Molecular mechanisms of angiogenic synergism between Fibroblast Growth Factor-2 and Platelet Derived Growth Factor-BB

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Summary

Tumors produce several angiogenic factors to promote blood vessel growth. These angiogenic factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and angiopoietin (Ang). In an advanced tumor tissues, these growth factors are often expressed at high levels. Although, the individual role of these factors in promoting tumor angiogenesis is relatively well studied, their joint effect in induction of tumor neovascularization is poorly understood. Prof. Cao and colleges has recently showed that two of these angiogenic factors, FGF-2 and PDGF-BB can synergistically induce angiogenesis in the mouse corneal model. To further elucidate the molecular mechanisms by which these two factors synergistically induce blood vessel growth, the signaling pathways induced by these two factors in endothelial cells was studied in endothelial cells. For functional analysis, the effect of the two factors on T241 wt (fibrosarcoma), 3T3 (fibroblast) and smooth muscle cells (SMC) was studied for proliferation and migration in vivo was also studied. Together with other findings by Prof. Cao's and colleges, it was concluded that FGF-2 and PDGF-BB synergistically induce tumor angiogenesis, tumor growth, metastasis, endothelial cell activities and angiogenic signaling activation. Thus, the studies have provided important information for current and future development of antiangiogenic drugs for the treatment of cancer and other angiogenesis-dependent diseases.

Background

Blood vessels can be formed in a number of different ways, one way is vasculogenesis that is the formation of blood vessels, it involves differentiation from stem cells to endothelial cells, and is crucial during the early stage of embryonic development [1]. Another mechanism of blood vessel formation is a process of division of large "mother" vessels into smaller "daughter" vessels, capillaries. It is a process where endothelial cells grow into the lumen of the "mother" blood vessels and forms a bridge, where the process continues to separates the vessel into smaller ones [2]. Third, but not least important, is angiogenesis, which is a process of sprouting new blood vessels from the pre-existing vessels. The angiogenesis process consists of several defined steps, which include; degradation of the endothelial basement membrane, invasion by proliferation and migration of endothelial cells, tube formation, branch formation, vessel remodeling, and reconstitution of the basement membrane [3] (see figure 1.).
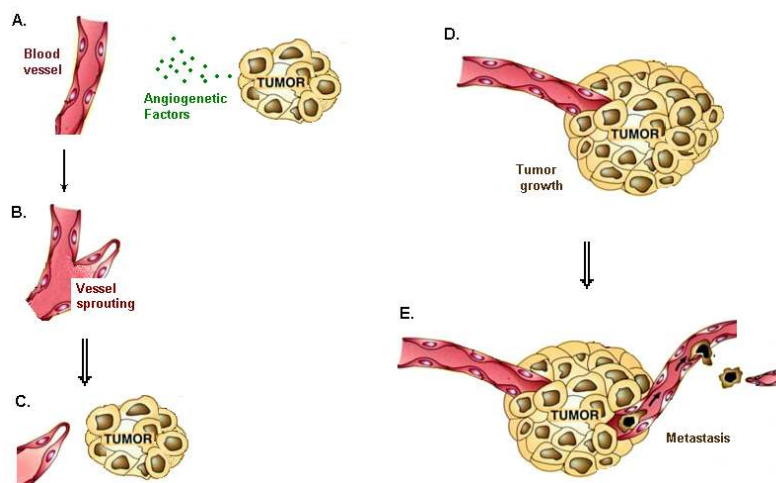

Figure 1. The process of tumor induced angiogenesis to tumor metastasis.
A.) The tumor releases angiogenic factors including VEGF, FGF and PDGF
to pre-existing blood vessels. B.) The endothelial cells start to proliferate
and migrate. C.) The sprouting vessel invade the tumor. D.) The tumor
grows and attracts more vessels. E.) Metastasis; tumor cells migrated
thorough the vessel and thereby are spreading. Figure adapted from [a].

In 1971, Judah Folkman and colleagues proposed that all solid tumor growth is dependent on angiogenesis and that suppression of angiogenesis might be useful for cancer therapy. In his early studies, he showed that a tumor could not grow beyond the size of 2-3 mm^3 without

angiogenesis [4]. Further, the first isolated factor, which "induced growth of new capillaries", was named tumor-angiogenesis factor (TAF) [5]. These findings were very important because beginning of a new era in prevention of tumor growth and metastasis. TAFs have been studied and today known to belong to several different families of TAFs. It is well known that tumors produce several angiogenic factors such as vascular endothelial growth factor (VEGF), angiopietin (Ang), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF). VEGF is the most known and well-studied tumor growth factor. Tumors produce several angiogenesis factors to switch on an angiogenic phenotype. The switch also requires simultaneous down-regulation of the expression levels of endogenous angiogenesis inhibitors [6]. For example, angiostatin, endostatin, thrombospondin [7, 8] and several other angiogenesis inhibitors have to be down regulated. Normal tissue has homeostasis between growth factors and inhibitors, but this is not the case for tumors, which have an imbalanced expression level [6].

Several angiogenic factors have been studied but only as single factors, and of no one in combinations. Single angiogenic factors such as vascular endothelial growth factor (VEGF) and FGF-2 have been tested alone in the treatment diseases, to induce angiogenesis. However, none of these trials have produced any beneficial effects (induction of angiogenesis) in patients [9]. For an example, VEGF was found to induce atherosclerotic plaque in atherosclerosis (thickening and hardening of the artery walls due to e.g. fat) [10]. Tumors on the other hand produce several factors, which indicates that the angiogenic factors act cooperatively and synergistically.

Synergism between FGF-2 and PDGF-BB

Recently it has been published that PDGF and FGF have been found to synergistically induce angiogenesis. First method was performed by implantation of micro pellets into the cornea, where the pellet contained growth factors alone or in combination (of PDGF-AA, PDGF-AB, PDGF-BB, FGF-2 and VEGF). A clear synergistic effect was visible between FGF-2 and PDGF-BB, while no significant effects occurred for single growth factors or the other combinations of growth factors (see figure 2). A long-lasting functional vessel assay was performed in the mouse cornea, by insertion of matrigel containing single or combined growth factors in the cornea. The mice were observed during long time. After a few days a

strong effect was visible, by much vessel growth in the cornea due to induced angiogenesis, and the effect lasted even after a year in the FGF-2 and PDGF-BB treated cornea (see figure 3) [9].
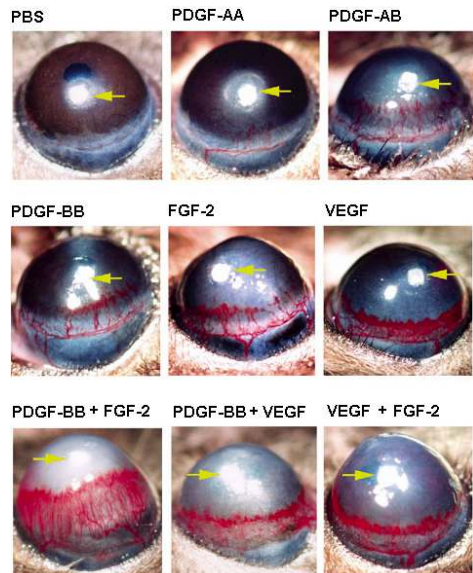

Figure 2. Mouse corneal micropocket; arrows indicating position of the insertion. An induction of angiogenesis is visible in PDGF-BB and FGF-2 treated mouse. Figure from [9].
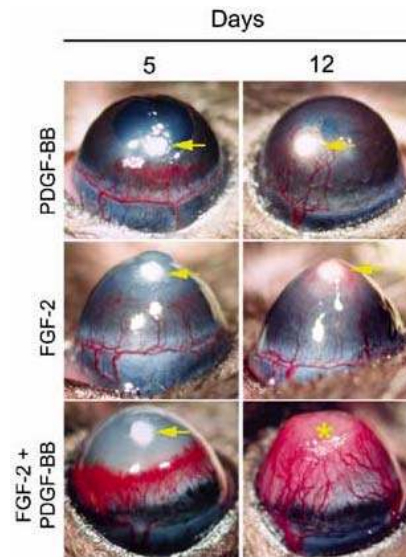

Figure 3. Mouse cornea matrigel; arrows/stars indicating location of matrigel and the star is a lost matrigel. Even though the loss, the synergistic induction is very strong and last for more than 12 days (data not shown). Figure from [9].

Fibroblast Growth Factor, FGF

The FGF family has several members including FGF-1 and FGF-2, which was first named acidic and basic FGF according to their properties [11]. FGF-2 was first purified in 3T3 fibroblast cells in 1975 [12] and since then much more is known; FGF-2 is found in the sub endothelial basement of blood vessels in all organs [13]. Delayed senescence and induction of division are two effects of FGF-1 and FGF-2 in cultured cell lines, such as endothelial cells, fibroblasts and smooth muscle cells [11]. FGF-1 and FGF-2 induce angiogenic phenotypes under physiological and pathological conditions [14]. FGFs are pleiotropic factors acting on different cell types including endothelial cells [14]. The FGF-2 consists of several different isoformes derived from the *fgf-2* gene [15]. Additionally, there are two different FGF-2 proteins of different size, where the larger proteins translocated into the nucleus (function unknown) [16] and the smaller protein is released by an autocrine mechanism [17]. FGF-2 mainly acts as an extra cellular factor; it is bound to the heparan-sulfate protoglycans

(HSPGs), which forms a reservoir in the body [14, 16]. The HSPG are found to be located on the surface of most cells and in the extracellular matrix [18]. It is activated of the FGF-HSPG-complex by binding to the tyrosine kinase FGF receptors (FGFRs) [19, 14, 16]. The FGF receptor is structurally similar to the Ig receptor super family [20], which also includes receptors such as the Platelet Derived Growth Factor Receptor, PDGFR [40]. The activation of the FGFR is due to binding of the FGF-HSPG complex on the surface and the three-unit complex is then internalized [22]. The dimerizised receptors get autophosphorylated of the tyrosine residues, which leads to the resulting activation of the intra cellular signal transduction pathways [16].

Signal transduction of FGFR

There are several signal transduction pathways (see figure 4); the first pathway is through the activation of the phospholipase C-gamma, PLCγ. The PLCγ was identified as a protein of 150 kDa associated to the FGFR after FGF-1 ligation [23]. The protein of PLCγ binds to the FGFR-1 and is tyrosine phosphorylated and thereby activated. Thereafter the PLCγ hydrolyses the phosphatidylinositol 4,5 bisphosphate to inositol 1,4,5 trisphosphate (IP$_3$) and diaglycerol (DAG). Two different pathways follow this, where IP$_3$ leads to the release of calcium from internal storages [24], which can induce migration. The other pathway by diaglycerol activates the protein kinase C, PKC, family [24, 25]. PKC is divergent and act on many different cell types, involving in cellular activities, such as proliferation, differentiation and apoptosis [25].

An additional pathway is the FGF receptor substrate 2, FRS2, signal transduction pathway. FRS2 is a docking molecule for the receptor and is associated with the SHP-2, which is needed for continuation of the Ras pathway [26, 27]. The phosphorylated FRS2 bind the small adaptor molecule Grb2, which by assistance by Sos activates Ras [28] that recruits Raf a serin/threonin kinas, which starts the cascade where it activates MEK, which then activates MAPK, mitogen activated protein kinases, including Extracellular signal-regulated kinase (Erk). These when activated translocates to the nucleus and activates the transcription factors, and thereby activates cellular activities [29].

All signaling pathways are not fully established. Yet two of these are including Src and the adaptor protein Crk. The activated FGFR-1 does phosphorylate Crk and when it is inactivated

the cells does not proliferate [30]. Likewise, when the Src is inactivated the endothelial cells do not migrate [31]. Also, the Src and PLCγ might inhibit each other [32].
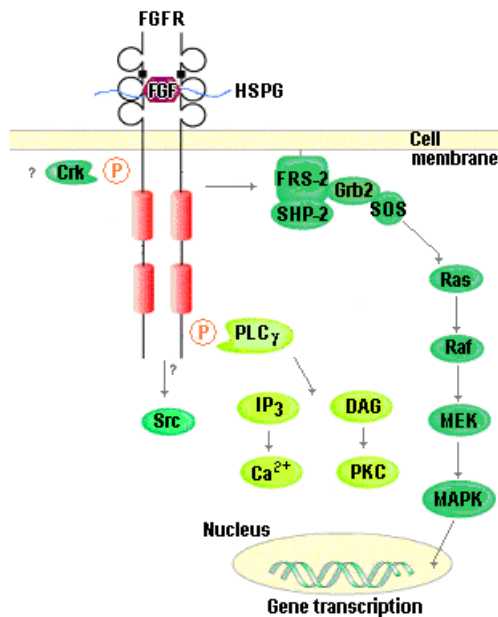

Figure 4. Signaling pathways of activated FGFR. Together in a complex the FGF and HSPG activate the FGFR, which undergo autophosphorylation and phosphorylates different signaling molecules, ending up with different activations of the cells. The FGFR also activates signaling molecules, which is of unknown function and further signaling transduction pathways. Figure adapted from [29, 33].

Platelet Derived Growth Factor, PDGF

The members of the PDGF family are important factors in the process of angiogenesis [34] and lymphangiogenesis [35] and function as a secondary vascular system in the higher vertebrates [36].

