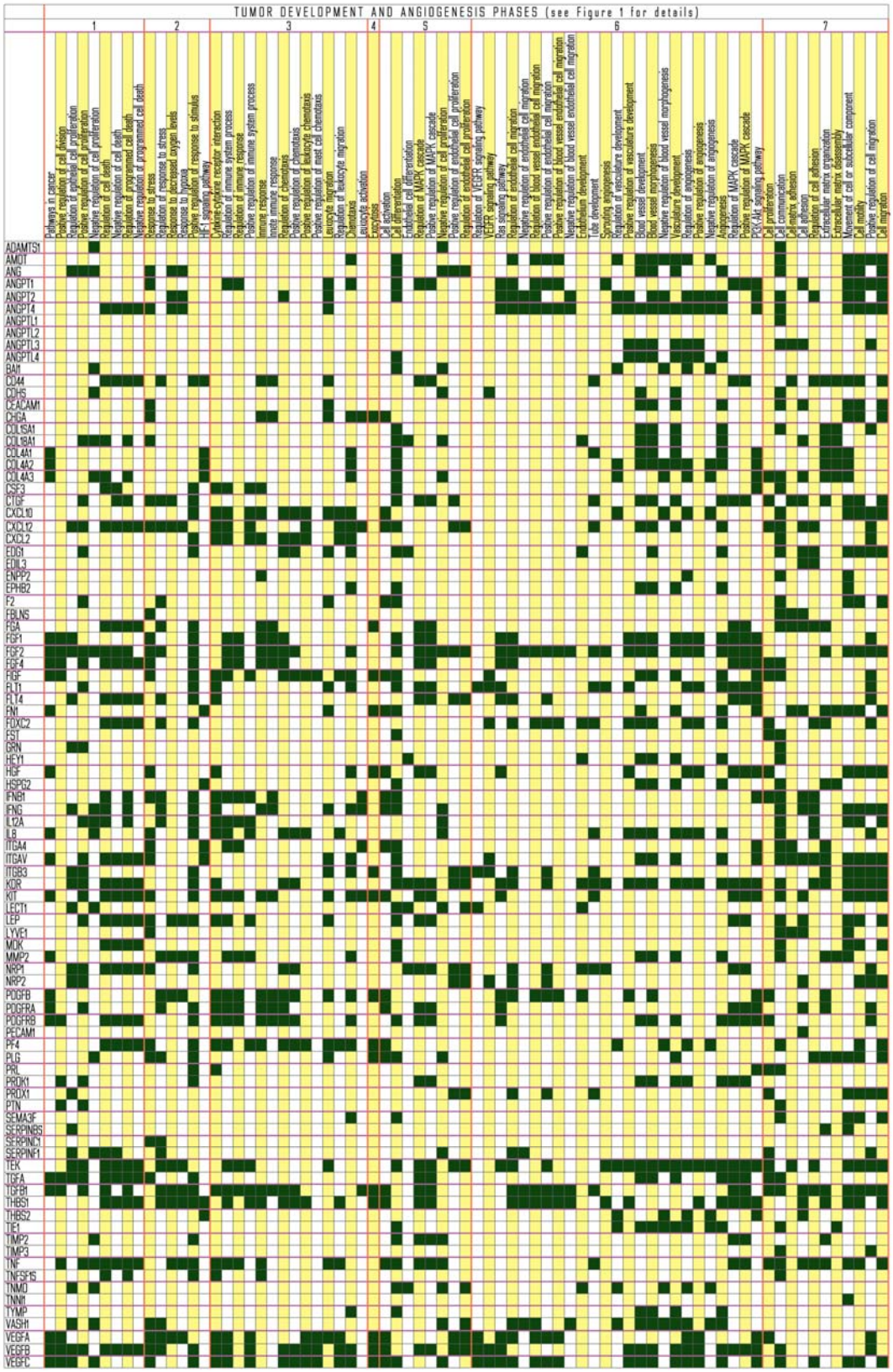


Figure 3. The overall relationships between the products of the 92 genes composing the The TaqMan® Array for Human Angiogenesis, from Applied Biosystems.

