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A comparison of homo- versus heterodimeric PDGF receptor signaling

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Title (English)

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Abstract

Platelet derived growth factor (PDGF) and its receptors are involved in signal transduction and over expression of PDGF and both receptors has been shown in several tumors. PDGF consists of disulphide bonded A- and B-chains (forming the three isoforms AA, AB and BB) that induce the formation of αα-, αβ- and ββ-receptor dimers. The aim of my project has been to compare the signaling between the homodimeric and heterodimeric PDGF receptors by use of DNA microarray technique. Foreskin fibroblasts have been used as a model system. Cells were either stimulated with the different isoforms of PDGF (to activate the different receptor combinations) or left untreated (controls). RNA was extracted from the cells and hybridized to the arrays. The results obtained are preliminary but show that there are some differences in homo- and heterodimeric PDGF receptor signaling.

Keywords

Platelet derived growth factor, PDGF, microarray, gene array, heterodimer

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A comparison of homo- versus heterodimeric PDGF receptor signaling.

Katarina Linde

Sammanfattning

Platelet derived growth factor (PDGF) är en tillväxtfaktor och utsöndras från cellen. Den består av två molekyler, A och B, som kan bilda tre olika par (dimerer): AA, AB och BB. De olika dimerena kan binda till sk mottagarmolekyler (receptorer) som sitter fast på cellens yta. I många cancertumörer är PDGF och dess receptorer överaktiva vilket gjort dem intressanta för vidare studier.

Det finns två olika mottagarmolekyler som PDGF kan binda till, nämligen α- och β-receptorer. När PDGF binder till receptorn sker en parbildning av två receptorer. Då kan αα-, αβ- och ββ-receptorpar bildas (beroende på vilken PDGF-dimer som binder). När dessa par bildas aktiveras receptorparet och börjar förmedla signaler till cellen, t.ex. att cellen ska börja dela sig.

I mitt projekt har jag gjort en jämförelse mellan αβ-receptorns och αα-, ββ-receptorernas signalering för att se om αβ receptorn förmedlar unika signaler till cellen eller endast summan av αα- och ββ-receptorernas signaler. Det har jag gjort m.h.a. den sk microarraytekniken där man kan se vilka gener i cellen som påverkats av de signaler som receptorerna har förmedlat. På så sätt kan man se om det finns någon skillnad i signalering mellan de olika receptorkombinationerna.

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1 Introduction

1.1 Platelet derived growth factor and its receptors

Cell growth, migration and apoptosis, important events in cancer development, are partly regulated by different growth factors. Platelet-derived growth factor (PDGF) is one such factor. It consists of disulphide-bonded A- and B-chains, which can exist as AA, AB or BB dimers. PDGF binds to cell surface receptors, with tyrosine kinase activity, called PDGF receptors. These receptors belong to the super-family of protein-tyrosine kinase receptors, which also contains for example the epidermal growth factor (EGF) receptor family and the fibroblast growth factor (FGF) receptor family.

PDGF and its receptors are normally expressed in the developing embryo. Inactivation of the genes for the A- and B-chain and the receptors lead to embryonic or perinathal dead (Heldin et al., 1999). In the adult, PDGF is involved in the process of wound healing (Heldin et al., 1999). PDGF is also involved in several diseases, i.e. in human oncogenesis. PDGF and PDGF receptors are expressed in several tumors and it has been observed that particularly in more malignant, high grade tumors, over expression of PDGF and its receptors is common (Westermark et al., 1995).

As mentioned above PDGF exists in three different isoforms: AA, AB and BB. They bind to two structurally related protein tyrosine kinases, the α– and β-receptors, with different specificities. The dimeric nature of PDGF allows it to interact simultaneously with two receptor monomers, inducing receptor dimerization. The A-chain only recognizes the α-receptor whereas the B-chain can bind both the α– and β-receptors (see figure 1).

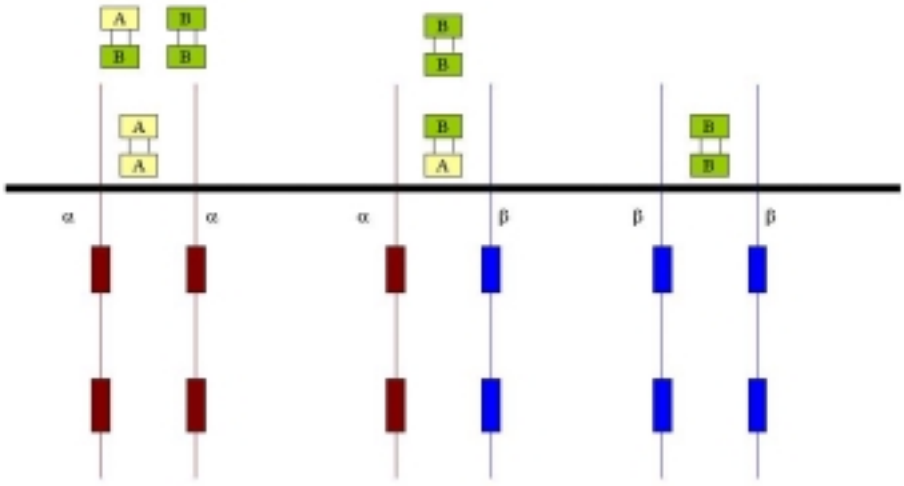

Figure 1. PDGF ligand specificity for the different PDGF receptor combinations.

Ligand-induced dimerization of the PDGF receptors leads to activation of the receptor kinase, a common theme among many cell surface receptors (Heldin, 1995). Then the intracellular part of the receptors gets autophosphorylated on tyrosine residues. One important function of autophosphorylation is to regulate the catalytic activity of the kinase by phosphorylation of the Tyr residue inside the kinase domain. Mutation of this residue (Y857 in the β-receptor) to a phenylalanine residue gives a receptor with a lowered kinase activity (Heldin et al., 1999). The other known phosphorylation sites lie outside the kinase domain. These tyrosine residues provide docking sites for signaling molecules containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains that initiate different signal pathways resulting in different cellular responses (Heldin et al., 1999).

The αα– and ββ–homodimeric receptors mediate similar but not identical cellular responses when activated (see figure 2).

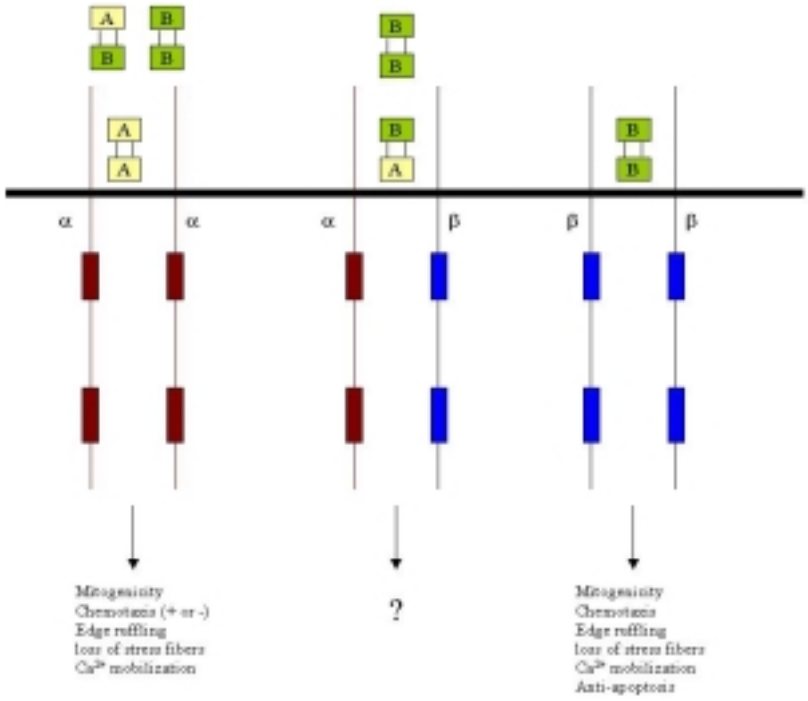

Figure 2. Cellular responses due to PDGF αα- and ββ-receptor signaling.

One interesting question is then whether the αβ heterodimer mediates a unique signal or just the sum of the α– and β-receptor signals. It has been shown that signaling via the heteromeric complex give a more potent mitogenic signal but not so much is known. The aim of my project has therefore been to compare the signaling between the homodimeric and heterodimeric PDGF receptors by stimulating cells with either PDGF-AA, -AB or -BB (to activate the receptor combinations) or nothing (controls) and see what similarities and differences there are on the transcriptional level by using the microarray technique. Since there is a low expression of α-receptors compared to the β-receptor expression in

the cell type used, PDGF-AB will activate almost only αβ-receptors, PDGF-AA will exclusively activate αα-receptors and PDGF-BB will activate a mix of the receptor combinations.