PDGF was discovered in serum as factor derived from platelets, where the factor stimulated proliferation in smooth muscle cells, SMC, [37] and has later been found to be involved in several cellular responses comprising proliferation, survival and migration [38]. The DPGF family has four members: PDGF-A, PDGF-B, PDGF-C and PDGF-D [39-43]. The PDGF-A and B are both synthesized as precursor molecules and undergo proteolytic cleavage at the NH_2 terminus. PDGF-B also undergoes cleavage at another site, close to the COOH terminus. The proteins form an anti parallel disulphide-bond dimer. The known isoformes include PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD [42, 44]. The intra cellular signals depend on the isoform of the dimer and are transferred to the cell by activation of the PDGF receptor upon binding.
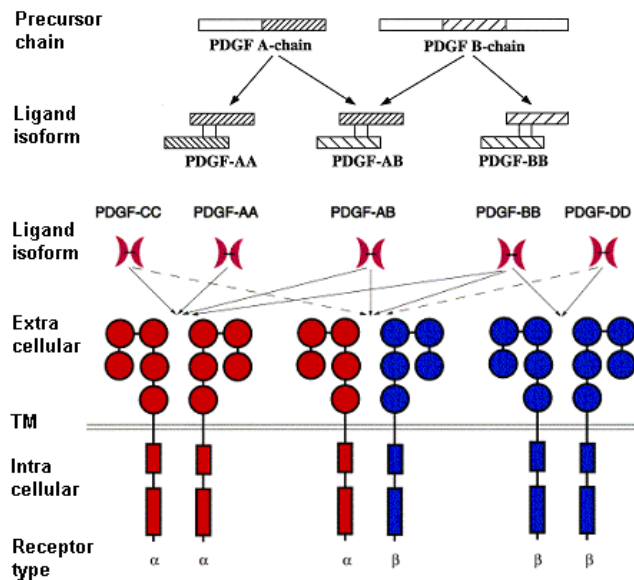
Figure 5. The PDGF ligands and receptors. Overview of ligand isoformes if PDGF-AA, -AB and –BB. The composition of CC and DD is not indicted. Also overview of ligand and receptor binding of PDGF and PDGFR, respectively. Figure adaptive from [44, 45].

Platelet Derived Growth Factor Receptor, PDGFR

The expression of PDGFR isotypes depends on the cell type, some cell types for an example vascular smooth muscle cells express both receptors [44], while other cell types for an example the human endothelial express only one receptor; the α-receptor [45], and the mouse capillary endothelial cells express the PDGF-β receptor [44]. Expression of the isotypes of the PDGFR due to stimulation of FGF-2 also depends on cell type, for an example in vascular smooth muscle cells; the α receptor expression is increased, but not the β receptor, [47]. The PDGF receptors are activated by dimerization by the binding of the dimeric PDGF ligand to hetero or homodimeric complexes of the α- and β-isoforms of PDGFR. The PDGF-AA activates the homodimer α and also the heterodimer of the receptor [44], while PDGF-BB is able to activate all kinds of receptor isoformes. The PDGF-CC can activate both the hetero dimer and the homodimer of PDGFR-β, whereas PDGF-DD only has the ability to activate the homodimer. The AB isoform of PDGF activates the heterodimer or the homodimer of PDGFR-α [48] (see figure 5).

The PDGFR is found on the cell membrane in caveolae, which is a membrane invagination involved in endocytosis [49]. When a ligand bind the receptor, the complex is internalized into endosomes [50], where the receptor complex dissociates and recycles to the surface, or the complex is degraded due to fusion of the endosomes with lysosomes, or the receptors are ubiquitinylated and undergo proteolysis [4, 51]. The kinase activity controls the internalization process [50]. Before this happens the ligand transfer its signal by the activation of the receptor followed by signal transduction.

Signal transduction pathways of PDGF

The tyrosine residues of the receptors are autophosphorylated [52], the autophosphorylation occurs in the kinase part of the receptor, which serves as docking sites for signal transduction molecules containing Src Homology 2 (SH2) domains [44]. This leads to signal transduction. The signal transduction is very complex, the pathways overlap each other and there is a lot of cross talking [53], and therefore a simplified picture will be described (see figure 6).

The activated PDGFR phosphorylates PLCγ [54, 55] and, as described, PLCγ regulates the activation of IP_3 and DAG, leading to the release of calcium and activation of PKC, respectively. For some cell types the PLCγ is important for migration [56]. Another pathway is phosphatidylinositol 3-kinase (PI3K) activates the Rac/Rho pathway leading to actin reorganization; involved in migration [57, 58]. Apart from signaling by the FGFR induced pathway, Grb-2, bind the autophosphorylated PDGFR directly, or via Shc, and thereafter Grb-2 activates the pathway of SOS activating Ras, which recruits RAF that activates the MAPK cascade, involving ERK, leading to gene transcription and cellular activities [59]. Ras can interestingly cross activate the PI3K pathway [58]. PI3K has another down stream molecule Akt/PKB, which is involved in survival in prevention of induced apoptosis [60, 61].

Src, is activated by the phosphorylated PDGFR, although the effect by Src is not fully clear [59]. Additionally a molecule with no known by the activation of PDGF function that also is activated by the PDGFR is Stat, Signal transducers and activators of transcription [59].
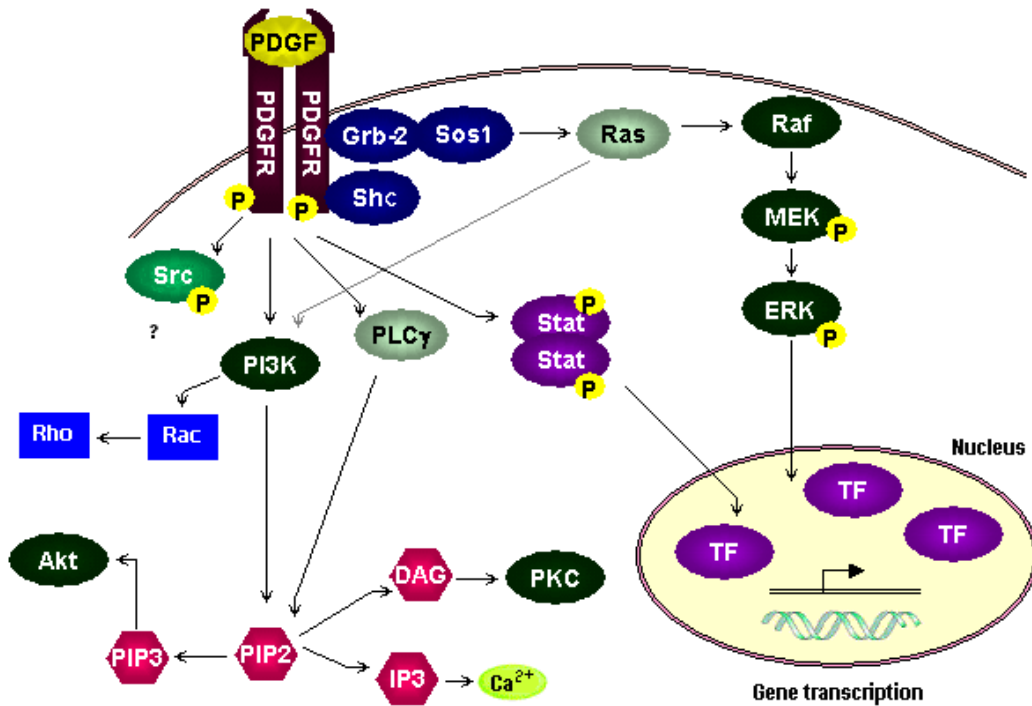
Figure 6. Signal transduction pathways induced by activated PDGFR due to PDGF binding. The autophosphorylated receptors activate signal transduction molecules upon phosphorylation. The sign of ? indicates unknown further pathway and TF is transcription factor. Figure adapted from [60, 64].

Additionally, it has recently been described in human SMC, that PDGF-BB is needed for activation of FGFR-1 in cell proliferation in presence of FGF-2 [62, 63]. A relationship between the endothelial cells and smooth muscle cells now occurs, where the growth factors regulates different cells (see figure 7). SMCs are known for their functional role as support for the sprouting angiogenic vessels, which are build by endothelial cells [1].
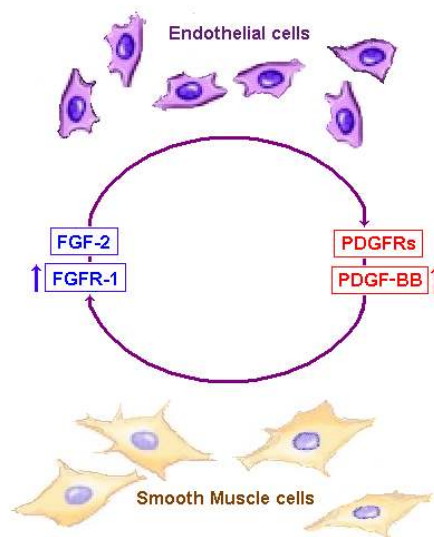

Figure 7. A hypothetical relationship between the Endothelial and Smooth Muscle Cells. The Bovine endothelial cells have induced cell activity by the activation of the PDGF-R due to FGF-2, in presence of PDGF-BB. On the other hand, the Smooth Muscle Cells have induced activation of the FGFR-1 due to PDGF-BB in presence of FGF-2.

Aim

In a mouse model the FGF-2 and PDGF-BB synergistically induces angiogenesis. One hypothesis is that when FGF-2 bind the FGFRs it activates PDGFRs, which will lead to cell proliferation, migration and differentiation in presence of PDGF-BB. It has been proved for bovine endothelial cells, BCE, that the growth factors induce proliferation and migration (unpublished data).

The aim was to further examine if other cell lines are also affected by the FGF-2 and PDGF-BB in proliferation and migration. A second task was to study the signal transduction pathways of the BCE, to se what really occurs in the cells due to the synergistically induced cell activity.

Material and Methods

Cell lines

Experiment were conducted using four cell lines; the T241 wt cells which are wild type Murine T241 fibro sarcoma cells and the SMCs, which are a primary mouse aortic smooth muscle cells, isolated by Prof. Cao and colleges. The 3T3 cells are fibroblasts and the BCE-HT⁺s, which is a bovine capillary endothelial cell line, immortalized with human telomerase, hTERT [64].

Growth factors

Throughout the project the Platelet Derived Growth Factor, PDGF-BB (PeproTech, Rocky Hill, NJ) and the Fibroblast Growth Factor-2, FGF-2 (Phamacia & UpJohn, Milan, Italy) was used.

Chemotaxis assay

Confluent cells on a 10-cm plate were washed, trypsinized, resuspended in media (10% FCS) and seeded in four wells on a 6-well plate. After two hours incubation at 37°C, 5.0% CO_2 the cells were washed and new media (10% serum to control and 1% or 0% serum to the experiments) and growth factors (FGF-2, concentration of 10 ng/ml per well, or PDGF-BB concentration of 10 ng/ml or 100 ng/ml per well), was added followed by incubation overnight. A membrane (Polycarbonate, Osmonics INC) was incubated in 1% gelatine (Difco)

solution over night in room temperature or incubated for 30 minutes in 37°C before drying 30-60 minutes in room temperature. Chemo-attractants, control media and growth factors, were added to the lower wells of a Boyden chamber [65]. Six wells were used for each sample. The membrane was put on top of the wells (see table 1) and the Boyden Chamber was assembled, cell suspension was added to the upper tubes $20\text{-}60 \cdot 10^4$ cells per tube. The chamber was incubated and after 10 minutes and a glass lid was placed on top of the chamber, which was followed by 3.5-hours incubation. The cells on the membrane were then incubated in cold methanol for 15 minutes followed by 15-minutes incubation in Giemsa dye (Sigma-Aldrich). The non-migrated cells were wiped off with a paper towel or a cotton swab and the membrane was then mounted between two glass lids with Nail polish. Each experiment contained six replicates and the experiment was repeated trice [66].

Table 1. Overview of the pre-treated cells and chemo-attractants, respectively.

	Experiment		Controls
Cells pre-treated:	FGF-2	PDGF-BB	Serum 10%
Chemo-attractant:	FGF-2	PDGF-BB	Serum 10%
Cells pre-treated:	FGF-2	PDGF-BB	Serum 1%
Chemo-attractant:	PDGF-BB	FGF-2	Serum 1%

Proliferation assay

Confluent cells on a 10-cm dish were washed, trypsinized and resuspended in media (10% FCS) and the cells were counted in a Bürker Chamber, and 10^4 cells were seeded in each well on a 24-well plate. After 2-hours incubation in 37°C the cells were washed and pretreated by addition of new media (10% FBS to the control and 1% or 0% FBS for the experiments) and growth factors (FGF-2, to concentration of 10 ng/ml or 100 ng/ml per well, or PDGF-BB to concentration of 100 ng/ml per well) (see table 2). After overnight, specific wells were washed, new media and new growth factors were added, and this is referred as treatment. The cells were further incubated for a totally of 72-hours in 37°C, 5.0% CO_2 and trypsinized and resuspended in IsotonII solution (Beckman coulter) and counted in a Coulter Counter (Beckman Coulter), also 92 hour assay was performed. Each experiment was repeated in four replicates and the experiments were repeated twice or trice [67].

Table 2. Proliferation set-up, treatment of cells, showing the four replicates.

