PDGFRA transcriptional regulation in human physiology and disease

PDGFRA transcriptional regulation in human physiology and disease

Een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
dinsdag 22 mei 2007
om 13.30 uur precies

door

Mascha Toepoel

geboren op 22 juli 1977
te Olst

Promotor:	prof. dr. Everardus J. J. van Zoelen
Manuscriptcommissie:	prof. dr. Gerard J. M. Martens
	dr. Henk J. Blom
	dr. Pieter Wesseling

ISBN: 978-90-9021797-0
Print: PrintPartners Ipskamp B.V. Enschede, The Netherlands
Cover: adapted from Klinghoffer et al., Dev Cell. 2002 Jan;2(1)

The research presented in this thesis was performed at the Department of Cell Biology, Faculty of Science at the Radboud University Nijmegen. The studies were supported financially by the Prinses Beatrix Fonds and the Hersenstichting Nederland.

Contents

Chapter 1

General Introduction

1. Basic mechanisms of transcriptional regulation

1.1 Introduction

For cells to function properly within a certain tissue of an organism, they must be able to respond correctly to signals they receive from neighboring cells and, vice versa, to give the proper signals to them. This cell-cell communication is for a large part mediated by secreted growth factors and their receptors, which have to be expressed in the right amount and at the right time and place for transduction of the correct signals. Regulation of protein expression takes place at several levels (1). First, each gene contains regulatory sequences that control the cell specificity, amount and time of DNA transcription. These regulatory sequences include a promoter, which is located just 5' of the coding region, and in addition so-called enhancer and silencer elements that can be located several kb away from the gene itself, either 5' or 3', or within intron sequences. Second, the full length mRNA must be processed by splicing, in order to remove the introns or to produce alternatively spiced mRNAs that encode protein variants. Third, the processed mRNA has to be transported correctly from the nucleus to the cytoplasm where the ribosomes are located. A fourth regulatory step is provided by mRNA stability; stable mRNAs can be translated multiple times, in contrast to mRNAs that are quickly degraded. A fifth control point is the efficiency by which mRNA is translated by ribosomes. Recently, a new regulating mechanism, which affects approximately one third of all human genes (2), has been identified that is based on short RNA fragments called microRNAs, which induce mRNA cleavage or inhibit its translation (3). Sixth, the protein must be transported to its proper site of action, including specific cell organelles, the cytoplasm, the plasma membrane or the extracellular space. A seventh step is regulation of protein activity by dimerization, phosphorylation, proteolytic processing from precursor forms etc., while finally the rate of protein degradation and recycling determines the amount of functional protein in a cell. All these processes together influence how a cell can function in its cellular environment (1).

1.2 The core promoter and basal transcription machinery

The first regulatory step in protein expression, i.e. transcriptional control, is mediated by specific DNA sequences that function as promoter, enhancer or silencer elements. The promoter can be functionally divided into the core promoter and the upstream promoter region. The core promoter is able to drive basal transcription of the gene, whereas the upstream promoter region can further stimulate or repress transcription in a cell type specific manner.

The core promoter can be defined as the 5' sequence of a gene that specifically binds RNA polymerase and thereby determines the transcription start site. Eukaryotic RNA polymerase II (polII), which transcribes protein-encoding genes, cannot initiate transcription by itself, but is dependent on a set of general transcription factors, named TFIIA to TFIIH, which guide polII to the proper position on the DNA, as shown in Figure 1. The complex of polII with these general transcription factors is called the basal transcriptional machinery or preinitation complex (PIC), and is able to perform transcription at a low rate. PIC formation starts by binding of TFIID to the core promoter. TFIID is a multi-protein complex consisting of TBP (TATA binding protein) and 14 TBP-associated factors (TAFs). The interaction of TFIID with the promoter is subsequently stabilized by TFIIA, although TFIIA binding is not required for transcription

from all promoters. Next TFIIB, which in contrast to all other PIC components consists of only a single protein, joins the complex. Binding of TFIIB as well as of TBP to the DNA is essential for basal transcription. TFIIB binding stabilizes the interaction of TBP with the DNA and allows the subsequent recruitment of polII, which is escorted by TFIIF. Next, the complex is joined by TFIIE which in turn recruits TFIIH. TFIIH contains helicase activity which is modulated by TFIIE, resulting in facilitated melting of the promoter DNA. Upon phosphorylation by THFIIH, polII is finally released from the complex together with TFIIF, which also contains helicase activity, and the actual transcription will start. This release of polII together with TFIIF is referred to as promoter clearance (4, 5).

PIC recruitment is mediated by specific sequences in the DNA surrounding the transcription initiation site. Four of these binding sites are currently known, which are recognized by TFIID and TFIIB. TFIID can be recruited to promoters via a TATAAA sequence located 30-25 bp upstream of the transcription initiation site. This so-called TATA-box, which is present in many but not all promoters, is specifically recognized by TBP present in TFIID. Some promoters also use other motives to recruit TFIID, such as the initiator (Inr) sequence or the downstream core promoter element (DPE). Inr is found in both TATA-containing and TATA-less promoters. The mammalian Inr consensus sequence is Py-Py(C)-A_{+1}-N-T/A-Py-Py, where the +1 designates the transcription start site and Py stands for a pyrimidine (C or T). The DPE is mainly found in TATA-less promoters and has the consensus sequence A/G_{+28}-G-A/T-C/T-G/A/C. The DPE must be located precisely at position +28 to +32 relative to the +1 of the Inr in order for the two elements to cooperatively bind TFIID, and mutation of either motive or altering the space between them results in a loss of TFIID binding and a substantial decrease in transcriptional activity. Both the Inr and the DPE are not bound by TBP itself, but by one of the TAFs of TFIID. TFIIB can bind the core promoter at the TFIIB recognition element (BRE), which is located immediately 5' of some TATA-boxes and has a consensus sequence of G/C-G/C-G/A-C-G-C-C. TFIIB is essential for TFIID-mediated transcription. No specific sequence requirements are known for the other general transcription factors (4, 5).

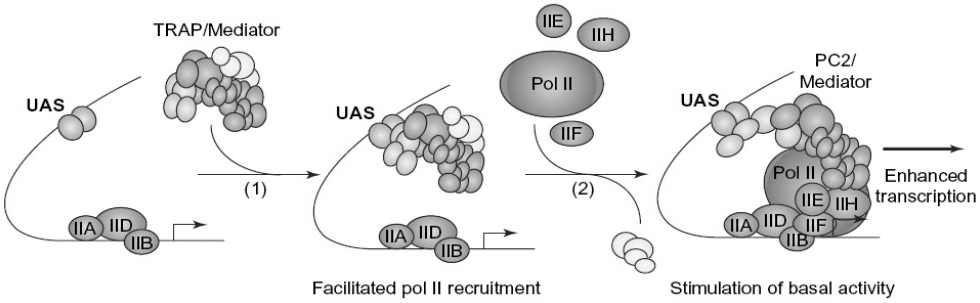

Figure 1: PIC formation and Mediator function (from (7)). Transcription initiation starts by binding of TFIIB to the promoter, which is subsequently joined by TFIIA and TFIIB. Next, polII and TFIIF enter the complex, followed by TFIIE and TFIIH to start basal transcription. Transcription factors bound to upstream activator sequences (UAS) can interact with the Mediator coactivator complex. Mediator then facilitates entry of polII into the complex, thus stimulating basal transcription.

1.3 Cis- and trans-acting factors

Each gene is flanked by and contains regulatory DNA sequences that are able to bind transcriptional activators and repressors, generally referred to as transcription factors, that are necessary for proper expression of the gene in a given cell type. Thus, the expression of a gene is dependent on regulatory DNA sequences (*cis* regulation) as well as the presence of diffusible proteins that bind these regulatory sequences (*trans* regulation). Most regulatory sequences necessary for proper expression are present in the upstream promoter region, i.e. the region 5' of the transcription initiation site, but as stated earlier they may also be located in enhancers or silencers several kb upstream or downstream of the gene or even in introns.

Transcription factors typically contain a DNA binding domain and a transactivation domain. The surface of a DNA binding domain precisely complements the structure of the major groove of a specific DNA sequence. Therefore, transcription factors show very high DNA sequence specificity, generally recognizing a consensus sequence of 5-8 nucleotides that is characteristic for each family of transcription factors (6). Various types of DNA binding domains can be discriminated, such as helix-turn-helix motives, as in homeodomains proteins, and zinc-finger motives. Other motives that are often found in transcription factors are leucine zippers and helix-loop-helix motives, which are used not only for DNA binding, but also for protein-protein interaction in transcription factors that function in dimers. After DNA binding, the transactivation domain of a transcription factor communicates with the basal transcription machinery to influence the rate of transcription.

Interaction between activators and repressors with the PIC does not take place directly but occurs through coregulator complexes, i.e. protein complexes that are involved in transcriptional regulation but that do not interact themselves with DNA. Mediator is an example of such a complex that is essential for transcriptional regulation. It was first discovered in yeast, but appears to be highly conserved in all organisms. Mediator complexes can consist of approximately 30 subunits reaching a molecular mass of up to 2 MDa, depending on the species. The mechanism by which Mediator influences transcription is not entirely clear yet, but it is believed that DNA-bound activator proteins bind specific Mediator subunits, which can be gene specific, while other Mediator subunits subsequently interact with RNA pol II to facilitate its recruitment into the PIC, thereby stimulating transcription, as outlined in Figure 1 (7).

1.4 Epigenetic: histone modifications and DNA methylation

Transcription is also regulated on the level of chromatin. DNA tightly packed in so-called heterochromatin is inaccessible to transcription factors and can thus not be transcribed even if the proper transcriptional activators are present in the nucleus. On the other hand, the more loosely packaged euchromatin is readily accessible and can be transcribed if the proper transcription factors are available. The packaging of DNA into so-called nucleosomes is mediated by specific DNA binding proteins called histones. A nucleosomal particle consists of 147 bp of DNA that is wrapped around a histone core complex made up of a histone (H3/H4) tetramer and two histone (H2A/H2B) dimers, thereby creating an 11 nm euchromatin fiber, referred to as "beads on a string". The chromatin structure can be further compacted into a 30 nm heterochomatin fiber by incorporation of the linker histone H1 into the complex (8).

Control of nucleosomal architecture is a very dynamic process that is regulated by specific modifications of a number of residues in the protruding tails of

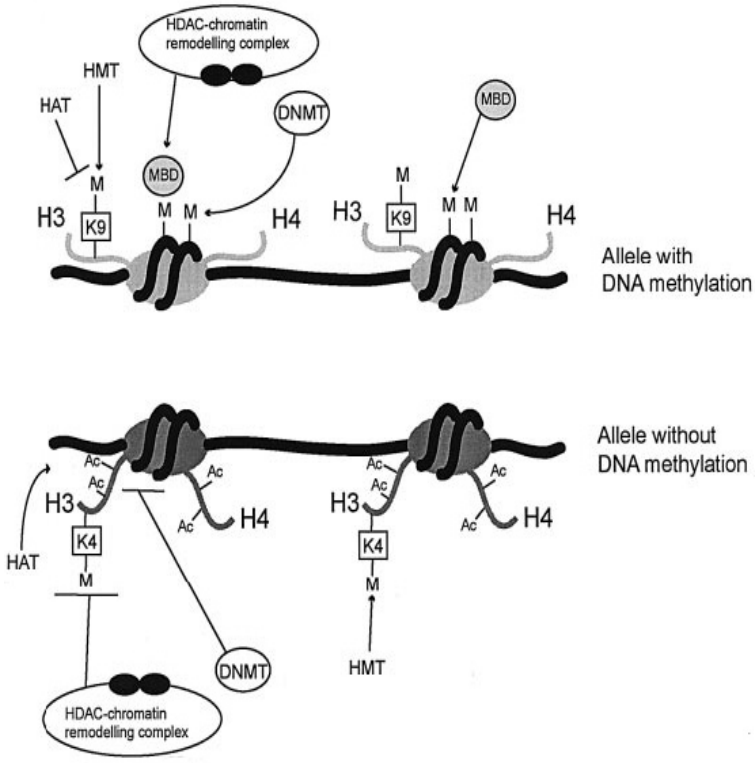

Figure 2: Epigenetic regulation of gene expression (from (123)). A silenced allele (top) is characterized by DNA methylation that is performed by DNA methyltransferase (DNMT) and methylation of H3-K9 by histone methyltransferases (HMT). Methyl-CpG-binding-domain (MBD) proteins bind the methylated DNA and recruit histone deacetylases (HDAC) containing complexes that keep histone tails dacetylated. An active allele (bottom) is not metylated and displays histone acyltransferases (HAT) driven histone acetylation (Ac) and HMT directed methylation of H3-K4.

histone molecules. Of these modifications, acetylation (of Lys residues) and methylation (Lys and Arg) have been studied most, but histone phosphorylation (Ser and Thr), ubiquitination (Lys), sumoylation (Lys), ADP ribosylation, glycosylation, biotinylation and carbonylation also take place. In addition to histone modifications, methylation of the DNA strand itselfs is also involved in chromatin compaction and thereby affects the transcription process. Hypermethylation of promoter regions is generally found in inactive DNA, whereas promoter hypomethylation correlates with gene transcription. These DNA and histone marks are maintained throughout the cell cycle and are transferred to daughter cells, thereby forming an epigenetic level of gene expression control (figure 2) (8).

Acetylation of histone tails is generally associated with active transcription, whereas histone hypoacetylation is found particularly in heterochromatin regions. Histone acetylation takes place on lysine residues and is performed by enzymes known as histone acyltransferases (HATs). Histone acetylation is believed to have a positive

influence on gene transcription in two ways. Firstly, the acetyl groups reduce the interaction between the negatively charged DNA and the positively charged histone tails, thus creating an open chromatin structure that allows transcription. Furthermore, acetylated histones recruit positive effectors for the transcription process, including certain TAFs. Deacetylation of histone tails is performed by histone deacetylases (HDACs), and their action results in transcriptional repression by reversing the acetylation effects (8).

Histone methylation is performed by histone methyltransferases and takes place on lysines, which can be mono-, di- and trimethylated, and on arginines, which can be monomethylated or (symmetrically or asymmetrically) dimethylated. At present, 24 sites are known to be methylation targets on histones, of which 17 are lysines and 7 arginines, thus in principle enabling 3×10^{11} distinct methylation states of histone proteins. Histone methylation was previously believed to be an irreversible process such that methylation marks could only be removed by histone replacement, but this view has changed recently by the discovery of two histone demethylating enzymes; a histone lysine demethylase (LSD1), and a peptidyl arginine deiminase (PAD4) which converts mono-methylated arginines to citrulline (8).

The effects of histone methylation on gene activity are not as straightforward as those of acetylation. Methylation of lysine 9 of histone H3 (H3-K9), as well as H3-K27 and H3-K79 methylation are associated with gene repression, whereas H3-K4 and H3-K36 methylation are specific for active genes (9). Furthermore, asymmetric dimethyl-arginine methylation is involved in gene activation, whereas symmetric dimethyl-arginine methylation has been associated with gene repression (10). There is also a strong interplay between the various types of histone modification in such a way that one modification can facilitate or inhibit other modifications. For instance, mono-ubiquitination of H2B-K123 is required for subsequent di-methylation of H3-K4 or H3-K79 (11), while phosphorylation of H3-S10 facilitates H3-K9 and H3-K14 acetylation, thereby inhibiting H3-K9 methylation (8).

DNA methylation in mammals takes place on the cytosine residue of CpG dinucleotides. The nucleotide combination CpG is relatively underrepresented in the genome when regarding the overall occurrence of cytosines and guanidines, but they tend to cluster in so-called CpG islands in promoter regions. CpG methylation of promoters can repress transcription in two ways: (1) it can directly interfere with transcription factor binding, or (2) it can recruit methyl-CpG-binding-domain (MBD) proteins which subsequently recruit HDACs that cause the chromatin to condense. DNA can be methylated by DNA methyltransferases which catalyze *de novo* methylation of unmethylated DNA or methylate the newly synthesized strand complementary to an already methylated strand after DNA replication. DNA methylation is indispensable for development, as is indicated for example by the observation that mouse mutants of the *de novo* DNA methyltransferases genes *Dnmt3a* or *Dnmt3b*, which are both highly expressed in the embryo but downregulated in differentiated cells, die before or around birth (12). Also, mice deficient in methyl CpG-binding proteins display a variety of developmental defects (13), thus underlining the importance of proper DNA methylation for development.

It is not known precisely what triggers *de novo* DNA methylation, but several factors have been proposed to be involved. First, information in the DNA itself, such as the nucleotide sequence, overall base composition and secondary DNA structure, may play a role. In certain fungi, TA-rich sequences of minimally 75 bp stimulate DNA

methylation, whereas G-C base pairs in this system tend to inhibit DNA methylation. Also certain regions of imprinted genes in the mouse can induce DNA methylation when placed in non-imprinted DNA regions (14).

Second, histone modification can influence DNA methylation. Histone H3-K9 methylation seems to control DNA methylation since murine embryonic stem cells lacking the H3-K9 histone methyltransferase Suv39h show decreased DNA methylation of major satellite DNA, and this specific transferase can be coprecipitated with the DNA methyltransferase Dnmt3b. This suggests that DNA methylating enzymes and histone modification enzymes work cooperatively (14). Moreover, the human methyl CpG-binding protein MBD1 has been found to interact with the H3-K9 histone methyltransferase SETDB1 such that H3-K9 methylation is lost in the absence of MBD1 (15). Thus, there appears to be a reciprocal interaction between histone modification and DNA methylation during regulation of gene repression.

Third, DNA methylation can be induced by so-called RNA-dependent DNA methylation, a process which involves the RNAi machinery. Small interfering RNA molecules have been found to target methylation of homologous DNA sequences mediated through Dnmt3b. Furthermore, mice that lack the RNAi processing molecule DICER are defective in DNA methylation and histone modification. This indicates that DNA methylation, histone modifications and RNAi are interconnected processes (16).

It has recently been established that DNA demethylation can be actively performed by some DNA glycosylases, which can excise methylated cytosines (17-20).

2. Regulatory polymorphisms, allele specific expression and disease susceptibility

2.1 Introduction

DNA polymorphisms are usually defined as sequence variations that occur in at least 1% of the population. Polymorphisms include substitutions of one nucleotide, referred to as single nucleotide polymorphisms (SNPs), and insertions or deletions (ins/dels) of one or more nucleotides. Sequence variation also occurs as a result of nucleotide rearrangements and insertion of repeating sequences. The latter are known as microsatellites, if the repeat sequence is not more than six nucleotides, and as minisatellites if this sequence is longer (21). SNPs occur with an average frequency of approximately one in every thousand nucleotides (6). When a polymorphism is present in a coding sequence, its effect may be evident if it results in an amino acid substitution or introduction of a premature stopcodon. Variation in putative regulatory sequences, on the other hand, may influence the expression of nearby genes by altering the binding affinity of transcription regulating proteins or by introducing new transcription factor recognition sites, resulting in altered rate or specificity of gene transcription, as shown schematically in Figure 3. Therefore it is assumed that such regulatory polymorphisms form the genetic basis for not only the phenotypic variation between individuals but also for the susceptibility of individuals to diseases.

2.2 Identification of regulatory polymorphisms

The effect of a polymorphism on the DNA binding affinity of transcription factors

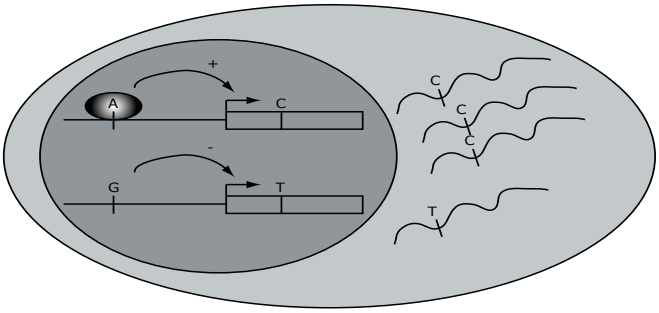

Figure 3: Regulatory variation and allele specific expression. A promoter SNP influences binding of a transcription factor and thus expression level of the two alleles, of which transcripts can be distinguished by a marker SNP in the coding region.

can relatively easy be studied by *in vitro* DNA-protein binding experiments, such as electrophoretic mobility shift assays (EMSAs) and DNA footprinting. An EMSA is a fast and sensitive method to study DNA-protein interactions, in which short radiolabeled DNA sequences are used to specifically bind transcription factors, usually out of a large pool of nuclear proteins, which are subsequently identified by gel electrophoresis. The disadvantage of this method is the small size of the oligonucleotides that can be used (typically 20-30 nt), which does not allow to study the effect of possible interactions of multiple polymorphisms. Furthermore, there is no straightforward method to identify the bound nuclear protein, unless specific antibodies are available against candidate proteins. DNA footprinting is based on the principle that DNA binding proteins protect otherwise naked DNA regions from cleavage by DNAseI. Differential protein binding thus results in different cleavage fragments, allowing specific protein binding regions in the DNA to be recognized. The advantage of the technique is that several hundred nucleotides can be studied at the same time, but protein concentrations must be high enough to ensure saturated DNA binding, while the resolution of this technique is limited because of the size of the DNAseI enzyme (6).

The most commonly used method to study the effects of regulatory polymorphisms on gene expression levels is transient transfection of promoter-reporter gene constructs into a cell line of interest. The amount of reporter gene protein produced thus reflects the corresponding promoter activity. Based on this technique, it has been shown on the basis of 170 randomly selected genes with polymorphisms in their proximal promoter region, that in 34% of the cases such polymorphisms modulate reporter gene expression by at least 1.5-fold in at least one of three human cell lines tested (22). Thus, promoter polymorphisms seem to have a direct effect on the expression level of a substantial number of genes.

2.3 Endogenous allele specific expression

The above techniques can give valuable information in terms of the function of specific polymorphisms in transcription factor binding and gene expression levels, since it is easy to study the effect of mutations on promoter activity and/or transcription factor binding. In addition, many different cell types can be tested for the presence of

cell specific *cis*- and *trans*-acting factors. A disadvantage of these techniques is that they study protein binding to DNA outside its normal chromatin environment, and as a result the endogenous epigenetic regulation is likely to go unnoticed. Furthermore, these approaches focus only on a limited number or even a single polymorphism, whereas *in vivo* a combination of polymorphisms present in the promoter region may determine the effect on transcriptional regulation. Such a genetically transmittable DNA region with a specific combination of polymorphisms is generally referred to as a haplotype.

The effect of promoter polymorphisms and haplotype differences on endogenous gene expression can be studied by analyzing allele-specific expression levels in samples that are heterozygous for a promoter haplotype. In this way it is ensured that *trans*-acting factors are equally available for each allele and as a result that differences in allelic expression must result from sequence differences between the alleles themselves. However, this approach requires the presence of a linked, so-called marker polymorphism in the coding region, which enables transcripts from different promoter haplotypes to be distinguished (figure 3). In that case, mRNA from individual alleles can be discriminated and quantified by SNP-microarray or by RT-PCR based methods. The latter include restriction fragment length polymorphism (RFLP) analysis, which is a method to determine allelic abundance based on the introduction or disappearance of a restriction site by a polymorphism. Another method is single base primer extension analysis, which makes use of (fluorescently labeled) dideoxynucleotides as chain terminators of an extended primer in order to discriminate both alleles on the basis a polymorphism, either by mass spectrometry or by sequence analysis (6).

Using the above techniques a number of studies have been carried out to investigate the effect of sequence variation on gene expression levels. Yan et al. (23) reported that six out of the 13 genes showed variation in allelic expression in lymphoblastoid cell lines with a maximum difference of 4.3 fold for the P73 gene. Furthermore, in a study based on 193 single-nucleotide polymorphisms (SNPs) from 129 genes expressed in lymphoblastoid cell lines it has been shown that 23 genes (18%) display allele-specific expression with significant deviation from an equimolar ratio (24). Based on a SNP oligonucleotide array on RNA material from seven individuals, Lo et al. (25) demonstrated that from the 602 heterozygous genes analyzed 326 (54%) showed preferential expression of one allele in at least one individual. Although in these cases the evidence for regulatory variation is indirect, it does clearly illustrate that many genes display variation in their regulatory sequences that will significantly influence the endogenous mRNA levels of those genes.

A very elegant and sensitive method to measure allele-specific expression without the need of a marker SNP has been described by Knight et al. (26). This so-called haplotype-specific chromatin immunoprecipitation (haplo-ChIP) method is based on the principle that the allele with the highest expression must also bind most phosphorylated RNA polII. In this assay, cells are treated with formaldehyde in order to fix protein-DNA interactions and subsequently the chromatin is sonicated to create short fragments. After immunoprecipitation with an antibody directed against phosphorylated RNA polII, the relative abundance of each allele is then measured by PCR, primer extension and mass spectrometry. Such haplo-ChIP analysis also allows the identification of polymorphism-dependent protein binding *in vivo,* by immunoprecipitating a candidate transcription factor instead of polII by a specific

antibody. Using this method the authors have demonstrated that the +80A/C SNP of the lymphotoxin-α (*LTA*) gene influences allelic expression *in vivo* as a result of differential binding of ABF-1 (transcriptional repressor activated B cell factor-1) (27). The limitation of the haplo-ChIP applied to polII method is that the polymorphism of interest must lie within approximately 800 bp of the transcription start site, which corresponds to the size of the DNA fragments after sonication.

2.4 Regulatory variation and disease susceptibility

Many examples have shown that regulatory polymorphisms may affect the susceptibility of individuals for common diseases. For instance, the G allele of a G/A SNP substitution in the promoter of the CCR5 gene, which encodes a chemokine co-receptor necessary for the HIV1 virus to enter the cell, appears to correlate with low expression and low propagation of the HIV1 virus. Furthermore, minisatellites near the insulin-encoding gene *INS* have been shown to influence *INS* expression levels and differentially bind the transcription factor PUR1 *in vitro*, and furthermore have been associated with type I diabetes (21). Moreover, variation in the promoter region of the macrophage migration inhibitory factor gene *MIF* is associated with differences in MIF production and with risk of juvenile idiopathic arthritis and psoriasis (28, 29), while SNP dependent binding of OCT-1 to the *TNF* promoter appears to influence promoter activity and susceptibility to malaria (30) Finally, polymorphisms in the LTA gene have been associated with susceptibility to a.o. asthma and cancer (21). These examples show that nucleotide variation in regulatory sequences can influence gene expression and thereby disease susceptibility. It should well be realized that it often remains unclear whether a disease-associated polymorphism is really functional or only acts as a marker due to linkage with other polymorphisms.

3. Platelet-derived growth factor alpha-receptor (PDGFRA)

3.1 Introduction

Platelet-derived growth factors (PDGFs) are dimeric polypeptide molecules that have initially been identified in platelets as mitogens for vascular smooth muscle cells. Thus far, four PDGF chains have been identified, called PDGF-A, PDGF-B, PDGF-C and PDGF-D, and two receptors, called the PDGF alpha-receptor and PDGF beta-receptor (PDGFRA and PDGFRB). The ligands and receptors are expressed in overlapping but also in distinct cell types. *PDGFRA* is mainly expressed in cells of mesodermal and neural origin and is involved in the regulation of cell migration, proliferation and differentiation. PDGFRA plays an important role in various biological processes such as embryonic development, wound healing and atherosclerosis, but also in tumorigenesis (31).

3.2 PDGFRA gene and protein structure

The human PDGFRA gene is located on chromosome 4q11-12, spans approximately 65 kb of DNA and contains 23 exons. The gene structure is very similar to that of the related receptor tyrosine kinase family members *PDGFRB*, *c-KIT* and *c-FMS*. It has a non-coding exon 1 followed by a large first intron of 23 kb, while the translation start codon is located in exon 2. *PDGFRA* and *c-KIT* are located in a head to tail orientation on chromosome 4, whereas *PDGFRB* and *c-FMS* are similarly orientated on chromosome

5 (32). The PDGFRA gene encodes a full length protein of 140 kDa which consists of an extracellular ligand binding domain containing five immunoglobulin-like domains, a transmembrane domain and a cytoplasmic split tyrosine kinase domain. When fully glycosylated, the molecular mass of the protein increases to approximately 170 kDa (33).

The PDGFRA gene encodes at least four different transcripts, which are formed as a result of alternative splicing and promoter use. A promoter located upstream of exon 1, called P1, gives rise to the full length 6.4 kb transcript as well as a 3.0 kb alternatively spliced transcript that putatively encodes a truncated receptor protein. Similarly, a promoter located in intron 12, termed P2, drives the expression of a 5.0 and 1.5 kb transcript and is particularly active in early embryonic human cells (34). The 1.5 kb transcript has been shown to be a prognostic marker for testicular germ cell tumors in humans, but protein products for these transcripts have not been established yet (35).

3.3 Ligands and downstream signaling

The four PDGF ligands act mainly as homodimers, but heterodimers of PDGF-A and PDGF–B are also known. Upon binding to their receptors they induce the formation of receptor homo- or heterodimers, depending on the ligand specificity and the relative abundance of receptor types expressed. PDGFRA can bind PDGF-

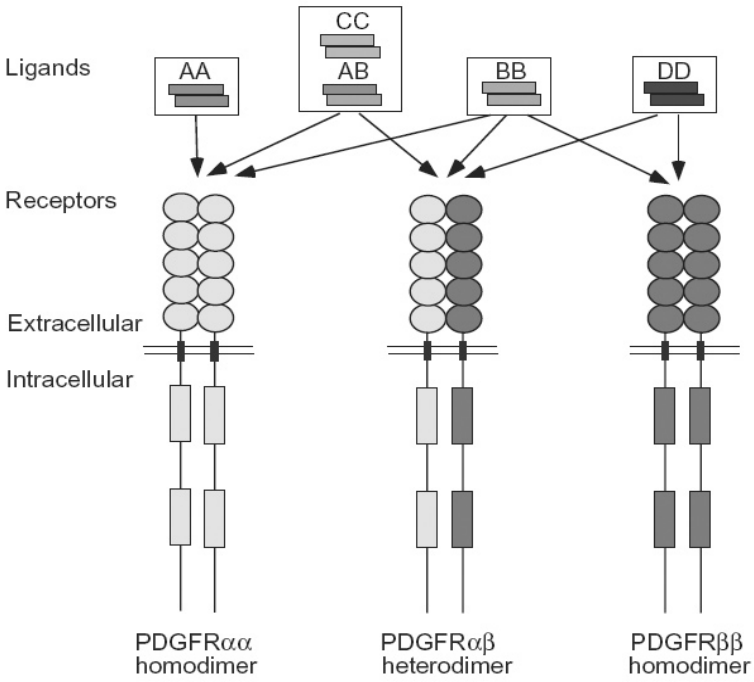

Figure 4: Interaction between PDGF receptor and ligand dimers (from (31)).

A, PDGF-B and PDGF-C, whereas PDGFRB can only bind PDGF-B and PDGF-D (figure 4). Receptor dimerization induces transphosphorylation of multiple tyrosine residues in the cytoplasmic region, after which a variety of intracellular signaling pathways are activated, depending on the type and concentration of the ligand (36) and the type of receptor dimer that is formed (31, 37).

Signaling downstream of PDGFRA involves the activation of phospholipase Cγ (PLCγ), phosphatidylinositol kinase (PI3K) and RAS/mitogen-activated protein kinase (MAPK) pathways (reviewed by (38, 39)). PLCγ binds the autophosphorylated receptor at Tyr-988 and Tyr-993, and converts phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) into inositol(1,4,5)trisphosphate and diacylglycerol (DAG), resulting in an increase in the intracellular Ca^{2+} concentration and activation of members of the protein kinase C (PKC) family respectively. PLCγ is particularly involved in PDGF-stimulated cell growth and motility.

PI3K binds the autophosphorylated receptor at Tyr-731 and Tyr-742, and phosphorylates PI(4,5)P2 to give phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3). Among the downstream targets of PI3K are protein kinase B/AKT, members of the Rho family of small GTPases and several PKC members, thereby mediating a variety of cellular responses which include actin reorganization, chemotaxis, cell growth, and anti-apoptosis (38, 39).

Activation of the RAS/MAPK pathway takes place through the tyrosine phosphatase SHP2 and the adapter protein SHC. These two molecules subsequently bind GRB2/SOS which can induce RAS into the active GTP-bound form, thereby activating the MAPK cascade via the serine/threonine kinase RAF1. Besides MAPK activation, RAS can also activate PI3K. The RAS/MAPK pathway has been implicated in cell growth, migration and differentiation (38, 39).

.

3.4 Developmental expression of Pdgfra and ligands

In the mouse *Pdgfra* is expressed from the two cell stage onwards by all cells of the blastocyst, but later in development its expression becomes restricted to cells of mesodermal and neural origin. In early post-implantation embryos, at embryonic day E6-E7.5, *Pdgfra* mRNA can be found in extra-embryonic and embryonic endoderm, as well as in the embryonic mesoderm (40-42). At E8.0-8.5 expression in the embryo becomes more regionalized to the somites and mesenchyme, while somite expression subsequently becomes restricted to the sclerotome and dermatome at E9.5 (40, 41). In the developing neural tube, *Pdgfra* is expressed in neuroepithelial stem cells as early as E8.5 and continues to be expressed by developing neurons (43-45). Furthermore, Pdgfra expression is found in oligodendrocytes precursor cells (OPCs, also referred to as O2A cells), which arise in the mouse around E12.5 in the periventricular zone of the neuroepithelium. From E12.5-E15.5, these OPCs proliferate and migrate to populate the entire neural tube/spinal cord (46). Expression of *Pdgfa* during this period is found in the surface ectoderm, the myotome of the somites and the floor plate of the neural tube, resulting in alternating layers of ligand and receptor expression (47). *Pdgfb* is mainly expressed in vascular endothelial cells (48), while its protein Pdgfb has also been detected in various neurons of the embryonic and adult brain (49). *Pdgfc* is expressed in the myotome and sclerotome of the somites, the surface ectoderm and the notochord, as well as in the developing brain and the neural tube, where its expression becomes restricted to the ventral horn and the floorplate at E16.5 (50).

3.5 Pdgfra and ligand mouse mutants

The first *Pdgfra* mouse mutant described was the naturally occurring *Patch* (*Ph*) mouse (51). The *Ph* mutation involves a variable deletion on mouse chromosome 5 that includes the entire Pdfgra gene (52, 53). While *Ph*/+ mice appear phenotypically normal apart from white patches in their fur (hence also its name), the *Ph* mutation is lethal in its homozygous form. Although *Pdfgra* is normally expressed from the two-cell stage onward, developmental defects in homozygous *Ph* mice are only observed from the primitive streak stage onward, indicating that *Pdfgra* function is dispensable until then. *Ph*/*Ph* mice survive until E10.5-E18, depending on their genetic background (C57/BL6/J, CBA or Balb/C), and display various developmental defects, mainly in mesodermal and neural crest-derived tissues and the neural tube. These include an overall smaller body size, spina bifida and a cleft face and/or palate, in addition to many axial skeletal defects such as malformed vertebrae and ribs (40, 54-56) (51).

Targeted *Pdfgra* null mutants display more or less the same phenotype as homozygous *Patch* mice, also involving mesoderm, neural crest and neural tube malformations (57). *Pdfgra* (-/-) mice survive until E16, are also smaller than their wild-type littermates and display a.o. a cleft face, spina bifida (figure 5) and various additional vertebral and rib cage malformations. The defects seen in both *Pdgfra* (-/-) and *Pdgfra* (+/-) mice generally occur in tissues that show physiological *Pdgfra* expression. Notably, the development of surrounding tissues and cell types that lack Pdgfra expression, e.g. the myotome and neurons, can also be affected. This is probably due to an altered interaction between these receptor negative cell types and cell types that normally express Pdgfra (41, 55, 57). The few differences between *Ph* and *Pdgfra* mutant mice, including the absence of patches in *Pdfgra* (+/-) mice and the milder heart defects in *Pdgfra* (-/-) mice, can most likely be ascribed to the deletion of sequences in the *Ph* mutation that affect the expression of other genes. Introduction of a YAC transgene carrying the entire human PDGFRA gene almost completely rescued the *Pdgfra* (-/-) phenotype, while the survival of *Ph*/*Ph* mice was not affected by this YAC, indicating that the *Ph* mutation indeed involves more than *Pdgfra* alone (58). Also in Xenopus, disabling Pdgfra function results in aberrant mesoderm development and neural tube closure (59).

Since the *Ph*/*Ph* and *Pdgfra* (-/-) mutants are both embryonically lethal, Pdgfra function in these mice cannot be studied later in development. Knock-in mice in which the endogenous Pdgfra gene was replaced by a transgene encoding mutant receptors deficient in activating specific downstream signaling molecules, allowed a study of the function of Pdgfra at later time points, as well as the unraveling of the contributions of specific Pdgfra-activated signaling pathways (60). Transgenic mice expressing a Pdgfra mutant that lacks the ability to activate PI3K ($\alpha^{PI3K}/\alpha^{PI3K}$) displayed prolonged survival until birth compared to *Pdgfra* (-/-) mice, but died soon after probably as a result of respiratory failure. The developmental defects of these $\alpha^{PI3K}/\alpha^{PI3K}$ mice are also much milder than those observed in the homozygous null mutants, but do include several skeletal malformations (a.o. spina bifida) and impaired oligodendrocyte development. Blocking only the receptor's ability to activate Src family kinases resulted in an even less severe phenotype. In these $\alpha^{Src}/\alpha^{Src}$ mice only oligodendrocyte development seemed to be affected, resulting in a severe hypomyelination of the central nervous system. Surprisingly, mice hemizygous for the so-called F7 allele (F7/-), a mutation that leads to a disruption of binding sites for PI3K, SRC family kinases, PLCγ and SHP2, do not show additional defects compared to the $\alpha^{PI3K}/\alpha^{PI3K}$ mice, indicating

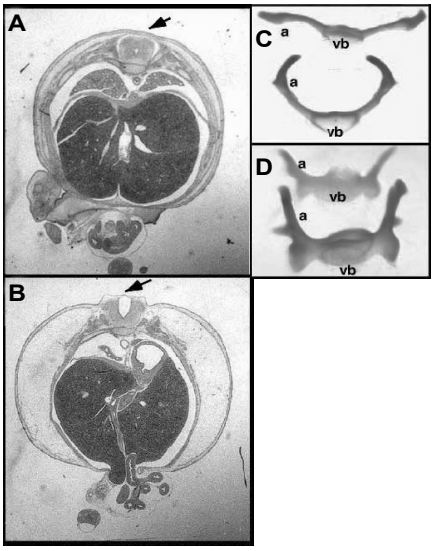

Figure 5: Spina bifida in *Pdgfra* (-/-) mice (from (57)). Cross-section of wildtype (A) and mutant (B) E15.5 embryos. The arrow indicates the neural tube. Ninth thoracic (C) and first sacral vertebrae (D) from wildtype (top) and mutant (bottom) embyos are shown in detail. (a) represents neural arch, (vb) vertebral body.

that PI3K is the main intracellular signaling molecule for Pdgfra during embryogenesis (61). The observation that the phenotype of both the PI3K and F7 mutant is milder than that of *Pdgfra* (-/-) mice, suggests that these mutant receptors are probably still able to activate PI3K at a low level by forming dimeric complexes with Pdgfrb. Disabling PI3K binding capacity in both Pdgfra and Pdgfrb indeed resulted in the same phenotype as observed for *Pdgfra* (-/-) mice (60). McKinnon et al. (36) confirmed the importance of Pdgfra signaling via PI3K and also through PLCγ for oligodendrocyte development. They demonstrated that PI3K is activated at relatively low ligand concentrations, is required for OPC migration and survival, and promotes proliferation, whereas PLCγ activation takes place at relatively high ligand concentration and promotes OPC proliferation but is not required for migration.

Mutations in the genes for the Pdgfra binding ligands Pdgfa, Pdgfb and Pdgfc are likely to display at least some of the defects seen in the receptor mutants. *Pdgfa* knockout mice die perinatally and show severe hypomyelination due to defective oligodendrocyte development, but lack the axial skeleton defects seen in the *Ph*/*Ph* and *Pdgfra* (-/-) mice (62). So although Pdgfa is expressed in neurons and appears to be the main Pdgfra ligand for proliferation and migration of OPCs *in vivo,* it does not seem necessary for proper bone formation. In contrast, the phenotype of *Pdgfb* deficient mice looks very similar to that of *Pdgfrb* knockout but not of *Pdgfra* knockout mice. *Pdgfb* (-/-) as well as *Pdgfrb* (-/-) mice die around birth, displaying defects in cardiovascular, hematological and renal development (48, 63-66). Also, the phenotype of *Pdgfa*/*Pdgfb* double mutant mice does not resemble that of Pdgfra (-/-) mice (67). Pdgfb therefore does not seem to play an important role as a Pdgfra ligand, at least not during embryonic development. In contrast, *Pdgfc* (-/-) mice resemble *Pdgfa* (-/-)

mice, in that they show some but not all of the characteristics of *Pdgfra* null mice; they die perinatally displaying a cleft palate and spina bifida. *Pdgfa*/*Pdgfc* double mutants on the other hand, fully recapitulate the *Pdgfra* (-/-) phenotype, indicating that a combination of these two ligands is required for proper activation of Pdgfra during development (67).

3.6 PDGFRA expression regulation: *cis*- and *trans*-acting factors

The 2.1 kb 5' flanking region of the human PDGFRA gene was first to characterized in our laboratory by Afink et al. (68). This region acts as a functional promoter since it can drive reporter gene expression in an orientation dependent manner in a variety of human and murine cell lines. Of the various deletion mutants tested, the -441/+118 promoter region displayed the highest activity, indicating the presence of both positive and negative regulatory elements in the -2100/+118 promoter region tested. Moreover, all deletion mutants, including the -52/+118 promoter fragment, appeared to be inducible by retinoic acid (RA) and cAMP in undifferentiated human embryonal carcinoma cells, a process accompanied by differential binding of nuclear proteins to this -52/+118 region *in vitro*. This region does not contain any putative RA or cAMP responsive element, however, which suggests that RA and cAMP induce *PDGFRA* expression indirectly via a currently unknown transcription factor (68).

A proximal 2.2 kb human *PDGFRA* promoter fragment appeared to be able to faithfully drive expression of a LacZ reporter gene in transgenic mice in a great number of tissues *in vivo*, indicating that it contains most regulatory elements necessary for proper PDGFRA transcription (69). However, the LacZ transgene was not expressed in OPCs, suggesting that the element driving PDGFRA expression in glia cells is located outside this 2.2 kb promoter region. Similar observations have been made for a murine 6.0 kb promoter fragment, suggesting that this putative glia element is not located in the direct upstream promoter region (70). In contrast, a yeast artificial chromosome (YAC) containing the entire human PDGFRA gene including 3.6 kb of the proximal promoter region was faithfully expressed in OPCs of *Ph*/*Ph* mice, indicating that this glia element is probably located in intronic or 3' sequences (58).

A number of nuclear proteins are known to influence *PDGFRA* expression, although for most of them a direct DNA target sequence has not been established. Joosten et al. (71) previously showed that the homeobox transcription factor PAX1 can influence *PDGFRA* promoter activity in human embryonal carcinoma and osteosarcoma cells. Moreover, the digenic mouse mutant that is homozygous for the *Pax1* mutation *undulated* and heterozygous for the *Patch* mutation (*un*/*un*, *Ph*/+), shows severe neural tube defects, whereas the two corresponding single gene mutants do not. This indicates that also *in vivo* Pax1 and Pdgfra act within the same or related pathways (72). Mice deficient in the gene for the related transcription factor Pax3 showed altered *Pdgfra* expression, indicating that this transcription factor may also act as an upstream regulator of *Pdgfra* expression (73, 74), although direct interaction sites for both Pax1 and Pax3 with the *Pdgfra* promoter have not been identified yet. The transcription factor GATA-4 can upregulate *Pdgfra* expression in F9 murine embryonic carcinoma cell, probably by binding to a sequence element located in the -799 to -672 murine promoter region (75), but so far this has only been shown in mouse cells. For three transcription factors direct binding site are known, i.e. C/EBPs (CCAAT/enhancer-binding proteins), Sp1 and ZNF148. C/EBPδ can upregulate *Pdgfra* expression in vascular smooth muscle cells (VSMCs) by binding to a C/EBP

binding site located in the -165 to -138 promoter of the rat Pdgfra gene (76, 77), while in humans a conserved C/EBP site at position -162 has been shown to be essential for high basal level *PDGFRA* promoter activity in osteogenic sarcoma MG-63 cells (78). The zinc finger transcription factor Sp1 can repress *Pdgfra* transcription in rat VSMCs when phosphorylated in response to FGF2, whereas dephosphorylated Sp1 activates *Pdgfra* transcription, both by binding to an atypical Sp1-binding site at position -61 to -52 in the rat promoter (79). Recently, is has been shown that the zinc finger transcription factor ZNF148 can bind the human *PDGFRA* promoter around position -1074 *in vitro*, but an influence on *PDGFRA* promoter activity has not been established yet (80).

