Human Molecular Genetics, 2012, Vol. 21, No. 9 ***1968–1978***
doi:10.1093/hmg/dds006
Advance Access published on January 13, 2012

Genetic and epigenetic mechanisms collaborate to control *SERPINA3* expression and its association with placental diseases

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Received December 7, 2011; Revised December 7, 2011; Accepted January 10, 2012

SERPINA3 (Serpin peptidase inhibitor clade A member 3), also known as a1-antichymotrypsin, is a serine protease inhibitor involved in a wide range of biological processes. Recently, it has been shown to be up-regulated in human placental diseases in association with a hypomethylation of the 5′ region of the gene. In the present study, we show that the promoter of *SERPINA3* is transcriptionally activated by three transcription factors (TFs) (SP1, MZF1 and ZBTB7B), the level of induction being dependent on the rs1884082 single nucleotide polymorphism (SNP) located inside the promoter, the T allele being consistently induced to a higher level than the G, with or without added TFs. When the promoter was methylated, the response to ZBTB7B was allele specific (the G allele was strongly induced, while the T allele was strongly down-regulated). We propose an adaptive model to explain the interest of such a regulation for placental function and homeostasis. Overexpression of SERPINA3 in JEG-3 cells, a trophoblast cell model, decreased cell adhesion to the extracellular matrix and to neighboring cells, but protects them from apoptosis, suggesting a way by which this factor could be deleterious at high doses. In addition, we show in different human populations that the T allele appears to predispose to Intra Uterine Growth Restriction (IUGR), while a G allele at a second SNP located in the second exon (rs4634) increases the risk of preeclampsia. Our results provide mechanistic views inside the involvement of SERPINA3 in placental diseases, through its regulation by a combination of epigenetic, genetic and TF-mediated regulations.

INTRODUCTION

Serine protease inhibitors (SERPINs) are a superfamily consisting of at least 37 proteins in humans (1). They have been shown to regulate a wide range of biological processes, including coagulation, inflammation and wound healing. SERPINs mainly act by inhibiting the activity of proteases, even though some of them are non-inhibitory and function instead as chaperones or hormone transporters in the circulation. These proteins are therefore very important for maintaining body homeostasis in a broad sense (2). SERPINA3 (Serpin peptidase inhibitor clade A member 3), initially named alpha-1-antichymotrypsin (ACT), like other SERPINs, has a characteristic three-dimensional structure and employs a peculiar mode of action referred to as suicide substrate-like inhibition. The SERPIN contains an exposed active site, the

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reactive center loop (RCL) that serves as a substrate for the target protease. Upon cleavage of the RCL, the SERPIN undergoes dramatic conformational changes resulting in the formation of a highly stable and irreversible covalent complex with its target. The SERPIN is therefore consumed through achieving its function.

SERPINA3 is a typical acute-phase protein secreted into the circulation during acute and chronic inflammation. SERPINA3 is able to inhibit several proteases, including mast cell chymase, pancreatic chymotrypsin, human glandular kallikrein 2, kallikrein 3 (prostate-specific antigen), pancreatic cationic elastase and an uncharacterized lung serum protease (3–7). However, its major target is probably the neutrophil cathepsin G (8), a pro-inflammatory enzyme released at sites of inflammation contributing to activation of inflammatory cytokines, degradation of pathogens and remodeling of tissues (9). Cathepsin G contributes to wound repair by digesting extracellular matrix (ECM) proteins and by releasing growth factors from the ECM (10). Further, cathepsin G promotes platelet aggregation (11). At sites of vascular injuries and atherosclerosis, this enzyme is able to convert angiotensin I into active angiotensin II (12). Additionally, it increases the permeability of endothelial barriers contributing to perivascular lymphocytic migration and activation (13). Cathepsin G has also a pro-apoptotic activity (14). Therefore, by inhibiting cathepsin G, SERPINA3 should limit inflammation, coagulation, ECM remodeling and should inhibit apoptosis.