1.2 The microarray technique

DNA microarrays are microscopic, physically ordered arrays of thousands of DNA sequences. They can be arrayed in an area no larger than a standard microscope slide. To survey the expression of genes, RNA transcripts are isolated from cells, labeled with a fluorescent dye during cDNA synthesis and hybridized to a DNA microarray. During the hybridization process the immobilized DNA sequences (each representing one gene) can bind to the complementary cDNAs in the fluorescent probe mixture. The fluorescent signal at each DNA sequence or "spot" then represents a quantitative signal of the expression level of this gene. This data is read by a scanner for all spots on the array and further analyzed in the computer. In this way one can monitor expression of tens of thousands genes simultaneously (Diehn et al., 2000). Both the control RNA as well as the RNA from experimental conditions (ligand stimulated) are hybridized to the same chip (but labeled with different dyes, in this case green for control and red for stimulated RNA). Therefore yellow spots on the array are genes whose expression level did not change after stimulation, red spots show genes with an elevated expression and green spots show genes with a lowered expression.

The microarray technique is used when you want to study the gene expression pattern in a cell during certain conditions. For example you might want to study the expression pattern for individuals with a certain disease and compare with the gene expression pattern for healthy individuals to see which genes behave differently (Alizadeh et al., 2000, Golub et al., 1999). In my case I want to see what differences there are in signaling between homo- and heterodimeric PDGF receptors. Are there genes that are more strongly expressed or less expressed and do this lead to different cellular responses? Since over expression of PDGF and its receptors are common in several diseases this could provide important knowledge for further research.

The DNA microarrays I have used contain about 5000 genes. Often genes are represented by oligo nucleotides (15-20 nucleotides) on the array but on these arrays the DNA sequences spotted are about 1 kb. Because of the great length of these sequences you get very good hybridization (of the labeled cDNA to the array) and therefore the gene is only represented in one spot (but in multiple copies in that spot). As controls, to see whether the hybridization has been successful, spots that recognize bacterial mRNA is located on the microarray in different dilutions. As seen in figure 3 the chip is divided into four quadrants with landmarks in each corner (bright shining spots) of the quadrants. The genes are spotted all over the rest of the chip where each quadrant contain 36*36 spots.

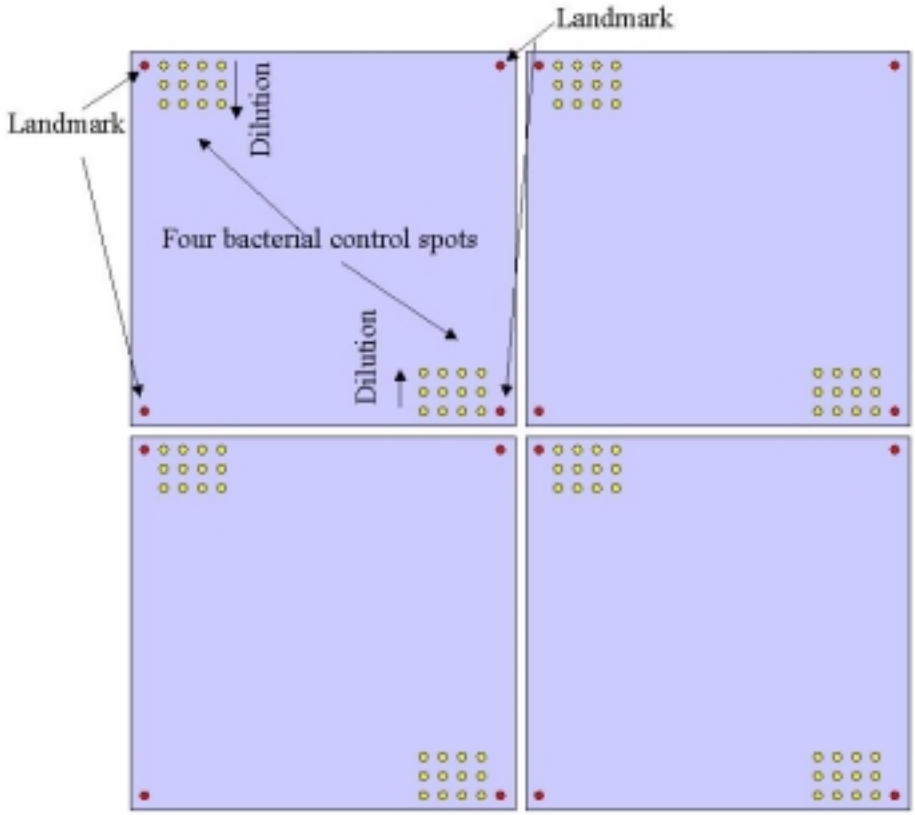

Figure 3. An overview of the microarrays used. The internal controls (the bacterial RNA) are marked yellow and the landmarks that define the quadrants are marked red.

1.3 Project plan

The plan in the beginning was to use cells stimulated with PDGF-AA, -AB and –BB at different time points and in that way be able to see which genes and how these genes changed by time after stimulation and to compare this for the different stimulations. Unfortunately the amount of microarrays was very limited so the experiment had to be done for a single time point. The plan was as follows:

- Culturing of human foreskin fibroblasts.
- Check of the PDGF receptor expression in these cells.
- Stimulation of the cells with either PDGF-AA, -AB or -BB for 2 hours. Also unstimulated cells to use as controls.
- Extraction of RNA from the stimulated and unstimulated cells.
- RNA gel and Northern blot for check of quality of total RNA.
- Generation of fluorescently-labeled single-stranded (ss) cDNA target.
- Competitive hybridization of labeled (ss) cDNA onto microarrays.
- Washing and scanning of the microarrays.
- Computer analysis of the microarrays to compare the gene expression between the PDGF-AA, -AB and -BB stimulated cells.

2 Materials & Methods

2.1 Cell culturing

Human foreskin fibroblasts (AG01518 from Coriell Cell Repositories) were cultured in minimal essential medium (MEM) Eagle-Earle with vitamins & essential and nonessential amino acids (M-5650 from Sigma) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. Trypsin-EDTA was used for detachment of cells during subcultivation. Cells were splitted 1:4 when confluent.

2.2 Stimulation of cells

The cells were washed with MEM without FBS and then starved for about 24 h in 15 ml MEM supplemented with 0.3% FBS and 2 mM glutamine. Then ortovanadate (Na_3VO_4), a phosphatase inhibitor, was added to the cells to a final concentration of 100 μM and the cells incubated for 30 minutes at 37°C. The cells were stimulated with 100 ng/ml of PDGF-AA, -AB, -BB or nothing (control) and incubated for 10 minutes (in the case of the check of PDGF receptor levels) or 2 hours (when used for RNA extraction) at 37°C. The medium was then removed and the cells were washed with cold phosphate buffer saline (PBS) which then was removed carefully. Then 1 ml of lysis buffer (1% Triton x100, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 20 mM Hepes) supplemented with protease inhibitors (1% trasylol, 1 mM PMSF, 10 μg/ml leupeptin) and the phosphatase inhibitor (200 μM Na_3VO_4) was added to the cells to lyse them when used for the check of PDGF receptor levels. When using the cells for RNA extraction the cells were instead lysed with Total RNA Isolation reagent (TRIZOL) from Life Technologies.

2.3 Check of receptor levels

2.3.1 Protein concentration

Unstimulated cells and cells stimulated with either PDGF-AA, -AB or -BB were lysed with the lysis buffer supplemented with protease inhibitors (see stimulation of cells). The cells were then transferred to different Eppendorf tubes and centrifuged at 13000 rpm for 10-15 minutes at 4°C. The supernatants were transferred to new Eppendorf tubes. The protein concentration for the different conditions was determined by using BCA Protein Assay kit from PIERCE. With this kit a standard curve was made by serial dilution of BSA where the reagent was added in the end. The reagent was also added to 50 μl of the samples and the concentration could be read out from the standard curve. The variation of protein concentration between the different samples was corrected for by different dilution with lysis buffer (about 300 μl) up to 1 ml before immunoprecipitation (IP) and pulldown.

2.3.2 Immunoprecipitation and pulldown

For immunoprecipitation (IP), 70% of each lysate was used. The IP was done with a home made antibody that recognizes the C-terminal of the PDGF α– and β-receptors. In the pulldown with Wheat Germ Agglutinin (WGA Sepharose, Amersham Pharmacia Biotech), 10% of the samples was used. WGA recognizes glycosylated membrane proteins (the receptor).

In the immunoprecipitation, 10 µl of the antibody (CED) was added to the samples, which were vortexed and left on ice for 1.5 hours. Then 50 µl protein A Sepharose beads was added to the samples which were incubated end over end for 30 minutes at 4°C. The samples were washed three times with 1 ml lysis buffer and one time with 1 ml ddH_2O. Then 50 µl sample buffer (8 mg dithiothreitol (DTT), 800 µl Laemmli buffer, 200 µl 20% sodium docedyl sulfate (SDS)) was added to the samples.

Before the pulldown experiment the WGA beads was washed three times with 2 ml lysis buffer. Then 50 µl of the washed beads was added to the samples and incubated end over end for 30 minutes at 4°C and then treated the same way as the immunoprecipitation samples.