	Positive control	Negative control	Experimental controls		Experiments	
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2

Cell extracts

BCE-HT^{+} cells were grown to 90-100% confluence in 60-mm dishes and DMEM (10% serum) containing 10 ng/ml FGF-2 for 24 hours. The cells were pretreated differently according to the following scheme; controls with serum, experiments with serum free media containing 10 ng/ml FGF-2, 100 ng/ml PDGF-BB or without any growth factor, followed by washed and the procedure of treatment was 24-hours incubation in serum free media containing 10 ng/ml FGF-2, 100 ng/ml PDGF-BB or without any growth factor (see figure 8). The cellular activity was stopped by addition of Whole Cell Lysis Buffer and Inhibitors containing: 20 mM Hepes buffer, pH 7.1, supplemented with 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 30 mM $Na_4P_2O_7$, 5 μM $ZnCl_2$, 20 mM β-glycerophosphate, 10 mM 4-nitrophenyl phosphate, 1 mM DTT, 200 mM Na_3VO_4, 0.5 mM PMSF [67]. The activity was stopped at different times points: after 0, 15, 30, 60, 90, 120 minutes and 6 hours (see figure 8). To the cells of 0 minutes not treated with growth factors, but served as controls. The cells were harvested by scraping with a cell scrape, vortexed for 10 seconds, centrifuged for 15 minutes at 13000 rpm in 4°C and the supernatant containing the proteins was transferred to in liquid nitrogen and snap freezed and stored of -80°C. The protein concentration was measured in duplicates in a 96-well plate and as a control BSA was used in different concentrations diluted in PBS. Colorimetric reagent solution (Bio Rad D_c Protein Assay, BIO-RAD) was added to all samples. After 10 minutes incubation the protein concentration was measured by measuring the absorbance at 960. A table of absorbance versus concentration was made of the BSA samples. Out of this the concentration of the protein samples was calculated due to its

absorbance. Although, it was followed by an extra balancing after the electrophoresis, which will be further described in the Electrophoresis and Immuno blotting section.
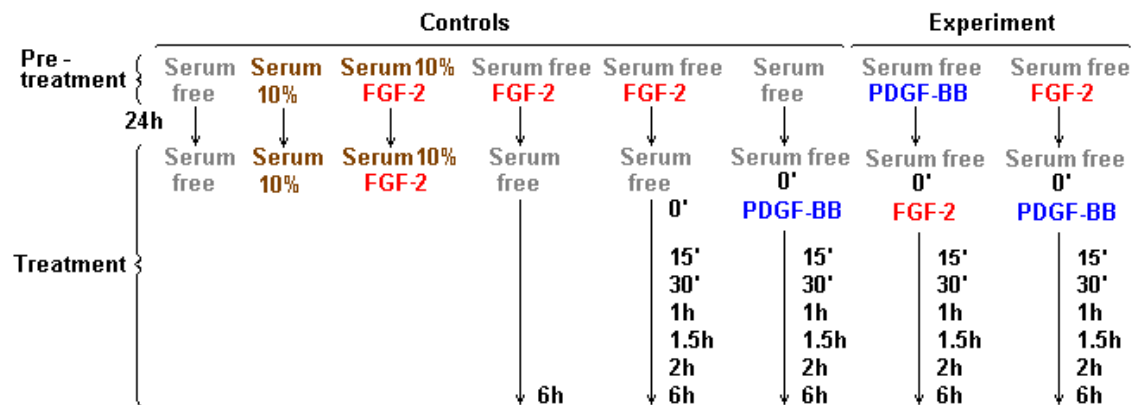

Figure 8. Set up of cell treatment for cell extracts. Time starts at 0 minutes (0´) is when the media is changed and growth factor are added cells and is incubated first for 15 minutes (15´) up to 6 hours (6h), except for the 0´ that got new media and no growth factor.

Western blot; Electrophoresis and Immuno blotting

The cell extract samples were boiled, thus denaturating the proteins, and NuPAGE LOS sample buffer was added, followed by heating and centrifugation for 10 seconds at 14000 rpm. 15-20 μg protein was separated by electrophoresis (MiniCell, Invitrogen) with a 4-12% BIS-Tris gel (Invitrogen) and MES SDS Buffer (Invitrogen) and the proteins were transferred in 1X transfer buffer (20X; Bicine 0.5M, Bis-Tris 0.5M and EDTA 20.5mM) in 14.25% Methanol (BDH AnalaR®), for 90 minutes at 24 V, to a Protan® nitrocellulose membrane (Perkin-Elmer). For blockage of nonspecific binding the membrane was incubated in 3% milk (fat-free, Semper) in T-PBS (PBS with 0.05% Tween-20), at room temperature for a few hours or at 4°C was used followed by washing in T-PBS and incubation with primary antibody: Anti-β-actin. The membrane was then washed and incubated with a secondary antibody; anti-mouse peroxidase-conjugated rabbit immunoglobulin. Photos were developed by addition of reagent solution, an existing chemiluminescent detection system (Amersham ECL Plus™ Western Blotting Detection Reagents) on top of the membrane. The membranes were kept in T-PBS in 4°C and were used repeatedly. For repeated use the membrane were stripped with stripping solution (0.25 M citric acid and 1% SDS in dH$_2$O) and incubation 1 hour with primary rabbit antibody followed by, 1-2 hours incubation with secondary goat anti-rabbit antibody and photography, every step was followed by washing in T-PBS or PBS with 0.05% TritonX-100. The antibodies were diluted in blocking buffer, if different sizes of

targeted proteins antibodies were placed together, and the antibody solutions were collected and frozen. For repeated use, the antibody solution could be used up to ten times. For an overview of the antibodies used, see table 3.

Antibodies

Table 3. The antibodies used in Immunoblotting.

	Antibody	Dilution	Origin
Primary antibody	Anti β-actin	1:5000	Monoclonal Anti-β-actin Clone AC-15 (Mouse ascites fluid, Mouse IgG1) Sigma (Lot: 064K4790)
Primary antibody	Anti Erk	1:1000	Phosphos-p44/42 MAP Kinase (Thr202/Tyr204) Antibody Cell Signaling Technology (#9101)
Primary antibody	Anti Src	1:5000	Rabit Pab Anti-Src (PY^{418}) Phosphospecific Antibody Camarillo, Ca USA (Lot: 0902)
Primary antibody	Anti p38	1:5000	Phosphos-p38 MAP Kinase (The180/Tyr182) Antibody Cell Signaling Technology (#9211)
Primary antibody	Anti Akt	1:5000	Phosphos-Akt (Ser473) Antibody Cell Signaling Technology (#92713)
Primary antibody	Anti JNC	1:5000	Phosphos-SAPK/JNK (Thr183/Tyr185) Antibody Cell Signaling Technology (#9251)
Primary antibody	Anti Stat3	1:2500	Phosphos- Stat3 (Ser727) Antibody Cell Signaling Technology (#9134)
Primary antibody	Anti Stat5	1:2500	Phosphos- Stat5 (Tyr694) Antibody Cell Signaling Technology (#9351)
Primary antibody	Anti PLCγ	1:1000	Rabbit antiserum against PLCγ-1 By Carl-Henrik Heldin, Uppsala, Sweden [68]
Secondary antibody	Anti Mouse	1:1000	Peroxidase Labeled anti-mouse IgG (H&L) Vector Laboratories Inc. Burlingame, CA 94010 (Lot: L0807)
Secondary antibody	Anti Rabbit	1:1000	Anti-Rabbit Antibody HRP-linked IgG (H&L) Cell Signaling Technology
Secondary antibody	Anti Rabbit	1:5000	HRP-Goat Anti-Rabbit IgG (H&L) Zymed (Lot: 50900549)

Statistics

Statistical analyses of the result were conducted, by using a Standard error and Standard Student one-tailed T test and ANOVA in Microsoft EXCEL 5. The *Probability* values <0.05: *, <0.01: **, <0.005: ***, and <0.001: **** were determined to be significant, well significant, highly significant and extremely significant, respectively [69]. Normalization was performed of all samples and all each experiments.

Results

Proliferation assay

To found out whether the different cell lines proliferates due to the growth factors proliferation assay was performed. Treating cells in serum or no serum as control samples and experimental samples was pretreated with growth factor followed by removal and then treatment a second growth factor of FGF-2 or PDGF-BB, respectively. The T241 wt cells showed no increased cell number, proliferation; due to addition of the growth factors FGF-2 or PDGF-BB neither alone nor in combination compared to the serum treated cells (see figure 9).
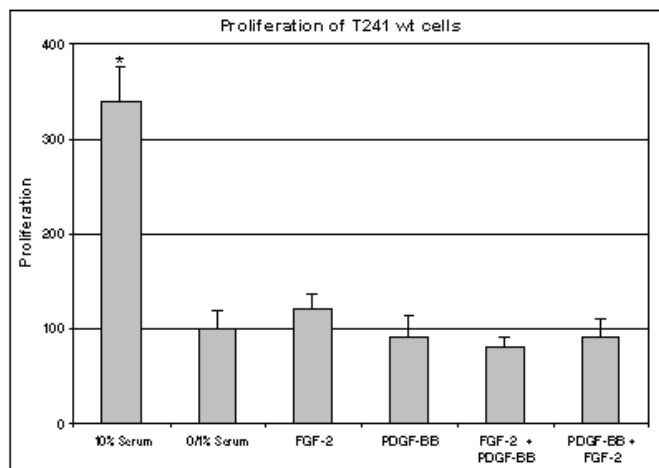

Figure 9. Proliferation of T241 wt cells. Normalized values of proliferated T241 wt cells, start number of cells is 10.000. The growth factors have no increasing effect on the cells number, compared to the background media. The P value is <0.05.

The proliferative effect, increase of cells, of the growth factors on the 3T3 cells is non or very low compared to the serum grown cells, therefore the conclusion drawn is that the growth factors have no effect, alone nor in combination, on the 3T3 cells (see figure 10). Statistics by an ANOVA in Excel was performed; by this test there is no differences between the cells treated with the growth factors. More interestingly, the SMC might indicate to have a decrease of proliferation due to FGF-2, alone and in pre-treatment, followed by treatment of PDGF-BB (see figure 11). This was not expected, since described the cells are known to become activated by PDGF-BB, which induce cell proliferation in presence of FGF-2 in human SMC [62]. What is seen though, is that after pretreatment with FGF-2 and treatment with PDGF-BB the cell number is in the same level as the background control, which could indicates on that PDGF-BB rescues the cells from the FGF-2 induced decreased proliferation.
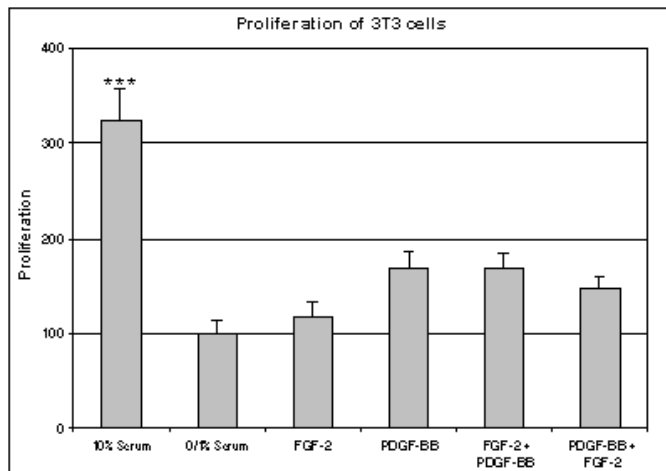
Figure 10. Proliferation of 3T3 cells. 10,000 cells are seeded and there is no increase of the cells treated with the growth factors compared to the control of background serum treated cells. ANOVA and TTEST in Excel was performed he P value is <0.005.
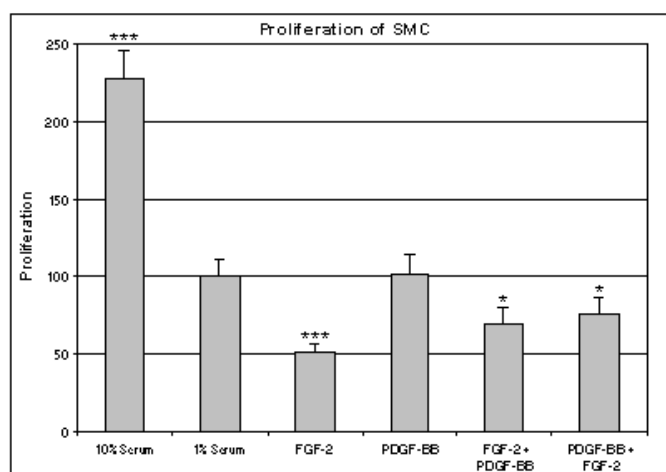

Figure 11. Proliferation of SMC, Smooth Muscle Cells. The FGF-2 seems to have a very small negative effect on the cells, where the cell number is decreased from start number of 10,000. The statistical comparison is based on the 1% serum, background media, and the P values are <0.05: * and <0.005: ***.