3.7 PDGFRA and diseases: glial defect

The mammalian central nervous system is comprised of two cell types: neurons and glia cells. The main glia subtypes are oligodendrocytes, i.e. glia cells which produce myelin sheaths and support axons, and star-shaped astrocytes, which control the extracellular environment of neurons and provide them with support and nutrients. Neurons, oligodendrocytes and astrocytes all descend from a neuroepithelial stem cell, but there is still controversy regarding the lineage relationship and fate restriction of the intermediate precursor cells. There are indications for a common precursor cell of both oligodendrocytes and astrocytes (the so-called glial-restricted precursor: GRP), but also the existence of a common precursor for oligodendrocytes and motorneurons (the motorneuron-oligodendrocyte precursor: MNOP) has been suggested (81). Recently a model has been proposed in which motorneuron and oligodendrocyte precursors are sequentially generated in vivo from neuroepithelial stem cells, without a common lineage-restricted progenitor. In this model neuroepithelial stem cells that do not differentiate into motorneuron or oligodendrocyte precursors will eventually become astrocytes (82). However, it is as yet unclear which precursors actually exist *in vivo*, since most experiments investigating the potency of precursors are performed on explants, whereby the positional cues present *in vivo* are lost and artificial positional cues may be created *in vitro* (83).

In the mouse, Pdgfra is highly expressed by OPCs, which arise in the ventral region of the neural tube (46, 84, 85), but recently more dorsally derived OPCs have been identified as well (86, 87). Such Pdgfra expressing precursors are sometimes referred to as O2A cells, because they can give rise to both oligodendrocytes and type 2 astrocytes *in vitro* (88). *In vivo*, however, generation of type 2 astrocytes has been difficult to establish (89, 90). It has been shown that GRPs, which can generate oligodendrocytes as well as astrocytes, may also express Pdgfra, although not in an initial stage (91, 92). Mature astrocytes also express Pdgfra (93), which suggests that PDGFRA is involved in both oligodendrocyte and astrocyte development. Very recently, Pdgfra has been found to be expressed in the adult brain in a population of subventricular zone astrocyte-like cells that are the primary precursors of newly formed neurons. These so-called B cells have now been shown, like adult OPCs, also to be able to generate oligodendrocytes in response to demyelination in vivo (94)), while forming tumor-like growths in response to increased PDGF signaling (95).

PDGFs are known to act as potent mitogens and chemo-attractants for OPCs both *in vitro* and *in vivo* (62, 96-98). It is therefore not surprising that some *Pdgfra*-related mutants suffer from defective oligodendrocyte development. Reduced Pdgfra signaling can result in decreased OPC proliferation and premature differentiation, leading to decreased OPC as well as oligodendrocyte numbers and ultimately to

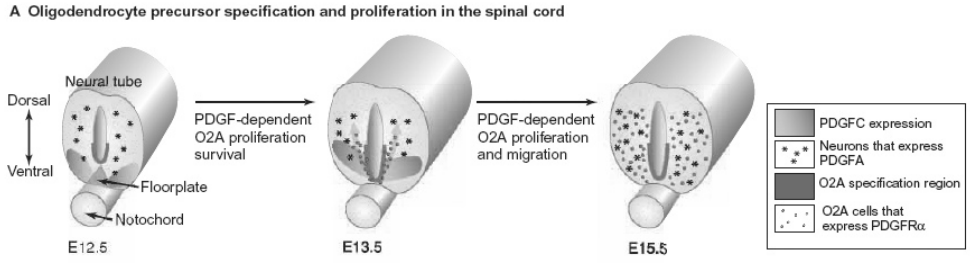

Figure 6: Pdgfra dependent oligodendrocyte development in the mouse neural tube (from (31)) Oligodendrocyte precursor cells (OPC/O2A) expressing Pdgfra arise in the ventral periventricular zone of the neural tube from where they proliferate and migrate throughout the neural tube in response to Pdgfa and Pdgfc.

severe hypomyelination. Since OPCs fail to reach the most dorsal parts of the neural tube, these regions most distant from the site of OPC origin show the highest degree of hypomyelination (60, 62)(figure 6).

Overexpression of Pdgfra signaling molecules has been shown to induce hyperproliferation of OPCs (46) and is associated with the formation of various gliomas, i.e. brain tumors of glial origin, in both mice and humans (99). Gliomas comprise more than 70% of all brain tumors and can be divided into tumors that are composed predominantly of astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas), mixtures of various glial cells (e.g. oligoastrocytomas) and ependymal cells (ependymomas). These tumors can be further classified according to the WHO grading system into four clinical grades according to their malignancy. Grade I gliomas are benign and can usually be surgically resected, whereas grade II to IV are malignant and diffusely infiltrate throughout the brain, making surgical resection virtually impossible. With 65 %, glioblastoma multiforme (GBM, also known as grade IV astrocytoma) is the most frequent and most malignant type of glioma. The median survival of patients diagnosed with GBM is less than a year, and less than 3% survive after five years (100, 101). Two types of GBM can be discriminated. Primary GBMs arise *de novo* and are generally associated with amplification and mutation of *ERBB1*, which encodes the receptor for EGF, and mutation or deletion of the tumor suppressor gene *PTEN*. Secondary GBMs have progressed from lower grade astrocytomas and are generally characterized by mutations in the tumor suppressor gene *TP53* as well as overexpression or amplification of *PDGFRA*. This progression process is often associated with additional genetic alteration such as loss of *RB* or amplification of *CDK4* (100).

Many gliomas and glioma cell lines of various tumor stages do not only overexpress PDGFRA but also express PDGFs, indicating that autocrine PDGFRA signaling may play an important role in tumorigenesis (102-104). Moreover *PDGFRA* amplification has been found in various glioblastomas and oligodendrogliomas (105-109). In low grade astrocytoma, the level of PDGFRA expression is correlated with poor patient prognosis (110). In glioblastomas, mutant forms of PDGFRA have been discovered, including one with an in-frame deletion in the extracellular domain, as a result of loss of exons 8 and 9 (PDGFR-$\alpha^{\Delta 8,9}$), resulting in an constitutively active, oncogenic isoform (111), and another one with a 2 bp deletion in exon 23 resulting in a mutant truncated

protein (112). Moreover, it has been shown in mice that Pdgf overexpression alone is sufficient to induce the formation of gliomas with, depending on the experimental model, properties similar to glioblastoma multiforme, primitive neuroectodermal tumor, oligodendroglioma or oligoastrocytoma (113-117). These tumor cells displayed OPC-like characteristics and constitutively activated Pdgfra, whereby the extent of Pdgfra signaling correlated with tumor malignancy. Moreover, PDGF receptor inhibiters as well as dominant negative mutations of PDGFs could antagonize tumor growth and reverse the malignant phenotype (102, 118, 119). Together, this suggests that also in humans PDGFRA expression levels, which determine the responsiveness to PDGF, may directly influence the risk of tumorigenesis. In conclusion, these data indicate that insufficient signaling via PDGFRA in the glial lineage may lead to hypomyelination and a reduction in the number of OPCs and mature oligodendrocytes, while excessive signaling may result in glioma formation.

3.8 PDGFRA and diseases: neural tube defects

In mice, defective Pdgfra signaling can lead to defects in vertebral arch fusion and neural tube closure (figure 5), commonly referred to as neural tube defects (NTDs) (40, 41, 51, 54-57, 60, 67). NTDs can be defined as congenital malformations which involve incomplete development of the brain, spinal cord, and/or their protective coverings. The most common forms of NTD are anencephaly, a cranial neural tube closure defect, encephalocele, a cranial bone defect, and spina bifida, a caudal closure defect. Anencephalic children generally die before or shortly after birth, but spina bifida is compatible with life. *Pdgfra* (-/-) and *Ph*/*Ph* mice, as well as several other mice with Pdgfra-signaling mutations, display spina bifida. Spina bifida can occur in two variants, called occulta ("hidden") and aperta ("open"). Spina bifida occulta is characterized by an incomplete fusion of one or more vertebral arches that can lead to a damaged spinal chord, but the defective site is overgrown by skin. It has been estimated that over 17% of the general population has unrecognized mild forms of spina bifida occulta (120, 121). Spina bifida aperta is a more severe form, in which either the meninges alone (meningocoel) but in most cases (90%) also the spinal cord (myelomeningocoel) can protrude from within the malformed vertebral column in a sack-like structure filled with cerebrospinal fluid. Children born with spina bifida aperta need to undergo surgery and mostly remain paralyzed from below the site of the defect (122).

The spina bifida seen in the mouse Pdgfra signaling mutants involves a skeletal defect, i.e. failure of neural arch fusion (41, 54, 60, 67), but development and closure of the neural tube are also affected (51, 55, 57). In wild-type mice, *Pdgfra* is expressed by the sclerotome of the somites, which will later develop into the various components of the axial skeleton. Lack of *Pdgfra* expression makes the sclerotome cells unable to respond to PDGF signals from the surrounding cells. Therefore, proliferation and migration of the sclerotomal precursor cells are impaired which results in improper positioning of a.o. the vertebral arches (41). The malformation of the neural tube itself may involve improper glial and neuronal development due to lack of PDGF responsiveness of the respective cell types, as well as reduced support of the vertebral arches. In conclusion, insufficient Pdgfra expression and signaling can result in spina bifida.

4. Aim of the thesis

The data presented indicate that in mice decreased Pdgfra signaling can lead to neural tube defects and myelination defects, while on the other hand increased Pdgfra signaling can be involved in the initiation as well as the progression of gliomas. It is therefore anticipated that in humans factors that influence PDGFRA expression may play a role in risk of developing PDGFRA-related diseases. The aim of the research presented in this thesis was to identify both *cis*- and *trans*-acting factors that influence *PDGFRA* transcription and subsequently to establish how these factors contribute to the susceptibility to and pathogenesis of PDGFRA-related diseases, in particular neural tube defects and gliomas. Understanding these mechanisms is very important for (prenatal) diagnostics, and ultimately for treatment and prevention of these types of diseases. We identified *cis*-acting factors by the use of two strategies, i.e. by investigating the effects of natural sequence variation (polymorphisms) in the *PDGFRA* promoter region on reporter gene activity and endogenous mRNA expression levels and by analyzing the inducible activity of various truncated promoter constructs in transient transfection assays, in various cell types. Subsequent identification of *trans*-acting factors was performed using electrophoretic mobility shift assays. The effects of natural sequence variation on disease susceptibility was studied by comparing the distribution of *PDGFRA* promoter haplotypes between groups of healthy control individuals and groups of patients suffering from spina bifida and glioblastoma respectively in case-control studies.

Chapter 2 identifies polymorphisms in the *PDGFRA* promoter that give rise to five different haplotypes and studies how these haplotypes influence reporter gene expression and predisposition to neural tube defects. **Chapter 3** describes an extended study in which also the influence of the interaction of *PDGFRA* promoter haplotypes with environmental factors on NTD risk is investigated. In **Chapter 4** we show haplotype specific binding of transcription factors to two polymorphic sites in the *PDGFRA* promoter that correspond with differences in promoter activity. **Chapter 6** describes how endogenous expression of one of the main *PDGFRA* haplotypes is specifically repressed by epigenetic mechanisms in glioblastoma cell lines. Furthermore, we show that this particular haplotype is associated with a decreased risk of glioblastomas. In **Chapter 5** we show that NTD-associated homeobox transcription factors can regulate *PDGFRA* promoter activity during differentiation of undifferentiated human embryonal carcinoma cells. Finally, **chapter 7** discusses the mechanisms whereby *PDGFRA* promoter haplotypes affect the pathogenesis of the disorders described in this thesis as well as other PDGFRA-related diseases.

References

1. Alberts, B, Bray, D, Lewis, J, Raff, M, Roberts, K, and Watson, JD. Molecular bioloy of the cell. 3rd edition, New York: Garland Publishing Inc.; 1994.
2. Lewis, BP, Burge, CB, and Bartel, DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15-20.
3. Bartel, DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116: 281-97.
4. Butler, JE and Kadonaga, JT. The RNA polymerase II core promoter: a key component in the regulation of gene expression. Genes Dev 2002;16:2583-92.
5. Martinez, E. Multi-protein complexes in eukaryotic gene transcription. Plant Mol Biol 2002;50:925-47.
6. Knight, JC. Functional implications of genetic variation in non-coding DNA for disease susceptibility and gene regulation. Clin Sci (Lond) 2003;104:493-501.
7. Malik, S and Roeder, RG. Dynamic regulation of pol II transcription by the mammalian Mediator complex. Trends Biochem Sci 2005;30:256-63.
8. Margueron, R, Trojer, P, and Reinberg, D. The key to development: interpreting the histone code? Curr Opin Genet Dev 2005;15:163-76.
9. Bannister, AJ and Kouzarides, T. Reversing histone methylation. Nature 2005;436:1103-6.
10. Wysocka, J, Allis, CD, and Coonrod, S. Histone arginine methylation and its dynamic regulation. Front Biosci 2006;11:344-55.
11. Peters, AH and Schubeler, D. Methylation of histones: playing memory with DNA. Curr Opin Cell Biol 2005;17:230-8.
12. Okano, M, Bell, DW, Haber, DA, and Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247-57.
13. Zhao, X, Ueba, T, Christie, BR, Barkho, B, McConnell, MJ, Nakashima, K, Lein, ES, Eadie, BD, Willhoite, AR, Muotri, AR, Summers, RG, Chun, J, Lee, KF, and Gage, FH. Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function. Proc Natl Acad Sci U S A 2003;100:6777-82.
14. Freitag, M and Selker, EU. Controlling DNA methylation: many roads to one modification. Curr Opin Genet Dev 2005;15:191-9.
15. Sarraf, SA and Stancheva, I. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. Mol Cell 2004;15:595-605.
16. Wassenegger, M. The role of the RNAi machinery in heterochromatin formation. Cell 2005;122:13-6.
17. Kapoor, A, Agius, F, and Zhu, JK. Preventing transcriptional gene silencing by active DNA demethylation. FEBS Lett 2005;579:5889-98.
18. Gehring, M, Huh, JH, Hsieh, TF, Penterman, J, Choi, Y, Harada, JJ, Goldberg, RB, and Fischer, RL. DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. Cell 2006;124:495-506.
19. Jost, JP, Oakeley, EJ, Zhu, B, Benjamin, D, Thiry, S, Siegmann, M, and Jost, YC. 5-Methylcytosine DNA glycosylase participates in the genome-wide loss of DNA methylation occurring during mouse myoblast differentiation. Nucleic Acids Res 2001;29:4452-61.
20. Zhu, B, Benjamin, D, Zheng, Y, Angliker, H, Thiry, S, Siegmann, M, and Jost, JP.

Overexpression of 5-methylcytosine DNA glycosylase in human embryonic kidney cells EcR293 demethylates the promoter of a hormone-regulated reporter gene. Proc Natl Acad Sci U S A 2001;98:5031-6.
21. Knight, JC. Regulatory polymorphisms underlying complex disease traits. J Mol Med 2005;83:97-109.
22. Hoogendoorn, B, Coleman, SL, Guy, CA, Smith, K, Bowen, T, Buckland, PR, and O'Donovan, MC. Functional analysis of human promoter polymorphisms. Hum Mol Genet 2003;12:2249-54.
23. Yan, H, Yuan, W, Velculescu, VE, Vogelstein, B, and Kinzler, KW. Allelic variation in human gene expression. Science 2002;297:1143.
24. Pastinen, T, Sladek, R, Gurd, S, Sammak, A, Ge, B, Lepage, P, Lavergne, K, Villeneuve, A, Gaudin, T, Brandstrom, H, Beck, A, Verner, A, Kingsley, J, Harmsen, E, Labuda, D, Morgan, K, Vohl, MC, Naumova, AK, Sinnett, D, and Hudson, TJ. A survey of genetic and epigenetic variation affecting human gene expression. Physiol Genomics 2004;16: 184-93.
25. Lo, HS, Wang, Z, Hu, Y, Yang, HH, Gere, S, Buetow, KH, and Lee, MP. Allelic variation in gene expression is common in the human genome. Genome Res 2003;13:1855-62.
26. Knight, JC, Keating, BJ, Rockett, KA, and Kwiatkowski, DP. In vivo characterization of regulatory polymorphisms by allele-specific quantification of RNA polymerase loading. Nat Genet 2003;33:469-75.
27. Knight, JC, Keating, BJ, and Kwiatkowski, DP. Allele-specific repression of lymphotoxin-alpha by activated B cell factor-1. Nat Genet 2004;36:394-9.
28. Donn, R, Alourfi, Z, Zeggini, E, Lamb, R, Jury, F, Lunt, M, Meazza, C, De Benedetti, F, Thomson, W, and Ray, D. A functional promoter haplotype of macrophage migration inhibitory factor is linked and associated with juvenile idiopathic arthritis. Arthritis Rheum 2004;50:1604-10.
29. Donn, RP, Plant, D, Jury, F, Richards, HL, Worthington, J, Ray, DW, and Griffiths, CE. Macrophage migration inhibitory factor gene polymorphism is associated with psoriasis. J Invest Dermatol 2004;123:484-7.
30. Knight, JC, Udalova, I, Hill, AV, Greenwood, BM, Peshu, N, Marsh, K, and Kwiatkowski, D. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat Genet 1999;22:145-50.
31. Hoch, RV and Soriano, P. Roles of PDGF in animal development. Development 2003;130:4769-84.
32. Kawagishi, J, Kumabe, T, Yoshimoto, T, and Yamamoto, T. Structure, organization, and transcription units of the human alpha-platelet-derived growth factor receptor gene, PDGFRA. Genomics 1995;30:224-32.
33. Claesson-Welsh, L. Platelet-derived growth factor receptor signals. J Biol Chem 1994;269:32023-6.
34. Mosselman, S, Claesson-Welsh, L, Kamphuis, JS, and van Zoelen, EJ. Developmentally regulated expression of two novel platelet-derived growth factor alpha-receptor transcripts in human teratocarcinoma cells. Cancer Res 1994;54:220-5.
35. Mosselman, S, Looijenga, LH, Gillis, AJ, van Rooijen, MA, Kraft, HJ, van Zoelen, EJ, and Oosterhuis, JW. Aberrant platelet-derived growth factor alpha-receptor transcript as a diagnostic marker for early human germ cell tumors of the adult testis. Proc Natl Acad Sci U S A 1996;93:2884-8.
36. McKinnon, RD, Waldron, S, and Kiel, ME. PDGF alpha-receptor signal strength controls an RTK rheostat that integrates phosphoinositol 3'-kinase and phospholipase Cgamma

pathways during oligodendrocyte maturation. J Neurosci 2005;25:3499-508.
37. Rosenkranz, S and Kazlauskas, A. Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. Growth Factors 1999;16:201-16.
38. Heldin, CH, Ostman, A, and Ronnstrand, L. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta 1998;1378:F79-113.
39. Heldin, CH and Westermark, B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 1999;79:1283-316.
40. Orr-Urtreger, A, Bedford, MT, Do, MS, Eisenbach, L, and Lonai, P. Developmental expression of the alpha receptor for platelet-derived growth factor, which is deleted in the embryonic lethal Patch mutation. Development 1992;115:289-303.
41. Schatteman, GC, Morrison-Graham, K, van Koppen, A, Weston, JA, and Bowen-Pope, DF. Regulation and role of PDGF receptor alpha-subunit expression during embryogenesis. Development 1992;115:123-31.
42. Palmieri, SL, Payne, J, Stiles, CD, Biggers, JD, and Mercola, M. Expression of mouse PDGF-A and PDGF alpha-receptor genes during pre- and post-implantation development: evidence for a developmental shift from an autocrine to a paracrine mode of action. Mech Dev 1992;39:181-91.
43. Andrae, J, Molander, C, Smits, A, Funa, K, and Nister, M. Platelet-derived growth factor-B and -C and active alpha-receptors in medulloblastoma cells. Biochem Biophys Res Commun 2002;296:604-11.
44. Forsberg-Nilsson, K, Behar, TN, Afrakhte, M, Barker, JL, and McKay, RD. Platelet-derived growth factor induces chemotaxis of neuroepithelial stem cells. J Neurosci Res 1998;53: 521-30.
45. Erlandsson, A, Enarsson, M, and Forsberg-Nilsson, K. Immature neurons from CNS stem cells proliferate in response to platelet-derived growth factor. J Neurosci 2001;21:3483-91.
46. Calver, AR, Hall, AC, Yu, WP, Walsh, FS, Heath, JK, Betsholtz, C, and Richardson, WD. Oligodendrocyte population dynamics and the role of PDGF in vivo. Neuron 1998;20: 869-82.
47. Orr-Urtreger, A and Lonai, P. Platelet-derived growth factor-A and its receptor are expressed in separate, but adjacent cell layers of the mouse embryo. Development 1992;115:1045-58.
48. Lindahl, P, Johansson, BR, Leveen, P, and Betsholtz, C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 1997;277:242-5.
49. Sasahara, A, Kott, JN, Sasahara, M, Raines, EW, Ross, R, and Westrum, LE. Platelet-derived growth factor B-chain-like immunoreactivity in the developing and adult rat brain. Brain Res Dev Brain Res 1992;68:41-53.
50. Aase, K, Abramsson, A, Karlsson, L, Betsholtz, C, and Eriksson, U. Expression analysis of PDGF-C in adult and developing mouse tissues. Mech Dev 2002;110:187-91.
51. Grüneberg, H and Truslove, GM. Two closely linked genes in the mouse. Genet Res 1960;1:69-90.
52. Stephenson, DA, Mercola, M, Anderson, E, Wang, CY, Stiles, CD, Bowen-Pope, DF, and Chapman, VM. Platelet-derived growth factor receptor alpha-subunit gene (Pdgfra) is deleted in the mouse patch (Ph) mutation. Proc Natl Acad Sci U S A 1991;88:6-10.
53. Smith, EA, Seldin, MF, Martinez, L, Watson, ML, Choudhury, GG, Lalley, PA, Pierce, J, Aaronson, S, Barker, J, Naylor, SL, and et al. Mouse platelet-derived growth factor receptor alpha gene is deleted in W19H and patch mutations on chromosome 5. Proc

Natl Acad Sci U S A 1991;88:4811-5.
54. Payne, J, Shibasaki, F, and Mercola, M. Spina bifida occulta in homozygous Patch mouse embryos. Dev Dyn 1997;209:105-16.
55. Li, L, Schatteman, GC, Oppenheim, RW, Lei, M, Bowen-Pope, DF, and Houenou, LJ. Altered development of spinal cord in the mouse mutant (Patch) lacking the PDGF receptor alpha-subunit gene. Brain Res Dev Brain Res 1996;96:204-9.
56. Morrison-Graham, K, Schatteman, GC, Bork, T, Bowen-Pope, DF, and Weston, JA. A PDGF receptor mutation in the mouse (Patch) perturbs the development of a non-neuronal subset of neural crest-derived cells. Development 1992;115:133-42.
57. Soriano, P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997;124:2691-700.
58. Sun, T, Jayatilake, D, Afink, GB, Ataliotis, P, Nister, M, Richardson, WD, and Smith, HK. A human YAC transgene rescues craniofacial and neural tube development in PDGFRalpha knockout mice and uncovers a role for PDGFRalpha in prenatal lung growth. Development 2000;127:4519-29.
59. Ataliotis, P, Symes, K, Chou, MM, Ho, L, and Mercola, M. PDGF signalling is required for gastrulation of Xenopus laevis. Development 1995;121:3099-110.
60. Klinghoffer, RA, Hamilton, TG, Hoch, R, and Soriano, P. An allelic series at the PDGFalphaR locus indicates unequal contributions of distinct signaling pathways during development. Dev Cell 2002;2:103-13.
61. Rosenkranz, S, DeMali, KA, Gelderloos, JA, Bazenet, C, and Kazlauskas, A. Identification of the receptor-associated signaling enzymes that are required for platelet-derived growth factor-AA-dependent chemotaxis and DNA synthesis. J Biol Chem 1999;274: 28335-43.
62. Fruttiger, M, Karlsson, L, Hall, AC, Abramsson, A, Calver, AR, Bostrom, H, Willetts, K, Bertold, CH, Heath, JK, Betsholtz, C, and Richardson, WD. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development 1999;126:457-67.
63. Leveen, P, Pekny, M, Gebre-Medhin, S, Swolin, B, Larsson, E, and Betsholtz, C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 1994;8:1875-87.
64. Kaminski, WE, Lindahl, P, Lin, NL, Broudy, VC, Crosby, JR, Hellstrom, M, Swolin, B, Bowen-Pope, DF, Martin, PJ, Ross, R, Betsholtz, C, and Raines, EW. Basis of hematopoietic defects in platelet-derived growth factor (PDGF)-B and PDGF beta-receptor null mice. Blood 2001;97:1990-8.
65. Hellstrom, M, Kalen, M, Lindahl, P, Abramsson, A, and Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 1999;126:3047-55.
66. Soriano, P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev 1994;8:1888-96.
67. Ding, H, Wu, X, Bostrom, H, Kim, I, Wong, N, Tsoi, B, O'Rourke, M, Koh, GY, Soriano, P, Betsholtz, C, Hart, TC, Marazita, ML, Field, LL, Tam, PP, and Nagy, A. A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. Nat Genet 2004;36:1111-6.
68. Afink, GB, Nister, M, Stassen, BH, Joosten, PH, Rademakers, PJ, Bongcam-Rudloff, E, Van Zoelen, EJ, and Mosselman, S. Molecular cloning and functional characterization of the human platelet-derived growth factor alpha receptor gene promoter. Oncogene 1995;10:1667-72.

69. Zhang, XQ, Afink, GB, Svensson, K, Jacobs, JJ, Gunther, T, Forsberg-Nilsson, K, van Zoelen, EJ, Westermark, B, and Nister, M. Specific expression in mouse mesoderm- and neural crest-derived tissues of a human PDGFRA promoter/lacZ transgene. Mech Dev 1998;70:167-80.
70. Reinertsen, KK, Bronson, RT, Stiles, CD, and Wang, C. Temporal and spatial specificity of PDGF alpha receptor promoter in transgenic mice. Gene Expr 1997;6:301-14.
71. Joosten, PH, Hol, FA, van Beersum, SE, Peters, H, Hamel, BC, Afink, GB, van Zoelen, EJ, and Mariman, EC. Altered regulation of platelet-derived growth factor receptor-alpha gene-transcription in vitro by spina bifida-associated mutant Pax1 proteins. Proc Natl Acad Sci U S A 1998;95:14459-63.
72. Helwig, U, Imai, K, Schmahl, W, Thomas, BE, Varnum, DS, Nadeau, JH, and Balling, R. Interaction between undulated and Patch leads to an extreme form of spina bifida in double-mutant mice. Nat Genet 1995;11:60-3.
73. Dickman, ED, Rogers, R, and Conway, SJ. Abnormal skeletogenesis occurs coincident with increased apoptosis in the Splotch (Sp2H) mutant: putative roles for Pax3 and PDGFRalpha in rib patterning. Anat Rec 1999;255:353-61.
74. Henderson, DJ, Conway, SJ, and Copp, AJ. Rib truncations and fusions in the Sp2H mouse reveal a role for Pax3 in specification of the ventro-lateral and posterior parts of the somite. Dev Biol 1999;209:143-58.
75. Wang, C and Song, B. Cell-type-specific expression of the platelet-derived growth factor alpha receptor: a role for GATA-binding protein. Mol Cell Biol 1996;16:712-23.
76. Fukuoka, T, Kitami, Y, Okura, T, and Hiwada, K. Transcriptional regulation of the platelet-derived growth factor alpha receptor gene via CCAAT/enhancer-binding protein-delta in vascular smooth muscle cells. J Biol Chem 1999;274:25576-82.
77. Yang, ZH, Kitami, Y, Takata, Y, Okura, T, and Hiwada, K. Targeted overexpression of CCAAT/enhancer-binding protein-delta evokes enhanced gene transcription of platelet-derived growth factor alpha-receptor in vascular smooth muscle cells. Circ Res 2001;89:503-8.
78. Afink, G, Westermark, UK, Lammerts, E, and Nister, M. C/EBP is an essential component of PDGFRA transcription in MG-63 cells. Biochem Biophys Res Commun 2004;315:313-8.
79. Bonello, MR and Khachigian, LM. Fibroblast growth factor-2 represses platelet-derived growth factor receptor-alpha (PDGFR-alpha) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR-alpha promoter. J Biol Chem 2004;279:2377-82.
80. De Bustos, C, Smits, A, Stromberg, B, Collins, VP, Nister, M, and Afink, G. A PDGFRA promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumours and ependymomas. J Med Genet 2005;42:31-7.
81. Noble, M, Proschel, C, and Mayer-Proschel, M. Getting a GR(i)P on oligodendrocyte development. Dev Biol 2004;265:33-52.
82. Wu, S, Wu, Y, and Capecchi, MR. Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. Development 2006;133:581-90.
83. Gabay, L, Lowell, S, Rubin, LL, and Anderson, DJ. Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. Neuron 2003;40:485-99.
84. Pringle, NP, Mudhar, HS, Collarini, EJ, and Richardson, WD. PDGF receptors in the rat CNS: during late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. Development 1992;115:535-51.

85. Hall, A, Giese, NA, and Richardson, WD. Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors. Development 1996;122:4085-94.
86. Cai, J, Qi, Y, Hu, X, Tan, M, Liu, Z, Zhang, J, Li, Q, Sander, M, and Qiu, M. Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. Neuron 2005;45:41-53.
87. Vallstedt, A, Klos, JM, and Ericson, J. Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. Neuron 2005;45:55-67.
88. Raff, MC, Miller, RH, and Noble, M. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. Nature 1983;303:390-6.
89. Espinosa de los Monteros, A, Zhang, M, and De Vellis, J. O2A progenitor cells transplanted into the neonatal rat brain develop into oligodendrocytes but not astrocytes. Proc Natl Acad Sci U S A 1993;90:50-4.
90. Scolding, NJ, Rayner, PJ, and Compston, DA. Identification of A2B5-positive putative oligodendrocyte progenitor cells and A2B5-positive astrocytes in adult human white matter. Neuroscience 1999;89:1-4.
91. Rao, MS, Noble, M, and Mayer-Proschel, M. A tripotential glial precursor cell is present in the developing spinal cord. Proc Natl Acad Sci U S A 1998;95:3996-4001.
92. Gregori, N, Proschel, C, Noble, M, and Mayer-Proschel, M. The tripotential glial-restricted precursor (GRP) cell and glial development in the spinal cord: generation of bipotential oligodendrocyte-type-2 astrocyte progenitor cells and dorsal-ventral differences in GRP cell function. J Neurosci 2002;22:248-56.
93. Mudhar, HS, Pollock, RA, Wang, C, Stiles, CD, and Richardson, WD. PDGF and its receptors in the developing rodent retina and optic nerve. Development 1993;118:539-52.
94. Menn, B, Garcia-Verdugo, JM, Yaschine, C, Gonzalez-Perez, O, Rowitch, D, and Alvarez-Buylla, A. Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 2006;26:7907-18.
95. Jackson, EL, Garcia-Verdugo, JM, Gil-Perotin, S, Roy, M, Quinones-Hinojosa, A, VandenBerg, S, and Alvarez-Buylla, A. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. Neuron 2006;51:187-99.
96. Noble, M, Murray, K, Stroobant, P, Waterfield, MD, and Riddle, P. Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature 1988;333:560-2.
97. Armstrong, RC, Harvath, L, and Dubois-Dalcq, ME. Type 1 astrocytes and oligodendrocyte-type 2 astrocyte glial progenitors migrate toward distinct molecules. J Neurosci Res 1990;27:400-7.
98. Woodruff, RH, Fruttiger, M, Richardson, WD, and Franklin, RJ. Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. Mol Cell Neurosci 2004;25:252-62.
99. Shih, AH and Holland, EC. Platelet-derived growth factor (PDGF) and glial tumorigenesis. Cancer Lett 2006;232:139-47.
100. Zhu, Y and Parada, LF. The molecular and genetic basis of neurological tumours. Nat Rev Cancer 2002;2:616-26.
101. Ohgaki, H and Kleihues, P. Epidemiology and etiology of gliomas. Acta Neuropathol (Berl) 2005;109:93-108.

102. Lokker, NA, Sullivan, CM, Hollenbach, SJ, Israel, MA, and Giese, NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res 2002;62:3729-35.
103. Di Rocco, F, Carroll, RS, Zhang, J, and Black, PM. Platelet-derived growth factor and its receptor expression in human oligodendrogliomas. Neurosurgery 1998;42:341-6.
104. Hermanson, M, Funa, K, Hartman, M, Claesson-Welsh, L, Heldin, CH, Westermark, B, and Nister, M. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res 1992;52:3213-9.
105. Fleming, TP, Saxena, A, Clark, WC, Robertson, JT, Oldfield, EH, Aaronson, SA, and Ali, IU. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. Cancer Res 1992;52:4550-3.
106. Smith, JS, Wang, XY, Qian, J, Hosek, SM, Scheithauer, BW, Jenkins, RB, and James, CD. Amplification of the platelet-derived growth factor receptor-A (PDGFRA) gene occurs in oligodendrogliomas with grade IV anaplastic features. J Neuropathol Exp Neurol 2000;59:495-503.
107. Knobbe, CB and Reifenberger, G. Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol 2003;13:507-18.
108. Suzuki, T, Maruno, M, Wada, K, Kagawa, N, Fujimoto, Y, Hashimoto, N, Izumoto, S, and Yoshimine, T. Genetic analysis of human glioblastomas using a genomic microarray system. Brain Tumor Pathol 2004;21:27-34.
109. Joensuu, H, Puputti, M, Sihto, H, Tynninen, O, and Nupponen, NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol 2005;207:224-31.
110. Varela, M, Ranuncolo, SM, Morand, A, Lastiri, J, De Kier Joffe, EB, Puricelli, LI, and Pallotta, MG. EGF-R and PDGF-R, but not bcl-2, overexpression predict overall survival in patients with low-grade astrocytomas. J Surg Oncol 2004;86:34-40.
111. Clarke, ID and Dirks, PB. A human brain tumor-derived PDGFR-alpha deletion mutant is transforming. Oncogene 2003;22:722-33.
112. Rand, V, Huang, J, Stockwell, T, Ferriera, S, Buzko, O, Levy, S, Busam, D, Li, K, Edwards, JB, Eberhart, C, Murphy, KM, Tsiamouri, A, Beeson, K, Simpson, AJ, Venter, JC, Riggins, GJ, and Strausberg, RL. Sequence survey of receptor tyrosine kinases reveals mutations in glioblastomas. Proc Natl Acad Sci U S A 2005;102:14344-9.
113. Assanah, M, Lochhead, R, Ogden, A, Bruce, J, Goldman, J, and Canoll, P. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor-expressing retroviruses. J Neurosci 2006;26:6781-90.
114. Uhrbom, L, Hesselager, G, Nister, M, and Westermark, B. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. Cancer Res 1998;58:5275-9.
115. Uhrbom, L, Hesselager, G, Ostman, A, Nister, M, and Westermark, B. Dependence of autocrine growth factor stimulation in platelet-derived growth factor-B-induced mouse brain tumor cells. Int J Cancer 2000;85:398-406.
116. Shih, AH, Dai, C, Hu, X, Rosenblum, MK, Koutcher, JA, and Holland, EC. Dose-dependent effects of platelet-derived growth factor-B on glial tumorigenesis. Cancer Res 2004;64:4783-9.
117. Dai, C, Celestino, JC, Okada, Y, Louis, DN, Fuller, GN, and Holland, EC. PDGF autocrine

stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. Genes Dev 2001;15: 1913-25.

118. Kaetzel, DM, Reid, JDt, Pedigo, N, Zimmer, SG, and Boghaert, ER. A dominant-negative mutant of the platelet-derived growth factor A-chain increases survival of hamsters implanted intracerebrally with the highly invasive CxT24-neo3 glioblastoma cell. J Neurooncol 1998;39:33-46.
119. Shamah, SM, Stiles, CD, and Guha, A. Dominant-negative mutants of platelet-derived growth factor revert the transformed phenotype of human astrocytoma cells. Mol Cell Biol 1993;13:7203-12.
120. Boone, D, Parsons, D, Lachmann, SM, and Sherwood, T. Spina bifida occulta: lesion or anomaly? Clin Radiol 1985;36:159-61.
121. Fidas, A, MacDonald, HL, Elton, RA, Wild, SR, Chisholm, GD, and Scott, R. Prevalence and patterns of spina bifida occulta in 2707 normal adults. Clin Radiol 1987;38:537-42.
122. Groenen, PM.Nutritional and environmental factors in human spina bifida. Nijmegen: University of Nijmegen; 2004.
123. Fournier, C, Goto, Y, Ballestar, E, Delaval, K, Hever, AM, Esteller, M, and Feil, R. Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. Embo J 2002;21:6560-70.

Chapter 2

Promoter haplotype combinations of the platelet-derived growth factor α-receptor gene predispose to human neural tube defects

Published in: Nature Genetics 27 (2001) 215-218
By: Paul H.L.J. Joosten, Mascha Toepoel, Edwin C.M. Mariman and Everardus J.J. Van Zoelen.

Neural tube defects (NTDs), including anencephaly and spina bifida, are multifactorial diseases that occur with an incidence of 1 in 300 births in the United Kingdom (1). Mouse models have indicated that deregulated expression of the gene encoding the platelet-derived growth factor α-receptor (*Pdgfra*) causes congenital NTDs (2-4), whereas mutant forms of Pax-1 that have been associated with NTDs cause deregulated activation of the human *PDGFRA* promoter (2, 5). There is an increasing awareness that genetic polymorphisms may have an important role in the susceptibility for NTDs (6). Here we identify five different haplotypes in the human *PDGFRA* promoter, of which the two most abundant ones, designated H1 and H2α, differ in at least six polymorphic sites. In a transient transfection assay in human bone cells, the five haplotypes differ strongly in their ability to enhance reporter gene activity. In a group of patients with sporadic spina bifida, haplotypes with low transcriptional activity, including H1, were under-represented, whereas those with high transcriptional activity, including H2α, were over-represented. When testing for haplotype combinations, H1 homozygotes were fully absent from the group of sporadic patients, whereas H1/H2α heterozygotes were over-represented in the groups of both sporadic and familial spina bifida patients, but strongly underrepresented in unrelated controls. Our data indicate that specific combinations of naturally occurring *PDGFRA* promoter haplotypes strongly affect NTD genesis.

We carried out single-stranded conformation (SSC) analysis on the 2-kb region upstream of the transcription initiation site of human *PDGFRA* (7). In transgenic mice, this region is known to be responsible for the spatio-temporal expression pattern of this gene during development (8). We performed analysis on 76 patients with sporadic spina bifida, 49 patients with familial spina bifida and 77 unrelated controls. SSC analysis revealed the presence of at least eight polymorphic sites in this promoter region, whereas subsequent sequencing of PCR products and cloning of genomic promoter sequences revealed the existence of five different haplotypes of the *PDGFRA* promoter. These haplotypes can be divided into two subfamilies, designated H1 and H2, that differ in at least five positions, including a two nucleotide deletion/insertion in the noncoding region of exon 1 (Fig. 1). Additional polymorphisms may exist further upstream (9). We found four H2 variants, designated H2α–δ, of which H2β–δ differ by only a single nucleotide from

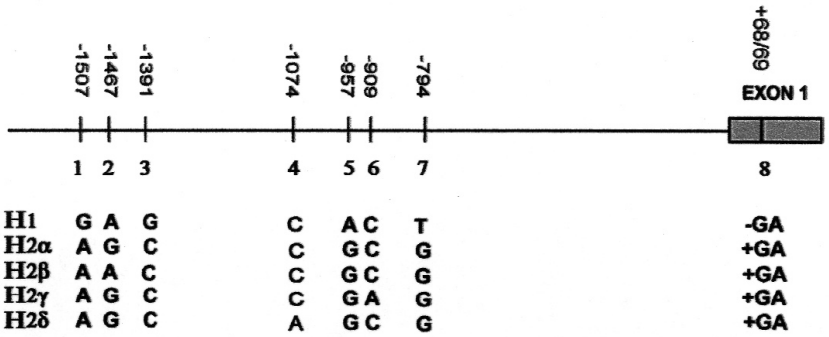

Fig. 1 Promoter haplotypes resulting from polymorphisms in the –1,589/+118 nucleotide sequence of *PDGFRA* (7). Top, the identified eight polymorphic sites are indicated together with their corresponding nucleotide position. Bottom, the five identified haplotypes (H1, H2α, H2β, H2γ and H2δ) are indicated together with their nucleotide sequence at the polymorphic sites.

H2α. Analysis of the control individuals revealed that 30.5% of the haplotypes were of type H1 and 57.1% of type H2α, with minor contributions of H2β–δ (Fig. 2*a*). The overall haplotype distribution in the group of familial spina bifida patients was not different from that of the control group, but the group of sporadic patients showed a 40% reduction in H1 haplotypes, combined with an over-representation of H2α and H2β haplotypes. We tested these groups for the presence of specific *PDGFRA* promoter haplotype combinations, and found that H1 homozygotes were fully lacking in the group of sporadic patients, whereas H2α/H2β heterozygotes were over-represented in this group (Fig. 2*b*). Heterozygotes of the two most abundant haplotypes, H1 and H2α, were significantly under-represented in the control group compared with Hardy–Weinberg equilibrium, whereas such heterozygotes were over-represented in the groups of both sporadic and familial patients (Fig. 2).

	FAMILIAL		SPORADIC		CONTROL	
H1	31	31.6%	28	18.4%	47	30.5%
H2α	54	55.1%	104	68.4%	88	57.1%
H2β	6	6.1%	13	8.6%	7	4.5%
H2γ	3	3.1%	4	2.6%	6	3.9%
H2δ	4	4.1%	3	2.0%	6	3.9%

	FAMILIAL		SPORADIC		CONTROL	
H1\H1	8.2%	10.0%	0.0%	3.4%	14.3%	9.3%
H1\H2α	40.8%	34.8%	34.2%	25.2%	23.4%	34.8%
H2α\H2α	30.6%	30.4%	43.4%	46.8%	40.3%	32.6%
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.

