Transcriptional regulation of the human Zfm1/Sf1 gene

Dissertation

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D7

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For my Father

Contents

Abbreviations

aa	amino acid
Acc. No.	accession number
AP-1	activator protein – 1
APS	ammonium persulfate
BASMC	bovine aortic SMC
bp	base pair
cDNA	complementary deoxyribose nucleic acid
ChIP	chromosome immunoprecipitation
DNA	deoxyribose nucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
ds	double-stranded
dODN	decoy oligonucleotide
ECM	extracellular matrix
EDTA	ethylendinitrilo-N, N, N', N'-tetra-acetate
EGR-1	early growth response 1
EMSA	electro-mobility shift assay
FCS	fetal calf serum
FGF-2	basic fibroblast growth factor-2
GTF	general transcription factors
HFM	histone fold motif
hSMC	Human smooth muscle cell
HUVEC	Human vascular endothelial cell
IL-1	interleukin -1
KH	hnRNP K homology
LB	Luria Bertani broth
LDL	low density lipoprotein
MEN1	multiple endocrine neoplasia 1
MMPs	matrix metalloproteinases
MCP-1	monocyte chemoattractant protein 1
MCS	multiple cloning site
mRNA	messenger ribose nucleic acid
NFAT	nuclear factor of activated T cells

NO	nitric oxide
NLS	nuclear localisation signal
NTD	N-terminal transcription activation domain
NTP	nuclear tri-phosphate
Nt	nucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PIC	pre-initiation complex
pol	polymerase
PVDF	polyvinylidene fluoride
RNA	ribose nucleic acid
RT	room temperature
RT-PCR	reverse transcription- polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	standard deviation
Sf1	splicing factor 1
SP-1	specificity protein 1
siRNA	small interfering RNA
SMC	smooth muscle cell
ss	single stranded
$TAF_{II}s$	TATA-binding protein associated factors
TBP	TATA-binding protein
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	transcription factor
TLS/FUS	translocated in liposcarcoma
TNFα	tumour necrosis factor α
Tris	Tris-(hydroxymethyl)-aminomethan
VCAM-1	vascular cell adhesion molecule 1
WT1	Willm's tumour 1
Zfm1	zinc finger motif 1

1. Atherosclerosis and inflammation

1.1 The pathogenesis of atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipids and fibrous elements in the vessel wall of large arteries (Ross, 1999 (a) and Plutzky, 2001). Despite lifestyle changes and new pharmacological approaches to lower plasma cholesterol concentrations; cardiovascular disease continues to be the principle cause of death in many industrialized nations (Ross, 1999 (a)).

The response-to-injury hypothesis of atherosclerosis states that the principally protective inflammatory response to vascular injury, followed by a fibroproliferative process, by way of continuous insult, may become excessive (Ross, 1999 (b)); where in which both the inflammatory response and proliferation of the fibrous connective tissue become the disease process.

However, endothelial dysfunction is proposed to be the first step in atherosclerosis. The possible causes are elevated or modified LDLs; free radicals caused by smoking; hypertension; diabetes mellitus and infectious microorganisms such as *Chlamydia pneumoniae* (Fig. 1a, Ross, 1999 (b)).

The earliest nature of atherosclerotic lesions are called ‘fatty streaks’ and can be described as purely inflammatory, consisting of only monocyte derived macrophages and T lymphocytes (Fig. 1b). If the inflammatory response does not neutralize or remove offending agents, it can continue indefinitely, leading to migration and proliferation of SMC that eventually combine within the site of injury forming an advanced lesion (Fig. 1c, Lavezzi *et al.,* 2005 and Ross, 1999 (b)).

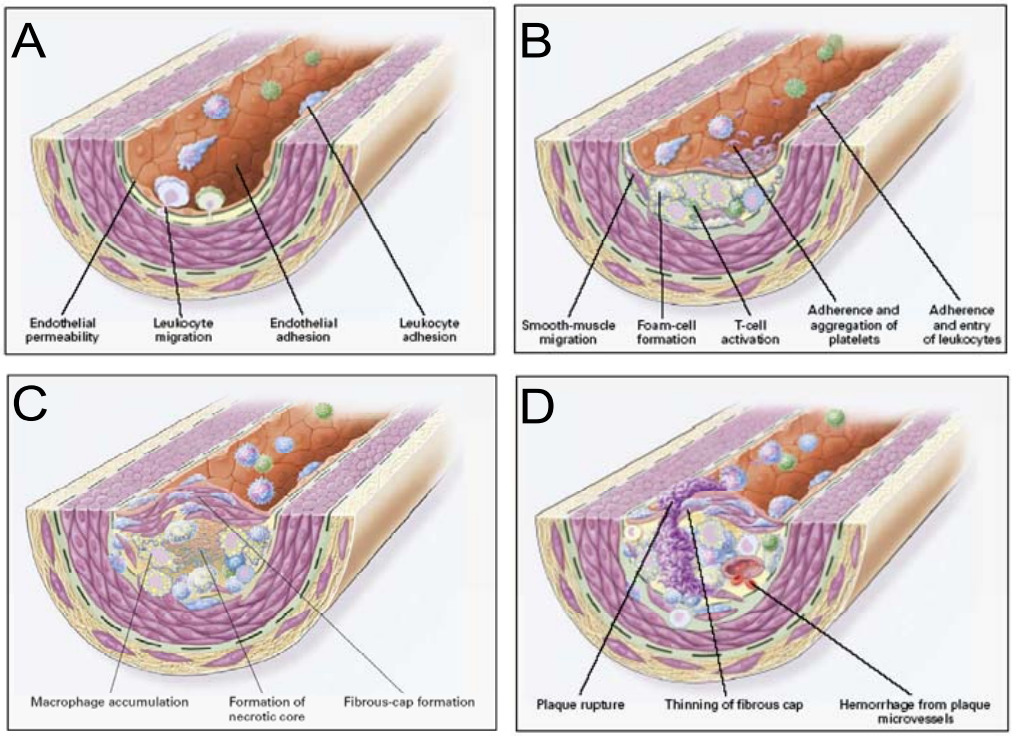


Figure 1. The response-to-injury hypothesis of atherosclerosis (taken from Ross, 1999 (B). A) Endothelial dysfunction - caused by offending agents e.g. increase in plasma LDL concentrations, free radicals and hypertension. **B)** Fatty streaks – earliest recognizable lesions of atherosclerosis, the aggregation of lipids, macrophages and T lymphocytes can be observed. **C)** Advanced lesion - fatty streaks progress to form intermediate/ advanced lesions. A fibrous cap tends to form due to an increase in proliferating SMC mediated by PDGF and pro-inflammatory cytokines. The fibrous cap conceals the lesion from the lumen and represents a 'wound healing response' to the injury. **D)** Unstable fibrous plaques - where rupture can occur due to thinning of the fibrous cap caused by continuous influx and activation of macrophages and release of metalloproteinases at theses sites; resulting in thrombus formation and occlusion of the artery.

Mediators in response to injury involve a vast number of cytokines implicated in immunity and inflammation along with growth factors implicated in chemotaxis and proliferation (Plutzky, 2001). In cardiovascular disease, these two roles are closely inter-related, where in which the same mediator acts as a pro-inflammatory agent and as a growth factor. Examples of mediators that have such pleiotropic effects are PDGF, IL1β, TNF-α and TGF-β. All these stimuli induce SMC proliferation and are usually not expressed in healthy arteries, but are up-regulated in atherosclerotic lesions (Ross, 1999 (b)).

Whilst IL1β/TNFα are released by macrophages at the site of inflammation; on the other hand, PDGF is a potent mitogen and is generally not expressed in normal healthy arteries, but is up-regulated in atherosclerotic lesions.

These pro-inflammatory cytokines in turn stimulate secondary gene expression of other growth regulatory molecules such as PDGF in endothelial cells; which evidently increase connective tissue proliferation, SMC proliferation and chemotaxis (Pintucci *et al.,* 2005). The dysfunctional endothelium releases more pro-inflammatory cytokines and PDGF, which continues to drive secondary gene expression of PDGF in the endothelium and SMC; leading to long-term stimulatory effects of these cytokines and growth factors on the SMC and endothelium (Ross, 1999. (a)).

An important point to consider is that long-term effects of these cytokines, growth factors and adhesion molecules can lead to changes in gene expression patterns of macrophages and SMC. Thus, the long-lasting phenotypic changes are important for the pathogenesis and disease progression of atherosclerosis (Ross, 1999 (a) and Plutzky 2001).

1.1.2 Plasticity and phenotypes of SMC

A principle function of mature vascular SMC is regulation of vascular tone. And like a majority of somatic cells, SMC contain a complete set of genetic material, but express only a sub-set of genes (Owens, 1995). Once vascular SMC are fully mature and differentiated, they proliferate at a very low rate and produce a very small amount of ECM proteins. However, during atherosclerosis vascular SMC phenotypically have accelerated rates of proliferation, and produce a large amount of matrix constituents such as collagen and fibronectin.

Based on the distribution of myosin filaments and formation of large amounts of secretory organelles such as ER and golgi apparatus, at least two different phenotypes of SMC have been described (Ross*,* 1993). Contractile, fully differentiated SMC respond to agents that induce either vasoconstriction or dilation such as catecholamines or NO. In contrast, SMC in the synthetic phenotypic state are capable of expressing genes for various growth regulatory molecules and cytokines, and can respond to growth factors via expression of appropriate receptors. Numerous observations suggest that SMC in atherosclerotic lesions have changed from a contractile to a synthetic phenotype (Ross*,* 1993 (b)). Thus, phenotypic changes can

lead to extreme effects on the capacity of cells within an atherosclerotic lesion to respond to various agonists. Since SMC plasticity is important under normal physiological conditions, and the contractile phenotype is essential for haemodynamic stability of the vessel wall, it would be important to discover genes essential in maintaining the contractile phenotype of vascular SMC.

1.2 Structure and functions of the Sf1/Zfm1 gene

Cattaruzza *et al*. (2002) have previously shown that stimulating rat SMC with pro-inflammatory cytokines or PDGF leads to a decrease in Zfm1/Sf1 gene expression, an increase in cell proliferation both *in vitro* and *in vivo*, as well as an up-regulation of pro-inflammatory gene products. This data suggests that Zfm1 may play a role in preventing the pathogenesis of atherosclerosis, by repressing pro-inflammatory gene expression in vascular SMC, stabilizing the differentiated state of these cells.

Zinc Finger Motif 1 (Zfm1), also known as Splicing Factor 1 (Sf1) was first cloned as a nuclear protein at a locus linked to Multiple Endocrine Neoplasia Type 1 (MEN1), within the region q13 of chromosome 11 (Toda *et al.,* 1994). The Zfm1/ Sf1 gene spans approximately 15 kb which consists of 14 exons and encodes a protein comprising approximately 623 amino-acids. Two major splice variants of Zfm1 exist: Zfm1 A~ 70 kDa and Zfm1 E~ 55 kDa which differ in length of the C-terminal proline rich region (Fig. 2; Toda *et al.,* 1994 and Arning *et al.,* 1996). The first functional characterization of Zfm1/Sf1 was made by Kramer *et al.* (1992) following purification of Zfm1 as a heat stable protein from HeLa cells, and revealed its ability to function as a kinetic factor in the assembly of the ATP dependent pre nuclear-mRNA splicing complex (Guth *et al.,* 2000, Liu *et al.,* 2001, Covini *et al.*, 1999).

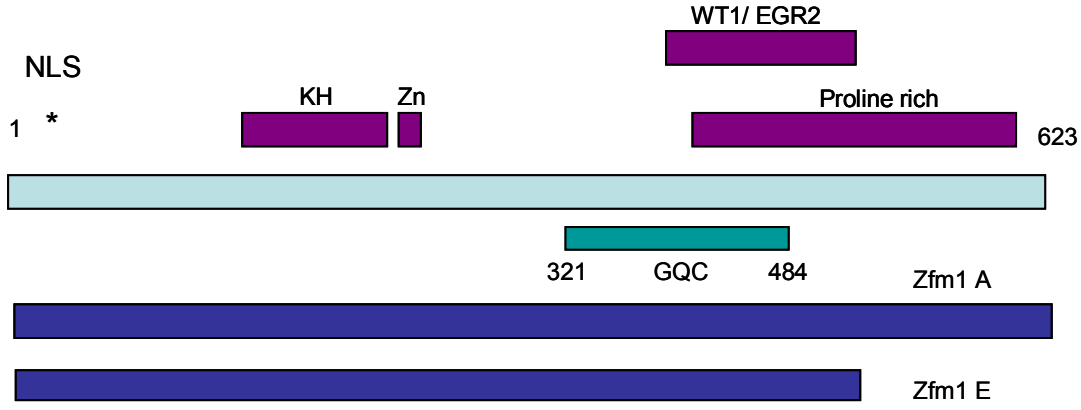


Figure 2. Schematic representation of the domains of Zfm1/Sf1. The protein domains of Zfm1/Sf 1 consists of a C-terminal proline rich domain, a zinc knuckle and a hnRNP K homology (KH) domain both of which are implicated in binding nucleic acid and a putative nuclear localization signal (NLS) at the N-terminus (Berglund *et al.,* 1998). Additionally a GQC-interaction domain exists that recognizes sequences rich in glutamine, glycine, serine and threonine. A functionally important region homologous to Willms tumour 1 suppressor (WT1) gene and the early growth response (Egr-2) gene also exists

A second function of Zfm1/Sf1 (defined in sea urchins) was its ability to be a repressor of the stage specific activator protein (SSAP) transcription factor which is essential for embryogenesis (Fig. 2; Zhang *et al.,* 1998 (a)). Zfm1 repression of SSAP requires both a repression domain found in the N-terminus consisting of 137 amino acids as well as the GQC-interaction region.

A human protein homologous to SSAP is EWS, known to be involved in cellular transformation in Ewing's sarcoma tumours. EWS contains an N-terminal transcription activation domain (NTD) which resembles that of SSAP in amino acid sequence (Zhang *et al.,* 1998 (a)). Interestingly, Zhang *et al*. have also shown that Zfm1/Sf1 represses EWS through an interaction between a region of 37 amino acids situated within the NTD of EWS. Two other proteins, translocated in liposarcoma (TLS/FUS) and the human TBP-associated factor 68 ($hTAF_{II}68$) have extensive homology to EWS and also interact with Zfm1. Both EWS and TLS/FUS are human pro-oncoproteins and products of genes which are commonly translocated in human sarcomas (Ladomery *et al.,* 2002 and Lee *et al.,* 2004). EWS/TLS/$hTAF_{II}68$

are also present in the basal transcription machinery, more commonly in distinct TFIID complexes (see below; Bertolotti *et al.,*1996).

1.3 Regulation of gene transcription

1.3.1 Basal transcription machinery

Gene transcription can be divided into several functional sequences of events that include promoter recognition and binding, assembly of the pre-initiation complex (PIC), RNA chain initiation, RNA chain elongation and finally termination of RNA synthesis. In higher eukaryotes, transcription initiation at promoters recognized by RNA polymerase II (RNA pol II) is carried out by the ordered binding of general transcription factors (GTFs) and the RNA polymerase core subunits; recruitment of Srb/Med proteins allow RNA polymerases to respond to activators or repressors (Gaston *et al.,* 2003). GTFs are initially recruited to promoters in an ordered fashion, and aid RNA polymerase binding to promoter DNA. The first GTF to bind is TFIID which is a complex containing TATA box-binding protein (TBP) and up to 13 TBP-associated factors (TAF_{II}s). Assembly of GTFs forms the PIC, which can initiate transcription and transcription elongation in the presence of nuclear tri-phosphates (NTPs) (Gaston *et al.,* 2003). A low level of transcription directed by these proteins is also known as basal transcription, but activated transcription levels require the presence of TAF_{II}s where different transcriptional activation domains, present in members of the multi-subunit TFIID complex, interact with distinct TAF_{II}s (Bertolotti *et al.,* 1996). TAF_{II}s can also function as co-activators for upstream DNA-binding TFs, and can recognize different elements such as Srb/Med proteins and/ or interact with other transcriptional activators, depending on their TFIID-TAF composition. Thus, specific stimuli may possibly drive transcriptional levels higher, via directing assembly of distinct TAF_{II}s at the basal transcriptional level.

1.3.2 TAF_{II}S can define functionally distinct TFIID complexes

Association of Zfm1/Sf1 with $hTAF_{II}$68/EWS/TLS implies that normal cellular function of Zfm1/Sf1 may be to negatively modulate transcription of target genes specifically coordinated by these cofactors (Zhang *et al.,* 1998 (b))

(Fig. 3). A few examples of TAF_{II}s that define functionally distinct TFIID complexes are $hTAF_{II}68$ and $hTAF_{II}80\delta$. $hTAF_{II}68$ can bind RNA and single stranded (ss) DNA homologous with the TLS/FUS and EWS binding sites. It has also been shown that $hTAF_{II}68$ can enter the PIC together with RNA polymerase II (Bertolotti *et al.,* 1996). TLS/FUS is further associated with a sub-population of TFIID complexes which are distinct from those containing $hTAF_{II}68$ (Bertolotti *et al.,* 1996) and therefore, it is possible that TLS/FUS and EWS may play specific roles during transcription initiation at distinct promoters.

Another interesting discovery was the linkage of apoptotic signaling pathways to TFIID function via an $hTAF_{II}80$ isoform termed '$hTAF_{II}80\delta$', which differs from $hTAF_{II}80\delta$ due to a 10-amino acid (aa) deletion (Bell *et al.,* 2001). It was shown that $hTAF_{II}80\delta$ could form distinct TFIID complexes lacking $hTAF_{II}31$ under apoptotic stimuli. Moreover, an increase in expression of $hTAF_{II}80\delta$ could trigger apoptosis and induce expression of pro-apoptotic target genes such as gadd45 and p21. Thus, $hTAF_{II}80\delta$ can couple apoptotic signals in order to reprogram RNA polymerase II transcription at the 'transcriptome' level. Therefore, TAF_{II}s may define disctinct TFIID complex formation and function under specific stimuli, and transcription itself is globally regulated at the so-called 'basal transcription machinery'.

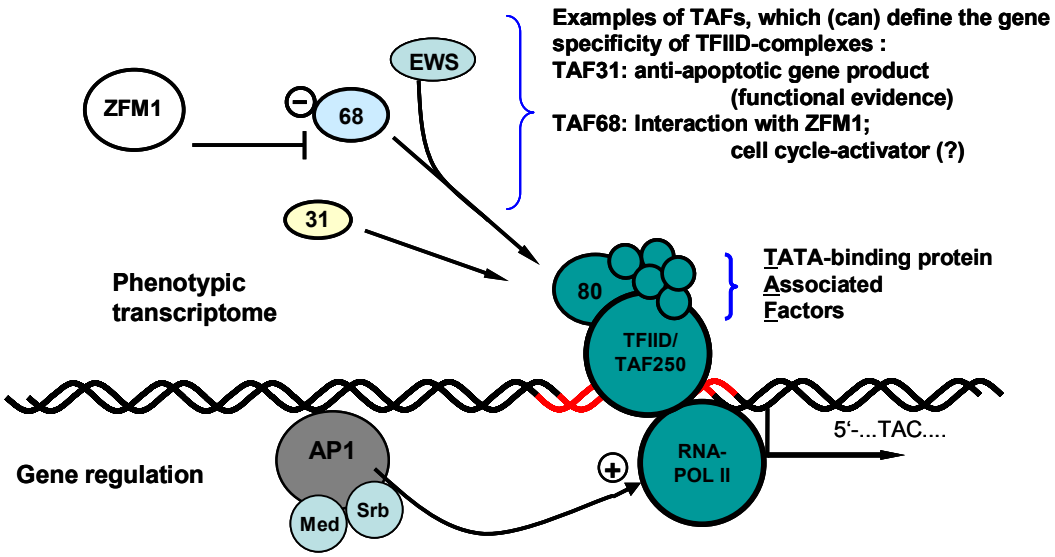


Figure 3. Phenotypic 'transcriptome' regulation of gene expression. Figure represents a schematic model of TAF_{II}s that may be able to define the formation of distinct TFIID complexes at the basal transcriptional level and how Zfm1/Sf1 may play a role as a modulator of transcription.

1.4 Aims of the study

Taking into account the previous findings, Zfm1/Sf1 seems to be important in maintaining the phenotype of non-proliferating by-stander cells by acting as a 'brake' on the expression of pro-inflammatory gene products. The first aim of this study was to observe if pro-inflammatory cytokines more specifically IL-1β plus TNFα and PDGF have a similar effect on Zfm1/Sf1 gene expression in human vascular SMC; whether an increase in cell proliferation can be observed and whether the function of Zfm1 observed in rSMC holds true for human vascular SMC (see Fig. 4 below). The second aim was to characterize the human Zfm1/Sf1 promotor and to understand how the human Zfm1/Sf1 gene is regulated under pro-inflammatory conditions in hSMC. In this context, regions of the promoter essential for transcription factor (TF) binding should be identified, as well as the stimulus and signaling pathway responsible for Zfm1/Sf1 gene expression.

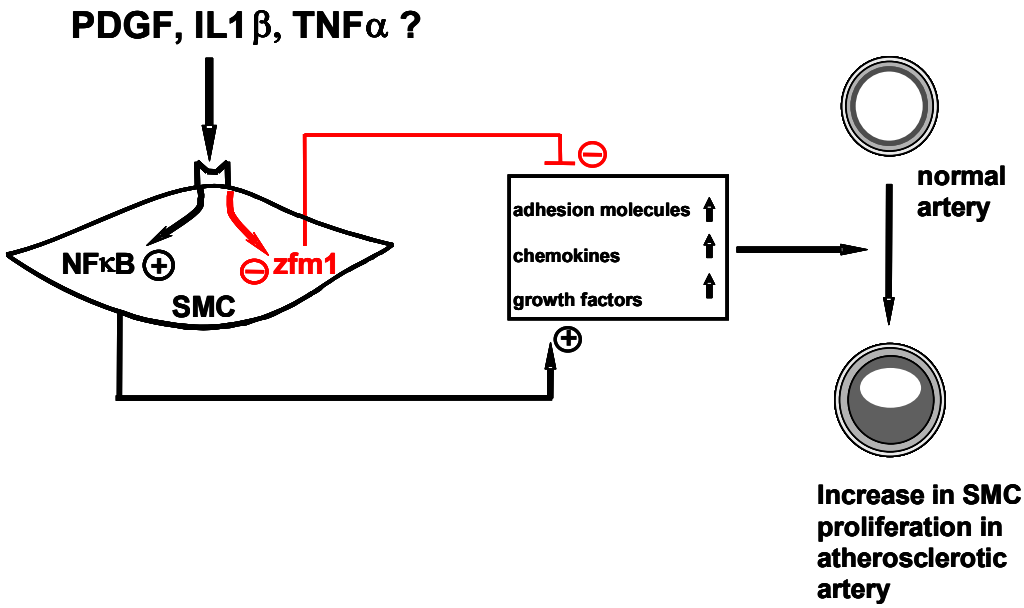


Figure 4. A schematic diagram of how PDGF or pro-inflammatory cytokines such as IL1β/TNFα may play a role in the pathogenesis of atherosclerosis. Expression of Zfm1/Sf1 may act as a 'brake' on the expression of various pro-inflammatory gene products; whose expression under pro-inflammatory stimuli may contribute to the fibroproliferative remodeling of the vessel wall in atherosclerosis.