Table 1. Summary of the 74 networks, displaying the biological processes in which are involved the 92 gene products. On the left side are the gene product names, and on top, there are displayed phase marks and the biological processes.



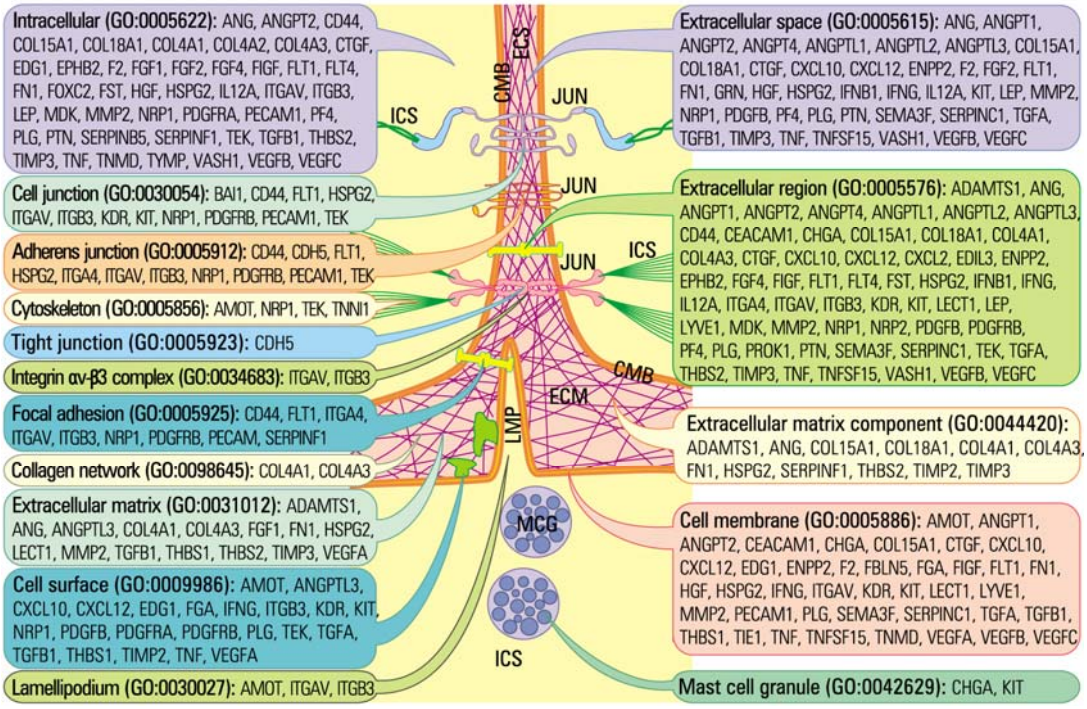


Figure 4. Spatial distribution of proteins involved in the main phases of tumoral development and angiogenesis. ECS, extracellular space, ICS, intracellular space, JUN, junction, CMB, cell membrane, ECM, extracellular matrix, MCG, mast cell granule, LMP, lammelipodium. The indicatives between the round brackets are the Gene Ontology (GO) Consortium indicatives for every location.

In order to see the overall relationships between the products of the 92 genes, we used highest confidence score (0.9), actions view, and no additional players. The built network is shown in Figure 3 and contains all the 92 genes and 248 interactions.

Then, to identify the gene products involved in different biological processes specific to each tumor development phase with respect to angiogenesis, we used the 'Biological Processes (GO)' and 'KEGG Pathways' from 'Analysis' menu of String® program to build 74 networks (not shown here). Each network was analyzed and the proteins were clustered by tumoral- and angiogenesis phase. The summary of these networks is available in Table 1. For spatial distribution and function of each protein, we used the 'Cellular Component (GO)' from 'Analysis' menu and we built 16 networks, summarized on the 'Cellular component' of Table 2 (Supplementary data). A drawing of this section is shown in Figure 4. Each protein's molecular functions were defined by building 20 networks, using the 'Molecular Functions (GO)' on the 'Analysis' menu of String® program, whose results are displayed on the 'Molecular function' section of Table 1. The Biological Processes, the Cellular Components and the Molecular Functions sections in the String® program are distributed according to the GO (Gene Ontology) terminology[10, 11] that was developed by the Gene Ontology Consortium in order to describe the activity of different gene products in specific locations: inside/outside the cells or in the cell membrane.