	H1	H2α	H2β	H2γ	H2δ
H1	T=15(7.4%) C=11(14%) S=0(0%) F=4(8%)				
H2α	T=64(31.6%) C=18(23%) S=26(34%) F=20(40%)	T=79(39.1%) C=31(40%) S=33(43%) F=15(30%)			
H2β	T=7(3.4%) C=4(5%) S=1(1.3%) F=2(4%)	T=15(7.4%) C=3(4%) S=9(12%) F=3(6%)	T=1(0.5%) C=0(0%) S=1(1.3%) F=0(0%)		
H2γ	T=2(1%) C=0(0%) S=1(1.3%) F=1(2%)	T=7(3.4%) C=4(5%) S=2(2.6%) F=1(2%)	T=2(1%) C=0(0%) S=1(1.3%) F=1(2%)	T=1(0.5%) C=1(1.3%) S=0(0%) F=0(0%)	
H2δ	T=3(1.5%) C=3(4%) S=0(0%) F=0(0%)	T=2(1%) C=1(1.3%) S=1(1.3%) F=0(0%)	T=0(0%) C=0(0%) S=0(0%) F=0(0%)	T=0(0%) C=0(0%) S=0(0%) F=0(0%)	T=4(2%) C=1(1.3%) S=1(1.3%) F=2(4%)

Fig. 2 Distribution of the different PDGFRA promoter haplotypes in a group of 49 familial spina bifida patients, 76 sporadic spina bifida patients and 77 control individuals. Sporadic patients had no first-, second- or third-degree relative with neural tube defects, whereas familial patients had at least one known affected relative within the third degree. Controls were unrelated to any of these patients. **a**, The distribution of the various haplotypes over the three groups is indicated in both absolute numbers and percentages, assuming diploid chromosome numbers. Based on χ^2 analysis, H1 haplotypes are significantly under-represented in the sporadic patients compared with controls ($P<0.01$; $\chi^2=6.05$). **b**, The distribution of haplotype combinations in the three groups is indicated. The total (T) number of individuals identified with a specific haplotype combination is indicated in both absolute numbers and percentages, as well as for the group of controls (C), sporadic patients (S) and familial patients (F). Note that H1 homozygotes are absent from the group of sporadic patients ($P<0.01$ compared with controls; $\chi^2=11.7$), whereas in this group H2α/H2β heterozygotes have a tendency towards over-representation ($P<0.1$ compared with controls; $\chi^2=3.34$). **c**, The observed distribution of combinations of the two major haplotypes (H1 and H2α) in the investigated groups (Obs.) is compared with the expected Hardy-Weinberg equilibrium (Exp.). The observed distribution of H1 homozygotes, H1/H2α heterozygotes and H2a homozygotes corresponds in all three groups to 77–78% of all haplotype combinations present. When considering specific haplotype combinations, H1 homozygotes were significantly higher in both controls ($P<0.01$; $\chi^2=11.7$) and familial patients ($P<0.02$; $\chi^2=6.4$) compared with sporadic patients. Moreover, H1/H2α heterozygotes were significantly more abundant in familial patients compared with controls ($P<0.05$; $\chi^2=4.3$). When testing the various groups for significant deviation of genotype frequencies from Hardy–Weinberg equilibrium according to a three-allele χ^2 analysis (with alleles H1, H2a, and the pooled group H2β+H2γ+H2δ), we observed a significant deviation in controls ($P<0.05$; $\chi^2=7.91$ with three degrees of freedom), with a significant deficit of heterozygotes ($P<0.01$; FIS=+0.248) according to the U-test (28). The group of sporadic patients showed significant deviation from Hardy–Weinberg equilibrium ($P<0.05$; $\chi^2=8.34$) with a non-significant excess of heterozygotes (FIS=–0.089), whereas we observed no significant deviation from equilibrium in the group of familial patients.

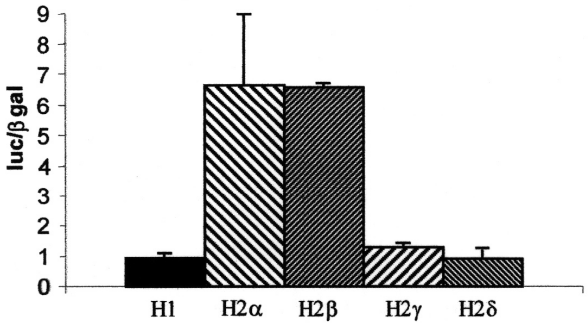

Fig. 3 Distribution of promoter activity of transfected 5′ PDGFRA haplotypes in U2-OS cells. Activity is presented as the ratio of luciferase and β-galactosidase activities relative to H1 (set to 1), including standard deviation. Repeated transfections were carried out in duplicate with different batches of DNA. A similar activity pattern of haplotypes was observed in two other human osteosarcoma cell-lines: SAOS and HOS (not shown).

Improper proliferation and differentiation of the mesodermal cartilage precursor cells that form the neural arches are major events in the genesis of spinal NTDs (1-4, 8, 10, 11). We therefore tested the activity of the various promoter haplotypes by transient transfection of promoter haplotype–luciferase constructs into human U2-OS osteosarcoma cells, which are an *in vitro* model system for bone formation during development. We observed strong differences in transcriptional activity between the various haplotypes, with an up to six-fold higher activity of haplotypes H2α and H2β compared with that of H1, H2γ and H2δ (Fig. 3). The two haplotypes with high activity are those that were found to be overrepresented in sporadic spina bifida patients, whereas those with low activity were found to be under-represented in these patients (Fig. 2*a*). The frequency of the high-activity haplotypes H2α and H2β, compared with that of the low-activity haplotypes H1, H2γ and H2δ, showed a strongly significant difference between the sporadic spina bifida patients and the control group ($P<0.01$). These data provide a direct correlation between the distribution of haplotypes in a patient population and a functional biochemical parameter of these haplotypes *in vitro*.

NTDs are multifactorial diseases that occur as a complex interaction between environmental and a variety of predisposing genetic factors (12-14). The heritability of human NTDs, which can be defined as the relative contribution of genetic factors, has been estimated to be approximately 60% (15). In the case of spina bifida, a severe form with open lesions exists (spina bifida aperta), as well as a less severe form in which the lesion is covered with skin (spina bifida occulta). This latter form is not always recognized and it has been suggested that latent lesions exist in at least 15% of the total human population (16, 17). Moreover, severe forms of NTDs will lead to embryonic lethality without contributing to the patient population. Various mouse models have indicated that aberrant expression of *Pdgfra* may result in spina bifida. Mice with a targeted null mutation in *Pdgfra* and patch mutant mice, with a natural deletion of the chromosomal region that includes *Pdgfra*, both show severe spina bifida combined with embryonic lethality in their homozygous form (3, 4). We have previously shown that gain-of-function mutations in the gene encoding Pax-1, an upstream transcriptional regulator of *Pdgfra*, are also associated with spina bifida (5, 18). It has been

proposed that reduced PDGF signaling during development may lead to premature differentiation of the neural glia and cartilage precursor cells, whereas upregulated PDGF signaling may result in a prolonged, undifferentiated, proliferative status of these cells (19, 20), in both cases resulting in improper closing of the neural tube. Our current results show that combinations of naturally occurring *PDGFRA* promoter haplotypes contribute to the adverse factors that eventually determine the manifestation of spina bifida in humans.

The observation that the H2 haplotype shows multiple variants, in contrast to H1, may indicate that H1 has only recently been introduced in the population investigated. The observation that H1/H2α heterozygotes are under-represented in the control population could indicate that H1 and H2 haplotypes are not randomly mixed in the population, as has been shown for promoter haplotypes of the β2-adrenergic receptor (21). This possibility seems unlikely, however, in the light of the over-representation of such heterozygotes in the patient population. Our results therefore indicate that, most likely in combination with other adverse factors, H1/H2α heterozygotes have an increased risk for malformations resulting in either embryonic lethality or NTDs. The fact that no H1 homozygotes were found in the group of sporadic patients indicates that this haplotype combination selects against the formation of spina bifida. No such protective effect was observed in the group of familial patients, indicating that in these patients additional adverse factors are involved for which H1 homozygosity cannot compensate.

Studies on the human, mouse and rat PDGF a-receptor promoter have provided evidence for the involvement of GATA-4 (22), PAX3-FKHR (23), Pax-1 (5) and C/EBPs (24) in transcriptional activation of this gene. Further investigations are needed to indicate whether these and other transcription factors are expressed in the relevant cell types and are able to activate the human *PDGFRA* promoter in a haplotype-specific manner (9). The observed correlation between haplotype variant activity in U2-OS cells and their over- or under-representation in spina bifida patients is indicative for the relevance of these functional data, but does not explain why H1/H2α heterozygosity predisposes to spina bifida. H2α homozygosity seems to be a neutral factor in the genesis of NTDs. It is known from embryonic developmental processes in lower organisms that promoters on different chromosomes may physically interact and can have a combined activity that is different from the average activity of the two promoters (25). Little is known about similar processes in mammals (26), particularly because *in vitro* detection assays for such so-called 'transvection' phenomena are still lacking. It will be worth investigating if such processes may underlie the observed improper matching of H1 and H2α haplotypes of the *PDGFRA* promoter, resulting in enhanced embryonic lethality and NTDs.

Methods

Automated sequencing

We analyzed the DNA sequences of PCR products and DNA constructs using the automated ABI310 automated sequencer according to the manufacturer's protocols.

Cell culture
U2-OS was seeded at a density of 5.0×104 cells/cm^2 and grown in Dulbecco's MEM/ nutrient mix F12 (1:1) supplemented with 10% (v/v) fetal calf serum (Gibco) and $NaHCO_3$ (44 mM) in a 7.5% CO2 atmosphere at 37 °C.

Haplotype cloning
PCR products of the 5´ flanking sequences of *PDGFRA* were obtained using *Pfu* polymerase with proofreading (New England Biolabs), and were ligated and cloned using a PCR cloning vector (Invitrogen). Clones were analysed and *Pst*1/*Nco*1 (Fermentas) restriction fragments were subsequently ligated into the pSLA4 luciferase reporter vector (Promega).

SSC analysis
DNA samples of 76 sporadic patients, 49 familial NTD patients and 77 control people were analysed by PCR (Fermentas) and subsequent SSC analysis with the Phast System (Promega) according to the manufacturer's protocols. We carried out SSC at 4 °C on native gels with native buffer strips using 0.3 µl PCR reaction mix containing the amplified fragment for each sample.

Transfection, luciferase and β-galactosidase assays
The –1589/+118 *PDGFRA* promoter haplotype constructs were each transiently transfected into U2-OS cells using the calcium phosphate coprecipitation method(27), in 10 cm2 wells seeded with 3.5×105 U2-OS cells one day before transfection. Luciferase activity was detected 48 h post-transfection (Luciferase assay kit, Promega). The activity of 1 µg transfected luciferase construct was corrected for transfection efficiency by measuring the β-galactosidase activity obtained from co-transfected SV40-driven *lacZ* gene construct PCH110 (50 ng; Promega).

Acknowledgments

We thank F. Hol, B. Franke, D. van Oosterhout and B. Hamel for contributions; and J. Ouborg for statistical advice. This study was supported by the Dutch Prinses Beatrix fonds.

Note

During following studies, including a case-control study on the interaction of *PDGFRA* promoter haplotypes with environmental risk factors for spina bifida described in chapter 3 and a case-control study on the role of *PDGFRA* promoter haplotypes in glioblastoma risk described in chapter 5, it appeared that the haplotype distribution of the control population used in this study deviated from that of our as well as other subsequent studies on *PDGFRA* promoter haplotype distributions. Therefore, our conclusion that H1/H2α predisposes to NTDs appeared to be biased as a result of this improper control population, as will be discussed in detail in chapter 7 (General discussion).

References

1. Dolk, H, De Wals, P, Gillerot, Y, Lechat, MF, Ayme, S, Cornel, M, Cuschieri, A, Garne, E, Goujard, J, Laurence, KM, and et al. Heterogeneity of neural tube defects in Europe: the significance of site of defect and presence of other major anomalies in relation to geographic differences in prevalence. Teratology 1991;44:547-59.
2. Helwig, U, Imai, K, Schmahl, W, Thomas, BE, Varnum, DS, Nadeau, JH, and Balling, R. Interaction between undulated and Patch leads to an extreme form of spina bifida in double-mutant mice. Nat Genet 1995;11:60-3.
3. Payne, J, Shibasaki, F, and Mercola, M. Spina bifida occulta in homozygous Patch mouse embryos. Dev Dyn 1997;209:105-16.
4. Soriano, P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997;124:2691-700.
5. Joosten, PH, Hol, FA, van Beersum, SE, Peters, H, Hamel, BC, Afink, GB, van Zoelen, EJ, and Mariman, EC. Altered regulation of platelet-derived growth factor receptor-alpha gene-transcription in vitro by spina bifida-associated mutant Pax1 proteins. Proc Natl Acad Sci U S A 1998;95:14459-63.
6. Fleming, A and Copp, AJ. A genetic risk factor for mouse neural tube defects: defining the embryonic basis. Hum Mol Genet 2000;9:575-81.
7. Afink, GB, Nister, M, Stassen, BH, Joosten, PH, Rademakers, PJ, Bongcam-Rudloff, E, Van Zoelen, EJ, and Mosselman, S. Molecular cloning and functional characterization of the human platelet-derived growth factor alpha receptor gene promoter. Oncogene 1995;10:1667-72.
8. Zhang, XQ, Afink, GB, Svensson, K, Jacobs, JJ, Gunther, T, Forsberg-Nilsson, K, van Zoelen, EJ, Westermark, B, and Nister, M. Specific expression in mouse mesoderm- and neural crest-derived tissues of a human PDGFRA promoter/lacZ transgene. Mech Dev 1998;70:167-80.
9. Herrmann, SM, Ricard, S, Nicaud, V, Brand, E, Behague, I, Blanc, H, Ruidavets, JB, Evans, A, Arveiler, D, Luc, G, Poirier, O, and Cambien, F. Polymorphisms in the genes encoding platelet-derived growth factor A and alpha receptor. J Mol Med 2000;78:287-92.
10. Peters, H, Wilm, B, Sakai, N, Imai, K, Maas, R, and Balling, R. Pax1 and Pax9 synergistically regulate vertebral column development. Development 1999;126:5399-408.
11. Wallin, J, Wilting, J, Koseki, H, Fritsch, R, Christ, B, and Balling, R. The role of Pax-1 in axial skeleton development. Development 1994;120:1109-21.
12. Campbell, LR, Dayton, DH, and Sohal, GS. Neural tube defects: a review of human and animal studies on the etiology of neural tube defects. Teratology 1986;34:171-87.
13. Barber, RC, Lammer, EJ, Shaw, GM, Greer, KA, and Finnell, RH. The role of folate transport and metabolism in neural tube defect risk. Mol Genet Metab 1999;66:1-9.
14. Barber, RC, Shaw, GM, Lammer, EJ, Greer, KA, Biela, TA, Lacey, SW, Wasserman, CR, and Finnell, RH. Lack of association between mutations in the folate receptor-alpha gene and spina bifida. Am J Med Genet 1998;76:310-7.
15. Copp, AJ and Bernfield, M. Etiology and pathogenesis of human neural tube defects: insights from mouse models. Curr Opin Pediatr 1994;6:624-31.
16. Boone, D, Parsons, D, Lachmann, SM, and Sherwood, T. Spina bifida occulta: lesion or anomaly? Clin Radiol 1985;36:159-61.
17. Fidas, A, MacDonald, HL, Elton, RA, Wild, SR, Chisholm, GD, and Scott, R. Prevalence and patterns of spina bifida occulta in 2707 normal adults. Clin Radiol 1987;38:537-42.

18. Hol, FA, Geurds, MP, Chatkupt, S, Shugart, YY, Balling, R, Schrander-Stumpel, CT, Johnson, WG, Hamel, BC, and Mariman, EC. PAX genes and human neural tube defects: an amino acid substitution in PAX1 in a patient with spina bifida. J Med Genet 1996;33:655-60.
19. Kondo, T and Raff, M. The Id4 HLH protein and the timing of oligodendrocyte differentiation. Embo J 2000;19:1998-2007.
20. Shimizu-Nishikawa, K, Tazawa, I, Uchiyama, K, and Yoshizato, K. Expression of helix-loop-helix type negative regulators of differentiation during limb regeneration in urodeles and anurans. Dev Growth Differ 1999;41:731-43.
21. Drysdale, CM, McGraw, DW, Stack, CB, Stephens, JC, Judson, RS, Nandabalan, K, Arnold, K, Ruano, G, and Liggett, SB. Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. Proc Natl Acad Sci U S A 2000;97:10483-8.
22. Wang, C and Song, B. Cell-type-specific expression of the platelet-derived growth factor alpha receptor: a role for GATA-binding protein. Mol Cell Biol 1996;16:712-23.
23. Epstein, JA, Song, B, Lakkis, M, and Wang, C. Tumor-specific PAX3-FKHR transcription factor, but not PAX3, activates the platelet-derived growth factor alpha receptor. Mol Cell Biol 1998;18:4118-30.
24. Fukuoka, T, Kitami, Y, Okura, T, and Hiwada, K. Transcriptional regulation of the platelet-derived growth factor alpha receptor gene via CCAAT/enhancer-binding protein-delta in vascular smooth muscle cells. J Biol Chem 1999;274:25576-82.
25. Pirrotta, V. Transvection and chromosomal trans-interaction effects. Biochim Biophys Acta 1999;1424:M1-8.
26. Ashe, HL, Monks, J, Wijgerde, M, Fraser, P, and Proudfoot, NJ. Intergenic transcription and transinduction of the human beta-globin locus. Genes Dev 1997;11:2494-509.
27. Sambrook, J, Fritsch, EF, and Maniatis, T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
28. Rousset, F and Raymond, M. Testing heterozygote excess and deficiency. Genetics 1995;140:1413-9.

Chapter 3

PDGFRA promoter haplotypes differentially interact with maternal environmental factors in predisposition to neural tube defects

Submitted to: Genetic Epidemiology
By: Mascha Toepoel, Regine P.M. Steegers-Theunissen, N. Joop Ouborg, Barbara Franke, Paul H.L.J. Joosten and Everardus J.J. van Zoelen

Summary

Background: Neural tube defects (NTDs), including spina bifida, are multifactorial diseases in which environmental factors, such as maternal nutrition, and genetic components interact. Aberrant expression of the gene encoding the platelet-derived growth factor alpha-receptor (PDGFRA) has been implicated in NTD etiology in both mice and humans. We and others have previously shown that the human *PDGFRA* promoter haplotype H1 is overrepresented and H2 is underrepresented in spina bifida patients, suggesting that genetically determined differences in *PDGFRA* expression levels affect the risk of NTDs. In the current study we investigated possible interactions between *PDGFRA* promoter haplotypes and periconceptional maternal glucose, myo-inositol and zinc levels in relation to the child's risk of spina bifida. In addition, we studied the effects of *PDGFRA* promoter haplotypes on physiological parameters such as maternal length and birth weight of the child.

Materials and methods: We analyzed a Dutch cohort of 88 children with spina bifida aperta and 74 control children, as well as 56 case mothers and 72 control mothers for their *PDGFRA* promoter haplotypes. This cohort had previously been analyzed for their plasma glucose and myo-inositol concentrations as well as their red blood cell zinc levels.

Results: A significantly higher frequency of the low activity *PDGFRA* promoter haplotype H1 was found in children with spina bifida than in controls (30.1% vs 20.3 %; p=0.043). High body-mass index (BMI) and glucose levels of the mother were strongly associated with spina bifida, irrespective of the child's *PDGFRA* promoter haplotype. Mothers of H2 homozygous cases (sbH2) showed a significantly lower myo-inositol concentration (median 14.3 μM) than mothers of H1 homozygous or H1/H2 heterozygous patients (sbH1; median 16.7 μM; p=0.0094) as well as mothers of H2 homozygous controls (cH2; median 16.2 μM; p=0.0081). Zinc levels were significantly higher in cH2 (median 227.7 μM) than in cH1 (median 210.7 μM; p=0.0062) and sbH2 (median 210.0 μM; p=0.013). Logistic regression analysis revealed a significant association with maternal BMI for both H1 and H2 cases, but of glucose only for H2 cases. Stepwise multiple logistic regression analysis showed that high maternal glucose in combination with low myo-inositol is the main risk factor for H2 cases, while a high BMI of the mother appeared the dominant risk factor for H1 cases. Finally, we observed that the presence of an H1 allele is associated with reduced body growth.

Conclusion: These data show that periconceptional BMI, glucose and myo-inositol status of the mother differentially associate with the child's *PDGFRA* promoter haplotype in determining the risk of spina bifida.

Introduction

Spina bifida belongs to the neural tube defects (NTDs), which are common congenital malformations that involve defective formation of the brain and/or spinal cord and its protective coverings. The birth prevalence rate of spina bifida is highly variable, ranging from 0.7 in central France to 11.7 in South America per 10,000 births (1), but the reason for the observed variation is not well understood. Spina bifida is a multifactorial disease, which implies that both genetic and environmental factors contribute to the

etiology.

Many candidate genes for NTDs, including spina bifida, have been identified in studies on mutant and transgenic mice (reviewed in 2, 3), but only few of these genes have actually been confirmed in humans (4). Among the genes thus identified is that encoding the platelet-derived growth factor alpha-receptor (PDGFRA). From mouse studies it is known that Pdgfra plays an important role in the development of the neural tube, since Pdgfra-deficient mice are embryonically lethal and show severe spina bifida over the entire spine (5-8). We have previously shown that the human *PDGFRA* –3600/+118 proximal promoter region contains a total of 10 polymorphic sites which directly affect its transcriptional activity (9, 10). Based on these polymorphisms at least five different haplotypes could be discriminated of which H1 and H2α are the most abundant, displaying allele frequencies of 21% and 68%, respectively, in the Western European population (9, 11, 12). H1 and H2α differ on eight of these ten polymorphic positions, and transient transfection assays have indicated that in most cell lines tested H1 induces low and H2α high transcriptional activity (9, 10, 13). We and others have shown that the H1 allele is overrepresented in cohorts of spina bifida patients, suggesting that low PDGFRA activity during human embryonic development increases the risk of NTDs (9, 13, 14).

During embryonic development, the mother can be considered as the environment of the child and therefore many maternal factors are known to influence NTD risk. It is well established that maternal peri-conceptional supplementation with folic acid prevents NTDs, although approximately 30% of the cases appear to be resistant to this treatment (15, 16). In the folic acid-resistant *curly tail* mutant mouse (17), 70% of NTDs could be prevented by myo-inositol (18) or D-chiro-inositol (19) supplementation, an effect that appears to be mediated by activation of PKC (20). These data suggest that myo-inositol supplementation may also reduce NTD risk in humans. Cellular uptake of myo-inositol is competitively inhibited by high levels of glucose (21, 22), and therefore the higher risk for diabetic women of an NTD-affected pregnancy may be caused by the low myo-inositol levels in their cells as a result of hyperglycemia (23, 24). Conversely, myo-inositol significantly reduced the NTD incidence in diabetic rats and reversed the inhibitory effect of glucose on murine neural tube closure (25, 26).

We previously demonstrated that low myo-inositol, high glucose and low zinc status of the mother 2-4 fold increased the risk of a child with spina bifida (27). In the present study, we analyzed these cohorts of mothers and children for their *PDGFRA* promoter haplotypes, in order to investigate how *PDGFRA* promoter haplotypes, as a genetic factor involved in NTDs, interplay with these environmental factors in determining the child's risk of developing spina bifida. Our results indicate that the effect of maternal risk factors for spina bifida depends on the *PDGFRA* promoter haplotype of the child.

Materials and Methods

Sample population

A case control study comprising spina bifida cases and their mothers as well as healthy control children and their mothers has been performed previously by us (27). Briefly, between August 1999 and December 2001, mothers and their Caucasian children

with spina bifida (non-syndromic meningo(myelo)cele) were recruited in collaboration with the Dutch Spina Bifida Teams of various Dutch University Medical Centers and hospitals. Control subjects, i.e. children in the same age range without major congenital malformations and their mothers, were recruited from midwiferies, nurseries and acquaintances from the case mothers to resemble the cases. Exclusion criteria were pregnancy or breastfeeding at the moment op blood sampling, consanguinity, familiar relationship between case and control subjects, maternal diabetes mellitus and blood transfusion within 3 months prior to blood sampling. Ultimately, this resulted in 134 spina bifida children with 127 of their mothers and 80 control children with 72 of their mothers. The study protocol was approved by the Institutional Review Board of the University Medical Center Nijmegen, The Netherlands.

Blood sampling and DNA isolation
Blood sampling, DNA isolation, and measurement of myo-inositol, glucose and zinc levels were performed as described (27, 28).

PDGFRA haplotyping
PCR and direct DNA sequencing was performed as described (chapter 5, this thesis). We were able to perform haplotype analysis on 88 spina bifida and 74 control children, from which DNA of sufficiently high quality was available. Subsequently haplotype analysis was performed on their corresponding mothers, resulting in analysis of 56 spina bifida and 72 control mothers with DNA of sufficiently high quality.

Statistical analyses
Differences in *PDGFRA* promoter haplotype distribution between case and control children and their mothers were evaluated by odds ratios and 95% confidence intervals. The interaction between individual maternal and child parameters with *PDGFRA* promoter haplotypes in relation to spina bifida risk was analyzed using the non-parametric one tailed Wilcoxon U-test (http://eatworms.swmed.edu/~leon/ stats/ utest.html). In order to analyze the relative contribution of maternal parameters in the child's risk of developing spina bifida, logistic regression analysis was performed for individual haplotypes and their significance was expressed by odds ratios. Stepwise multiple logistic regression was subsequently performed in order to test the relative risk of becoming a patient as a function of the measured variables.

For logistic regression analysis, a binomial response variable (patient versus non-patient) was tested against the continuous variables (BMI, glucose, zinc and myo-inositol) and the categorical variable haplotype (H1 and H2). A general linear model with a binomial error distribution and a logit link function was fitted to the data. In order to test the independence of the variables, the correlation structure of the predictor variables was first investigated for the entire dataset (n=113). Significant but low correlations were found between glucose and BMI (0.198, $p<0.05$) and between glucose and zinc (-0.184, $p <0.05$). A maximum of 4% of the variation in BMI could be explained by variation in glucose, and therefore we further treated the predictor variables in the multiple logistic regression procedure as being effectively independent.

In order to be able to compare the impact of various predictor variables in a stepwise multiple logistic regression analysis, the distribution of the continuous variables was standardized to a mean of 0 and a standard deviation of 1, prior to the analysis.

Table 1: Distributions of *PDGFRA* haplotypes in spina bifida cases and their mothers compared to control children and their mothers.

A

haplotype combination	*sb child*		*c child*		*sb mother*		*c mother*	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*
H1/H1	11	12.5	3	4.1	5	8.9	3	4.2
H1/H2α	25	28.4	23	31.1	18	32.1	23	31.9
H1/H2β	3	3.4	0	0.0	1	1.8	0	0.0
H1/H2γ	2	2.3	1	1.4	2	3.6	0	0.0
H1/H2β	1	1.1	0	0.0	1	1.8	0	0.0
H2α/H2α	38	43.2	36	48.6	20	35.7	37	51.4
H2α/H2β	4	4.5	8	10.8	7	12.5	4	5.6
H2α/H2γ	4	4.5	2	2.7	0	0.0	3	4.2
H2α/H2δ	0	0.0	0	0.0	1	1.8	0	0.0
H2β/H2β	0	0.0	1	1.4	0	0.0	1	1.4
H2β/H2γ	0	0.0	0	0.0	1	1.8	0	0.0
H2γ/H2γ	0	0.0	0	0.0	0	0.0	1	1.4
total	88		74		56		72	

B

	sb child		*c child*		*sb mother*		*c mother*	
haplotype	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
H1	53	30.1	30	20.3	32	28.6	29	20.1
H2α	109	61.9	105	70.9	66	58.9	104	72.2
H2β	7	4.0	10	6.8	9	8.0	6	4.2
H2γ	6	3.4	3	2.0	3	2.7	5	3.5
H2δ	1	0.6	0	0.0	2	1.8	0	0.0
total	176		148		112		144	

C

	n					
child	*sb*	*c*	*OR*	*95% CI*		*p*
H1/H1	11	3	3.746	1.046	13.245	0.042*
H1/H2	31	24	1.32	0.678	2.57	0.417
H2/H2	46	47	1			
	88	74				
H1	53	30	1.695	1.016	2.826	0.043*
H2	123	118	1			
pH1	0.301	0.203				

D

	n					
mother	*sb*	*c*	*OR*	*95% CI*		*p*
H1/H1	5	3	2.644	0.642	10.79	0.193
H1/H2	22	23	1.517	0.723	3.186	0.273
H2/H2	29	46	1			
	56	72				
H1	32	29	1.586	0.893	2.817	0.116
H2	80	115				
pH1	0.285	0.201				

Sb = spina bifida, c = control, OR = odds ratio, CI = confidence interval, * = p<0.05

In a stepwise selection procedure the best fitting multiple logistic regression model was searched for, i.e. the model with maximum fit for the fewest predictor variables. Inclusion criterion for deviance was set to 0.05. This procedure was carried out for both the H1 and H2 subset of data are expressed in standardized logistic regression coefficients for the variables included in the best fitting model, with their significance indicated by odds ratio. All analyses were performed with SAS for Windows 9.3.

Results

PDGFRA haplotypes as risk factors for NTDs

After our initial identification and characterization of human *PDGFRA* promoter haplotypes (9), at least two additional studies have confirmed that the H1 haplotype is overrepresented in spina bifida patients (13, 14). To strengthen these findings, and to subsequently investigate the interaction between *PDGFRA* promoter haplotypes and environmental factors influencing NTD risk, we analyzed the *PDGFRA* promoter haplotype distribution in a cohort of Dutch spina bifida patients and their mothers, of which in previous studies body length, body weight, blood myo-inositol and glucose concentrations, as well as red blood cell levels of zinc had been determined (27). Only patients with sporadic spina bifida aperta were considered, and only those individuals were included of which the *PDGFRA* promoter haplotypes could be determined unequivocally.

Table 1A shows the *PDGFRA* promoter haplotype distribution of these patients and their mothers, in comparison with a cohort of matched controls. Table 1B shows that the haplotype distribution of the control mothers and children is very similar, giving rise to an H1 frequency (pH1) of 0.201 and 0.203, respectively. These numbers are very similar to the value of pH1=0.211 that we have determined recently for a large (n=998) combined Western European control group (chapter 5, this thesis). In contrast, a much higher frequency of H1 was observed for both the cases (pH1=0.301) and their mothers (pH1=0.286). Table 1C shows that the frequency of H1 in the cases was significantly enhanced compared to the control children (OR=1.695; p=0.043), particularly because of the increased frequency of H1 homozygotes in the case group (OR=3.746; p=0.042). The overrepresentation of H1 in the mother group showed a similar odds ratio but was not significant (OR=1.586; p=0.116), most likely because of the smaller sample size (Table 1D). These data agree with previous studies showing that both spina bifida patients and their mothers have an increased frequency of the *PDGFRA* H1 allele (13, 14).

Maternal parameters as risk factors for spina bifida offspring

In order to analyze the interaction between genetic and environmental factors in the risk of spina bifida, we compared maternal BMI, glucose, myo-inositol and zinc values between cases and controls for both the H1 and the H2 group of children. The H1 group consisted of both H1 homozygotes and H1/H2 heterozygotes, while the H2 group consisted of H2 homozygotes irrespective of the their specific subtype (α,β,γ,δ). Table 2A shows medians with 5th-95th percentile of each maternal parameter, as a function of the genetic and disease status of their child. Note that the number of mothers in the analysis is not necessarily identical to that of Table 1A, since only mothers were included

for whom the respective parameter as well as the haplotype of the child could be determined. Table 2B shows the corresponding statistical analysis, on the basis of the non-parametric Wilcoxon U-test. The data show that case mothers have significantly higher BMI (median 25.6 vs 23.2 kg/m^2; p=0.0006), higher serum glucose (4.30 vs 4.05 mM; p=0.0001) and reduced red blood cell zinc (210.3 vs. 220.1 μM; p=0.0414) values than control mothers, in agreement with previous data (27).

When H1 and H2 children were analyzed separately, maternal BMI and glucose were found to be significantly enhanced in both groups. Myo-inositol was significantly reduced in mothers of H2 cases, both when compared to mothers of H1 cases and to mothers of H2 controls (14.3 vs. 16.7 and 16.2 μM, p=0.0094 and 0.0081 respectively), indicating that a haplotype specific effect of maternal myo-inositol exists for spina bifida. Intriguingly, mothers with a control H2 child had significantly higher zinc levels than mothers with a control H1 child (227.7 vs. 210.0 μM, p=0.0062) or with a spina bifida H2 child (210.7 μM, p=0.0130), while maternal zinc was also significantly higher for pooled H2 than for pooled H1 haplotypes (224.3 vs. 211.4 μM, p=0.0414), indicating

Table 2: Interaction between *PDGFRA* promoter haplotypes of the child and maternal variables BMI, glucose, myo-inositol and zinc analyzed by the one tailed Wilcoxon U-test.

A

Maternal parameter		*Child status* sb	c	sbH1	cH1	sbH2	cH2	H1	H2
BMI (kg/m^2)	median (range)	25.6 (19.6-36.6)	23.2 (19.6-29.1)	25.3 (19.4-35.7)	22.8 (19.3-28.3)	25.7 (20.8-38.7)	23.7 (20.2-29.6)	23.2 (19.2-35.6)	24.1 (20.5-36.7)
	n	61	72	30	26	31	46	56	77
Glucose (mM)	median (range)	4.30 (3.71-5.62)	4.05 (3.69-4.61)	4.27 (3.57-6.31)	4.04 (3.52-4.75)	4.31 (3.85-4.97)	4.05 (3.71-4.51)	4.19 (3.52-5.77)	4.14 (3.72-4.72)
	n	54	68	25	26	29	42	51	71
Myo-inositol (μM)	median (range)	15.9 (11.5-22.3)	15.9 (12.0-22.5)	16.7 (13.2-21.1)	15.1 (11.6-22.6)	14.3 (11.1-22.5)	16.2 (13.6-21.2)	15.7 (12.0-22.4)	15.9 (11.5-22.5)
	n	54	68	25	26	29	42	51	71
Zinc (μM)	median (range)	210.3 (147.2-256.5)	220.1 (170.7-260.4)	212.1 (145.3-253.8)	210.7 (178.1-249.7)	210.0 (157.7-267.6)	227.7 (170.0-264.6)	211.4 (161.2-253.4)	224.3 (166.9-268.1)
	n	49	64	24	22	25	42	46	67

B

Maternal parameter		*Child status* sb/c	sbH1/cH1	sbH2/cH2	sbH1/sbH2	cH1/cH2	H1/H2
BMI (kg/m^2)	median	25.6/23.2	25.3/22.8	25.7/23.7	25.3/25.7	22.8/23.7	23.2/24.1
	p	0.0006**	0.0225*	0.0043**	0.2476	0.1003	0.1797
Glucose (mM)	median	4.30/4.05	4.27/4.04	4.31/4.05	4.27/4.31	4.04/4.05	4.19/4.14
	p	0.0001**	0.0329*	0.0004**	0.4186	0.3932	0.3039
Myo-inositol (μM)	median	15.9/15.9	16.7/15.1	14.3/16.2	16.7/14.3	15.1/16.2	15.7/15.9
	p	0.1793	0.1131	0.0081**	0.0094**	0.0582	0.3144
Zinc (μM)	median	210.3/220.1	212.1/210.7	210.0/227.7	212.1/210.0	210.7/227.7	211.4/224.3
	p	0.0414*	0.3122	0.0130*	0.4339	0.0062**	0.0295*

Sb = spina bifida, c = control, range = 5-95% percentile, * = p<0.05, ** = p<0.01

Table 3: Interaction between *PDGFRA* promoter haplotypes and physiological parameters of mother and child analyzed by the one tailed Wilcoxon U-test .

A

Maternal parameter		*Maternal status*							
		sb	c	sbH1	cH1	sbH2	cH2	H1	H2
Length (cm)	median (range)	166.0 (158.4-174.0)	168.5 (159.1-179.0)	164.5 (160.2-173.9)	166.5 (158.0-177.4)	168.8 (157.1-175.0)	169.4 (161.6-179.5)	165.5 (159.4-174.6)	169.1 (158.4-178.0)
	n	46	71	24	25	22	46	49	68

B

Maternal parameter		*Maternal status*					
		sb/c	sbH1/cH1	sbH2/cH2	sbH1/sbH2	cH1/cH2	H1/H2
Length (cm)	median	166.0/168.5	164.5/166.5	168.8/169.4	164.5/168.8	166.5/169.4	165.5/169.1
	p	0.0706	0.4173	0.1831	0.4172	0.0211*	0.0029**

C

Child parameter		*Maternal status*							
		sb	c	sbH1	cH1	sbH2	cH2	H1	H2
Birth weight (g)	median (range)	3160 (2153-4038)	3320 (2728-4086)	3300 (2338-3921)	3255 (2568-3685)	3130 (1925-4090)	3430 (2771-4250)	3260 (2464-3852)	3350 (2479-4169)
	n	56	72	27	26	29	46	53	75

D

Child parameter		*Maternal status*					
		sb/c	sbH1/cH1	sbH2/cH2	sbH1/sbH2	cH1/cH2	H1/H2
Birth weight (g)	median	3160/3320	3300/3255	3130/3430	3300/3130	3255/3430	3260/3350
	p	0.0271*	0.4835	0.0140*	0.4612	0.0129*	0.0220*

E

Child parameter		*Child status*							
		sb	c	sbH1	cH1	sbH2	cH2	H1	H2
Birth weight (g)	median (range)	3160 (2290-467)	3328 (2733-4095)	3218 (2290-3986)	3370 (2790-3949)	3160 (2322-4120)	3300 (2715-4189)	3320 (2334-3982)	3253 (2533-4164)
	n	87	74	42	27	45	47	69	92

F

Child parameter		*Child status*					
		sb/c	sbH1/cH1	sbH2/cH2	sbH1/sbH2	cH1/cH2	H1/H2
Birth weight (g)	median	3160/3328	3218/3370	3160/3300	3218/3160	3370/3300	3320/3253
	p	0.0097**	0.0293*	0.0804	0.2951	0.149	0.4975

Range = 5-95% percentile, sb = spina bifida, c = control, * = p<0.05, ** = p<0.01 (**).

that also maternal blood zinc levels may differentially influence children with an H1 and H2 genotype.

Very similar results were obtained when the above data were analyzed with a parametric t-test. Median and mean values never differed by more than 5%, indicating that the data presented are close to a normal distribution (data not shown).

Effect of PDGFRA promoter haplotypes on physiological parameters of mother and child

PDGFRA deficient mice are smaller than their wild-type littermates (5-7), suggesting a role for Pdgfra in body growth. Therefore we investigated the association of *PDGFRA* haplotypes with growth-related parameters such as maternal length and birth weight of the child, as is depicted in table 3. We observed that H1 control mothers are significantly shorter than H2 control mothers (166.5 vs.169.4 cm, p=0.0211). This difference was even more pronounced for pooled case and control mothers (165.5 vs. 169.1 cm, p=0.0029,

table 3A and B), which suggests that H1-driven low PDGFRA expression inhibits body growth. Also, the haplotype of the mother appeared to affect the child's birth weight, as is depicted in table 3C and D. Control H1 as well as pooled H1 mothers gave birth to a significantly lighter child than control and pooled H2 mothers respectively (3255 vs. 3430 g, p=0.0129 and 3260 vs. 3350 g, p=0.0220), while the lower birth weight for cases as observed in the total population (3160 vs.3320 g, p=0.0271) remained statistically significant for the H2 mother subset only (3130 vs. 3430 g, p=0.0140). Furthermore, table 4E and F show that the haplotype of the child itself also affected birth weight. We observed a reduced birth weight for cases compared to control children (3160 vs. 3328 g, p=0.0097), which remained for the H1 subset (3218 vs. 3370, p=0.0293), but not for the much larger H2 subset (3160 vs. 3300, p=0.0804), indicating that particularly for H1 children spina bifida is associated with a low birth weight. Taken together, these data indicate that *PDGFRA* promoter haplotypes differentially affect body growth.

Logistic regression analysis on maternal risk factors for spina bifida offspring

In order to analyze which maternal parameters show the best prognostic indication for spina bifida risk in the child, we carried out logistic regression analyses for both the H1 and H2 cohorts. Table 4A shows that according to such analysis maternal BMI is the only significantly associated parameter for H1 children, while for H2 children both glucose and BMI are significantly associated with spina bifida.

In order to evaluate the relative contribution of the various maternal parameters

Table 4: Logistic regression (A) and stepwise multiple logistic regression (B) analysis on maternal variables stratified for *PDGFRA* haplotype of the child.

A

H1 (n=46)	*OR*	*95% CI*	
BMI	2.107*	1.04	4.27
Glucose	2.009	0.928	4.348
Myo-inositol	1.097	0.609	1.976
Zinc	0.944	0.525	1.697
H2 (n=67)			
BMI	2.243*	1.236	4.072
Glucose	2.859*	1.445	5.655
Myo-inositol	0.533	0.258	1.103
Zinc	0.604	0.352	1.035

B

H1 (n=46)	*part.st.regr.coeff.*	*OR*	*95% CI*	
BMI	0.7454	2.107*	1.04	4.27
Glucose	not included			
Myo-inositol	not included			
Zinc	not included			
H2 (n=67)				
BMI	0.6297	1.877	0.943	3.735
Glucose	1.0695	2.914*	1.315	6.456
Myo-inositol	-0.851	0.427*	0.185	0.984
Zinc	not included			

OR = odds ratio, CI = confidence interval, part.st.regr.coeff = partial standard regression coefficient

in the risk of spina bifida, we performed stepwise multiple regression analysis on this set of data and determined for each parameter its partial standardized regression coefficient. Table 4B shows that for H1 children BMI is by far the most prominent predictive parameter. After taking into account the contribution of BMI, none of the other three parameters contributed to a better fit of the model to the experimental data and were therefore excluded by the analysis. For H2 children glucose showed the highest association with spina bifida, with a minor but significant contribution of myo-inositol. Introduction of BMI as an additional parameter resulted in only a small further improvement of the fit. In conclusion, enhanced BMI of the mother appears the best prognostic parameter for the risk of spina bifida of an H1 child, while enhanced maternal glucose, particularly in combination with reduced maternal myo-inositol, is the best prognostic parameter for an H2 child.

Discussion

NTDs are common congenital malformations of multifactorial etiology. In the present study we searched for interactions between genetic and environmental factors that are known to influence the risk of spina bifida. As a genetic factor we studied the *PDGFRA* promoter haplotype of the child, and as environmental factors the BMI and periconceptional levels of blood glucose, myo-inositol and red blood cell zinc of the mother. First, we confirmed that spina bifida children show an enhanced incidence of the *PDGFRA* promoter haplotype H1. Second, we observed that high maternal BMI, high maternal glucose and low maternal zinc each predispose to spina bifida in the offspring, irrespective of their *PDGFRA* promoter haplotype. Third, when analyzing the relative risk of developing spina bifida, we observed by stepwise multiple logistic regression analysis that high BMI is the most prominent maternal risk factor for H1 children, and high glucose in combination with low myo-inositol the most prominent for H2 children. Fourth, spina bifida appeared to be associated with a low birth weight, particularly for H1 children. Finally, when investigating the role of *PDGFRA* promoter haplotypes on maternal and child physiology, we found that H1 mothers are significantly shorter than H2 mothers, while also giving birth to lighter children. These data show that *PDGFRA* promoter haplotypes differentially affect both physiological and pathological processes.

Our observation that the frequency of the H1 allele is increased in spina bifida patients as well as in their mothers agrees with previous studies by Zhu (13) and Au (14). These authors studied particularly Hispanic populations in the USA, who show a very high H1 incidence (pH1=0.31) in their control population. Our current data, showing a pH1=0.203 for the control children and pH1=0.201 for their mothers, is in agreement with values obtained from a very large Western European control population (pH1=0.211). In a previous study we concluded that particularly H1/H2 heterozygosity is associated with spina bifida (9), but these results were obtained on the basis of much smaller and less well defined cohorts, of which the controls clearly deviated from the Western European control population.

PDGFRA is involved in the growth of various, mostly mesenchymal cell types, such as fibroblast, osteoblasts, smooth muscle, neural crest and oligodendrocyte precursor cells (29). Many Pdgfra signaling mutants in the mouse display a smaller

overall body size than their wild type littermates (5-7, 30-32). In line with this observation, we now show that the low expressing H1 haplotype negatively influences the body length of humans. *PDGFRA* is thus one of the few genes that can directly be linked to general body size in humans, although a relation with the well described hypothalamus-pituitary-GH axis remains unclear (33).

Multifactorial diseases such as NTDs have been described in terms of a liability/threshold model, in which discontinuous phenotypes are explained by assuming that the sum of adverse genetic and/or environmental factors must exceed a threshold before the abnormal phenotype becomes manifest (34, 35). In terms of this model the low activity H1 allele can be considered as an adverse genetic factor for NTDs, and consequently for this haplotype less environmental factors (a.o. BMI, glucose, myo-inositol and zinc) require an aberrant value, before it results in spina bifida. This is in agreement with our finding that high BMI alone is already a substantial maternal risk factor for H1 children, while for H2 children a combination of high glucose, low myo-inositol and high BMI is required. It is important to realize that only live-born children with spina bifida have been included in our study, and therefore it is likely that in combination with multiple adverse environmental parameters H1 children have a higher risk of prenatal death than H2 children. Alternatively, the possibility should be considered that the genetic and environmental factors discussed in this study have a common mechanism of action, e.g. in mediating PDGFRA signaling, this in spite of the observation that they behave as independent parameters in the statistical analysis.