SERPINA3 has been implicated in the pathology of several devastating human diseases, including chronic obstructive pulmonary disease, Parkinson's disease, Alzheimer's disease, cystic fibrosis, stroke and cerebral hemorrhage. SERPINA3 is also expressed at high levels in several malignant melanomas and carcinomas where it might have a role in regulating apoptosis and invasiveness (15,16). In addition, plasma levels of SERPINA3 have been found to be increased in patients with liver, pancreatic and prostate cancers (17,18). Some SERPIN mutations within key structural regions are able to affect the conformational stability of the protein, leading to a group of diseases called serpinopathies (19,20). Experimental and clinical data suggest that some single nucleotide polymorphisms (SNPs) in regulatory regions subtly alter gene expression and plasma levels of SERPINA3, thus disturbing the SERPIN-protease equilibrium. For instance, studies have reported that the rs1884082 SNP (G/T) located in the *SERPINA3* gene promoter is directly correlated with basal gene expression in human cell lines and with SERPINA3 plasma levels (21). This same T has also been shown to increase luciferase activity at a higher level than the G allele in two cell models after induction by oncostatin M (22). Among the two lines, one was T98G (glial/neuronal) and the other astrocytic (U373 MG), and in the second the difference in induction by the two alleles was maximum (up to 7-fold more for the T allele) 6 h after oncostatin M induction. Recently, a significant association has been found between a germ-line *SERPINA3* SNP and overall survival in pancreatic cancer (23).

Preeclampsia (PE) and vascular intrauterine growth restriction (IUGR) are two common obstetrical complications which represent major causes of materno-fetal morbidity and mortality. PE is a maternal multi-systemic disorder characterized by pregnancy-induced hypertension, proteinuria and endothelial dysfunction. It can lead to life-threatening clinical conditions, such as hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome or eclampsia (seizures). IUGR is defined by a falling off in the fetal growth curve. Both diseases are thought to share a common etiology in placental malperfusion, secondary to shallow invasion of uterine spiral arteries by invasive extravillous cytotrophoblats. Defective invasion results in a deficient maternal arterial remodeling at the feto-maternal interface, consecutively leading to intermittent vascular flow, hypoxia-reoxygenation injuries, localized ischemic lesions and oxidative stress in pathological placentas. The most classical hypothesis for explaining the preeclamptic syndrome is that the release of placental debris or microparticles and secreted molecules into the maternal blood flow triggers maternal systemic inflammation, endothelial lesions and subsequent symptoms. Plasma levels of SERPINA3 are increased in women with PE and/or IUGR (24,25). We have previously demonstrated that *SERPINA3* mRNA is overexpressed in the placentas of preeclamptic/IUGR pregnancies compared with controls, along with hypomethylation of the 5′ gene region. However, while hypomethylation was present among both isolated PE and PE + IUGR cases, SERPINA3 was only mildly overexpressed in isolated PE (∼2-fold) and strongly overexpressed (∼10-fold) in IUGR pregnancies, regardless of the presence of PE (26). Since understanding the regulation of *SERPINA3* is imperative to dissecting the role of SERPINA3 in the pathology, we explored herein the molecular bases underlying expressional changes observed in these placental diseases. In addition, we performed a preliminary case–control study that shows in two different populations that a T allele in the promoter (rs1884082) and a G allele in the second exon (rs4934) predispose to placental diseases and especially IUGR.

RESULTS

The rs1884082 SNP located in the promoter correlates to mRNA levels of SERPINA3 in placentas

SERPINA3 mRNA quantity was monitored by quantitative reverse transcription–polymerase chain reaction (RT–PCR) in placentas from individuals that underwent either non-pathological (controls, $n = 14$) or pathological pregnancies (isolated preeclampsia: PE, $n = 14$, isolated intra-uterine growth restriction: IUGR, $n = 17$ and preeclampsia combined with intra-uterine growth restriction: PE + IUGR, $n = 5$). The results are presented in Figure 1A. Compared with non-pathological placentas, *SERPINA3* mRNA levels were ∼2-fold higher in isolated PE, 7-fold higher in isolated IUGR and 14-fold higher in PE + IUGR. Then, among the complete set of placentas, 36 for which DNA was available were genotyped at the rs1884082 SNP, a G/T polymorphism located within the SERPINA3 promoter, in order to determine the average expression level of *SERPINA3* according to the placenta genotype regardless of the pathological status (Fig. 1B). Interestingly, mRNA expression correlated with rs1884082 promoter genotypes. Average expression was 10 times higher in placentas harboring the TT compared with the GG genotypes, while the GT genotype had an intermediate