Chemotaxis assay

The cell migratory ability was studied by the use of a Boyden chamber, where cells pretreated with growth factor FGF-2 or PDGF-BB migrated into a membrane, toward the chemo-attractants FGF-2 or PDGF-BB. The migrated cells were counted and the result of the chemotaxis of T241 wt cells could possibly have a synergistically effect, increasing migration number, of pretreatment of the PDGF-BB and chemoattractant of the FGF-2, although according to statistics there is no significans between these samples compared to the other treatments of the growth factors. Therefore no real conclusions can be drawn in the direction of a possible migratory effect. The data is normalized, due to the experiments were performed in different conditions, the cells where grown in serum free or 1% serum, respectively (see figures 12).
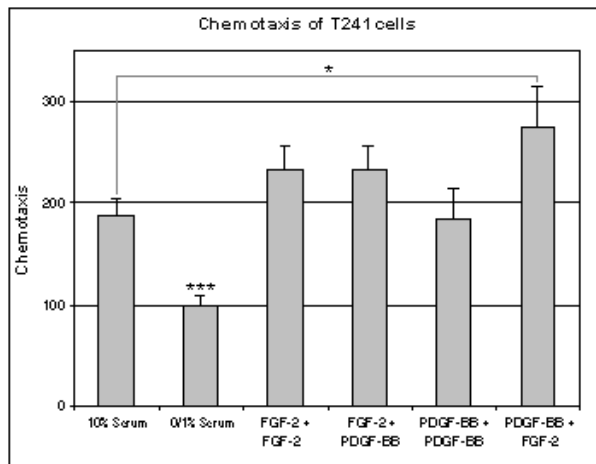
Figure 12. Chemotaxis of T241 wt cells. The media control is used as background. *P* value is 0.05 between 10% serum and PDGF-BB pretreated cells and with chemo attractant FGF-2. Although, these cells are not increased comparable to the other growth factor treated cells. The *P* values are <0.05: *.

The graphs of the 3T3 cell migration indicates a very small increase of migrated cells in the growth factor treated group compared to the background, the media control. According to the statistics, the only statistically significance is the background media control, which has few migrated cells compared to the other samples. The conclusion is no effect of the growth factors FGF-2 and PDGF-BB on migration of the 3T3 cells (see figure 13).
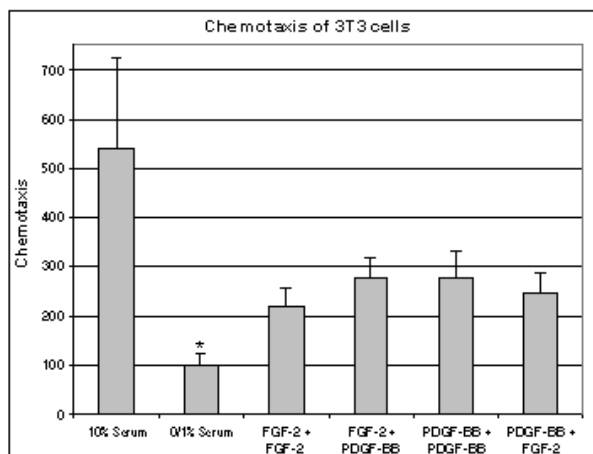

Figure 13. Chemotaxis of 3T3 cells. According to the statistics there is no significant difference between the data, except for the shown star of the background serum control. The *P* values are <0.05: *.

The chemotactic effect, the increased cell migration, of the SMC showed no effect of the FGF-2, but a small increased migration of the PDGF-BB treated cells, both to the chemo-attractants of FGF-2 and PDGF-BB, respectively. The FGF-2 treated cells seems to have decreased migration with the FGF-2 chemo-attractant, although this is not statistically significant compared to the media control. These two data might indicate that there is no migrational effect of the FGF-2 alone on the SMC, whereas the PDGF-BB has an increased migratory effect on the cells (se figure 14).
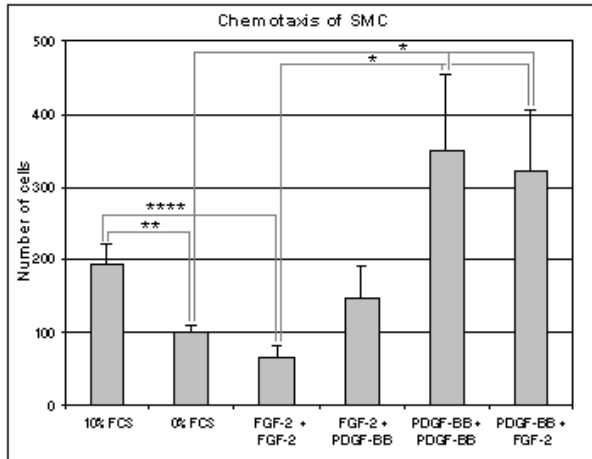
Figure 14. Chemotaxis of SMC. The PDGF-BB treated cells have induced migration, while the FGF-2 indicates to have no migratory effect of the cells. The P values are <0.05: *, <0.01: ** and <0.001: ****.

Western Blot

To study the signal transduction pathways the cells were pretreated with growth factor FGF-2 or PDGF-BB, which was removed followed by treatment of the growth factors. The proteins was extracted, separated due to size by electrophoresis and immuno blotted by antibodies to specific phosphorylated signal transduction molecules. Monoclonal mouse antibody against the β-actin was used to see the amount of proteins on the membrane and as a comparison to the polyclonal rabbit antibody that targeted the signal transduction proteins.

The BCE has high expression of Erk, Src and PLCγ when treated in both serum and FGF-2 (Figure 15). The Src, p38 and Stat remain unchanged through the different treatments of the cells. The Akt is increased after 15-30 minutes of FGF-2 treatment (figure 16) but not affected by PDGF-BB treatment (figure 17). The Erk is activated by PDGF-BB for a short time (figure 17); while FGF-2 treated cells have the Erk still activated after 6 hours (figure 16). The Erk is still activated after pretreatment for 24 hours with FGF-2 when treatment of PDGF-BB is added the Erk activation increases and interestingly remains activated after 6 hours (figure 18). PLCγ is induced by FGF-2 (figure 15) and have a slight indication of increased activation by time due to pretreatment of FGF-2 and treatment of PDGF-BB figure 19).
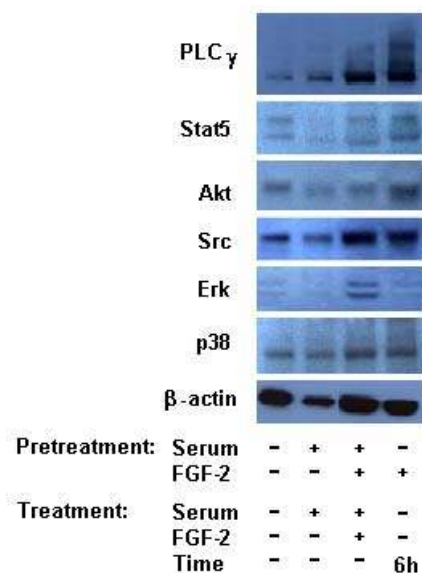

Figure 15. 24-hour pretreatment of cells, with or without serum and/or FGF-2 and treatment for 0 or 6 hours.
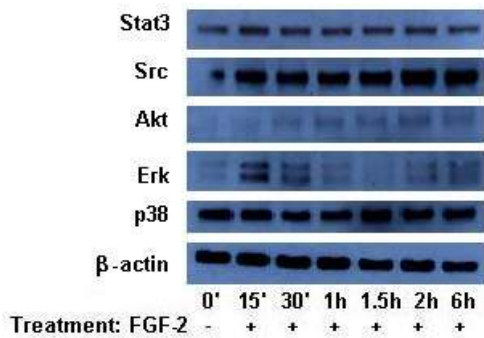
Figure 16. 24-hour serum free treatment followed by FGF-2 treatment for 15 minutes up to 6 hours Comment: the lower amount of Src protein in sample 0' is unfortunately due to unequal secondary antibody distribution.
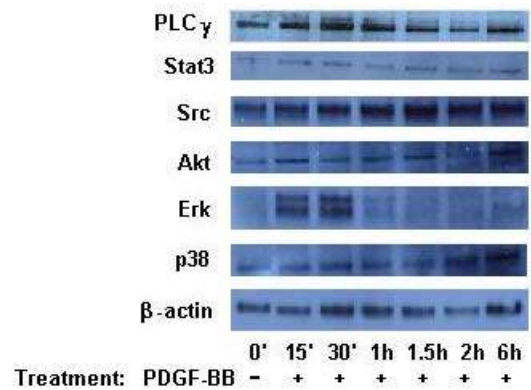

Figure 17. 24-hour serum free treatment followed by PDGF-BB treatment for 15 minutes up to 6 hours.
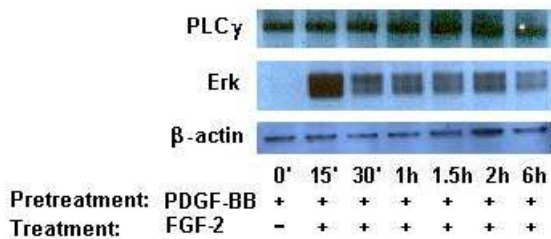

Figure 18. Pretreatment for 24 hours with PDGF-BB followed by treatment of FGF-2 for 15 minutes up to 6 hours.

Figure 19. Pretreatment for 24 hours with FGF-2 followed by treatment of PDGF-BB for 15 minutes up to 6 hours.

Discussion

Proliferation assay was carried out because it visualizes one of the early steps in the process of angiogenesis. The method is well established in this laboratory and not much can go wrong due to the human factor. The T241 wt cells do not show any effect of the growth factors, FGF-2 and PDGF-BB, neither alone nor in combination. This could be due to production by the tumors of these factors and their receptors and therefore external addition of the growth factors gives no effect. The 3T3, fibroblasts, cells treated with growth factors had no

significant effect compared to the serum grown cells. Prof. Cao and colleges indicates there is a synergistically effect by the growth factor on the BCE cells, the effect is above the effect of the serum treated cells. The tendency in the fibroblast could be due to the serum and in retrospect the cells should have probably have been grown in serum free media.

It has been published that PDGF-BB activates the SMC to release FGF-2, which then activates FGFR-1 [62, 63]. This should have been seen by increased cell migration in treatment of PDGF-BB and chemo-attractant FGF-2, instead decreased number of cells was observed (figure 14). What are seen are indications of negative effects of the cells. This is probably due to different reasons: 1) that the cells are very sensitive to serum free media, maybe they ought to have been treated in 1% serum, 2) the most reliable reason is that the SMC where treated as they where a cell line and had passage above 20, while the SMC should not have had passage number above 5 maximum up to 10, and new cells should have been isolated. Indeed, the cell morphology did change over time and from being slow and difficult grown; they changed and became smaller, simpler to trypsinate, and faster grown.

Another early step in the process of angiogenesis is migration, which is when vessel elongates. The process of migration was examined by chemotaxis assay, performed in the Boyden chamber is well established in this laboratory. Unlike the proliferation assay, which is a very precise method, the migration assay has a number of factors and uncertainties, which can affect the result. The migrated T241 wt cells show no effect of the growth factors, neither alone nor in combination. The 3T3 cells migrated well, but it was not due to the growth factors, but rather due to the cells general migratory ability. The SMC on the other hand did not migrate well. The cells looked less already when pre-treated in growth factors, as well as in the serum free control. Usually, with other cell lines, no changes are visible before counting the cells, except for the SMC. The results indicate no induction of the FGF-2 of the SMC; on the other hand the PDGF-BB pre-treated cells had an indication of higher migration ratio. Although, as described above, the cells where passaged to long and had changed morphology as well as migratory ability. The SMC migration has to be repeated in a manner of primary cells.

To distinguish the signal transduction events in the BCE cells protein assay was performed by Western blot. The antibodies are against the phsophorylated form of the proteins, which is the activated form by the FGF and PDGF receptors, and therefore shows the cellular activity. The starvation has probably activated the cells; the control views almost no completely zero limits of the proteins (figure 15). This indicates on activation of the certain signal transduction

pathways due to the non-serum pretreatment, and if the additional growth factor affect the signaling pathways are not very clear. Interestingly, both FGF-2 and PDGF-BB induces Erk, respectively. Although, pretreatment of FGF-2 might induce the PDGFR activation of Erk, in presence of PDGF-BB, to last longer time. The other signal transduction molecules seem to be activated as well, especially PLCγ and Src. For some cell types the pathways of PLCγ is important, especially in migration, it would be interesting to do new cell extract due to these data, by using a PLCγ inhibitor, to see whether the cells migrates or not. It would also be very interesting to examine if the PI3K pathway is activated, in comparison to the PLC γ, by the usage of an anti-PI3K antibody.

Conclusion

The results of proliferation and chemotaxis indicate that FGF-2 and PDGF-BB might not have any effect on the T241 wt and 3T3 cells. The PDGF-BB might have a migratory effect of the SMC. The hypothesis of FGF-2 inducing the endothelial cells to activate the PDGF receptor and in presence of PDGF-BB to induce strong activation of the PDGFR signal transduction pathways seems to be true for the Erk protein and maybe the PLCγ. Although, the FGF-2 pretreatment still affect the result of the PDGF-BB treatment, and therefore the cells need to be starved between treatment and pretreatment, but without the loss of the PDGF receptor induction due to the pretreatment of FGF-2. Longer time of starvation have to be done for the starvation control, which should have no induction of any activated proteins, and thereby decreased cellular activity. In future prospective the growth factor, FGF-2 and PDGF-BB, might be used for the treatment of diseases where angiogenesis is needed. On the other hand, the two growth factors together synergistically induces angiogenesis and this can be of inhibitory target for example in cancer.

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a. Figure 1. Adapted from www.google.se, Bilder, and searching word: Angiogenesis, Date: 060601.