Previously published data by Zhang *et al.* (1998, b) show that Zfm1 interacts with hTAF$_{II}$68/ EWS/TLS, suggesting the possibility that Zfm1 may play a role in directing the formation of a functional phenotypic 'transcriptome' under pro-inflammatory cytokine and/or PDGF stimulation. Zfm1 may thus negatively modulate the transcription of certain target genes, specifically co-ordinated by hTAF$_{II}$68/EWS/TLS factors. Therefore, a third aim of the study was to understand the function of Zfm1 by investigating whether Zfm1 interacts with other distinct hTAF$_{II}$s such as hTAF$_{II}$80 or hTAF$_{II}$31.

If successful, this study should provide new insights into Zfm1/Sf1 gene expression, function and stabilization of phenotype in human vascular SMC. It would thus contribute to a new understanding of how vascular SMC maintain their contractile, non-proliferative phenotype, paving the way towards reduction of fibroproliferative remodeling of the vessel wall, and slowing down progression of atherosclerosis and its complications.

Materials

2.1 Bacteria

Bacterial strains used for cloning and maintenance of plasmids constructs are listed in Table 2.1

Table 2.1 Bacterial E. coli strains.

Strain	Company	Genotype
Top 10 F	Invitrogen	F'{LacI^{q} Tn10(Tet^{R})}, Mer A, Δmrr-hsdRMS-mcr BC), φ 80 LacZΔx74, deoR, recA1, araD139, Δ(ara-leu) 7697, galU, gal K, rpsL (str^{R}), end A1, nupG
XL 1 Blue	Stratagene	RecA1, endA1, gyrΔ96, thi-1, hsdR17, supE44, relA1, lac[F' proAB lacI^{q} ZΔM15 Tn10 (Tet^{R})]

2.2 Plasmid Vectors

Table 2.2 Plasmid vectors

Vector	Properties	Company
pCR® 2.1-Topo® 3.9 Kb	pUC origin, Ampicillin & Kanamycin resistance genes, lacZα reporter, T7 RNA polymerase promoter, TA cloning site.	Invitrogen
pGL3 Basic	ColE1 origin, Ampicillin & Kanamycin resistance genes, luciferase gene (luc+).	Promega

2.3 Primers

Table 2.3 Primers

A. Primers for cloning			
Primer name	**Expected fragment length (bp)**	**Annealing temperature**	**Primer (forward/ reverse)**
Primer 1	2999	60˚C	AGCTGGGCATGATGGCAGGTGG TGCGGCGGCGGGTACGAG
Primer 2	1500	58˚C	CACCCAACTCCCTTTGCCTCTC AGCCAGCGTGTTCCGATTCC
Primer 3	860	58˚C	CACCGTGTTGGTCAGGTTGG CTCTCTCGGCCCGACTCACCTTC
Primer 4	670	58ºC	CGCGTTTGCTCCATCTACGCATGCGCAA GGCGCCCCCGGGGACAG

B. Primers for RT-PCR			
Gene product	**Expected fragment length (bp)**	**Annealing temperature**	**Primer (forward/ reverse)**
GAPDH	581	58˚C	TCACCATCTTCCAGGAGCG CTGCTTCACCACCTTCTTGA
Zfm1	100	58˚C	CACCCAACTCCCTTTGCCTCTC AGCCAGCGTGTTCCGATTCC

C. Primers for sequencing		
Name of primer	**Vector**	**Primer sequence (5'-3')**
T7 Forward	2.1 Topo ®	CCAGCACTTTGACAGGC
SP6 Reverse	2.1 Topo ®	GCCTGTCAAAGTGCT
RV3 Forward	pGL3 Basic	CTAGCAAAATAGGCTGTCCC
GL2 Reverse	pGL3 Basic	GGAAGACGCCAAAAACATAAAG

Table 2.3

D. Primers for mutagenesis and ChIP-analysis		
Primer name	**Mutation site**	**Primer sequence (forward/ reverse)**
∆1	-191 to -186	CCCCGGTCCCGCCGGCCCGCCCCCGCGGTGC GCACCGCGGGGGCGGGCCGGCGGGACCGGGG
∆2	-179 to – 174	CGCCCCCGGCCCGCGGTGCCCTCTCTCC GGAGAGAGGGCACCGCGGGCCGGGGGCG
Double Mutant	-191 to -186 -179 to - 174	CCCCGGTCCCGCCGGCCCGCGGTGCCCTCTC GAGAGGGCACCGCGGGCCGGCGGGACCGGGG
ChIP 1	-216 to -193	CTCAGTTCACGCAGTAACAAATG CGGCGGCTTCTCCTTCGCAAG
ChIP 2	-173 to -152	TCCCAGCCCACCGAACTCCGC AAGGCACCGGCACCTGCTTTTC

2.4 Enzymes

All restriction enzymes were purchased from NEB GmbH (Frankfurt am Main, Germany). Various DNA polymerases were used according to the application (the polymerase used in each application is detailed in Methods) and were purchased from GeneCraft (Lüdinghausen, Germany) and Stratagene (Amsterdam Zuidoost, The Netherlands). T4 ligase & Calf intestinal alkaline phosphatase were purchased from Fermentas MBI (St. Leon-Rot, Germany).

2.5 Cell Culture

Table 2.5 Medium and substances for cell culture

Medium and substance	Company
SMC growth medium	Promocell (Karlsruhe, Germany)
DMEM	Life Technologies (Karlsruhe, Germany)
0.05% Trypsin/ 0.2% EDTA	Life Technologies
Penicillin	Life Technologies
Streptomycin	Life Technologies
FCS	Life Technologies
Heparin	Sigma-Aldridge (Deisenhofen, Germany)
Gelatine	Sigma-Aldridge
Collagenase	Sigma-Aldridge

2.6 siRNA

All ds siRNAs were purchased from Dharmacon RNA technologies (Lafayette, CO, USA)

Table 2.6

Name	Sequence (5' – 3')
Zfm1 Sense	CAG AAG ACA GUG AUU CCA G.dT.dT
Zfm1 anti-sense	CUG GAA UCA CUG UCU UCU G.dT.dT

2.7 Decoy oligonucleotides

Decoy oligonucleotides used for decoy experiments are listed in table 2.7. Sequence specific decoys were manufactured by Eurogentec (Seraign, Belgium).

Table 2.7 Decoy oligonucleotides

Name of decoy	Sequence (forward/ reverse)
EGR1	G*G*A*TCCAGCGGGGGCGAGCGGGGG*C*G*A T*C*G*CCCCCGCTCGCCCCCGCTGGA*T*C*C
SP1	A*T*T*CGATCGGGGCGGGGCG*A*G*C G*C*T*CGCCCCGCCCCGATCG*A*A*T
NFAT	C*G*C*CCAAAGAGGAAAATTTGTTTC*A*T*A T*A*T*GAAACAAATTTTCCTCTTTGG*G*C*G
Scrambled	G*C*G*AGTGCGAGCGTGCGAG*T*G*C G*C*A*CTCGCACGCTCGCACT*C*G*C

2.8 Antibodies

All antibodies used for protein detection are listed in Table 2.8. In the case of using the SuperSignal® West Femto Maximum Sensitivity Kit, antibodies specifically for this kit were used according to the manufacturer's instructions.

Table 2.8 Antibodies used for protein dection

Name	Source
Zfm1 rabbit polycolonal	Custom made by: Sigma-Genosys (Sigma-Aldridge, Haverhill, UK). See results: 4.1.
β-Actin mouse monoclonal	Abcam plc. (Cambridge, UK)
$hTAF_{II}80$ mouse monoclonal	Prof. L. Tora (Strasbourg, France)
$hTAF_{II}68$ mouse monoclonal	Prof. L. Tora (Strasbourg, France)
Anti-Rabbit A6154	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)
Anti-Mouse A5278	Santa Cruz Biotechnology Inc. (Heidelberg, Germany)

2.9 Kits

All kits used are outlined in Table 2.9.

Table 2.9 Kits used throughout this project

A. Nucleic acid purification	
Name	**Source**
QIAQuick® Gel Extraction	Qiagen (Hilden, Germany)
QIAPrep® Spin Miniprep Kit	Qiagen
Endofree® Plasmid Maxi Kit	Qiagen
RNeasy® Mini Kit	Qiagen

B. PCR cloning kits	
Name	**Source**
Topo TA Cloning® Kit	Ínvitrogen (Karlsruhe, Germany)
DNA Blunting and Ligation	Fermentas MBI (St. Leon-Rot, Germany)

C. Cell biology	
Name	**Source**
CyQuant® Cell Proliferation	Molecular Probes (Eugene, OR, USA)
RNAifect®	Qiagen (Hilden, Germany)
Human AoSMC	Amaxa GmbH (Cologne, Germany)
Luciferase Assay Kit	Promega (Mannheim, Germany)

D. Mutagenesis	
Name	**Source**
QuikChange Site-directed	Stratagene (Amsterdam Zuidoost, The Netherlands)

E. Protein detection	
Name	**Source**
SuperSignal® West Femto	Pierce (Perbio Science GmbH, Bonn, Germany)
SuperSignal® West Pico	Pierce

F. Chromosome immunoprecipitation	
Name	**Source**
ChIP-IT™ Enzymatic	Active Motif (Active Motif, Rixensart, Belgium)

2.10 Sequence analysis tools & software

Table 2.10 Software and sequence analysis tools

A. Software		
Program	**Use**	**Reference**
GeneRunner	Primer design	Hastings Software Inc.
InStat© version 3.0	Statistical analysis	Graph Pad Software Inc.
One D Scan®	Densitometry analysis	Scanalytics
Photo Finish®	Imaging system	Wordstar
SoftMax Pro® version 1.3.1	Fluoro-imaging analysis	Molecular Devices Corporation

B. Online sequence analysis servers		
Program	**Use**	**Reference**
SDS Biology Workbench	Multiple sequence alignments	http://www.workbench.sdsc.edu/
Webcutter	Restriction Maps	http://www.medkem.gu.se/cutter
BLAST	Finding similar sequence	http://www.ncbi.nlm.nih.gov/BLAST/
ClustalW	Multiple sequence alignments	http://www.ebi.ac.uk/clustalw/
Genomatix	TF binding site analysis	http://www.Genomatix.de/shop/index.html

Methods

3.1 Cell biology methods

3.1.1 Culturing of human SMC from thymus veins

Smooth muscle cells were obtained from the thymus of young children undergoing heart surgery. Parents of all young children had given their written and informed consent prior to the surgical procedure. The consent was approved by the Local Ethical Committee (Ethics document number 13/5/01). Small veins were excised from the organ, freed of adjacent fat and connective tissue and transferred to a 60 mm Petri dish containing 250 µl of 1 % collagenase solution and 1.4 ml DMEM medium with 5 % FCS. Petri dishes were placed in an incubator at 37˚C with 5 % CO_2 to allow hydrolysis of the extra cellular matrix overnight (14-16 h). Isolated cells were then centrifuged for 5 min at 1000 rpm and ambient temperature; the cell sediment was re-suspended in 2-3 ml of SMC growth medium and allowed to adhere to a Petri dish previously coated with 2 % gelatine. The medium was changed every 2 days thereafter.

3.1.2 Plating and Passage of cells (enzymatic hydrolysis)

After reaching 90-100 % confluence (approximately after 5-6 days), cells were passaged or plated into a 24 well plate. Cells were washed with serum free DMEM medium, incubated with trypsin/EDTA (5 min, 37˚C, 5 % CO_2), re-suspended in 15 % serum DMEM and spun at 1000 rpm for 5 min. Thereafter, the supernatant was carefully removed and cells were re-suspended in 5 ml 1% serum DMEM and counted using a haemocytometer. Calculation for cell counting proceeded as follows:

$C = N \times 10^4$ where C = cells per ml
N = cells counted
10^4 = volume conversion factor for $1mm^2$

Total yield = C x V where V = total volume of cells (ml)

For the experiments described, cells of passage 3 to 4 were used throughout.

3.1.3 Transfection with siRNA

Two wells of a 6-well plate of hSMC 80-90 % confluence were transfected with siRNA as listed in Table 2.6 (the antisense sequence was annealed prior to use, see 3.2.5) and two wells with a mock transfection (no RNAi) using the RNAifect Kit according to the manufacturers instructions. Cells were kept in an incubator (37˚C, 5 % CO_2) for 2 days.

RNAifect transfection mix (for 1 well in a 6-well plate. Concentration of RNAi used according to the manufacturers instructions)

	RNAi		EC-Buffer		Transfection Reagent
siRNA	5 µg	+	up to 100 µl	+	15 µl
Mock	-	+	100 µl	+	15 µl

3.1.4 Pre-stimulation of hSMC with cytokines and growth factors

Cells were stimulated with cytokines (see 3.1.5) of a 1:1 mix of IL-1β and TNFα ($6x10^3$ and $1x10^3$ units/ ml respectively, both from R&D systems) or with PDGF **(**60 units/ ml R&D systems). For one well of a 6-well plate 2 µl was added to a media volume of 2 ml. For one well of a 24-well plate, 0.5 µl was added to a media volume of 500 µl. The plates were kept in an incubator (37˚C, 5 % CO_2) for up to 7 days and the medium was replaced with fresh DMEM containing 1 % serum every two days.

3.1.5 hSMC cell proliferation assay

Cattaruzza *et al.* 2002 showed that transfection of siRNA into rSMC and stimulation with pro-inflammatory cytokines IL-1β/TNFα or PDGF increased cell proliferation, along with up-regulation of adhesion molecules VCAM-1 and MCP-1. The following hSMC cell proliferation assays were performed to test the effect of siRNA on cell proliferation under PDGF or cytokine stimulation in hSMC.

15,000 cells per well were seeded in a 24-well plate in 1 % serum DMEM. Cells were incubated (37˚C, 5 % CO_2) for 6 hours before cells were

transfected with siRNA. After 4 days, cell proliferation was measured using the CyQuant® cell proliferation assay kit according to the manufacturer's instructions. Thereafter, lysed cell samples were transferred to a 96-well microplate and the level of fluorescence was read using the Fmax Fluorescent Micro plate Reader® (Molecular Devices Corp.) and results were analysed using the Softmax Pro® version 1.3.1 program.

3.1.6 Harvesting of cells

Medium was removed from the wells and cells were washed twice with ice-cold PBS. One ml of PBS was added to the wells and the cells were scraped off with a cell scraper. They were then transferred to Eppendorf tubes and kept on ice. Cells were centrifuged for 5 min at 3000 rpm and 4˚C. Thereafter the supernatant was removed and 50 µl of lysis buffer (freshly made, see 3.4.1) was added, and the sample mixed. Samples were incubated on ice for 30 min and mixed every 5 min. Thereafter, 2.5 µl of 10 % Triton-X-100 was added to each sample and mixed for 30 sec. Samples were further incubated on ice for 15 min and mixed every minute. All samples were then centrifuged for 5 min at 2000 rpm at 4˚C. The supernatant was transferred to fresh Eppendorf tubes (this sample was labelled as cytosol). Fifty µl of Lysis Buffer was added to the remaining pellet and mixed until well dissolved. Protein concentrations of all cytosol samples were then determined by the Bradford Protein Assay (see 3.3.1).

3.1.7 Antibody generation

Zfm1 serum antibodies were generated by Sigma-Genosys. The following peptide was injected subcutaneously into two New Zealand white rabbits MATGAMATPLDFPSC (sequence obtained from Guth *et.al,* 2000). During a 77 day schedule the rabbits were immunised six times with the peptide and bled four times each. The serum antibodies from both rabbits were tested against several different cells types by western blot analysis (see 3.3.4).

3.2 Molecular biology methods

3.2.1 PCR

The reagents for PCR amplification were premixed to ensure identical conditions in each reaction [20 mM Tris/ HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U Taq DNA polymerase (all from Genecraft, Lüdinghausen, Germany) and 20 pmol primers (both forward and reverse primers), in a total volume of 50 µl, each reaction containing 1 µg of DNA]. The PCR was performed in an Eppendorf Thermal Mastercycler® (Eppendorf, Hamburg, Germany) programmed as follows:

Pre-denaturation:	2 min at 95˚C
Denaturation:	10 s at 94˚C
Annealing:	30 s at temperatures shown in Table 2.3
Extension:	2 min (depending on fragment size) at 72˚C
Final extension:	10 min at 72˚C

3.2.2 Isolation of total RNA

Messenger RNA from cultured hSMC was isolated using the RNeasy Kit® according to the manufacturer's instructions. An aliquot of 13 µl of RNA was used to make single-stranded (ss) cDNA for RT-PCR analysis (see 3.2.3) and the remaining RNA stored at -70˚C.

3.2.3 Semi-quantitative RT-PCR

For each sample, 13 µl of RNA was mixed with 1 µl of Oligo-dT (Promega, Mannheim, Germany) (500 ng/µl) and denatured by incubation at 70˚C for 10 min. After brief centrifugation, 6 µl of cDNA synthesis mix [50 mM Tris/ HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2.5 mM dNTP (all from Genecraft, Lüdinghausen, Germany), and 200 U MMLV reverse transcriptase (Promega, Mannheim, Germany)] was added and cDNA synthesis was continued for 50 min at 42˚C. The reaction was stopped by a further incubation for 10 min at 70˚C. Each cDNA sample was diluted with 180 µl of dH_2O and stored at -20˚C.

Semi-quantitative PCR was performed by normalising the relative amount of cDNA of a house-keeping gene. For this purpose, glyceraldehyde phosphate dehydrogenase (GAPDH) was chosen as an internal standard. The reagents for PCR were premixed as in 3.2.1. The PCR reaction was performed in an Eppendorf Thermal Mastercycler (Eppendorf, Hamburg, Germany) programmed as follows:

Pre-denaturation:	2 min at 94˚C
Denaturation:	30 s at 94˚C
Annealing:	40 s at the temperatures shown in Table 2.3
Extension:	1 min at 72˚C
Final extension:	5 min at 72˚C

After amplification, PCR products were separated on 1-1.5% agarose gels (depending on fragment size) with ethidium bromide staining. The densities of the detected bands were determined by using the PhotoFinish® imaging system and the One-Dscan® software. These results permitted adjustments for the volume of cDNA administered in order to obtain (in a PCR reaction of 20 cycles) the identical quantity of the product for the housekeeping gene (GAPDH). With that amount of cDNA fixed, the PCR reactions were performed with specific primers for the genes of interest.

3.2.4 Sequencing of the PCR product

A 10 µl sequencing reaction was setup by mixing 5 µl of purified PCR product (100 ng), 3 µl of BigDye Terminator DNA-sequencing-mix (Applied Biosystems, Damstadt, Germany); 1 µl of primer (see Table 2.3 for primers) and 1 µl of sterilised water. The sequencing PCR was performed as follows: denaturation at 94˚C for 30 sec; annealing temperature was primer specific as shown in Table 1 for 15 sec; and extension at 60˚C for 4 min.
After the sequencing PCR, the products were precipitated by adding 50 µl 100 % ethanol and 1.5 µl of 2M NaAc (pH 4.0). The samples were left overnight at -20˚C. Thereafter, the samples were centrifuged at 4˚C, 13,000 rpm for 20 min. The supernatant was carefully removed, and washed with 250 µl 70 % ethanol. The pellet was then dissolved in 25 µl of dH_2O. The

products were sequenced by using a model 3100 Genetic Analyser (Applied Biosystems, Damstadt, Germany).

3.2.5 siRNA technique

Double-stranded (ds) RNAi were prepared from the complementary single-stranded 2'-OH RNAi (Dharmacon, RNA Technologies, Lafayette, CO, USA) according to the manufacturer's instructions. The single stranded sequences of the RNAi are listed in Table 2.6

3.2.6 Cloning

3.2.6.1 Topo-cloning

Large and small fragments of the promotor region were cloned into the pCR® Topo 2.1 vector (3.9 Kb) using the Topo TA Cloning® Kit according to the manufacturer's instructions. Plasmids containing the inserts were further amplified as in 3.2.6.3. The plasmids were sequenced as in 3.2.4 to check for the correct insert.

3.2.6.2 Cloning into pGL3

Empty pGL3 vector containing the luciferase reporter gene, was digested with restriction enzyme SmaI. The hydrolysed vector was then de-phosphorylated using calf alkaline phosphatase in order to minimise the chances of the vector re-annealing on itself during the ligation procedure. Both large and small fragments of the Zfm1 promotor were excised from the pCR® Topo 2.1 vector with EcoRI, which produced 'sticky ends' on the insert. The 'sticky ends' of the promotor insert were 'polished' to produce 'blunt ends' using the DNA Blunting and Ligation Kit. The blunt ended promotor insert and SmaI digested pGL3 vector were ligated in a reaction mixture [~80 ng of plasmid DNA; ~300 ng of insert DNA; 2 µl of 10 X ligation buffer; 2 µl PEG4000; dH_2O up to 20 µl and 2 µl T4 DNA ligase] at 14˚C overnight. Transformation of Top10™ competent cells were carried out as stated in 3.2.6.3.1. Colonies containing the insert of interest were screened via mini-culture; plasmid DNA analysis (see 3.2.6.3-4) and restriction digest

with KpnI and HindIII restriction enzymes. The orientation of the insert was confirmed via sequencing (see 3.2.4) with primers specific for the pGL3 multiple cloning site (MCS) (see Table 2.3).