The samples were then run on a 7% SDS-PAGE (Hoefer) midi gel at 5 mA over night.

2.3.3 Western blot

The filter used for blotting (Immobilon-P from Millipore) was first wet in methanol and then put in transfer buffer (56.3 g glycine, 15 g Tris-HCl, 1 l methanol, 4 l ddH_2O) and incubated for 5-10 minutes at room temperature on a shaker. Just before starting the transfer 10 ml of 20% SDS was added to the transfer tank. The transfer was run at 400 mA for 3.5 hours.

After the transfer the filter was blocked in 5% BSA in PBS for 1 hour. The home made anti-PDGF α– and β–receptor antibody CED (rabbit) was used as primary antibody in the blot. The incubation was done overnight at 4°C but can also be done for 1.5 hour at room temperature. The filter was washed 3x5 minutes with 0.5% Tween20 PBS (PBST). A secondary anti-rabbit antibody coupled to horseradish peroxidase (HRP) was then used. The filter was incubated with the antibody solution for 1 hour and then the filter was washed 3x5 minutes with 0.5% PBST. Lumi-Light^{PLUS} Western Blotting Substrate from ROCHE was used for detection of antigens on the membrane. A CCD camera was used to "develop" the picture.

The filter was then stripped by incubation in 2% SDS, 62.5 mM Tris-HCl pH 6.8, 0.7 ml β-mercaptoethanol/100 ml solution in ddH_2O for 30 minutes at 55°C (waterbath). Then the filter was blocked and the procedure above repeated but this time the primary antibody used was PY99 (Santa Cruz, mouse antibody) that recognizes phosphorylated

tyrosine residues. The secondary antibody used in this case was an anti-mouse antibody coupled to HRP (Amersham LIFE SCIENCE). The filter was then stripped again and reprobed with a primary antibody against PDGFR-α (TIE2, home made) followed by the anti-rabbit antibody used the first time. This time, after developing the picture, the filter was not stripped but reprobed with an antibody against PDGFR-β (PELE) directly coupled to HRP.

2.4 Extraction of RNA

Unstimulated and stimulated cells were lysed with 6 ml TRIZOL/T175 flask. For each condition (stimulation with PDGF-AA, -AB or -BB and unstimulated) two T175 flask were cultured which made a total of 12 flasks (one control per stimulation). The cells were incubated for 5 minutes at room temperature. The lysates from the different conditions were split into two 14 ml polypropylene tubes (RNAse free). Then 0.2 ml chloroform per 1 ml of TRIZOL reagent used was added and the samples were vortexed for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were then centrifuged at 4000 rpm for 15 minutes at 4°C to separate the phases. The aqueous upper phase (about 6 ml/condition) was transferred to 8 microcentrifuge tubes (2 ml) and 0.5 ml isopropanol per 1 ml of TRIZOL reagent used was added to precipitate the RNA. The samples were mixed by inversion, incubated at room temperature for 10 minutes and centrifuged at 13000 rpm for 15 minutes at 4°C. The RNA was now seen as a pellet at the bottom of each tube. The supernatants were removed and the pellets washed with 75% ethanol (1 ml per 1 ml TRIZOL reagent used). The samples were vortexed and centrifuged at 8000 rpm for 5 minutes at 4°C. The supernatants were removed and the RNA pellets were air-dried. The pellets were then resuspended in 50 µl diethylpyrocarbonate (DEPC) H_2O and incubated at 55-60°C until the pellets were completely dissolved. Total RNA was quantitated by using a spectrophotometer (at 280 nm) and the quality of the RNA was assessed by gel electrophoresis of 2 µg and also by Northern blot. Finally 3 volumes of 100% ethanol was added to the aqueous samples that were stored at -70°C.

2.5 Check of quality of total RNA

2.5.1 RNA gel

A 1% agarose gel was made by mixing 1.07 g RNAse free agarose with 90 ml DEPC water, 2 ml 50x Northern runner (209.4 g MOPS, 18.6 g EDTA, 20.3 g NaAc, water to 1000 ml, adjustment of pH to 7) and adding 15 ml of formaldehyde when the solution had cooled down after dissolving the agarose. Then 2 µg of total RNA in water (see RNA extraction) were mixed with 30 µl sample buffer (500 µl formamide, 180 µl formaldehyde, 20 µl 50x Northern Runner, 50 µl brom-phenyl-blue (BFB), 10 µl EtBr). The samples were heated to 65°C for 10 minutes and then loaded on the gel that was run at 80V in 1x Northern runner until the front had moved about 10 cm (approximately 1.5 hours). Then the gel was removed and a photo taken.

2.5.2 Northern blot

A 1% agarose gel was made (1% agarose, 84% DEPC H_2O, 1.9% 50x Northern Runner, 14% formaldehyde). Then 10 µg of the RNA samples (from the extraction of RNA) dissolved in 10 µl water were mixed with 30 µl sample buffer (500 µl formamide, 180 µl formaldehyde, 20 µl 50x Northern Runner, 50 µl brom-phenyl-blue (BFB), 10 µl EtBr). The samples were then heated for 10 minutes at 65°C. The gel was run at 80 V in 1x Northern Runner. When the front had moved about 10 cm, the gel was removed and a photo taken. The gel was incubated in 20x SSC (175.3 g NaCl, 88.2 g Sodium Citrat-2 hydrat, water up to 1000 ml, autoclave) for 10 minutes and then a transfer was run overnight (using a Hybond-N filter). Then a photo of the gel was taken to see how efficient the transfer had been. The filter was left to dry for 10 minutes and then wrapped in plastic film and UV-crosslinked. The filter was then prehybridized by incubating it with 10 ml hybridization solution (1.5x SSPE (20x SSPE (20 mM EDTA, 160 mM Na_2HPO_4, 3M NaCl, adjust to 1000 ml, adjust pH to 7), 10% PEG 8000, 7% SDS) and 100 µg/ml salmon sperm DNA (that had been denatured by heating to 95°C for 5 minutes followed by cooling in ice water for one minute) for 2-4 hours at 65°C to prevent unspecific binding to the filter. Glyceraldehyde-3-phosphate, GAPDH (5 ng in 45 µl water) was used as probe. The DNA probe was denatured by heating to 95°C for 5 minutes. Rediprime labeling mix (Amersham LIFE SCIENCE) was added to the denatured DNA and mixed gently until the blue color was uniform. Then the tube was centrifuged briefly and 5 µl of radioactive ^{32}P dCTP was added and the mixture incubated for 10 minutes. The reaction was stopped by adding 5 µl 0.2 M EDTA.The labeled probe was heated to 95°C for 5 minutes and then cooled on ice for 1 minute. Then 10 µl of the hot probe was added to the hybridization solution mix and incubated overnight at 65°C. The filter was then washed with following: 1x SSC + 0.1 % SDS for 30 minutes at 65°C, 0.5x SSC + 0.1 % SDS for 30 minutes at 65°C and 0.1x SSC + 0.1 % SDS for 30 minutes at 65°C. The filter was now melted into a plastic bag and exposed overnight on the phosphoimager and then scanned.

2.6 Microarray

2.6.1 Generation of fluorescently-labeled single-stranded (ss) cDNA target

Bacterial mRNA "cocktail" (2 µl per sample, provided as controls) was added to 25 µg (or 10 µg the first time) of total RNA. The RNA was precipitated by addition of 1/40 volume of 3 M NaAc, pH 5.2 to the RNA (already in 75% ethanol) and incubated at -70°C for 20-30 minutes. The samples were centrifuged at 13000 rpm to pellet the RNA. The supernatants were removed and the RNA pellets were resuspended in 12.9 µl DEPC ddH_2O and 2.5 µl anchored oligo-dT_{17}. The RNA/oligo mixtures were heated to 70°C for 10 minutes and snap-chilled on ice. Then 6.0 µl 5x first strand buffer, 3.0 µl 0.1 M DTT, 0.6 µl dNTP mix 25 mM, 3.0 µl dCTP-Cy3 or dCTP-Cy5 (Cy3 for reference RNA, Cy5 to stimulated RNA) and 2.0 µl SuperscriptII was added to the RNA/oligo mixtures and incubated at 42°C for 2 hours in the dark to produce labeled cDNA. Then 15 µl 0.1 M NaOH was added and the samples were incubated at 70°C for 20 minutes to hydrolyze

the RNA. The reaction was neutralized by adding 15 µl 0.1 M HCl. The two corresponding ss cDNA samples were then pooled. The cDNA was precipitated by addition of 1/10 volume of 3 M NaAc pH 5.2 and 3 volumes 100% ethanol at room temperature and incubation for 20-30 minutes. The samples were then centrifuged for 10 minutes at 13000 rpm to pellet the ss cDNA. The supernatants were removed and 100 µl of 70% ethanol was added. The samples were centrifuged for 3-5 minutes at 13000 rpm. This wash step was then repeated until the supernatants appeared clear. The supernatants were removed after the last wash and the pellets left to air dry.