Molecular mechanisms of angiogenic synergism between FGF-2 and PDGF-BB

Author Eva-Maria Hedlund

Supervisor *Professor* Yihai Cao

Summary

Tumors produce several angiogenic factors to promote blood vessel growth. These angiogenic factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and angiopoietin (Ang). In an advanced tumor tissues, these growth factors are often expressed at high levels. Although, the individual role of these factors in promoting tumor angiogenesis is relatively well studied, their joint effect in induction of tumor neovascularization is poorly understood. Prof. Cao and colleges has recently showed that two of these angiogenic factors, FGF-2 and PDGF-BB can synergistically induce angiogenesis in the mouse corneal model. To further elucidate the molecular mechanisms by which these two factors synergistically induce blood vessel growth, the signaling pathways induced by these two factors in endothelial cells was studied in endothelial cells. For functional analysis, the effect of the two factors on T241 wt (fibrosarcoma), 3T3 (fibroblast) and smooth muscle cells (SMC) was studied for proliferation and migration in vivo was also studied. Together with other findings by Prof. Cao's and colleges, it was concluded that FGF-2 and PDGF-BB synergistically induce tumor angiogenesis, tumor growth, metastasis, endothelial cell activities and angiogenic signaling activation. Thus, the studies have provided important information for current and future development of antiangiogenic drugs for the treatment of cancer and other angiogenesis-dependent diseases.

Background

Blood vessels can be formed in a number of different ways, one way is vasculogenesis that is the formation of blood vessels, it involves differentiation from stem cells to endothelial cells, and is crucial during the early stage of embryonic development [1]. Another mechanism of blood vessel formation is a process of division of large "mother" vessels into smaller "daughter" vessels, capillaries. It is a process where endothelial cells grow into the lumen of the "mother" blood vessels and forms a bridge, where the process continues to separates the vessel into smaller ones [2]. Third, but not least important, is angiogenesis, which is a process of sprouting new blood vessels from the pre-existing vessels. The angiogenesis process consists of several defined steps, which include; degradation of the endothelial basement membrane, invasion by proliferation and migration of endothelial cells, tube formation, branch formation, vessel remodeling, and reconstitution of the basement membrane [3] (see figure 1.).
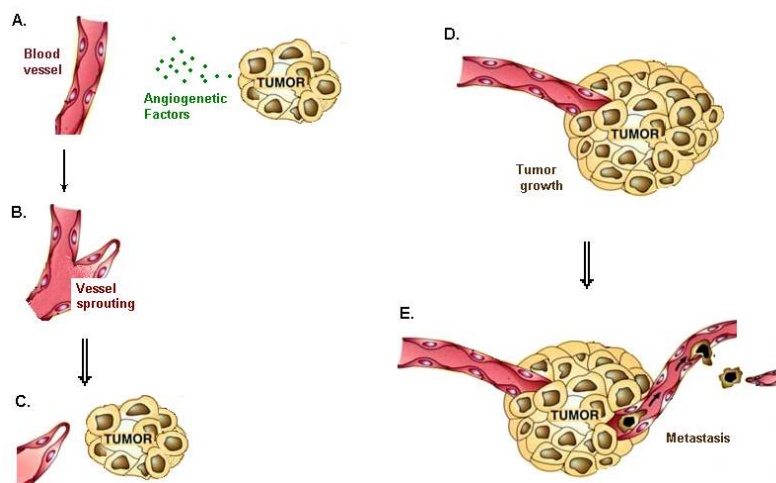

Figure 1. The process of tumor induced angiogenesis to tumor metastasis.
A.) The tumor releases angiogenic factors including VEGF, FGF and PDGF
to pre-existing blood vessels. B.) The endothelial cells start to proliferate
and migrate. C.) The sprouting vessel invade the tumor. D.) The tumor
grows and attracts more vessels. E.) Metastasis; tumor cells migrated
thorough the vessel and thereby are spreading. Figure adapted from [a].

In 1971, Judah Folkman and colleagues proposed that all solid tumor growth is dependent on angiogenesis and that suppression of angiogenesis might be useful for cancer therapy. In his early studies, he showed that a tumor could not grow beyond the size of 2-3 mm^3 without

angiogenesis [4]. Further, the first isolated factor, which "induced growth of new capillaries", was named tumor-angiogenesis factor (TAF) [5]. These findings were very important because beginning of a new era in prevention of tumor growth and metastasis. TAFs have been studied and today known to belong to several different families of TAFs. It is well known that tumors produce several angiogenic factors such as vascular endothelial growth factor (VEGF), angiopietin (Ang), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF). VEGF is the most known and well-studied tumor growth factor. Tumors produce several angiogenesis factors to switch on an angiogenic phenotype. The switch also requires simultaneous down-regulation of the expression levels of endogenous angiogenesis inhibitors [6]. For example, angiostatin, endostatin, thrombospondin [7, 8] and several other angiogenesis inhibitors have to be down regulated. Normal tissue has homeostasis between growth factors and inhibitors, but this is not the case for tumors, which have an imbalanced expression level [6].

Several angiogenic factors have been studied but only as single factors, and of no one in combinations. Single angiogenic factors such as vascular endothelial growth factor (VEGF) and FGF-2 have been tested alone in the treatment diseases, to induce angiogenesis. However, none of these trials have produced any beneficial effects (induction of angiogenesis) in patients [9]. For an example, VEGF was found to induce atherosclerotic plaque in atherosclerosis (thickening and hardening of the artery walls due to e.g. fat) [10]. Tumors on the other hand produce several factors, which indicates that the angiogenic factors act cooperatively and synergistically.

Synergism between FGF-2 and PDGF-BB

Recently it has been published that PDGF and FGF have been found to synergistically induce angiogenesis. First method was performed by implantation of micro pellets into the cornea, where the pellet contained growth factors alone or in combination (of PDGF-AA, PDGF-AB, PDGF-BB, FGF-2 and VEGF). A clear synergistic effect was visible between FGF-2 and PDGF-BB, while no significant effects occurred for single growth factors or the other combinations of growth factors (see figure 2). A long-lasting functional vessel assay was performed in the mouse cornea, by insertion of matrigel containing single or combined growth factors in the cornea. The mice were observed during long time. After a few days a

strong effect was visible, by much vessel growth in the cornea due to induced angiogenesis, and the effect lasted even after a year in the FGF-2 and PDGF-BB treated cornea (see figure 3) [9].
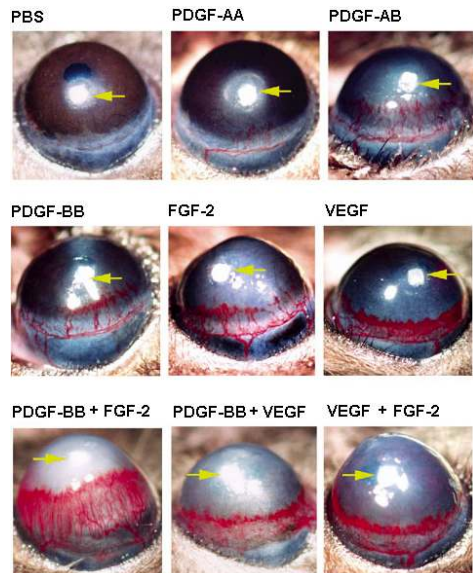

Figure 2. Mouse corneal micropocket; arrows indicating position of the insertion. An induction of angiogenesis is visible in PDGF-BB and FGF-2 treated mouse. Figure from [9].
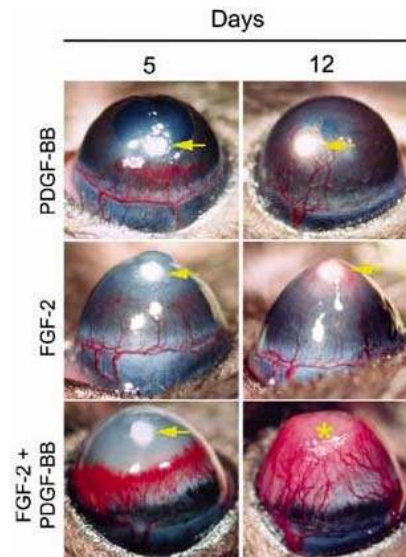

Figure 3. Mouse cornea matrigel; arrows/stars indicating location of matrigel and the star is a lost matrigel. Even though the loss, the synergistic induction is very strong and last for more than 12 days (data not shown). Figure from [9].

Fibroblast Growth Factor, FGF

The FGF family has several members including FGF-1 and FGF-2, which was first named acidic and basic FGF according to their properties [11]. FGF-2 was first purified in 3T3 fibroblast cells in 1975 [12] and since then much more is known; FGF-2 is found in the sub endothelial basement of blood vessels in all organs [13]. Delayed senescence and induction of division are two effects of FGF-1 and FGF-2 in cultured cell lines, such as endothelial cells, fibroblasts and smooth muscle cells [11]. FGF-1 and FGF-2 induce angiogenic phenotypes under physiological and pathological conditions [14]. FGFs are pleiotropic factors acting on different cell types including endothelial cells [14]. The FGF-2 consists of several different isoformes derived from the *fgf-2* gene [15]. Additionally, there are two different FGF-2 proteins of different size, where the larger proteins translocated into the nucleus (function unknown) [16] and the smaller protein is released by an autocrine mechanism [17]. FGF-2 mainly acts as an extra cellular factor; it is bound to the heparan-sulfate protoglycans

(HSPGs), which forms a reservoir in the body [14, 16]. The HSPG are found to be located on the surface of most cells and in the extracellular matrix [18]. It is activated of the FGF-HSPG-complex by binding to the tyrosine kinase FGF receptors (FGFRs) [19, 14, 16]. The FGF receptor is structurally similar to the Ig receptor super family [20], which also includes receptors such as the Platelet Derived Growth Factor Receptor, PDGFR [40]. The activation of the FGFR is due to binding of the FGF-HSPG complex on the surface and the three-unit complex is then internalized [22]. The dimerizised receptors get autophosphorylated of the tyrosine residues, which leads to the resulting activation of the intra cellular signal transduction pathways [16].

Signal transduction of FGFR

There are several signal transduction pathways (see figure 4); the first pathway is through the activation of the phospholipase C-gamma, PLCγ. The PLCγ was identified as a protein of 150 kDa associated to the FGFR after FGF-1 ligation [23]. The protein of PLCγ binds to the FGFR-1 and is tyrosine phosphorylated and thereby activated. Thereafter the PLCγ hydrolyses the phosphatidylinositol 4,5 bisphosphate to inositol 1,4,5 trisphosphate (IP_3) and diaglycerol (DAG). Two different pathways follow this, where IP_3 leads to the release of calcium from internal storages [24], which can induce migration. The other pathway by diaglycerol activates the protein kinase C, PKC, family [24, 25]. PKC is divergent and act on many different cell types, involving in cellular activities, such as proliferation, differentiation and apoptosis [25].

An additional pathway is the FGF receptor substrate 2, FRS2, signal transduction pathway. FRS2 is a docking molecule for the receptor and is associated with the SHP-2, which is needed for continuation of the Ras pathway [26, 27]. The phosphorylated FRS2 bind the small adaptor molecule Grb2, which by assistance by Sos activates Ras [28] that recruits Raf a serin/threonin kinas, which starts the cascade where it activates MEK, which then activates MAPK, mitogen activated protein kinases, including Extracellular signal-regulated kinase (Erk). These when activated translocates to the nucleus and activates the transcription factors, and thereby activates cellular activities [29].

All signaling pathways are not fully established. Yet two of these are including Src and the adaptor protein Crk. The activated FGFR-1 does phosphorylate Crk and when it is inactivated

the cells does not proliferate [30]. Likewise, when the Src is inactivated the endothelial cells do not migrate [31]. Also, the Src and PLCγ might inhibit each other [32].
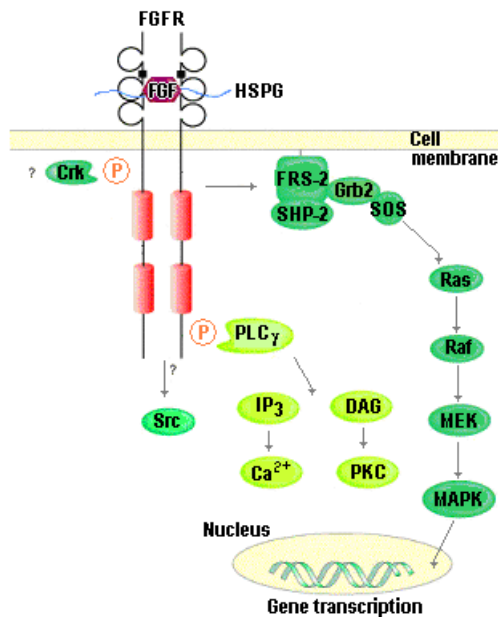

Figure 4. Signaling pathways of activated FGFR. Together in a complex the FGF and HSPG activate the FGFR, which undergo autophosphorylation and phosphorylates different signaling molecules, ending up with different activations of the cells. The FGFR also activates signaling molecules, which is of unknown function and further signaling transduction pathways. Figure adapted from [29, 33].

Platelet Derived Growth Factor, PDGF

The members of the PDGF family are important factors in the process of angiogenesis [34] and lymphangiogenesis [35] and function as a secondary vascular system in the higher vertebrates [36].