3. Results

In the network displayed in Figure 3, one can observe some nodes having complex interactions with various other nodes (VEGFA, KDR, ANGPT1, FLT1, FLT4, FGF2, TEK, MMP2, ITGB3, PLG, CXCL12, COL18A1 etc.), some have fewer complex interactions than

the first ones (THBS2, TNF, LEP, ANGPTL3, SERPINC1, FGA, IL8, IFNG, PDGFRA etc.), some having only one connection (IL12A, PROX1, HEY1, PRL, LYVE1, HGF, FOXC2), and other nodes being disconnected, having no interaction with any of the displayed proteins (AMOT, ANGPTL4, GRN, TNNI, ANG, TYMP, IFNB1, ENPP2, TNMD etc.).

Because the network comprises only the 92 analyzed gene products, those observations have validity only for them, and they could not be extended over the interactions of those proteins with any other players.

The most potent angiogenic known molecules belong to the VEGF family, e.g. VEGFA, VEGFB, VEGFC, VEGFD. In our network, those proteins proved to have the most numerous interactors, e.g., 33 for VEGFA, 12 for VEGFB, 14 for VEGFC and 12 for FIGF (VEGFD). It could be predicted that nodes having various and numerous links with other nodes are involved in many signaling pathways, which can provide some hints for potential strategies for targeting them in anti-cancer therapies. One way suggested by the network in Figure 3 for inhibiting the expression of VEGFA can be restoring the normal expression of SERPINF1 protein. Its expression is inhibited by hypoxia, but, once the normal oxygen level is restored in the tumoral microenvironment, SERPINF1 protein has the ability to balance the effects of VEGFA, by negative regulation of tumor cell proliferation and by inhibiting the endothelial cell growth and migration [12].

TNF was described as a dual potential cytokine: having angiostatic activity[13] by caspase 3 activation and endothelial cells apoptosis induction[14], and also angiogenic activity[15,16]. Our network shown in Figure 3 gives some clues for the latter point of view, because TNF induces angiogenic cytokine IL8 activation and inhibits the activity of angiostatic IFNG cytokine. IL8 has angiogenic activity by induction of MMP2 expression and activation, acting sinergically with LEP, VEGFA and CXCL12. As it is already known, MMP2 has angiogenic and pro-tumoral activity by cell to cell junctions weakening and by endothelial and tumor cells migration facilitating.

Another interesting fact resulting from the built network is that expression of FGA gene product can be inhibited or diminished by SERPINC1. As fibrin cleavage product, FGA has an angiogenic, immunomodulatory and chemotactic effect [17]. The SERPINC1 protein has inhibitory activity over the F2 protein expression, the latter being linked to tumor cell invasion, adhesion and metastasis, as well as to angiogenesis [18].

According to the network in Figure 3, COL18A1 has inhibitory activity over KDR and FGF2 and dual action over MMP2. COL18A1 is a member of collagen family, found both in the intracellular compartment and extracellular matrix (Figure 4). KDR (FLK1, VEGFR2) is the most important receptor for VEGFA[19], VEGFB and VEGFC[20], transducing stimulatory signals for endothelial cells survival, proliferation and migration[19, 21, 22]. The binding affinity of these proteins is amplified by the interaction of VEGFA with NRP1[19].

In the network in Figure 3, 22 nodes appear totally disconnected from the central network. This fact doesn't necessarily mean that they are unrelated to the angiogenic or tumoral processes, but for the selected settings (protein partners, confidence level, the added interactors) their interactions are not visible. Their role for these processes can be seen in Table 1, which shows the involvement of the analyzed proteins for the tumoral and angiogenic processes. The majority of proteins have more than one function, being pleiotropic. Only ANGPTL1 and TNMD appear to have only one function, different for each of them, and we have not seen any significant involvement of ANGPTL2 in the selected processes. Nevertheless, according to the network in Figure 3, ANGPTL1 has complex interactions with TIE1 and TEK (TIE2) receptors. ANGPTL2 seems to bind and to interact in a complex manner with TEK (TIE2) receptor, transducing the signal through PI3K pathway, and MAPK cascade[23], both being involved in

endothelial cell proliferation and migration as well as new vessel sprouting (Table 1). As shown in Figure 4, TNMD is a transmembrane glycoprotein [24] and, in secreted form seems to have angiostatic and antitumoral activities, at least *in vitro* [24, 25].