2.6.2 Competitive hybridization of labeled (ss) cDNA onto microarray

The cDNA pellets were resuspended in 20 µl of hybridization buffer (5x SSC, 6x Dehnarts, 0.12% sacrosyl, 48% formamide). Then 2 µl polyA DNA and 2 µl human $C_o t$ was added to the samples. The cDNA mixtures were then placed in 100°C water bath for 5 minutes and then removed and left to cool to room temperature. The samples were centrifuged briefly to remove evaporated liquid from the tube walls. The microarray slides were pre-cooled at 4°C and the samples (in hybridization buffer) were applied to the center of the coverslips. The slides containing the microarray were gently placed on top of the coverslips by placing the end of the array at approximately 20° angle, gradually lowering the entire array slide (creation of bubbles had to be avoided). The microarrays were then placed in humid chambers (2 ml 40% formamide, 2x SSC on a piece of Whatman paper) to prevent buffer from evaporating during hybridization. The chambers were sealed with parafilm and incubated at 47°C for 12-24 hours in a dark oven.

2.6.3 Washing

The microarrays were removed from the humid chambers in a fume hood and placed in a glass slide rack containing 100-200 ml of room temperature wash solution 1 (2x SSC) where the coverslips fell off. The slides were removed carefully and placed in a new rack and washed for 5 minutes at room temperature with gentle shaking. The microarray slides were then transferred to wash solution 2 (0.1x SSC, 0.1% SDS) and washed at room temperature for 15 minutes with gentle shaking. This step was repeated once more but at 37°C. The slides were then transferred to wash solution 3 (0.1x SSC) and washed at room temperature for 15 minutes. This step was also repeated once more. The microarray slides were then transferred to 50 ml Falcon tubes and centrifuged at 500 rpm for 1-2 minutes to dry the slides.

2.6.4 Scanning

The slides were scanned using a scanner from GSI Lumonics. Image files for each channel (Cy3 and Cy5) were generated with the software Scanarray. The images were then imported to another program called Quantarray. There an overlay picture of the Cy3 channel (control RNA) and the Cy5 channel (stimulated RNA) was generated. To check that the overlay was correct the four internal control spots (the bacterial mRNA) should turn yellow. If not the channels had to be rescanned on different laser power for the two channels to generate a spot intensity (at the bacterial RNA spots) that was the same for

the two channels giving yellow spots (green + red) in the overlay picture (could not be modified in Quantarray). Then gridding based on the specific array was performed and artifactual spots were flagged out. This could be spots where dust or something else was disturbing the signal and therefore not reliable. Finally the data was ready to be analyzed. This was a time consuming part since the result was presented in a way that made you click through a lot of points in a diagram where each point represented a gene (shown as a co-ordinate in the array). When clicking on a point, information came up describing the spot- and background intensity for the two channels and a value calculated by subtracting the background intensity from the spot intensity for the channels and then dividing the Cy5 channel number with the Cy3 channel number. This value described whether the gene was up- or down-regulated. In the Quantarray software the reference channel (control RNA) was always marked in red (opposite of the labeling) which meant that green spots were up regulated and red spots down regulated.

3 Results

3.1 Protein concentration

The standard curve obtained in the determination of the protein concentration is seen in figure 4. By applying linear regression, a formula for calculating the protein concentration for the samples was obtained.

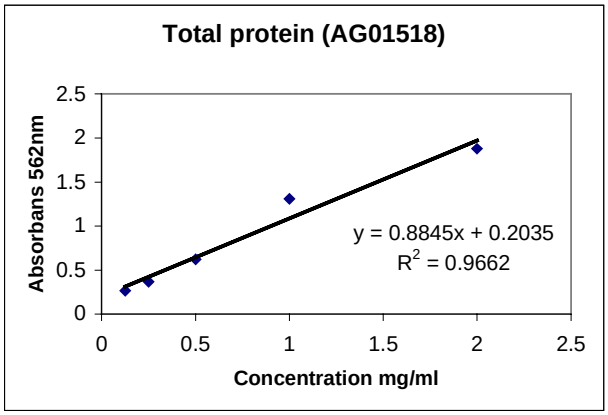

Figure 4. Standard curve representing the absorbance as a function of the concentration of total protein in AG01518 cells.

Since the absorbance for the samples were known the concentrations could be calculated (see table 1). Before the immunoprecipitation and pulldown the samples were diluted with lysis buffer slightly differently so the final protein concentration in all samples became the same.

	Absorbance	**Concentration of protein mg/ml**
Unstimulated cells	0.238	0.039
PDGFAA stimulated cells	0.248	0.050
PDGF AB stimulated cells	0.285	0.092
PDGF BB stimulated cells	0.249	0.051

Table 1. The protein concentration obtained from the fibroblasts stimulated with PDGF-AA, -AB and -BB and unstimulated fibroblast cells.

3.2 Check of receptor levels

In the first blot the home made antibody recognizing the C-terminal of the PDGF α- and β-receptors was used. As seen in figure 5a, only one band represents the PDGF α- and β-receptors. This is due to that the α- and β-receptors have approximately the same size (170-180 kDa) and could not be separated on the gel. Since only 10% of the samples was used in the WGA pulldown only weak or no bands are seen in these lanes (figure 5a-d). The antibody PY99 was used in the next blot. This antibody recognizes phosphorylated residues. In the PDGF-BB stimulated cells the receptors are much more phosphorylated than in the PDGF-AA and -AB stimulated cells (see figure 5b). The unstimulated cells show no receptor phosphorylation as expected since PDGF receptors only get activated and phosphorylated when the ligand binds and there is no ligand added in this case (see figure 5b). In figure 5c the antibody TIE2 was used. It only recognizes the PDGF α-receptor and quite weak bands were seen in the IP. In the next figure, the filter wasn't stripped, only reprobed with the antibody PELE-HRP that only recognizes the PDGF β-receptor. Then there was an increased signal of PDGF receptor compared to figure 5c since both the α- and β-receptors were recognized (see figure 5d).

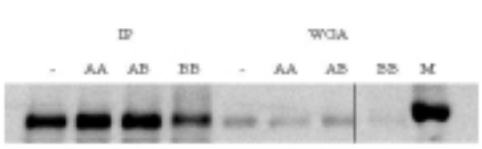

Figure 5a.

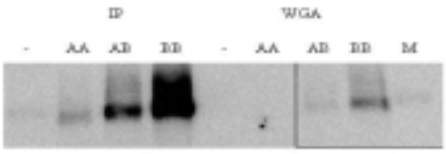

Figure 5b.

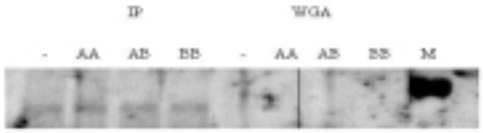

Figure 5c.

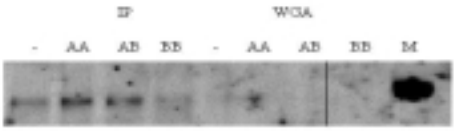

Figure 5d.

Figure 5a-d. These figures show the immunoprecipitation and WGA pulldown of the α- and β-receptors. The AA, AB and BB lanes show fibroblasts stimulated with PDGF-AA, -AB and -BB and the – lanes represent unstimulated fibroblasts. The M lane shows the marker and the band seen represents 207 kDa. Figure 5a shows a blot with the CED antibody that recognize both the α- and β-receptors. In figure 5b the antibody PY-99 is used in the blot. This antibody recognizes phosphorylated tyrosine residues in the α- and β-receptors. In figure 5c the antibody TIE-α is used that recognizes the α-receptor. Figure 5d shows a blot with the antibody PELE-HRP that recognizes the β-receptor. In this blot the filter wasn't stripped from the bands seen in figure 5c.

3.3 RNA extraction

The RNA extraction was successful and gave the amounts of RNA showed in table 2.

	PDGF-AA	**PDGF-AB**	**PDGF-BB**	**Unstimulated**
Amount RNA	112.5 µg	116.4 µg	122.1 µg	346.4 µg

Table 2. The total amount of extracted RNA from the fibroblast cells stimulated with PDGF-AA, -AB and -BB and unstimulated fibroblast cells.

3.4 Quality of RNA

3.4.1 RNA gel

As seen in figure 6, the RNA gel that was run, showed that the RNA was intact and not degraded and could be used for the microarray experiments. The bands seen on the gel are the big and the small subunits of rRNA. This is a good quality control for the mRNA as well.