3.2.6.3 Amplification of plasmid

3.2.6.3.1 Transformation of competent cells

One µl of pGL3 vector with insert or pCR® Topo vector with insert was mixed with 20 µl of Top10™ competent cells in a 1.5 ml Eppendorf tube. The mix was then incubated on ice for 30 min. Thereafter, the cells were subjected to a heat shock at 42˚C for 20 s, and placed on ice for a further 2 min. Two hundred and fifty µl of SOC Medium was added and cells were incubated for 1 h at 37˚C with shaking. Sterile LB-agar (see 3.4.3) was melted and cooled until 'warm to the touch'. Ampicillin was then added to give a final working concentration of 20 µg/ml. While still warm, the LB-agar ampicillin solution was poured equally into 100 mm petri dishes and left to set at room temperature. Before evenly plating transformed competent cells, the LB-agar plates were pre-warmed to 37˚C. Thereafter, up to 200 µl of transformed competent cells were plated on pre-prepared LB-agar plates. Plates were then incubated at 37˚C overnight to allow bacterial colonies to grow.

3.2.6.4 Plasmid mini-cultures

Plasmid mini-cultures were set up in 15 ml falcon tubes. One transformed colony of Top10 cells was added to 5 ml of LB Broth (with appropriate antibiotic) via sterile technique. Holes were perforated on the cap of the falcon tubes to allow air to circulate through the tube. These cultures were then incubated at 37˚C with shaking overnight.

3.2.6.5 Plasmid purification

Plasmids grown in mini-cultures were purified using the Qiagen Plasmid Mini Spin Kit according to the manufacturer's instructions.

3.2.7 Nucleofection

Nucleofection is a new technique for transfecting plasmids into cells produced by Amaxa™ Biosystems (Köln, Germany). To transfect hSMC with our reporter plasmid, 2 µg of the plasmid of interest was mixed with 100 µl containing approximately 0.5-1 x 10^6 cells of hSMC harvested (as in 3.1.2) suspended in Nucleofector™ Solution optimised for human aortic smooth muscle cells. The cells were then transferred to a cuvette especially manufactured for the Nucleofector™ apparatus and transfected under the optimised transfection program A-33. The transfection program was optimised according to the manufacturer. After transfection, 500 µl of pre-warmed 15 % FCS DMEM was added to the cuvette and transferred to an appropriate well in a 6-well plate containing 1.5 ml of 15 % FCS DMEM pre-warmed to 37˚C. The cells were then kept at 37˚ C with 5 % CO_2.

3.2.8 Preparation of decoy oligonucleotides

3.2.8.1 Rehydration of decoy oligonucleotides

Decoy ODN (dODN) were manufactured by Eurogentec, Seraign, Belgium and each dODN had a specific molecular weight, stated on the accompanying information sheet. An appropriate volume of TEN Buffer (see 3.4.1) was added to give a final concentration of 2 µM per dODN oligonucleotide. Each oligonucleotide was then mixed and incubated at ambient for 40 min. mixing every 2 min.

3.2.8.2 Hybridisation of decoy oligonucleotides

Equal volumes of forward and reverse decoy oligonucleotides were mixed carefully in an Eppendorf tube. The mix was placed in a boiling (100˚C) waterbath for 2-4 min. The waterbath was then switched off and the decoy oligonculeotides were left in the waterbath until the temperature reached approximately 30˚C. Decoy ODN were then confirmed for hybridisation by running single stranded and hybridised decoys on a 2.5 % agarose gel (if hybridised the dODN should be a higher molecular weight compared to its single stranded counterpart) and stored at -20˚C.

In order to test whether the dODNs were successfully hybridised, single-stranded oligonucleotides and hybridised dODN were run on a 2.5 % agarose gel. Figure 5 confirms that our single-stranded oligonucleotides were successfully hybridised.

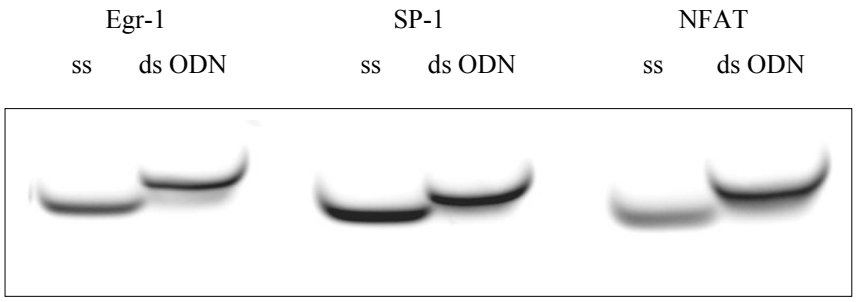


Figure 5. Hybridisation of dODNs. Exemplary agarose gel depicting single stranded (ss oligonucleotide) vs Hybridised dODN. Less than 500 ng of DNA was loaded on the gel.

3.2.8.3 Decoy oligonucleotide experiments

All experiments containing decoy oligonucleotides had a final experimental concentration of 10 µM. All dODN stock solutions used were at 100 fold concentration.

3.2.9 Site-directed mutagenesis

The short promotor fragment cloned into the pGL3 vector was mutated at two transcription factor (TF) binding sites of interest. Primers for mutagenesis (Table 2.3) were designed and manufactured by IBA nucleic acids research (Göttingen, Germany). Site-directed mutagenesis was carried out using the QuikChange® Site-Directed Mutagenesis Kit according to the manafacturer's instructions. Mutations were confirmed by mini-culture, plasmid purification and sequencing as outlined in 3.2.4, 3.2.6 and 3.2.6.3 respectively.

3.2.10 luciferase reporter gene analysis

Transfected cells to be analysed for luciferase gene activity were lysed and prepared according to the Luciferase Assay Kit instructions. The luminescence was recorded from an average of 15 readings using the MicroLumat LB 96P EG & G luminometer EG & G Berthold Winglow© software version 1.22 (1992-1997) both from Berthold Technologies GmbH & Co KG (Bad Wildbad, Germany). And luciferase activity was calculated against the protein concentration of each sample and represented as level of fluorescence per µg protein.

3.3 Protein biochemistry methods

3.3.1 Bradford protein assay

hSMC samples harvested as in 3.1.6 were diluted 1:100 with dH_2O. In a 96-well micro-plate, 200 µl of Bradford Protein Assay solution (BioRad, München, Germany) was added. Eighty µl of each diluted sample along with pre-prepared protein standards (10, 20, 30 and 40 µg/ml protein) were added and mixed with the assay solution. The samples were left for 10 min at room temperature. Samples were analysed using the ELX808 micro-plate reader (Bio-Tek Instruments, Inc.) and the MikroWin version 3.0 program (Mikrotek Laborsysteme GmbH, Germany).

3.3.2 Ethanol prepcipitation of protein samples

100 % ice-cold ethanol was added nine times the volume of the protein sample to be precipitated. Samples were mixed and incubated at -20˚C overnight. Thereafter, samples were centrifuged at 10,000 rpm for 10 min. Ethanol was removed and samples were left to air dry and resuspended in dH_2O. 3 X sample loading buffer (see 3.4.2.3) was added, and the samples incubated at 95˚C for 5 min.

3.3.3 Protein analysis by SDS-PAGE

SDS-polyacrylamide gels were made according to the Laemmli method (Laemmli, 1977). Gels from 10 – 15 % SDS were used. For recipies and buffers see chapter 3.4. Proteins were denatured by the addition of 3 X sample loading buffer (see 3.4.2.3) and heated for 5 min at 95˚C. Thereafter, proteins were separated by electrophoresis at 100 V through the stacking gel, and 200 V through the separating gel.

3.3.4 Western blot analysis

Proteins were separated by SDS-PAGE (see 3.3.3) and transferred onto a hydrated PVDF membrane (5 min in 100 % methanol and 45 min ddH_2O) at 350 mA for 45 min with transfer buffer (see 3.4.2.3). The membrane was then dried for 2 h at 50˚C or directly blocked overnight (or for 2 h) with blocking buffer (see 3.4.2.3).
The primary antibody (diluted 1:1000 in Washing Buffer) was added to the membrane and incubated at ambient temperature for 2 h. Thereafter the membrane was washed 3 times for 10 min. with washing buffer. The appropriate secondary antibody conjugated to peroxidase (see Table 2.8, diluted 1:10,000 in Washing Buffer) was added and the membrane was further incubated at ambient temperature for 1 h. After 1 h, the membrane was washed 3 times for 10 min with Washing Buffer.

3.3.4.1 Development of membrane

The membrane was developed using ECL solution (Amersham Pharmacia Biotech, Freiburg, Germany), according to the manufacturer's instructions.

3.3.5 Immuno-precipitation (IP) of hSMC

Human SMC were harvested as in 3.1.6. Cells were then resuspended in 1 ml of IP buffer (see 3.4.2.1) and divided into two Eppendorf tubes, 500 µl per tube (each tube containing between approximately 350 - 400 µg of protein). Five hundred µl of antibody was added to each tube and samples were incubated at ambient temperature with rotation for 1 h. Thereafter, 25 µl of

protein-A sepharose beads (Sigma-Aldridge, München, Germany, pre-prepared according to manufacturer's instructions) was added and the samples further incubated at ambient temperature with rotation for 2 h.
Samples were then centrifuged for 1 min at 1000 rpm at 4˚C and a 90 µl aliquot of the supernatant was kept for analysis. All samples were then washed consecutively with 100 µl of washing buffer I, washing buffer II and washing buffer III (see 3.4.2.1), for 10 min at ambient temperature with rotation and centrifuged for 1 min at 1000 rpm and 4 ˚C. Ninety µl from each supernatant was kept for analysis, along with the pellet samples, respectively.

3.4 Chromosome immuno-precipitation (ChIP)

ChIP was performed on different batches of hSMC, using the ChIP IT™ Enzymatic Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions.

3.5 Statistical analysis

Results are expressed as the mean ± SEM of 'n' independent observations (i.e samples from different batches of cells), unless otherwise stated. Statistical evaluation was performed by employing a One-way non-parametric ANOVA with the appropriate student t-test using the Instat™ version 3.00 for Windows 95 statistics software package (Graph Pad Software, San Diego, CA, USA). A 'p' value < 0.05 was considered statistically significant.

3.6 Buffers and stock solutions

3.6.1 Cell biology buffers and stock solutions

CyQUANT Cell Lysis mix

19 µl RNAse-free water
1 ml 20 X CyQuant Lysis buffer
50 µl CyQuant dye

TEN Buffer

100 mM NaCl
10 mM Tris pH 7.5
1mM EDTA

Cell Lysis Buffer

905 µl Solution A buffer
20 µl of DTT
25 µl of Protease Inhibitor Mix
50 µl of Pefabloc

Solution A Buffer (in a final volume of 10 ml)

0.1 mM EDTA
0.1 mM EGTA
10 mM KCl
10 mM HEPES pH 7.5

3.6.2 Protein biochemistry buffers and stock solutions

3.6.2.1 Immuno-precipitation buffers

IP Lysis buffer

20 mM TRIS pH 7.5
100 mM NaCl
0.5 % Triton-X-100
0.5 mM EDTA
0.5 mM Pefabloc
0.5 % Protease inhibitor mix

Washing buffer I

100 mM TRIS pH 7.5
150 mM NaCl
2 mM EDTA
0.2 % Triton-X-100

Washing buffer II

40 mM TRIS pH 8.5
50 mM NaCl
5 mM EDTA
1.25 % Triton-X-100

Washing buffer III

40 mM TRIS pH 8.5
300 mM NaCl
10 mM EDTA
1.25 % Triton-X-100
0.2 % SDS

3.6.2.2 SDS-PAGE buffers and recipies

	10 % SDS-PAG	4 % Stacking Gel
Acrylamide stock (30%)	3.3 ml	0.65 ml
1.5 M Tris.HCl pH 8.8	2.5 ml	-
0.5 M Tris.HCl pH 6.8	-	1.25 ml
dH_2O	4.1 ml	3.05 ml
20 % SDS	50 µl	50 µl
10 % APS	50 µl	25 µl
TEMED	10 µl	10 µl

3.6.2.3 Buffers for western blot

Transfer buffer

Tris	25 mM
Glycine	0.192 M
Methanol AR	20 %

Wash Buffer

Triton X 100	2.5 g
PBS 1 X	1 L

Blocking Buffer

Milk powder	2.5 g
Wash buffer	50 ml

3 X Loading Buffer

Tris.HCl pH 6.8	150 mM
DTT	300 mM
SDS	6 %
Bromophenol blue	0.3 %
Glycerol	30 %

3.6.3 Molecular biology recipies

LB (Luria-Bertani) Medium

To 950 ml dH_2O add:

Bacto-tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Dissolved solutes and adjust pH to 7.0 with 5 M NaOH. Adjust the volume to 1L with dH_2O. Sterilise by autoclaving for 20 min at 15 lb/square inch or liquid cycler.

LB agar

Add 15 g of agarose to 1L of LB-medium.
Sterilise as above.

4. Results

4.1 Production of the Zfm1 antibody

In order to analyse Zfm1 protein expression, first antibodies against the human protein has to be generated as they were not available. Thus, suitable peptides were selected and antibodies against Zfm1 were produced in two different rabbits (numbered 1675 and 1676 respectively) by Sigma-Genosys (Sigma-Aldridge, Haverhill, UK) using the following sequence: MATGAMATPLDFPSC (obtained from Guth *et al.* 2000). Approximately 6 months after the peptides were injected, the rabbits were bled for a maximum of five times and the blood sera were analysed and tested for antibody quality with protein samples derived from several different species. Western blot analysis revealed that Zfm1 antibody number 1676 detects two isoforms, Zfm1 A and Zfm1 E, with a molecular mass of approximately 55 and 70 kDa respectively, in hSMC and HUVECs. The Zfm1 antibody number 1675 only detected the 55 kDa isoform (Fig. 6). From this point, the Zfm1 antibody number 1676 was used for all further protein expression analyses.

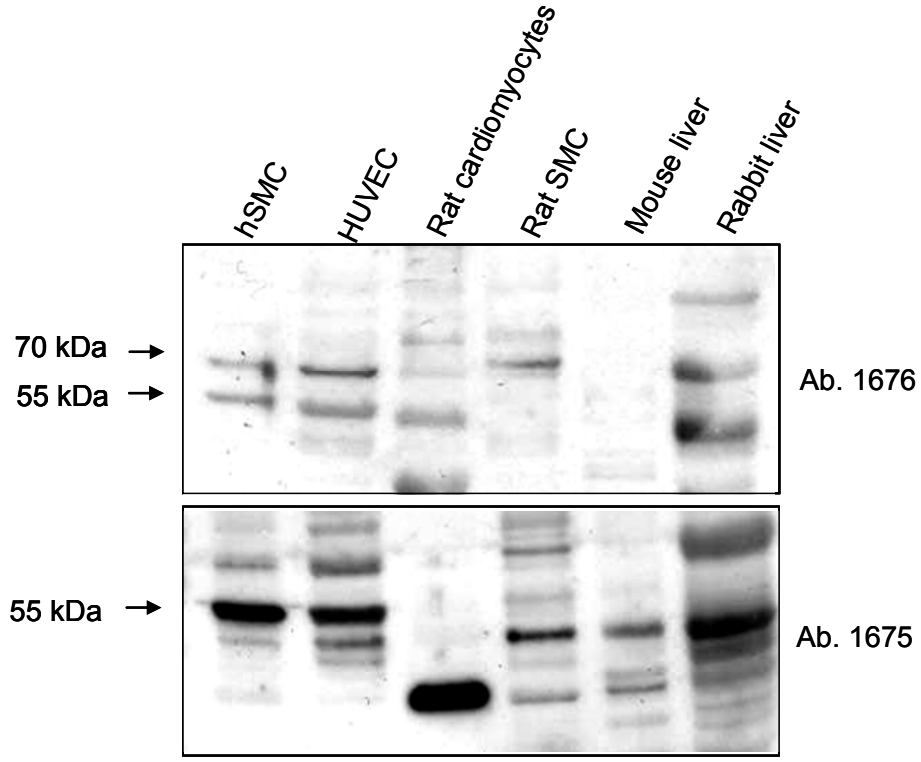
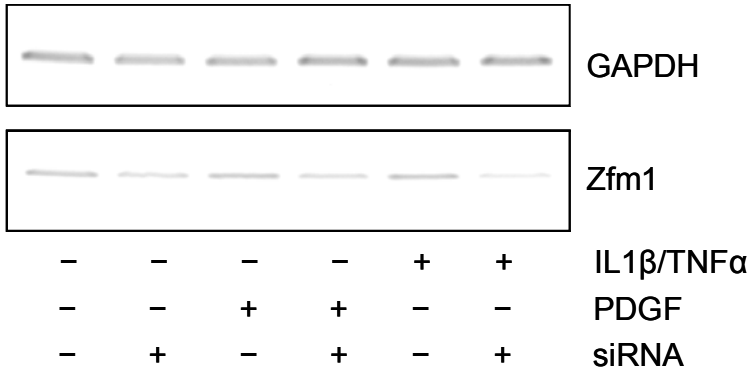


Figure 6. Zfm1 serum antibody test. Expression of Zfm1 was tested using custom made serum antibodies in several different species; rat, human and mouse. The figure depicts a representative Western blot analysis. The expected band sizes of Zfm1 are approximately 55 and 70 kDa which corresponds to Zfm1 isoforms E and A, respectively.

4.2 Zfm1 siRNA

The siRNA technique was chosen in order to analyse the role of Zfm1 in cell proliferation. Expression of Zfm1 was monitored from 24 to 72 h post transfection of Zfm1 siRNA. Subsequent analysis confirmed that the expression of Zfm1 RNA (Fig. 7) and protein (Fig. 8) was decreased effectively following Zfm1 siRNA transfection.

A



B

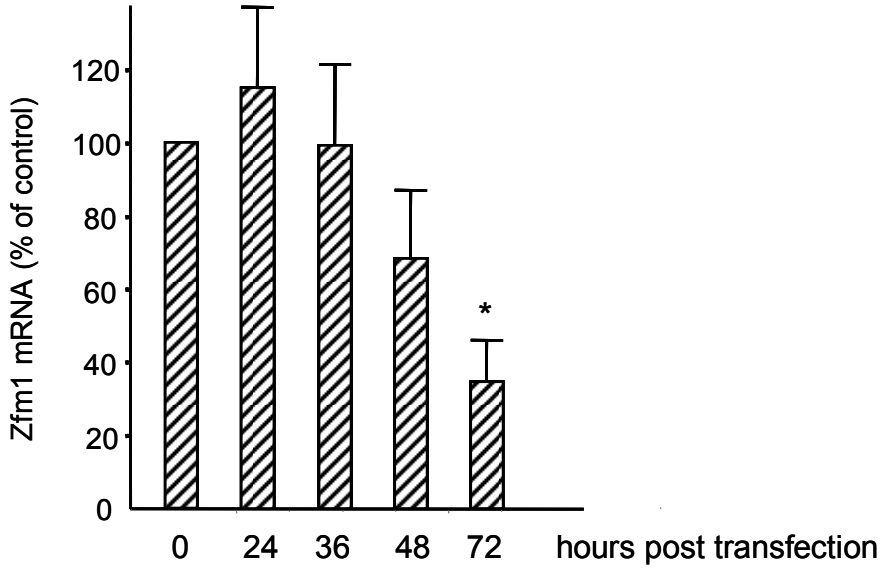


Figure 7. Analysis of Zfm1 siRNA effects in hSMC. A) Representative RT-PCR analysis result. Primers against GAPDH were used as an internal control. **B)** Statistical analysis of RT-PCR timecourse of Zfm1 siRNA in hSMC. **(***P<0.05, control vs. 72 h; n=5).

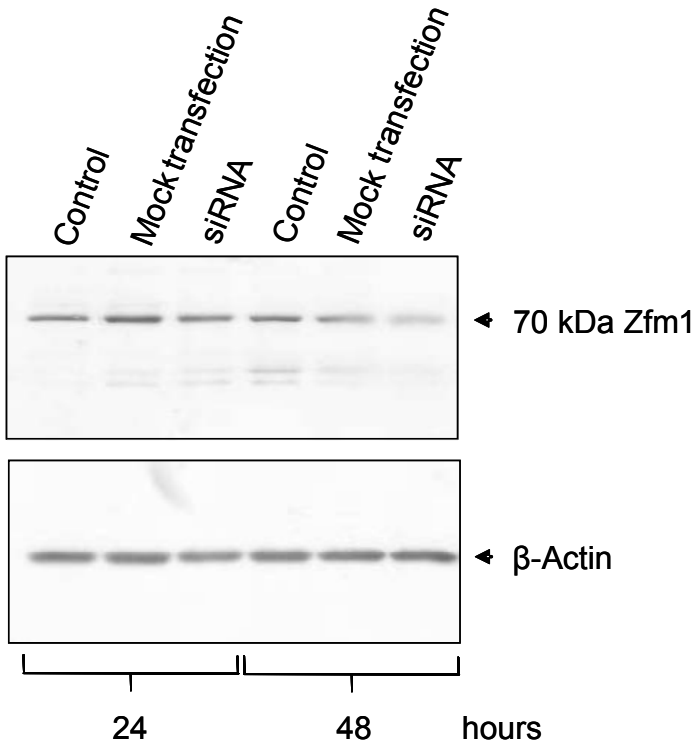


Figure 8. Western blot analysis of Zfm1 siRNA effects on Zfm1 protein expression. Representative Western blot analysis, similar experiments were obtained in 3 different batches of hSMC. B-actin was used to verify equal loading of each lane

4.3 Proliferation of human vascular smooth muscle cells

Proliferation of hSMC pre-treated with Zfm1 siRNA was analysed following stimulation with PDGF or IL-1β/TNFα. This analysis confirmed that stimulation of hSMC with pro-inflammatory cytokines IL-1β/TNFα alone is not sufficient to induce cell proliferation. However, an approximate 25% increase in cell proliferation was observed in response to IL-1β/TNFα stimulation in cells treated with Zfm1 siRNA (Fig. 9). This result is different from previously published data obtained with rat SMC (rSMC) showing that both exposure to IL-1β/TNFα and decreasing Zfm1 abundance alone stimulates proliferation.