The most pleiotropic of the 92 proteins can be clustered in seven groups (Table 1):

• ANGPT group, consisting of ANGPT1, ANGPT2 and ANGPT4. At least one member of this group is involved in 6 of 7 phases in tumor development, with respect to angiogenic process;

• FGF group, comprising FGF1, FGF2 and FGF4, all of them are spread over 6 of 7 phases of tumor development and angiogenic processes;

• ITGB3-KDR-KIT group. ITGB3 and KIT are engaged in all the phases displayed in Table 1, and KDR is not essential for synthesis and release of angiogenic factors;

• IFNG-IL12A group. These proteins are engaged in tumor cell proliferation, in loss of adhesion and in migration, invasion and metastasis;

• PDGF-PDGFR group, including PDGFB, PDGFRA, and PDGFRB. PDGFB protein appears to be involved in all 7 phases of tumor development, as well in angiogenic process, while PDGFRA and PDGFRB are not involved in synthesis and release of angiogenic factors;

• TEK-TGF-THBS1 group, consisting of TEK, TGFA, TGFB1, and THBS1. TGFB1 is engaged in biological processes of all phases, while TEK and THBS1 are involved in most of the phases, excepting synthesis and release of angiogenic factors. TGFBA is not involved in leukocyte chemoattraction, synthesis and release of angiogenic molecules;

• VEGF group, comprising VEGFA, VEGFB, and VEGFC, all of them being involved in all the seven phases. The same behavior appears to have FLT4 (VEGFD), even if it has a lower pleiotropy.

Temporal distribution of protein actions on specific biological processes for each phase of tumor development with respect to angiogenesis, are displayed in Table 1. As it can be observed, some gene products, as FGA, FIGF, FN1, HGF, ITGB3, KIT, PDGFB, PF4, PLG, TGFB1 and VEGFs, seems to be involved in one or more biological processes specific for each phase. On the other hand, many gene products (CD44, COL18A1, COL4A1, COL4A2, COL4A3, CSF3, CXCL12, EDG1, FGFs, FLTs, IFNB1, IL8, ITGAV, KDR, LEP, MMP2, NPP1, PDGFRs, TEK, THBS1 and TNF) appear to exert some specific actions in 6 of the 7 phases, in tumor and vasculature development, with no involvement in phase number 4, 'Synthesis and release of angiogenic factors'. This can be explained by the fact that we considered only exocytosis of angiogenic factors as the main feature for this phase. Other gene expression products have temporal specific action patterns, as ADAMTS1, F2, FST, HSPG2, SEMA3F etc. and, interestingly, ANGPTL2 seems to have no interference with the defining biological processes for tumoral development and angiogenesis.

But, for describing the interaction puzzle of the proteins exerting some actions for tumoral and vasculature development, it is of high importance to check the spatial distribution of their actions. In order to approach this goal, we have built 16 networks using String® software, whose summary is displayed in Figure 4. One can observe that the proteins are spread into extracellular and intracellular compartments.

Using data shown in Figure 3, the Table 1 and Figure 4, the interaction type among the 92 analysed gene products, the specific phase and the location where they occur, can be evidentiated.