Our data indicate that high BMI and glucose are the main maternal risk factors for spina bifida. Periconceptional intake of simple sugars and foods with a high glycemic index have already been described to influence NTD risk in humans (36), while high concentrations of glucose inhibit neural tube closure in mice (26). Also obesity, generally defined as a BMI above 29kg/m^2, has previously been associated with elevated risk of NTDs, as well as with other anomalies in offspring (37-42). Although the underlying mechanism for correlation is unknown, several possibilities have been suggested. Obese women might suffer from poorer nutrition than non-obese women, resulting in higher glucose and lower myo-inositol blood levels. Alternatively, they may have metabolic alterations that increase NTD risk, such as hyperglycemia, increased insulin or estrogen levels (40).

The role of myo-inositol in the risk of spina bifida is less well documented. Our current data indicate that low maternal myo-inositol is a risk factor particularly for H2 children. Myo-inositol provides the basis for various inositol phospholipids, of which phosphatidylinositol (PI) is the most abundant in mammalian cells (~5-20% (21)). The PI derivative PIP_2 is used as an intracellular signaling molecule by many growth factor and hormone receptors, including PDGFRA (21). NTDs are prevented by myo-inositol suppletion in the curly tail mouse (18), via a PKC dependent mechanism (20), thus providing a possible link between myo-inositol and PDGFRA, which also signals through PKC. In the case of low maternal myo-inositol levels and consequently low myo-inositol supply to the developing embryo, in combination with the high expressing H2 allele, the amount of PIP_2 for PDGFRA signaling may become limited in fast growing tissues such as the neural tube, resulting in reduced PDGFRA signaling and consequently a higher risk of spina bifida. In addition, low myo-inositol supply in combination with low H1 driven embryonic PDGFRA expression may result in reduction of PDGFRA signaling capacity to such a low level that these embryos die prenatally. In line with this hypothesis, the mothers of the live-born H1 cases analyzed in this study have significantly higher myo-

inositol levels in their blood than do mothers of H2 cases. Since glucose inhibits cellular uptake of myo-inositol, high glucose levels may enhance the effect of low maternal myo-inositol supply, which in combination may affect PDGFRA signaling. It must be noted, however, that is unclear how the myo-inositol levels in serum as measured in this study correlate to its intracellular availability.

Maternal zinc status has also previously been implicated in the etiology of neural tube defects (43-46). Zinc is an essential element for the structure and function of a large number of macromolecules including 300 enzymes. It plays a role in e.g. insulin biosynthesis and is a structural component of many transcription factors (47, 48). In this study we observed that maternal zinc levels are higher for H2 than for H1 children, and higher in controls than in cases. A number of zinc finger transcription factors have been implicated in *PDGFRA* transcriptional regulation, including GATA4, Sp1 and ZNF148 (12, 49-52), but it is unclear to what extent zinc concentrations limit the efficiency of their action, and thereby of PDGFRA signaling.

In conclusion, we found that *PDGFRA* promoter haplotypes are differentially involved in the etiology of neural tube defects and interact with different environmental factors to influence NTD risk.

Acknowledgements

We would like to thank Angela M. Gonzalez-Zuloeta Ladd and Cornelia M. van Duijn of the Erasmus University Medical Center in Rotterdam for help with the statistics and the Hersenstichting Nederland for financial support.

References

1. Detrait, ER, George, TM, Etchevers, HC, Gilbert, JR, Vekemans, M, and Speer, MC. Human neural tube defects: developmental biology, epidemiology, and genetics. Neurotoxicol Teratol 2005;27:515-24.
2. Juriloff, DM and Harris, MJ. Mouse models for neural tube closure defects. Hum Mol Genet 2000;9:993-1000.
3. Greene, ND and Copp, AJ. Mouse models of neural tube defects: investigating preventive mechanisms. Am J Med Genet C Semin Med Genet 2005;135:31-41.
4. Boyles, AL, Hammock, P, and Speer, MC. Candidate gene analysis in human neural tube defects. Am J Med Genet C Semin Med Genet 2005;135:9-23.
5. Soriano, P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997;124:2691-700.
6. Schatteman, GC, Morrison-Graham, K, van Koppen, A, Weston, JA, and Bowen-Pope, DF. Regulation and role of PDGF receptor alpha-subunit expression during embryogenesis. Development 1992;115:123-31.
7. Payne, J, Shibasaki, F, and Mercola, M. Spina bifida occulta in homozygous Patch mouse embryos. Dev Dyn 1997;209:105-16.
8. Li, L, Schatteman, GC, Oppenheim, RW, Lei, M, Bowen-Pope, DF, and Houenou, LJ. Altered development of spinal cord in the mouse mutant (Patch) lacking the PDGF receptor alpha-subunit gene. Brain Res Dev Brain Res 1996;96:204-9.
9. Joosten, PH, Toepoel, M, Mariman, EC, and Van Zoelen, EJ. Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects. Nat Genet 2001;27:215-7.
10. Toepoel, M, Ackerschott, B, and van Zoelen, EJ. Haplotype-dependent binding of nuclear proteins to the promoter of the neural tube defects-associated platelet-derived growth factor alpha-receptor gene. Biochim Biophys Acta 2005;
11. Herrmann, SM, Ricard, S, Nicaud, V, Brand, E, Behague, I, Blanc, H, Ruidavets, JB, Evans, A, Arveiler, D, Luc, G, Poirier, O, and Cambien, F. Polymorphisms in the genes encoding platelet-derived growth factor A and alpha receptor. J Mol Med 2000;78:287-92.
12. De Bustos, C, Smits, A, Stromberg, B, Collins, VP, Nister, M, and Afink, G. A PDGFRA promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumours and ependymomas. J Med Genet 2005;42:31-7.
13. Zhu, H, Wicker, NJ, Volcik, K, Zhang, J, Shaw, GM, Lammer, EJ, Suarez, L, Canfield, M, and Finnell, RH. Promoter haplotype combinations for the human PDGFRA gene are associated with risk of neural tube defects. Mol Genet Metab 2004;81:127-32.
14. Au, KS, Northrup, H, Kirkpatrick, TJ, Volcik, KA, Fletcher, JM, Townsend, IT, Blanton, SH, Tyerman, GH, Villarreal, G, and King, TM. Promotor genotype of the platelet-derived growth factor receptor-alpha gene shows population stratification but not association with spina bifida meningomyelocele. Am J Med Genet A 2005;139:194-8.
15. Wald, NJ and Gilbertson, MP. Folic acid in prevention of neural tube defects. Lancet 1995;345:389.
16. Czeizel, AE and Dudas, I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 1992;327:1832-5.
17. Seller, MJ. Vitamins, folic acid and the cause and prevention of neural tube defects. Ciba Found Symp 1994;181:161-73; discussion 73-9.
18. Greene, ND and Copp, AJ. Inositol prevents folate-resistant neural tube defects in the mouse. Nat Med 1997;3:60-6.

19. Cogram, P, Tesh, S, Tesh, J, Wade, A, Allan, G, Greene, ND, and Copp, AJ. D-chiro-inositol is more effective than myo-inositol in preventing folate-resistant mouse neural tube defects. Hum Reprod 2002;17:2451-8.
20. Cogram, P, Hynes, A, Dunlevy, LP, Greene, ND, and Copp, AJ. Specific isoforms of protein kinase C are essential for prevention of folate-resistant neural tube defects by inositol. Hum Mol Genet 2004;13:7-14.
21. Holub, BJ. The nutritional importance of inositol and the phosphoinositides. N Engl J Med 1992;326:1285-7.
22. Scalera, V, Natuzzi, D, and Prezioso, G. myo-inositol transport in rat intestinal brush border membrane vesicles, and its inhibition by D-glucose. Biochim Biophys Acta 1991;1062:187-92.
23. Hendricks, KA, Nuno, OM, Suarez, L, and Larsen, R. Effects of hyperinsulinemia and obesity on risk of neural tube defects among Mexican Americans. Epidemiology 2001;12:630-5.
24. Mills, JL. Malformations in infants of diabetic mothers. Teratology 1982;25:385-94.
25. Reece, EA, Khandelwal, M, Wu, YK, and Borenstein, M. Dietary intake of myo-inositol and neural tube defects in offspring of diabetic rats. Am J Obstet Gynecol 1997;176: 536-9.
26. Baker, L, Piddington, R, Goldman, A, Egler, J, and Moehring, J. Myo-inositol and prostaglandins reverse the glucose inhibition of neural tube fusion in cultured mouse embryos. Diabetologia 1990;33:593-6.
27. Groenen, PM, Peer, PG, Wevers, RA, Swinkels, DW, Franke, B, Mariman, EC, and Steegers-Theunissen, RP. Maternal myo-inositol, glucose, and zinc status is associated with the risk of offspring with spina bifida. Am J Obstet Gynecol 2003;189:1713-9.
28. Groenen, PM, Klootwijk, R, Schijvenaars, MM, Straatman, H, Mariman, EC, Franke, B, and Steegers-Theunissen, RP. Spina bifida and genetic factors related to myo-inositol, glucose, and zinc. Mol Genet Metab 2004;82:154-61.
29. Heldin, CH and Westermark, B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 1999;79:1283-316.
30. Grüneberg, H and Truslove, GM. Two closely linked genes in the mouse. Genet Res 1960;1:69-90.
31. Ding, H, Wu, X, Bostrom, H, Kim, I, Wong, N, Tsoi, B, O'Rourke, M, Koh, GY, Soriano, P, Betsholtz, C, Hart, TC, Marazita, ML, Field, LL, Tam, PP, and Nagy, A. A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. Nat Genet 2004;36:1111-6.
32. Klinghoffer, RA, Hamilton, TG, Hoch, R, and Soriano, P. An allelic series at the PDGFalphaR locus indicates unequal contributions of distinct signaling pathways during development. Dev Cell 2002;2:103-13.
33. Castro-Feijoo, L, Quinteiro, C, Loidi, L, Barreiro, J, Cabanas, P, Arevalo, T, Dieguez, C, Casanueva, FF, and Pombo, M. Genetic basis of short stature. J Endocrinol Invest 2005;28:30-7.
34. Hol, FA, van der Put, NM, Geurds, MP, Heil, SG, Trijbels, FJ, Hamel, BC, Mariman, EC, and Blom, HJ. Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects. Clin Genet 1998;53:119-25.
35. Hol, FA.Genetic factors in human neural tube defects. Nijmegen: University of Nijmegen; 1999.

36. Shaw, GM, Quach, T, Nelson, V, Carmichael, SL, Schaffer, DM, Selvin, S, and Yang, W. Neural tube defects associated with maternal periconceptional dietary intake of simple sugars and glycemic index. Am J Clin Nutr 2003;78:972-8.
37. Shaw, GM, Todoroff, K, Finnell, RH, and Lammer, EJ. Spina bifida phenotypes in infants or fetuses of obese mothers. Teratology 2000;61:376-81.
38. Shaw, GM, Velie, EM, and Schaffer, D. Risk of neural tube defect-affected pregnancies among obese women. Jama 1996;275:1093-6.
39. Werler, MM, Louik, C, Shapiro, S, and Mitchell, AA. Prepregnant weight in relation to risk of neural tube defects. Jama 1996;275:1089-92.
40. Watkins, ML, Rasmussen, SA, Honein, MA, Botto, LD, and Moore, CA. Maternal obesity and risk for birth defects. Pediatrics 2003;111:1152-8.
41. Kallen, K. Maternal smoking, body mass index, and neural tube defects. Am J Epidemiol 1998;147:1103-11.
42. Watkins, ML, Scanlon, KS, Mulinare, J, and Khoury, MJ. Is maternal obesity a risk factor for anencephaly and spina bifida? Epidemiology 1996;7:507-12.
43. Cavdar, AO, Bahceci, M, Akar, N, Erten, J, Bahceci, G, Babacan, E, Arcasoy, A, and Yavuz, H. Zinc status in pregnancy and the occurrence of anencephaly in Turkey. J Trace Elem Electrolytes Health Dis 1988;2:9-14.
44. Srinivas, M, Gupta, DK, Rathi, SS, Grover, JK, Vats, V, Sharma, JD, and Mitra, DK. Association between lower hair zinc levels and neural tube defects. Indian J Pediatr 2001;68:519-22.
45. Warkany, J and Petering, HG. Congenital malformations of the central nervous system in rats produced by maternal zinc deficiency. Teratology 1972;5:319-34.
46. Buamah, PK, Russell, M, Bates, G, Ward, AM, and Skillen, AW. Maternal zinc status: a determination of central nervous system malformation. Br J Obstet Gynaecol 1984;91: 788-90.
47. Taylor, CG. Zinc, the pancreas, and diabetes: insights from rodent studies and future directions. Biometals 2005;18:305-12.
48. Tapiero, H and Tew, KD. Trace elements in human physiology and pathology: zinc and metallothioneins. Biomed Pharmacother 2003;57:399-411.
49. Fukuoka, T, Kitami, Y, Okura, T, and Hiwada, K. Transcriptional regulation of the platelet-derived growth factor alpha receptor gene via CCAAT/enhancer-binding protein-delta in vascular smooth muscle cells. J Biol Chem 1999;274:25576-82.
50. Yang, ZH, Kitami, Y, Takata, Y, Okura, T, and Hiwada, K. Targeted overexpression of CCAAT/enhancer-binding protein-delta evokes enhanced gene transcription of platelet-derived growth factor alpha-receptor in vascular smooth muscle cells. Circ Res 2001;89:503-8.
51. Afink, G, Westermark, UK, Lammerts, E, and Nister, M. C/EBP is an essential component of PDGFRA transcription in MG-63 cells. Biochem Biophys Res Commun 2004;315:313-8.
52. Bonello, MR and Khachigian, LM. Fibroblast growth factor-2 represses platelet-derived growth factor receptor-alpha (PDGFR-alpha) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR-alpha promoter. J Biol Chem 2004;279:2377-82.

Chapter 4

Haplotype-dependent binding of nuclear proteins to the promoter of the neural tube defects-associated platelet-derived growth factor alpha-receptor gene

Published in: Biochimica et Biophysica Acta 1741 (2005) 350 – 357
By: Mascha Toepoel, Bart Ackerschott and Everardus J.J. van Zoelen

Abstract

We have previously shown that polymorphisms in the promoter of the human platelet-derived growth factor α-receptor (PDGFRA) gene can be grouped into five distinct haplotypes, designated H1, H2α, H2β, H2γ and H2δ, and that specific combinations of these promoter haplotypes predispose to neural tube defects (NTDs). These promoter haplotypes differ strongly in their ability to drive reporter gene expression in various human cell lines, with highest activity for H2α and H2β. Here we show that the haplotype-linked *PDGFRA* promoter region extends to 3.6 kb upstream from the transcription start site, and contains a total of ten polymorphic sites. For two of these polymorphic sites, i.e. –909C/A and +68GAins/del, we observed differential binding of nuclear proteins from human osteosarcoma (HOS) cells. The protein complex binding specifically to –909C, which is present in all haplotypes except the low activity haplotype H2γ, contained members of the upstream stimulatory factor (USF) family of transcription factors. Furthermore we identified a protein complex of 125 kDa which bound specifically to the low activity haplotype H1 at position +68GAdel and may represent an H1-specific *PDGFRA* transcriptional repressor. The current identification of *cis*-acting elements in the *PDGFRA* promoter and the transcription factors that bind them, provides a new strategy for the identification of genes that are potentially involved in neural tube defects.

Introduction

With the recent completion of the overall sequence of the human genome, current genomic research now focuses on the estimated 0.1% difference in DNA sequences between individuals. Emphasis is put particularly on the role of single nucleotide polymorphisms (SNPs) and small insertion/deletions (ins/dels) within regulatory DNA sequences, since such polymorphisms may directly affect the expression levels of the nearby genes. Regulatory polymorphisms in multiple genes may not only determine the specific genetic characteristics of an individual, but also his/her predisposition for complex diseases.

In a previous study, we have identified seven SNPs and one dinucleotide ins/del in the 1.6 kb promoter region of the gene encoding the human platelet-derived growth factor α-receptor (PDGFRA). These polymorphisms appeared to be linked and gave rise to five distinct haplotypes, designated H1, H2α, H2β, H2γ and H2δ (1). H1 and H2α are the two most common haplotypes (approximately 22% and 65% of the Western European population (1-3) and differ from each other on six positions in this promoter region, while H2β, H2γ and H2δ differ each on only a single nucleotide position from H2α. Upon transient transfection of these *PDGFRA* promoter haplotypes into the human osteosarcoma cell lines U2OS, HOS and SAOS, we observed an up to six-fold higher promoter activity for H2α and H2β when compared to H1, H2γ and H2δ (1). This suggests that the corresponding polymorphisms may also influence gene expression in vivo and, as a consequence, may contribute to the susceptibility to PDGFRA-related diseases.

Deregulated expression of *PDGFRA* has been linked to neural tube defects (NTDs) in mice (4,5) as well as in humans (1,6). NTDs, including anencephaly and spina bifida, are common congenital malformations that involve defective development of

the brain and/or spinal cord as a result of incomplete closure of the neural tube. NTDs occur with an average frequency of one per thousand births in the Western world and are considered to be multifactorial in origin, implying that both environmental and genetic factors contribute to their etiology (7).

In mice, *Pdgfra* deficiency leads to prenatal lethality and various developmental defects including severe spina bifida along the entire spinal column (4,5(. Moreover, some *PDGFRA* upstream regulators have been associated with NTDs, such as Pax1 (8-10), Pax3 (11-13) and PRX1/PRX2 (14,15), both in mice and men. This suggests that aberrant *PDGFRA* expression may predispose to NTDs in humans. Interestingly, we (1) and others (6) have shown that the H1 promoter haplotype of *PDGFRA* is associated with an increased risk of NTDs. In order to understand the role of *PDGFRA* haplotypes in NTDs at the molecular level, it will be important to identify transcription factors that are able to bind the *PDGFRA* promoter in a SNP dependent manner. Recently, De Bustos et al. (3) have shown that the C to A substitution specific for the H2δ haplotype results in a loss of binding capacity of the zinc finger transcription factor ZNF148.

The *PDGFRA* haplotype-linked promoter SNPs identified thus far are located in the promoter region between +118 and -1589 relative to the transcription start site according to Afink et al. (16), which is the most conserved part of the promoter between human, mouse and rat. Studies on transgenic mice containing *PDGFRA* promoter constructs have shown, however, that the promoter region between -1589 and -3600 may contain additional elements necessary for proper *PDGFRA* transcription (17,18). Analyses by Herrmann et al. (2) have indicated that this region contains at least a five base pairs ins/del at position –1631, which according to Zhu et al. (6) is haplotype coupled. In the present study we show that this ins/del and an additional T/C SNP at position -2795 are linked to the H1 and H2 haplotypes, thereby extending the length of the various promoter haplotypes to at least 3600 bp upstream of the transcription initiation site. In order to identify haplotype-specific binding of transcription regulating proteins, we subsequently analyzed all eight haplotype-coupled SNPs and the two ins/dels present in the –3600/+118 *PDGFRA* promoter region for allele-specific binding of nuclear proteins from human osteosarcoma (HOS) cells. Here we show by electrophoretic mobility shift assays (EMSAs) that the –909C/A SNP and the +68GAins/del show haplotype specific protein binding. Using supershift assays the protein binding to the –909C oligonucleotide could be identified as a member of the upstream stimulatory factor (USF) family of transcription factors, while the +68del oligonucleotide was shown to bind specifically a thus far unidentified protein complex with a molecular weight of 125 kDa. These data offer a possible mechanistic explanation for the different promoter activities of the various *PDGFRA* promoter haplotypes, and are discussed in terms of the relation between promoter haplotypes and susceptibility to PDGFRA-related diseases such as NTDs.

Methods

Automated DNA sequencing

For analysis of the -3600 to -1535 region of the *PDGFRA* promoter, two overlapping fragments were amplified by PCR using the following primer pairs: forward primer F1 5'-

AGGGAACTTTCACCTCAAGC-3' with reverse primer R1 5'-CAGGCCACCATATGTACCC-3' and forward primer F2 5'-CCCACATTCTTAACCCCTTG-3' with reverse primer R2 5'-GGAATGCTAACAGGATACCG-3'. PCR mixtures contained 1x PCR buffer, 1 mM $MgCl_2$, 1 unit of Biotherm DNA polymerase (GeneCraft), 0.4 mM of each dNTP (Fermentas), 100 ng of each primer and 15 ng of genomic DNA in a total volume of 25 μl. Cycling parameters were 5 min at 95°C, followed by 50 cycles of 30 sec at 95°C, 30 sec at 60°C and 2 min at 72°C, with a subsequent extension of 10 min at 72°C. PCR products were purified using the PCR purification kit (QIAgen). Automated sequencing was performed on the ABI310 genetic analyzer using the Big Dye terminator sequencing kit (Applied Biosystems).

Cell culture

Human osteosarcoma cells (MNNG/HOS TE85 Clone F-5, ATCC CRL1547) were maintained in bicarbonate-buffered Dulbecco's MEM/nutrient mix F12 (1:1) medium supplemented with 10% fetal calf serum (FCS, Life Technologies) in a 7.5% CO_2 atmosphere at 37°C.

Preparation of nuclear extracts

HOS cells were grown to confluency on 56.7 cm^2 culture plates, washed once with ice-cold phosphate-buffered saline (PBS) and harvested on ice by gentle scraping in 1 ml PBS. Cells were centrifuged for 15 sec at maximum speed in an Eppendorf centrifuge and the pellet containing the cells was resuspended in 400 μl buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). The lysate was subsequently incubated for 15 min on ice after which 25 μl of 10% NP40 were added. After vortexing for 15 sec and centrifugation for another 30 sec at maximum speed in an Eppendorf centrifuge, the pellet containing the nuclei was resuspended in 40 μl buffer B (20 mM Hepes pH 7.9, 10 mM NaCl, 1 mM EDTA, 1mM EGTA, 1 mM DTT, 1 mM PMSF) and vortexed for 15 min at 4°C. The lysate was subsequently centrifuged for 5 min at 4°C at maximum speed in an Eppendorf centrifuge and the pellet discarded. Protein concentration of the nuclear extract was determined using the Bio-Rad protein assay (Bio-Rad) and fractions were frozen in liquid nitrogen.

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides were annealed and subsequently end-labeled using T4 polynucleotide kinase (Fermentas) and (γ-^{32}P)dATP (ICN). Sequences of the forward oligonucleotides used are depicted in Table 1. For the EMSA, 10 μg of nuclear extract were incubated with 5.0 fmol of the labeled probe and 2 μg poly(dIdC) (Amersham) in 1x binding buffer (10 mM Hepes pH 7.9, 60 mM KCl, 4% Ficoll, 1 mM EDTA), in a final volume of 20 μl. Unlabeled competitor DNA was added to the binding reactions at 100-fold molar excess where indicated. After incubating for 5 min at room temperature, protein-DNA complexes were separated on a pre-electrophoresed 4% non-denaturing polyacrylamide gel in 0.5x TBE buffer (19) at 180 V. After electrophoresis, gels were dried and protein-DNA complexes visualized by autoradiography. For supershift assays, 2 or 4 μg of specific antibody (sc-229X for USF1 and sc-13842X for HES-1, Santa Cruz Biotechnology) were added 5 min prior to addition of the probe.

UV-crosslinking

UV-crosslinking experiments were performed using the same DNA probes as in the gel

Table 1
Sequences of EMSA probes

Oligonucleotide	DNA sequence
+68ins/del	GGGAGAGAAACA $^{GA}/_{-}$GGAGGAGACTGC
−794G/T	TGCGAGATAGGAA $^{G}/_{T}$ CCAGGGCAACCT
−909C/A	GGGTTCGACCCA $^{C}/_{A}$ GCGGCGGGAGGG
−957G/A	CTTGGGCGCAGG $^{G}/_{A}$ TTTGGGGCTCAGC
−1074C/A	CGCTCCCCTCCC $^{C}/_{A}$ CGCTGTCGCTGG
−1391C/G	GTAGAAGAACTG $^{C}/_{G}$ GGCAATGGGACC
−1467/G/A	TTTCCTGAAGG $^{G}/_{A}$ ACATGTGGAAGT
−1507A/G	GCATTCCGAACA $^{A}/_{G}$ GGGGCTTCAT
−1631ins/del	AACCCGGTTCTC $^{AACTT}/_{-}$AACAGCATGTGG
−2795T/C	TCTCCCCAACTTC $^{T}/_{C}$ CGGCCCGGCTCA
USF	CACCCGGTCACGTGGCCTACACC
+68ins/del-BrdU-R	GCAGTCUCCUCC $^{TC}/_{-}$UGUUTCTCTCCCT

Only the forward sequences are given (5′ to 3′), except where indicated with -R, which represents a reverse sequence.

shift experiments, except that five of the thymidine residues in the reverse sequence were substituted for bromo-deoxyuridine residues (Table 1). Binding reactions were performed similarly as in the EMSA except that the reactions were scaled up two-fold. Reaction mixtures were exposed to UV light (254 nm) for 30 min (GS Gene Linker UV chamber, Bio-Rad) after which samples were split in two. One half was analyzed on a 4% non-denaturing polyacrylamide gel and, after addition of Laemni buffer, the other half on a 12.5% denaturing SDS-polyacrylamide gel. Gels were dried and proteins crosslinked to the radiolabeled probe were visualized by autoradiography.

Results

Analysis of the upstream PDGFRA promoter region

In a Dutch control population we have previously identified five distinct *PDGFRA* promoter haplotypes, based on analysis of polymorphisms in the +118 to –1589 promoter region (see Fig. 1). We have now analyzed the -1535 to -3600 *PDGFRA* promoter region for additional polymorphisms, and observed a T/C SNP at position -2795 (NCBI SNP database rs2114039) which is strictly haplotype-coupled, such that the T allele corresponds to all H2 haplotypes and the C allele to H1 (Fig. 1). Also, the strict correlation of the -1631AACTTins/del (rs10563643) with the known haplotypes was confirmed, such that all H2 haplotypes contained this five-nucleotide insertion, while the H1 allele fully lacked this sequence. The NCBI SNP data base contains five additional putative polymorphic sites in this region (rs17084067 at –1820; rs13114391 at –2563; rs13140747 at –2666; rs17084065 at –3180; rs17084062 at –3433), but we could not detect any of these polymorphisms in the set of individuals tested (data not shown). These data therefore extend the region in which polymorphisms in the *PDGFRA* promoter are linked to the H1 and H2 haplotypes to at least 3600 nucleotides upstream from the transcription start site. In total this region contains eight SNPs and two ins/dels that are linked to the five haplotypes identified (see survey Fig. 1)

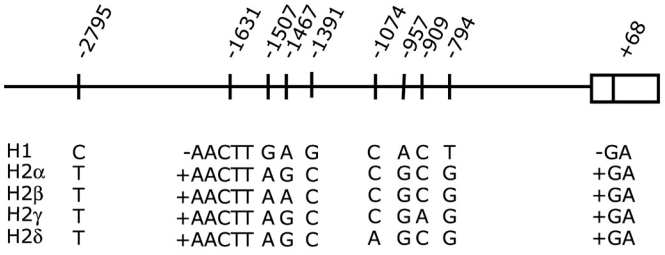

Fig. 1. Promoter haplotypes resulting from polymorphisms in the -3600 /+118 region of *PDGFRA*. At the top, the nucleotide positions of the polymorphisms are indicated, and at the bottom the possible nucleotides at these positions and their corresponding haplotypes. The analysis was performed on genomic DNA from at least two individuals of each of the following haplotype combinations selected from a previously described control population (1): H1/H1, H1/H2α, H2α/H2α, H2α/H2β, H2β/H2β, H2α/H2γ and H2α/H2δ.

Differential protein binding to the -909A/C PDGFRA promoter polymorphism

We have previously shown that the five *PDGFRA* promoter haplotypes differ in their ability to drive luciferase expression after transient transfection into human osteosarcoma (HOS) cells. Osteosarcoma cells form a relevant model system, since improper bone formation during axial development forms a general characteristic of NTDs. In order to investigate if these differences in promoter activity correlate with altered binding of nuclear proteins to these haplotypes, we performed electrophoretic mobility shift assays (EMSAs) on HOS nuclear extracts using double stranded oligonucleotides of 24-29 bp length, each containing one of the ten identified haplotype-coupled polymorphisms in the –3600/+118 PDGFRA promoter region. Our data revealed differential binding of HOS nuclear proteins on the polymorphisms –909C/A and +68GAins/del only. The -909A allele is specific for the low activity H2γ haplotype, whereas the GA deletion is a characteristic of the low activity H1 haplotype.

Fig. 2a shows a protein complex that binds the -909C, but not the -909A allele (lanes 1 and 4). Addition of 100x molar excess of unlabeled -909C oligonucleotide as competitor resulted in an almost complete disappearance of the radiolabeled complex (lane 2), whereas addition of a similar excess of unlabeled -909A oligonucleotide failed to do so (lane 3). Addition of unlabeled competitor DNA to the -909A allele had no effect (lanes 5 and 6). These studies therefore identify a protein (complex) from HOS cells that specifically binds the -909C allele.

To identify the protein(s) in this -909C complex, we performed in silico analyses on the sequences surrounding the -909A/C polymorphic site with TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). We identified USF (upstream stimulatory factor) and N-MYC as potential binders to the -909C, but not to the -909A allele. When considering non-vertebrate transcription factors as well, the –909C but not the –909A allele also contains a potential binding site for Hairy, the Drosophila homolog of the vertebrate Hes (Hairy and Enhancer of split) family of transcription factors. Interestingly, both N-Myc and Hes1 have been associated with NTDs in mice (20,21), while proper expression of both members of the Usf family, Usf1 and Usf2, is known to be important for normal brain function (22).

In order to investigate the presence of USF proteins in the -909C complex,

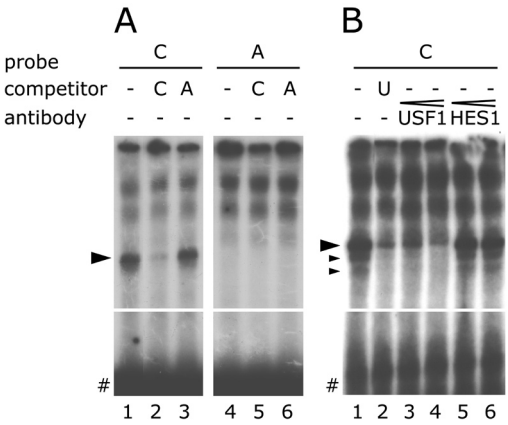

Fig. 2. Characterization of differential protein binding at the -909 polymorphic site. [^{32}P]-labeled probes containing either -909C or -909A (see Table 1) were incubated with HOS nuclear extracts and protein-DNA complexes were resolved by non-denaturing gel electrophoresis. A: Protein-DNA complexes were competed with 100x molar excess of either unlabeled -909C (C) or -909A (A). All samples were run on the same gel. B: Protein-DNA complexes were either competed with 100x molar excess of an unlabeled oligonucleotide containing a USF-1 consensus binding site (U), or alternatively pre-incubated with increasing amounts (2 and 4 μg respectively) of antibodies against USF-1 or HES-1. All samples were run on the same gel. A major and two minor -909C specific complexes are indicated by the large and small arrow heads, respectively. Free probe is indicated by the hash.

a competition EMSA was performed with an oligonucleotide that contains the USF consensus binding sequence as competitor. Fig. 2b shows that this oligonucleotide was able to fully compete with the radiolabeled -909C probe for binding of the HOS protein complex (lane 2), which suggests that the -909C complex may indeed contain USF proteins. Also, an EMSA with this labeled USF consensus oligonucleotide and HOS nuclear extracts displayed the same competition pattern as the -909C oligonucleotide (not shown). In addition to the major -909C binding protein complex (large arrow head), two minor DNA-protein complexes of higher mobility were present (small arrow heads) that were also specifically competed away by the USF consensus oligonucleotide, suggesting that these complexes contain USF proteins as well. The appearance of these minor complexes was only visible after a longer exposure time. Supershift analysis with a specific antibody against USF1 resulted in a dose-dependent disappearance of both the major and the minor radiolabeled complexes. However, no supershifts could be detected, most likely because the antibody interferes with DNA binding or alternatively because the supershifted complexes are not visible on the gel since they comigrate with other, non-specific complexes (lanes 3-4). Antibodies directed against the transcription factors HES1 (lanes 5-6) or N-MYC (not shown) did not result in a disappearance or supershift of the radiolabeled complexes. These data therefore identify USF1 as a component of the -909C complexes.

Differential protein binding tot the +68GAins/del PDGFRA promoter polymorphism

Fig. 3a shows a protein complex that binds specifically to +68del oligonucleotide but not the +68ins oligonucleotide (lanes 1 and 4). This complex could be fully abolished by an excess of unlabeled +68del allele, but not or only very poorly by a similar excess of

+68ins oligonucleotide (lanes 2 and 3), while addition of competitor DNA to the +68ins allele had no effect (lanes 5 and 6). These data indicate that HOS nuclear extracts contain a protein (complex) that binds with high affinity to the +68del, but with no or only low affinity to the +68ins allele. The eight remaining polymorphisms did not display differential protein binding that could be competed in a specific manner.

TFSEARCH analysis of the sequence surrounding the +68 polymorphic site did not reveal potential binding sites for known transcription factors. Therefore, we determined the molecular weight of the protein complex by a UV-crosslinking experiment in which five of the thymidines in the +68del and +68ins oligonucleotides had been replaced by bromo-deoxyuridines (BrdUs). To verify the specificity of the UV-crosslinked complex, we first carried out EMSAs to confirm that thymidine replacement by bromo-deoxyuridine and subsequent UV-crosslinking did not alter the EMSA competition pattern.

Fig. 3b shows that the +68del complex can still be detected after UV-crosslinking and that the formation of the radiolabeled complex is prevented if prior to crosslinking an excess of unlabeled +68del but not of +68ins oligonucleotide is added, similarly as in the non-crosslinked EMSA (lanes 1-3). Addition of specific competitor DNA after crosslinking left most of the +68del complex intact, indicating that crosslinking was successful (lane 4).

Subsequent SDS-PAGE analysis of the crosslinked material showed multiple radiolabeled bands (Fig. 3c) of which the one with molecular weight around 140 kDa displayed a similar competition pattern as in the EMSA. This suggests that this band contains proteins that are also present in the +68del EMSA complex. Since this 140

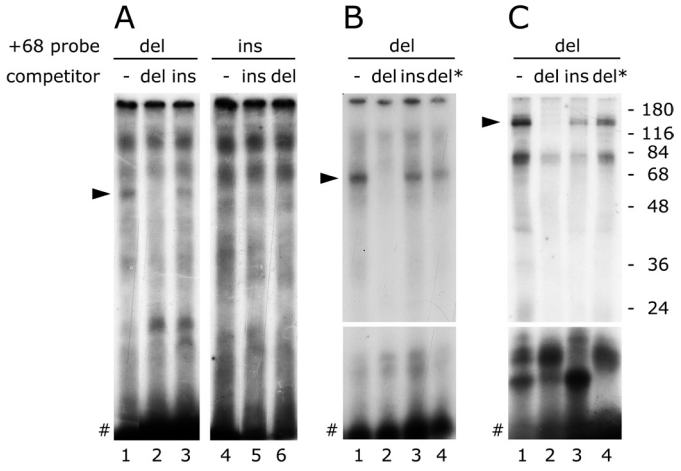

Fig. 3. Characterization of differential protein binding at the +68 polymorphic site.
A: [^{32}P]-labeled probes containing either the +68GAins or +68GAdel (see Table 1) were incubated with HOS nuclear extracts. Protein-DNA complexes were competed with 100x molar excess of unlabeled +68GAdel (del) or +68GAins (ins) oligonucleotides and resolved by non-denaturing gel electrophoresis. All samples were run on the same gel. B, C: HOS nuclear proteins binding to the +68GAdel probe containing five bromodeoxyuridine substitutions (see Table 1) were UV-crosslinked and resolved by non-denaturing gel electrophoresis (B) and denaturing gel electrophoresis (C) as described. The asterisk indicates addition of 100x fold molar excess of unlabeled +68GAdel oligonucleotide after crosslinking. The +68GAdel specific complex is indicated by the arrowhead and the free probe by the hash. Molecular weights are indicated in kDa.

kDa band reflects the weight of the bound protein together with the double stranded probe (15 kDa), the molecular weight of the +68del binding protein(s) will most likely be around 125 kDa. UV crosslinking of the -909C interacting proteins was unsuccessful, and therefore we were unable to confirm the molecular weight of USF1 complexes.

Discussion

Single nucleotide polymorphisms in regulatory DNA sequences may alter gene expression by influencing the binding affinity of transcription factors. Therefore, such SNPs could play an important role in an individual's susceptibility to complex diseases. Based on our previous observation that polymorphisms in the promoter of the human PDGFRA gene can be correlated with the risk of neural tube defects, we have extended the investigated promoter region to –3600 bp relative to the transcription start site and shown that it contains a total of eight SNPs and two ins/dels, which give rise to five distinct promoter haplotypes. It has been established that stable, heritable haplotypes can extend over many centi-Morgans (23). In a recent whole-genome analysis of common DNA variation patterns, it has been shown that at least in Caucasians *PDGFRA* promoter haplotypes extend from –36 to +8 kb relative to exon 1 (24). We have confirmed that three SNPs located approximately 7 kb downstream from the transcription start site (rs7678144, rs6554163 and rs6836215) are indeed still coupled to these promoter haplotypes.

EMSA analyses revealed that two of the polymorphic sites in the -3600/+118 *PDGFRA* promoter region, i.e. -909C/A and +68GAins/del, show differential binding of nuclear proteins from the HOS human osteosarcoma cells, an in vitro model system for bone formation during axial development. USF1 could be identified as a component of the –909C complexes, while the protein complex binding to +68GAdel was characterized to have a molecular weight of approximately 125 kDa. The differential protein binding at these two sites correlates with the low promoter activities of both the H1 and H2γ haplotypes compared to H2α and H2β. Zhu et al. (6) also observed a low promoter activity for H2γ relative to H2α upon transfection into U2OS cells, but found a similarly high promoter activity for both haplotypes in MCF-7 and HeLa cells, indicating that the activity of *PDGFRA* promoter haplotypes is clearly cell type dependent (see also (3)) . HeLa cells are known to contain functional USF proteins, while on the other hand USF proteins obtained from human SAOS osteosarcoma cells, although capable of binding to DNA, are not transcriptionally active (25). It is therefore well realized that our data do not provide direct functional evidence that USF proteins are involved in the different activities of the *PDGFRA* promoter haplotypes.

USF proteins are ubiquitously expressed transcription factors of the basic-helix-loop-helix/leucine zipper (bHLH-zip) family and are encoded by two genes, *USF1* and *USF2*. Both genes can give rise to two proteins by means of alternative splicing; USF1 and USF1/BD are encoded by the *USF1* gene, whereas USF2a and USF2b are encoded by the *USF2* gene (26,27,28). USFs bind DNA as dimers, such that under in vivo conditions over 66% of the USF binding activity is present as USF1-USF2a heterodimers, whereas homodimers of either USF1 or USF2a contribute for approximately 10% and 7% respectively, while USF1-USF2b heterodimers can account for up to 15% in some cells (28). Moreover, it is known that USF1-USF2a heterodimers display the lowest mobility on

EMSA of all USF1 containing dimers, including USF1-USF1, USF1-USF2b, USF1-USF1/BD and USF1/BD-USF1/BD (25,28,27). Most likely, the identified major USF1-containing complex that binds the -909C polymorphic site is therefore a USF1-USF2a heterodimer, while the two minor complexes may represent two of the higher mobility USF1 dimers.

We have previously postulated that aberrant transcription of *PDGFRA* can enhance the risk of neural tube defects. Aberrant transcription may result from a combination of promoter polymorphisms and the proper availability of trans-acting factors. As a result, not only *PDGFRA* itself but also genes encoding upstream regulators of *PDGFRA* can be associated with such diseases. Although a number of nuclear transcription factors are known that affect *PDGFRA* expression, such as PAX1 (10), PAX3 (11-13), GATA4 (29) and C/EBPs (30,31), a direct interaction of these factors with specific promoter regions has only been shown in a limited number of studies (15,16). The present approach differs from these previous studies, in that we have used DNA-protein complex formation as a starting point of our analysis to identify controlling elements in the promoter. In addition to our observation that USF1 can specifically bind the *PDGFRA* promoter at the –909C position, it has recently been shown in PSFK-1 cells that the zinc finger protein ZNF148 can differentially bind the -1074C/A SNP, whereby it recognizes all haplotypes except H2δ. Interestingly, H2δ has been shown to be overrepresented in patients with PNETs and ependymoma brain tumors (3).

Loss of one *Pdgfra* allele in combination with a mutated upstream regulator Pax1 results in extensive neural tube defects (8). In the light of the role of both *Pdgfra* and *Usf* genes in axial and neural development, it would be very interesting to study if *Pdgfra* (+/-)/*Usf1* (-/-) and *Pdgfra* (+/-)/*Usf2* (-/-) mice indeed show neural tube defects. Since H1 has a low promoter activity compared to H2a, the +68GAdel binding protein complex potentially acts as an H1 specific repressor of *PDGFRA* transcription. Using a transcription factor trapping approach (32), we are currently isolating larger amounts of this protein(s), to allow its identification by the use of mass spectrometry techniques. It will be very interesting to see if the corresponding gene can be associated directly with neural tube defects. Deregulated expression of PDGFRA has also been associated with various neoplasias, including osteosarcomas (33), chondrosarcomas (34), gastrointestinal stromal tumors (35) and glia brain tumors (36-40). It will be of great importance to study if the risk for development of such tumors can also be associated with *PDGFRA* promoter haplotypes and upstream regulators of this gene.

Acknowledgements

We would like to thank Dr. Paul H.L.J. Joosten for fruitful discussions, and the Hersenstichting Nederland for financial support.

References

1. P.H. Joosten, M. Toepoel, E.C. Mariman and E.J. Van Zoelen, Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects, Nat Genet 27 (2001) 215-7.
2. S.M. Herrmann, S. Ricard, V. Nicaud, E. Brand, I. Behague, H. Blanc, J.B. Ruidavets, A. Evans, D. Arveiler, G. Luc, O. Poirier and F. Cambien, Polymorphisms in the genes encoding platelet-derived growth factor A and alpha receptor, J Mol Med 78 (2000) 287-92.
3. C. De Bustos, A. Smits, B. Stromberg, V.P. Collins, M. Nister and G. Afink, A PDGFRA promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumours and ependymomas, J Med Genet 42 (2005) 31-7.
4. J. Payne, F. Shibasaki and M. Mercola, Spina bifida occulta in homozygous Patch mouse embryos, Dev Dyn 209 (1997) 105-16.
5. P. Soriano, The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites, Development 124 (1997) 2691-700.
6. H. Zhu, N.J. Wicker, K. Volcik, J. Zhang, G.M. Shaw, E.J. Lammer, L. Suarez, M. Canfield and R.H. Finnell, Promoter haplotype combinations for the human PDGFRA gene are associated with risk of neural tube defects, Mol Genet Metab 81 (2004) 127-32.
7. A.J. Copp and M. Bernfield, Etiology and pathogenesis of human neural tube defects: insights from mouse models, Curr Opin Pediatr 6 (1994) 624-31.
8. U. Helwig, K. Imai, W. Schmahl, B.E. Thomas, D.S. Varnum, J.H. Nadeau and R. Balling, Interaction between undulated and Patch leads to an extreme form of spina bifida in double-mutant mice, Nat Genet 11 (1995) 60-3.
9. F.A. Hol, M.P. Geurds, S. Chatkupt, Y.Y. Shugart, R. Balling, C.T. Schrander-Stumpel, W.G. Johnson, B.C. Hamel and E.C. Mariman, PAX genes and human neural tube defects: an amino acid substitution in PAX1 in a patient with spina bifida, J Med Genet 33 (1996) 655-60.
10. P.H. Joosten, F.A. Hol, S.E. van Beersum, H. Peters, B.C. Hamel, G.B. Afink, E.J. van Zoelen and E.C. Mariman, Altered regulation of platelet-derived growth factor receptor-alpha gene-transcription in vitro by spina bifida-associated mutant Pax1 proteins, Proc Natl Acad Sci U S A 95 (1998) 14459-63.
11. E.D. Dickman, R. Rogers and S.J. Conway, Abnormal skeletogenesis occurs coincident with increased apoptosis in the Splotch (Sp2H) mutant: putative roles for Pax3 and PDGFRalpha in rib patterning, Anat Rec 255 (1999) 353-61.
12. D.J. Henderson, S.J. Conway and A.J. Copp, Rib truncations and fusions in the Sp2H mouse reveal a role for Pax3 in specification of the ventro-lateral and posterior parts of the somite, Dev Biol 209 (1999) 143-58.
13. J.A. Epstein, B. Song, M. Lakkis and C. Wang, Tumor-specific PAX3-FKHR transcription factor, but not PAX3, activates the platelet-derived growth factor alpha receptor, Mol Cell Biol 18 (1998) 4118-30.
14. D. ten Berge, A. Brouwer, J. Korving, J.F. Martin and F. Meijlink, Prx1 and Prx2 in skeletogenesis: roles in the craniofacial region, inner ear and limbs, Development 125 (1998) 3831-42.
15. P.H. Joosten, M. Toepoel, D. van Oosterhout, G.B. Afink and E.J. van Zoelen, A regulating element essential for PDGFRA transcription is recognized by neural tube defect-associated PRX homeobox transcription factors, Biochim Biophys Acta 1588 (2002) 254-60.