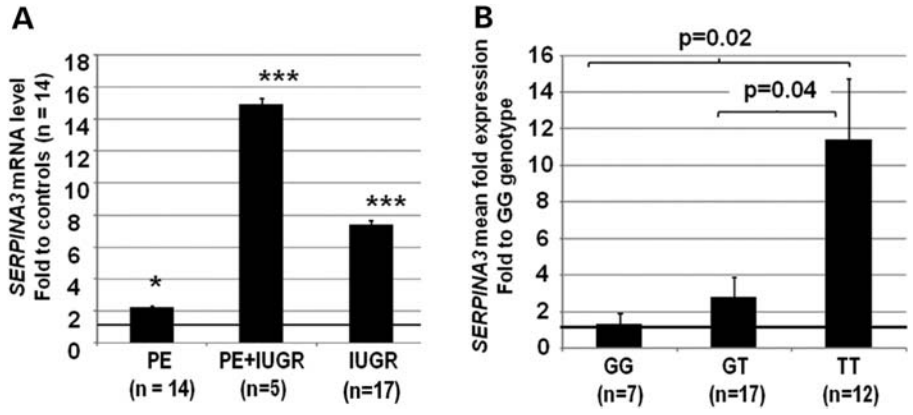

Figure 1. The rs1884082 polymorphism influences placenta mRNA levels of SERPINA3. (**A**) Quantitative RT–PCR of the *SERPINA3* gene on normal and pathological placentas. The expression level was normalized using *SDHA* as reference gene. The results are represented as the fold induction level in pathological placentas (PE $n = 14$, PE + IUGR $n = 5$, vIUGR $n = 17$) compared with controls ($n = 14$). Data are shown as mean ± SEM. $^{***}P < 0.001$ $^{*}P < 0.05$ compared with control placentas. (**B**) *SERPINA3* mRNA levels calculated according to the genotype at the rs1884082 SNP for 36 placentas. The results are represented as the fold expression compared with GG genotypes.

level of expression (about twice that of GG placentas, the apparent non-proportionality being probably due to interindividual variability).

The rs180482 SNP influences the basal levels of SERPINA3 promoter activity as well as its transactivation by zinc fingers that are up-regulated in placental diseases

To further evaluate the involvement of this promoter polymorphism on the regulation of *SERPINA3* expression level, the transcriptional activity of the two promoter variants was measured in controlled situations using JEG-3 choriocarcinoma cells, a classical model of trophoblast cells. Constructs containing either the T or G version of the *SERPINA3* promoter upstream of the firefly Luciferase reporter gene were transiently transfected (along with the internal normalizing plasmid pRL-RSV) into JEG-3 cells. Interestingly, the luciferase activity of the T variant was ∼6-fold the level of the empty pGL3enh-Luc vector, while the G variant only reached a 3.5-fold induction. Thus, the T allele has a higher basal expression than the G allele (Fig. 2A, $P < 0.0001$) in this cellular context. These data confirmed that the T allele of the promoter contributes to the higher expression of the *SERPINA3* gene in TT placentas.

Bioinformatic analysis of the *SERPINA3* promoter using Genomatix^{TM} software indicated putative binding sites for three Zinc-finger containing transcription factors (TFs), overlapping the rs1884082 SNP, namely MZF1, ZBTB7B and SP1. Thus, to assess the effects of these TFs, a series of transactivation assays with either T or G version of the *SERPINA3* promoter was carried out, in the presence or absence of *MZF1*, *ZBTB7B* or *SP1* expressing vectors. Variance analysis revealed an effect of the allele ($P < 0.05$) an effect of the inducers ($P < 0.003$) but no effect of the interaction between the type of promoter (harboring a G or a T) and the inducers. To summarize, the three zinc-finger TFs induced an increase in luciferase activity, on both alleles, the highest induction being obtained with ZBTB7B and the lowest with SP1 (Fig. 2A). We assume that this induction reflects the ability of these zinc fingers TF to bind on *SERPINA3* promoter, even though we cannot exclude the simultaneous existence of indirect effects. The resulting fold induction which remained systematically higher for the T allele than for the G allele seemed to only maintain the difference existing at the basal level.