In the interaction network, ANGPTL2 and TEK seem to have a complex list of interactions, but since ANGPTL2 has no relevance for tumor development and angiogenesis, its position appears as blank in Table 1. These proteins can interact in the extracellular compartment, being involved in other metabolic pathways. The complex interaction between

ANGPTL1 and TIE1 is possible only in the 7th phase, when both proteins are active and when the tumor is growing up vigorously and it is spreading by invasion into the surrounding tissues; meanwhile some transformed cells can intravasate into bloodstream or into lymph and can generate metastases. Since ANGPTL1 is found only in the extracellular space (GO:0005615) and in extracellular region (GO:0005576), and TIE1 is bound to the membrane, their interaction seems to be somehow limited. In similar situations are the interactions among COL18A1 and THBS1, COL18A1 and VEGFA, CSF3 and CXCL12, all the interactions of IL8, EDG1 and CXCL2, FGA and PRL, FGF1 and PDGFRB, FGF1 and KDR, FGF2 and KDH5, FGF2 and THBS1, FGF2 and VEGFA, FGF2 and KDR, FGF2 and PDGFRB, FLT4 and VEGFA, FN1 and ITGA4, HEY1 and KDR, ITGA4 and THBS1, ITGA4 and FN1, KDR and FGF1, KDR and CDH5, LEP and VEGFA, MMP2 and TIMP2, NRP2 and VEGFA, PRGFRB and FGF1, PF4 and VEGFA, PRL and FGA, PROX1 and FLT4, SERPINF1 and VEGFA, THBS1 and FGF2, THBS1 and ITGA4, TIE1 and ANGPT4, TIE1 and ANGPTL1, TIPM2 and MMP2, VEGFA and SERPINf1, VEGFA and FLT4, VEGFA and NRP2, VEGFA and TEK, VEGFA and PF4, VEGFA and ANGPT2.

Some proteins are found in numerous cellular compartments having the chance to interact with multiple partners (e.g., TEK, NRP1, ITGAV, ITGB3, FLT1, HSPG2, KIT, COL15A1, ANG, PECAM1, PDGFRB, TIMP3, TNF, TGFB1, FN1, MMP2 etc.). Among these proteins, ITGAV and ITGB3 are found to interact in 7 locations (extracellular region, intracellular space, cell junctions, intercellular anchors, focal adhesions, lammelipodia and integrin alphav-beta3 complexes), being involved in biological processes common to 6 of 7 phases. The complex interactions among NRP1 and FLT1 can take place into 6 locations (extracellular region, intracellular space, extracellular space, junctions, intercellular anchors and focal adhesions), being involved in biological processes corresponding to 6 of 7 phases. ITGB3 and PDGFRB are found to interact in 5 locations (extracellular region, cell surface, junctions, intercellular anchors and focal adhesions). All the other proteins interact in 4 or less locations, their involvement in tumoral and angiogenic processes spreading over 7 phases or less, e.g. FIGF (VEGFD) binds and reacts with VEGFB and VEGFC during all the phases and in three locations (intracellular space, cell membrane and extracellular regions). Together with VEGFC, FIGF is the most potent inductor of lymphangiogenesis and lymph nodes metastases[26, 27, 28, 29, 30, 31]; PLG reacts with FIGF in the same locations and their effect is spread also during all the 7 phases; KIT and FGF2 are activating each other in extracellular space, during 6 phases etc.

4. Discussion

The protein expression levels are regulated by microenvironmental conditions, through regulatory factors. In order to have a more complete image about these regulatory proteins, with respect to angiogenic process, HIF1A was added as angiogenesis driving force and confidence level in the String Program was reduced from highest (0.9) to high (0.7) in order to build the interactive network, whose summary is displayed in Figure 5. Due to the fact that HIF1 release and leukocyte chemoattraction can take place in the same time, the phases 3 and 4 were merged. Here, we assumed that quasinormal oxygen replenishment could be restored only when new blood vessels are formed. Until that moment, the transformed cells release HIF1A, which increases expression of ANGPT1, CXCL12, FLT1, PDGFB and VEGFA. The production of HIF1A increases with the level of hypoxia.