16. G.B. Afink, M. Nister, B.H. Stassen, P.H. Joosten, P.J. Rademakers, E. Bongcam-Rudloff, E.J. Van Zoelen and S. Mosselman, Molecular cloning and functional characterization of the human platelet-derived growth factor alpha receptor gene promoter, Oncogene 10 (1995) 1667-72.
17. T. Sun, D. Jayatilake, G.B. Afink, P. Ataliotis, M. Nister, W.D. Richardson and H.K. Smith, A human YAC transgene rescues craniofacial and neural tube development in PDGFRalpha knockout mice and uncovers a role for PDGFRalpha in prenatal lung growth, Development 127 (2000) 4519-29.
18. X.Q. Zhang, G.B. Afink, K. Svensson, J.J. Jacobs, T. Gunther, K. Forsberg-Nilsson, E.J. van Zoelen, B. Westermark and M. Nister, Specific expression in mouse mesoderm- and neural crest-derived tissues of a human PDGFRA promoter/lacZ transgene, Mech Dev 70 (1998) 167-80.
19. J. Sambrook, E.F. Fritsch and T. Maniatis, Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
20. J. Charron, B.A. Malynn, P. Fisher, V. Stewart, L. Jeannotte, S.P. Goff, E.J. Robertson and F.W. Alt, Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene, Genes Dev. 6 (1992) 2248-57.
21. M. Ishibashi, S.L. Ang, K. Shiota, S. Nakanishi, R. Kageyama and F. Guillemot, Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects, Genes Dev. 9 (1995) 3136-48.
22. M. Sirito, Q. Lin, J.M. Deng, R.R. Behringer and M. Sawadogo, Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice, Proc Natl Acad Sci U S A 95 (1998) 3758-63.
23. A. Bergman, Z. Einbeigi, U. Olofsson, Z. Taib, A. Wallgren, P. Karlsson, J. Wahlstrom, T. Martinsson and M. Nordling, The western Swedish BRCA1 founder mutation 3171ins5; a 3.7 cM conserved haplotype of today is a reminiscence of a 1500-year-old mutation, Eur J Hum Genet 9 (2001) 787-93.
24. D.A. Hinds, L.L. Stuve, G.B. Nilsen, E. Halperin, E. Eskin, D.G. Ballinger, K.A. Frazer and D.R. Cox, Whole-genome patterns of common DNA variation in three human populations, Science 307 (2005) 1072-9.
25. Y. Qyang, X. Luo, T. Lu, P.M. Ismail, D. Krylov, C. Vinson and M. Sawadogo, Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation, Mol Cell Biol 19 (1999) 1508-17.
26. M. Sirito, Q. Lin, T. Maity and M. Sawadogo, Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells, Nucleic Acids Res 22 (1994) 427-33.
27. T. Saito, T. Oishi, K. Yanai, Y. Shimamoto and A. Fukamizu, Cloning and characterization of a novel splicing isoform of USF1, Int J Mol Med 12 (2003) 161-7.
28. B. Viollet, A.M. Lefrancois-Martinez, A. Henrion, A. Kahn, M. Raymondjean and A. Martinez, Immunochemical characterization and transacting properties of upstream stimulatory factor isoforms, J Biol Chem 271 (1996) 1405-15.
29. C. Wang and B. Song, Cell-type-specific expression of the platelet-derived growth factor alpha receptor: a role for GATA-binding protein, Mol Cell Biol 16 (1996) 712-23.
30. T. Fukuoka, Y. Kitami, T. Okura and K. Hiwada, Transcriptional regulation of the platelet-derived growth factor alpha receptor gene via CCAAT/enhancer-binding protein-delta in vascular smooth muscle cells, J Biol Chem 274 (1999) 25576-82.
31. Z.H. Yang, Y. Kitami, Y. Takata, T. Okura and K. Hiwada, Targeted overexpression of CCAAT/enhancer-binding protein-delta evokes enhanced gene transcription of

platelet-derived growth factor alpha-receptor in vascular smooth muscle cells, Circ Res 89 (2001) 503-8.

32. H. Gadgil and H.W. Jarrett, Oligonucleotide trapping method for purification of transcription factors, J Chromatogr A 966 (2002) 99-110.
33. I. Sulzbacher, P. Birner, K. Trieb, M. Traxler, S. Lang and A. Chott, Expression of platelet-derived growth factor-AA is associated with tumor progression in osteosarcoma, Mod Pathol 16 (2003) 66-71.
34. I. Sulzbacher, P. Birner, K. Trieb, M. Muhlbauer, S. Lang and A. Chott, Platelet-derived growth factor-alpha receptor expression supports the growth of conventional chondrosarcoma and is associated with adverse outcome, Am J Surg Pathol 25 (2001) 1520-7.
35. H. Yamamoto, Y. Oda, K. Kawaguchi, N. Nakamura, T. Takahira, S. Tamiya, T. Saito, Y. Oshiro, M. Ohta, T. Yao and M. Tsuneyoshi, c-kit and PDGFRA mutations in extragastrointestinal stromal tumor (gastrointestinal stromal tumor of the soft tissue), Am J Surg Pathol 28 (2004) 479-88.
36. J. Andrae, C. Molander, A. Smits, K. Funa and M. Nister, Platelet-derived growth factor-B and -C and active alpha-receptors in medulloblastoma cells, Biochem Biophys Res Commun 296 (2002) 604-11.
37. A. Das, W.L. Tan, J. Teo and D.R. Smith, Glioblastoma multiforme in an Asian population: evidence for a distinct genetic pathway, J Neurooncol 60 (2002) 117-25.
38. C.B. Knobbe and G. Reifenberger, Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas, Brain Pathol 13 (2003) 507-18.
39. L. Uhrbom, G. Hesselager, A. Ostman, M. Nister and B. Westermark, Dependence of autocrine growth factor stimulation in platelet-derived growth factor-B-induced mouse brain tumor cells, Int J Cancer 85 (2000) 398-406.
40. J.S. Smith, X.Y. Wang, J. Qian, S.M. Hosek, B.W. Scheithauer, R.B. Jenkins and C.D. James, Amplification of the platelet-derived growth factor receptor-A (PDGFRA) gene occurs in oligodendrogliomas with grade IV anaplastic features, J Neuropathol Exp Neurol 59 (2000) 495-503.

Chapter 5

Haplotype-specific expression of the human PDGFRA gene correlates with the risk of glioblastomas

Submitted to: Oncogene
By: Mascha Toepoel, Paul H.L.J. Joosten, Christiane B. Knobbe, Gijs B. Afink, Rainer B. Zotz, Regine P.M. Steegers-Theunissen, Guido Reifenberger, and Everardus J.J. van Zoelen

Abstract

Aberrant expression of the platelet-derived growth factor α-receptor (PDGFRA) gene has been associated with various diseases, including neural tube defects and glioma malignancies. We have previously shown that the promoter region of the human PDGFRA gene contains several polymorphisms which can be grouped into five distinct haplotypes, designated H1, H2α, H2β, H2γ and H2δ, of which H1 and H2α are the most common. Transient transfection studies have indicated that H1 mediates low and H2α high transcriptional activity. In the present study we have investigated the role of these *PDGFRA* promoter polymorphisms in gliomagenesis, both at the cellular and genetic level. Our results show that in three out of four H1/H2α heterozygous human glioblastoma cell lines tested, the H1-derived mRNA levels are more than 10-fold lower than those from H2α, resulting in near monoallelic expression of the H2α allele. This appeared to result at least in part from haplotype-specific differences in epigenetic regulation, i.e. DNA methylation and histone acetylation. Furthermore, in a case-control study on a panel of 71 glioblastoma patients we observed an underrepresentation of H1 alleles which was significant when compared to a combined Western European control group (n=998, $p<0.05$). Together, these results indicate that *PDGFRA* promoter haplotypes may provide a genetic predisposition for gliomagenesis. In combination with published data on *Pdgfra* transgenic mice, we propose a model in which PDGFRA promoter polymorphisms control the upregulation of PDGFRA during differentiation of neural stem cells into glia cells, and thereby the pool size of cells that can undergo gliomagenesis.

Introduction

Platelet-derived growth factor α-receptor (PDGFRA) signaling plays an important role in normal neural development, as well as in the pathogenesis of various disorders of the central nervous system. Platelet-derived growth factors (PDGFs) act as potent mitogens and chemo-attractants for oligodendrocyte precursor cells (OPCs/O2A cells) both *in vitro* (1-3) and *in vivo* (4, 5). Mouse OPCs express Pdgfra at high levels (6, 7) and its expression level directly affects OPC proliferation and differentiation *in vivo* (8, 9). Defective Pdgfra signaling in transgenic mice leads to various developmental defects, including neural tube defects (NTDs) such as spina bifida, and severe hypomyelination as a result of impaired oligodendrocyte development (4, 8, 10-13). On the other hand, different types of human gliomas and glioma cell lines co-express PDGFs with PDGFRA (14-16). In the mouse, constitutive Pdgfra activation by overexpression of its ligand gene Pdgfb results in gliomagenesis, whereby the amount of PDGF signaling correlates with glioma grade (17-20), whereas in humans malignant gliomas often show amplification of *PDGFRA* (21-25). This suggests that reduced PDGFRA signaling may lead to neural tube defects and hypomyelination, while on the other hand increased PDGFRA signaling may be involved in the initiation and progression of gliomas.

DNA polymorphisms such as small DNA insertion/deletions (ins/del) and single nucleotide polymorphisms (SNPs) in regulatory DNA sequences are believed to play an important role in the regulation of expression levels of the corresponding genes, and as a consequence they may directly affect the susceptibility of individuals for diseases

related to those genes. We have previously shown that the –3600/+118 promoter region of *PDGFRA* contains a total of ten polymorphic sites that give rise to five distinct haplotypes, designated H1, H2α, H2β, H2γ and H2δ (26, 27). H1 and H2α, which differ in eight out of these ten positions (26-29), are the two most common haplotypes, with allele frequencies of 21% and 68%, respectively, in the Western European population (26, 29-31). Upon transient transfection into human osteosarcoma cells, H2α displayed a three- to six-fold higher promoter activity than H1 (26, 28). We ((26); M. Toepoel, R.P.M. Steegers-Theunissen and E.J.J. van Zoelen, unpublished) and others (28, 29) have found an increased H1 frequency in a population of patients with NTDs, suggesting that individuals with the H1 haplotype have an increased risk of developing diseases that are characteristic for reduced PDGFRA signaling.

In the present study we have tested the hypothesis that polymorphisms in the *PDGFRA* promoter region may contribute to the genetic predisposition of individuals to develop glioblastoma multiforme, the most common and most aggressive form of primary brain tumors (32). In H1/H2 heterozygous glioblastoma cell lines in culture we determined the endogenous activity of both promoter alleles by the use of a newly developed technique. Our data show that in three out of four cell lines tested the H1 allele displays very low transcriptional activity compared to the H2α allele, which is paralleled by differences in epigenetic regulation. Moreover, in a panel of glioblastoma patients, we observed a reduced frequency of the H1 allele, which suggests that the low activity H1 allele may protect against gliomagenesis. These results identify *PDGFRA* promoter haplotypes as an important genetic factor in the predisposition for gliomas.

Materials and Methods

Cell culture and drug treatment

U-251 MGsp, U-343 MGa 31L, U-343 MGa Cl2:6 and U-410 MG human glioblastoma cells (33-35) were maintained in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS; Invitrogen, Carlsbad, CA), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 7.5% CO_2. For treatment of cells with 5-azacytidine (5AC) and trichostatin A (TSA), both obtained from Sigma-Aldrich (St-Louis, MO), cells were seeded at a density of 2.0 x 10^5 cells/cm^2, with daily medium refreshment. One day after seeding, cells were incubated for 48 hours in medium with or without 5AC (1 μM), and subsequently for an additional 24 hours with or without 5AC and/or TSA (0.5 μM).

DNA, RNA isolation and cDNA synthesis

Cultured cells were grown to confluency in a 9.6 cm^2 dish, genomic DNA (gDNA) was isolated using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Total RNA was isolated from cells grown in monolayer using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. DNA and RNA concentrations were determined by measuring the absorbance at 260 nm. Total RNA (1 μg) was reverse transcribed using random hexamer primers (Amersham–Pharmacia, United Kingdom) in a total volume of 50 μl, using the SUPERSCRIPT II reverse transcriptase kit (Invitrogen). RNA from glioblastoma tumor samples was extracted, purified and reverse transcribed as described elsewhere (36).

Polymerase Chain Reaction (PCR) for allele specificity
Forward primer exon1F (5'-GGAGCTACAGGGAGAGAAAC-3') was end-labeled using T4 polynucleotide kinase (Fermentas, Burlington, Ontario) and [γ-^{32}P]dATP (ICN, Irvine, CA), and subsequently purified over a Sephadex G50 column. *PDGFRA* cDNA and gDNA was amplified using labeled forward primer exon1F and reverse primer exon3R (5'-AAAGCTGGCAGAGGATTAGG-3') or reverse primer intron1R (5'-GACACACAACTGGAATCCAG-3') respectively. PCR mixtures contained 1x PCR buffer, 1 mM $MgCl_2$, 1 unit of Biotherm DNA polymerase (GeneCraft, Germany), 0.4 mM of each dNTP (Fermentas), 100 ng of each primer and 15 ng of genomic DNA or 1-5 µl of cDNA in a total volume of 25 µl. Cycling parameters were 5 min at 95°C, followed by 30-40 cycles of 30 sec at 95°C, 30 sec at 62°C and 1 min at 72°C, with a subsequent extension of 10 min at 72°C.

Sample preparation and polyacrylamide gel electrophoresis
PCR products were digested for one hour at 37°C with 2 units of BsuRI/HaeIII (Fermentas). Subsequently, 5 µl of digested PCR product were precipitated by addition of 15 µl H_2O (milliQ), 2 µl of 3 M NaAc pH 5.2 and 50 µl of 96% ethanol for at least 30 min at -20°C, and subsequently centrifuged for 30 min at maximum speed in an Eppendorf centrifuge. The pellet was washed twice with 70% ethanol, dried and dissolved in 3 µl of formamide loading buffer (98% deionized formamide, 10 mM EDTA, bromophenolblue and xylene cyanole). Just prior to electrophoresis, the samples were incubated for 5 min at 96°C and immediately chilled in an ice-water bath. Samples were analyzed on a pre-electrophoresed 13% acrylamide gel containing 8 M urea (37) at 75W, until the xylene cyanole had run to the bottom of the gel. After electrophoresis, the gel was fixed for 45 min in 10% methanol/10% HAc and vacuum dried for 30 min at 85°C. Digested PCR products were visualized and quantified by analysis on a Personal Molecular Imager FX System with Quantity One software (BioRad, Hercules, CA).

Real-time quantitative PCR
Real-time quantitative RT-PCR on 2-5 µl cDNA with primers PDGFRA-F (5'-AAAGAAGTTCCAGACCATCCC-3') x PDGFRA-R (5'-AGGTGACCACAATCGTTTCC-3') and β-actin-F (5'-GAAGAGCTACGAGCTGCCTGA-3') x β-actin-R (5'-GAACCGCTCATTGCCAATG-3') and calculation of expression values was performed as described (38).

Bisulphite sequencing
A total of 1 µg of gDNA was converted with the EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the manufacturer's protocol, after which a PCR reaction was performed on 50 ng of the converted DNA with primers F-1064CpG (5'-GTTTAGGTT TGGTTTTTGGAGTATTAG-3') and R-732CpG (5'-ACCTTTACTCCCTTAAAATCACTTATC-3') designed to recognize bisulphite-modified DNA only. PCR conditions were the same as above. Next, 1-2 µl of PCR product were ligated into pCR2.1 using the TA-cloning kit (Invitrogen) and individual clones were sequenced on an ABI310 genetic analyzer using the Big Dye terminator sequencing kit (Applied Biosystems, Foster City, CA).

Population studies and haplotype determination
Tumor and peripheral blood samples from German glioblastoma multiforme (WHO

grade IV) patients, as well as from age and sex matched German blood donors were obtained as described (23), following the approval of the local institutional review board of the Heinrich-Heine-University (Düsseldorf, Germany). DNA was extracted from these samples as described (36). Genomic DNA from Dutch and Northern Irish controls were obtained as described (30, 39, 40). For haplotype determination, a PCR reaction was performed on genomic DNA with primer -1651F (5'-AGCCTAAAAACCCGGTTCTC-3') and -727R (5'-TCCCTTGAAGTCACTTATCTG-3') under similar PCR conditions as above. PCR products were purified using the PCR purification kit (QIAgen, Germany). Automated sequencing was performed on the ABI310 genetic analyzer using the Big Dye terminator sequencing kit (Applied Biosystems).

Results

Haplotype-specific *PDGFRA* expression and gene amplification in human glioblastoma cell lines

Transient transfection assays are frequently used to study the role of promoter polymorphisms in transcriptional activity, but such assays do not take into account the epigenetic control of gene expression within its physiological chromatin context. We have therefore developed an assay that permits quantification of endogenous haplotype-specific *PDGFRA* expression levels, as well as of haplotype-specific *PDGFRA* gene amplification. This assay is based on the observation that one of the polymorphisms that discriminates H1 from all H2 haplotypes, the GA insertion/deletion (ins/del) in non-coding exon 1, is transcribed and can thus be used to determine the haplotype origin of *PDGFRA* transcripts (26-29). Following a PCR reaction with a radiolabeled forward primer that anneals just 5' of the GA ins/del and an unlabeled reverse primer that anneals in exon 3 (for cDNA) or in intron 1 (for genomic DNA, gDNA), and subsequent cleavage with BsuRI/HaeIII just 3' of the ins/del, a blunt product of 49 bp product was obtained for H1 and a 51 bp product for all H2 haplotypes (Fig. 1A), of which the relative amounts were quantified by molecular imager analysis after separation by denaturing gel electrophoresis. Most reliable results were obtained with a polymerase that has the ability to add adenosines to the 3' end of each formed product, and consequently we obtained products of both 51 and 52 nt for H2, and of 49 and 50 nt for H1, depending on the efficiency of the polymerase after cleavage. To determine the relative intensities of H1 and H2, we summed up the intensities of the two bands for each haplotype.

When applying this technique to primary human fibroblasts obtained from healthy individuals of three different *PDGFRA* genotypes, i.e. H1/H1, H1/H2α and H2α/H2α, the two homozygous cell lines showed only expression of their corresponding haplotype, whereas in the heterozygous fibroblasts the H1 and H2α promoter allele induced equal transcript levels, as shown in Fig. 1B and quantified in Fig. 1C (upper panel). At the genomic level, the homo- and heterozygosity of the three cell lines was confirmed, with equal amounts of H1 and H2α DNA in the heterozygous cells (Fig. 1B and 1C, lower panel). Thus, unlike previous observations with promoter transfection studies in human osteosarcoma cells, no difference was observed in endogenous H1 and H2α activity in primary fibroblasts.

To study the role of *PDGFRA* expression in gliomagenesis, we subsequently

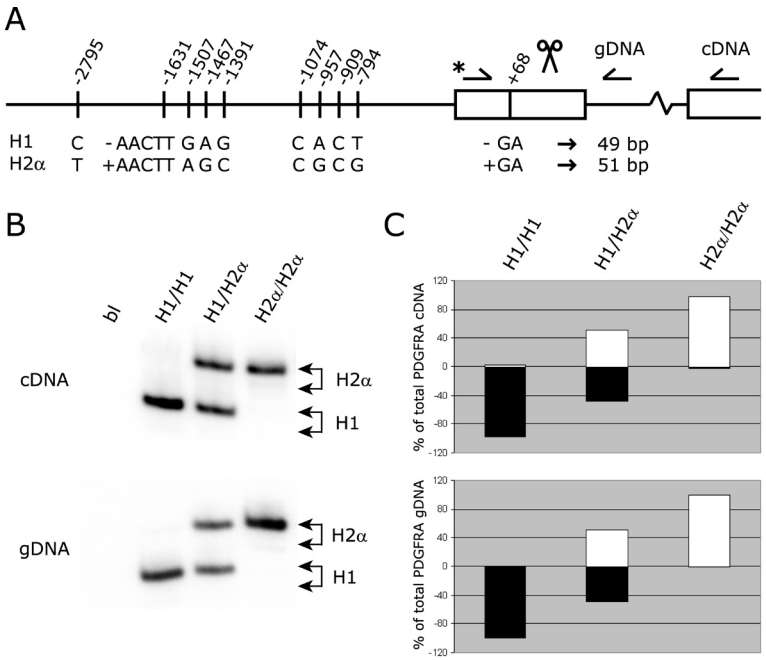

Figure 1. Haplotype-specific *PDGFRA* expression and gene amplification. **A:** Schematic representation of the two main *PDGFRA* haplotypes H1 and H2α and the location of the primers for amplification of cDNA and genomic DNA (gDNA). Due to the haplotype coupled GA ins/del in exon 1, HaeIII/BsuRI (indicated by the scissors) digested amplicons of H1 are 49/50 bp and of H2α 51/52 bp respectively (see arrows in B). **B:** Denaturing gel electrophoresis of digested PCR fragments from cDNA and gDNA isolated from primary fibroblasts with haplotype combinations H1/H1, H1/H2α and H2α/H2α. In the first lane (bl) no template was used. **C:** Quantification of band intensities by molecular imager analysis. The sum of the intensities of the H1 and H2α bands was set to 100%. The percentage of H1 is represented by the black bars and the percentage of H2α by the white bars.

analyzed H1/H2 heterozygous human glioblastoma cell lines for haplotype-specific *PDGFRA* expression. Three out of the four H1/H2α heterozygous cell lines thus identified, designated U-251 MGsp, U-343 MGa 31L and U-343 MGa Cl2:6 (hereafter referred to as U-251, 31L and Cl2:6 respectively), displayed only very low H1 activity, resulting in near monoallelic expression of the H2α allele (see Fig. 2A, upper panel). Only the U-410 MG cell line (hereafter referred to as U-410) exhibited equal expression levels of both alleles. Quantification revealed that only 2, 15 and 13%, respectively, of the total *PDGFRA* mRNA level in U-251, 31L and Cl2:6 cells resulted from H1 activity, compared to 52% in U-410 cells (see Fig. 2B, upper panel). Thus, it appears that in three out of the four heterozygous glioblastoma cell lines tested the H1 allele is strongly repressed compared to H2α.

Since *PDGFRA* is often amplified in gliomas, we also analyzed these cells for haplotype-specific *PDGFRA* amplification. It is known that these cell lines show aberrant karyotypes with amplifications of the 4q12 chromosomal region which includes *PDGFRA* (41). Figure 2A (lower panel) shows that in U-251 and 31L cells, H2α DNA is clearly more

abundant than H1 (33% and 31% H1, respectively; see Fig. 2B, lower panel), whereas in Cl2:6 H1 is more abundant than H2α (60% H1), while equal copy numbers appear to be present in U-410 cells (50% H1). It therefore appears that in spite of the H1 amplification in Cl2:6 cells, the H2α allele gives rise to higher *PDGFRA* mRNA levels than the H1 alleles combined. On the other hand the relative amplification of the H2α allele in U-251 and 31L cells does not account for the relatively low activity of H1 in these cells.

In order to verify that the different expression levels of H1 and H2α are an intrinsic property of the U-251, 31L and Cl2:6 cell lines and do not result from cell heterogeneity in culture, we subcloned each of these cell lines by limiting dilution. Twelve clones of each cell line were analyzed and all appeared to display the same haplotype-specific expression profile as the parental cells (data not shown), indicating that either all cells in the original culture behaved similarly in terms of haplotype-specific expression, or that the cell populations grown from the individual clones had differentiated again into the same heterogeneous population.

The present assay gives only information about the ratio of H1 and H2 activity, and therefore we combined this analysis with real-time quantitative RT-PCR to examine total *PDGFRA* expression levels in these cells. This revealed great differences in basal *PDGFRA* expression levels between the various cell lines, with the highest value relative to the β-actin house-keeping gene for U-410 (0.2), followed by 31L (0.02), U-251 (0.001) and Cl2:6 (0.0004). It therefore appears that in U-410 cells both the H1 and H2α allele are much higher expressed than in each of the other cell lines. These data agree with

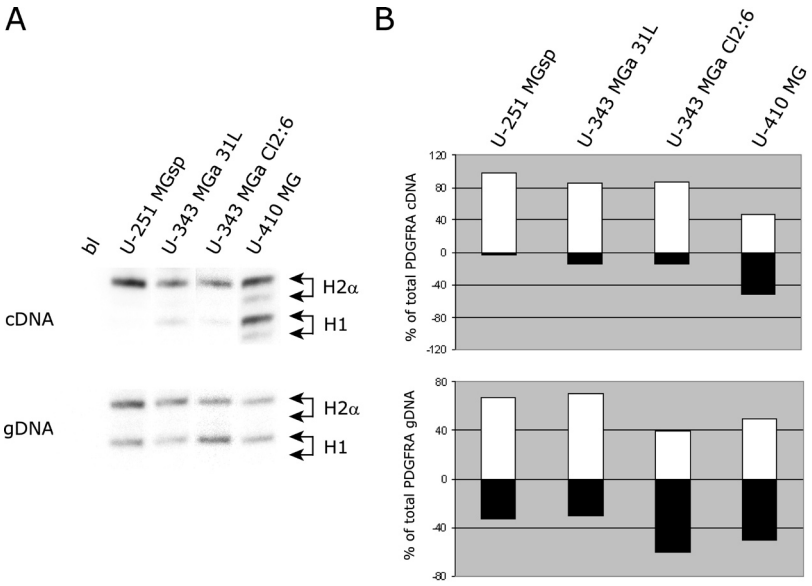

Figure 2. Haplotype-specific *PDGFRA* expression and gene amplification in four H1/H2α heterozygous human glioblastoma cell lines. **A:** Denaturing gel electrophoresis of digested PCR fragments from cDNA and gDNA isolated from Cl2:6, U-251, 31L and U-410 cells; bl: no template used. **B:** Quantification of the relative intensities of the H1 and H2α bands. The percentage of H1 is represented by the black bars and the percentage of H2α by the white bars.

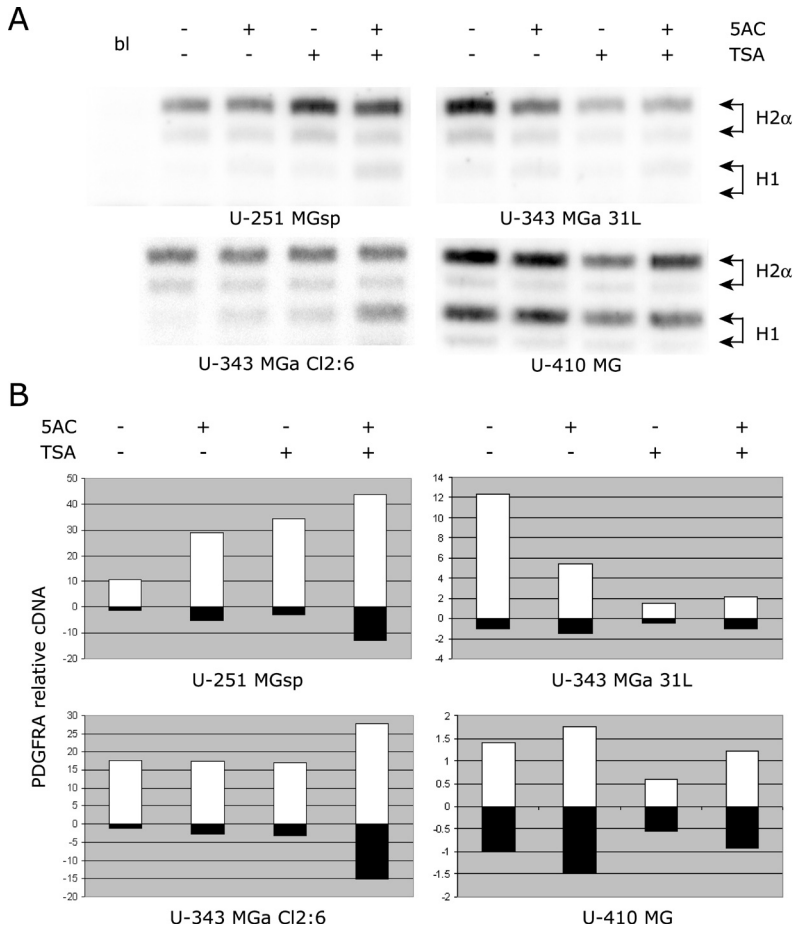

Figure 3. Effect of 5-azacytidine (5AC; 1 μM) and trichostatin A (TSA; 0.5 μM) on haplotype-specific and total *PDGFRA* expression. Glioblastoma cells were cultured for 2 days in the presence or absence of 5AC followed by an additional day in the presence or absence of 5AC and/or TSA. **A:** Gel electrophoretic separation of expressed *PDGFRA* haplotypes; bl: no template used. **B**: Quantitative analysis of haplotype-specific *PDGFRA* expression. Total *PDGFRA* expression relative to β-actin was determined by quantitative RT-PCR, multiplied by the fraction of haplotype-specific expression and corrected for the haplotype copy number (with the untreated H1 activity set to 1). H1 is represented by the black bars and H2α by the white bars.

previous studies on the expression level of PDGFRA in these cells at both the protein and mRNA level (42), with the exception of the 31L cell line for which we have used another subclone.

Haplotype-specific epigenetic regulation of PDGFRA expression

To investigate whether epigenetic regulation is involved in the observed repression of the H1 allele in U-251, 31L and Cl2:6 cells, cultures were treated with the DNA demethylating agent 5-azacytidine (5AC) and the histone deacetylase inhibitor trichostatin A (TSA). Figure 3A shows that particularly in U-251 and Cl2:6 cells the relative

expression of H1 is enhanced by this treatment.

Figure 3B shows the absolute expression levels of the individual H1 and H2α alleles in these glioblastoma cell lines, relative to β-actin, corrected for the observed differences in relative haplotype copy number (obtained from Fig. 2B). In the absence of 5AC and TSA, an 11-fold enhanced expression of H2α compared to H1 is observed in U-251 cells, reaching a 12-fold difference in 31L cells and a 17-fold difference in Cl2:6 cells. Upon treatment of the cells with 5AC and TSA, not only the expression level of both the H1 and H2α allele is increased, particularly in U-251 and Cl2:6 cells, but also the relative expression of H1 compared to H2α, resulting in only a two-fold difference in expression level in Cl2:6 cells after the combined treatment. TSA appeared toxic for the 31L and U-410 cells, and 5AC particularly for 31L cells, as judged from the decrease in total *PDGFRA* expression and the large amount of dead cells after treatment. These data indicate that epigenetic regulation controls at least in part the difference in activity between the H1 and H2α allele in these glioblastoma cells, although under all conditions tested H2α remained more active than H1.

Haplotype-specific PDGFRA promoter methylation

The observed low activity of H1 as compared to H2α in these heterozygous glioblastoma cells may result from differences in CpG methylation of the respective promoter alleles. The -3600/+118 *PDGFRA* promoter contains two putative CpG islands, i.e. regions with a relatively high CpG content, ranging from -2482 to -2172 and from -1119 to -722 (http://www.ebi.ac.uk/emboss/cpgplot/). Only the latter CpG island contains polymorphic sites that discriminate between H1 and H2α (-957G/A and -794G/T), and therefore we analyzed this promoter region for haplotype-specific CpG methylation by bisulphite sequencing.

Figure 4 shows the CpG methylation patterns of the H1 and H2α -1020/-748 promoter region, which contains a total of 23 CpG residues, of the four glioblastoma

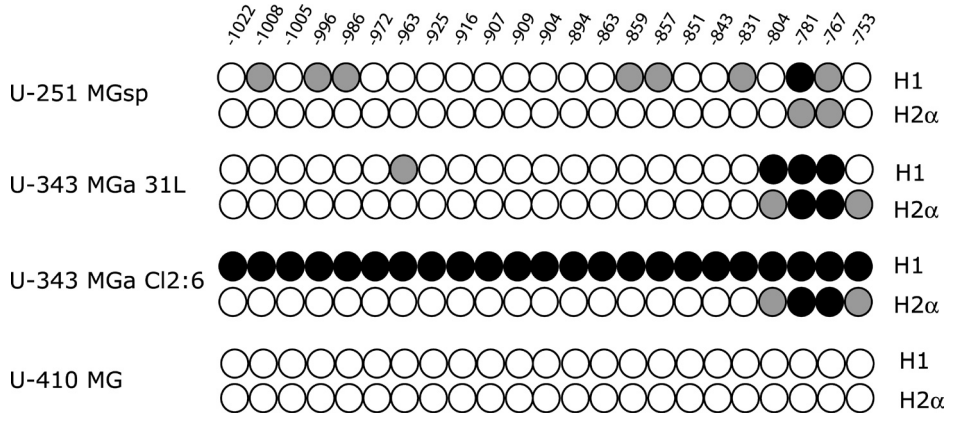

Figure 4. Haplotype-specific CpG methylation in the -1020/-748 *PDGFRA* promoter region. Methylation patterns were determined from at least ten clones for each normal or overrepresented allele, and at least four clones for underrepresented alleles. Results have been averaged for each CpG position, in which open circles represent less than 25% methylation, gray circles represent methylation between 25% and 75%, and black circles represent methylation of 75% and more. Nucleotide positions of the CpGs are indicated.

cell lines. Haplotype-specific CpG methylation patterns were observed in all three H1 repressing cell lines U-251, 31L and Cl2:6, whereby H1 was methylated more frequently and on more positions than H2α. Differential methylation was particularly evident in Cl2:6 cells, where all 23 CpG residues of the H1 allele were mostly methylated, whereas H2α showed only frequent methylation on the four most 3' located CpG residues. These data show that in heterozygous cells the two alleles of a specific gene can strongly differ in their methylation status. By contrast, none of the 23 CpG residues were methylated in U-410 cells on either allele, which corresponds well with the high total *PDGFRA* expression level in these cells, equally distributed over both haplotypes. In conclusion, hypermethylation of H1 compared to H2α can explain at least in part the differential expression of both haplotypes in the three H1 repressing glioma cell lines.

H1 frequencies and risk of glioblastoma formation

We subsequently analyzed the *PDGFRA* promoter haplotype distribution in blood samples of a group of 71 patients with glioblastoma multiforme and a group of 44 matched controls, both from Germany (Düsseldorf). The H1 is a relatively rare allele in Caucasian control populations (approximately 21%), and therefore large patient and matched control groups are required to establish a significant deviation. We therefore used the strategy to show that the present control group is highly similar to a large Western European control group, while the patient group significantly deviates from this Western European control group. Table I shows the distribution of three new control cohorts (146 from Nijmegen, the Netherlands; 98 from Belfast, UK; and 44 from Düsseldorf, Germany), that were each selected as matched controls for comparison with a specific patient cohort (suffering from spina bifida, coronary heart disease and

Table 1: *PDGFRA* haplotype combinations.

Haplotype combination	Dutch controls	Northern Irish controls	German controls	German glioblastoma patients
H1/H1	6 (4.1%)	4 (4.1%)	1 (2.3%)	1 (1.4%)
H1/H2α	46 (31.5%)	28 (28.5%)	14 (31.8%)	14 (19.7%)
H1/H2β	0	7 (7.1%)	1 (2.3%)	1 (1.4%)
H1/H2γ	1 (0.7%)	0	1 (2.3%)	3 (4.2%)
H2α/H2α	73 (50.0%)	45 (45.9%)	15 (34.1%)	35 (49.3%)
H2α/H2β	12 (8.2%)	8 (8.2%)	6 (13.6%)	8 (11.3%)
H2α/H2γ	5 (3.4%)	4 (4.1%)	5 (11.4%)	7 (9.9%)
H2α/H2δ	0	0	1 (2.3%)	1 (1.4%)
H2β/H2β	2 (1.4%)	1 (1.0%)	0	0
H2β/H2γ	0	1 (1.0%)	0	0
H2γ/H2γ	1 (0.7%)	0	0	0
H2δ/H2δ	0	0	0	1 (1.4%)
Total	146	98	44	71
H1 frequency	0.202	0.219	0.205	0.141

Table 2: Distribution of H1 and H2 alleles in glioblastoma patients compared to a combined European control group.

	Glioblastomas		Western European controls			
Haplotype	**n**	**freq**	**n**	**freq**	**OR**	**95% CI**
H1	20	0.141	421	0.211	0.613	0.379; 0.992
H2	122	0.859	1575	0.789		
Total	142	1.000	1996	1.000		

n = number, freq = frequency, OR = odds ratio, CI = confidence interval

glioblastoma, respectively). The data show that these three control groups have a very similar overall distribution of haplotype combinations, although intriguing differences were observed in the frequency of some rare allele combinations, such as H1/H2β in the Northern Irish group, and of H2α/H2β and H2α/H2γ in the German control group. The frequency of H1 in these control populations ranged from 0.202 to 0.219, which is very similar to comparable studies on a Swedish (pH1= 0.197; 91 individuals) (31) and French (pH1= 0.214; 619 individuals) (30) control population. It can therefore be concluded that the H1 incidence in the Western European population is very constant, with an average value of pH1=0.211 based on 998 individuals.

Table I also shows that the *PDGFRA* promoter haplotype distribution in blood samples of the 71 patients with glioblastoma multiforme corresponds to an H1 frequency of only 0.141, which is significantly lower ($p<0.05$; see Table II) than that in the Western European control population (pH1=0.211) and also lower than in the matched German matched control group (pH1=0.205). These data show that the H1 allele is underrepresented in glioblastoma patients. Because of the low incidence of H2γ and H2δ, similar statistics were obtained when the frequencies of alleles with high (H2α and H2β) and low (H1, H2γ and H2δ) activity were compared (data not shown). Although larger groups of patients and matched controls will be required to further optimize the statistical analysis, still these data indicate that individuals with an H1 allele show a decreased risk of developing glioblastomas.

Haplotype-specific PDGFRA expression in glioblastoma tumors

Increasing evidence indicates that in addition to a large excess of more differentiated glia-like cells, glioblastomas also contain a small fraction of tumor stem cells from which these more differentiated cells have been derived. Such glioma stem cells have been shown to resemble neural stem cells or early glia progenitor cells in many respects (43). To investigate total and haplotype-specific *PDGFRA* expression in surgically removed glioblastoma tissue, we analyzed specifically the H1/H2 heterozygous glioblastoma samples from the German patient group (14 out of the above 71 cases). Based on histological criteria, the tissue samples contained more than 80% tumor cells (data not shown). Proper RNA was available from 12 out of the 14 heterozygous tumors. As shown in Figure 5A, all samples expressed both haplotypes with at maximum a 2-fold higher expression of H2 compared to H1 (GB26D). Only in the case of GB96D expression of H1 appeared to be higher than that of H2. Total *PDGFRA* expression levels relative to β-actin in these tumors varied from 0.02 (GB5D) to 0.4 (GB4D), as shown in Figure 5B.

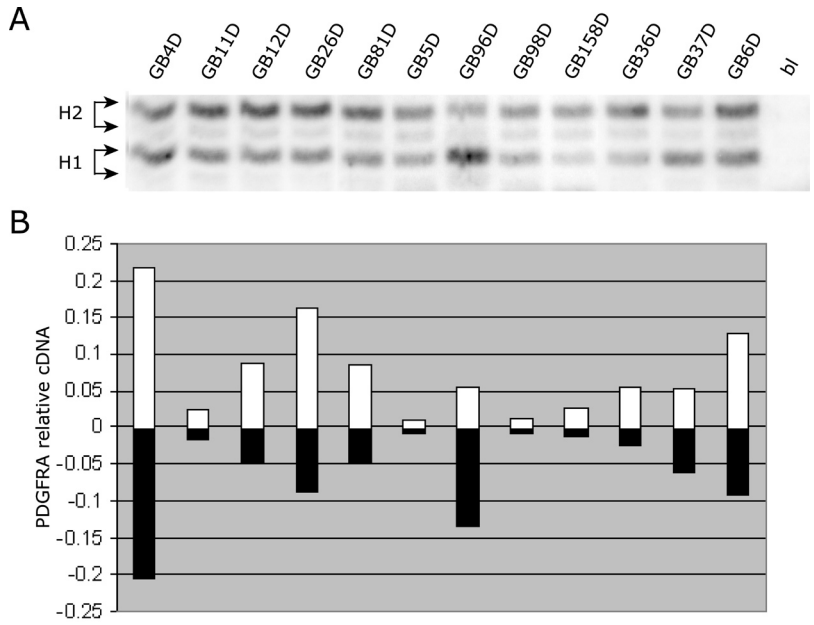

Figure 5. Quantitative haplotype-specific *PDGFRA* expression in H1/H2 heterozygous glioblastoma samples. **A:** Gel electrophoretic separation of expressed *PDGFRA* haplotypes; bl: no template used. **B:** Quantitative analysis of haplotype-specific *PDGFRA* expression, relative to β-actin. H1 is represented by the black bars and H2 by the white bars.

These high expression levels in combination with the observation that both alleles are active, indicate that with respect to *PDGFRA* expression the majority of cells in these tumors show more resemblance to the U-410 cells than to the other three cell lines studied.

Discussion

In the present study we have shown that in H1/H2α heterozygous primary human fibroblasts both *PDGFRA* alleles show similar transcriptional activity, while in three out of four heterozygous human glioblastoma cell lines the H1 allele has more than 10-fold lower activity than H2α, resulting in near monoallelic expression of the H2α allele. Mono-allelic expression is known to occur as a result of genomic imprinting, during X-chromosome inactivation and upon allelic exclusion of genes in the immune and olfactory system (44). The lower activity of the H1 allele in the glioblastoma cell lines cells is associated with allele-specific DNA methylation and histone deacetylation, thus providing an epigenetic basis for the transcriptional repression. To our knowledge, the present study is the first to show that promoter polymorphisms can result in an almost complete silencing of one of the alleles. In combination with our observation of a reduced H1 frequency in a group of glioblastoma patients, our results suggest that the epigenetic repression of the *PDGFRA* H1 allele is correlated with a reduced risk of

glioblastoma development.

Promoter methylation and gene silencing

The removal of DNA methylation and inhibition of histone deacetylation in the glioblastoma cell lines U-251, 31L and Cl2:6 by treatment with 5AC and TSA resulted in a derepression of H1 expression to approximately 50% of the level of H2a. It is known that CpG methylation can repress transcription by interfering with binding of transcription factors and by recruiting histone deacetylase (HDAC) family members via methyl-CpG binding proteins (44). The observation that both *PDGFRA* promoter alleles are fully demethylated in U-410, but partly methylated in U-251, 31L and Cl2:6 cells, parallels the observed differences in total *PDGFRA* expression levels. When comparing the methylation status of the H1 and H2α alleles in these various cell lines with their transcriptional activity, a clear parallel is observed in Cl2:6 and U-410 cells, but much less so in 31L and U-251 cells. However, it has been shown previously that methylation of only a few CpGs can significantly downregulate promoter activity (45), and therefore even minor differences in the haplotype-specific *PDGFRA* methylation in 31L cells might explain the observed 12-fold lower expression of H1 compared to H2α. The observation that treatment with 5AC and TSA results in only a partial derepression of H1, when compared to H2α, indicates that besides epigenetic regulation also additional mechanisms may be involved in the differential expression of H1 and H2α. Due to the polymorphic sites, transcription factors could differentially bind the *PDGFRA* promoter haplotypes, as has been shown for USF-1 in human osteosarcoma cells (27) and ZNF-148 in PNET cells (31). However, thus far we have not observed qualitative differences in nuclear protein binding to any of the polymorphisms in the -3600/+118 promoter region based on electrophoretic mobility shift assays with nuclear extracts from U-251, 31L and Cl2:6 cells (data not shown).