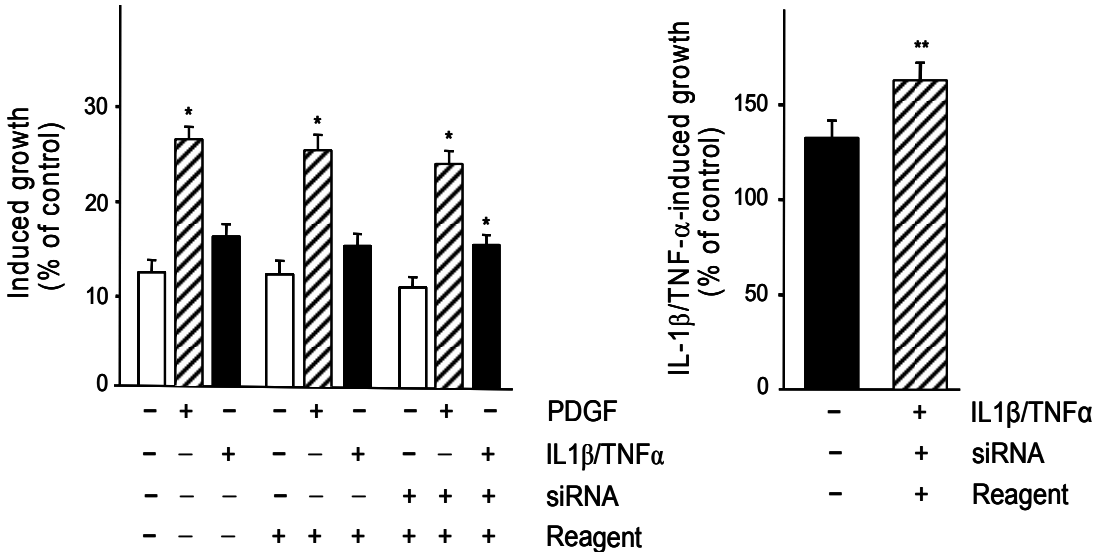


Figure 9. Proliferation of human vascular smooth muscle cells pre-treated with Zfm1 siRNA stimulated with either PDGF (60 u/ml) or IL-1β/TNFα (1000 u/ml) for 72 h. Cell proliferation was measured in hSMC using the CyQuant™ method, which determines cell number by the amount of fluorescent DNA. A mock transfection (transfection reagent only) was performed as a control for potential cell cytotoxicity. (**P*<0.05 vs. respective controls;***P*<0.01 vs. mock-transfected cells; n=14).

Besides proliferation, Zfm1 also inhibits pro-inflammatory gene expression in rSMC (Cattaruzza *et al.,* 2002). VCAM-1 is such a pro-inflammatory gene product which was shown to be up-regulated by Zfm1 knock down alone and in the presence of pro-inflammatory cytokines. Therefore, endogenous VCAM-1 expression in hSMC pre-treated with Zfm1 siRNA following stimulation with PDGF or IL-1β/TNFα was analysed. In contrast to rSMC, knock down of Zfm1 with or without exposure to PDGF had no effect on VCAM-1 expression. Only after stimulation with IL-1β/TNFα is VCAM-1 expression was significantly increased and this effect was independent of the abundance of Zfm1 (Fig. 10). Thus Zfm1 does not play a role in modulating pro-inflammatory gene expression in human SMC.

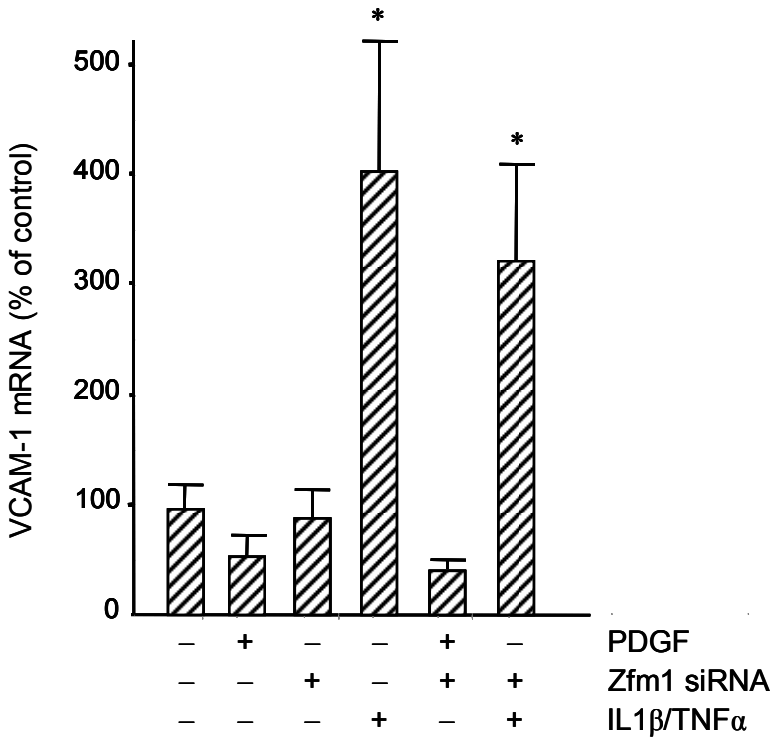


Figure 10. RT-PCR analysis of VCAM-1 expression in hSMC pre-treated with Zfm1 siRNA and stimulated with PDGF (60 u/ml) or IL-1β/TNFα (1000 u/ml) for 48 h post treatment with siRNA, (**P*<0.01 vs. control; n=5).

4.4 Effects of PDGF and IL-1β/TNFα on Zfm1 protein expression

Cell proliferation assays revealed that stimulating hSMC with IL-1β/TNFα alone is not sufficient to induce proliferation, while a growth promoting effect of these cytokines was observed following knock down of Zfm1. On the other hand, PDGF induced cell proliferation to a maximum extent, and this effect was not dependent on Zfm1 abundance. Because of this, it was hypothesised that PDGF itself down-regulates endogenous Zfm1 gene expression, and in order to test this, Western blot and RT-PCR analyses were performed. IL-1β/TNFα effects (Fig. 11) on endogenous Zfm1 gene expression were analysed as well to support their lack of effect in the cell proliferation assay.

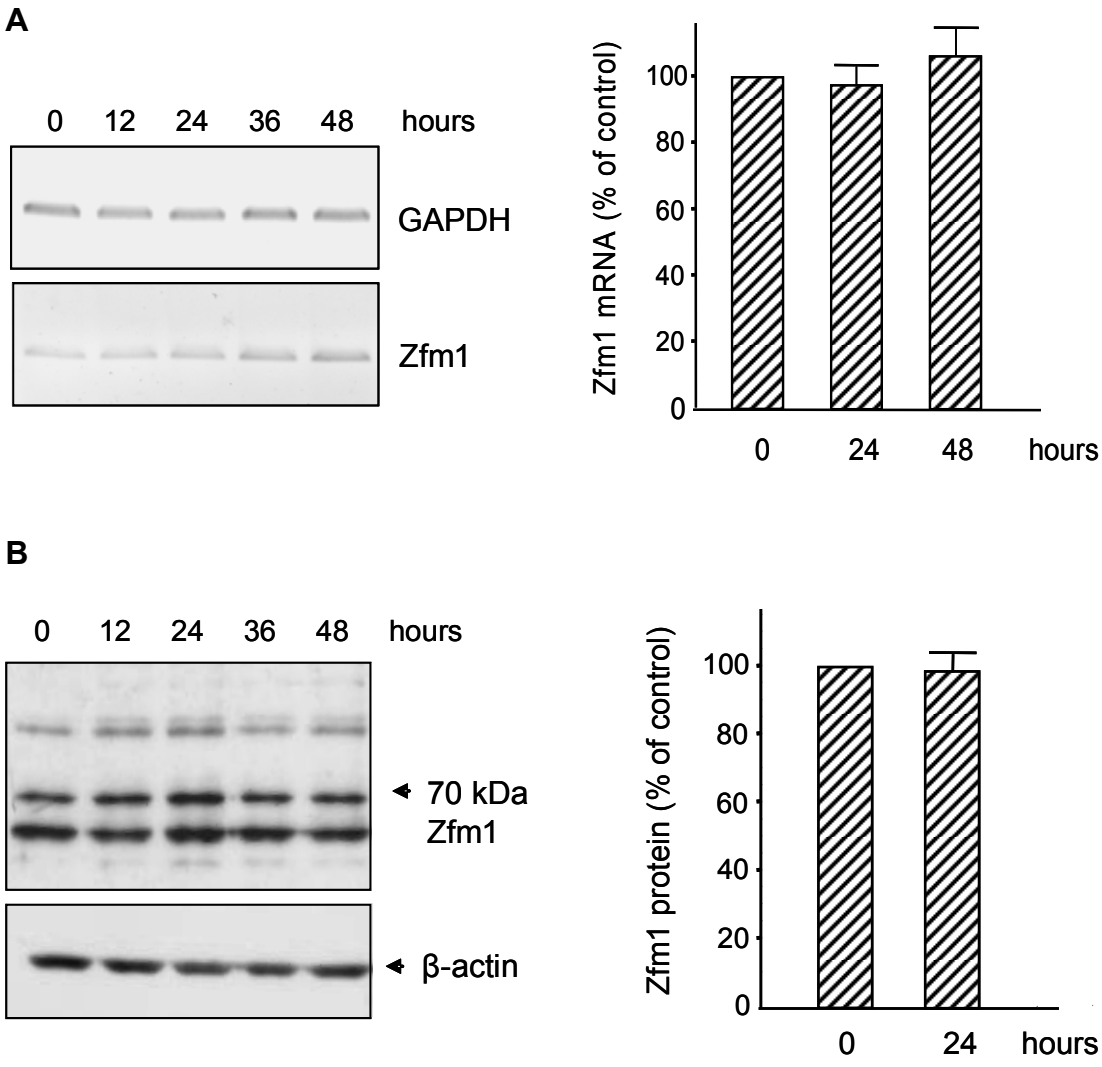


Figure 11. Effects of IL-1-β/TNFα on endogenous Zfm1 expression at the RNA and protein level in hSMC. A) IL-1β/TNFα (1000 u/µl) has no effect on Zfm1 mRNA levels after 24 and 48 h exposure. GAPDH served as an internal control. **B)** IL-1β/TNFα has no effect on Zfm1 mRNA and protein abundance. Actin was used as a loading control. Figures depict the result of one representative experiment (left panel) and the statistical summary of five independent experiments (right panel).

Messenger RNA and protein time course analyses confirmed that IL-1β/TNFα alone does not down regulate endogenous Zfm1 in hSMC. In contrast, hSMC stimulated with PDGF revealed a significant decrease in Zfm1 expression, both at the mRNA and protein levels (Fig. 12).

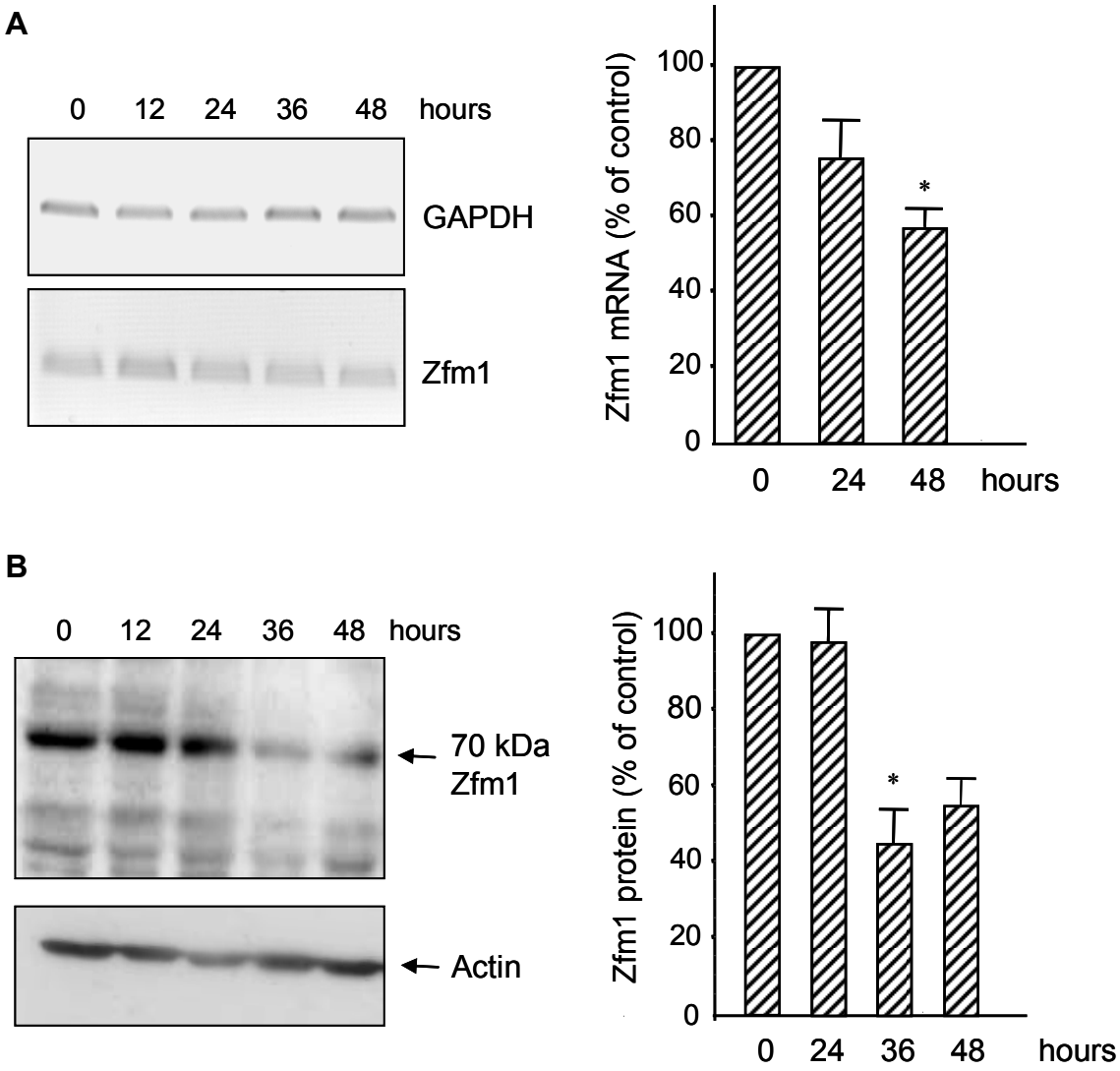


Figure 12. Time-dependent PDGF-induced knockdown of endogenous Zfm1 expression at the RNA and protein level in hSMC. A) PDGF (60 u/ml) down regulates Zfm1 at the RNA level approximately after 24 and 48 h exposure. GAPDH served as an internal control (**P*<0.05 vs. control;n=5). **B)** PDGF down regulates Zfm1 at the protein level at approximately 36 and 48 h after stimulation. Actin was used as a loading control. Figures depict results of one representative experiment (**P*<0.05 vs. control;n=5).

As Zfm1 down-regulation was likely to be important for PDGF-induced hSMC cell proliferation, we set out to analyse the mechanisms of this PDGF effect. A first step in this analysis was to clone the human Zfm1 promotor and to create a Zfm1 promotor reporter-gene construct.

4.5 Cloning of the Zfm1 promotor

The Zfm1 promotor was cloned as two fragments (large and small) into the pGL3 basic luciferase reporter gene vector (see Annex Fig. 1 for full Sf1/Zfm1 sequence as published in GenBank, Accession no. AJ000051). During our attempt to amplify the Zfm1 promotor directly from a genomic DNA template, numerous PCR products whose sizes were approximately within the right range were obtained. These products were sequenced and came back negative for Zfm1 and were labelled as 'false positives'. Thus, a second approach to cloning the promoter was employed (outlined in Fig. 13) and involved screening of the human chromosome 11 clone library no. LANL-LA11NCO1 (obtained from the *Deutsche Resourcenzentrum für Genomforschung GmbH* (DRG, Berlin, Germany). The chromosome 11 library is organised in hierarchical clone pools. In a first step of the search for a single clone therefore primary pools (up to a total of 4608 single clones) were screened by PCR for the sequence of interest (Fig. 13).

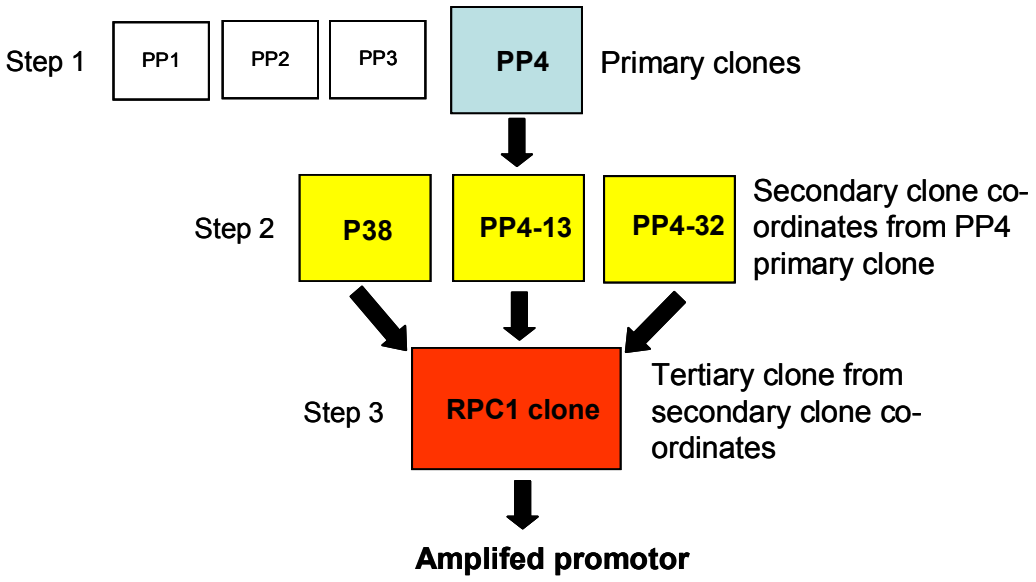


Figure 13. Outline of the Zfm1 promotor amplification from a chromosome 11 BAC clone library. Secondary clone co-ordinates p38, PP4-13 and PP4-32 (in bold) were used to elect the tertiary clone (RPC1-11) for further analysis by PCR. Coloured boxes represent the clones that gave positive products for Zfm1.

A 96-well plate of primary pool 4 containing redundant pools of secondary clones (up to 54 single clones per well) was then obtained and screened (see Annex Fig. 10 for the PP4-secondary clone map). As these pools are redundant and every single clone is obtained by a unique ('co-ordinate') combination of secondary clone pools, with this step, a single clone can be unambiguously identified. In this case, the 'co-ordinate' combination P38, PP4-13 and PP4-32 pointed towards the single clone RPC1 of chromosome 11 (Fig. 15). Details of the screening procedure are outlined in chapter 4.5.1.

4.5.1 Screening the chromosome 11 BAC clone library

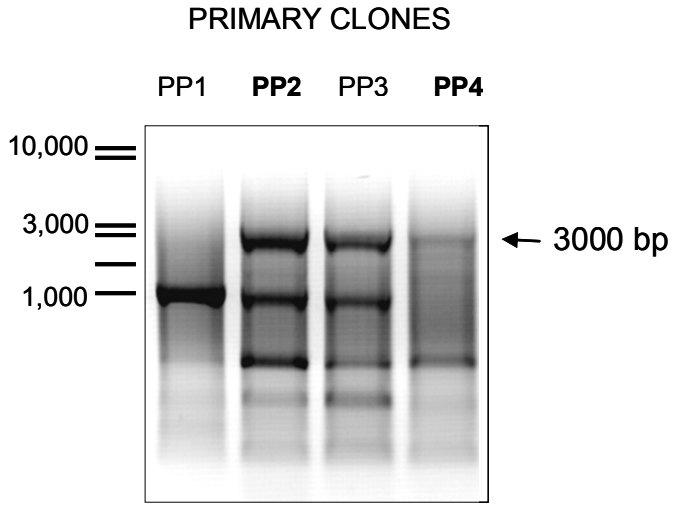


Figure 14. Screening of primary clones. The bands indicated from clones PP2 and PP4 (in bold) were excised and purified. The DNA was sequenced and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) results were positive for Zfm1. Co-ordinate numbers PP2 and PP4 were then used to order the secondary clones of human chromosome 11.

Both plates of secondary clones named PP2 and PP4 were then screened. Screening of the PP4 plate was successful and three co-ordinates from the secondary clones were matched (Fig. 15). In the next step, the secondary clone pools 'PP-4' were screened for Zfm1-specific sequences (as outlined above).

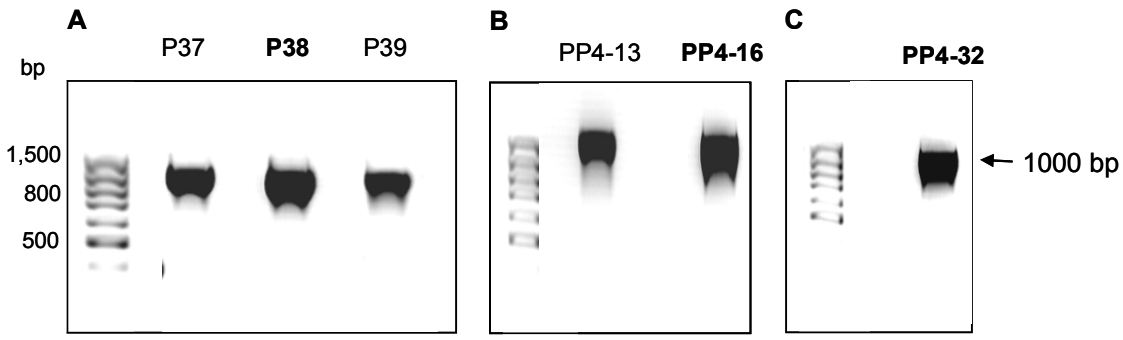


Figure 15. Screening of 13 plate pools, 16 pooled rows and 24 pooled columns in the PP4 secondary clone. A) 13 plate pools. B) 16 pooled rows. C) 24 pooled columns. The candidate fragments (in bold) were excised, purified and sequenced. BLAST results indicated that the fragments were positive for human Sf1/Zfm1. These were then chosen as co-ordinates to obtain the tertiary clone.