PDGF was discovered in serum as factor derived from platelets, where the factor stimulated proliferation in smooth muscle cells, SMC, [37] and has later been found to be involved in several cellular responses comprising proliferation, survival and migration [38]. The DPGF family has four members: PDGF-A, PDGF-B, PDGF-C and PDGF-D [39-43]. The PDGF-A and B are both synthesized as precursor molecules and undergo proteolytic cleavage at the NH_2 terminus. PDGF-B also undergoes cleavage at another site, close to the COOH terminus. The proteins form an anti parallel disulphide-bond dimer. The known isoformes include PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD [42, 44]. The intra cellular signals depend on the isoform of the dimer and are transferred to the cell by activation of the PDGF receptor upon binding.
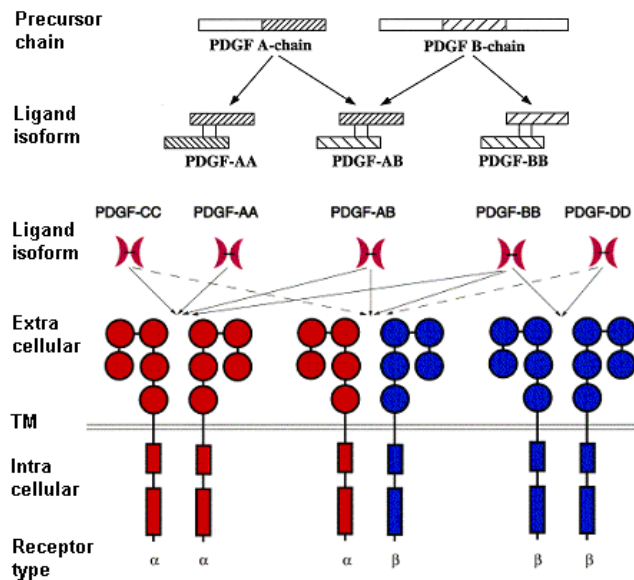
Figure 5. The PDGF ligands and receptors. Overview of ligand isoformes if PDGF-AA, -AB and –BB. The composition of CC and DD is not indicted. Also overview of ligand and receptor binding of PDGF and PDGFR, respectively. Figure adaptive from [44, 45].

Platelet Derived Growth Factor Receptor, PDGFR

The expression of PDGFR isotypes depends on the cell type, some cell types for an example vascular smooth muscle cells express both receptors [44], while other cell types for an example the human endothelial express only one receptor; the α-receptor [45], and the mouse capillary endothelial cells express the PDGF-β receptor [44]. Expression of the isotypes of the PDGFR due to stimulation of FGF-2 also depends on cell type, for an example in vascular smooth muscle cells; the α receptor expression is increased, but not the β receptor, [47]. The PDGF receptors are activated by dimerization by the binding of the dimeric PDGF ligand to hetero or homodimeric complexes of the α- and β-isoforms of PDGFR. The PDGF-AA activates the homodimer α and also the heterodimer of the receptor [44], while PDGF-BB is able to activate all kinds of receptor isoformes. The PDGF-CC can activate both the hetero dimer and the homodimer of PDGFR-β, whereas PDGF-DD only has the ability to activate the homodimer. The AB isoform of PDGF activates the heterodimer or the homodimer of PDGFR-α [48] (see figure 5).

The PDGFR is found on the cell membrane in caveolae, which is a membrane invagination involved in endocytosis [49]. When a ligand bind the receptor, the complex is internalized into endosomes [50], where the receptor complex dissociates and recycles to the surface, or the complex is degraded due to fusion of the endosomes with lysosomes, or the receptors are ubiquitinylated and undergo proteolysis [4, 51]. The kinase activity controls the internalization process [50]. Before this happens the ligand transfer its signal by the activation of the receptor followed by signal transduction.

Signal transduction pathways of PDGF

The tyrosine residues of the receptors are autophosphorylated [52], the autophosphorylation occurs in the kinase part of the receptor, which serves as docking sites for signal transduction molecules containing Src Homology 2 (SH2) domains [44]. This leads to signal transduction. The signal transduction is very complex, the pathways overlap each other and there is a lot of cross talking [53], and therefore a simplified picture will be described (see figure 6).

The activated PDGFR phosphorylates PLCγ [54, 55] and, as described, PLCγ regulates the activation of IP$_3$ and DAG, leading to the release of calcium and activation of PKC, respectively. For some cell types the PLCγ is important for migration [56]. Another pathway is phosphatidylinositol 3-kinase (PI3K) activates the Rac/Rho pathway leading to actin reorganization; involved in migration [57, 58]. Apart from signaling by the FGFR induced pathway, Grb-2, bind the autophosphorylated PDGFR directly, or via Shc, and thereafter Grb-2 activates the pathway of SOS activating Ras, which recruits RAF that activates the MAPK cascade, involving ERK, leading to gene transcription and cellular activities [59]. Ras can interestingly cross activate the PI3K pathway [58]. PI3K has another down stream molecule Akt/PKB, which is involved in survival in prevention of induced apoptosis [60, 61].

Src, is activated by the phosphorylated PDGFR, although the effect by Src is not fully clear [59]. Additionally a molecule with no known by the activation of PDGF function that also is activated by the PDGFR is Stat, Signal transducers and activators of transcription [59].
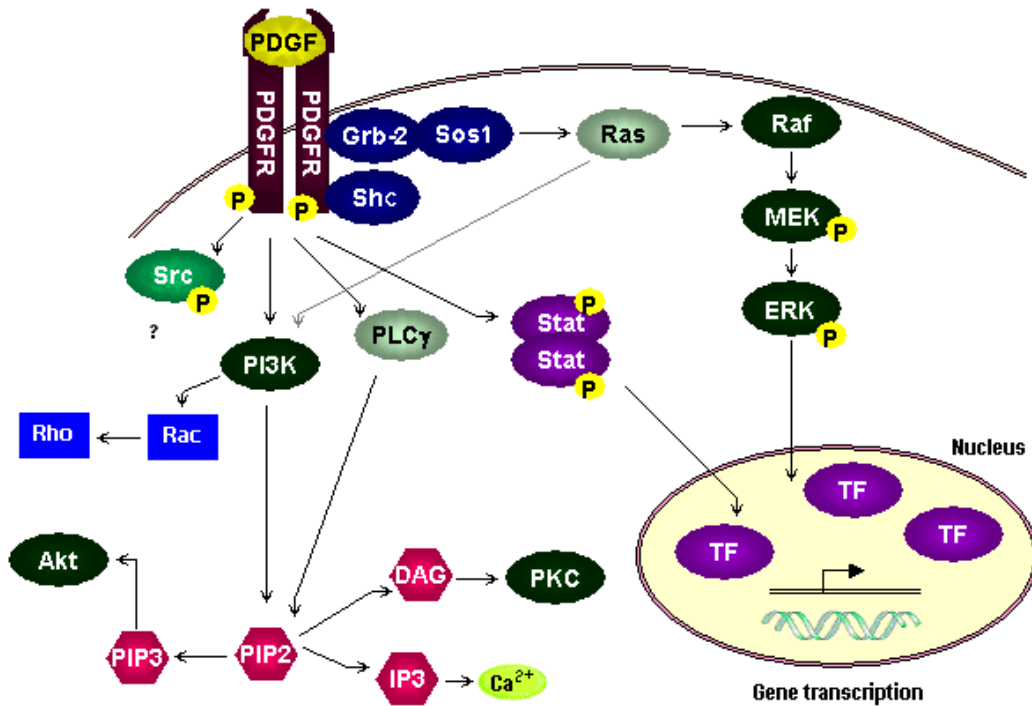
Figure 6. Signal transduction pathways induced by activated PDGFR due to PDGF binding. The autophosphorylated receptors activate signal transduction molecules upon phosphorylation. The sign of ? indicates unknown further pathway and TF is transcription factor. Figure adapted from [60, 64].

Additionally, it has recently been described in human SMC, that PDGF-BB is needed for activation of FGFR-1 in cell proliferation in presence of FGF-2 [62, 63]. A relationship between the endothelial cells and smooth muscle cells now occurs, where the growth factors regulates different cells (see figure 7). SMCs are known for their functional role as support for the sprouting angiogenic vessels, which are build by endothelial cells [1].
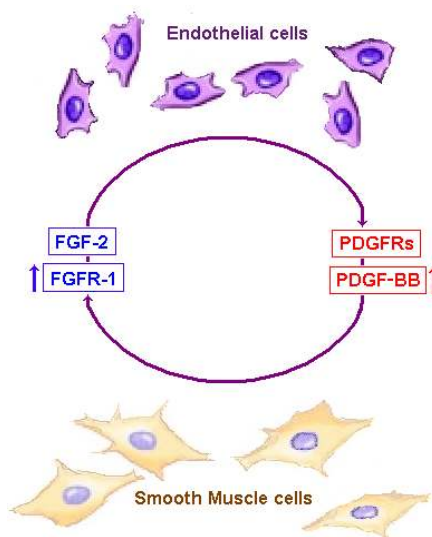

Figure 7. A hypothetical relationship between the Endothelial and Smooth Muscle Cells. The Bovine endothelial cells have induced cell activity by the activation of the PDGF-R due to FGF-2, in presence of PDGF-BB. On the other hand, the Smooth Muscle Cells have induced activation of the FGFR-1 due to PDGF-BB in presence of FGF-2.

Aim

In a mouse model the FGF-2 and PDGF-BB synergistically induces angiogenesis. One hypothesis is that when FGF-2 bind the FGFRs it activates PDGFRs, which will lead to cell proliferation, migration and differentiation in presence of PDGF-BB. It has been proved for bovine endothelial cells, BCE, that the growth factors induce proliferation and migration (unpublished data).

The aim was to further examine if other cell lines are also affected by the FGF-2 and PDGF-BB in proliferation and migration. A second task was to study the signal transduction pathways of the BCE, to se what really occurs in the cells due to the synergistically induced cell activity.

Material and Methods

Cell lines

Experiment were conducted using four cell lines; the T241 wt cells which are wild type Murine T241 fibro sarcoma cells and the SMCs, which are a primary mouse aortic smooth muscle cells, isolated by Prof. Cao and colleges. The 3T3 cells are fibroblasts and the BCE-HT$^+$s, which is a bovine capillary endothelial cell line, immortalized with human telomerase, hTERT [64].

Growth factors

Throughout the project the Platelet Derived Growth Factor, PDGF-BB (PeproTech, Rocky Hill, NJ) and the Fibroblast Growth Factor-2, FGF-2 (Phamacia & UpJohn, Milan, Italy) was used.

Chemotaxis assay

Confluent cells on a 10-cm plate were washed, trypsinized, resuspended in media (10% FCS) and seeded in four wells on a 6-well plate. After two hours incubation at 37°C, 5.0% CO_2 the cells were washed and new media (10% serum to control and 1% or 0% serum to the experiments) and growth factors (FGF-2, concentration of 10 ng/ml per well, or PDGF-BB concentration of 10 ng/ml or 100 ng/ml per well), was added followed by incubation overnight. A membrane (Polycarbonate, Osmonics INC) was incubated in 1% gelatine (Difco)

solution over night in room temperature or incubated for 30 minutes in 37°C before drying 30-60 minutes in room temperature. Chemo-attractants, control media and growth factors, were added to the lower wells of a Boyden chamber [65]. Six wells were used for each sample. The membrane was put on top of the wells (see table 1) and the Boyden Chamber was assembled, cell suspension was added to the upper tubes $20\text{-}60\cdot10^4$ cells per tube. The chamber was incubated and after 10 minutes and a glass lid was placed on top of the chamber, which was followed by 3.5-hours incubation. The cells on the membrane were then incubated in cold methanol for 15 minutes followed by 15-minutes incubation in Giemsa dye (Sigma-Aldrich). The non-migrated cells were wiped off with a paper towel or a cotton swab and the membrane was then mounted between two glass lids with Nail polish. Each experiment contained six replicates and the experiment was repeated trice [66].

Table 1. Overview of the pre-treated cells and chemo-attractants, respectively.

	Experiment		Controls
Cells pre-treated:	FGF-2	PDGF-BB	Serum 10%
Chemo-attractant:	FGF-2	PDGF-BB	Serum 10%
Cells pre-treated:	FGF-2	PDGF-BB	Serum 1%
Chemo-attractant:	PDGF-BB	FGF-2	Serum 1%

Proliferation assay

Confluent cells on a 10-cm dish were washed, trypsinized and resuspended in media (10% FCS) and the cells were counted in a Bürker Chamber, and 10^4 cells were seeded in each well on a 24-well plate. After 2-hours incubation in 37°C the cells were washed and pretreated by addition of new media (10% FBS to the control and 1% or 0% FBS for the experiments) and growth factors (FGF-2, to concentration of 10 ng/ml or 100 ng/ml per well, or PDGF-BB to concentration of 100 ng/ml per well) (see table 2). After overnight, specific wells were washed, new media and new growth factors were added, and this is referred as treatment. The cells were further incubated for a totally of 72-hours in 37°C, 5.0% CO_2 and trypsinized and resuspended in IsotonII solution (Beckman coulter) and counted in a Coulter Counter (Beckman Coulter), also 92 hour assay was performed. Each experiment was repeated in four replicates and the experiments were repeated twice or trice [67].

Table 2. Proliferation set-up, treatment of cells, showing the four replicates.