H1 frequency and disease incidence

There is increasing evidence that promoter polymorphisms form the genetic basis for differences in gene expression patterns between individuals and thereby affect their predisposition for various multifactorial diseases. Previous studies have shown that patients with NTDs show an enhanced frequency of the *PDGFRA* H1 allele (26, 28, 29), while our current case-control study indicates that glioblastoma patients have a reduced frequency of the H1 allele. This suggests that low PDGFRA activity as a result of promoter polymorphisms may increase the risk of improper neural tube development, but at the same time reduce the risk of gliomagenesis later in life. These observations agree with data on the incidence of these diseases in various racial groups in the USA. From a recent study on genome wide distribution of haplotypes in USA control populations, H1 frequencies of 0.30, 0.25 and 0.20 could be calculated for African Americans, European Americans and Asian Americans respectively (46). Interestingly, African Americans have a significantly lower incidence of brain tumors than Caucasians (32, 47, 48). Furthermore, Hispanics have the highest incidence of neural tube defects in the USA (49, 50), which is paralleled by a high H1 frequency of 0.31 in the control population and of 0.36 in spina bifida patients (28). Interestingly, a significant overrepresentation of the rare H2δ haplotype has recently been observed in patients with primitive neuroectodermal tumors and ependymomas (31). Taken together, these observations provide a strong link between PDGFRA expression levels and the occurrence of central nervous system diseases.

PDGFRA expression and tumorigenesis

Our results indicate that PDGFRA expression levels provide a predisposition for gliomagenesis in humans. Studies on transgenic mice have shown that constitutive autocrine Pdgfra activation is sufficient to induce gliomagenesis, whereby the extent of PDGF signaling corresponds with tumor malignancy (17-20). Pdgfra expression is strongly upregulated upon differentiation of neural stem cells into OPCs (51, 52), which are the precursor cells for both oligodendrocytes and type-2 astrocytes (51). Our molecular and pharmacological data indicate that increased PDGFRA expression during OPC development may involve activation of the PDGFRA gene by DNA demethylation and histone modification, including acetylation. Activation of Pdgfra is essential for the subsequent proliferation of these progenitor cells, thereby preventing their premature differentiation. *Pdgfra* (+/-) mice show only 50% Pdgfra protein levels compared to wild type mice, and brain explants of such hemizygous mice display significantly lower OPC numbers than explants from wild type mice. In addition, *Pdgfra* (+/-) mice display decreased OPC pool expansion and oligodendrocyte numbers as a result of accelerated OPC maturation (8, 9). This indicates that Pdgfra gene dosage and consequently Pdgfra expression levels directly influence the number of OPCs and mature oligodendrocytes.

Based on our results, we propose that in a similar manner in humans the low activity H1 allele induces accelerated OPC maturation, resulting in a reduced OPC pool size and therefore a smaller risk that one of these cells may eventually transform into a tumor cell. In our case-control studies we have not discriminated between primary and secondary glioblastomas. In general PDGFRA overexpression is more often associated with secondary than with primary glioblastomas (53), but based on our model in which the OPC pool size plays a central role, we expect that both types of glioblastomas will be equally affected by *PDGFRA* promoter haplotypes. Recently, it has been shown that Pdgfra is already expressed in the astrocyte-like neural stem cells, which are present in the subventricular zone of adult murine and human brain. These cells can still generate both neurons and oligodendrocytes, are dependent on Pdgfra signaling for proliferation and form glioma-like hyperplasias upon continuous activation by PDGF (54). It will be interesting to study if in heterozygous human cells of this type *PDGFRA* promoter haplotypes are differentially expressed, since this could indicate that PDGFRA levels affect gliomagenesis not only by controlling the pool size of OPCs but also of neural stem cells.

Glioblastomas have been shown to contain a small fraction of cells, generally referred to as cancer stem cells, which have the capacity to self-renew and are essential for the continuous outgrowth of the tumor (18, 55, 56). Isolated human glioma stem cells have been shown to be highly tumorigenic in nude mice, while the more differentiated glia-like cells that form the majority of cells in glioblastoma tumors are only poorly tumorigenic (43). These glioma stem cells resemble neural stem cells in that they express similar markers and have in addition the ability to grow in so-called neurospheres Addition of serum induces glioma stem cells in vitro to differentiate into more mature glia-like cells. Our preliminary results indicate that at least U-251 and Cl2:6 cells form highly infiltrative intracranal tumors in nude mice, while U-410 cells are unable to do so (A. Claes, P. Wesseling, E.J.J. van Zoelen; unpublished). This agrees with previous data showing that astrocytoid and small polygonal-shaped glioma cell lines, such as the Cl2:6, readily form tumors when injected into the brain of nude rats, while glioma cell lines with a fibroblastic morphology, such as the U-343 MG (57) and U-410,

are unable to do so (57). These data suggest that the U-251, 31L and Cl2:6 cells used in the present study have maintained important characteristics of glioma stem cells, in spite of the fact that they have been cultured in serum-containing medium for a prolonged period of time. In contrast, the U-410 cell line with its high expression levels of both the PDGFRA H1 and H2α allele, shows all the characteristics of more differentiated glia-like tumor cells, similar to the majority of cells in the analyzed glioblastoma tissue samples.

In conclusion, we propose that during differentiation of neural stem cells into OPCs, PDGFRA expression is gradually upregulated whereby the H2α allele is more readily demethylated than the H1 allele. As a result, it will take longer to fully activate H1 than H2α, which will result in a smaller size of the OPC pool due to reduced PDGF induced proliferation. The highly tumorigenic U-251, 31L and Cl2:6 cell lines with their allele-specific *PDGFRA* expression would thereby more resemble transformed neural stem cells or early glia progenitor cells. Upon subsequent differentiation to proliferating glia cells, PDGFRA expression is further upregulated to the high, allele-independent expression observed in the non-tumorigenic U-410 cells and the majority of cells in the analyzed glioblastoma tissue. Deregulated expression of PDGFRA has also been associated with other neoplasias than glioblastomas, in particular osteosarcomas, chondrosarcomas, breast cancer and ovarian carcinomas (58-61). It will be of interest to study if the risk for development of such tumors is also associated with specific *PDGFRA* promoter haplotypes.

Acknowledgements

We thank Dr. B. Franke and Dr. F.P.M. Cremers (Radboud University Nijmegen Medical Centre) for providing us with the fibroblasts, Dr. A. Evans (University of Belfast) for DNA from a Northern Irish control population, Dr. A.M. Cleton-Jansen and Dr. M. Lombaerts (LUMC) for their advice in bisulphite sequencing, Mr. M. Franssen (Radboud University Nijmegen) for assistance in EMSA experiments, Dr. C. Logie (Radboud University Nijmegen) for fruitful discussions and Dr. M. Bots for critical reading of the manuscript. Furthermore, we would like to acknowledge the Hersenstichting Nederland for financial support.

References

1. Armstrong, RC, Harvath, L, and Dubois-Dalcq, ME. Type 1 astrocytes and oligodendrocyte-type 2 astrocyte glial progenitors migrate toward distinct molecules. J Neurosci Res 1990;27:400-7.
2. Noble, M, Murray, K, Stroobant, P, Waterfield, MD, and Riddle, P. Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature 1988;333:560-2.
3. Zhang, H, Vutskits, L, Calaora, V, Durbec, P, and Kiss, JZ. A role for the polysialic acid-neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells. J Cell Sci 2004;117:93-103.
4. Fruttiger, M, Karlsson, L, Hall, AC, Abramsson, A, Calver, AR, Bostrom, H, Willetts, K, Bertold, CH, Heath, JK, Betsholtz, C, and Richardson, WD. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development 1999;126:457-67.
5. Woodruff, RH, Fruttiger, M, Richardson, WD, and Franklin, RJ. Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. Mol Cell Neurosci 2004;25:252-62.
6. Richardson, WD, Pringle, N, Mosley, MJ, Westermark, B, and Dubois-Dalcq, M. A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. Cell 1988;53:309-19.
7. Pringle, NP, Mudhar, HS, Collarini, EJ, and Richardson, WD. PDGF receptors in the rat CNS: during late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. Development 1992;115:535-51.
8. McKinnon, RD, Waldron, S, and Kiel, ME. PDGF alpha-receptor signal strength controls an RTK rheostat that integrates phosphoinositol 3'-kinase and phospholipase Cgamma pathways during oligodendrocyte maturation. J Neurosci 2005;25:3499-508.
9. Murtie, JC, Zhou, YX, Le, TQ, Vana, AC, and Armstrong, RC. PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. Neurobiol Dis 2005;19:171-82.
10. Li, L, Schatteman, GC, Oppenheim, RW, Lei, M, Bowen-Pope, DF, and Houenou, LJ. Altered development of spinal cord in the mouse mutant (Patch) lacking the PDGF receptor alpha-subunit gene. Brain Res Dev Brain Res 1996;96:204-9.
11. Payne, J, Shibasaki, F, and Mercola, M. Spina bifida occulta in homozygous Patch mouse embryos. Dev Dyn 1997;209:105-16.
12. Soriano, P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997;124:2691-700.
13. Klinghoffer, RA, Hamilton, TG, Hoch, R, and Soriano, P. An allelic series at the PDGFalphaR locus indicates unequal contributions of distinct signaling pathways during development. Dev Cell 2002;2:103-13.
14. Hermanson, M, Funa, K, Hartman, M, Claesson-Welsh, L, Heldin, CH, Westermark, B, and Nister, M. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res 1992;52:3213-9.
15. Di Rocco, F, Carroll, RS, Zhang, J, and Black, PM. Platelet-derived growth factor and its receptor expression in human oligodendrogliomas. Neurosurgery 1998;42:341-6.
16. Lokker, NA, Sullivan, CM, Hollenbach, SJ, Israel, MA, and Giese, NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in

glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res 2002;62:3729-35.

17. Uhrbom, L, Hesselager, G, Nister, M, and Westermark, B. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. Cancer Res 1998;58:5275-9.
18. Uhrbom, L, Hesselager, G, Ostman, A, Nister, M, and Westermark, B. Dependence of autocrine growth factor stimulation in platelet-derived growth factor-B-induced mouse brain tumor cells. Int J Cancer 2000;85:398-406.
19. Dai, C, Celestino, JC, Okada, Y, Louis, DN, Fuller, GN, and Holland, EC. PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. Genes Dev 2001;15: 1913-25.
20. Shih, AH, Dai, C, Hu, X, Rosenblum, MK, Koutcher, JA, and Holland, EC. Dose-dependent effects of platelet-derived growth factor-B on glial tumorigenesis. Cancer Res 2004;64:4783-9.
21. Fleming, TP, Saxena, A, Clark, WC, Robertson, JT, Oldfield, EH, Aaronson, SA, and Ali, IU. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. Cancer Res 1992;52:4550-3.
22. Smith, JS, Wang, XY, Qian, J, Hosek, SM, Scheithauer, BW, Jenkins, RB, and James, CD. Amplification of the platelet-derived growth factor receptor-A (PDGFRA) gene occurs in oligodendrogliomas with grade IV anaplastic features. J Neuropathol Exp Neurol 2000;59:495-503.
23. Knobbe, CB and Reifenberger, G. Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol 2003;13:507-18.
24. Suzuki, T, Maruno, M, Wada, K, Kagawa, N, Fujimoto, Y, Hashimoto, N, Izumoto, S, and Yoshimine, T. Genetic analysis of human glioblastomas using a genomic microarray system. Brain Tumor Pathol 2004;21:27-34.
25. Joensuu, H, Puputti, M, Sihto, H, Tynninen, O, and Nupponen, NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol 2005;207:224-31.
26. Joosten, PH, Toepoel, M, Mariman, EC, and Van Zoelen, EJ. Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects. Nat Genet 2001;27:215-7.
27. Toepoel, M, Ackerschott, B, and van Zoelen, EJ. Haplotype-dependent binding of nuclear proteins to the promoter of the neural tube defects-associated platelet-derived growth factor alpha-receptor gene. Biochim Biophys Acta 2005;
28. Zhu, H, Wicker, NJ, Volcik, K, Zhang, J, Shaw, GM, Lammer, EJ, Suarez, L, Canfield, M, and Finnell, RH. Promoter haplotype combinations for the human PDGFRA gene are associated with risk of neural tube defects. Mol Genet Metab 2004;81:127-32.
29. Au, KS, Northrup, H, Kirkpatrick, TJ, Volcik, KA, Fletcher, JM, Townsend, IT, Blanton, SH, Tyerman, GH, Villarreal, G, and King, TM. Promotor genotype of the platelet-derived growth factor receptor-alpha gene shows population stratification but not association with spina bifida meningomyelocele. Am J Med Genet A 2005;139:194-8.
30. Herrmann, SM, Ricard, S, Nicaud, V, Brand, E, Behague, I, Blanc, H, Ruidavets, JB, Evans, A, Arveiler, D, Luc, G, Poirier, O, and Cambien, F. Polymorphisms in the genes encoding platelet-derived growth factor A and alpha receptor. J Mol Med 2000;78:287-92.
31. De Bustos, C, Smits, A, Stromberg, B, Collins, VP, Nister, M, and Afink, G. A PDGFRA

promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumours and ependymomas. J Med Genet 2005;42:31-7.
32. Ohgaki, H and Kleihues, P. Epidemiology and etiology of gliomas. Acta Neuropathol (Berl) 2005;109:93-108.
33. Nister, M, Heldin, CH, and Westermark, B. Clonal variation in the production of a platelet-derived growth factor-like protein and expression of corresponding receptors in a human malignant glioma. Cancer Res 1986;46:332-40.
34. Nister, M, Wedell, B, Betsholtz, C, Bywater, M, Pettersson, M, Westermark, B, and Mark, J. Evidence for progressional changes in the human malignant glioma line U-343 MGa: analysis of karyotype and expression of genes encoding the subunit chains of platelet-derived growth factor. Cancer Res 1987;47:4953-60.
35. Nister, M and Westermark, B. Human glioma cell lines. In: Atlas of Human Tumor Cell Lines. R. J. Hay. San Diego: Academic Press; 1994. p. 17-42.
36. van den Boom, J, Wolter, M, Kuick, R, Misek, DE, Youkilis, AS, Wechsler, DS, Sommer, C, Reifenberger, G, and Hanash, SM. Characterization of gene expression profiles associated with glioma progression using oligonucleotide-based microarray analysis and real-time reverse transcription-polymerase chain reaction. Am J Pathol 2003;163: 1033-43.
37. Sambrook, J, Fritsch, EF, and Maniatis, T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
38. Vaes, BL, Dechering, KJ, van Someren, EP, Hendriks, JM, van de Ven, CJ, Feijen, A, Mummery, CL, Reinders, MJ, Olijve, W, van Zoelen, EJ, and Steegenga, WT. Microarray analysis reveals expression regulation of Wnt antagonists in differentiating osteoblasts. Bone 2005;36:803-11.
39. Groenen, PM, Peer, PG, Wevers, RA, Swinkels, DW, Franke, B, Mariman, EC, and Steegers-Theunissen, RP. Maternal myo-inositol, glucose, and zinc status is associated with the risk of offspring with spina bifida. Am J Obstet Gynecol 2003;189:1713-9.
40. Groenen, PM, Klootwijk, R, Schijvenaars, MM, Straatman, H, Mariman, EC, Franke, B, and Steegers-Theunissen, RP. Spina bifida and genetic factors related to myo-inositol, glucose, and zinc. Mol Genet Metab 2004;82:154-61.
41. Kubota, H, Nishizaki, T, Harada, K, Oga, A, Ito, H, Suzuki, M, and Sasaki, K. Identification of recurrent chromosomal rearrangements and the unique relationship between low-level amplification and translocation in glioblastoma. Genes Chromosomes Cancer 2001;31:125-33.
42. Nister, M, Claesson-Welsh, L, Eriksson, A, Heldin, CH, and Westermark, B. Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines. J Biol Chem 1991;266:16755-63.
43. Lee, J, Kotliarova, S, Kotliarov, Y, Li, A, Su, Q, Donin, NM, Pastorino, S, Purow, BW, Christopher, N, Zhang, W, Park, JK, and Fine, HA. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9:391-403.
44. Jones, PA and Takai, D. The role of DNA methylation in mammalian epigenetics. Science 2001;293:1068-70.
45. Gonzalgo, ML, Hayashida, T, Bender, CM, Pao, MM, Tsai, YC, Gonzales, FA, Nguyen, HD, Nguyen, TT, and Jones, PA. The role of DNA methylation in expression of the p19/p16 locus in human bladder cancer cell lines. Cancer Res 1998;58:1245-52.
46. Hinds, DA, Stuve, LL, Nilsen, GB, Halperin, E, Eskin, E, Ballinger, DG, Frazer, KA, and Cox,

DR. Whole-genome patterns of common DNA variation in three human populations. Science 2005;307:1072-9.

47. Barnholtz-Sloan, JS, Sloan, AE, and Schwartz, AG. Racial differences in survival after diagnosis with primary malignant brain tumor. Cancer 2003;98:603-9.
48. Hess, KR, Broglio, KR, and Bondy, ML. Adult glioma incidence trends in the United States, 1977-2000. Cancer 2004;101:2293-9.
49. Lary, JM and Edmonds, LD. Prevalence of spina bifida at birth--United States, 1983-1990: a comparison of two surveillance systems. MMWR CDC Surveill Summ 1996;45:15-26.
50. Feuchtbaum, LB, Currier, RJ, Riggle, S, Roberson, M, Lorey, FW, and Cunningham, GC. Neural tube defect prevalence in California (1990-1994): eliciting patterns by type of defect and maternal race/ethnicity. Genet Test 1999;3:265-72.
51. Hall, A, Giese, NA, and Richardson, WD. Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors. Development 1996;122:4085-94.
52. Hu, JG, Fu, SL, Zhang, KH, Li, Y, Yin, L, Lu, PH, and Xu, XM. Differential gene expression in neural stem cells and oligodendrocyte precursor cells: a cDNA microarray analysis. J Neurosci Res 2004;78:637-46.
53. Zhu, Y and Parada, LF. The molecular and genetic basis of neurological tumours. Nat Rev Cancer 2002;2:616-26.
54. Jackson, EL, Garcia-Verdugo, JM, Gil-Perotin, S, Roy, M, Quinones-Hinojosa, A, Vandenberg, S, and Alvarez-Buylla, A. PDGFRalpha-Positive B Cells Are Neural Stem Cells in the Adult SVZ that Form Glioma-like Growths in Response to Increased PDGF Signaling. Neuron 2006;51:187-99.
55. Galli, R, Binda, E, Orfanelli, U, Cipelletti, B, Gritti, A, De Vitis, S, Fiocco, R, Foroni, C, Dimeco, F, and Vescovi, A. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004;64:7011-21.
56. Yuan, X, Curtin, J, Xiong, Y, Liu, G, Waschsmann-Hogiu, S, Farkas, DL, Black, KL, and Yu, JS. Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene 2004;23:9392-400.
57. Enblad, P, Hesselager, G, Bongcam-Rudloff, E, Hallin, I, Westermark, B, and Nister, M. Modulation of phenotype and induction of irregular vessels accompany high tumorigenic potential of clonal human glioma cells xenografted to nude-rat brain. Int J Cancer 2000;85:819-28.
58. Sulzbacher, I, Birner, P, Trieb, K, Traxler, M, Lang, S, and Chott, A. Expression of platelet-derived growth factor-AA is associated with tumor progression in osteosarcoma. Mod Pathol 2003;16:66-71.
59. Sulzbacher, I, Birner, P, Trieb, K, Muhlbauer, M, Lang, S, and Chott, A. Platelet-derived growth factor-alpha receptor expression supports the growth of conventional chondrosarcoma and is associated with adverse outcome. Am J Surg Pathol 2001;25: 1520-7.
60. Charron, J, Malynn, BA, Fisher, P, Stewart, V, Jeannotte, L, Goff, SP, Robertson, EJ, and Alt, FW. Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. Genes Dev 1992;6:2248-57.
61. Lassus, H, Sihto, H, Leminen, A, Nordling, S, Joensuu, H, Nupponen, NN, and Butzow, R. Genetic alterations and protein expression of KIT and PDGFRA in serous ovarian carcinoma. Br J Cancer 2004;91:2048-55.

Chapter 6

A regulating element essential for *PDGFRA* transcription is recognized by neural tube defect-associated PRX homeobox transcription factors

Published in: Biochimica et Biophysica Acta 1588 (2002) 254–260
By: Paul H.L.J. Joosten, Mascha Toepoel, Dirk van Oosterhout, Gijs B. Afink, and Everardus J.J. van Zoelen

Abstract

We have previously shown that deregulated expression of the platelet-derived growth factor α-receptor (PDGFRA) can be associated with neural tube defects (NTDs) in both men and mice. In the present study we have investigated the transcription factors that control the upregulation of *PDGFRA* expression during differentiation of early embryonic human cells in culture. In Tera-2 embryonal carcinoma cells *PDGFRA* expression is strongly enhanced upon differentiation induced by retinoic acid and cAMP treatment. Here we show that the corresponding increase in promoter activity is controlled by an ATTA-sequence containing element located near the transcription initiation site, which is bound by a transcriptional complex that includes PBX and PRX homeobox transcription factors. Mutation of the putative binding sites for these transcription factors results in strong impairment of *PDGFRA* promoter activity in differentiated cells. Since functional inactivation of *Prx* genes has been associated with NTDs in mice, these data support a model in which improper *PDGFRA* expression as a result of mutations in or altered binding of its upstream regulators may be causally related to NTDs.

Introduction

Neural tube defects (NTDs), including anencephaly and spina bifida, form a major group of congenital malformations with an average incidence of 1 per 1,000 pregnancies in the Western world. NTDs are multifactorial traits with both genetic and environmental factors contributing to their etiology. Several candidate genes for NTDs have emerged from studies on mouse models, but only few of them have actually been associated with related diseases in humans. Mice with a targeted null mutation in the gene encoding the platelet-derived growth factor α-receptor (*Pdgfra*) and also *Patch* mutant mice, which contain a natural deletion of the chromosomal region that includes *Pdgfra*, both show severe spina bifida combined with embryonic lethality in their homozygous form (1, 2). We have recently shown that this gene also plays a prominent role in the genesis of NTDs in humans. Based on an analysis of naturally occurring single nucleotide polymorphisms in the *PDGFRA* promoter, five different promoter haplotypes could be discriminated which strongly differed in their transcriptional activity. Specific combinations of such haplotypes correlated directly with a predisposition for NTDs in a group of sporadic spina bifida patients (3). Based on these observations we have postulated that aberrant transcriptional regulation of *PDGFRA* may also play a central role in the genesis of NTDs in humans.

The above hypothesis suggests that transcription factors that act as upstream regulators of *PDGFRA* expression may also be associated with NTDs. In line with this concept, it has been shown that mice heterozygous for the *Patch* deletion show spina bifida only in combination with the *undulated* mutation in the *Pax1* gene (4). In a previous study we have shown that Pax1 indeed acts as an upstream transcriptional regulator of *PDGFRA* (5). Moreover we have shown that Pax1 carrying the *undulated* mutation, as well as a mutant form of PAX1 found in a patient with spina bifida (6), both show a gain of function in their ability to stimulate *PDGFRA* transcription (5). Also other transcription factors that in mouse models have been associated with NTDs, including Pax3 (7), Gli2/Gli3 (8), HoxD4 (9, 10) and Mfh1 (11) have been associated directly with

Pdgfra transcription or at least coexpress with *Pdgfra* during development.

Expression of the platelet-derived growth factor (PDGF) α-receptor and thus responsiveness of cells to the various PDGF isoforms (PDGF-A, -B, -C and -D) is strictly regulated during embryonic development. Although present in all cells of the pre-implantation embryo from the two cell stage onwards (12), *Pdgfra* expression becomes limited to mesodermal and certain neural crest-derived structures after implantation (13-17). Human embryonal carcinoma (EC) cells, which represent the undifferentiated stem cells of non-seminomatous testicular germ cell tumors, are widely used as an in vitro model system for studying differentiation-dependent regulation of gene expression during early human development (18). In previous studies we have shown that the Tera-2 cell line in its undifferentiated state (Tera-EC) expresses *PDGFRA* from an internal promoter, giving rise a set of alternative transcripts that have been used as a specific marker for early detection of testicular germ cell tumors (19, 20). Upon in vitro treatment with retinoic acid (RA), differentiated cell populations are obtained with neuronal and endodermal characteristics, designated Tera-RA, which abundantly express the 6.4 kb *PDGFRA* transcript that encodes the full length functional PDGF α-receptor. Using the cloned 2 kb promoter region of human *PDGFRA* that drives the formation of this 6.4 kb transcript, we have shown (21) in transient transfection assays with a luciferase read-out, that *PDGFRA* transcription is strongly upregulated upon treatment of Tera-EC cells with RA, particularly in combination with dibutyryl-cAMP (Bt_2cAMP) and theophylline, this in spite of the fact that the promoter does neither contain a consensus RA-responsive element nor a consensus cAMP-responsive element.

Based on the hypothesis that aberrant expression of *PDGFRA* during development can be associated with NTDs, we have investigated in the present study the transcription factors that are involved in the upregulation of *PDGFRA* promoter activity during RA-induced differentiation of Tera-EC cells. Here we show that an ATTA-sequence containing element (parATTA) is essential for *PDGFRA* transcriptional activity by binding a new complex of transcription factors that includes the homeobox proteins PBX2 and PRX2. Intriguingly, in mouse models *Prx* genes have been found to be associated directly with NTDs (22). These data underline the hypothesis that improper *PDGFRA* expression during development may be a major cause for the genesis of NTDs.

Materials and methods

Reporter constructs

The construction of the truncated *PDGFRA* promoter-luciferase (LUC) reporter gene vectors -2120/ +118 LUC, -441/+118 LUC, -275/+118 LUC, –197/+118 LUC, -175/+118 LUC and -52/+118 LUC has been described previously (21). Mutant forms of these promoter-luciferase constructs were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene).

Cell culture

The human embryonal carcinoma cell line Tera-2 clone 13 (23) was maintained in α-modification of minimal essential medium lacking nucleosides and deoxynucleosides, supplemented with 10% ES-approved fetal calf serum (Gibco BRL). Undifferentiated

cells were seeded one day prior to transfection at high density ($5.0x10^4$ cells/cm^2) in a 0.1% gelatin-coated tissue culture dish. Differentiation was induced by the addition of 5 mM of retinoic acid (RA), 12h after the cells were seeded at low density ($5.0x10^3$/cm^2), and maintained in this medium for 7 days before further use. Transfections were carried out using the calcium-phosphate method (24) upon addition of 1 μg/well of the promoter-luciferase vector. In addition, 50 ng/well of the lacZ-expressing vector pCH110 (Pharmacia) were cotransfected to correct for differences in transfection efficiency. After the appropriate incubation times, cells were lysed in reporter lysis buffer (Promega). Luciferase activity in the lysate was determined using the luciferase assay kit (Promega), according to the manufacturer's protocol. β-Galactosidase activity was assayed as described (25) using Galacton plus (Tropix) as the substrate.

Electrophoretic mobility shift assays

Nuclear extracts from Tera-2 cells were prepared as described by Schreiber et al. (26). Extracts (10-15 μg) were incubated with $3x10^4$ cpm ^{32}P-labelled DNA, 2 μg dI-dC and, if necessary, unlabelled competitor DNA in a reaction buffer (20 mM Hepes pH 7.9, 50 mM NaCl, 1mM EDTA, 0.15 mM EGTA, 1 mM DTT, 0.15 mM PMSF, 4% (w/v) Ficoll and 50 mM KCl) for 5 min at room temperature. The resulting protein-DNA complexes were separated on a 4% polyacrylamide gel using 0.5xTBE as running buffer (24) and visualized on X-ray film (Kodak X-Omat, Fuji RX).

Synthetic oligonucleotides

The following synthetic oligonucleotides were obtained in single-stranded form in both the sense and anti-sense direction: parATTA (5'-CTATAACATTGAATCAATTACAA-3'), m-PBX (5'-CTATAACAGGGAAGCAATTACAA-3'), m-PRX (5'-CTATAACATTGAATCTAGAGCAA-3'), DM (5'- CTATAACAGGGAAGCTAGAGCAA-3'), consensus CRE (5'-AGAGATTGCCTGACGTCAGAGAGAAG-3'), consensus AP1 (5'-CGGATGACTCAGCCGGAA-3') and consensus AP2 (5'-GATCGAACTGACCGCCC GCGGCCGT-3'). Equal amounts of the sense and anti-sense oligonucleotides were mixed in distilled water, heated to boiling temperature and slowly cooled to room temperature. If necessary, the resulting double stranded product was subsequently purified by non-denaturating polyacrylamide gel electrophoresis (24).

Antibodies

Anti-PBX polyclonal antibodies were directed against the C-terminus of the human protein, recombinantly prepared as a GST fusion protein (a generous gift from Dr. C. Murre, UCSD). Polyclonal antibodies specific for PRX2 (αPrx2) were directed against a synthetic peptide derived from mouse Prx2 (IKSYGQEAAIEQPVAPRPTT), while polyclonal antibodies recognizing both PRX1 and PRX2 (αPrx1+2) were generated against the *E.coli* recombinant protein AGRRAAGPVSGPAEARVGAAREPSGGSSGTEAAPQDGDCP SPGRGTKRKKKQRRNRTTFNSSQLQALERVFERTHYPDAFVREELARRVNLSEARVQVWFQNRRAK FRRNERAMLATRSASLLKSYGQEAAIEQPVAPRPTTMSPDYLSWPASSPYSSVPPYSPGGSSPATPG VNMANSIASLRLKAKEFSLHHSQVPTVN. Both antibodies were a generous gift from Dr. F. Meijlink, Hubrecht Laboratory, Utrecht.

Results

We have previously shown that the activity of the so-called *PDGFRA* P1-promoter is strongly upregulated upon RA treatment of undifferentiated Tera-2 human embryonal carcinoma cells. Upregulation was also observed in the presence of Bt_2cAMP, such that synergy in promoter activation was observed by a combination of RA and this cAMP analogue (21). Since the promoter region tested does not contain sequences corresponding to consensus RA- or cAMP-responsive elements, a panel of progressive 5'-deletion mutants of the promoter, cloned into a luciferase reporter gene vector, was used to map the cis-element(s) involved in the RA- and cAMP-induced promoter activity. Upon transient transfection into undifferentiated Tera-EC cells, the promoter activity of the deletion mutants varied in agreement with previous data (21), but for each mutant a 9- to 20-fold induction of activity was observed upon treatment of the cells for 48 hours with RA alone or with RA in combination with Bt_2cAMP and the phosphodiesterase inhibitor theophylline (RACT), as shown in figure 1. Interestingly, similar results were obtained when the transfections were performed in Tera-2 cells that had already been differentiated by RA treatment for 7 days (Tera-RA cells). Since these differentiated cells already display a high basal *PDGFRA* promoter activity (21), RA and RACT seem to enhance, rather than induce, promoter activity. The observation that also the smallest element tested is still RA and Bt_2cAMP responsive, suggests that these stimuli mediate their effects through regulating elements within the -52/+118 region of the *PDGFRA* gene.

To characterize the transcription factors that bind to this specific promoter region, electrophoretic mobility shift assays (EMSAs) were performed using the -52/+118 promoter fragment as a probe. As shown in figure 2 (lane 1) at least four different complexes were observed upon incubation of this fragment with nuclear extracts of Tera-EC cells. Upon 24 hours treatment of these cells with RA, quantitative changes were observed in the three low mobility complexes, while the high mobility

construct	RA	RACT	RA	RACT
-52/+118LUC	9.3 ± 0.1	16.5 ± 0.0	2.5 ± 0.2	10.7 ± 0.2
-175/+118LUC	7.9 ± 0.1	22.4 ± 1.0	3.1 ± 0.3	11.8 ± 0.3
-197/+118LUC	6.1 ± 0.7	25.8 ± 0.7	2.6 ± 0.0	13.6 ± 1.7
-275/+118LUC	9.3 ± 0.4	16.3 ± 0.9	2.2 ± 0.4	18.6 ± 0.6
-441/+118LUC	9.2 ± 0.7	21.9 ± 1.8	3.8 ± 0.3	8.8 ± 0.2
-825/+118LUC	6.2 ± 0.0	18.8 ± 2.1	3.2 ± 0.1	11.9 ± 1.0

Fig. 1. Retinoic acid (RA) and cAMP-induced activity of *PDGFRA* promoter deletion mutants. Indicated 5' promoter-luciferase (LUC) deletion mutants were transiently transfected into undifferentiated Tera-EC or differentiated Tera-RA cells. Transfected cells were treated for 48 hours with either 5 μM RA or a combination of 5 μM RA, 1 mM Bt_2cAMP and 350 μM theophylline (RACT). Subsequently, luciferase activity in the cells was determined and corrected for differences in transfection efficiency. The values represent the fold induction of luciferase activity after treatment relative to that of unstimulated cells, including the sample standard deviation based on two independent experiments. Unstimulated values were in agreement with Afink et al. (21).

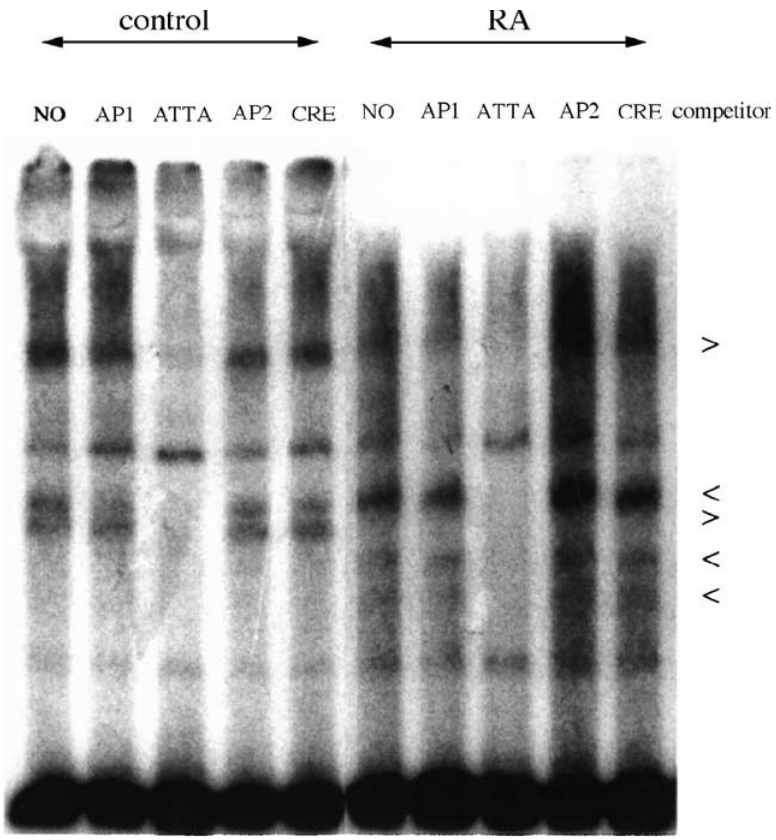

Fig. 2. Electrophoretic mobility shift assay on nuclear extracts of Tera-EC cells before (control) and 48 hours after incubation with 5 μM retinoic acid (RA). Use was made of the ^{32}P-labelled –52/+118 *PDGFRA* promoter fragment, either in the absence of any specific competitor (NO), or in the presence of a 500-fold excess of consensus AP1, parATTA, consensus AP2 or consensus CRE oligonucleotide (see Materials and Methods for sequences). The five distinct DNA-protein complexes that can be competed with unlabelled parATTA are indicated by arrows, specified as complexes upregulated by RA (<) and complexes with decreased intensity following RA treatment (>).

complex had disappeared in favour of two new complexes with even higher mobility. Qualitatively similar differences, although often more pronounced, were observed 24 hours after treatment with RACT (data no shown; see also ref (21)). These data show that RA treatment of Tera-2 cells results in a complex but characteristic change in nuclear protein binding to the –52/+118 promoter fragment of *PDGFRA*.

The *PDGFRA* promoter does not contain a consensus RA-responsive element, but it contains various putative binding sites for RA-inducible transcription factors such as AP2 (21), and one such AP2 site ($^{+97}$GCCGTGGG^{+104}) is located within the -52/+118 region. In addition, an ATTA-containing sequence ($^{+4}$ATTGAATCAATTA^{+16}), designated here as parATTA, is present which shows some homology to both a cAMP-responsive element (TGACGTCA) and to binding sites for the AP1 transcription factor complex (TGASTCA) (27, 28). In order to investigate the involvement of these sequences in the the -52/+118 promoter region in binding of nuclear proteins, we carried out oligonucleotide

competition studies, as shown in figure 2. Competition with the unlabelled parATTA oligonucleotide resulted in a complete loss of five of the six earlier mentioned protein-DNA complexes bound to the -52/+118 probe. This shows that this parATTA element is essential for the formation of protein-DNA complexes in both Tera-EC and Tera-RA cells. No binding competition was observed with a consensus API oligonucleotide, an AP2 oligonucleotide or an oligonucleotide containing a cAMP-responsive element (CRE), which is indicative for the specificity of nuclear protein binding to the parATTA sequence in this promoter element (figure 2).

ATTA motifs are known to be involved in the DNA binding of homeobox-containing transcription factors. A search for transcription factor binding sites in the parATTA sequence present in the –52/+118 fragment of the *PDGFRA* promoter (TFSEARCH analysis) revealed the highest correlation with binding sites for members of the PBX and PRX families of homeobox proteins (TTGAAT and AATTA respectively). In order to identify if such factors indeed bind the parATTA sequence in Tera-2 cells, we used specific antibodies directed against PBX and PRX proteins in EMSA studies. Figure 3 shows that upon incubation of parATTA with nuclear extracts of Tera-EC cells, a small increase in gel mobility of the DNA-protein complex was observed in the presence of antibodies specific for PBX2. More pronounced, a clear supershift was observed when the experiment was carried out in the presence of antibodies specific for PRX2, while

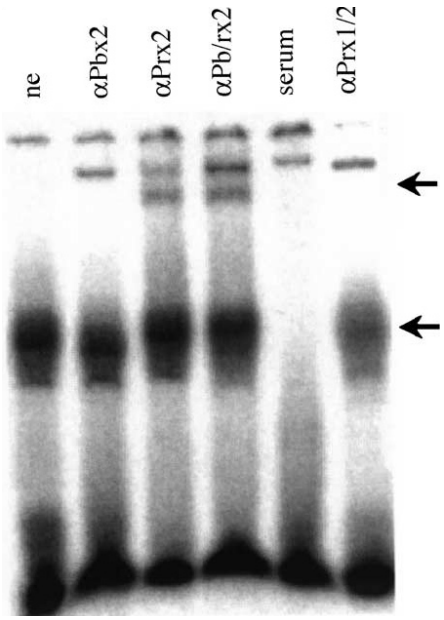

Fig. 3. Identification of parATTA binding proteins in Tera-EC cells. Electrophoretic mobility shift assays were performed on the ^{32}P-labelled *PDGFRA* promoter-derived parATTA oligonucleotide, using nuclear extracts of Tera-EC cells in the presence of anti-PRX and anti-PBX antibodies, as follows: ne, control nuclear extracts; αPbx2, antibodies specific for PBX2; αPrx2, antibodies specific for PRX2; αPb/rx2, a combination of αPbx2 and αPrx2; serum, control rabbit serum in the absence of nuclear extract; αPrx1/2, antibodies recognizing both PRX1 and PRX2. Arrows denote the DNA-protein complexes that are formed or altered upon antibody binding.

antibodies that recognise both PRX2 and its transcriptional partner PRX1 (22, 29, 30) strongly reduce DNA-protein complex formation. These data suggest that the parATTA sequence is bound by PRX2, and possibly also PRX1, most likely in a protein complex with PBX2. Similar results were obtained using nuclear extracts from Tera-RA cells (data not shown).

In order to analyse the specific function of the bound nuclear proteins, we introduced inactivating mutations into the parATTA oligonucleotide on positions that are specific for binding of either PBX2 or PRX1/2 (based on TFSEARCH analysis). Figure 4 shows that mutation of the putative PBX binding site in the parATTA oligonucleotide results in a strongly reduced ability to bind Tera-2 nuclear proteins. Similar results were obtained upon mutation of the ATTA site involved in PRX1/2 binding, while no detectable complex formation was observed when both sites were mutated simultaneously. The similarity in effect observed upon mutation of the putative PBX and PRX site suggests that inhibition of binding of one of these factors may already block formation of the entire complex. It has been described that HOX gene products may modulate the DNA binding activity of PBX transcription factors and consequently that removal of either PBX itself or of the homeobox-containing transcription factor is sufficient for dissociation of the DNA-protein complex (31). In agreement with this observation we

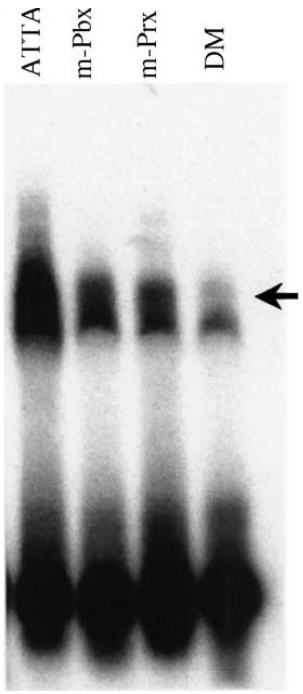

Fig. 4. Effect of mutations in the parATTA sequence on nuclear protein binding. Electrophoretic mobility shift assays were performed with nuclear extracts of Tera-EC cells using ^{32}P-labelled oligonucleotides with different mutations. The sequences of the wild-type parATTA oligonucleotide and of the mutant forms aimed at preventing PBX and PRX binding (designated m-PBX, m-PRX, and the double mutant DM) are shown in the Materials and Methods. The arrow denotes the major specific DNA-protein complex.

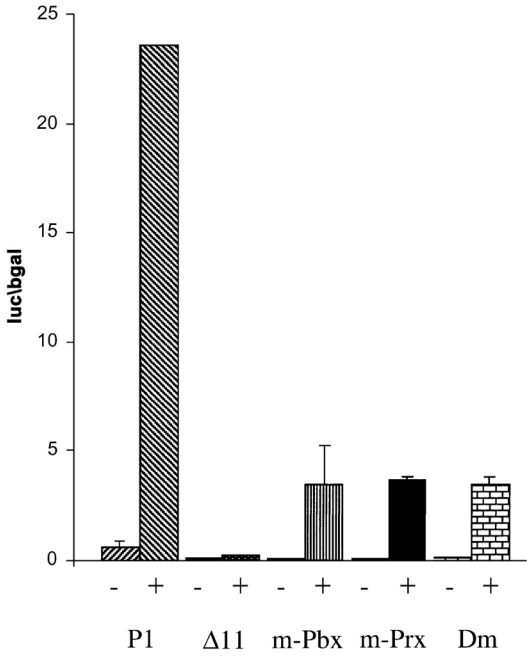

Fig. 5. Effect of mutations in the parATTA element on *PDGFRA* promoter activity. In the –441/+118 *PDGFRA* promoter construct linked to luciferase, the following mutations were made: P1, no mutation; Δ11, P1 carrying a deletion of the 11 nucleotide inverted repeat $^{+4}$ATTGAATCAAT $^{+14}$; m-Pbx, P1 carrying the PBX sensitive mutation similarly as in the m-PBX oligonucleotide; m-Prx, P1 carrying the PRX sensitive mutation similarly as in the m-PRX oligonucleotide; Dm, P1 carrying both sets of mutations similarly as in the DM oligonucleotide. Constructs were transfected into Tera-EC cells and luciferase (luc) activity was measured in control cells (-) and in cells treated for 48 hours with RACT (+). Promoter activity values were corrected for differences in transfection efficiency, expressed in β-galactosidase (bgal) activity. Error bars represent the standard deviation of duplicate independent experiments.

find that PBX2 only forms stable complexes with specific DNA sequences when other homeobox-containing transcription factors, such as in this case PRX1/2, are bound in addition.