Then, expression of the three zinc-finger encoding genes was measured in normal and pathological placentas by quantitative RT–PCR. The mRNA levels of *SP1*, *MZF1* and *ZBTB7B* were consistently higher in the IUGR groups compared with normal placentas (Fig. 2B). In PE + IUGR, *ZBTB7B* and *SP1* were significantly up-regulated while upregulation of *MZF1* was not statistically significant. In isolated PE, only *SP1* was found up-regulated. In all cases, *SP1* was much more strongly up-regulated than MZF1 and ZBTB7B. The fact that in IUGR, either accompanied or not by PE, there was a strongest mRNA increase of these zinc-finger genes in pathological placentas indicated that they probably all contribute to the sharp overexpression of *SERPINA3* expression in placentas of the IUGR (± PE) category.

Hypoxia-induced transcription of SP1, MZF1 and ZBTB7B precedes SERPINA3 increase

The impact of oxygen pressure on *SERPINA3* expression was investigated since a prominent role has been evidenced for hypoxia in placental diseases (27–29). JEG-3 cells were placed in a Lwoff chamber and exposed either to an oxygen-depleted atmosphere (considered a hypoxic condition, $2\%O_2$) or maintained as control in atmospheric oxygen pressure (considered a normoxic condition). After exposure, the endogenous mRNA level of *SERPINA3* was determined by quantitative RT–PCR in cells harvested at 3, 6, 12, 24, 36 and 48 h. Results are represented on the Figure 3A as *SERPINA3* fold induction in hypoxic condition relative to normoxic condition at each time point. The kinetics showed that under hypoxia the mRNA quantity increased significantly at 24 h (>3-fold), and that later on, the induction level progressively decreased. Logically, we also analyzed the effect of

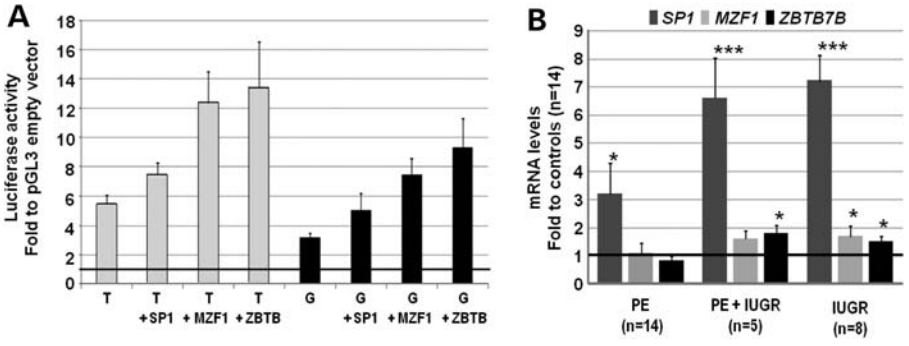

Figure 2. The rs180482 SNP influences the basal levels of SERPINA3 promoter activity as well as its transactivation by zinc-finger proteins transcriptionally up-regulated in placental diseases. (**A**) Biological activity of *SERPINA3* promoter either containing a T or a G at the rs180482 SNP following transient transfection into JEG-3 cells was measured by luciferase assays. Transactivation assays with human SP1, MZF1 or ZBTB7B whose putative binding sites overlap the rs1884802 SNP were additionally performed. Results are represented as fold induction compared with the corresponding pGL3enh-Luc empty vector. Data are shown as mean fold ± SEM of three independent experiments (including three to six replicates per condition). (**B**) Placenta mRNA levels of SP1, MZF1 and ZBTB7B were assessed by quantitative RT–PCR on normal and pathological placentas. The expression level was normalized using SDHA as reference gene. The results are represented as the fold induction level in pathological placentas compared with control placentas. Data are shown as mean ± SEM. $^{***}P < 0.001$ $^{*}P < 0.05$.