4.5.2 Amplification of Zfm1 promotor fragments

A large fragment approximately 870 bp long, of the hZfm1 promotor was amplified (Fig. 16) using primer pair 3 (Table 2.3).

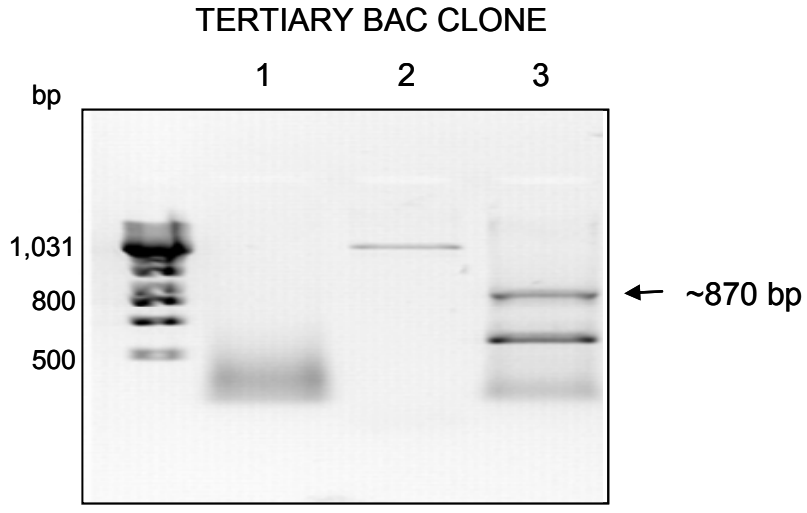


Figure 16. PCR and amplification of the large Zfm1 promotor fragment. The upper band (marked by the arrow), was excised, purified and sequenced.

Because the large fragment amplified fell 672 bp upstream of the transcriptional start site, new primers had to be designed in order to amplify the missing 672 bp. Primer pair 4 (Table 2.3) was used to amplify the missing 672 bp romoter fragment from the same human chromosome 11 BAC clone RPC1-11 (Fig. 17).

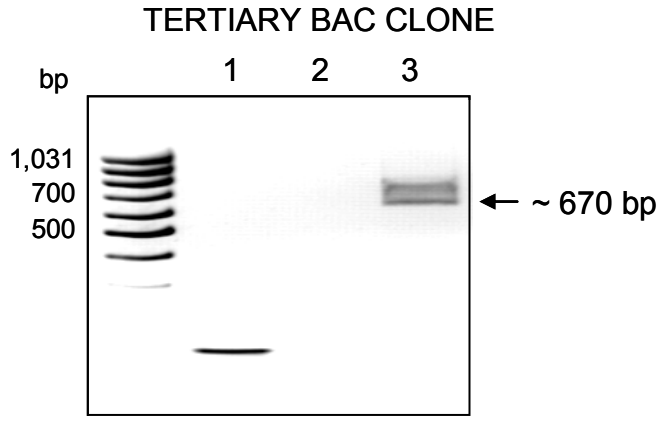


Figure 17. PCR and amplification of small Zfm1 promotor fragment. The fragment amplified was approximately 670 bp long, and termed 'small romoter fragment'.

Of the 672 bp, 629 bp belonged to the romoter while the remaining 43 bp corresponded to the coding region of the hZfm1 gene.

4.6 Generation of the Zfm1 promotor reporter gene construct

The Zfm1 promoter sequence cloned into pGL3 was 629 bp long and had no sequence mismatches in any known transcription factor binding sites (Fig.18). Details of the full amplified sequence are provided in the Annex (Fig. 5).

```
-629 tcccccgccg taaatctcgt tccggctggg cctttcccgc cgcgactctc
-579 gcttaatccc ggagaaactg ccccctgggg gaggggagta gaaaaaggct
-529 ggaaccagcg acagccaatc ccgcgacact acaacgcagg cgagattgat
-479 tgagtccacc accgcagcca atgagagagc tcgccgtcgc tccgtcatag
-429 agttcgcccc accccatccc ctcctttctg gactcggagc tcagttcacg
-379 cagtaacaaa tgaagtgcgc gctgcgacac ctcccagccc accgaactcc
-329 gccgccattt cctcgcttgg cctaacggtt cggccaatcc cagcgcgcat
-279 caagaaggac tgaggctccg ccaatcggag gccgccgatt tcgacccttc
-229 gcctcggccc ggcccaatcc aggccccggt cccgccgccc ccggcccgcc
-179 cccgcggtgc cctctctcct ccctctttgt gcgtctcgcg ccgccgccgc
-129 ccgccgcgtg agaggacggg ctccgcgcgc tccggcagcg cattcgggtc
 -79 ccctcccccc gggaggcttg cgaaggagaa gccgccgcag aggaaaagca
 -29 ggtgccggtg cctgtccccg ggggcgccat g
```

Figure 18. Sequence of the Zfm1/Sf1 short promotor fragment. The fragment cloned into pGL3 basic was sequenced using the GL2 reverse sequencing primer. And the orientation and confirmation of the fragments cloned were confirmed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The transcription startsite is highlighted in yellow. When compared to the wild-type Zfm1 promotor sequence deposited in GenBank (Acc. No. AJ000051), two base differences were detected which are depicted in red (see Annex Fig. 5 for the alignment sequence).

In order to amplify the promoter by PCR a reverse primer was chosen well within the coding region of the Zfm1 gene resulting in a reporter gene construct containing the 14 N-terminal amino acids of Zfm1. This fragment was blunt cloned via the SmaI restriction enzyme into the MCS of the pGL3 basic vector (Fig. 19). The sequence of the short Zfm1 promotor and a map of the pGL3-basic reporter gene vector can seen in Annex Fig. 2 and Fig. 9, respectively.

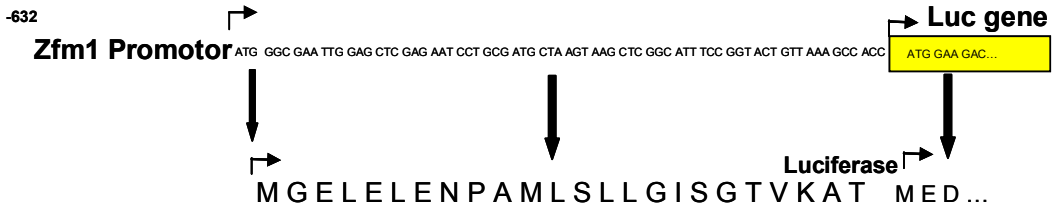


Figure 19. Map of the Zfm1 small promotor construct in pGL3. The amino acid sequence derived from the N-terminal sequence of the Zfm1 gene, along with some sequences of the multiple cloning site (MCS) is emphasised.

4.7 Analysis of the Zfm1 promotor

4.7.1 PDGF down-regulates Zfm1 promotor activity

In order to analyse how PDGF down-regulates Zfm1 expression, the Zfm1 promotor luciferase reporter-gene construct was transfected into hSMC. The transfected hSMC were stimulated for 48 h with PDGF and Zfm1 promotor activity was measured by chemiluminesence analysis. This, revealed that PDGF decreases Zfm1 promotor activity 48 h after PDGF stimulation (Fig. 20) confirming that the construct, similar to the endogenous gene, is PDGF-sensitive.

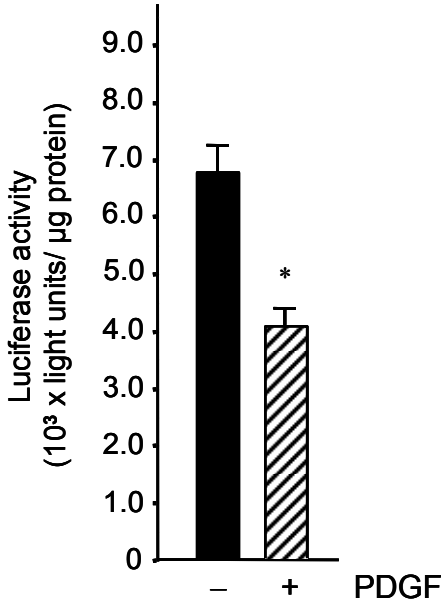


Figure 20. Exposure to PDGF decreases Zfm1 promotor activity in hSMC. Plasmid was transfected into hSMC via nucleofection and the transfected hSMC were stimulated with PDGF (60 u/ml) for 48 h, (**P*<0.01 control vs. PDGF; n=16)

4.7.2 TRANSFAC analysis of the hZfm1 short promotor

TRANSFAC analysis was performed in order to identify candidate transcription factors that may bind to the short promotor sequence of the hZfm1 gene. Several transcription factor binding sites for transcription factors (TFs) known to be activated by PDGF were identified (Fig. 21).

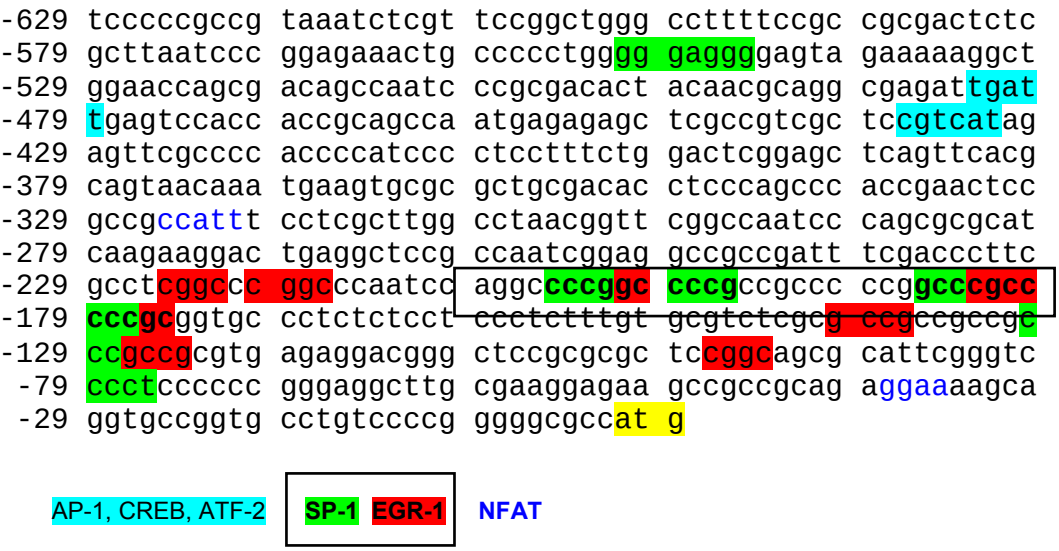


Figure 21. Putative TF binding site analysis of the hZfm1 short promotor by TRANSFAC. All binding sites for transcription factors known to be activated by PDGF are highlighted (http://www.Genomatix.de/shop/index.html). Three overlapping SP-1/ Egr-1 consensus sequences were revealed. The transcription start site is highlighted in yellow. Only conserved binding sites indicated in this figure were considered for further analysis (parameters: core and matrix similarities equaling 1.00 and 0.90, respectively).

4.8 Analysis of the Zfm1 promotor with decoy ODN

The transcription factors Egr-1, SP-1 and NFAT were chosen for further analysis. The following experiment was designed to identify which of these TFs is involved in PDGF inhibition of Zfm1 gene expression. Decoy oligonucleotides ((dODN) for a short discussion on the mechanism of dODN actions on target-TFs, see 5.4) against Egr-1, SP-1 and NFAT were designed and hybridised (see 3.2.8). AP-1 and ATF-2/CREB dODNs had been previously tested without significant effects (not shown). The hSMC were again transfected with the reporter gene construct. The respective dODNs (10 µmol/L) were added 12 h later. After another 4 h, the cells were stimulated with PDGF (60 u/ml) and luciferase activity analysed 48 h post PDGF exposure (Fig. 22).

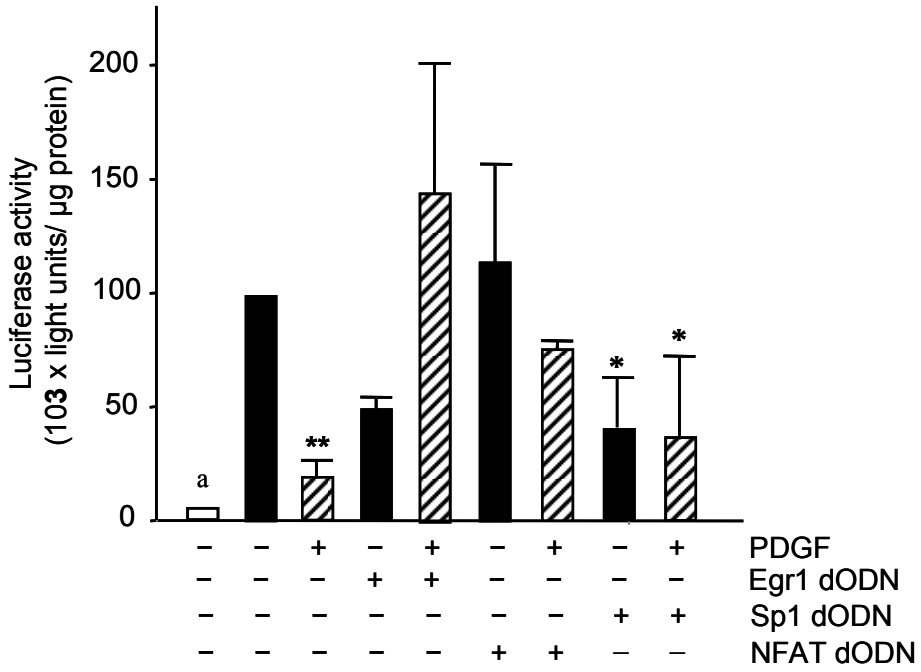


Figure 22. Effects of different dODNs on hZfm1 reporter gene activity in hSMC, in the absence and presence of PDGF (60 u/ml) over a period of 48 h. (***P*<0.005 vs. control,* *P*<0.05 vs. control; n=5; a: non-transfected negative control).

Data derived from these experiments suggested that SP-1 may be responsible for basal expression of Zfm1, while Egr-1 may be responsible for PDGF induced down-regulation of Zfm1 gene expression. In order to confirm whether dODNs targeting these two TFs exert similar effects on endogenous (i.e. genomic) Zfm1 gene expression, these were added to non-transfected hSMC, followed by PDGF stimulation for 48 h. Subsequent RT-PCR analyses (Fig. 23) revealed that addition of either dODN resulted in an increase in basal Zfm1 expression. This finding seems to contradict those obtained in the Zfm1 reporter gene assays (Fig. 22). Several reasons exist as to why apparently opposing results were obtained at the genomic level and these will be further discussed in chapter 5.4.

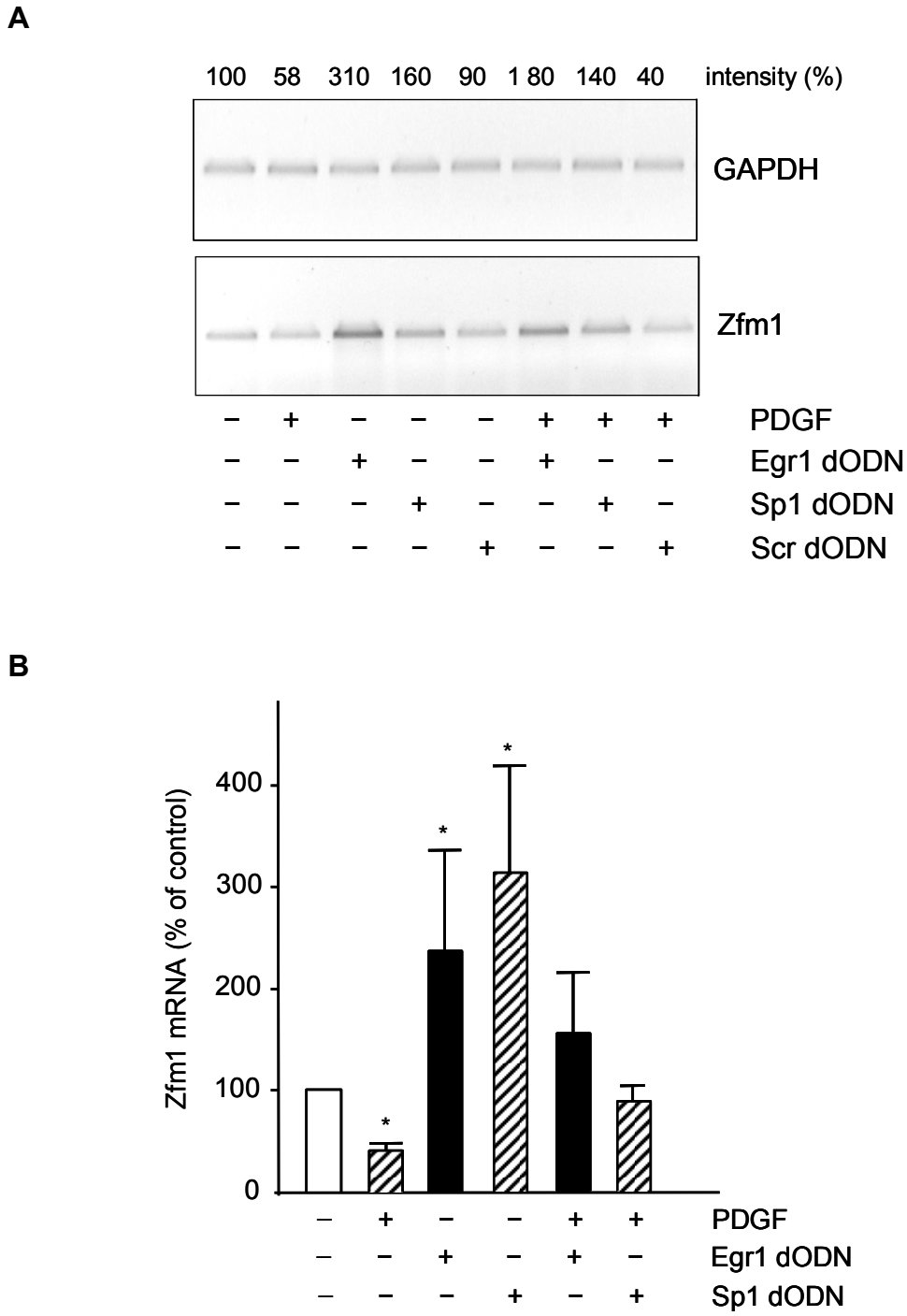


Figure 23. RT-PCR analysis of the effects of Egr-1 and SP-1 dODN on the expression of the endogenous Zfm1 gene. A) Exemplary RT-PCR analysis. **B)** Statistical summary). (**P*<0.05 vs. control; n=8.). A scrambled dODN (ScODN), as seen in A, 10 µmol/L) was used as a control and had no effect on Zfm1 expression (not shown

4.8.1 Deletion constructs of the Zfm1 reporter gene

In an attempt to characterise the role of Egr-1 and SP-1 in Zfm1 gene expression, Zfm1 reporter gene deletion constructs were generated using the Quik Change site-directed mutagenesis kit (Stratagene). The deletion constructs (Fig. 24) targeting the most probable putative Egr-1/SP-1 binding sites were labelled Δ1 and Δ2 respectively. For further details refer to Figs 3, 4 and 6 in the Annex.

```
Zfm1 mutation 1     CCCCGGTCCC GCCG------GCCCGCCCCCGCGGTGCCCTCT
Zfm1 WT promotor    CCCCGGTCCC GCCGCCCCCGGCCCGCCCCCGCGGTGCCCTCT
Zfm1 mutation 2     CCCCGGTCCC GCCGCCCCCGGCCCGC------GGTGCCCTCT
```

(Δ1 – 191 to − 186 bp and Δ2 −179 to −174 bp from the start site)

Figure 24. Sequence of the Zfm1 promotor with the missing Δ1 and Δ2 binding sites (missing basepairs are represented by dashes). The mutation was achieved by using the QuikChange Site Directed-Mutagenesis Kit using the Δ1 and Δ2 primers shown in Table 2.3 (WT: wild-type).

The deletion constructs were then transfected into hSMC which were stimulated for 48 h with PDGF 4 h post transfection (Fig. 25). The results indicate that both the Δ1 and the Δ2 sites are responsible for basal expression of Zfm1. After stimulation with PDGF, no further effect was seen with the Δ1 construct, suggesting that sensitivity to PDGF had been lost. On the other hand, this PDGF sensitivity seems to have been preserved in the Δ2 construct.

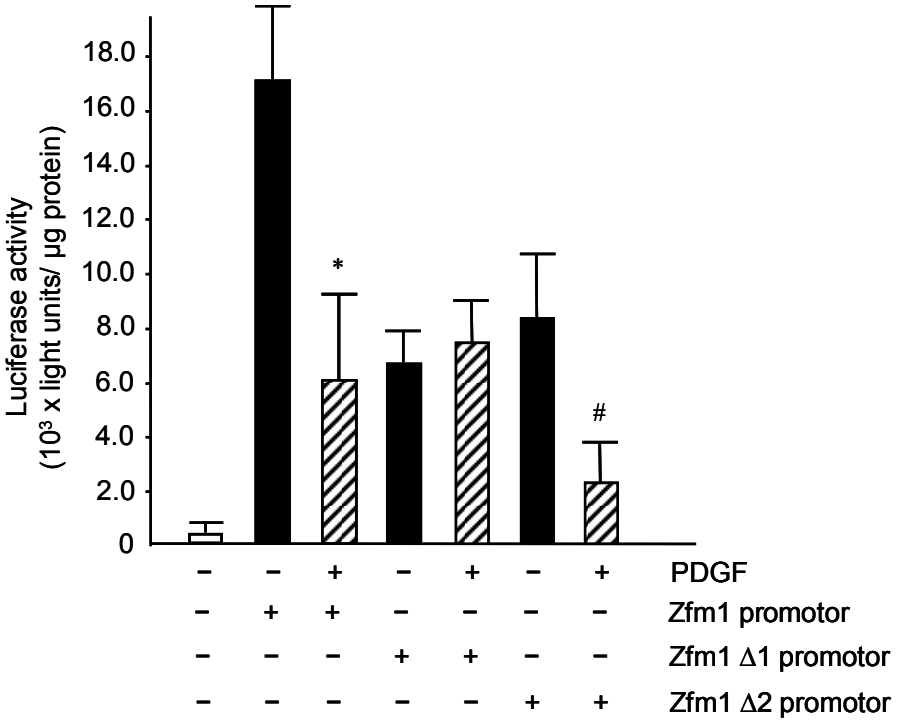


Figure 25. Effects of the Δ1 and Δ2 deletion on Zfm1 reporter gene activity in hSMC in the absence and presence of PDGF (60 u/ml, 48 h). (*P<0.01 vs. control; n=9. #P<0.05 vs. Δ2-control; n=12).