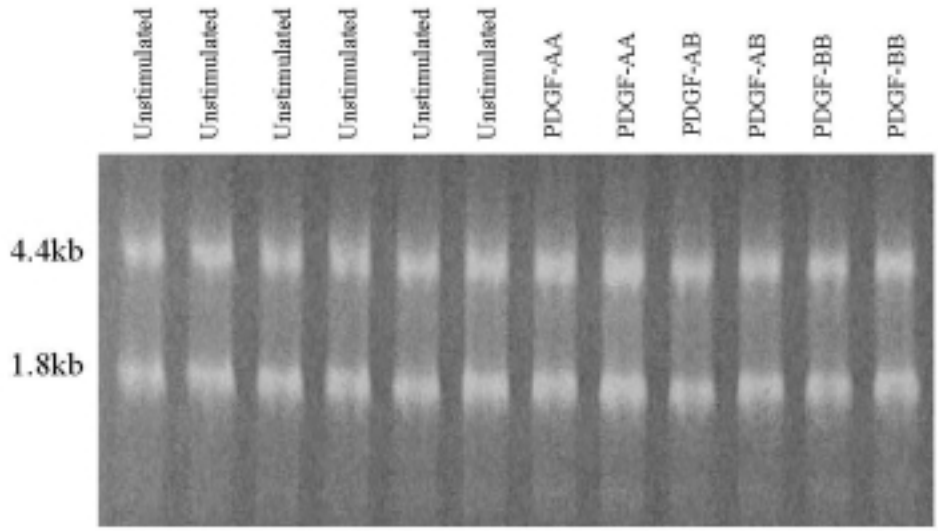

Figure 6. The lanes show the RNA content (big and small subunits of ribosomal RNA) from unstimulated fibroblasts and fibroblasts stimulated with PDGF-AA, -AB and -BB.

3.4.2 Northern Blot

To be sure that the mRNA, used for the hybridization to the microarrays, was not degraded a Northern blot was also performed (see figure 7). The probe glyceraldehyde-3-phosphate (GAPDH) was used (this is a “housekeeping” gene whose expression is not affected by the stimulation) and as seen in the blot the RNA wasn’t degraded.

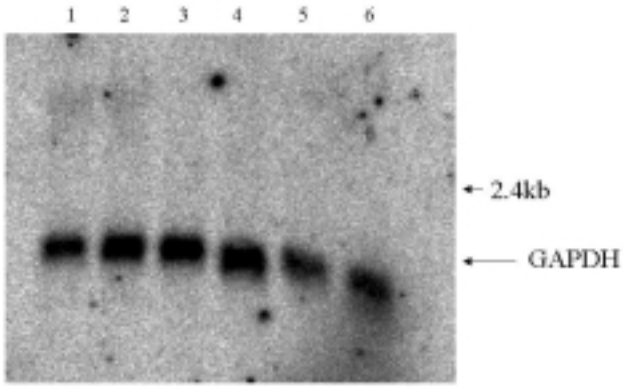

Figure 7. In lanes 1-3 RNA from unstimulated cells was run. In lane 4, and RNA from PDGF-AA stimulated cells were run, in lane 5 RNA from PDGF-AB stimulated cells was run in lane 6, RNA from PDGF-BB stimulated cells was run. The Northern transfer was uneven but all the bands in the blot correspond to GAPDH.

3.5 Microarray

Out of the 6 arrays I got, only four turned out nice (see appendix A, figure1-4). The big blobs of color seen in some of them are dust that was difficult to get rid of. The first three arrays were done using only 10 μg RNA (from each condition) to see if this was enough for the hybridization. Only the array with PDGF-AB stimulated RNA versus control RNA turned out well (see appendix A, figure 1). But as seen the signal in this array was quite weak and not so many spots were detected (green spots in the array represent up regulated genes, red spots down regulated genes and yellow spots are genes that didn't change in expression after stimulation.).

In the next attempt 25 μg RNA was used instead. This time the PDGF-AB array (PDGF-AB stimulated RNA versus control RNA, appendix A, figure 2) had a lot of background (Cy-5 dye that stuck to the array). This array was used for analysis anyway since stronger signals could be detected. As seen in the array a lot more spots were detected compared to the array with 10 μg due to the higher RNA amount used. The PDGF-AA and -BB arrays (appendix A, figure 3 and 4) turned out very nice and did not have background problems like the PDGF-AB array. Also in this case many more spots could be detected. One would expect that the pattern between the PDGF-AA, -BB and -AB arrays would be quite similar. This is true for the PDGF-AA and -BB arrays but not if you compare with the PDGF-AB arrays. This was due to that different batches (5K-1, AA, BB and 5K-2, AB) of the arrays had been used for the different conditions (because of the limited amount of arrays) and the genes were not spotted at the same locations on the different batches. There were also quite a lot of genes that differed between the array batches, which was a problem.

As seen in figure 1-4 in the appendix A, the bacterial controls did not hybridize correctly to the array. They hybridized with different efficiency to the array and the same bacterial RNA also hybridized differently between the different arrays (see figure 8a-d, see also figure 3 to see where the controls are located on the array).

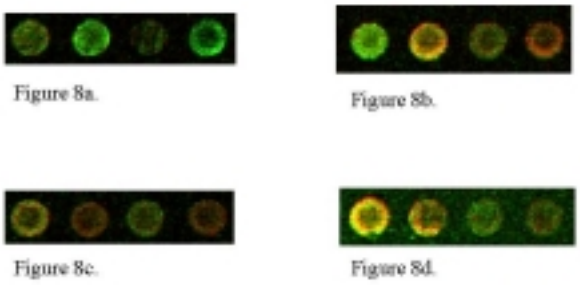

Figure 8a-d. In figure 8a the bacterial controls in the PDGF-AA are shown. In figure 8b the bacterial controls in the PDGF BB array are shown. In figure 8c the bacterial controls in the PDGF-AB (10 μg) array are shown. In figure 8d the bacterial controls in the PDGF-AB (25 μg) are shown.

The result of the most up regulated and down regulated genes in the different arrays (PDGF-AA, -AB, -BB RNA versus 3 control RNA, one each) and a comparison to the other arrays are shown in table 3a-g. Genes with low intensity are marked **Low**. Genes with maximum intensity and therefore saturated are marked **Max**. Genes with signals almost having maximum intensity are marked **High**. Genes marked with B have too much background to be detected. Genes marked with – are genes that are not spotted on that array batch (5K-1 for PDGF-AA and -BB arrays and 5K-2 for the PDGF-AB arrays). The values were calculated by subtracting background intensity from the spot intensity for each spot in each channel and then the Cy5 channel (stimulated RNA) value was divided with the Cy3 channel value (control RNA). If the value > 1 the gene is over expressed otherwise it is down regulated compared to control conditions. The PDGF-AA values are much lower than the PDGF-AB and -BB values.

In table 3a the most up regulated genes in the PDGF-AB (25 μg) array are shown. A comparison to the PDGF-AA and -BB arrays is also shown. In table 3b the most up regulated genes in the PDGF-AB array are shown that were not spotted on the 5K-1 batch and hence could not be compared to the PDGF-AA and -BB arrays. In table 3c the most up regulated genes in the PDGF-AB (10 μg) array are shown. This table also shows a comparison to the PDGF-AB (25 μg), -AA and -BB arrays. The most up regulated genes

in the PDGF-AA array and a comparison to the PDGF-AB (25 μg) and -BB arrays are shown in table 3e. The most down regulated genes in the PDGF-AA array are shown in table 3d. These were very few compared to up regulated genes. Table 3f and 3g shows the most up regulated and down regulated genes in the PDGF-BB array compared to the other arrays (PDGF-AA and -AB).

Gene	Description	Value AB 25 µg	Value AA	Value BB
COX-2	Cytoplasmic cyclooxygenase, induced by cytokines and mitogens.	3.76 Max	1.85	4.85
NR4A1	Nuclear receptor, induction by growth-stimulating agents.	3.62	1.24 Low	3.83
DUSP1	Dual specificity phosphatase 1. Dephosphorylates MAP kinase erk2 on both thr-183 and tyr-185.	3.56	1.21	2.64
IER	Immediate early response, membrane protein.	3.12 High	0.85	2.00
ID3	DNA binding protein.	3.05	1.14 Low	1.54
CREM	Transcription factor, stimulated by phosphorylation.	2.80 High	0.97 Low	B
IGFBP5	Igf binding protein 5 ,dimerizing with Igf, prolong the half-life of igf.	2.76	1.00 Max	1.01 Max
DLST	Dihydrolipoamide S-succinyltransferase mitochondrial.	2.68 Max	1.23 Low	1.18 Low
DIPA	Hepatitis delta antigen-interacting protein A.	2.66 High	1.53	2.75
DUSP4	Dual specificity phosphatase 4. Dephosphorylates MAP kinase Erks.	2.60	1.14 Low	1.54 Low
KIAA0127	Unknown function, expressed in chronic myeloid leukemia.	2.51	1.45 Low	1.89
NFKB1A	Involved in regulation of transcriptional responses to NfKb.	2.40	1.24 Low	2.56
EMD	Cytoskeletal protein, defects in emd are a cause of Emery-Dreifuss muscular dystrophy.	2.20	0.99 Low	0.74
KIAA0653	Unknown function, expressed in chronic myeloid leukemia.	2.18	1.12 Low	1.11 Low
GADD45A	Growth arrest and DNA-damage-inducible , alpha.	2.16	1.50	2.44
ABCB2	ATP-binding cassette, sub-family B (MDR/TAP), member 2. Involved in the transport of antigens from the cytoplasm to a membrane bound compartment for association with MHC class I molecules.	2.12	1.08	2.46
AP1S2	Adaptor protein, involved in clathrin mediated endocytosis.	2.09	1.14 Low	1.23 Low
DGAT	Diacylglycerol O-acyltransferase.	2.00	1.11 Low	1.29 Low
PC4	Cofactor involved in RNA polymerase II activation.	1.66 High	1.30	1.57
HMG17	Chromatin regulator.	1.48 High	1.19 Max	1.14

Table 3a. The most up regulated genes in the PDGF-AB (25 µg) array that also were spotted in the 5K-1 batch arrays are shown. The values in the PDGF-AB array are compared to the values in the PDGF-AA and -BB arrays for the genes.