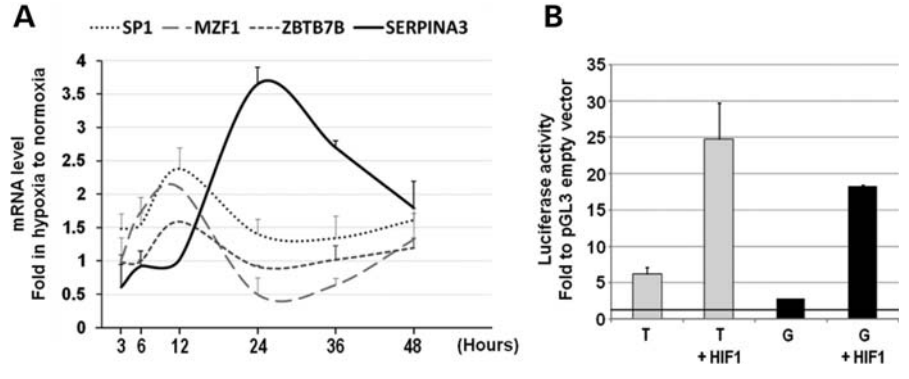

Figure 3. *SERPINA3* regulation under hypoxia. (**A**) JEG-3 cells were maintained in hypoxia (2% O_2) in a Lwoff chamber during 3, 6, 12, 24, 36 and 48 h. SERPINA3 expression was monitored by quantitative RT–PCR. Results are represented as fold in hypoxia compared with normoxia (20% O_2). (**B**) The two alleles of the SERPINA3 promoter were co-transfected in JEG-3 cells with a vector encoding a stable form of HIF1α. The results were measured by luciferase assays. Despite the absence of canonical HIF1-binding site in this SERPINA3 promoter element, HIF was able to induce the T and the G alleles.

hypoxia on SP1, MZF1 and ZBTB7B expression (Fig. 3A). Interestingly, all three TFs were transcriptionally induced before 24 h, peaking at 12 h, thus at a time point consistent with their putative involvement in *SERPINA3* induction.

Induction of SERPINA3 promoter is HIF1 mediated

To assess the effect of hypoxia on the *SERPINA3* promoter, the luciferase aforementioned constructs were transiently co-transfected in JEG-3 cells with a vector encoding HIF1-TM, a mutated version of HIF1α (hypoxia inducible factor 1 α-subunit) which cannot be targeted by prolyl-hydroxylases and is therefore constitutively stabilized. HIF1α overexpression led to a strong induction of the luciferase activity ∼4-fold on the T allele and 7-fold on the G allele (Fig. 3B). Again, the basal difference between the *SERPINA3* promoter T and G alleles was maintained. Since the bioinformatic analysis of *SERPINA3* promoter did not predict any HIF1α-binding site, the HIF1-triggered induction observed might be indirect and mediated through the activation of SP1, MZF1 and/or ZBTB7B, consistently with their transient induction by hypoxia.

The response of the methylated promoter is allele dependant

Since we have previously shown that pathological placentas harbor a hypomethylation of the *SERPINA3* 5′ region, we also investigated the role of this parameter on *SERPINA3* regulation. Luciferase assays were performed with *Sss*I-methylated versions of *SERPINA3* promoter either in the presence or the absence of HIF1α, ZBTB7B and SP1 inductors (Fig. 4), as well as MZF1 (not shown in the figure). As expected, methylation of the plasmids, whatever the allele, resulted in a decrease in luciferase activity compared with the unmethylated situation. These results suggested that DNA hypomethylation in pathological placentas contribute to *SERPINA3* overexpression. When proceeding to transactivation assays, SP1 remained able to induce both alleles, the T allele demonstrating a higher transcriptional activity than the G allele. MZF1 presented a quite similar pattern, being