In order to confirm the data obtained with the single deletion constructs, a double mutant Zfm1 promotor construct was generated, which lacks both the Δ1 and the Δ2 motif (Fig. 26). The double mutant construct was created using the double mutant primers as outlined in Table 2.3 and the Quik Change™ site-directed mutagenesis kit (see Annex Fig. 8 for the full double deletion and wild-type sequence alignment). As before, the mutant constructs were then transfected into hSMC which were stimulated with PDGF for 48 h.

4.8.2 Zfm1 double deletion construct

Zfm1 double mutant CCCGGTCCCGCCG-----GGCCCGC------GGTGCCCTCTCTC
Zfm1 WT promoter CCCGGTCCCGCCGCCCCCGGCCCGCCCCCGCGGTGCCCTCTCTC

Figure 26. Sequence of the double mutant Zfm1 promotor luciferase reporter gene construct. The remaining base (highlighted in red) was unexpected from the mutagenesis strategy. Missing base pairs are represented as dashes.

Deleting both Δ1 and the Δ2 sites resulted in a similar basal reporter gene activity as seen in the wild-type construct (Fig. 27). This result is in contrast to what was expected, i.e that with the double mutant, both basal reporter gene activity and PDGF sensitivity are lost. Possible reasons for this rather curious finding are discussed in chapter 5.6.

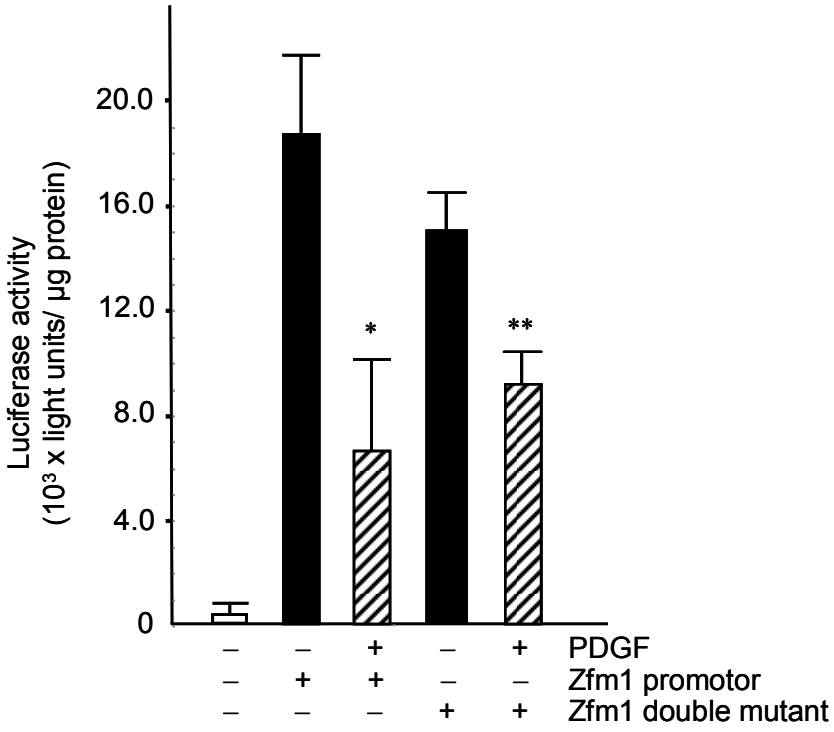


Figure 27. Luciferase reporter-gene analysis of the Zfm1 promotor double mutant construct. Cells were stimulated for 48 h with PDGF (60 u/ml) post-transfection. (**P*≤0.01 control vs. PDGF ; n=12 ; ***P*≤0.01 control vs. Double mutant + PDGF ; n=6).

4.9 Chromosome immuno-precipitation (ChIP) analysis

In order to define the TFs regulating basal expression and PDGF-induced down-regulation of the endogenous Zfm1 gene expression, ChIP analysis was performed with hSMC in the absence and presence of PDGF. ChIP analysis revealed that the combined Egr-1/SP-1 binding site at position approximately at -190 to -170 bp of the Zfm1 gene promotor (Fig. 21) was predominantly associated with SP-1 under basal conditions. In contrast, in response to PDGF stimulation SP-1 was effectively replaced by Egr-1 for this site (Fig. 28). This confirms that SP-1 maintains basal expression of Zfm1, whereas in the presence

of PDGF, Egr-1 is responsible for repressing Zfm1 gene expression. This will be further discussed in 5.7.

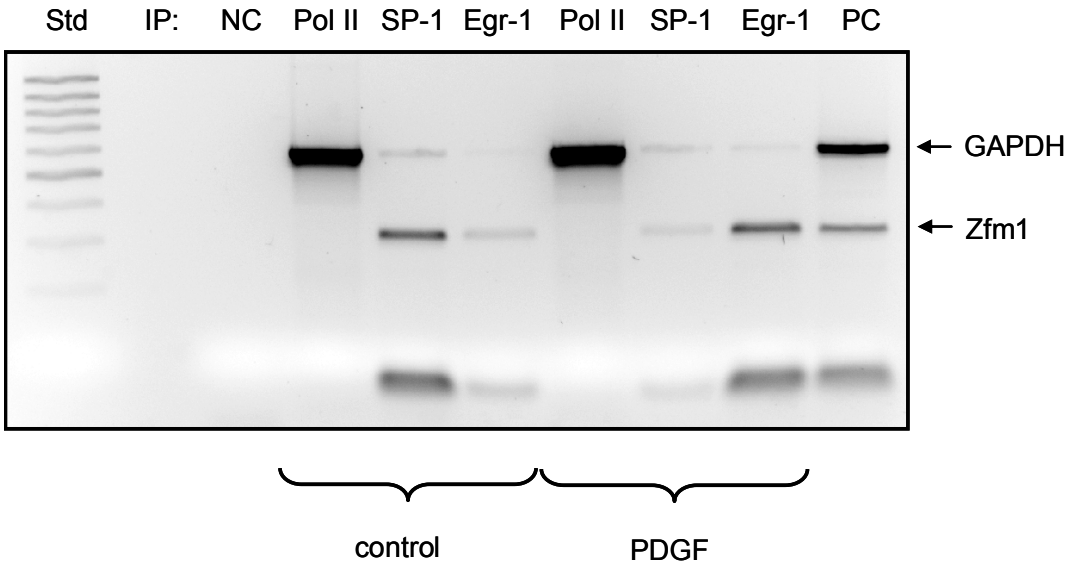


Figure 28. ChIP analysis of the Zfm1 gene promoter. Human SMC were incubated for 4 h in the presence or absence of PDGF (60 u/ml). RNA-polymerase II was immuno-precipitated and part of the *gapdh* gene promoter was amplified as a positive control for the ChIP procedure itself. **Egr-1**: Egr-1 precipitation with amplification of the Zfm1 gene promoter. **IP**: immuno-precipitation; **NC**: control without DNA load. **PC**: positive PCR control. **Pol II**: RNA-polymerase precipitation with amplification of the *gapdh* gene promoter. **SP-1**: SP-1 precipitation with amplification of the Zfm1 gene promoter; **Std**: DNA length standard.

4.10 Co-immunoprecipitation of Zfm1

Co-immunoprecipitation assays are employed to characterise protein-protein interactions of a specific protein of interest. In order to characterise new Zfm1-binding proteins and to deduce a functional role for Zfm1 in hSMC; co-immunoprecipitation of Zfm1 followed by immuno-blots using different antibodies was performed with cell lysates from hSMC. The Zfm1 antibody (No. 1676) that is able to detect both isoforms of Zfm1 was used for the assay, and Western blot analysis revealed that co-immunoprecipitation of Zfm1 from hSMC was successful (Fig. 29).

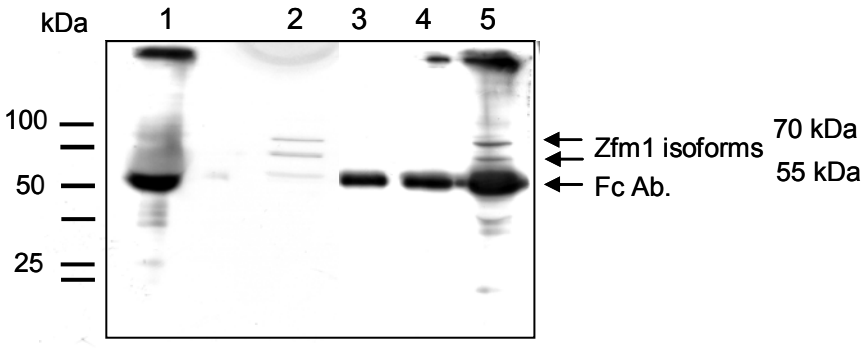


Figure 29. Western Blot analysis of the Zfm1 protein after co-immunoprecipitation. Lane 1 – Zfm1 serum antibody; lane 2 – hSMC cell lysate; lanes 3 and 4 – supertantants from the immunoprecipitation washes; **lane 5** – co-immunoprecipitated pellet

Western blot analysis was also performed on Zfm1 co-immunoprecipitated samples, using antibodies against protein components of the basal transcription factor TFIID (Fig. 30), namely $hTAF_{II}68$ and $hTAF_{II}80$ (a kind gift from Prof. Tora, Strasbourg, France). The data obtained confirm that $hTAF_{II}68$ does interact with Zfm1, as previously published (Zhang *et al.,*1998 (b)). The results presented also reveal that Zfm1 interacts with $hTAF_{II}80$.

A

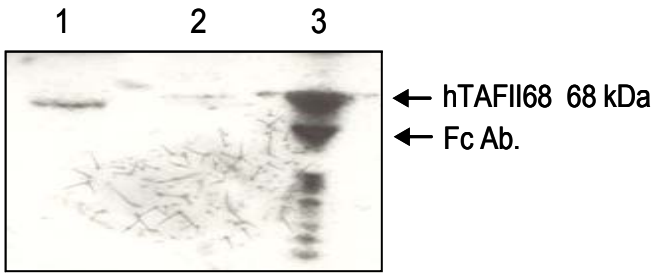


Figure 30. $hTAF_{II}68$ and $hTAF_{II}80$ interact with Zfm1, representative co-immunoprecipitation analyses. **A)** Lane 1- hSMC cell lysate; lanes 2 and 3 **–** supernatants from the immuno-precipitation washes; **lane** 4 – immuno-pellet.

B

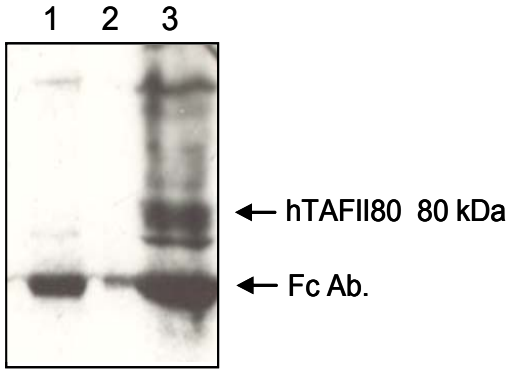


Figure 30. $hTAF_{II}68$ and $hTAF_{II}80$ interact with Zfm1, representative co-immunoprecipitation analyses. **B)** Lane 1**–** hSMC cell lysate with antibody; lane 2 – supernatant from the immuno-precipitation wash; Lane 3 – Co-immunoprecipitated pellet.

5. Discussion

During the last decade there has been increasing evidence ranging from *in vitro* experiments to pathological evidence that pro-inflammatory cytokines and growth factors such as IL-1β/TNFα and PDGF play major roles in the pathogenesis of atherosclerosis (Plutzky, 2001). Smooth muscle cell (SMC) proliferation induced by these factors is one of the major hallmarks in the pathophysiology of atherosclerosis whereby the SMC phenotype changes from a contractile phenotype, predominantly located in the media to a proliferative synthetic phenotype, present in the neo-intima. In rat, one gene important for preventing the development of a synthetic SMC phenotype might be Zfm1/Sf1. Thus, it has been shown that stimulation of rat SMC with pro-inflammatory cytokines leads to an increase in cell proliferation and an up-regulation of pro-inflammatory gene products such as VCAM-1 and MCP-1 that is paralleled by a decrease in Zfm1 gene expression (Cattaruzza *et al.*, 2002). Interestingly, even Zfm1 down-regulation alone without cytokine or growth factor stimulation led to an increase in cell proliferation and expression of VCAM-1. Thus, it seems that Zfm1/Sf1 plays an important role in maintaining the phenotype in non-proliferating rat SMC by acting as a 'repressor' of pro-inflammatory and growth-promoting gene products.

As outlined above, a better understanding of the regulatory mechanisms that govern the remodeling process is crucial for developing new strategies to prevent atherosclerosis and hence the majority of cardiovascular disease. One purpose of this present work thereafter was to investigate whether Zfm1/Sf1 plays a role (if any) also in human SMC proliferation. Moreover, the human Zfm1 promotor was cloned for the first time in order to understand how pro-inflammatory cytokines and growth factors affect Zfm1 gene expression.

5.1 Zfm1 antibodies and Zfm1 siRNA – the development of experimental tools.

Antibodies against Zfm1 were not available, and thus had to be generated in two New Zealand white rabbits by using the peptide MATGAMATPLDFPSC (previously published by Guth *et al.*, 2000). The Zfm1 anti-sera were required for Zfm1 protein expression analysis. The quality of each anti-

serum was tested and confirmed that anti-serum 1676 is able to detect both Zfm1 isoforms A (70 kDa) and E (55 kDa), whilst anti-serum 1675 only detected isoform E. Thus, only anti-serum 1676 was chosen for all subsequent analysis.

RNA interference (RNAi) is an evolutionary conserved cellular defense mechanism for controlling the gene expression of foreign (e.g., viral) genes, occurring in most eukaryotes including humans (Tuschl *et al.*, 2002; Hammond, 2005). The Zfm1 siRNA was tested for its efficiency to knock down the Zfm1 gene at the protein and mRNA level. Although basal Zfm1 expression was not completely suppressed (approximately to 35% of control), this was sufficient to analyse the function of Zfm1 e.g in SMC proliferation.

5.2 Zfm1 is involved in the repression of cell proliferation in hSMC

Stimulating rat SMC with pro-inflammatory cytokines and PDGF led to an increase in cell proliferation and an up-regulation of pro-inflammatory gene products (Cattaruzza *et al.,* 2002), and this effect was partially dependent o a down-regulation of Zfm1 gene expression. Whether or not Zfm1 plays a similar role in human SMC, and if Zfm1 could be a potential target for maintaining the SMC contractile phenotype under pro-atherosclerotic conditions, was elucidated by the analysis of pro-inflammatory gene expression and cell proliferation assays.

Whereas pro-inflammatory gene expression (the cell adhesion molecule VCAM-1 was chosen as a read out) was not dependent on Zfm1 expression, cell proliferation assays revealed that in contrast to rat SMC, in human SMC IL-1β/TNFα alone is not sufficient to induce cell proliferation but requires the knock down of Zfm1 expression by pretreatment with a Zfm1-specific siRNA. Thus, Zfm1 appears to be a repressor of IL-1β/TNFα mediated proliferation also in human SMC. Stimulation of these cells with PDGF on the other hand, induced a maximum increase in cell growth, paralleled by a PDGF-induced down-regulation of Zfm1 expression. This suggests that suppression of Zfm1 in fact is crucial for the growth-promoting effects of PDGF in hSMC as will be discussed below.

One logical approach to confirm this role of Zfm1 would be to over-express the protein by transfecting a gene construct with a strong and PDGF-independent gene promoter into primary hSMC. One anticipated result of such an experiment may be a decreased proliferative response to PDGF in transfected hSMC. Regrettably, it was not feasible to perform such an experiment, since primary cells are highly sensitive to culture conditions and the harsh transfection conditions would most probably spoil all consecutive analyses on hSMC proliferation. The use of stable cell lines for such an assay also proves to be unfavourable, because of their stable proliferation rate, possibly leading to invalid growth effects. But how could Zfm1 potentially inhibit PDGF-induced proliferation as suggested by the data? Besides playing a role in mRNA splicing, Zfm1 is known to act as a transcriptional repressor by interacting with $hTAF_{II}68$/EWS/TLS TFIID complexes (Zhang *et al,.* 1998 (b)).

It is important to consider that PDGF induces proliferation via *de novo* expression of growth-promoting gene products, e.g., the up-regulation of amino acid transporters in order to fulfill the metabolic requirements of cell growth (Durante *et al.,* 1996). Taking into account that the association of Zfm1 with $hTAF_{II}68$ and homologous factors in active transcriptional complexes has been implicated in cell proliferation (Zhang *et al.,* 1998 (b)) and cancer progression (Ranallo *et al.,* 1999), it is conceivable that PDGF-induced cell proliferation may require the down-regulation of Zfm1. Zfm1 may act as a repressor of growth-promoting gene transcription by sustaining the transcriptome architecture, thus preserving the non-proliferative hSMC phenotype. As previously stated, Zhang et al. also revealed that Zfm1 repression of the SSAP transcription factor occurs through its Gal4-GQC interaction region, which recognizes sequences rich in glutamine-glycine/ serine-threonine. Interestingly, $hTAF_{II}68$/EWS/TLS binds RNA polymerase II via sequences rich in glycine, arginine and proline repeats. Thus, the GQC-interaction domain of Zfm1 may repress $hTAF_{II}68$/EWS/TLS mediated gene transcription by interacting with the glycine repeats in RNA polymerase II. This possibility has however not been tested here. Nonetheless, taking into account these ideas as to how Zfm1 may maintain the non-proliferative hSMC phenotype at the level of transcriptional, and the fact that PDGF effectively represses Zfm1 gene expression, it became of interest to deduce

the mechanism underlying the latter effect. To this end, the Zfm1 gene promoter was cloned and different promoter-reporter gene constructs were generated.

5.3 Cloning of the human Zfm1 promotor

Luciferase reporter gene analysis proves to be a useful method in studying how a specific romoter of interest is regulated. The romoter of interest is cloned and situated in front of a gene encoding a reporter protein. Here the gene encoding the light-generating enzyme luciferase was used (Wood *et al.,* 1984). Activation of the romoter induces luciferase gene expression and translation into the active enzyme. Thus, in this assay system romoter activity is proportional to the level of light generated from luciferin. Therefore, in order to understand PDGF induced down regulation of the Zfm1 gene, parts of the human Zfm1 promotor were cloned into a luciferase reporter gene construct.

However, no detailed information was available on the sequence of the human Zfm1 gene romoter. Our literature analysis revealed that approximately 90% of the romoter contains CpG islands. CpG islands are target motifs for DNA methylation and represent approximately 1% of the human genomic DNA. Generally they are found in the romoter region of house keeping genes (Caiafa *et al.,* 2005). Cloning of the hZfm1 promotor reported here posed a number of problems. Addition of organic additives such as dimethyl sulfoxide (DMSO) to the reaction mixture has been previously used to optimize amplification, but results can be unpredictable (Hube *et al.,* 2005*).* More than 30 different primer pairs, along with differing concentrations, annealing temperatures and cycles were used in a futile attempt to directly amplify the romoter from a human genomic DNA template, but only random annealing of the primers and random amplification was achieved.

Therefore, the alternative selective strategy of isolating the romoter from a chromosome 11 BAC clone was elected. For this purpose, a special screening procedure was employed starting from a primary BAC clone pool containing 4,608 single clones. This approach proved successful, and the

Zfm1 promotor was cloned into two separate fragments of approximately 860 and 670 bp in length. Although it was hoped to amplify a longer 1500-bp fragment of the romoter, this was impossible due to the high redundancy of the GC-rich romoter sequence.

Both large and short romoter fragments of the Zfm1 gene were cloned separately into the pGL3 basic vector. The short fragment composing of approximately 670 bps was chosen for analysis since important transcription factors are more likely to bind within this region downstream of the start site. Moreover, the Zfm1 gene is situated close to the locus of the MEN-1 gene, which is associated with an inherited form of endocrine neoplasia (Toda *et al.*, 1994). The start sites of both genes are approximately 1500 bp apart. Choosing to analyse a larger promoter fragment (spanning -670 up to -1530 bp from the start site, cloned alternatively) may have posed problems due to overlapping promoter sequences with the MEN 1 gene. Thus, it made sense to analyse the short Zfm1 promotor fragment first.

5.4 Analysis of the human Zfm1 promotor

As anticipated, the 670 bp Zfm1 promotor reporter gene construct worked well with a clearly detectable enzyme activity in hSMC. Moreover, exposure of the transfected hSMC with PDGF resulted in a decrease in luciferase activity, further confirming that the Zfm1 promotor reporter-gene construct is fully operational. Therefore, in order to deduce which transcription factors might be involved in PDGF-induced down-regulation of Zfm1 expression, two strategies were employed: The decoy ODN approach and the generation of Zfm1 promotor deletion constructs.

Decoy ODN (dODN) provide a powerful experimental tool to target transcription factors. These short double-stranded DNA molecules usually mimic the consensus binding site of their target transcription factor, thereby effectively neutralising the protein following uptake into the target cell. As a consequence, gene expression controlled by this transcription factor is effectively blocked. Therefore, the dODN technique can also be considered as a gene silencing approach (or in the case of the Zfm1 gene, maintaining gene expression), which is less specific than the siRNA or anti-sense ODN

approach. However, dODN are highly specific for their target transcription factor.