Gene	Description	Value AB 25 µg	Value AB 10 µg
T1A-2	Transmembrane protein.	4.16	2.9
VEGF	Vascular endothelial growth factor, secreted.	3.20	2.75
TNFAIP3	Tumor necrosis factor, alpha induced protein 3. Inhibitor of programmed cell death.	3.12	1.92 Low
ADORA3	Integral membrane protein, receptor for adenosine. Activation is mediated by G proteins.	3.11	1.04 Low
GEM	GTP-binding protein over expressed in skeletal muscle, also expressed in mitogen stimulated T-cells.	3.07	2.34 Low
PHLDA1	Pleckstrin homology-like domain, family A, member 1).	2.96	1.66 Low
FOXC1	Forkhead box C1, can bend the DNA at an angle of 80-90 degrees.	2.87	4.27
NFIX	Nuclear factor I/X (CCAAT-binding transcription factor).	2.67	1.04 Low
TFP12	Tissue factor pathway inhibitor 2, seems to inhibit trypsin.	2.61	2.39
GCMB	Glial cells missing (Drosophila) homolog b.	2.47	2.63
SOX9	Sex determing region Y box 9.	2.39	1.82 Low
S100A9	S100 calcium-binding protein A9 (calgranulin B).	2.38	3.52
PLAU	Plasminogen activator, urokinase.	2.35	2.97
RGS3	Regulator of G-protein signalling 3, inhibits signal transduction by increasing the GTPase activity of G-protein alpha subunits thereby driving them into their inactive GDP-bound form.	2.32	1.32 Low
PLP2	Proteolipid protein 2(colonic epithelium-enriched).	2.31	2.39 Low
GSPT1	G1 to S phase transition 1. Involved in regulation of mammalian cell growth.	2.28	?
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related). Involved in programming of differentiation and concomitant of viability but not of proliferation (probably). Inhibitor of apoptosis.	2.21	2.52
NCBP2	Nuclear cap binding protein subunit 2, 20kD.	2.21	?
VASP	Vasodilator-stimulated phosphoprotein. May act in concert with profilin to convey signal transduction to actin filament production.	2.18	1.42 Low
TOB2	Transducer of ERBB2,2.	2.18	1.59 Low
TSSC3	Tumor suppressing subtransferable candidate 3.	2.18	2.39
PTP4A1	Protein tyrosine phosphatase type IVA, member 1.	2.07	1.89 Low
KLF7	Kruppel-like factor 7. Transcription factor.	2.03	?
DDX21	DEAD/H box polypeptide 21.	2.00	?

DOC1	Down regulated in ovarian cancer.	1.93	1.62 Low
TIEG	TGFB inducible early growth response. Induced by TGFB, acts as transcriptional repressor involved in the regulation of cell growth. Inhibits cell growth.	1.99	2.00 Low
RAGA	Ras-related GTP-binding protein.	1.82	1.67 Low

Table 3b. Up regulated genes in the PDGF-AB (25 μg) array that were not spotted on the 5K-1 batch arrays. A comparison of the PDGF-AB (25 μg) values to the PDGF-AB (10 μg) values are shown. Some values in the PDGF AB (10 μg) array couldn't be detected (because of problems with the computer file) and are marked ?.

Gene	Description	Value AB 10 µg	Value AB 25 µg	Value AA	Value BB
SCYA2	Small inducible cytokine A2 (monocyte chemotactic protein 1).Augments monocyte anti-tumor activity.	4.79	2.10 Max	-	-
COX-2	Cytoplasmic cyclooxygenase, induced by cytokines and mitogens.	4.56	3.76 Max	1.85	4.85
FOXC1	Forkhead box C1, can bend the DNA at an angle of 80-90 degrees.	4.27	2.87	-	-
SCYA7	Small inducible cytokine A7 (monocyte chemotactic protein 3). Augments monocyte anti-tumor activity.	4.19	no signal	1.24 Low	1.46 Low
U2AF1RS2	Small nuclear ribonucleoprotein auxiliary factor.	3.96 High	1.00 Max	-	-
MT3	Metallthionein 3 (growth inhibitory factor (neurotrophic)).	3.89 High	1.00 Max	-	-
PLAG1	Pleiomorphic adenoma gene 1.	3.78 High	1.00 Max	1.21 Low	B Low
MT1X	Metallothionein 1X.	3.74 High	1.00 Max	1.55	1.78
GADD45A	Growth arrest and DNA-damage inducible, alpha.	3.71	2.16	1.50	2.44
SCYA13	Small inducible cytokine subfamily A (Cys-Cys), member 13.	3.58	no signal	1.11 Low	1.19 Low
S100A9	S100 calcium-binding protein A9 (calgranulin B).	3.52	2.38	-	-
MT1G	Metallthionein 1G.	3.50 High	1.06 Max	-	-
JUNB	Jun B proto-oncogene, transcription factor.	3.40	1.94 Max	0.95	2.23
TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor). Secreted.	3.07 High	1.00 Max	1.54	1.47

Table 3c. The most up regulated genes in the PDGF-AB (10 µg) array are shown. A comparison of its values with the PDGF-AB (25µg), the PDGF-AA and the PDGF-BB values are also shown.

Gene	Description	Value AA	Value BB	Value AB 25 µg
DTR	Diphteria toxin receptor (heparin binding epidermal growth factor-like growth factor). Mitogenic for fibroblasts and smooth muscle cells. Can bind EGF receptors with higher affinity than EGF itself and is a far more potent mitogen for smooth muscle cells than EGF.	2.10	4.69	-
DUSP6	Dual specificity phosphatase 6. Inactivates MAP kinases. Has specificity for the Erk family.	2.06	3.77	-
PLAUR	Plasminogen activator, urokinase receptor.	1.88	2.85	B
COX-2	Cytoplasmic cyclooxygenase, induced by cytokines and mitogens.	1.85	4.85	3.76
SLC20A1	Solute carrier family 20(phosphate transporter) member 1.	1.82	2.17	-
SPRY2	Sprouty (Drosophila) homolog 2.	1.81	3.04	-
ANXA6	Annexin 6May associate with CD21. May regulate the release of Ca^{2+} from intracellular stores.	1.68	0.99	-
H2AFZ	H2A histone family member Z.	1.64	1.32	-
ITPR3	Inositol 1,4,5-triphosphate receptor type 3.	1.63	1.56	1.77
FOSL1	Fos-like antigen 1.	1.62	2.80	-
ALDOC	Aldolase C, fructose bisphosphate.	1.61	1.48	-
FXYD3	FXYD domain-containing ion transport regulator 3.	1.60	1.40	-
NR1H2	Nuclear receptor subfamily 1, group H, member 2.	1.60	1.60	B
SFRS7	Splicing factor, arginine/serine rich 7 (35kDa).	1.59	1.83	-
ARHGDIA	Rho GDP dissociation inhibitor (GDI9 alpha).	1.59	1.93	1.10
RPS6KB2	Ribosomal protein S6 kinase.	1.59	1.57	-

Table 3d. The most up regulated genes in the PDGF-AA array are shown. A comparison of the PDGF-AA values with the PDGF-AB (25 µg) and PDGF-BB values are shown.

Gene	Description	Value AA	Value BB	Value AB 25 µg
MUSK	Muscle, skeletal, receptor tyrosine kinase. Expression in embryonic muscle, at the neuromuscular junction and after injury.	0.29	1.93	-
SGK	Serum/glucocortoid regulated kinase.	0.65	0.92	1.33
BDKRB2	Bradykinin receptor B2. It is associated with a G protein that activates a phosphatidylinositol-calcium second messenger system.	0.77	1.12	-
GNS	Glucoseamine (N-acetyl)-6-sulfatase. Defect in this gene gives Sanfilippo disease IIID.	0.74	2.39	-

Table 3e. The most down regulated genes in the PDGF-AA array are compared to the PDGF-AB (25 µg) and the PDGF-BB arrays.