	Positive control	Negative control	Experimental controls		Experiments	
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2
Pre-treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	FGF-2	PDGF-BB
Treatment	Serum 10%	Serum free	FGF-2	PDGF-BB	PDGF-BB	FGF-2

Cell extracts

BCE-HT^{+} cells were grown to 90-100% confluence in 60-mm dishes and DMEM (10% serum) containing 10 ng/ml FGF-2 for 24 hours. The cells were pretreated differently according to the following scheme; controls with serum, experiments with serum free media containing 10 ng/ml FGF-2, 100 ng/ml PDGF-BB or without any growth factor, followed by washed and the procedure of treatment was 24-hours incubation in serum free media containing 10 ng/ml FGF-2, 100 ng/ml PDGF-BB or without any growth factor (see figure 8). The cellular activity was stopped by addition of Whole Cell Lysis Buffer and Inhibitors containing: 20 mM Hepes buffer, pH 7.1, supplemented with 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 30 mM $Na_4P_2O_7$, 5 µM $ZnCl_2$, 20 mM β-glycerophosphate, 10 mM 4-nitrophenyl phosphate, 1 mM DTT, 200 mM Na_3VO_4, 0.5 mM PMSF [67]. The activity was stopped at different times points: after 0, 15, 30, 60, 90, 120 minutes and 6 hours (see figure 8). To the cells of 0 minutes not treated with growth factors, but served as controls. The cells were harvested by scraping with a cell scrape, vortexed for 10 seconds, centrifuged for 15 minutes at 13000 rpm in 4°C and the supernatant containing the proteins was transferred to in liquid nitrogen and snap freezed and stored of -80°C. The protein concentration was measured in duplicates in a 96-well plate and as a control BSA was used in different concentrations diluted in PBS. Colorimetric reagent solution (Bio Rad D_c Protein Assay, BIO-RAD) was added to all samples. After 10 minutes incubation the protein concentration was measured by measuring the absorbance at 960. A table of absorbance versus concentration was made of the BSA samples. Out of this the concentration of the protein samples was calculated due to its

absorbance. Although, it was followed by an extra balancing after the electrophoresis, which will be further described in the Electrophoresis and Immuno blotting section.
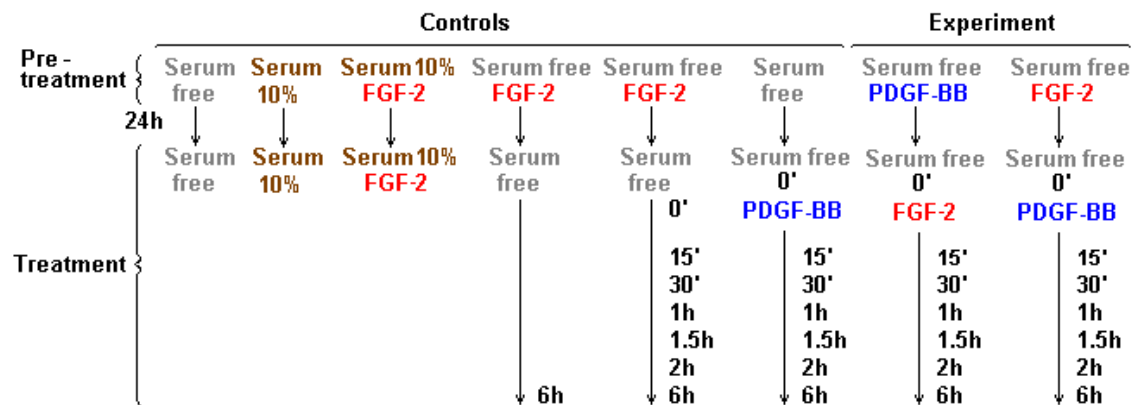

Figure 8. Set up of cell treatment for cell extracts. Time starts at 0 minutes (0´) is when the media is changed and growth factor are added cells and is incubated first for 15 minutes (15´) up to 6 hours (6h), except for the 0´ that got new media and no growth factor.

Western blot; Electrophoresis and Immuno blotting

The cell extract samples were boiled, thus denaturating the proteins, and NuPAGE LOS sample buffer was added, followed by heating and centrifugation for 10 seconds at 14000 rpm. 15-20 μg protein was separated by electrophoresis (MiniCell, Invitrogen) with a 4-12% BIS-Tris gel (Invitrogen) and MES SDS Buffer (Invitrogen) and the proteins were transferred in 1X transfer buffer (20X; Bicine 0.5M, Bis-Tris 0.5M and EDTA 20.5mM) in 14.25% Methanol (BDH AnalaR®), for 90 minutes at 24 V, to a Protan® nitrocellulose membrane (Perkin-Elmer). For blockage of nonspecific binding the membrane was incubated in 3% milk (fat-free, Semper) in T-PBS (PBS with 0.05% Tween-20), at room temperature for a few hours or at 4°C was used followed by washing in T-PBS and incubation with primary antibody: Anti-β-actin. The membrane was then washed and incubated with a secondary antibody; anti-mouse peroxidase-conjugated rabbit immunoglobulin. Photos were developed by addition of reagent solution, an existing chemiluminescent detection system (Amersham ECL Plus™ Western Blotting Detection Reagents) on top of the membrane. The membranes were kept in T-PBS in 4°C and were used repeatedly. For repeated use the membrane were stripped with stripping solution (0.25 M citric acid and 1% SDS in dH$_2$O) and incubation 1 hour with primary rabbit antibody followed by, 1-2 hours incubation with secondary goat anti-rabbit antibody and photography, every step was followed by washing in T-PBS or PBS with 0.05% TritonX-100. The antibodies were diluted in blocking buffer, if different sizes of

targeted proteins antibodies were placed together, and the antibody solutions were collected and frozen. For repeated use, the antibody solution could be used up to ten times. For an overview of the antibodies used, see table 3.

Antibodies

Table 3. The antibodies used in Immunoblotting.

	Antibody	Dilution	Origin
Primary antibody	Anti β-actin	1:5000	Monoclonal Anti-β-actin Clone AC-15 (Mouse ascites fluid, Mouse IgG1) Sigma (Lot: 064K4790)
Primary antibody	Anti Erk	1:1000	Phosphos-p44/p42 MAP Kinase (Thr202/Tyr204) Antibody Cell Signaling Technology (#9101)
Primary antibody	Anti Src	1:5000	Rabit Pab Anti-Src (PY^{418}) Phosphospecific Antibody Camarillo, Ca USA (Lot: 0902)
Primary antibody	Anti p38	1:5000	Phosphos-p38 MAP Kinase (The180/Tyr182) Antibody Cell Signaling Technology (#9211)
Primary antibody	Anti Akt	1:5000	Phosphos-Akt (Ser473) Antibody Cell Signaling Technology (#92713)
Primary antibody	Anti JNC	1:5000	Phosphos-SAPK/JNK (Thr183/Tyr185) Antibody Cell Signaling Technology (#9251)
Primary antibody	Anti Stat3	1:2500	Phosphos- Stat3 (Ser727) Antibody Cell Signaling Technology (#9134)
Primary antibody	Anti Stat5	1:2500	Phosphos- Stat5 (Tyr694) Antibody Cell Signaling Technology (#9351)
Primary antibody	Anti PLCγ	1:1000	Rabbit antiserum against PLCγ-1 By Carl-Henrik Heldin, Uppsala, Sweden [68]
Secondary antibody	Anti Mouse	1:1000	Peroxidase Labeled anti-mouse IgG (H&L) Vector Laboratories Inc. Burlingame, CA 94010 (Lot: L0807)
Secondary antibody	Anti Rabbit	1:1000	Anti-Rabbit Antibody HRP-linked IgG (H&L) Cell Signaling Technology
Secondary antibody	Anti Rabbit	1:5000	HRP-Goat Anti-Rabbit IgG (H&L) Zymed (Lot: 50900549)

Statistics

Statistical analyses of the result were conducted, by using a Standard error and Standard Student one-tailed T test and ANOVA in Microsoft EXCEL 5. The *Probability* values <0.05: *, <0.01: **, <0.005: ***, and <0.001: **** were determined to be significant, well significant, highly significant and extremely significant, respectively [69]. Normalization was performed of all samples and all each experiments.

Results

Proliferation assay

To found out whether the different cell lines proliferates due to the growth factors proliferation assay was performed. Treating cells in serum or no serum as control samples and experimental samples was pretreated with growth factor followed by removal and then treatment a second growth factor of FGF-2 or PDGF-BB, respectively. The T241 wt cells showed no increased cell number, proliferation; due to addition of the growth factors FGF-2 or PDGF-BB neither alone nor in combination compared to the serum treated cells (see figure 9).
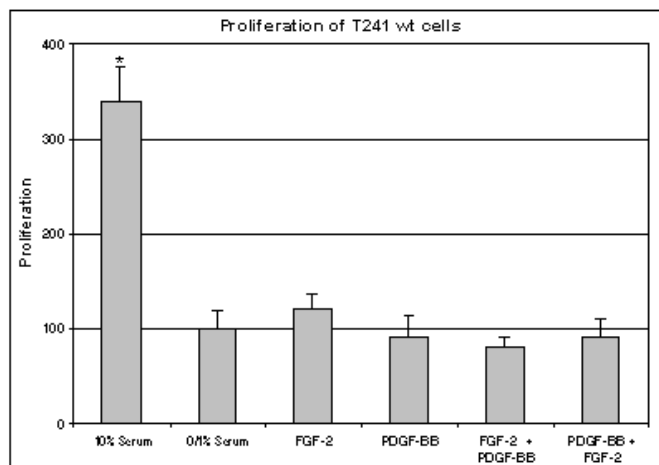

Figure 9. Proliferation of T241 wt cells. Normalized values of proliferated T241 wt cells, start number of cells is 10.000. The growth factors have no increasing effect on the cells number, compared to the background media. The P value is <0.05.

The proliferative effect, increase of cells, of the growth factors on the 3T3 cells is non or very low compared to the serum grown cells, therefore the conclusion drawn is that the growth factors have no effect, alone nor in combination, on the 3T3 cells (see figure 10). Statistics by an ANOVA in Excel was performed; by this test there is no differences between the cells treated with the growth factors. More interestingly, the SMC might indicate to have a decrease of proliferation due to FGF-2, alone and in pre-treatment, followed by treatment of PDGF-BB (see figure 11). This was not expected, since described the cells are known to become activated by PDGF-BB, which induce cell proliferation in presence of FGF-2 in human SMC [62]. What is seen though, is that after pretreatment with FGF-2 and treatment with PDGF-BB the cell number is in the same level as the background control, which could indicates on that PDGF-BB rescues the cells from the FGF-2 induced decreased proliferation.
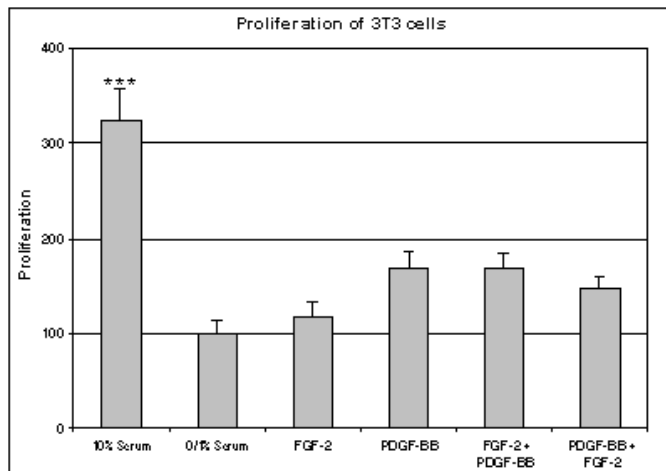
Figure 10. Proliferation of 3T3 cells. 10,000 cells are seeded and there is no increase of the cells treated with the growth factors compared to the control of background serum treated cells. ANOVA and TTEST in Excel was performed he P value is <0.005.
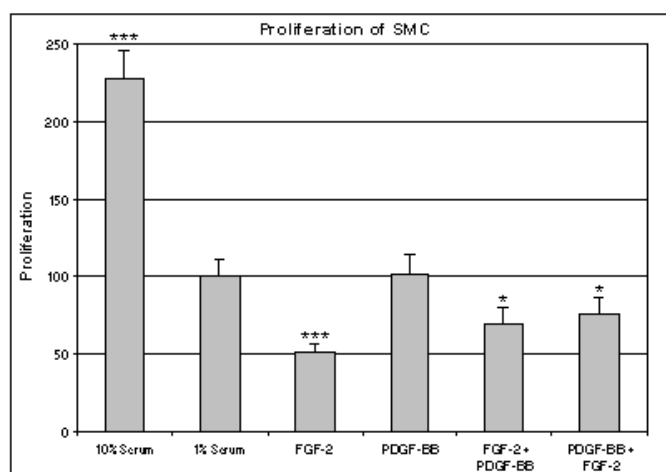

Figure 11. Proliferation of SMC, Smooth Muscle Cells. The FGF-2 seems to have a very small negative effect on the cells, where the cell number is decreased from start number of 10,000. The statistical comparison is based on the 1% serum, background media, and the P values are <0.05: * and <0.005: ***.