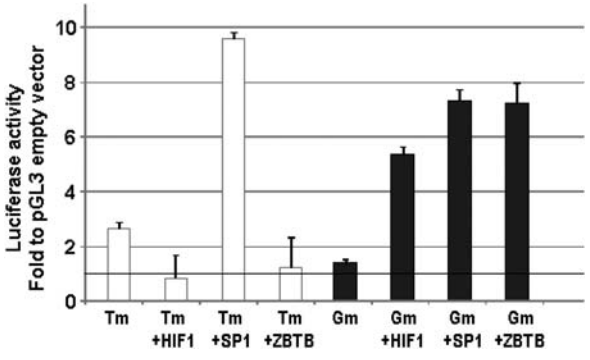

Figure 4. The transactivation of the methylated promoter is allele-dependent. JEG-3 cells were co-transfected with *Sss*I-methylated versions of the *SERPINA3* promoter together with expression vectors encoding for HIF1α, SP1, ZBTB7B and MZF1 TFs. HIF1α and ZBTB7B had differential effects on the two methylated promoter versions contrary to SP1 or MZF1 (not shown) that induced both T and G allele transcription. When the promoter was methylated, HIF1α and ZBTB7B were not able to transactivate the T allele, while they induced the G allele. Since there are apparently no HIF1α-binding sites in this promoter, one should assume that the effect of this TF was indirect.

able to induce both methylated alleles (data not shown). Strikingly, HIF1 and ZBTB7B induced a clear dichotomous and allele-dependant response. HIF1α and ZBTB7B were not able to transactivate the T allele and even tended to decrease its activity, whereas they were able to up-regulate the methylated G allele. Since there is no HIF1α-binding site on the promoter element analyzed, we postulate that the allele-dependant effect observed with HIF1α could be mediated through ZBTB7B TF in a sequential series of events.

Case–control study reveals that the rs1884082 SNP located inside the SERPINA3 promoter is associated with IUGR

To investigate the possible association between the rs1884082 SNP and placental diseases, a case–control study was performed. First, a French collection constituted by 134 DNA samples extracted from placentas collected from Caucasian individuals that underwent either non-pathological (controls, $n = 44$) or pathological pregnancies (PE, $n = 30$, IUGR, $n = 38$ and PE + IUGR, $n = 26$) was genotyped for the rs1884082 SNP. The frequencies of the three possible genotypes (GG, GT and TT) were determined and a Chi-square test comparing the observed genotype frequencies in pathological conditions with expected genotype frequencies calculated based on the observed allelic repartition in the control group was performed (Fig. 5A). A significant difference was found when the complete set of pathological samples was considered ($P = 0.008$). The difference remained significant when considering IUGR with or without PE ($P = 0.017$). In this category, the TT genotype appeared overrepresented, while the GG genotype was underrepresented. These data suggested that the promoter T allele is associated with an increased risk of developing IUGR. These data were confirmed when comparing the observed allele frequencies to the HapMap frequencies in the Caucasian population ($P = 0.04$ for IUGR, data not shown). Since the absence of association in the PE group could be due to the limited number of samples constituting the French collection, we attempted to extend the number of placenta DNAs from this category. Therefore, samples collected at Los Angeles from 166 controls and 120 preeclamptic individuals of Hispanic origin, as well as 32 controls and 185 cases of PE accompanied by HELLP syndrome were analyzed. Again, no significant association was found when the PE samples were considered separately or all together (Table 1).

Case–control study reveals that the rs4934 SNP located in the Exon2 is associated with PE

According to the literature, in a number of complex diseases such as Alzheimer disease, genetic association studies using single SNPs have failed to provide clear evidence for the involvement of the *SERPINA3* gene, suggesting that the analysis looking at multiple SNP patterns might be more revealing (30). Therefore, we decided to additionally analyze the rs4934 SNP, an A/G polymorphism located in the signal peptide region in Exon2 of the *SERPINA3* gene which converts amino acid alanine to threonine. In the French cohort, the GG and AG genotypes were found significantly overrepresented only when placental diseases were taken as a whole ($P = 0.016$, Fig. 5B). When analyzing the Los Angeles collection, a significant association was found with the PE group. The risk of PE ($\pm$ HELLP) was increased when a G allele was present. The relative risk was estimated at 1.66 when a G was present (1.01–2.76) and 1.8 with the AG genotype (1.06–3.06).