Functional analysis of the wild-type and various mutated parATTA elements was subsequently studied within the context of the highly active -441/+118 promoter-luciferase reporter construct. When transfected into Tera-EC cells the various mutations in the parATTA element caused a severe reduction in promoter activity compared to the wild-type promoter, as shown in figure 5. Mutation of the putative PBX binding site resulted in a more than 5-fold reduction in luciferase activity, both before and after RACT treatment. A similar reduction was observed upon mutation of the ATTA site or when both sets of mutations were combined. A complete loss of promoter activity was observed upon deletion of 11 nucleotides from the palindromic sequence within parATTA that is involved in binding of PBX and PRX1/2. Since this deletion leaves the transcription initiation intact, these data underline the essential role of the parATTA element in the basal transcriptional activity of the *PDGFRA* promoter.

Discussion

In the present study we have shown that the upregulation of *PDGFRA* expression during differentiation of early embryonic human cells requires a promoter element, designated parATTA, which binds a complex of PRX and PBX proteins. Various studies have shown that improper expression of the PDGF α-receptor during embryonic development can result in NTDs, and therefore both *PDGFRA* itself and genes encoding upstream regulators of its expression may be associated with such malformations. We have previously shown that PAX1, as product of a NTD-associated gene, is an upstream regulator of *PDGFRA* expression, while the current study identifies PBX2, PRX2 and possibly also PRX1 as regulators of *PDGFRA* expression that are potentially involved in NTDs. These observations are in line with mouse studies, where severe NTDs have been observed particular in *Prx1*(-/-); *Prx2*(-/-) mice, with similar vertebrae defects as seen in the *Pdgfra* null mutant (22).

The exact nature of the protein complex that binds the parATTA element in Tera-2 cells is unclear, but the observation that five distinct DNA–protein complexes have been identified in this study that are competed in electrophoretic mobility shift assays by unlabelled parATTA containing oligonucleotides, indicates that multiple complexes are formed. This suggests that in addition to PBX2 and PRX1/2, also other proteins may be present in the complexes formed. The EMSAs indicate that PBX2 and PRX1/2 containing complexes can be formed with nuclear extracts from both undifferentiated and differentiated Tera-2 cells, indicating that the increase in *PDGFRA* promoter activity upon RA treatment is most likely not due to the induction of these homeotic genes. These observations suggest that PBX-PRX proteins form the core of a larger complex that is required for transcriptional activation, in agreement with recent data on the binding of such transcription factors to a collagen promoter (32). The formation of this complex is at most only partly regulated by the mere availability of the transcription factors involved and most likely requires an additional activation step. This is confirmed by the observed synergy between Bt_2AMP and RA, which in comparison with RA alone gives rise to only quantitative, and not to qualitative changes in transcription factor binding to the –52/+118 region (21). Also the observation that RACT still stimulates *PDGFRA* transcription in Tera-RA cells, supports this hypothesis.

The present data indicate that the parATTA binding proteins PBX2 and PRX1/2 are essential for formation of the complex that regulates *PDGFRA* expression during embryonic cell differentiation. Homeobox transcription factors are known to control expression of other homeotic genes, but only few examples are available in which they control expression of non-homeotic genes such as *PDGFRA* (32). Amino acid alignment reveals that members of the PBX and PRX families are 93-99% conserved between mouse and human, while the parATTA element itself is 100% conserved between rat, mouse and human. This underlines the importance of this mechanism for normal vertebrate development.

Studies on the promoters of the human, mouse and rat PDGF α-receptor gene have provided evidence for the involvement of GATA4 (33), PAX3-FKHR (7), Pax1 (5), C/EBPs (34) and Gli1 (35) in transcriptional control. Potentially, the genes encoding these transcription factors can all be involved in the development of NTDs. In mice, *Pdgfra* itself as well as *Pax1*, *Pax3*, *Prx1* and *Prx2*, which all encode upstream regulators of *Pdgfra* expression, have been associated directly with NTDs. In humans, specific combinations of *PDGFRA* promoter haplotypes have been associated with

predisposition to NTDs, while also mutations in *PAX1* and *PAX3* are considered to be risk factors for human NTDs. Based on our present results, *PBX2*, *PRX2* and maybe also *PRX1* should also be considered as candidate genes in the genesis of NTDs in humans. Further studies will have to indicate if mutations in these genes or alterations in their expression levels are indeed observed in patients suffering from anencephaly and spina bifida.

In conclusion, we have shown that *PDGFRA* promoter activity in human Tera-2 cells is strongly upregulated upon RA and cAMP treatment. The element involved is located in the -52/+118 region of the promoter, and electrophoretic mobililty shift assays show that multiple protein complexes that can bind this region which are all competed by a 23 bp parATTA oligonucleotide. These protein complexes include PRX and PBX, but since these complexes are present in both Tera-EC and Tera-RA cells, RA/cAMP-induced induction of *PDGFRA* promoter activity seems to require an additional, as yet unknown activation step. Point mutations in parATTA reduce binding affinity for PBX en PRX proteins and thereby the induction of promoter activation by RA/cAMP. It is therefore concluded that the parATTA sequence is essential for normal *PDGFRA* transcription regulation and for its activation upon RA/cAMP treatment.

Acknowledgements

The present studies were supported by grants from the Dutch Prinses Beatrix Fonds. We thank Dr. E. Mariman (Dept. of Anthropogenetics, University of Nijmegen) for fruitful discussion, and Drs. C. Murre and F. Meijlink for the generous gifts of antibodies.

References

1. Soriano, P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997;124:2691-700.
2. Payne, J, Shibasaki, F, and Mercola, M. Spina bifida occulta in homozygous Patch mouse embryos. Dev Dyn 1997;209:105-16.
3. Joosten, PH, Toepoel, M, Mariman, EC, and Van Zoelen, EJ. Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects. Nat Genet 2001;27:215-7.
4. Helwig, U, Imai, K, Schmahl, W, Thomas, BE, Varnum, DS, Nadeau, JH, and Balling, R. Interaction between undulated and Patch leads to an extreme form of spina bifida in double-mutant mice. Nat Genet 1995;11:60-3.
5. Joosten, PH, Hol, FA, van Beersum, SE, Peters, H, Hamel, BC, Afink, GB, van Zoelen, EJ, and Mariman, EC. Altered regulation of platelet-derived growth factor receptor-alpha gene-transcription in vitro by spina bifida-associated mutant Pax1 proteins. Proc Natl Acad Sci U S A 1998;95:14459-63.
6. Hol, FA, Geurds, MP, Chatkupt, S, Shugart, YY, Balling, R, Schrander-Stumpel, CT, Johnson, WG, Hamel, BC, and Mariman, EC. PAX genes and human neural tube defects: an amino acid substitution in PAX1 in a patient with spina bifida. J Med Genet 1996;33:655-60.
7. Epstein, JA, Song, B, Lakkis, M, and Wang, C. Tumor-specific PAX3-FKHR transcription factor, but not PAX3, activates the platelet-derived growth factor alpha receptor. Mol Cell Biol 1998;18:4118-30.
8. Aruga, J, Mizugishi, K, Koseki, H, Imai, K, Balling, R, Noda, T, and Mikoshiba, K. Zic1 regulates the patterning of vertebral arches in cooperation with Gli3. Mech Dev 1999;89:141-50.
9. Horan, GS, Kovacs, EN, Behringer, RR, and Featherstone, MS. Mutations in paralogous Hox genes result in overlapping homeotic transformations of the axial skeleton: evidence for unique and redundant function. Dev Biol 1995;169:359-72.
10. Horan, GS, Ramirez-Solis, R, Featherstone, MS, Wolgemuth, DJ, Bradley, A, and Behringer, RR. Compound mutants for the paralogous hoxa-4, hoxb-4, and hoxd-4 genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. Genes Dev 1995;9:1667-77.
11. Furumoto, TA, Miura, N, Akasaka, T, Mizutani-Koseki, Y, Sudo, H, Fukuda, K, Maekawa, M, Yuasa, S, Fu, Y, Moriya, H, Taniguchi, M, Imai, K, Dahl, E, Balling, R, Pavlova, M, Gossler, A, and Koseki, H. Notochord-dependent expression of MFH1 and PAX1 cooperates to maintain the proliferation of sclerotome cells during the vertebral column development. Dev Biol 1999;210:15-29.
12. Palmieri, SL, Payne, J, Stiles, CD, Biggers, JD, and Mercola, M. Expression of mouse PDGF-A and PDGF alpha-receptor genes during pre- and post-implantation development: evidence for a developmental shift from an autocrine to a paracrine mode of action. Mech Dev 1992;39:181-91.
13. Orr-Urtreger, A, Bedford, MT, Do, MS, Eisenbach, L, and Lonai, P. Developmental expression of the alpha receptor for platelet-derived growth factor, which is deleted in the embryonic lethal Patch mutation. Development 1992;115:289-303.
14. Orr-Urtreger, A and Lonai, P. Platelet-derived growth factor-A and its receptor are expressed in separate, but adjacent cell layers of the mouse embryo. Development 1992;115:1045-58.

15. Schatteman, GC, Morrison-Graham, K, van Koppen, A, Weston, JA, and Bowen-Pope, DF. Regulation and role of PDGF receptor alpha-subunit expression during embryogenesis. Development 1992;115:123-31.
16. Morrison-Graham, K, Schatteman, GC, Bork, T, Bowen-Pope, DF, and Weston, JA. A PDGF receptor mutation in the mouse (Patch) perturbs the development of a non-neuronal subset of neural crest-derived cells. Development 1992;115:133-42.
17. Takakura, N, Yoshida, H, Ogura, Y, Kataoka, H, and Nishikawa, S. PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5. J Histochem Cytochem 1997;45:883-93.
18. Andrews, PW. Human teratocarcinomas. Biochim Biophys Acta 1988;948:17-36.
19. Mosselman, S, Looijenga, LH, Gillis, AJ, van Rooijen, MA, Kraft, HJ, van Zoelen, EJ, and Oosterhuis, JW. Aberrant platelet-derived growth factor alpha-receptor transcript as a diagnostic marker for early human germ cell tumors of the adult testis. Proc Natl Acad Sci U S A 1996;93:2884-8.
20. Mosselman, S, Claesson-Welsh, L, Kamphuis, JS, and van Zoelen, EJ. Developmentally regulated expression of two novel platelet-derived growth factor alpha-receptor transcripts in human teratocarcinoma cells. Cancer Res 1994;54:220-5.
21. Afink, GB, Nister, M, Stassen, BH, Joosten, PH, Rademakers, PJ, Bongcam-Rudloff, E, Van Zoelen, EJ, and Mosselman, S. Molecular cloning and functional characterization of the human platelet-derived growth factor alpha receptor gene promoter. Oncogene 1995;10:1667-72.
22. ten Berge, D, Brouwer, A, Korving, J, Martin, JF, and Meijlink, F. Prx1 and Prx2 in skeletogenesis: roles in the craniofacial region, inner ear and limbs. Development 1998;125:3831-42.
23. Thompson, S, Stern, PL, Webb, M, Walsh, FS, Engstrom, W, Evans, EP, Shi, WK, Hopkins, B, and Graham, CF. Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic acid. J Cell Sci 1984;72:37-64.
24. Sambrook, J, Fritsch, EF, and Maniatis, T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
25. Jain, VK and Magrath, IT. A chemiluminescent assay for quantitation of beta-galactosidase in the femtogram range: application to quantitation of beta-galactosidase in lacZ-transfected cells. Anal Biochem 1991;199:119-24.
26. Schreiber, E, Matthias, P, Muller, MM, and Schaffner, W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
27. Angel, P, Imagawa, M, Chiu, R, Stein, B, Imbra, RJ, Rahmsdorf, HJ, Jonat, C, Herrlich, P, and Karin, M. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 1987;49:729-39.
28. Lee, W, Mitchell, P, and Tjian, R. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 1987;49:741-52.
29. Lu, MF, Cheng, HT, Kern, MJ, Potter, SS, Tran, B, Diekwisch, TG, and Martin, JF. prx-1 functions cooperatively with another paired-related homeobox gene, prx-2, to maintain cell fates within the craniofacial mesenchyme. Development 1999;126:495-504.
30. Bergwerff, M, Gittenberger-de Groot, AC, Wisse, LJ, DeRuiter, MC, Wessels, A, Martin, JF, Olson, EN, and Kern, MJ. Loss of function of the Prx1 and Prx2 homeobox genes alters architecture of the great elastic arteries and ductus arteriosus. Virchows Arch 2000;436:

12-9.
31. van Dijk, MA, Peltenburg, LT, and Murre, C. Hox gene products modulate the DNA binding activity of Pbx1 and Pbx2. Mech Dev 1995;52:99-108.
32. Penkov, D, Tanaka, S, Di Rocco, G, Berthelsen, J, Blasi, F, and Ramirez, F. Cooperative interactions between PBX, PREP, and HOX proteins modulate the activity of the alpha 2(V) collagen (COL5A2) promoter. J Biol Chem 2000;275:16681-9.
33. Wang, C and Song, B. Cell-type-specific expression of the platelet-derived growth factor alpha receptor: a role for GATA-binding protein. Mol Cell Biol 1996;16:712-23.
34. Fukuoka, T, Kitami, Y, Okura, T, and Hiwada, K. Transcriptional regulation of the platelet-derived growth factor alpha receptor gene via CCAAT/enhancer-binding protein-delta in vascular smooth muscle cells. J Biol Chem 1999;274:25576-82.
35. Xie, J, Aszterbaum, M, Zhang, X, Bonifas, JM, Zachary, C, Epstein, E, and McCormick, F. A role of PDGFRalpha in basal cell carcinoma proliferation. Proc Natl Acad Sci U S A 2001;98:9255-9.

Chapter 7

General Discussion

Role of *PDGFRA* promoter polymorphisms in normal physiology and disease

With the recent completion of the human genome sequence, current emphasis in molecular research is now on the 0.1% of DNA in which individuals differ from each other. These differences in DNA sequences mainly result from the presence of single nucleotide polymorphisms (SNPs) and small nucleotide insertions and deletions. It is well accepted that human beings differ from each other, not because they make different proteins but because they make the same proteins in different amounts. Regulatory polymorphisms, such as those in promoter regions, can strongly contribute to the transcriptional activity of the corresponding gene and thereby to the amount of protein produced. Such variation in gene expression levels can, however, also predispose individuals to diseases in which the corresponding gene is involved. In studies on transgenic mice, the effects of gene depletion or overexpression can be studied relatively easily. Often the phenotypes obtained are very severe, because the expression level of the gene of interest is strongly impaired. Under those conditions a direct relation between gene defect and disease is observed. However, the promoter polymorphisms that occur in the normal human population will induce more subtle differences in gene expression levels than those observed in transgenic mice. As a result promoter polymorphisms may predispose individuals to diseases in which the gene of interest is involved, but generally only in combination with other genetic and environmental factors as part of a multifactorial disease. Our current data show that polymorphisms in the *PDGFRA* promoter contribute not only to the normal physiology (body length) of individuals but also to their risk of developing such diseases as neural tube defects and brain tumors.

PDGFRA promoter polymorphisms influence disease susceptibility

Studies on transgenic mice have shown that Pdgfra plays a role in various diseases, including neural tube defects (NTDs) and glioma tumors. Altered expression of this gene may influence cell proliferation, differentiation, survival, migration, chemotaxis and extracellular matrix deposition. In the present study we have investigated how expression of the human PDGFRA gene is regulated in various cell types and how *PDGFRA* expression levels contribute to the susceptibility of individuals for developing NTDs and glioblastomas. We have identified a number of *PDGFRA* upstream regulators, in particular USF, PRX and PBX, some of which are also themselves associated with NTDs in mice. In addition we identified 10 polymorphic sites in the human *PDGFRA* promoter, which give rise to five distinct haplotypes, of which the H1 and H2α alleles are the most prominent. Our *in vitro* studies on human osteosarcomas cells as well as our *in vivo* data on intact human glioblastoma cells indicated that these haplotypes strongly differ in promoter activity, such that the H1 allele may induce low and the H2α allele high *PDGFRA* mRNA levels. In case-control studies we subsequently observed that the low activity H1 allele predisposes individuals to NTDs while protecting them against glioblastomas. Figure 1 gives a survey of the *PDGFRA* promoter haplotype combinations and H1 frequency observed in our studies on Western European control populations, spina bifida patients and glioblastoma patients. The data show a gradual decrease in H1 frequency when going from neural tube patients to the control

population to glioblastoma patients, fully in line with the model that H1 is a low activity allele that predisposes to NTDs but protects against glioblastomas.

It generally requires large patient and matched control cohorts to observe a statistically significant enrichment for a specific polymorphism. In our studies the increased frequency of H1 in spina bifida patients and decreased incidence of H1 in glioblastoma patients both have p-values just below 0.05. However, when comparing the two patient groups of Fig.1 with each other, a difference with much higher significance is observed both when analyzing the number of H1/H1 homozygotes (OR=12.4, p<0.009; 95%CI: 2.0 - 77.1) and the frequency of the H1 allele (OR=2.6, p<0.001; 95%CI: 1.5 – 4.6). This shows that with respect to *PDGFRA* promoter haplotypes spina bifida and glioblastoma patients are genetically two highly distinct populations.

To further evaluate a possible inverse correlation between the risk of NTDs and glioblastomas, we subsequently studied the incidence of both diseases on a world-wide scale. From a recent study on genome wide distribution of haplotypes in the USA, an H1 frequency of approximately 0.30 could be calculated for African Americans, of 0.25 for European Americans and 0.20 for the Chinese population (1), while an

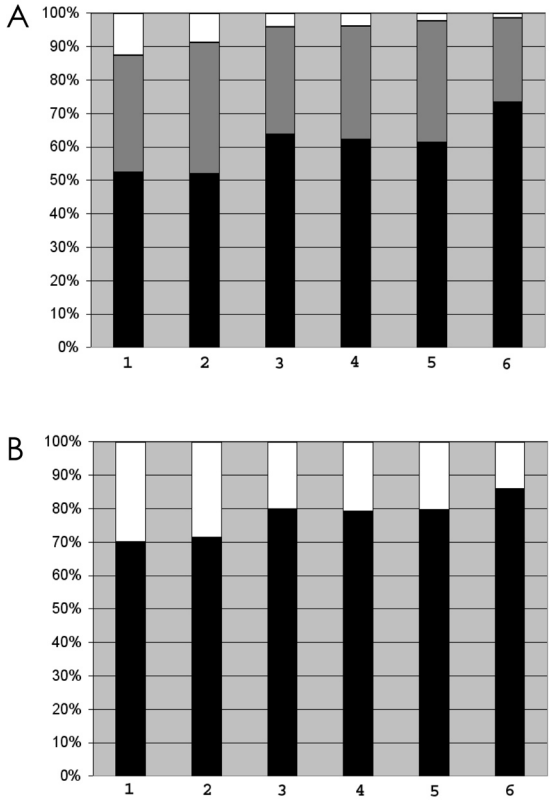

Figure 1: Distributions of *PDGFRA* promoter haplotypes. Depicted are percentages of PDGFRA haplotype combinations (A) and single haplotypes (B) in Dutch spina bifida cases (1), their mothers (2), Dutch controls (3), combined Western European controls (4), German controls (5), and German glioblastoma cases (6). Black represents H1/H1 (A) and H1 (B), gray H1/H2 and white H2/H2 (A) and H2 (B).

H1 frequency as low as 0.172 was reported for a Taiwanese control population (2). Other studies have indicated that particularly American Hispanics show a very high H1 frequency ranging from 0.32 to 0.47 (3, 4). When comparing these distributions with the incidence of PDGFRA-related diseases in these population groups, it has been observed that the incidence of NTDs in the USA is highest among Hispanics, followed by American Indians, Whites, Blacks, and finally Asians (5, 6). Thus, it appears that groups in the population with a high H1 frequency generally also have an increased incidence of NTDs. African Americans form an exception to this correlation, since they combine a higher H1 frequency with a lower risk of NTDs, when compared to European Americans. Interestingly, the length of the *PDGFRA* promoter haplotype is considerably shorter in African Americans (Fig 2), most likely because they represent a more ancient race, but it is unclear if that observation is in any way related to their relatively low NTD incidence. Alternatively, the African American race may be protected from NTDs by other factors.

When considering gliomas, Caucasians/Whites combine a lower H1 frequency with a significantly higher brain tumor incidence than African Americans (11.6 vs. 7.8 cases per 100,000 persons per years respectively (7), which is consistent with our hypothesis. Also in line is the observation that the incidence of central nervous system (CNS) tumors in most African countries lies below the 2.4 and 1.5 cases per 100,000 persons per year for males and females respectively (8). Furthermore, if it is assumed that Mexicans have a similarly high H1 incidence as determined for American Hispanics from Mexican descent in the studies of Zhu and Au, this could explain why the incidence of CNS tumors in Mexico is much lower than in the USA (<5.9 vs. <11.1 cases per 100.000 persons per year respectively for males, and <4.3 vs. <11.3 cases per 100.000 persons per year respectively for females, (8)). However, when considering the low H1 frequency in Asians, the CNS tumor incidence in Asian countries is unexpectedly low (<2.8 and <2.1 cases per 100.000 persons per year for males and females respectively, (8)). It could therefore be hypothesized that Asians are protected from gliomagenesis by other mechanisms. It has indeed been suggested that in the Asian population a distinct genetic pathway is involved in the etiology of glioblastoma multiforme (9).

H1 and glioblastoma risk

In our glioblastoma case-control study, we could only demonstrate a statistically significant difference in H1 frequency between the patient group and a combined Western European control group consisting of 998 individuals. The matched control group showed a similar haplotype distribution as this Western European control group, but at least a threefold increase in both cases and controls would have been required to obtain a significant difference in H1 frequency ($p<0.05$) between glioblastoma patients and the matched controls.

The most likely explanation for the protective effect of H1 against glioblastomas would be that the low H1-driven *PDGFRA* expression makes the tumor initiating cells less sensitive to PDGF-induced proliferation. These cells could be neural stem cells (NSCs), glial restricted precursors (GRPs), oligodendrocyte precursor cells (OPCs) or the recently identified astrocyte like adult neural stem cells called B cells (10-12). It has been shown that overstimulation of this receptor system is sufficient to induce the formation of glioma tumors in mice (13-17). However, this would only account for those tumors in which high PDGF signaling is indeed the oncogenic event. This may hold

true for secondary glioblastomas, which are associated with overexpression of both PDGF and PDGFRA, as opposed to primary glioblastomas, which are characterized by EGFR activating mutations and gene amplifications (18). Secondary glioblastomas often arise relatively early in life, at an average age of 45 years, whereas primary glioblastomas generally occur later in life at an average age of 62 years (8). In general, primary glioblastomas are the most prevalent type, constituting more than 80% of all glioblastomas (19). The glioblastoma patients tested in our study were on the average 60 years of age (unpublished) and the majority shows EGFR abnormalities (20), which suggests that these are mainly primary glioblastomas. This makes it unlikely that high PDGF signaling is the primary cause of tumor formation in most of these patients.

We therefore prefer an alternative hypothesis, one that can also account for the effect of *PDGFRA* promoter haplotypes on primary glioblastomas. Although in rodents *Pdgfra* expression has been observed in early NSCs (21)), its expression expression is strongly upregulated upon differentiation into OPCs (22, 23). Based on our data on haplotype specific expression in glioblastoma cell lines, we propose that following differentiation of their neural stem cells, H1 individuals display lower PDGFRA expression levels on their glial precursors (OPCs/O2As/GRPs) than H2 individuals due to differential promoter (de)methylation. This would result in a decreased precursor pool size, analogous to what has been observed in Pdgfra (+/-) and (-/-) mice when compared to wild-type mice (24). Assuming that each glia precursor cell has a specific risk of transforming into a malignant counterpart, lower precursor numbers will reduce the total risk of developing glial tumors later in life. In this way, *PDGFRA* promoter haplotypes could influence the risk of all glial precursor-derived tumors, irrespective of the PDGF levels at the actual time of tumorigenesis. In this respect, it would be very interesting to study haplotype specific expression in the recently identified B cells(10, 25), to investigate whether these cells also show a reduced H1 expression during their initial development.

H1 and NTD risk

Several mouse models have shown that decreased Pdgfra signaling, either by ligand or receptor deficiency, can lead to spina bifida, suggesting that low PDGFRA expression may be a risk factor for NTDs in humans. Based on our observation that H1 is a weak promoter in a number of cell lines, we therefore tested the hypothesis that the H1 allele confers a genetic predisposition to NTDs. In our initial study (26) we observed an underrepresentation of H1/H2 heterozygotes in the control group in combination with an overrepresentation of such heterozygotes in sporadic and familial NTD cases. Subsequent studies have indicated, however, that for unknown reasons the control group used in that study strongly deviated from the later established Western European control group. When compared with this latter control group (pH1=0.211), the patient data of Joosten et al. (26) show an increased H1 frequency in the group of familial NTD patients (pH1=0.316) but not in that of sporadic patients (pH1=0.184). The reason for this latter observation is unknown, but may be related to the less strict definition of the cases, since also cases with encephalocele, craniorachischisis and anencephaly were incuded (27, 28). Therefore, our initial conclusion that H1/H2 heterzygotes have an increased predisposition for NTDs appeared to be biased as a result of this improper control group and possibly also deviating case groups.

In our subsequent analysis we have used a cohort of both spina bifida patients

and controls and their respective mothers, in combination with data on their blood glucose, myo-inositol and zinc value. Cases consisted only of live-born spina bifida apertas, while control mothers and children were carefully selected to match with the cases and their mothers on the basis of race, geographical origin, time of birth of the child, and the absence of congenital malformations (29). Importantly this control group strictly obeyed the distribution that we and others had obtained for other Western European control cohorts. From all these data combined we could define a Western European control distribution of *PDGFRA* promoter haplotypes with an H1 frequency (pH1) of 0.211. This control cohort strictly obeyed Hardy-Weinberg equilibrium, so without any indication for a deficiency of H1/H2 heterozygotes. Moreover, we could conclude that children with an H1 allele have an increased risk of developing NTDs. These findings are now supported by two independent studies on American Hispanics (3, 4), although minor discrepancies in the conclusions remain, particularly as a result of differences in the experimental set-up and recruitment of the cases and controls. These differences and their accompanying limitations will now be discussed in order to show that they support our hypothesis that H1 increases the risk of NTDs.

In the study by Zhu et al. (3) on the association of *PDGFRA* promoter haplotypes with spina bifida in a group of American Hispanics from Mexican descent, the H1 allele was more abundant in cases (pH1=0.360) than in controls (pH1=0.319), but the observed difference was not within the $p<0.05$ significance level (OR = 1.2, 95% CI = 0.7-2.0). An increase in H1 frequency was also observed in case mothers (pH1=0.385), which was significant compared to the control mother group (pH1=0.319, OR = 1.3, 95% CI = 0.9–1.9). Moreover, the authors observed that H1 homozygotes were significantly overrepresented in case mothers, which is in agreement with our findings. From the observation that a better correlation was observed between spina bifida of the child and the haplotype of the mother than of the child itself the authors concluded that that homozygosity for low activity alleles in mothers increases the risk of NTD in their offspring, possibly by affecting placental growth. We believe that these findings can mainly be attributed to the selection criteria of the cases and case mothers. Zhu et al. (3) also included NTD mothers of which no child material was available (79 of 122), whereas we only included child-mother pairs in our second study. In our opinion, the increased risk for H1 homozygote mothers of an NTD-affected pregnancy could also be explained by an increased transmission of the H1 risk allele.

More recently, Au et al. (4) performed a similar study, in which not only American Hispanics from Mexican descent but also American Caucasians were included. The authors came to the conclusion that *PDGFRA* haplotypes are differently distributed in the two populations, but they found no significant association with spina bifida myelomeningocele. Similar to Zhu et al. (3), they made the distinction between low and high activity alleles. However it is known that H2γ can also have a high promoter activity in cell types where H1 still displays a low promoter activity (3), and therefore we prefer the distinction on sequence similarity, i.e. H1 vs. H2 haplotypes. When doing so, the data of Au et al. (4) show an overrepresentation of H1 alleles in the Caucasian cases compared to the corresponding controls which, very similar to our observation, which is on the verge of significance (29.9% H1 in cases vs. 21.9% in controls, OR = 1.523, 95% CI = 0.997-2.326). This suggests that only a small increase in the sample would be required to obtain a statistically significant difference in H1 frequency between the two populations. Nevertheless, these data are in support of our hypothesis that H1 increases the risk of spina bifida risk in Caucasians.

In the Hispanic population of the Au study, the H1 frequency in the control group appears extremely high (47.3%) when compared to the findings of Zhu et al. (31.9%) in a similar population. In cases and case mothers on the other hand, the H1 frequency is similar in both studies (cases Zhu=36.0%, Au=42.9%; case mothers Zhu=38.5%, Au=39.6%). We therefore doubt the representativeness of the Hispanic control group of the Au et al. study. When comparing the Au Hispanic cases with the Zhu Hispanic controls, the H1 allele is significantly more abundant in the cases than in the controls (OR=1.608 95% CI: 1.166 – 2.219). Furthermore, the authors observed an over-transmission of H1 in the Hispanic spina bifida triads, which suggests that H1 is indeed a risk factor for spina bifida, also in American Hispanics. In addition, in the Au study only mothers of cases were included, and it was observed that both in the Caucasians and the Hispanic groups the H1 frequency was higher in cases then in their mothers, just as in our study. This indicates that H1 is a risk factor for NTDs in the child itself and not for NTDs in offspring, as was concluded by Zhu et al. Thus, when considering all these data together it appears that the H1 allele contributes to the etiology of spina bifida, both in Caucasians and in American Hispanics.

Spina bifida and glioblastoma as multifactorial diseases

Both spina bifida and gliomagenesis can be considered as a multifactorial disease in which a combination of genetic and environmental factors plays a role. Our data show that *PDGFRA* contributes to the genetic factors for both diseases, such that high PDGFRA expression levels enhance the risk of glioblastoma formation, while low expression levels increase the risk of NTDs. Besides *PDGFRA*, only very few genes have been implicated in human NTD risk, including methylenetetrahydrofolate reductase (30). On the other hand, various environmental factors are known that contribute to the risk of spina bifida, including low maternal folic acid intake (31), high maternal BMI or maternal obesity (32-37), as well as high glucose and low myo-inositol and low zinc status of the mother (29) (and references therein). Such multifactorial diseases are best described in terms of a liability/threshold model, in which all factors that influence the development of a multifactorial disorder, either genetic or environmental, add up to the total liability for the disease. If the sum of all adverse genetic and environmental factors exceeds a certain threshold, this will result in the disease phenotype (38). Assuming that H1 is an adverse genetic factor for NTDs, this would imply that less adverse environmental factors would be required for an H1 child than for an H2 child to be affected with spina bifida.

Interpretation of our results in terms of this model is complicated by the fact that our analysis is restricted to live-born children with spina bifida. Most likely a too severe combination of adverse factors will result in prenatal death, and these cases are not included in our study. Our observation that high maternal glucose and low maternal myo-inositol are risk factors particularly for H2 children, but much less so for H1 children, most likely results from increased prenatal mortality of H1 children with a low myo- inositol or high glucose mother. On the other hand, it has been established that some 15% of control individuals show a very mild form of spina bifida occulta, indicating that in the case of NTDs an exact patient group is difficult to define (39, 40).

In the case of glioblastoma the situation is less complex, since the patient definition is more clear, while in addition no environmental factors are known that unequivocally predispose to gliomagenesis, with the exception of intracranial radiation

(8). Other genetic factors may act in concert with *PDGFRA* promoter haplotypes to exceed the threshold necessary for brain tumor formation to occur. These could include mutation or homozygous deletion of the tumor suppressor genes *PTEN* (20, 41) or *TP53,* or allelic variants of the genes encoding the carcinogen-metabolizing enzymes cytochrome P-450 (CYP2D6) and glutathione S-transferase (GSTT1 and GSTM1) (8). It has already been shown that deletion of the tumor suppressor gene encoding INK4a-ARF or that encoding p53 cooperates with a retrovirally expressed PDGFRA ligand PDGF-B regarding both tumor latency and frequency in a mouse brain tumor model for secondary glioblastoma (42). It should be noted however, that in our precursor pool size model high PDGFRA expression in itself is not necessarily tumorigenic, but the before mentioned factors could be responsible to transform cells out of the enlarged pool.

Future research

In our study we have shown that U251, 31L and Cl2:6 glioblastoma cells display a more than 10-fold lower expression of H1 than of H2α, whereas U410 glioblastoma cells show high expression of both alleles. Moreover, we have shown that the first three cell lines display low total *PDGFRA* expression, while U401 cells highly express *PDGFRA*. In combination with the observation that in rats *Pdgfra* expression is upregulated upon differentiation from NSC to OPC (22, 23), we hypothesized that the first three cell lines have maintained more stem cell characteristics than the fourth. Our recent preliminary results indicate that at least U-251 and Cl2:6 cells form highly infiltrative intracranal tumors in nude mice, while U-410 cells are unable to do so (A. Claes, P. Wesseling, E.J.J. van Zoelen; unpublished). This agrees with previous data showing that astrocytoid and small polygonal-shaped glioma cell lines, such as the Cl2:6, readily form tumors when injected into the brain of nude rats, while glioma cell lines with a fibroblastic morphology, such as the U-343 MG (43) and U-410, are unable to do so. Another possibility would be to study these cells for the expression of NSC, OPC and mature glia specific marker genes, such as Nestin (NSC (44)), A2B5 (early OPC (45)), NG2 (OPC (46)) and PLP (oligodendrocytes (46)) or MBP (oligodendrocytes (24)). It has recently been described that gliomas indeed contain stem cells which resemble NSCs in their gene expression profile (47).

We furthermore hypothesized that *PDGFRA* transcription is upregulated during differentiation of NSCs to OPCs by promoter demethylation, but such that H1 is demethylated at a later stage during this differentiation process than H2α. To investigate this possibility, homogeneous populations of H1/H2 heterozygous human NSCs would be required which could be stimulated to differentiate into OPCs and eventually into mature oligodendrocytes, during which haplotype-specific *PDGFRA* expression could be measured. At the moment it is not feasible, however, to obtain such NSCs from mature donor tissue. A possibility would be to use H1/H2 heterozygous human embryonic stem cells, which could first be induced to neural differentiation by growing them as neurospheres and subsequently be grown as homogeneous monolayes of NSCs. These cells could be studied for haplotype-specific *PDGFRA* expression following differentiation into glia cells, according to a recently described protocol (48). Alternatively, homogeneous monolayers of cancer stem cells can be isolated from glioblastoma tumors. If these cancer stem cells still resemble NSCs in that they only express low levels of *PDGFRA*, they can also be triggered to differentiate into glia-like cells by addition of serum or retinoic acid, as

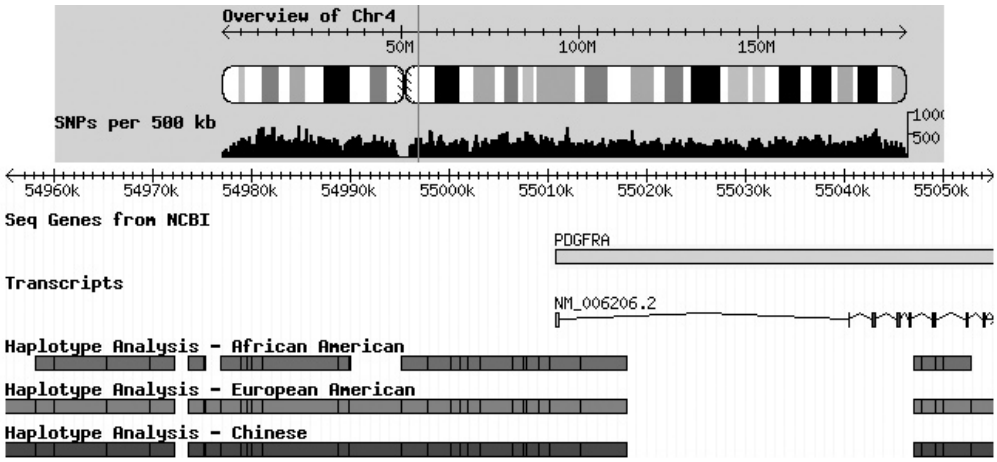

Figure 2: *PDGFRA* haplotype blocks in African Americans, European Americans and Chinese (from Perlegen.com). *PDGFRA* promoter haplotype block ranges from 54973631 to 55017967 bp in European Americans and Chinese and from 54995156 to 55017967 bp in African Americans on chromosome 4.

recently described (47). A third approach would be to generate transgenic mice in which the Pdfgra gene is replaced by the human *PDGFRA* H1 and/or H2 allele, or alternatively to use Pdgfra (-/-) mice which are complemented with human H1 and H2 *PDGFRA* YACs. In this way it can be studied whether the low activity H1 allele indeed induces premature OPC maturation resulting in lower OPC and oligodendrocyte numbers as well as hypomyelination, when compared to H2. In this respect it would also be interesting to compare individuals with the various haplotype combinations (H1/H1, H1/H2 and H2/H2) for their amount of myelin by MRI based techniques (49).

Repression of the H1 allele in glioblastoma cells could be mediated by the inability of certain transcription factors to bind methylated DNA. This hypothesis could be tested by studying differential protein binding to methylated vs. unmethylated promoters by EMSA techniques. Furthermore, haplo-ChIP experiments could be performed with antibodies against transcription factors that potentially bind the promoter region that is differentially methylated between H1 and H2, such as ZBP-89, EGR1, GATA-2 and FKHRL1 (identified by MatInspector analysis), as well as against factors that have been identified by methylation specific EMSAs to investigate haplotype and/or methylation specific promoter binding *in vivo*. However, it is unlikely that this will give insight into the origin of observed differential (de)methylation, since the cause of this phenomenon must lie within the sequential differences between H1 and H2. Unlike in the human osteosarcoma cells, however, we have been unable to detect any polymorphism-dependent protein binding in the -3600/+118 promoter region of *PDGFRA* using nuclear extracts from the three H1 repressing glioblastoma cell lines. As an alternative for EMSAs oligonucleotide trapping experiments could be used, in which nuclear proteins which are bound differentially to H1 and H2 are isolated by affinity chromatography (50). Using this method, we have recently shown by mass spectrometry analysis on nuclear extracts from osteosarcoma cells, that the chromatin remodeling complex SWI/SNF preferentially binds the H1 specific +68GAdel

allele. Subsequent chromatin immunoprecipitation (ChIP) analysis on the glioblastoma cell lines indicated that this allelic preference is dependent on promoter methylation (P. Joosten, unpublished data). This suggests that SWI/SNF may be involved in the differential expression and methylation of *PDGFRA* promoter alleles in glioblastoma cells.

It has been established that *PDGFRA* promoter haplotypes span approximately 40 kb of genomic DNA, at least in Caucasian Americans and Chinese (fig. 2), and therefore it cannot be excluded that polymorphisms outside the -3600/+118 region studied so far are involved in the haplotype-specific expression and methylation in these glioblastoma cell lines. In this respect, we could confirm that the haplotype of polymorphism rs7678144, which lies just on the 3' border of the haplotype block at approximately 7 kb downstream of the transcription start site, is linked to the *PDGFRA* promoter polymorphisms described in our studies, whereas rs4637467, which is located at approximately 29 kb downstream, does not appear to be coupled to these promoter polymorphisms (unpublished).

Spina bifida is not only a characteristic of *Patch* and *Pdgfra* (-/-) mice, but also of *Pdgfc* mutants and *Pdgfa*/*Pdgfc* double mutant mice (51), indicating that the genes for these two ligand genes might also be susceptibility genes for human NTDs. Thus, NTD case-control could be performed on these genes in humans, and multiple logistic regression analysis could be performed to test whether there is an additional or interactive effect of *PDGFRA* promoter haplotypes and *PDGFA* (52) and/or *PDGFC* alleles (51).

PDGFRA and other diseases

Airway remodeling by proliferation of lung smooth muscle cells is a mayor cause of the chronicity and severity of asthma(53), and PDGFRA signaling plays an important role in this process (54, 55). In a case control study on various types of asthma, Wu et al. (2) found that the frequency of H1 containing PDGFRA genotypes (H1/H1 + H1/H2) was increased nonallergic asthmatics, while the higher expression H2/H2 genotype was more frequent in patients with more severe types of childhood asthma, thus providing an association between *PDGFRA* promoter haplotypes with a third PDGFRA related disease.

Atherosclerosis is another disease in which PDGFRA signaling plays an important role, since PDGFRA regulates chemoattraction and proliferation of vascular smooth muscle cells (56). Therefore, Herrmann et al. (52) performed a large case-control study on coronary heart disease patients, but found no association with variation in the genes encoding PDGFRA, PDGFRB, PDGFA and PDGFB, including the H1/H2 linked -1631AACTTins/del PDGFRA polymorphism. Most likely this implies that the level of PDGFRA expression in pre-atherosclerotic lesions is not indicative for the disease risk.

Besides gliomas, also a number of other malignancies have been associated with PDGFRA, such as chondrosarcomas, osteosarcomas, breast cancer, prostate cancer and gastrointestinal stromal tumors (GISTs). Chondrosarcomas show a high expression of both *PDGFA* and *PDGFRA* which increases with tumor grade, whereas in healthy cartilage tissue no expression could be detected, except for the hypertrophic cartilage of callus which shows high expression. Interestingly, higher PDGFRA expression in chondrosarcomas has been associated with a significantly shorter patient survival time (57). This suggests that *PDGFRA* expression is involved in chondrocyte proliferation,

as well as in the aggressiveness of chondrosarcoma growth and thus that *PDGFRA* haplotypes may also influence susceptibility to chondrosarcomas. This may imply that the high expressing H2α allele may stimulate chondrocyte and chondrosarcoma growth whereas the low expressing H1 allele may do so less, or, like we hypothesize for glioblastomas, H2 and H1 differentially affect the chondrocyte precursor pool size. This would require analyzing haplotype-specific expression in proliferating chondrocytes, their precursor cells and in chondrosarcoma cells, as well as determining the haplotype distribution in chondrosarcoma patients.

The growth factor PDGF-AA stimulates the proliferation of osteoblastic cells by activation of PDGFRA. Both ligand and receptor are highly expressed in osteosarcomas suggesting that auto- and paracrine mechanisms are involved in osteosarcoma growth (58). Prostate carcinomas also overexpress both *PDGFA* and *PDGFRA* (59, 60), suggesting an involvement of PDGFRA expression in prostate cancer development as well. In breast carcinomas, PDGFRA expression correlates with tumor malignancy (61), while in ovarian carcinomas, PDGFRA expression is associated with high tumor grade, high proliferation index and poor patient outcome (62). Thus, similarly to chondrosarcomas, *PDGFRA* promoter haplotypes could also influence susceptibility to these types of tumors. Again, haplotype specific expression in the appropriate cells as well as haplotype distribution in patients has to be analyzed. By contrasts, GISTs mainly arise as a consequence of activating mutations in either *PDGFRA* or its neighbouring gene *c-KIT*, but not from overexpression of either gene (63). Therefore *PDGFRA* promoter haplotypes most likely will not affect GIST susceptibility. In the case of mesenchymal tumors such as osteosarcomas and chondrosarcomas, PDGFRA is already expressed in the normal cells and tends to be upregulated in the tumor cells. In the case of epithelial tumors, such as breast and prostate cancer, the normal cells are lined up in a continuous monolayer of epithelial cells which do not express PDGFRA. Also the tumor cells will not express PDGFRA as long as they show an epithelial morphology, but as soon as they undergo epithelial-mesenchymal transition (EMT), which is a prerequisite for metastasis, they obtain a mesenchymal morphology characterized by PDGFRA expression (64). It remains to be established if the extent of PDGFRA expression as a result of promoter polymorphisms will correlate with the metastatic behavior of such tumor cells. NSCs upregulate PDGFRA upon differentiation into the glial lineage, and in this respect this differentiation process resembles EMT.

Insufficient PDGFRA signaling can lead to myelination defects due to improper oligodendrocyte development, as is demonstrated by several mouse models (46, 65, 66). Therefore, *PDGFRA* expression may also play a role in myelination diseases such as multiple sclerosis (MS). MS is characterized by demyelination of the CNS with associated neurological deficits. Remyelination can occur but is often incomplete, and requires the proliferation and migration of OPC into the lesion from the neighboring areas (67). In *Pdgfra* (+/-) mice, which display a 50% reduced PDGFRA protein expression (68), OPC proliferation and oligodendrocyte repopulation after demyelination are impaired (46), suggesting that also in recovery from or progression of myelination disorders such as MS PDGFRA haplotypes may play a role.