Chemotaxis assay

The cell migratory ability was studied by the use of a Boyden chamber, where cells pretreated with growth factor FGF-2 or PDGF-BB migrated into a membrane, toward the chemo-attractants FGF-2 or PDGF-BB. The migrated cells were counted and the result of the chemotaxis of T241 wt cells could possibly have a synergistically effect, increasing migration number, of pretreatment of the PDGF-BB and chemoattractant of the FGF-2, although according to statistics there is no significans between these samples compared to the other treatments of the growth factors. Therefore no real conclusions can be drawn in the direction of a possible migratory effect. The data is normalized, due to the experiments were performed in different conditions, the cells where grown in serum free or 1% serum, respectively (see figures 12).
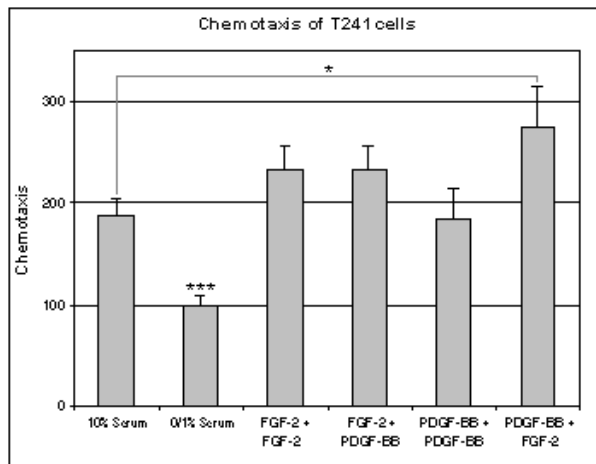
Figure 12. Chemotaxis of T241 wt cells. The media control is used as background. *P* value is 0.05 between 10% serum and PDGF-BB pretreated cells and with chemo attractant FGF-2. Although, these cells are not increased comparable to the other growth factor treated cells. The *P* values are <0.05: *.

The graphs of the 3T3 cell migration indicates a very small increase of migrated cells in the growth factor treated group compared to the background, the media control. According to the statistics, the only statistically significance is the background media control, which has few migrated cells compared to the other samples. The conclusion is no effect of the growth factors FGF-2 and PDGF-BB on migration of the 3T3 cells (see figure 13).
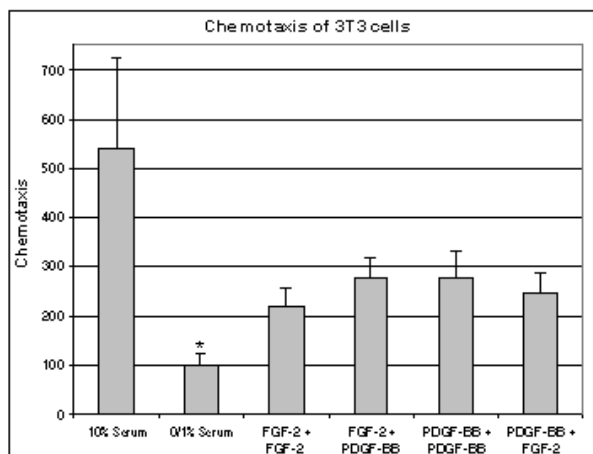

Figure 13. Chemotaxis of 3T3 cells. According to the statistics there is no significant difference between the data, except for the shown star of the background serum control. The *P* values are <0.05: *.

The chemotactic effect, the increased cell migration, of the SMC showed no effect of the FGF-2, but a small increased migration of the PDGF-BB treated cells, both to the chemo-attractants of FGF-2 and PDGF-BB, respectively. The FGF-2 treated cells seems to have decreased migration with the FGF-2 chemo-attractant, although this is not statistically significant compared to the media control. These two data might indicate that there is no migrational effect of the FGF-2 alone on the SMC, whereas the PDGF-BB has an increased migratory effect on the cells (se figure 14).
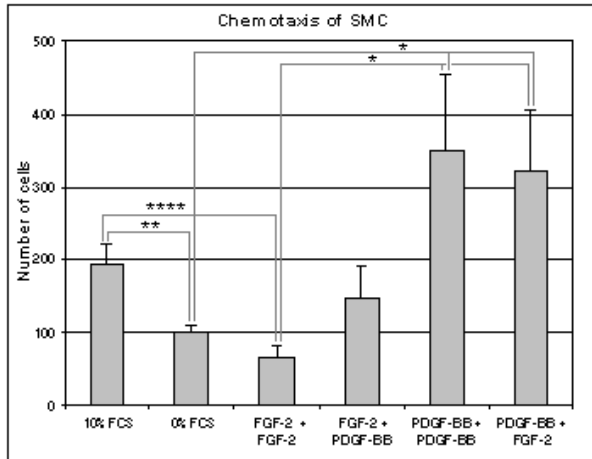
Figure 14. Chemotaxis of SMC. The PDGF-BB treated cells have induced migration, while the FGF-2 indicates to have no migratory effect of the cells. The P values are <0.05: *, <0.01: ** and <0.001: ****.

Western Blot

To study the signal transduction pathways the cells were pretreated with growth factor FGF-2 or PDGF-BB, which was removed followed by treatment of the growth factors. The proteins was extracted, separated due to size by electrophoresis and immuno blotted by antibodies to specific phosphorylated signal transduction molecules. Monoclonal mouse antibody against the β-actin was used to see the amount of proteins on the membrane and as a comparison to the polyclonal rabbit antibody that targeted the signal transduction proteins.

The BCE has high expression of Erk, Src and PLCγ when treated in both serum and FGF-2 (Figure 15). The Src, p38 and Stat remain unchanged through the different treatments of the cells. The Akt is increased after 15-30 minutes of FGF-2 treatment (figure 16) but not affected by PDGF-BB treatment (figure 17). The Erk is activated by PDGF-BB for a short time (figure 17); while FGF-2 treated cells have the Erk still activated after 6 hours (figure 16). The Erk is still activated after pretreatment for 24 hours with FGF-2 when treatment of PDGF-BB is added the Erk activation increases and interestingly remains activated after 6 hours (figure 18). PLCγ is induced by FGF-2 (figure 15) and have a slight indication of increased activation by time due to pretreatment of FGF-2 and treatment of PDGF-BB figure 19).
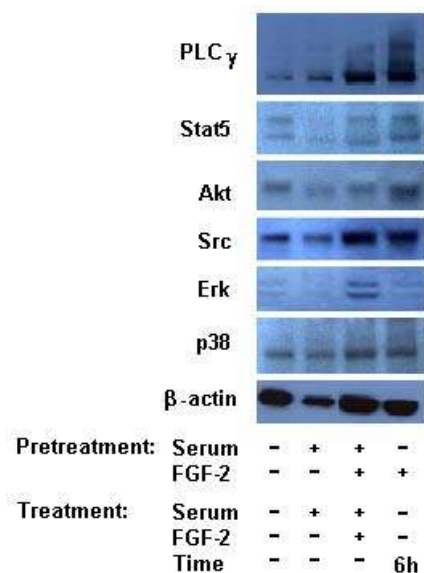

Figure 15. 24-hour pretreatment of cells, with or without serum and/or FGF-2 and treatment for 0 or 6 hours.
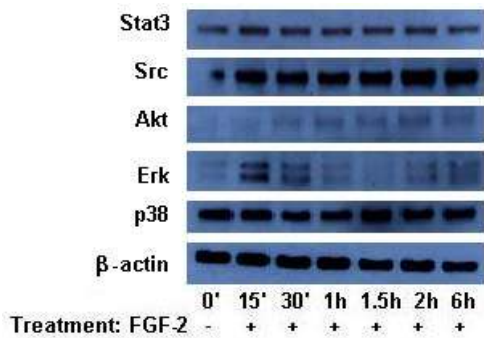
Figure 16. 24-hour serum free treatment followed by FGF-2 treatment for 15 minutes up to 6 hours Comment: the lower amount of Src protein in sample 0' is unfortunately due to unequal secondary antibody distribution.
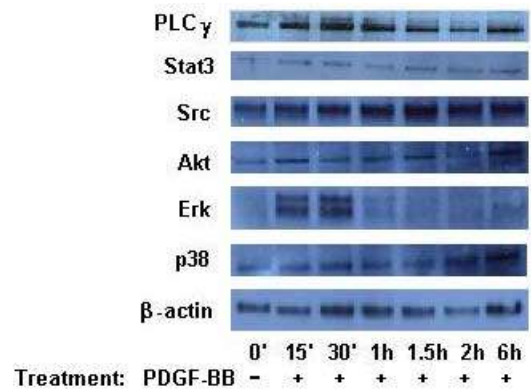

Figure 17. 24-hour serum free treatment followed by PDGF-BB treatment for 15 minutes up to 6 hours.
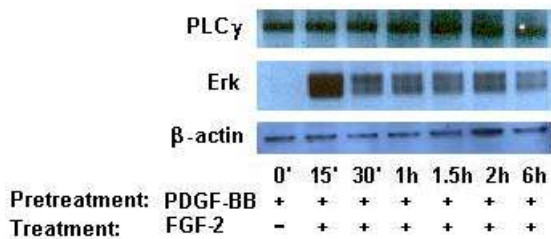

Figure 18. Pretreatment for 24 hours with PDGF-BB followed by treatment of FGF-2 for 15 minutes up to 6 hours.

Figure 19. Pretreatment for 24 hours with FGF-2 followed by treatment of PDGF-BB for 15 minutes up to 6 hours.

Discussion

Proliferation assay was carried out because it visualizes one of the early steps in the process of angiogenesis. The method is well established in this laboratory and not much can go wrong due to the human factor. The T241 wt cells do not show any effect of the growth factors, FGF-2 and PDGF-BB, neither alone nor in combination. This could be due to production by the tumors of these factors and their receptors and therefore external addition of the growth factors gives no effect. The 3T3, fibroblasts, cells treated with growth factors had no

significant effect compared to the serum grown cells. Prof. Cao and colleges indicates there is a synergistically effect by the growth factor on the BCE cells, the effect is above the effect of the serum treated cells. The tendency in the fibroblast could be due to the serum and in retrospect the cells should have probably have been grown in serum free media.

It has been published that PDGF-BB activates the SMC to release FGF-2, which then activates FGFR-1 [62, 63]. This should have been seen by increased cell migration in treatment of PDGF-BB and chemo-attractant FGF-2, instead decreased number of cells was observed (figure 14). What are seen are indications of negative effects of the cells. This is probably due to different reasons: 1) that the cells are very sensitive to serum free media, maybe they ought to have been treated in 1% serum, 2) the most reliable reason is that the SMC where treated as they where a cell line and had passage above 20, while the SMC should not have had passage number above 5 maximum up to 10, and new cells should have been isolated. Indeed, the cell morphology did change over time and from being slow and difficult grown; they changed and became smaller, simpler to trypsinate, and faster grown.

Another early step in the process of angiogenesis is migration, which is when vessel elongates. The process of migration was examined by chemotaxis assay, performed in the Boyden chamber is well established in this laboratory. Unlike the proliferation assay, which is a very precise method, the migration assay has a number of factors and uncertainties, which can affect the result. The migrated T241 wt cells show no effect of the growth factors, neither alone nor in combination. The 3T3 cells migrated well, but it was not due to the growth factors, but rather due to the cells general migratory ability. The SMC on the other hand did not migrate well. The cells looked less already when pre-treated in growth factors, as well as in the serum free control. Usually, with other cell lines, no changes are visible before counting the cells, except for the SMC. The results indicate no induction of the FGF-2 of the SMC; on the other hand the PDGF-BB pre-treated cells had an indication of higher migration ratio. Although, as described above, the cells where passaged to long and had changed morphology as well as migratory ability. The SMC migration has to be repeated in a manner of primary cells.

To distinguish the signal transduction events in the BCE cells protein assay was performed by Western blot. The antibodies are against the phsophorylated form of the proteins, which is the activated form by the FGF and PDGF receptors, and therefore shows the cellular activity. The starvation has probably activated the cells; the control views almost no completely zero limits of the proteins (figure 15). This indicates on activation of the certain signal transduction

pathways due to the non-serum pretreatment, and if the additional growth factor affect the signaling pathways are not very clear. Interestingly, both FGF-2 and PDGF-BB induces Erk, respectively. Although, pretreatment of FGF-2 might induce the PDGFR activation of Erk, in presence of PDGF-BB, to last longer time. The other signal transduction molecules seem to be activated as well, especially PLCγ and Src. For some cell types the pathways of PLCγ is important, especially in migration, it would be interesting to do new cell extract due to these data, by using a PLCγ inhibitor, to see whether the cells migrates or not. It would also be very interesting to examine if the PI3K pathway is activated, in comparison to the PLC γ, by the usage of an anti-PI3K antibody.

Conclusion

The results of proliferation and chemotaxis indicate that FGF-2 and PDGF-BB might not have any effect on the T241 wt and 3T3 cells. The PDGF-BB might have a migratory effect of the SMC. The hypothesis of FGF-2 inducing the endothelial cells to activate the PDGF receptor and in presence of PDGF-BB to induce strong activation of the PDGFR signal transduction pathways seems to be true for the Erk protein and maybe the PLCγ. Although, the FGF-2 pretreatment still affect the result of the PDGF-BB treatment, and therefore the cells need to be starved between treatment and pretreatment, but without the loss of the PDGF receptor induction due to the pretreatment of FGF-2. Longer time of starvation have to be done for the starvation control, which should have no induction of any activated proteins, and thereby decreased cellular activity. In future prospective the growth factor, FGF-2 and PDGF-BB, might be used for the treatment of diseases where angiogenesis is needed. On the other hand, the two growth factors together synergistically induces angiogenesis and this can be of inhibitory target for example in cancer.

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a. Figure 1. Adapted from www.google.se, Bilder, and searching word: Angiogenesis, Date: 060601.

Molecular mechanisms of angiogenic synergism between Fibroblast Growth Factor-2 and Platelet Derived Growth Factor-BB

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