Gene	Description	Value BB	Value AA	Value AB 25 µg
COX-2	Cytoplasmic cyclooxygenase, induced by cytokines and mitogens.	4.85	1.85	3.76 Max
DTR	Diphteria toxin receptor (heparin binding epidermal growth factor-like growth factor). Mitogenic for fibroblasts and smooth muscle cells. Can bind EGF receptors with higher affinity than EGF itself and is a far more potent mitogen for smooth muscle cells than EGF.	4.69	2.10	-
CD22	CD22 antigen. Mediates B-cell B-cell interactions. May be involved in the localization of B-cells in lymphoid tissues. Binds sialylated glycoproteins for example CD45.	3.88	0.91 Low	-
NR4A1	Nuclear receptor, induction by growth-stimulating agents.	3.83	1.24 Low	3.62
DUSP6	Dual specificity phosphatase 6. Inactivates MAP kinases. Has specificity for the Erk family.	3.77	2.06	-
EGR3	Early growth response 3. Putative transcription factor.	3.08	1.01 Low	-
EST	Unknown function.	3.06	1.53	-
SPRY2	Sprouty (Drosophila) homolog 2.	3.04	1.81	-
DUSP5	Dual specificity phosphatase 5. Inactivates MAP kinases. Has specificity for the erk1.	2.92	1.26 Low	B
PLAUR	Plasminogen activator, urokinase receptor.	2.85	1.88	B

LOC56851	Unknown function.	2.80	1.62	-
DIPA	Hepatitis delta antigen-interacting protein A.	2.75	1.53	2.66
DUSP1	Dual specificity phosphatase 1. Dephosphorylates MAP kinase erk2 on both thr-183 and tyr-185.	2.64	1.21	3.56
NFKB1A	Involved in regulation of transcriptional responses to NfKb.	2.56	1.24 Low	2.40
ABCB2	ATP-binding cassette, sub-family B (MDR/TAP), member 2. Involved in the transport of antigens from the cytoplasm to a membrane bound compartment for association with MHC class I molecules.	2.46	1.08	2.12

Table 3f. The most up regulated genes in the PDGF-BB array are shown. The values are compared to the values for the same genes in the PDGF-AB (25 µg) and PDGF-AA arrays.

Gene	Description	Value BB	Value AA	Value AB 25 µg
EST	Unknown function.	0.64	0.85	-
EMD	Cytoskeletal protein, defects in emd are a cause of Emery-Dreifuss muscular dystrophy.	0.74	0.99 Low	2.20

Table 3g. The most down regulated genes in the PDGF-BB array are shown. A comparison of the PDGF-BB values with the PDGF-AB (25 µg) and the PDGF-AA arrays are done.

4 Discussion

From the check of PDGF α- and β-receptor expression it could be confirmed that the fibroblasts had an endogenous expression of both PDGF α- and β-receptors. The expression of α-receptors was much lower than the expression of β-receptors though. This is best seen in figure 5b where the antibody PY99 is used, which recognizes phosphorylated tyrosine residues in the different conditions with the same affinity. As seen in the blot there is a very low signal of activated α-receptors compared to β-receptors. If there had been an equal amount of α- and β-receptors in the cell, the signal in the AA lane should have been half the signal of the BB lane (since PDGF-BB recognize all receptor combinations and PDGF-AA only recognize the PDGF αα-receptor). The lower expression of the α-receptor can also be seen if figure 5c and 5d are compared. In figure 5c only α-receptor expression is shown. In figure 5d also β-receptor expression is shown and an increased signal is seen.

The results of the changes in gene expression for the different conditions have to be seen as preliminary results. To get more reliable results one has to improve several things:

The fact that the arrays available were not from the same batch caused problems. Many genes that were found in the 5K-2 batch (the PDGF-AB arrays) were not spotted on the 5K-1 batch (the PDGF-AA and -BB arrays) and vice verse. As seen in table 3b some interesting genes on the PDGF-AB (25 μg) array could not be compared to the PDGF-AA and -BB arrays.

Moreover the internal controls (the bacterial RNA) have to work satisfactory which was not the case here. This meant that a correct overlay picture of the scanned Cy3 and Cy5 channel was very difficult to generate. All the bacterial RNA spots in the array were supposed to turn yellow in the overlay picture, meaning that an equal amount of control RNA and stimulated RNA had hybridized to these spots. This was expected because the same amount of bacterial RNA had been used both for the control and stimulated RNA. These controls should “normalize” the hybridization of the different dyes to the array. Since this was not the case I tried to “normalize” the overlay pictures by scanning the Cy3- and Cy5-channels for the different arrays with the same laser power (Cy3-80%, Cy5-60%) and only select the most up regulated and down regulated genes in the arrays. This only gave a qualitative result (what genes that were affected by the stimulation) and the quantitative numbers for the genes in the tables are not absolute values. But since hundred of genes were affected in the experiments (where almost all of them were up regulated) a selection of the 20 most up regulated genes and a small selection of down regulated genes was interesting to do.

The great background in the Cy5 channel in the PDGF-AB (25 μg) array also was a problem. This meant that only high intensity spots could be picked out and some lower intensity spots that could have been interesting “drowned” in the background signal.

There was also a saturation problem. When analyzing the PDGF-AB (10 μg) array some very up regulated genes (marked **High** in table 3b) were unchanged in the PDGF-AB (25 μg) array. In the PDGF-AB (25 μg) array these spots (genes) had maximum intensity (marked **Max**) and hence saturated and could not be detected correctly by the system. The saturation problem could depend on two things: The copy number of the DNA at the spot was too low and the spots were saturated by cDNA hybridization. Then one would have to redo the hybridization with a lower concentration of cDNA. The other explanation is that the system was saturated and could not detect the signal. This could be solved by rescanning the two channels at a lower laser power. Unfortunately I realized the saturation problem too late and when trying to rescan the PDGF-AA and -BB arrays, to see if this was solving the problem, the signal from the fluorescent dyes was gone.

Probably I missed a lot of up regulated genes in the arrays since there was quite a lot of saturated spots in both the PDGF-AA and -BB arrays that seemed to be "unchanged" because of saturation.

Another problem was the low expression of α-receptors in the cell type used. Most of the genes marked **Low** in the tables 3a-g are found in the PDGF-AA array. These genes have a low spot intensity. This is due to the fact that there was a low expression of the α-receptor in the cell type used. This meant a weaker signal and that less RNA was transcribed. Therefore the amount of RNA hybridizing to the array (giving the intensity) would be lower in the PDGF-AA array compared to the PDGF-AB and -BB arrays. The calculated values should not be so much lower in this array compared to the others which was the case here. Since the internal controls did not work and I "normalized" by scanning this was probably why the values were lower. The comparison of the arrays are based on if the gene is among the highest value genes (shown in tables) in several arrays or if there is a big difference in the value and not so dependent on the absolute value.

Another thing to keep in mind is that PDGF-AA exclusively activates the PDGF αα-receptor. When stimulating cells with PDGF-AB almost only the αβ-receptor will be activated since the α-receptor expression was very low compared to the β-receptor expression in the cells. When stimulating cells with PDGF-BB all three receptor combinations will be activated. This means that overlapping of signals are expected but when differences appear between the stimulations this will be due to the receptor combination.

Finally at least quadruplicates (four repetitions) of each array have to be done. This is because the RNA might hybridize slightly differently to different arrays. Small differences in the preparations of the RNA may also lead to different results in the end.

With these problems taken into account I still got some genes that could be compared for the homo- and heterodimeric receptors.

Some genes that behaved in a similar way in all stimulations were found.

The dual specificity phosphatases (DUSP) genes are expressed in all the PDGF stimulations and these genes inactivate the MAP kinase cascade by dephosphorylation of critical phosphothreonine and phosphotyrosine residues. The MAP kinase cascade is a pathway that mediate a mitogenic signal to the cell which means that the DUSP genes negatively control this signal (Camps et al., 2000). The DUSP genes up regulated in these arrays have a specificity towards the Erk MAP kinase. Since both the homo- and heterodimeric receptors mediates a mitogenic signal when expressed, the DUSP genes should be up regulated in all the arrays to inhibit (negative feedback mechanism) this signal. Different DUSP members are up regulated in the different arrays though. Perhaps there is a difference in efficiency of inhibiting the MAP kinase between the different receptor complexes.

Some differences between the heterodimeric (PDGF-AB) and homodimeric (PDGF-AA and -BB) receptor signals were also seen. The genes ID3, CREM, EMD, KIAA0653, KIAA0127, SCYA7, SCYA13 were all up regulated in the PDGF-AB array (heterodimeric receptor) compared to the PDGF-AA and -BB arrays (homodimeric receptors).