In conclusion, in multifactorial diseases such as NTDs and glioblastomas it is generally very difficult to establish the contribution of a single factor to the etiology. Therefore, the association of *PDGFRA* promoter haplotypes with both these diseases as observed by us provides a significant contribution to our understanding of the molecular basis of NTDs and glioblastomas.

References

1. Hinds, DA, Stuve, LL, Nilsen, GB, Halperin, E, Eskin, E, Ballinger, DG, Frazer, KA, and Cox, DR. Whole-genome patterns of common DNA variation in three human populations. Science 2005;307:1072-9.
2. Wu, LS, Tan, CY, Wang, LM, Lin, CG, and Wang, JY. Variant in promoter region of platelet-derived growth factor receptor-alpha (PDGFRalpha) gene is associated with the severity and allergic status of childhood asthma. Int Arch Allergy Immunol 2006;141: 37-46.
3. Zhu, H, Wicker, NJ, Volcik, K, Zhang, J, Shaw, GM, Lammer, EJ, Suarez, L, Canfield, M, and Finnell, RH. Promoter haplotype combinations for the human PDGFRA gene are associated with risk of neural tube defects. Mol Genet Metab 2004;81:127-32.
4. Au, KS, Northrup, H, Kirkpatrick, TJ, Volcik, KA, Fletcher, JM, Townsend, IT, Blanton, SH, Tyerman, GH, Villarreal, G, and King, TM. Promotor genotype of the platelet-derived growth factor receptor-alpha gene shows population stratification but not association with spina bifida meningomyelocele. Am J Med Genet A 2005;139:194-8.
5. Lary, JM and Edmonds, LD. Prevalence of spina bifida at birth--United States, 1983-1990: a comparison of two surveillance systems. MMWR CDC Surveill Summ 1996;45:15-26.
6. Feuchtbaum, LB, Currier, RJ, Riggle, S, Roberson, M, Lorey, FW, and Cunningham, GC. Neural tube defect prevalence in California (1990-1994): eliciting patterns by type of defect and maternal race/ethnicity. Genet Test 1999;3:265-72.
7. Surawicz, TS, McCarthy, BJ, Kupelian, V, Jukich, PJ, Bruner, JM, and Davis, FG. Descriptive epidemiology of primary brain and CNS tumors: results from the Central Brain Tumor Registry of the United States, 1990-1994. Neuro-oncol 1999;1:14-25.
8. Ohgaki, H and Kleihues, P. Epidemiology and etiology of gliomas. Acta Neuropathol (Berl) 2005;109:93-108.
9. Das, A, Tan, WL, Teo, J, and Smith, DR. Glioblastoma multiforme in an Asian population: evidence for a distinct genetic pathway. J Neurooncol 2002;60:117-25.
10. Menn, B, Garcia-Verdugo, JM, Yaschine, C, Gonzalez-Perez, O, Rowitch, D, and Alvarez-Buylla, A. Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 2006;26:7907-18.
11. Kesari, S and Stiles, CD. The bad seed: PDGF receptors link adult neural progenitors to glioma stem cells. Neuron 2006;51:151-3.
12. Jackson, EL, Garcia-Verdugo, JM, Gil-Perotin, S, Roy, M, Quinones-Hinojosa, A, Vandenberg, S, and Alvarez-Buylla, A. PDGFRalpha-Positive B Cells Are Neural Stem Cells in the Adult SVZ that Form Glioma-like Growths in Response to Increased PDGF Signaling. Neuron 2006;51:187-99.
13. Uhrbom, L, Hesselager, G, Nister, M, and Westermark, B. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. Cancer Res 1998;58:5275-9.
14. Uhrbom, L, Hesselager, G, Ostman, A, Nister, M, and Westermark, B. Dependence of autocrine growth factor stimulation in platelet-derived growth factor-B-induced mouse brain tumor cells. Int J Cancer 2000;85:398-406.
15. Shih, AH, Dai, C, Hu, X, Rosenblum, MK, Koutcher, JA, and Holland, EC. Dose-dependent effects of platelet-derived growth factor-B on glial tumorigenesis. Cancer Res 2004;64:4783-9.
16. Dai, C, Celestino, JC, Okada, Y, Louis, DN, Fuller, GN, and Holland, EC. PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and

oligoastrocytomas from neural progenitors and astrocytes in vivo. Genes Dev 2001;15: 1913-25.
17. Assanah, M, Lochhead, R, Ogden, A, Bruce, J, Goldman, J, and Canoll, P. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor-expressing retroviruses. J Neurosci 2006;26:6781-90.
18. Zhu, Y and Parada, LF. The molecular and genetic basis of neurological tumours. Nat Rev Cancer 2002;2:616-26.
19. Kleihues, P and Ohgaki, H. Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro-oncol 1999;1:44-51.
20. Knobbe, CB and Reifenberger, G. Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol 2003;13:507-18.
21. Andrae, J, Hansson, I, Afink, GB, and Nister, M. Platelet-derived growth factor receptor-alpha in ventricular zone cells and in developing neurons. Mol Cell Neurosci 2001;17: 1001-13.
22. Hall, A, Giese, NA, and Richardson, WD. Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors. Development 1996;122:4085-94.
23. Hu, JG, Fu, SL, Zhang, KH, Li, Y, Yin, L, Lu, PH, and Xu, XM. Differential gene expression in neural stem cells and oligodendrocyte precursor cells: a cDNA microarray analysis. J Neurosci Res 2004;78:637-46.
24. McKinnon, RD, Waldron, S, and Kiel, ME. PDGF alpha-receptor signal strength controls an RTK rheostat that integrates phosphoinositol 3'-kinase and phospholipase Cgamma pathways during oligodendrocyte maturation. J Neurosci 2005;25:3499-508.
25. Jackson, EL, Garcia-Verdugo, JM, Gil-Perotin, S, Roy, M, Quinones-Hinojosa, A, VandenBerg, S, and Alvarez-Buylla, A. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. Neuron 2006;51:187-99.
26. Joosten, PH, Toepoel, M, Mariman, EC, and Van Zoelen, EJ. Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects. Nat Genet 2001;27:215-7.
27. Hol, FA, Geurds, MP, Chatkupt, S, Shugart, YY, Balling, R, Schrander-Stumpel, CT, Johnson, WG, Hamel, BC, and Mariman, EC. PAX genes and human neural tube defects: an amino acid substitution in PAX1 in a patient with spina bifida. J Med Genet 1996;33:655-60.
28. Hol, FA, van der Put, NM, Geurds, MP, Heil, SG, Trijbels, FJ, Hamel, BC, Mariman, EC, and Blom, HJ. Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects. Clin Genet 1998;53:119-25.
29. Groenen, PM, Peer, PG, Wevers, RA, Swinkels, DW, Franke, B, Mariman, EC, and Steegers-Theunissen, RP. Maternal myo-inositol, glucose, and zinc status is associated with the risk of offspring with spina bifida. Am J Obstet Gynecol 2003;189:1713-9.
30. Boyles, AL, Hammock, P, and Speer, MC. Candidate gene analysis in human neural tube defects. Am J Med Genet C Semin Med Genet 2005;135:9-23.
31. Lumley, J, Watson, L, Watson, M, and Bower, C. Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects. Cochrane Database Syst Rev 2001;CD001056.

32. Shaw, GM, Velie, EM, and Schaffer, D. Risk of neural tube defect-affected pregnancies among obese women. Jama 1996;275:1093-6.
33. Shaw, GM, Todoroff, K, Finnell, RH, and Lammer, EJ. Spina bifida phenotypes in infants or fetuses of obese mothers. Teratology 2000;61:376-81.
34. Werler, MM, Louik, C, Shapiro, S, and Mitchell, AA. Prepregnant weight in relation to risk of neural tube defects. Jama 1996;275:1089-92.
35. Kallen, K. Maternal smoking, body mass index, and neural tube defects. Am J Epidemiol 1998;147:1103-11.
36. Watkins, ML, Scanlon, KS, Mulinare, J, and Khoury, MJ. Is maternal obesity a risk factor for anencephaly and spina bifida? Epidemiology 1996;7:507-12.
37. Watkins, ML, Rasmussen, SA, Honein, MA, Botto, LD, and Moore, CA. Maternal obesity and risk for birth defects. Pediatrics 2003;111:1152-8.
38. Hol, FA.Genetic factors in human neural tube defects. Nijmegen: University of Nijmegen; 1999.
39. Boone, D, Parsons, D, Lachmann, SM, and Sherwood, T. Spina bifida occulta: lesion or anomaly? Clin Radiol 1985;36:159-61.
40. Fidas, A, MacDonald, HL, Elton, RA, Wild, SR, Chisholm, GD, and Scott, R. Prevalence and patterns of spina bifida occulta in 2707 normal adults. Clin Radiol 1987;38:537-42.
41. Li, J, Yen, C, Liaw, D, Podsypanina, K, Bose, S, Wang, SI, Puc, J, Miliaresis, C, Rodgers, L, McCombie, R, Bigner, SH, Giovanella, BC, Ittmann, M, Tycko, B, Hibshoosh, H, Wigler, MH, and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943-7.
42. Hesselager, G, Uhrbom, L, Westermark, B, and Nister, M. Complementary effects of platelet-derived growth factor autocrine stimulation and p53 or Ink4a-Arf deletion in a mouse glioma model. Cancer Res 2003;63:4305-9.
43. Enblad, P, Hesselager, G, Bongcam-Rudloff, E, Hallin, I, Westermark, B, and Nister, M. Modulation of phenotype and induction of irregular vessels accompany high tumorigenic potential of clonal human glioma cells xenografted to nude-rat brain. Int J Cancer 2000;85:819-28.
44. Lendahl, U, Zimmerman, LB, and McKay, RD. CNS stem cells express a new class of intermediate filament protein. Cell 1990;60:585-95.
45. Liu, Y, Wu, Y, Lee, JC, Xue, H, Pevny, LH, Kaprielian, Z, and Rao, MS. Oligodendrocyte and astrocyte development in rodents: an in situ and immunohistological analysis during embryonic development. Glia 2002;40:25-43.
46. Murtie, JC, Zhou, YX, Le, TQ, Vana, AC, and Armstrong, RC. PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. Neurobiol Dis 2005;19:171-82.
47. Lee, J, Kotliarova, S, Kotliarov, Y, Li, A, Su, Q, Donin, NM, Pastorino, S, Purow, BW, Christopher, N, Zhang, W, Park, JK, and Fine, HA. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9:391-403.
48. Conti, L, Pollard, SM, Gorba, T, Reitano, E, Toselli, M, Biella, G, Sun, Y, Sanzone, S, Ying, QL, Cattaneo, E, and Smith, A. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 2005;3:e283.
49. Vidarsson, L, Conolly, SM, Lim, KO, Gold, GE, and Pauly, JM. Echo time optimization for linear combination myelin imaging. Magn Reson Med 2005;53:398-407.
50. Gadgil, H and Jarrett, HW. Oligonucleotide trapping method for purification of

transcription factors. J Chromatogr A 2002;966:99-110.
51. Ding, H, Wu, X, Bostrom, H, Kim, I, Wong, N, Tsoi, B, O'Rourke, M, Koh, GY, Soriano, P, Betsholtz, C, Hart, TC, Marazita, ML, Field, LL, Tam, PP, and Nagy, A. A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. Nat Genet 2004;36:1111-6.
52. Herrmann, SM, Ricard, S, Nicaud, V, Brand, E, Behague, I, Blanc, H, Ruidavets, JB, Evans, A, Arveiler, D, Luc, G, Poirier, O, and Cambien, F. Polymorphisms in the genes encoding platelet-derived growth factor A and alpha receptor. J Mol Med 2000;78:287-92.
53. Vignola, AM, Mirabella, F, Costanzo, G, Di Giorgi, R, Gjomarkaj, M, Bellia, V, and Bonsignore, G. Airway remodeling in asthma. Chest 2003;123:417S-22S.
54. Hoyle, GW, Li, J, Finkelstein, JB, Eisenberg, T, Liu, JY, Lasky, JA, Athas, G, Morris, GF, and Brody, AR. Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor. Am J Pathol 1999;154:1763-75.
55. Bonner, JC, Lindroos, PM, Rice, AB, Moomaw, CR, and Morgan, DL. Induction of PDGF receptor-alpha in rat myofibroblasts during pulmonary fibrogenesis in vivo. Am J Physiol 1998;274:L72-80.
56. Ross, R, Raines, EW, and Bowen-Pope, DF. The biology of platelet-derived growth factor. Cell 1986;46:155-69.
57. Sulzbacher, I, Birner, P, Trieb, K, Muhlbauer, M, Lang, S, and Chott, A. Platelet-derived growth factor-alpha receptor expression supports the growth of conventional chondrosarcoma and is associated with adverse outcome. Am J Surg Pathol 2001;25: 1520-7.
58. Sulzbacher, I, Birner, P, Trieb, K, Traxler, M, Lang, S, and Chott, A. Expression of platelet-derived growth factor-AA is associated with tumor progression in osteosarcoma. Mod Pathol 2003;16:66-71.
59. Fudge, K, Wang, CY, and Stearns, ME. Immunohistochemistry analysis of platelet-derived growth factor A and B chains and platelet-derived growth factor alpha and beta receptor expression in benign prostatic hyperplasias and Gleason-graded human prostate adenocarcinomas. Mod Pathol 1994;7:549-54.
60. Fudge, K, Bostwick, DG, and Stearns, ME. Platelet-derived growth factor A and B chains and the alpha and beta receptors in prostatic intraepithelial neoplasia. Prostate 1996;29:282-6.
61. Carvalho, I, Milanezi, F, Martins, A, Reis, RM, and Schmitt, F. Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. Breast Cancer Res 2005;7:R788-95.
62. Lassus, H, Sihto, H, Leminen, A, Nordling, S, Joensuu, H, Nupponen, NN, and Butzow, R. Genetic alterations and protein expression of KIT and PDGFRA in serous ovarian carcinoma. Br J Cancer 2004;91:2048-55.
63. Yamamoto, H, Oda, Y, Kawaguchi, K, Nakamura, N, Takahira, T, Tamiya, S, Saito, T, Oshiro, Y, Ohta, M, Yao, T, and Tsuneyoshi, M. c-kit and PDGFRA mutations in extragastrointestinal stromal tumor (gastrointestinal stromal tumor of the soft tissue). Am J Surg Pathol 2004;28:479-88.
64. Jechlinger, M, Sommer, A, Moriggl, R, Seither, P, Kraut, N, Capodiecci, P, Donovan, M, Cordon-Cardo, C, Beug, H, and Grunert, S. Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Invest 2006;116:1561-70.
65. Klinghoffer, RA, Hamilton, TG, Hoch, R, and Soriano, P. An allelic series at the PDGFalphaR locus indicates unequal contributions of distinct signaling pathways during development. Dev Cell 2002;2:103-13.

66. Fruttiger, M, Karlsson, L, Hall, AC, Abramsson, A, Calver, AR, Bostrom, H, Willetts, K, Bertold, CH, Heath, JK, Betsholtz, C, and Richardson, WD. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development 1999;126:457-67.
67. Tiwari-Woodruff, SK, Kaplan, R, Kornblum, HI, and Bronstein, JM. Developmental expression of OAP-1/Tspan-3, a member of the tetraspanin superfamily. J Neurosci Res 2004;77:166-73.
68. Soriano, P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997;124:2691-700.

Summary

Transcription is one of the first steps in the regulation of gene expression, and therefore it contributes greatly in determining how much of a protein is produced in time and space, which consequently has a great influence on cell function. Transcription is regulated by specific DNA sequences surrounding and within genes, so-called *cis*-acting factors. The promoter of a gene is a *cis*-acting sequence located just 5' of the transcription start site, which determines where RNA polymerase II initiates transcription and in addition controls the basal rate of transcription. RNA polymerase II is guided to the transcription start site by various proteins, together termed the basal transcriptional machinery or preinitiating complex. Basal transcription can be stimulated or inhibited by other regulatory sequences, termed enhancer and silencer elements. These enhancers and silencers recruit transcription factors, so-called *trans*-acting factors, which communicate with the basal transcriptional machinery by protein-protein interactions, resulting in a stimulation or inhibition of RNA polymerase II recruitment to the transcription initiation site. Additionally, transcriptional regulation takes place at the epigenetic level. DNA methylation and modification of histones, the proteins that package DNA into chromatin, act in concert to determine the accessibility of transcription factors to the DNA.

Binding of transcription factors to DNA is dependent upon specific DNA sequences. Consequently, variation in sequence of regulatory DNA elements may alter protein binding and thus transcription of neighboring genes. Natural variation in DNA sequence on a specific position is termed a polymorphism, and mainly occurs as the substitution of a single nucleotide, a so-called single nucleotide polymorphism (SNP). A specific combination of linked polymorphisms is referred to as haplotype. It has been postulated that most phenotypic variation between individuals is caused by variation in regulatory sequences, since this mostly leads to relatively subtle, non-lethal alterations, whereas variations in coding sequences often causes alterations of protein function and generally results in much more severe consequences. In agreement with this, many studies have shown that by altering protein-DNA binding, and subsequently the rate of transcription, regulatory variation can influence susceptibility to diseases such as asthma, diabetes and cancers.

The platelet-derived growth factor (PDGF) ligand-receptor system plays an important role in vertebrate development. The four PDGF isoforms PDGFA, -B, -C and -D differentially bind and activate the two receptor isoforms PDGFRA and PDGFRB. Both ligands and receptors act as homo- or heterodimers and display specific expression patterns and, as a consequence, influence various developmental processes. PDGFRA and its two specific ligands PDGFA and PDGFC are involved in development of the axial skeleton and central nervous system (CNS) by regulating proliferation, migration and survival of sclerotomal cells and oligodendrocyte precursor cells (OPCs). Mice deficient in Pdgfra signaling display various developmental defects including hypomyelination of the central nervous system and the neural tube defect (NTD) spina bifida. This suggests that also in humans reduced PDGFRA signaling may contribute to such conditions. On the other hand, overexpression of Pdgfra signaling molecules is associated with various types of brain tumors (gliomas) in both mice and humans, indicating that enhanced PDGFRA signaling may predispose to gliomas. We thus hypothesized that factors that influence PDGFRA transcription may contribute to the risk of PDGFRA-related diseases such as NTDs and gliomas. The studies described in this thesis were undertaken to

identify both *cis*- and *trans*-acting factors that regulate *PDGFRA* transcription and to determine their involvement in gliomas and NTDs (chapter 1).

In search of regulatory sequences that may influence NTD risk we performed a case-control study in which we identified five haplotypes of the –1,589/+118 promoter region of the human PDGFRA gene, designated H1, H2α, H2β, H2γ and H2δ. Of these haplotypes, H1 and H2α, which differ from each other on six of the eight polymorphic sites, are the most abundant. Importantly, in transient transfection assays on osteosarcoma cells H2α and H2β displayed a 3-5 fold higher promoter activity than H1, H2γ and and H2δ. Moreover, specific combinations of promoter haplotypes appeared to correlate with NTDs. Together, this shows that naturally occurring variation in regulatory sequences can indeed cause differences in gene expression and consequently influence disease susceptibility (chapter 2).

NTDs are multifactorial diseases that result from a combination of various genetic and environmental factors. We therefore investigated the interaction of *PDGFRA* promoter haplotypes as a genetic factor, with maternal nutrient status as environmental factor on the risk of spina bifida in a similar case-control study. We found that the low activity H1 haplotype was significantly overrepresented in cases and mildly in case mothers, suggesting that low PDGFRA expression predisposes to spina bifida. Furthermore, we could show that for H2 children high maternal glucose and low maternal myo-inositol levels are the main risk factors for spina bifida, while for H1 children the maternal body mass index (BMI) was the only prominent risk factor. Finally, we found that mothers with an H1 allele are significantly shorter than homozygous H2 mothers, indicative for a role of PDGFRA in body growth (chapter 3).

Knowing that differences in PDGFRA expression can influence the occurrence of NTDs, we subsequently analyzed the human *PDGFRA* promoter for haplotype specific differences in protein binding on the previously identified polymorphic sites. We also included two additional polymorphisms found by an extension of the promoter analysis to -3600. We found differential protein binding for two of these ten sites; -909C/A and +68GAins/del. The protein complex binding specifically to the C allele of the -909C/A SNP, which is present in all haplotypes but the low activity H2γ, could be identified as USF family members, most likely a USF1a/USF2a heterodimer. Both USF1 and USF2 are important for normal brain function, suggesting that USF proteins may also play a role in *PDGFRA* transcriptional regulation during development. Furthermore, a protein complex of 125 kDa which bound specifically to the low activity haplotype H1 at position +68GAdel was found which may represent an H1-specific *PDGFRA* transcriptional repressor.

Gliomas often overexpress PDGFRA, and in this study we investigated whether endogenous *PDGFRA* expression in four H1/H2α heterozygous human glioblastoma cell lines is haplotype dependent. With a newly developed technique, we could show that in three of these four cell lines H1 expression was more than ten-fold lower than H2α, which was accompanied by a low total *PDGFRA* expression. This difference in expression could be greatly reduced by DNA demethylating agents and histone deacetylase inhibitors, indicating that epigenetic regulation plays an important role in the differential expression of H1 and H2α in glioblastoma cells. The large difference in haplotype specific expression also suggested that PDGFRA promoter haplotypes might influence susceptibility to gliomas. In a case-control study on 71 glioblastoma patients we indeed found a reduced frequency of the H1 allele, which was significant when comparing to a combined Western European control group, thus suggesting that the

low activity H1 haplotype may protect against gliomas.

Human embryonal carcinoma (EC) cells are widely used as an in vitro model system for studying differentiation-dependent regulation of gene expression during early human development. In Tera-2 human embryonal carcinoma cells *PDGFRA* expression is strongly enhanced upon differentiation induced by retinoic acid and cAMP treatment. We could show that this upregulation of *PDGFRA* expression requires a promoter element located near the transcription initiation site, which binds a complex of PRX and PBX proteins. Interestingly, Prx-deficient mice show a severe NTD phenotype, similar to that observed for *Pdgfra* null mice, suggesting that also in vivo these two genes may interact in axial development and thus may play a role in NTD genesis.

Taken together, the data presented in this thesis show that *PDGFRA* expression can influence physiological (body growth) as well as pathological processes (genesis of NTDs and gliomas). We have provided evidence that low PDGFRA expression driven by the H1 promoter haplotype predisposes to spina bifida, while at the same time protecting against glioblastomas. This observation is further supported by data on geographical and racial incidences of these two disorders, which correlate well with haplotype frequencies, such that populations with a high H1 frequency show a high incidence of NTDs, while populations with a low H1 frequency show a high brain tumor incidence. Based on our data and published data on *Pdgfra* transgenic mice we hypothesize that due to slower promoter demethylation, the H1 promoter haplotype reduces the risk of gliomagenesis by limiting the glia precursor pool size, which is controlled by PDGFRA signalling. Since PDGFRA overexpression is also associated with osteosarcomas, chondrosarcomas and ovarian carcinomas, *PDGFRA* promoter haplotypes may also influence susceptibility to these diseases. Additionally, as PDGFRA signaling is essential for (re)myelination, *PDGFRA* promoter haplotypes may also play a role in demyelination diseases such as MS (chapter 7).

Samenvatting

Transcriptie is een van de eerste stappen in de regulatie van genexpressie en aangezien het een grote bijdrage levert aan het bepalen van de hoeveelheid eiwit die in tijd en plaats wordt geproduceerd, is dit proces van grote invloed op het functioneren van de cel.. Transcriptie wordt gereguleerd door specifieke DNA sequenties rondom en binnen genen, de zogenoemde *cis*-werkende factoren. De promoter van een gen is een *cis*-werkende sequentie die net voor de transcriptiestart ligt, de plaats waar het RNA polymerase II het transcriptieproces initieert, en die de basale transcriptiesnelheid reguleer. Het RNA polymerase II wordt begeleid door een aantal eiwitten, die tezamen de basale transcriptionele machinerie of het preinitiatiecomplex worden genoemd. Basale transcriptie kan gestimuleerd of geremd worden door andere regulerende sequenties, de zogenoemde enhancer en silencer elementen. Deze enhancers en silencers rekruteren transcriptiefactoren, die als *trans*-werkende eiwitten met de basale transcriptionele machinerie communiceren via eiwit-eiwitinteracties, en dit resulteert in een stimulatie of remming van rekrutering van het RNA polymerase II aan de transcriptiestart. Transcriptieregulatie vindt daarnaast ook plaats op epigenetisch niveau, waarbij methylering van het DNA en modificatie van histonen, de eiwitten die het DNA inpakken in chromatine, samen de toegankelijheid van het DNA voor transcriptiefactoren reguleren.

Binding van transcriptiefactoren aan DNA is afhankelijk van specifieke DNA sequenties. Als gevolg daarvan kan variatie in regulerende DNA sequenties leiden tot een veranderde DNA binding van transcriptiefactoren en dus ook tot veranderingen in transcriptie van nabijgelegen genen. Natuurlijke variatie in DNA sequentie op een bepaalde positie wordt een polymorfisme genoemd, en komt voornamelijk voor als een substitutie van één enkel nucleotide, hetgeen bekend staat als een single nucleotide polymorphism (SNP). Een specifieke combinatie van aan elkaar gekoppelde polymorfismen wordt ook wel een haplotype genoemd. Algemeen wordt aangenomen dat de meeste fenotypische verschillen tussen individuen het gevolg zijn van variatie in regulerende sequenties, aangezien polymorfismen in deze regio's meestal leiden tot relatief subtiele, niet lethale veranderingen, terwijl daarentegen variatie in eiwit-coderende sequenties vaak leidt tot veranderde eiwitfuncties hetgeen vaak ernstige gevolgen heeft. In overeenstemming hiermee is in veel studies aangetoond dat variatie in regulerende sequenties die resulteren in veranderde eiwit-DNA binding en als gevolg daarvan in veranderde expressie, het risico op het ontstaan van ziekten als als astma, diabetes en kanker sterk kan beïnvloeden.

Het platelet-derived growth factor (PDGF) ligand-receptor systeem speelt een belangrijke rol in de ontwikkeling van vertebraten. De vier ligand-isovormen PDGFA, PDGFB, PDGFC en PDGFD, binden en activeren de twee receptor-isovormen PDGFRA en PDGFRB ieder op een specifieke manier. Zowel de liganden als de receptoren werken in de vorm van homo- en heterodimeren en vertonen celspecifieke expressiepatronen, waardoor zij een grote diversiteit aan ontwikkelingsprocessen beïnvloeden. PDGFRA en zijn specifieke liganden PDGFA en PDGFC zijn betrokken bij de ontwikkeling van het axiale skelet en het centrale zenuwstelsel door het reguleren van de proliferatie, migratie en overleving van cellen van het sclerotoom en oligodendrocyt precursor cellen (OPCs). Muizen met een defect in Pdgfra signalisatie vertonen allerlei ontwikkelingsstoornissen, waaronder hypomyelinisatie van het centrale zenuwstelsel en een vorm van neuraal buisdefect (NBD) die bekend staat als

spina bifida (open ruggetje). Dit suggereert dat mogelijk ook in de mens factoren die signalering via PDGFRA remmen bij kunnen dragen aan het ontstaan van dergelijke afwijkingen. Aan de andere kant wordt overexpressie van PDGFRA en zijn liganden vaak aangetroffen in verschillende soorten hersentumoren (glioma's) bij zowel muizen als mensen, hetgeen suggereert dat verhoogde PDGFRA signalering aanleiding kan geven tot het ontstaan van glioma's. Wij hebben daarom de hypothese gesteld dat factoren die transcriptie van *PDGFRA* beïnvloeden, bij kunnen dragen aan het risico op het ontstaan van PDGFRA-gerelateerde aandoeningen zoals NBDs en glioma's. De studies beschreven in dit proefschrift zijn uitgevoerd om zowel *cis*- als *trans*-werkende factoren die *PDGFRA* transcriptie reguleren te identificeren en hun betrokkenheid bij glioma's en NBD te bepalen (hoofdstuk 1).

Om regulerende DNA sequenties te identificeren die het risico op NBD kunnen beïnvloeden, hebben wij een patiënt-controle studie gedaan waarin wij vijf verschillende haplotypes van de –1,589/+118 promoter regio van het humane PDGFRA gen hebben geïdentificeerd, die wij H1, H2α, H2β, H2γ en H2δ hebben genoemd. Van deze haplotypes komen H1 en H2α, die op zes van de acht polymorfe posities van elkaar verschillen, het meest voor en verschillen van elkaar op 6 van de 8 polymorfe posities. In een transiënte transfectie assay uitgevoerd op humane osteosarcoma cellen vertoonden H2α en H2β een 3-5 voudige hogere promoteractiviteit dan H1, H2γ en H2δ. Daarnaast bleken specifieke combinaties van promoterhaplotypes te correleren met het vóórkomen van NBD. Deze resultaten tonen aan dat variatie in regulerende sequenties inderdaad verschillen in genexpressie kan veroorzaken en dat dergelijke natuurlijk expressieverschillen de aanleg voor ziektes als NBDs kunnen beïnvloeden (hoofdstuk 2).

NBD zijn multifactoriële aandoeningen die het gevolg zijn van een combinatie van diverse genetische en omgevingsfactoren. Daarom hebben wij in een patiënt-controle studie onderzocht of er een interactie bestaat tussen *PDGFRA* promoter haplotypes als genetische factor en het voedingspatroon van de moeder als omgevingsfactor in relatie tot het risico op het ontstaan van spina bifida. Wij hebben aangetoond dat het H1 haplotype, dat aanleiding geeft tot lage *PDGFRA* expressie, significant oververtegenwoordigd is in spina bifida patiënten en mild oververtegenwoordigd is in moeders van deze patiënten, hetgeen suggereert dat lage PDGFRA expressie het risico op NBD verhoogt. Bovendien konden wij laten zien dat voor H2 kinderen een hoog glucose en een laag myo-inositolgehalte in het bloed van de moeder de belangrijkste risicofactoren zijn voor het ontstaan van spina bifida zijn, terwijl voor H1 kinderen de body mass index (BMI) van de moeder de meest prominente risicofactor is. Bovendien bleken moeders met een H1 allel significant kleiner te zijn dan H2 homozygote moeders, wat aangeeft dat PDGFRA expressieniveaus een direct effect hebben op de lichaamsgroei (hoofdstuk 3).

Wetende dat verschillen in PDGFRA expressie het vóórkomen van NBDs kan beïnvloeden, hebben wij de humane *PDGFRA* promoter onderzocht op haplotype specifieke verschillen in binding van transcriptiefactoren op de geïdentificeerde polymorfe posities. We hebben hierbij ook twee nieuwe promoter polymorfismen betrokken, die geïdentificeerd zijn bij een analyse van promotersequenties tot -3600 bp. Op twee van deze in totaal tien polymorfe posities, te weten -909A/C en +68GAins/del, hebben we differentiële eiwitbinding waargenomen. Van het eiwitcomplex dat specifiek aan het C-allel van de -909A/C SNP bindt, een nucleotide die in alle haplotypes behalve H2γ voorkomt, kon aangetoond worden dat het leden van de upstream stimulator factor (USF) familie bevat, hoogstwaarschijnlijk bestaande uit een

USF1a-USF2a heterodimeer. Daarnaast is er een eiwitcomplex gevonden dat specifiek het laag actieve H1 haplotype bindt op het +68GAdel allel, en potentieel zou dit complex een H1 specifieke *PDGFRA* repressor kunnen zijn (hoofdstuk 4).

Glioma's brengen vaak PDGFRA tot overexpressie, maar onbekend is of het expressieniveau afhangt van de aanwezige promoter haplotypen. Derhalve hebben wij endogene *PDGFRA* expressieniveaus bestudeerd in vier humane H1/H2a heterozygote glioblastoma cellijnen. Gebaseerd op een speciaal daarvoor door ons ontwikkelde techniek, hebben wij aangetoond dat in drie van de vier cellijnen de H1 expressie meer dan tien keer lager was dan die van H2a, hetgeen ook gepaard ging met een lagere totale *PDGFRA* expressie. Het verschil tussen H1 en H2α kon grotendeels opgeheven worden door inductie van DNA demethylatie en inhibitie van histon-deacetylatie, wat aangeeft dat epigenetische regulatie een belangrijke rol speelt in de differentiële expressie van H1 en H2a in glioblastoma cellen. Het grote verschil in activiteit van de haplotypen suggereert ook dat *PDGFRA* promoter haplotypen het ontstaan van glioma's kunnen beïnvloeden. In een patiënt-controle studie onder 71 glioblastoma patienten vonden we inderdaad een verminderde frequentie van het H1 allel, hetgeen significant was vergeleken met een gecombineerde West-Europese controlegroep. Dit wijst erop dat het laag actieve H1 haplotype beschermend zou kunnen werken tegen het ontstaan van glioma's.

Humane embryonale carcinoom (EC) cellen worden algemeen gebruikt als een *in vitro* modelsysteem voor het analyseren van differentiatie-afhankelijke regulatie van genexpressie tijdens vroege ontwikkeling. In Tera-2 humane EC cellen wordt de *PDGFRA* expressie sterk verhoogd door differentiatie te induceren via behandeling met retinolzuur en cAMP. Wij hebben aangetoond dat voor deze inductie van *PDGFRA* expressie een promoter-element nodig is dat de transcriptiefactoren PRX en PBX bindt en dichtbij de transcriptie-initiatie plaats ligt. Muizen zonder functioneel Prx hebben een sterk NBD fenotype, wat kan betekenen dat *PRX* en *PDGFRA* elkaar ook *in vivo* kunnen beïnvloeden tijdens axiale ontwikkeling en zo een rol kunnen spelen in het ontstaan van NBDs.

Samengevat tonen de resultaten in dit proefschrift aan dat *PDGFRA* expressie zowel fysiologische (lichaamsgroei) als pathologische (ontstaan van NBDs en glioma's) processen kan beïnvloeden. Onze data wijzen er op dat lage PDGFRA expressie gestuurd door het H1 promoter haplotype het risico op spina bifida verhoogt, maar tegelijkertijd beschermt tegen het ontstaan van glioblastoma's. Dit idee wordt ondersteund door studies aan de incidentie van deze ziektes in bepaalde bevolkingsgroepen en geografische locaties: in populaties met een hoge H1 frequentie komen NBDs relatief vaak voor, terwijl in populaties met een lage H1 frequentie relatief vaak hersentumoren voorkomen. Op basis van onze resultaten, in combinatie met gepubliceerde data aan *Pdgfra* transgene muizen, hebben wij de hypothese gepostuleerd dat, dankzij tragere promoter demethylering, het H1 haplotype het risico op het ontstaan van glioblastoma's vermindert doordat het de glia precursor-populatie, waarvan bekend is dat die wordt bepaald door PDGFRA signalering, klein houdt. Omdat PDGFRA overexpressie ook geassocieerd wordt met osteosarcoma's, chondrosarcoma's en eierstok-sarcoma's, is het zeer wel mogelijk dat *PDGFRA* promoter haplotypen ook de gevoeligheid voor dergelijke ziektes beïnvloeden. Aangezien PDGFRA signalering essentieel is voor (re)myelinatie, kunnen *PDGFRA* haplotypes daarnaast ook een rol spelen in ziektes die geassocieerd zijn met demyelinering zoals multiple sclerosis (hoofdstuk 7).

Curriculum Vitae

Mascha Toepoel werd geboren op 22 juli 1977 te Olst. Na het behalen van het VWO diploma aan de Alexander Hegius Scholengemeenschap te Deventer ging zij in 1995 Biologie studeren aan de Katholieke Universiteit Nijmegen. Daar liep zij een onderzoeksstage aan de afdeling Celbiologie (FNWI) onder supervisie van prof.dr. E.J.J. van Zoelen en aan de afdeling Biochemie (UMC) onder supervisie van prof.dr. W.J. de Grip en prof.dr. J.J.H.H.M. de Pont. In 2000 legde zij haar doctoraal examen af, waarna zij startte met een promotieonderzoek bij bovengenoemde afdeling Celbiologie. Sinds 1 mei 2006 is zij werkzaam als postdoc op de afdeling Medical Protein Research van het VIB aan de Universiteit Gent in België, waar zij onderzoek doet naar betacel-dysfunctie en -destructie bij diabetes.

List of publications

Joosten PH, **Toepoel M**, Mariman EC, Van Zoelen EJ. Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects. Nat Genet. (2001) 27:215-7.

Joosten PH, **Toepoel M**, van Oosterhout D, Afink GB, van Zoelen EJ. A regulating element essential for PDGFRA transcription is recognized by neural tube defect-associated PRX homeobox transcription factors. Biochim Biophys Acta (2002) 1588:254-60.

Toepoel M, Ackerschott B, van Zoelen EJ. Haplotype-dependent binding of nuclear proteins to the promoter of the neural tube defects-associated platelet-derived growth factor alpha-receptor gene. Biochim Biophys Acta (2005) 174:350-7.

Toepoel M, Joosten PH, Knobbe CB, Afink GB, Zotz RB, Steegers-Theunissen RP, Reifenberger G, van Zoelen EJ. Haplotype-specific expression of the human PDGFRA gene correlates with the risk of glioblastomas. Submitted to Oncogene.

Toepoel M, Steegers-Theunissen RP, Ouborg NJ, Franke B, Joosten PH, van Zoelen EJ. PDGFRA promoter haplotypes differentially interact with maternal environmental factors in predisposition to neural tube defects. Submitted to Genetic Epidemiology.

Dankwoord

En toen was er het dankwoord. Het schrijven van dit proefschrift is niet altijd even gemakkelijk geweest, en ook een dankwoord is niet iets dat je zo even uit je mouw schudt, want je wilt toch iedereen die de afgelopen periode iets voor je betekend heeft op passende manier bedanken, en vooral ook niemand vergeten. Allereerst natuurlijk Joop. Ik ben je heel erg dankbaar dat je me ertoe over hebt weten te halen om na mijn studie toch het onderzoek in te gaan. Zonder jou was dit boekje er dus letterlijk nooit gekomen. Dank je wel ook voor je stimulerende discussies, je structureringen in mijn chaos en de prettige samenwerking, die ik in de loop der tijd steeds meer ben gaan waarderen.

Een andere onmisbare factor uit mijn AIO-periode, tevens lieftallig nimfje, is natuurlijk Paul. Dank je wel dat je me als stagiaire hebt doen inzien dat moleculaire biologie helemaal niet zo abstract en ongrijpbaar is als het in eerste instantie lijkt. Met jou is het altijd giga-gecellig en je bent altijd een grote steun voor me geweest, zowel op het lab als daarbuiten. Wil je nog steeds m'n epje zijn?

Ook met Bart, mijn ander lieftallig nimfje, heb ik heel veel ups en downs, en ook menig drankje gedeeld. Of het nu gaat om het losmaken van grote gelen of het irriteren van vierdaagselopers, Bart is altijd van de partij. Super bedankt daarvoor! En daarom ben ik ook erg blij dat we nu aan elkaars zijde staan, en alleen wij twee weten wat er nu eigenlijk in die waterkan zit...

Dan de rest van de harde kern. Ester, dank je wel dat je er altijd voor me bent! Zonder jou zou het op MSN stukken minder gezellig zijn. En Monica's zijn hard nodig in deze wereld, dus ik weet zeker dat je een fijne nieuwe werkplek zult vinden. Bas, ik hoop dat het niet lang meer duurt voordat ook jij je boekje kunt verdedigen. Gaan we nog eens hakkuh? Laura, dank je wel voor je vrolijkheid en de rode wijntjes die we samen hebben gedronken. Leonie, ik bewonder je moed om iets totaal anders te gaan doen, heel veel succes! Suzanne, altijd behulpzaam, dank je wel voor je fijne gezelschap en erg handig dat je zo'n rijke vent hebt ;-). Sander, nog niet zo lang AIO maar nu al genoeg verhalen, nu ook nog een mooi boekje. Allemaal enorm bedankt voor de gezellige borrels, etentjes, housewarmings, logeerpartijen en weekendjes weg!

Wie ook een speciale plaats verdient, is Janny. Altijd klaar voor een babbel en een grote steun bij alle niet-wetenschappelijke zaken. Wetende dat ik alles met een gerust hart aan je kon overlaten, heb je heel veel stress bij me weggenomen in de laatste hectische maanden, duizend maal dank daarvoor.

Ook de rest van de collega's wil ik natuurlijk heel erg bedanken voor de goede werksfeer de afgelopen jaren. Catelijne (ik hoop dat je iets moois kunt opbouwen in Gent), Saskia, "grote" Ester, Miriam, Diana, Erik, Robert, Walter, Peter, Dorine, Wilma, José, Marc, Wadia, Husam, Frits, Monique, Jeroen, Lex, Koen, Wilbert, Dick en ook "mijn" studenten, Bart, Saskia en Maarten, hartstikke bedankt voor de fijne tijd op het lab.

Iedereen van de NBD-bespreking, en in het bijzonder Barbara en Regine, heel erg bedankt voor jullie hulp en stimulerende discussies. Jullie zijn een geweldige steun voor me geweest, vooral toen ik er eventjes alleen voor stond. Waar ik ook altijd terecht kon, was bij de mensen van Plantkunde, als er bij ons weer eens iets op of kapot was, voor advies over experimenten of voor een gezellige borrel of barbecue. Enorm bedankt daarvoor. Titti, ik vind het superleuk dat jij op de grote dag pro-rector bent! Ook wil ik graag Jelle en Monique heel erg bedanken voor de eindeloze sequenties die ze voor

me gelopen hebben, en Gerald voor de crosslinks die ik daar heb mogen doen. En Joop, hartstikke bedankt voor alle lastige maar onmisbare statistiek die je voor ons gedaan hebt.

Dan verlaten we nu de wetenschappelijke sferen. Met groot plezier heb ik me de afgelopen jaren gestort in de muziek, spelletjes, pubquizzen, festivals en uitgaan. En ook dat doe je niet alleen. Jochem, ik vind het geweldig dat we elkaar nog bijna elke ochtend een goeiemorgen wensen en de dingen des levens bespreken, ook al zitten we zo'n 200 km uit elkaar. Jij en Sandra heel veel geluk de komende spannende tijd! Maarten, ik mis onze nachtelijke avonturen in het Nijmeegse uitgaansleven, en ik wacht nog altijd op een bepaald feestje ;-). Geert, Henco, allebei heel veel geluk met jullie kleine spruiten, jullie zijn vast super-papa's! Michiel, ik mis ook onze vroege uurtjes in de Gonzo en Dio, en ik zal nooit aan jouw onaardse strakheid kunnen tippen. Stefan, ik hoop dat we nog veel mooie Tori-concertjes mogen meemaken. Johan, dit jaar weer naar Haldern? Supergeil! Imke, ook al ben je vaak ver weg, je bent toch altijd heel dichtbij. En ook Jeroen, Tim, Kim, Marian, Rudolf, Janine, Tom, Coco, Renee, Arjan en de rest in en rond The Kinky Party, Renegade Ostrich en Holgersson, super bedankt voor de geweldige tijd die ik heb gehad in Nijmegen! Lex en Bente, Joachim en Ilse, dank je wel voor de fijne feestjes, optredens en spelletjes!

Twee mensen die het een stuk aangenamer hebben gemaakt om een nieuw leven op te bouwen in Gent, zijn Tom en Vanessa. Tom, dank je wel voor je gezelligheid, je ondernemendheid en je luisterend oor. Vanessa, dank je wel dat je ons alle ins en outs van het Gentse leven leert kennen. En voor mijn nieuwe collega's; ik zal met veel plezier nog meer vla en pindakaas voor jullie meenemen.

Hans en Jeannette, ik weet dat ik altijd bij jullie terecht kan. Michael en Vanessa, super dat jullie er nog bij kunnen zijn, en heel veel geluk in Australië, maar dat spat er nu al vanaf.

Mijn "kleine" broertje Rik, je hebt je studie nu gevonden, dus maak er iets moois van! Dank je wel dat je me op de hoogte houdt van de nieuwe trends en dat je mijn gezeur aanhoort. Pap en mam, dank je wel voor jullie nimmer aflatende betrokkenheid bij alles wat me bezig houdt. Weet dat ik aan jullie denk en ik hoop er vanaf nu meer voor jullie te kunnen zijn. Ik hou van jullie.

Lieve Marc, juist voor degene die de meeste woorden verdient, zijn er geen woorden om te beschrijven hoeveel je voor me betekent. Ik hou van je, samen kunnen we alles aan!