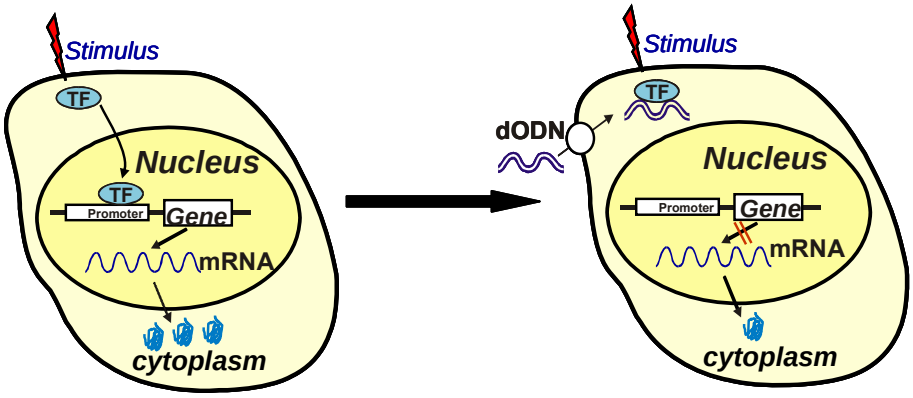


Figure 31. The gene silencing action of dODN. (adopted from Prof. M. Hecker, 2006). Decoy ODN bind to a specific transcription factor (TF), preventing the TF to interact with its regulatory binding site in the genome, thereby silencing gene expression.

5.5 Decoy ODN characterization of the Zfm1 promotor

First a TRANSFAC analysis of the Zfm1 short promoter was performed in order to identify possible transcription factors that could be involved in PDGF down-regulation of the Zfm1 gene. TFs known to be activated by PDGF are Egr-1, SP-1, NFAT, ATF-2, and AP-1 (Fos/ Jun heterodimers), and binding sites for all these transcription factors were found in the 630 bp promoter construct.

Egr-1 binds to the promoters of many genes involved in pro-artherogenic processes such as SMC proliferation and migration, and target genes activated by Egr-1 include PDGF-A and B (Kamimura *et al.,* 2004). Moreover, Zfm1 contains a region homologous to the WT1 gene, and WT1 is known to function as a repressor when bound by Egr-1 (Wang *et al.*, 1992). SP-1 on the other hand is a member of a family of zinc finger transcription factors that have highly conserved DNA binding domains which recognise GC boxes (GGGGCGGGC) and GT motifs (GGTTGTGGC) (Hagen *et al.,* 1994). Finally, NFAT is a transcription factor initially identified in T-cells, but has been shown to regulate other genes related to cell cycle progression, differentiation and apoptosis (Viola, *et al.*, 2005).

Since in previous experiments, dODN against AP-1 and ATF-2 were without effect on Zfm1 gene expression (data not shown), Egr-1, SP-1 and NFAT as the remaining candidates, were chosen for further analysis. Zfm1 promotor reporter-gene construct analysis in hSMC pre-treated with Egr-1, SP-1 or NFAT dODNs without PDGF stimulation revealed that Egr-1 and SP-1, but not NFAT may be important in maintaining basal expression of Zfm1. Furthermore, after stimulation with PDGF, it was confirmed that Egr-1 is the transcription factor responsible for PDGF induced down-regulation of Zfm1, whilst SP-1 is the transcription factor responsible for maintaining basal Zfm1 expression.

Interestingly, it has been shown that PDGF induces Egr-1 expression in bovine aortic SMC (BASMC), and that increased activity of Egr-1 leads to the displacement of SP-1 at a site 55 and 71 bp upstream of the PDGF-A chain promotor start site (Silverman *et al*., 1997). It was also confirmed that SP-1 is the transcription factor responsible for maintaining basal expression of the PDGF-A chain gene. Since PDGF is known to activate Egr-1, the question arose as to whether a similar mechanism could operate with respect to PDGF induced down-regulation of the Zfm1 gene. This was the more suggestive as the combined Egr-1/SP-1 binding site found approximately at position -191 to -174 in the Zfm1 gene promotor was virtually identical to the binding site functionally characterised by Silverman *et al.* in the PDGF-A gene promotor (Fig. 32). Therefore these binding sites were chosen for further analysis in the Zfm1 promotor reporter-gene construct.

```
Egr-1 con    gcgggggcga-gcgggggc
WT rev -191 AGAGAGGGCACCGCGGGGGCGGGCCGGGGGCGGCGGGA
PDGF-A - 71 GGGGGGGGGGGGGCGGGGGCGGGGGCGGGGGAGGGGCG
SP-1 con                 cgggg-cggggcgagc
```

Figure 32. Alignment of the Zfm1 wild type promotor with the PDGF-A Egr-1/SP-1 responsive promotor element (derived from Silverman et al., 1997) and Egr1/SP1 consensus sites. The highlighted bases in yellow represent homologous bases between the sequences.

5.6 Transcription factor binding site characterisation using Zfm1 promotor deletion construct.

In order to define the binding sites responsible for basal and PDGF-induced down-regulation of Zfm1 expression, the homologous putatitive Egr-1/SP-1 binding sites (shown in Fig. 32) were deleted in the Zfm1 promotor reporter-gene construct. These were labelled Δ1 and Δ2, respectively. Stimulation of hSMC transfected with the Δ1 or Δ2 construct with PDGF revealed that both sites are important for maintaining basal Zfm1 expression. However, loss of sensitivity to PDGF-induced down-regulation was observed only with the Δ1 construct. Thus, the Δ1 site seems to be important for the effects of PDGF on Zfm1 gene expression.

Considering that both Δ1 and Δ2 sites are important for maintaining the basal expression of Zfm1, it was speculated as to whether deleting both sites would result in almost complete knock-down of Zfm1 expression. In order to test this hypothesis, a double deletion Zfm1 promotor construct was created. Human SMC transfected with the double deletion construct and stimulated with PDGF revealed a restoration of basal Zfm1 promotor activity. This result was opposite to what was anticipated since both sites seemed to be important for maintaining basal Zfm1 promotor activity.

Taking into consideration the GC-rich promoter sequence, it is possible that a new artificial Egr1/SP1 binding site was created during the mutagenesis procedure. In order to assess the likelihood of this to have occurred, an alignment between the combined Egr-1/SP-1 consensus site, and the Zfm1 double deletion construct was made (Fig. 33).

```
EGR-1 CON:    GGATCCAGCGGGGGCGAGCGGGGGCGA
DM rev        CGCGGGCCCGGCGG-GACCGGGGCCTGGATT

SP-1 CON:       ATTCGATCGGGGCGGGGCGAGC
DMrev           CCTGGATTGGGCCGGGCCC
```

Figure 33. Alignment between the Zfm1 double mutant construct and recognition sites for Egr-1 and SP-1. Yellow highlighted bases represent homologous sites between the double mutant and the Egr-1 consensus site. Blue highlighted bases represent homologous sites between the SP-1 consensus sites.

The alignment revealed that a GC-rich motif highly homologous to both the Egr-1 and SP-1 consensus sites is present in the double mutant of Zfm1 promotor, suggesting that a new artificial and overlapping binding motif for Egr-1 and SP-1 was created. It is therefore feasible that indeed by chance a functionally similar binding motif was created by the double deletion.

5.7 Analysis of genomic Zfm1 gene expression

As previously stated, the Egr-1 and SP-1 dODNs down-regulate Zfm1 promotor reporter gene activity at the basal level. In order to test whether these dODNshave a similar effect at the genomic level, hSMC pre-treated with Egr-1 or SP-1 dODN were incubated in the absence and presence of PDGF. The genomic Zfm1 expression was then analysed by RT-PCR.

In contrast to the Zfm1 promotor construct, genomic Zfm1 expression analysis revealed that addition of either Egr-1 or SP-1 dODN leads to an increase in basal Zfm1 expression. The apparent discrepancy between genomic and plasmid gene expression data is conceivable since dODNs are not fully selective for transcription factors that recognise similar or almost identical binding sites. Moreover, since Egr-1 and SP-1 recognise highly homologous motifs, this suggests that overlapping effects between the Egr-1 and SP-1 dODN were observed, evidently leading to effective inactivation of SP-1 at the basal level.

Another reason to consider is the fact that only the 670 bp long Zfm1 promotor fragment was used for analysis. The endogenous gene would be regulated by a full length romoter that may contain additional regulatory elements, which could account for the increase in basal expression of the endogenous gene. As previously stated, analysis with the 860 bp long romoter fragment was not feasible, as this most likely contains nucleotides overlapping with the MEN 1 gene romoter. However as discussed above, the construct was fully sensitive to PDGF, suggesting that the romoter construct analysed was indeed fully functional.

A comparison between the amplified romoter sequence and the wild-type sequence deposited in GenBank (see Annex Fig. 1) revealed two nucleotide mismatches not present in any known binding sites. The GC-rich sequence of the romoter may account for the minor sequence mismatches that exist

which also posed problems in the PCR amplification. However, these mismatches are unlikely to be responsible for the differences in basal expression between the genomic Zfm1 gene and the plasmid construct as they do not affect any known transcription factor binding site.

The most likely reason for this experimental discrepancy is that previously published electro-mobility shift assay (EMSA) data by Silverman *et al.* revealed that Egr-1 is present and active in the nucleus of SMC also under basal conditions. Considering that SP-1 apparently maintains basal expression of Zfm1, and that Egr-1 and SP-1 recognise overlapping binding sites, it is tempting to speculate that the low basal activity of Egr-1 is in balance with the higher basal activity of SP-1, resulting in a relatively high, but not maximal endogenous Zfm1 expression. Inhibiting the low Egr-1 activity with a dODN may be sufficient to fully shift the balance towards SP-1, so that SP-1 is able to mediate maximal Zfm1 expression under such experimental conditions. In the case of the reporter gene assay, however, a significantly increased number of nuclear Zfm1 promotor-like binding sites are present relative to the endogenous sites. Therefore, the low basal activity of Egr-1 may not suffice to decrease SP-1-mediated basal luciferase expression. In this situation a dODN (not fully selective for SP-1 and Egr-1 as both factors have similar binding sites, see above), will not lead to a noticeable Egr-1 inhibition, but rather a significant inhibition of SP-1, thereby in fact decreasing the activity of the multiple constructs in the transfected cell. In conclusion, although the romoter construct is probably fully functional, the copy number of Egr-1/SP-1 binding sites per cell may result in such a mere discrepancy between the genomic and plasmid-driven gene expression.

Although suggestive, the data discussed so far do not show a real binding of SP-1 or Egr-1 to the Zfm1 gene romoter. One method to confirm such specific protein-DNA interactions is through chromosome immunoprecipitation (ChIP). In order to verify that Egr-1 and SP-1 are the transcription factors involved in regulation of the Zfm1 gene, and that the same mechanism of gene-regulation proposed by Silverman *et al.* holds true for the Zfm1 promotor, with ChIP analyses were performed on hSMC in the absence or presence of PDGF.

The analysis revealed that in the absence of PDGF, SP-1 predominantly binds to the Zfm1 promotor at an Egr-1/SP-1 recognition motif situated approximately 200 bp upstream of the start site. In the presence of PDGF however, Egr-1 is predominantly bound to the same motif whilst SP-1 interaction is lost. The ChIP analysis thus confirmed our model of how PDGF induces down-regulation of Zfm1 expression through Egr-1 displacement of SP-1 (Fig. 34).

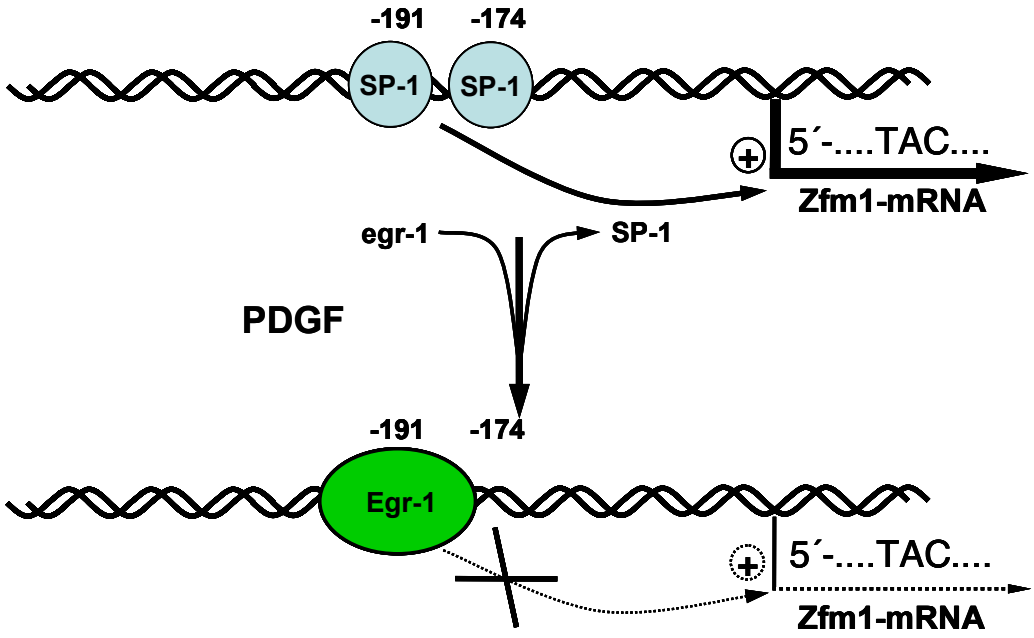


Figure 34. Model mechanism of PDGF induced down-regulation of the human Zfm1 gene. Under basal conditions SP-1 is the transcription factor maintaining Zfm1 gene expression. In the presence of PDGF, Egr-1 activity is up-regulated, and displaces SP-1 from its binding site, thereby inhibiting Zfm1 gene transcription.

In conclusion, Egr-1 induces transcription of growth-promoting genes (Santiago *et al.,* 1999) and also plays a role in repressing Zfm1 gene expression. Silverman *et al*. have suggested a similar mechanism to induce PDGF-A expression. This is the first experimental proof that PDGF, through the displacement of SP-1 by Egr-1, elicits down-regulation of the human Zfm1 gene, hence relieving its repressing effects on the expression.

5.8 Possible functional roles of Zfm1

As stated in the aims, it was hoped to elucidate Zfm1 protein-protein interactions by investigating whether Zfm1 interacts with other distinct TAF_{II}s known to play a role in directing the formation of a functional phenotypic 'transcriptome'/TFIID complex. Due to time constraints however, only experiments to analyse Zfm1-TAF interactions could be performed revealing that Zfm1 interacts with $hTAF_{II}80$. As previously demonstrated by Zhang *et al.,* Zfm1 interacts with $hTAF_{II}68$/EWS/TLS, suggesting that Zfm1 plays a regulatory role in the expression of genes controlled by these factors. Interaction with $hTAF_{II}68$ was also confirmed. Taking into account this finding, it may be feasible to conclude that Zfm1 possibly acts as a repressor of $hTAF_{II}68$/EWS/TLS TFIID complexes.

A new isoform of $hTAF_{II}80$ termed '$hTAF_{II}80\delta$' was recently reported by Bell *et al.* to form a specific TFIID complex that lacks $hTAF_{II}31$ and is sufficient to trigger induction of its target genes gadd45 and p21, both of which are pro-apoptotic. Thus, both a growth-promoting ($hTAF_{II}68$) and a pro-apoptotic TAF ($hTAF_{II}80$) may interact with Zfm1; indeed suggesting that Zfm1 can repress both processes alike and therefore, is a key factor in regulating the cellular response to PDGF.

5.9 A role of Zfm1 in atherosclerosis? An outlook.

Taking into account the findings presented here, the role of Zfm1 in atherosclerosis still remains incompletely understood, though the data strongly suggest that one main function of Zfm1 lies in maintaining SMC in an anti-proliferative state. Considering the pathogenesis of atherosclerosis, it may be an attractive concept to prolong Zfm1 gene expression during the early stages of disease progression, where SMC proliferation and migration contributes greatly to the formation of the neo-intima. SMC at this stage are in the synthetic proliferative state, and are highly susceptible to respond to pro-inflammatory cytokines and growth factors. Therapeutically maintaining the expression of Zfm1 at this stage may efficiently inhibit SMC cell proliferation, evidently slowing down the progression of plaque and neo-intima formation, and preserving the contractile, non-proliferative SMC phenotype. Nevertheless, the late stages of atherosclerosis are

characterised by vulnerable fibrous plaques, where matrix metalloproteinase's (MMPs) derived from SMC and various leukocytes, are naturally up-regulated by pro-inflammatory cytokines and growth factors. Eventually these MMPs degrade the ECM components of the fibrous cap in advanced lesions, causing the cap to thin and rupture, resulting in thrombosis and most often ischemia. At this point, it may be favourable to decrease Zfm1 expression in vascular SMC to promote a synthetic proliferative phenotype, in order to induce an increase in ECM formation and thus, to protect the vulnerable fibrous cap from degradation and rupture.

At this stage, it is important to increase our understanding of Zfm1 at the functional level in order to acquire an appreciation of its role in the phenotypic regulation of hSMC. To enhance our understanding of the functional role that Zfm1 plays in SMC, it would be important to clearly define proteins that interact directly with Zfm1 in the absence or presence of pro-inflammatory or growth promoting stimuli and to analyse their function in the course of atherogenic SMC proliferation.

6. Summary

The first aim of the present study was to elucidate whether IL-1β/TNFα and/or PDGF down-regulate Zfm1 gene expression and induce proliferation also in human SMC. The results revealed that IL-1β/TNFα alone is not sufficient to induce cell proliferation, but only after down-regulation of the Zfm1 gene through siRNA treatment proliferation was increased. In contrast, PDGF alone maximally induced cell proliferation. Thus, it was investigated whether PDGF directly down-regulates the endogenous Zfm1 gene in order to induce cell proliferation. The findings presented here revealed that PDGF does down-regulate endogenous Zfm1 gene expression, suggesting that this decrease in Zfm1 abundance may be important to allow PDGF induced growth-promoting gene transcription.

In order to understand the mechanism of PDGF induced down-regulation of Zfm1 expression in hSMC, the promotor of the human gene was cloned for the first time. Zfm1 promotor reporter gene analysis confirmed that PDGF down-regulates Zfm1 gene expression in hSMC.

Experiments employing decoy ODN directed against the transcription factors Egr-1 and SP-1 revealed that both factors are involved in the regulation of the Zfm1 gene; while Egr-1 is the transcription factor responsible for PDGF-induced down-regulation of Zfm1, SP-1 is important for maintaining basal expression of Zfm1. In order to define the exact binding sites and transcription factors important for basal expression and PDGF-induced down-regulation of the Zfm1 gene, deletion constructs of a combined Egr-1/SP-1 consensus site (situated approximately 200 bp upstream of the transcriptional start site) of the Zfm1 promotor were generated. Together with a paralleled chromosome immunoprecipitation analysis, data obtained confirmed that under basal conditions, SP-1 predominantly interacts with the complex binding site located at position -191 to -174 of the Zfm1 gene and maintains the basal expression of Zfm1. Interestingly, under PDGF stimulation, Egr-1 activity was up-regulated, whilst SP-1 activity was decreased, confirming that under PDGF stimulation, Egr-1 displaces SP-1 from the binding site.

Finally, analysis of Zfm1 protein-protein interactions through co-immunoprecipitation confirmed that Zfm1 interacts with both $hTAF_{II}68$ and

$hTAF_{II}80$, suggesting that Zfm1 plays a role as a regulator of SMC phenotype at the transcriptional level.

In summary, these findings reveal that Zfm1 is a repressor of growth promoting gene transcription possibly through modulating the 'transcriptome' architecture via specific Zfm1-TAF interactions. Zfm1 expression is down-regulated by PDGF through the displacement of SP-1 by Egr-1. A deepened understanding of SMC proliferation in atherosclerosis and the role of Zfm1 therein, may lead to new therapeutic strategies involving the stabilization of a contractile and non-proliferating SMC phenotype, in order to prevent or slow down neo-intima formation in the course of the disease.

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Appendix

Figure A1.