The SCY genes or monocyte chemotactic proteins (MCPs) are chemokines and both SCYA7 (MCP-3) and SCYA13 (MCP-4) augments monocyte anti-tumor activity. In dermal fibroblasts these genes are up regulated by TNF-α (Hein et al., 1999). SCYA13 and partly SCYA7 play a key role in selective eosinophil recruitment to sites of inflammation in allergies, asthma (Gangur et al., 2000) and tissue infection (Hein et al., 1999). SCYA13 may also be involved in the recruitment of monocytes into the arterial wall during the disease process of artherosclerosis (Reape et al., 2000). It is known that PDGF is involved in the process of wound healing and also is up regulated in artherosclerosis (Heldin et al., 1999). Interestingly though, is that the up regulation is seen only in the PDGF-AB stimulated cells which may indicate that this specific wound healing process is $\alpha\beta$-heterodimeric regulated.

The KIAA genes are genes that have been identified in chronic myeloid leukemia but the function is still unknown. Since these genes (especially KIAA0653) only are up regulated in the PDGF-AB stimulated cells these genes could be interesting to study further.

CREM is a transcription factor that regulates JUNB and many other transcription factors. Also ID3 is a transcription factor. Exactly how these genes affect the cell is very difficult to say.

The conclusion that can be drawn from this experiment is that there are some differences between the homo- and heterodimeric PDGF signaling but a clear difference in cellular responses due to these differences are not possible to see with the data obtained here. One would have to do more experiments to be sure of the results and still it would be very difficult to predict the cellular response. Of course also the same batch of arrays has to be used and all other problems that occurred in this experiment have to be eliminated.

Future experiments that can be done (except repeating this experiment) is to stimulate the cells for many different time points and see how the genes behave in a time dependent way and to compare if the behavior differ between the homo- and heterodimeric receptors. This would provide more information like in what order genes are turned on and off and a more clear view of which genes that induce transcription of other genes.

5 Acknowledgements

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6 References

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Appendix A

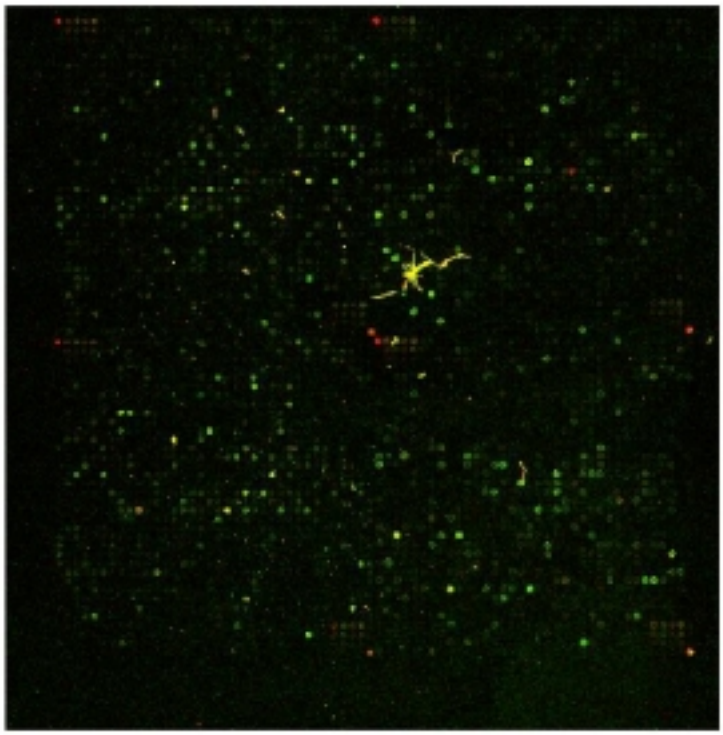

Figure 1. 2h PDGF-AB stimulation, 10 µg total RNA

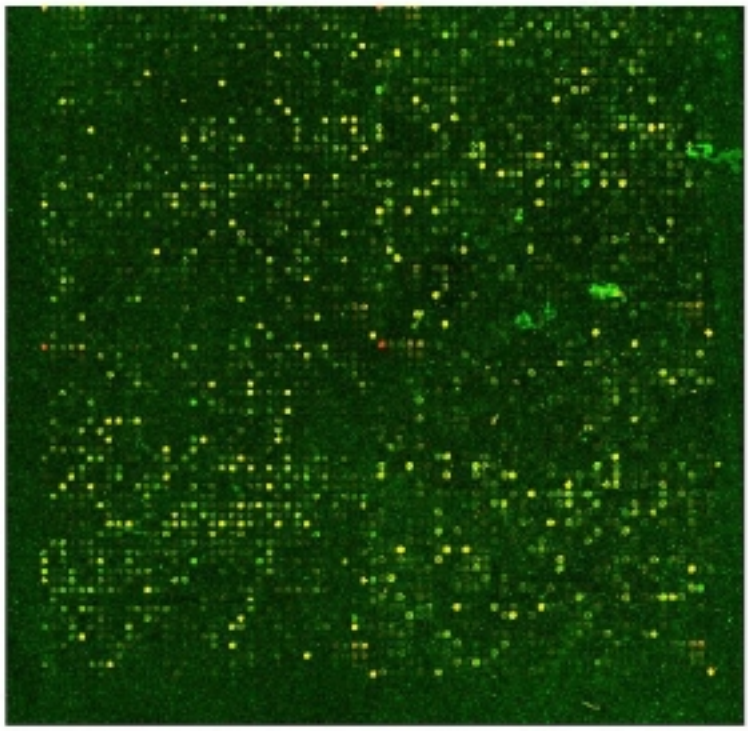

*Figure 2.*2h PDGF-AB stimulation, 25 µg total RNA (80-60)

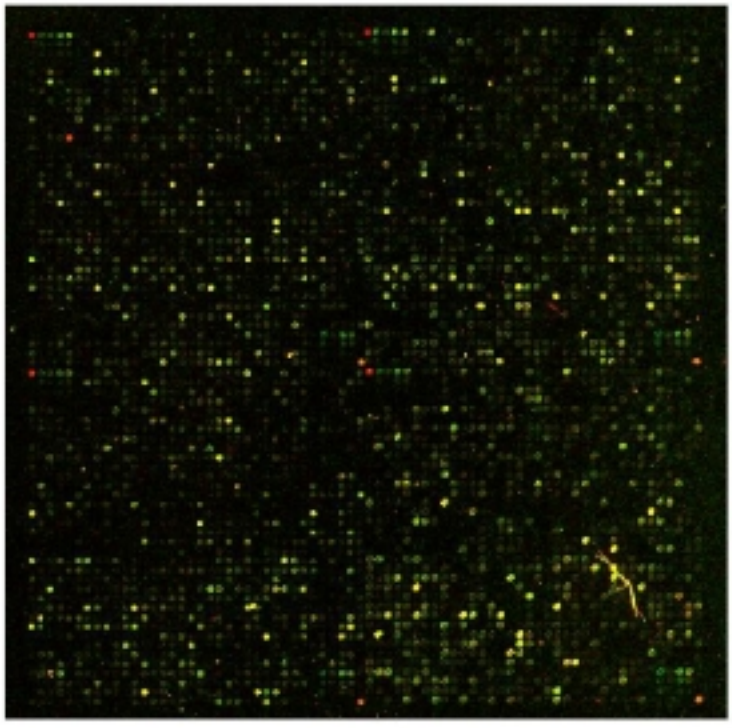

Figure 3. 2h PDGF-AA stimulation, 25 µg total RNA (80-60)

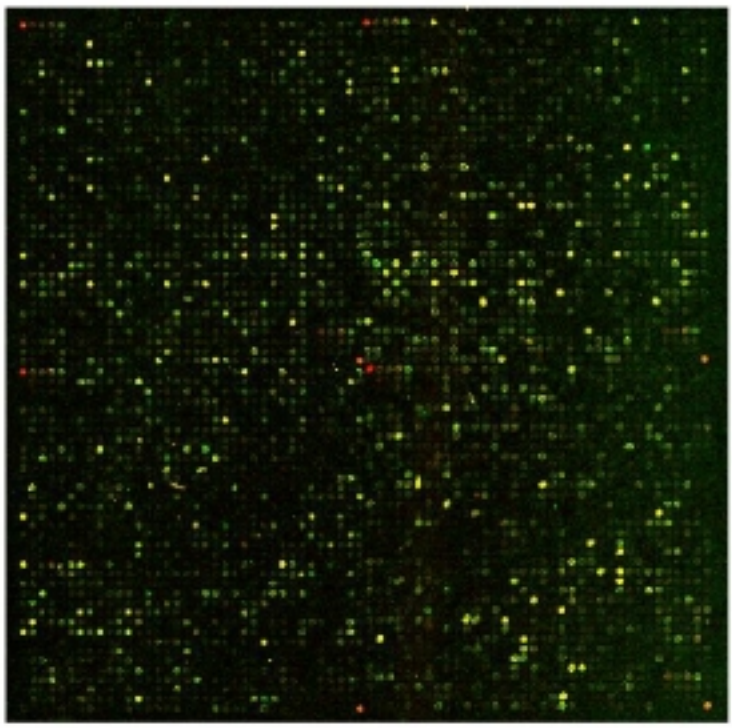

*Figure 4.*2h PDGF-BB stimulation, 25 µg total RNA (80-60)