As revealed in Figure 5, HIF1A sustains ANGPT1 expression during several phases, but, because ANGPT1 seems to have no effect on the expression or the activity of other given members, the link between the two proteins appears as irrelevant. Nevertheless, ANGPT1

positively regulates endothelial cell migration, MAPK, ERK cascades, Ras signaling pathway, cell motility, leukocyte migration, chemotaxis, cell proliferation, vascular development, response to stress, sprouting angiogenesis etc. By inducing VEGFA expression, HIF1A further modulates the expression of other proteins. VEGFA decreases VEGFB expression. VEGFA is able to induce angiogenesis development directly, by its own effects on endothelial cells, and indirectly, by preparing the pathway for these cells to migrate towards the oxygen lacking tissue, by MMP2 expression stimulation. MMP2 sustains CXCL12 expression, which promotes PDGFB and MMP2 expression. VEGFA is increasing KDR expression, but it is also able to decrease its expression, probably by a feed-back mechanism.

VEGFA sustains FOXC2 expression. This leads to two pathways, one inducing ANGPT2 expression, which diminishes VEGFA expression (probably by a feed-back pathway), and the second leading to overexpression of PROX1. This stimulates FLT4 overexpression, which inhibits NRP1 and FLT1 expression. FLT1 and NRP1 are able to reduce KDR expression. FLT1 receives overexpression signals from VEGFA and from HIF1A.

The activation network is far more complex and a summary of it can be seen on Table 2 (Supplementary data), with the remark that HIF1A activates FLT1, ANGPT2, MDK, MMP2, TGFA, CXCL12, VEGFA and LEP.

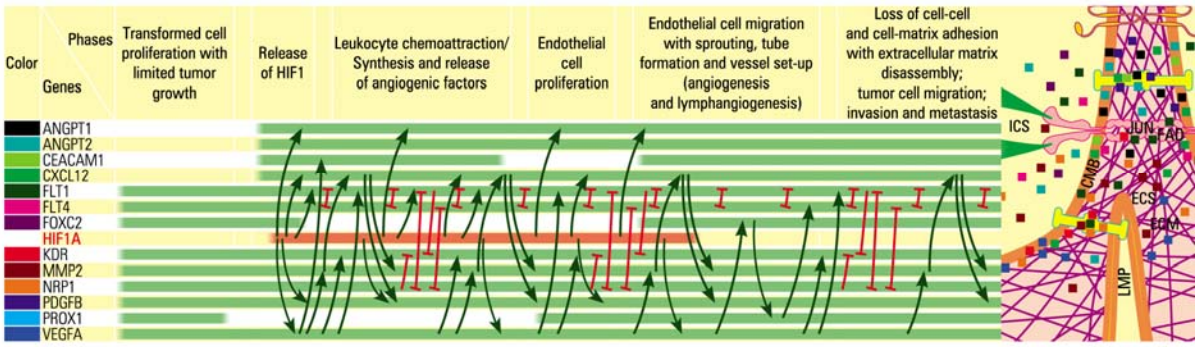


Figure 5. A predictive model for the temporal distribution of expression pathways involving HIF1A. The pale green horizontal lines mark the temporal distribution of proteins active in the main phases of tumor development and angiogenesis, as shown in table 2. Dark-green arrows mark proteins with induced expression and red lines those with inhibited expression.

5. Conclusions

Combining the interaction data with biological processes data and with the cellular distribution of proteins activity data could aid the researchers to develop an overview regarding the dynamics of positive and negative interactions of proteins in tumoral and angiogenic processes, at least at a predictive level. Using the String® software, 91 networks were built, one of them displaying the proteins interactions, 74 the involvement of those products in a similar number of biological processes, and 16 networks are displaying the cellular compartments where those products are most probably to interact. In our customized settings (highest confidence level and no others interactors added), 22 proteins showed no interaction and appeared as disconnected nodes. However, this could be due to the limited list of the analyzed gene products.

The biological activities of the 92 analyzed gene products were clustered depending on the involvement in the main phases of tumoral development and angiogenesis, allowing to generate a temporal distribution of each active protein. The 16 networks showing each protein spatial distribution were used, in addition to the temporal distribution data and to the

interaction type data, to identify the nature, the most probable temporal distribution and the most probable location for an interaction to take place. By adding HIF1A, as specific marker for hypoxia, the positive and negative expression regulation pathways in our network could be followed. The resulted model could be an useful instrument to determine the most appropriate treatment that could induce the expression/inhibition or activation/inactivation of some protein factors involved in various tumorigenic or angiogenic biological processes.

Acknowledgements

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