Sequence of *Homo sapiens* gene encoding splicing factor 1/ Zfm1, exon 1, as published in GenBank. Accession number AJ000051.

```
1    agatgcaggc gggccaggct ggtctcgaac tcctgacctc aggtgatcca cccacctcgg
61   cctcccaaag tgttggagcc accgcgcctg gatggagatt aagtttctaa cacatgagct
121  ttgggggaca cattcaaacc tgtccaccta aagtaatcaa aagggtcaga atctaatttc
181  aatagtttat tcaagcacaa aacatgaggg tggactacct ggaaacaccg actccaaatg
241  aatggagtca gtgtttcgaa gtagagaagt taaggtttca tttacacaca caaaggcaga
301  ggagttttag gattacattc ttcatatatg gtcagtgcgt acttacagta atttggttat
361  aggccgagtg cagtggtcca ctcctgtaat cccagcactt tgggaggccg aggcgggcag
421  atcacctgag gtcaggagtt caagaccagc ctggccaaca tggtgaaatt ccatctttac
481  aaaaatgtaa caattagctg ggcatgatgg caggtggctg taatcccggc tactcaggag
541  gctgaggtgg gagaatcgct tgaatctggg aggcagaggg tgcagtgagc cgagattgtg
601  ccattgcact ccagcctggg cgacagagcc agactctgtc tcaaattgaa ataataataa
661  taaataattt ggttgtaagc agtgtttctt tttgggaggc atacatttaa catttttac
721  agagggtgta atagtcatgg gtttgctgtc atctggtcta agtaaagcag gacaacaaaa
781  gggaagttat ctataagaag ggtcattaag aaggcagcag gtttttgtcc catgtttaat
841  tctctccaat aattgtacag aacaagaaaa acaagcaggt ctaatttatt atctcagaaa
901  aaattataac catacatggc tgagatcaca gtcacctctc tctccaggtt tcaagtgttg
961  tgggggttcc aacatctttt ctatttattt taaaaaacca cagcagaggg gttgtgcacc
1021 ctttcatatt tcttggctat ttggatttgt tcttctgaga catgtccatt cctatttttt
1081 gctcattttc tattgttttt gccttttttc taattagttt ttaagatgta tttgtatatt
1141 acaggtattg gctctttatc atgtatggca aatatttttt cctcatctat cattgtctct
1201 tgacttttat cagtgttaat tttcggggta atcaagtata tctttttaca gtttgaggtt
1261 cctgtctttg cagtctctca acccctagaa taccttata gtctcctaaa ttatattcta
1321 aatgttttgt tgtttaaatt taagattcta cgccatttat caacgaaatc atcttttcct
1381 gattgaaata aaatactaca tttgtggtat ttccggattc tgttgcacta atgtgtcttt
1441 cctaccaaca atatcatatt gttttattat tgtggttttt tgtttttgag acggagtctc
1501 atgctgttgc ccaggctgga gtacagtggt gcgatctcag ctcactacac ctccgcctcc
1561 tgggttcaag caattctcct gcctcagcct cctgagaagc tgggattata ggcgtctgcc
1621 accacgctcg gctaattttt gtacttttag tagagacggg ggtttcaccg tgttggtcag
1681 gttggtctcg aactcctgac ctcaggtgat ccccccgcct tggcctccca gtgccttggc
1741 actggcctat tgtgttttat atactatgtt tgtcatctgg aaaggcaagc tcctgctccg
1801 tcttgtaaag ttttcccagt actgtagtag ttatttttat ttttattttt gacagtctcg
1861 ctctgtcacc caggctgaag tgcagtggcc ccatctcggc tcactgcaac ctccgcctcc
1921 cgggttcaag cgattctctt gcctcatcct cctgggaagc tgggattgca ggagtgcacc
1981 actacaccca actaattttt gtatttttcg tagagacggg gtttcgccat gttggtcagg
2041 ctggtctcga ccttctgacc ttgtgattcg cccgcctcgg cctgtcaaag tgctgggatt
2101 acaggcgtga gccaccgcgc ccggccgtag ttattgtttt atacgatttt aagatcattt
2161 aattaaatcc tctgaactcc gtggagattc caatagaagt gtgtatttgt aatttttgaa
2221 ctgacaacct tctgttacct ttcatctaat atgttgttgc atcagttcaa atactgtttt
2281 aaatccttcg ataagtcgtc tctattcgta gctttttggg tcacttttt ttttttttt
2341 tttttccaaa aaaacaggaa gtctttttac ctgcagcgtc aactgagccg gttttccggc
2401 gcgcgccagc ccctcccctg ctcccgcccc aaggttctgc gttaccatgg gaacaaaggt
2461 tgacgtcact tagggcttcc ttgctggacc caagggtcga tggtgaaggg agacgggtgg
2521 agaggcggtt ttgctccatc tacgcatgcg cacttcctgc tccaggactc ccccgccgta
2581 aatctcgttc cggctgggcc ttttccgccg cgactctcgc ttaatcccgg agaaactgcc
2641 ccctggggga ggggagtaga aaaaggctgg aaccagcgac agccaatccc gcgacactac
2701 aacgcaggcg agattgattg agtccaccac cgcagccaat gagagagctc gccgtcgctc
2761 cgtcatagag ttcgccccac cccatcccct cctttctgga ctcggagctc agttcacgca
2821 gtaacaaatg aagtgcgcgc tgcgacacct cccagcccac cgaactccgc cgccatttcc
2881 tcgcttggcc taacggttcg gccaatccca gcgcgcatca agaaggactg aggctccgcc
2941 aatcggaggc cgccgatttc gacccttcgc ctcggcccgg cccaatccag gccccggccc
3001 cgccgccccc ggcccgcccc cgcggtgccc tctctcctcc ctctttgtgc gtctcgcgcc
3061 gccgccgccc gccgcgtgag aggacgggct ccgcgcgctc cggcagcgca ttcgggtccc
3121 ctcccccggg gaggcttgcg aaggagaagc cgccgcagag gaaaagcagg tgccggtgcc
3181 tgtccccggg ggcgccatgg cgaccggagc gaacgccacg ccgttgggta agctgggccc
     ccccgggctg cccccgctcc ccgggcccaa aggaggcttc gagccgggcc ctccgcctgc
     acccgggcct ggggcggggc tgctggctcc cgggccgccg ccgcccccgc ccgtgggctc
     gatgggggcc ctgaccgcgg ccttcccctt cgcggcgctg cctccgccgc ctccgccgcc
     gccccctccg cctccccagc agccgccgcc gcctccaccg ccaccgtccc ccggcgcctc
     gtacccgccg ccgcagccgc ccccTCCGCC GCCGCTCTAC CAGCGCGTGT CGCCGCCGCA
     GCCGCCGCCA CCCCAGCCGC CGCGTAAGGA CCAGCAGCCG GGCCCGGCCG GCGGCGGAGG
     AGGTGAGTCG GGCCGAGAGA GCGCGAGAGG CGTCGCGCGG ACGGGCCCGG CCGCCTGAGG
     GGCGCGCGGC GGCGGCGACG GCGGCTGCAC GCGGCAGGGG CGCGCGTGCG CGCACGGGGA
```

GGCCCGGGCG GAGGCGCCCC TGGGCGCGCG CGGCCTGGTT CTAGGGCCTC GAGTGCGCAC
GCGCGCTGTG GGGGGAGGGC TGGGCGGGCG GGAGGCGCTC TGTGCGCAGA CGCAGTGAGG
TGGCCGGGCT GTGCGGCGCA ACTGTATAGA CTGGGGGCCC ACTATTGCTG TCCAGTCCGC
TGGTGTCGGG AACAGGGGTG GCGGGTGTTC AGCGCTGGAC GGGGTGCTAG CTGTGAATGC
CAGCAGGGCG TGAAAGCCTC ACCTGATGAC GTGCAGCGAC TCCCTTCTGG TCTTGAGGAA
CCAGAATGGG AACCGTACCA AGGATAGCTC CTCTGCTTCT ACAGCAAGGC AGGGTTTTCA
TCCGGGAGAG GTTG

Yellow: (major) atg-site
Blue: defined zfm1-sequence (2463197); capitalized letters: chromosomal clone (4755212)

Figure A2.
Zfm1 short promoter cloned into pGL3-basic

-629 tcccccgccg taaatctcgt tccggctggg ccttttccgc cgcgactctc gcttaatccc
-569 ggagaaactg ccccctgggg gaggggagta gaaaaaggct ggaaccagcg acagccaatc
-509 ccgcgacact acaacgcagg cgagattgat tgagtccacc accgcagcca atgagagagc
-449 tcgccgtcgc tccgtcatag agttcgcccc accccatccc ctcctttctg gactcggagc
-389 tcagttcacg cagtaacaaa tgaagtgcgc gctgcgacac ctcccagccc accgaactcc
-329 gccgccattt cctcgcttgg cctaacggtt cggccaatcc cagcgcgcat caagaaggac
-269 tgaggctccg ccaatcggag gccgccgatt tcgacccttc gcctcggccc ggcccaatcc
-209 aggccccggc cccgccgccc ccggcccgcc cccgcggtgc cctctcctct ccctctttgt
-149 gcgtctcgcg ccgccgccgc ccgccgcgtg agaggacggg ctccgcgcgc tccggcagcg
-89 cattcgggtc ccctcccccc gggaggcttg cgaaggagaa gccgccgcag aggaaaagca
-29 ggtgccggtg cctgtccccg ggggcgccat g

Figure A3.
Zfm1 promotor with Delta 1 mutation

-629 tcccccgccg taaatctcgt tccggctggg ccttttccgc cgcgactctc gcttaatccc
-569 ggagaaactg ccccctgggg gaggggagta gaaaaaggct ggaaccagcg acagccaatc
-509 ccgcgacact acaacgcagg cgagattgat tgagtccacc accgcagcca atgagagagc
-449 tcgccgtcgc tccgtcatag agttcgcccc accccatccc ctcctttctg gactcggagc
-389 tcagttcacg cagtaacaaa tgaagtgcgc gctgcgacac ctcccagccc accgaactcc
-329 gccgccattt cctcgcttgg cctaacggtt cggccaatcc cagcgcgcat caagaaggac
-269 tgaggctccg ccaatcggag gccgccgatt tcgacccttc gcctcggccc ggcccaatcc
-209 aggccccggc cccgccgccc ccggcccgcc cccgcggtgc cctctcctct ccctctttgt
-149 gcgtctcgcg ccgccgccgc ccgccgcgtg agaggacggg ctccgcgcgc tccggcagcg
-89 cattcgggtc ccctcccccc gggaggcttg cgaaggagaa gccgccgcag aggaaaagca
-29 ggtgccggtg cctgtccccg ggggcgccat g

Pink highlighted sequences represent missing bp. Start site is highlighted yellow

Figure A4.
Zfm1 promotor with Delta 2 Mutation

-629 tcccccgccg taaatctcgt tccggctggg ccttttccgc cgcgactctc gcttaatccc
-569 ggagaaactg ccccctgggg gaggggagta gaaaaaggct ggaaccagcg acagccaatc
-509 ccgcgacact acaacgcagg cgagattgat tgagtccacc accgcagcca atgagagagc
-449 tcgccgtcgc tccgtcatag agttcgcccc accccatccc ctcctttctg gactcggagc
-389 tcagttcacg cagtaacaaa tgaagtgcgc gctgcgacac ctcccagccc accgaactcc
-329 gccgccattt cctcgcttgg cctaacggtt cggccaatcc cagcgcgcat caagaaggac
-269 tgaggctccg ccaatcggag gccgccgatt tcgacccttc gcctcggccc ggcccaatcc
-209 aggccccggc cccgccgccc ccggcccgcc cccgcggtgc cctctcctct ccctctttgt
-149 gcgtctcgcg ccgccgccgc ccgccgcgtg agaggacggg ctccgcgcgc tccggcagcg
-89 cattcgggtc ccctcccccc gggaggcttg cgaaggagaa gccgccgcag aggaaaagca
-29 ggtgccggtg cctgtccccg ggggcgccat g

Pink highlighted sequences represent missing bp. Start site is highlighted yellow

Figure A5.
Zfm1 short promotor alignment with Wildtype sequence (from BLAST)

```
THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   GAACAAAGGTTGACGTCACTTAGGGCTTCCTTGCTGGACCCAAGGGTCGA

THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   TGGTGAAGGGAGACGGGTGGAGAGGCGGTTTTGCTCCATCTACGCATGCG

THE_672_PROMOTOR          ------------------TCCCCCGCCGTAAATCTCGTTCCGGCTGGGCC
SF1_original_from_Blast   CACTTCCTGCTCCAGGACTCCCCCGCCGTAAATCTCGTTCCGGCTGGGCC
                                            ********************************

THE_672_PROMOTOR          TTTCCCGCCGCGACTCTCGGCTTAATCCCGGAGAAACTGCCCCCTGGGG
SF1_original_from_Blast   TTTTCCGCCGCGACTCTCG-CTTAATCCCGGAGAAACTGCCCCCTGGGG
                          *** *************** ****************************

THE_672_PROMOTOR          GAGGGGAGTAGAAAAAGGCTGGAACCAGCGACAGCCAATCCCCGCGACA
SF1_original_from_Blast   GAGGGGAGTAGAAAAAGGCTGGAACCAGCGACAGCCAATCCC-GCGACA
                          ****************************************** ******

THE_672_PROMOTOR          CTACAACGCAGGCGAGATTGATTGAGTCCACCACCGCAGCCAATGAGAGA
SF1_original_from_Blast   CTACAACGCAGGCGAGATTGATTGAGTCCACCACCGCAGCCAATGAGAGA
                          **************************************************

THE_672_PROMOTOR          GCTCGCCGTCGCTCCGTCATAGAGTTCGCCCCACCCCATCCCCTCCTTT
SF1_original_from_Blast   GCTCGCCGTCGCTCCGTCATAGAGTTCGCCCCACCCCATCCCCTCCTTT
                          **************************************************

THE_672_PROMOTOR          TGGACTCGGAGCTCAGTTCACGCAGTAACAAATGAAGTGCGCGCTGCGAC
SF1_original_from_Blast   TGGACTCGGAGCTCAGTTCACGCAGTAACAAATGAAGTGCGCGCTGCGAC
                          **************************************************

THE_672_PROMOTOR          ACCTCCCAGCCCACCGAACTCCGCCGCCTTTCCTCGCTTGGCC-AACGG
SF1_original_from_Blast   ACCTCCCAGCCCACCGAACTCCGCCGCCTTTCCTCGCTTGGCCTAACGG
                          ****************************************** *****

THE_672_PROMOTOR          TTCGGCCAATCCCAGCGCGCATCAAGAAGGACTGGGCTCCGCCAATCGG
SF1_original_from_Blast   TTCGGCCAATCCCAGCGCGCATCAAGAAGGACTGGGCTCCGCCAATCGG
                          **************************************************
THE_672_PROMOTOR          AGGCCGCCGATTTCGACCCTTCGCCTCGGCCCGGCCCAATCCAGGCCCCG
SF1_original_from_Blast   AGGCCGCCGATTTCGACCCTTCGCCTCGGCCCGGCCCAATCCAGGCCCCG
                          **************************************************

THE_672_PROMOTOR          GTCCCGCCGCCCCCGGCCCGCCCCCGCGGTGCCCTCTCTCCTCCCTCTTT
SF1_original_from_Blast   GCCCCGCCGCCCCCGGCCCGCCCCCGCGGTGCCCTCTCTCCTCCCTCTTT
                          * **********************************************

THE_672_PROMOTOR          GTGCGTCTCGCGCCGCCGCCGCCCGCCGCGTGAGAGGACGGGCTCCGCGC
SF1_original_from_Blast   GTGCGTCTCGCGCCGCCGCCGCCCGCCGCGTGAGAGGACGGGCTCCGCGC
                          **************************************************

THE_672_PROMOTOR          GCTCCGGCAGCGCATTCGGGTCCCCTCCCCCCGGGAGGCTTGCGAAGGAG
SF1_original_from_Blast   GCTCCGGCAGCGCATTCGGGTCCCCTCCCCCCGGGAGGCTTGCGAAGGAG
                          **************************************************

THE_672_PROMOTOR          AAGCCGCCGCAGAGGAAAAGCAGGTGCCGGTGCCTGTCCCCGGGGGCGCC
SF1_original_from_Blast   AAGCCGCCGCAGAGGAAAAGCAGGTGCCGGTGCCTGTCCCCGGGGGCGCC
                          **************************************************

THE_672_PROMOTOR          ATG-----------------------------------------------
SF1_original_from_Blast   ATGGCGACCGGAGCGAACGCCACGCCGTTGGGTAAGCTGGGCCCCCCCGG
                          ***

THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   GCTGCCCCCGCTCCCCGGGCCCAAAGGAGGCTTCGAGCCGGGCCCTCCGC
```

```
THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   CTGCACCCGGGCCTGGGGCGGGGCTGCTGGCTCCCGGGCCGCCGCCGCCC


THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   CCGCCCGTGGGCTCGATGGGGGCCCTGACCGCGGCCTTCCCCTTCGCGGC


THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   GCTGCCTCCGCCGCCTCCGCCGCCGCCCCCTCCGCCTCCCCAGCAGCCGC


THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   CGCCGCCTCCACCGCCACCGTCCCCCGGCGCCTCGTACCCGCCGCCGCAG


THE_672_PROMOTOR          --------------------------------------------------
SF1_original_from_Blast   CCGCCCCCTCCGCCGCCGCTCTACCAGCGCGTGTCGCCGCCGCAGCCGCC
```

Figure A6.
Zfm1 short promotor mutation 1 alignment with wildtype promotor

```
ZFM1 mutatation 1       CAATCCCAGCGCGCATCAAGAAGGACTGAGGCTCCGCCAATCGGAGGCCG
ZFM1 WT promotor        CAATCCCAGCGCGCATCAAGAAGGACTG-GGCTCCGCCAATCGGAGGCCG
Mutation 1 primer       --------------------------------------------------

ZFM1 mutatation 1       CCGATTTCGACCCTTCGCCTCGGCCCGGCCCAATCCAGGCCCCGGTCCCG
ZFM1 WT promotor        CCGATTTCGACCCTTCGCCTCGGCCCGGCCCAATCCAGGCCCCGGTCCCG
Mutation 1 primer       ----------------------------------------CCCCGGTCCCG
                                                                 **********
ZFM1 mutatation 1       CCG------GCCCGCCCCCGCGGTGCCCTCTCTCCCTCTTTGTGCGT
ZFM1 WT promotor        CCGCCCCCGGCCCGCCCCCGCGGTGCCCTCTCTCCCTCTTTGTGCGT
Mutation 1 primer       CCG------GCCCGCCCCCGCGGTGC----------------------
                        ***      ****************
```

Figure A7.
Zfm1 short promotor mutation 2 alignment with wildtype promotor

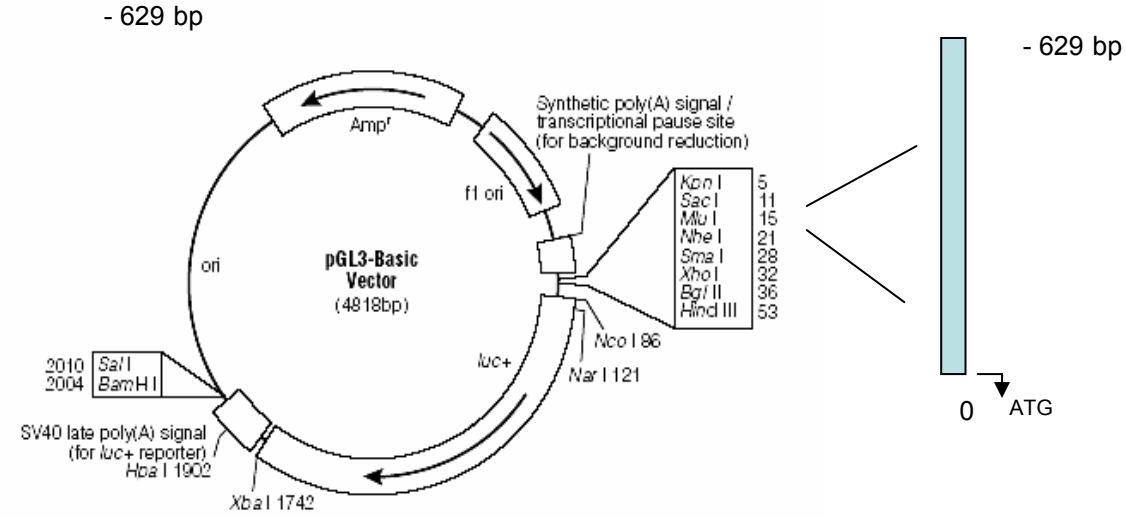
```
Mutation 2 primer       --------------------------------------------------
ZFM1 mutation 2         CGCGGCATCAAGAAGGACTGAGGCTCCGCCAATCGGAGGCCGCCGATTTC
ZFM1 WT promotor        GCG-CATCAAGAAGGACTG-GGCTCCGCCAATCGGAGGCCGCCGATTTC

Mutation 2 primer       -------------------------------------------CGCCCCC
ZFM1 mutation 2         GACCCTTCGCCTCGGCCCGGCCCAATCCAGGCCCCGGTCCCGCCGCCCCC
ZFM1 WT promotor        GACCCTTCGCCTCGGCCCGGCCCAATCCAGGCCCCGGTCCCGCCGCCCCC
                                                           *******
Mutation 2 primer       GGCCCGC------GGTGCCCTCTCTCCC----------------------
ZFM1 mutation 2         GGCCCGC------GGTGCCCTCTCTCCCTCCCTCTTTGTGCGTCTCGCGCC
ZFM1 WT promotor        GGCCCGCCCCCGCGGTGCCCTCTCTCCCTCCCTCTTTGTGCGTCTCGCGCC
                        *******      **************

Mutation 2 primer       --------------------------------------------------
ZFM1 mutation 2         GCCGCCGCCCGCCGCGTGAGAGGACGGGCTCCGCGCGCTCCGGCAGCGCA
ZFM1 WT promotor        GCCGCCGCCCGCCGCGTGAGAGGACGGGCTCCGCGCGCTCCGGCAGCGCA
```

Figure A8.
Zfm1 short promotor double mutant alignment with wildtype promotor

```
Zfm1 double mutant      CGCCTGGGCCCGGCCCAATCCAGGCCCCGGTCCCGCCG-----GGCCCGC
Zfm1 WT promotor        CGCCTCGGCCCGGCCCAATCCAGGCCCCGGTCCCGCCGCCCCCGGCCCGC
                        ***** ********************************     *******

Zfm1 double mutant      ------GGTGCCCTCTCTC-TCCCTCTTTGTGCGTCTCGCGCCGCCGCCG
Zfm1 WT promotor        CCCCGCGGTGCCCTCTCTCCTCCCTCTTTGTGCGTCTCGCGCCGCCGCCG
                              ************* ****************************

Zfm1 double mutant      CCCGCCGCGTGAGAGGACGGGCTCCGCGCGCTCCGGCAGCGCATTCGGGT
Zfm1 WT promotor        CCCGCCGCGTGAGAGGACGGGCTCCGCGCGCTCCGGCAGCGCATTCGGGT
                        **************************************************

Zfm1 double mutant      CCCCTCCCCCCGGGAGGCTTGCGAAGGAGAAGCCGCCGCAGAGGAAAAGC
Zfm1 WT promotor        CCCCTCCCCCCGGGAGGCTTGCGAAGGAGAAGCCGCCGCAGAGGAAAAGC
                        **************************************************
```

Figure A9.

Map of the human Zfm1 promotor fragment cloned into pGL3



Map of the human zfm1 promotor fragment. The fragment was blunt cloned via SmaI into the multiple cloning site (MCS) of the pGL3 basic vector (vector map, courtesy of Promega).

Figure A10.

p37	**p38**	p39	p40	p41	p42	P43	p44
p45	p46	p47	p48	p49			
PP4-1	PP4-2	PP4-3	PP4-4	PP4-5	PP4-6	PP4-7	PP4-8
PP4-9	PP4-10	PP4-11	PP4-12	**PP4-13**	PP4-14	PP4-15	PP4-16
PP4-17	PP4-18	PP4-19	PP4-20	PP4-21	PP4-22	PP4-23	PP4-24
PP4-25	PP4-26	PP4-27	PP4-28	PP4-29	PP4-30	PP4-31	**PP4-32**
PP4-33	PP4-34	PP4-35	PP4-36	PP4-37	PP4-38	PP4-39	PP4-40

13 plate pools

16 pooled rows

24 pooled columns

Map of the PP4 secondary clone plate LANLc154PP4. The secondary pool comprises of 53 pools in total: 13 plate pools, 16 pooled rows and 24 pooled columns. Co-ordinates p38, PP4-13 and PP4-32 (in bold) were used to chose the tertiary clone for further analysis by PCR.

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