Overexpression of SERPINA3 results in loss of adherence and delay of apoptosis

We wished to explore the biological function of *SERPINA3* in the context of trophoblast cells. Thus, JEG-3 cells were transiently transfected with a vector encoding SERPINA3. Strikingly, overexpression of SERPINA3 led to a strong increase in the number of floating cells. Therefore, the supernatant from JEG-3 culture, after transfection either by the expression vector encoding SERPINA3 or alternatively by the empty expression vector, was harvested 24 or 48 h post-transfection. Then the number of detached cells found in the supernatant was counted in a Malassez cytometer. As shown in Figure 6A, the number of non-adherent cells was significantly increased by SERPINA3 overexpression. To decipher if this increase of detachment was correlated with an increase in apoptosis, western blot analysis of the protein extracts from these cells was carried out using anti-cleaved caspase 3 antibody (monitoring apoptosis) and anti-α-actin antibody (to check for the amount of protein in each extract, Fig. 6B). Interestingly, the first wave of detachment was not due to induced apoptosis but rather due to a loss of adherence since SERPINA3-transfected cells as mock-transfected cells do not show apoptosis at 24 h. This loss of adherence could probably result from changes in cell adhesion to the ECM and to neighboring cells. At 48 h where apoptotic cells started to be detected, the level of cleaved caspase-3 was decreased in the cell overexpressing SERPINA3 compared with the control, indicating that these cells are *a priori* more resistant to apoptosis, or potentially protected from its action by SERPINA3.

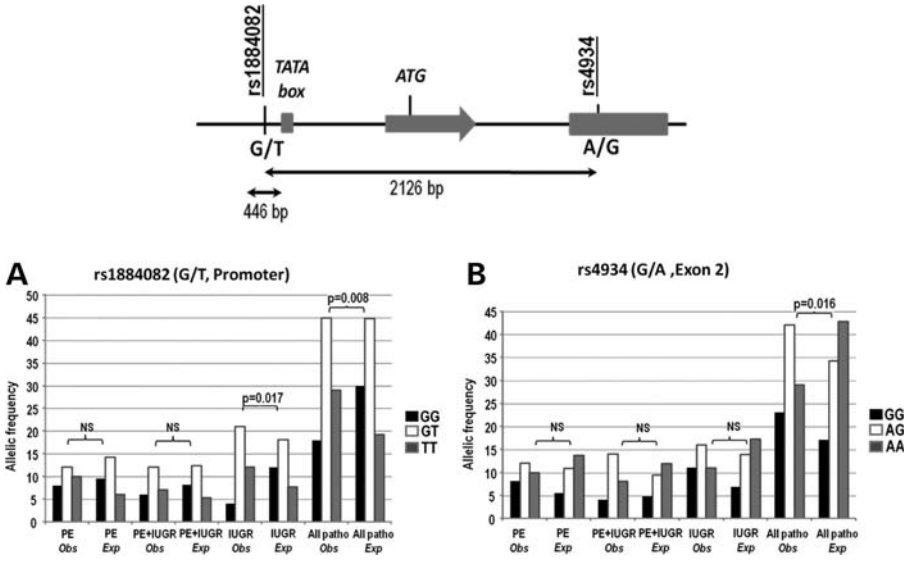

Figure 5. Analysis of the allelic distribution of the two SERPINA3 polymorphisms and of their haplotypes in placental diseases in the French collection. Positions of the two polymorphisms studied are represented in the below right panel. Rs1884082 is located upstream of the TATA box of the gene within a promoter element described by Morgan *et al*. (21). The rs4934 is present in the second exon. Both SNPs have two alleles of similar frequencies (near 0.5, according to HapMap). The graphs compare observed and expected frequencies (calculated according to the frequencies observed in the control placentas), for the SNPs located (**A**) in the promoter (**B**) in Exon2.

DISCUSSION

SERPINA3 is a typical acute-phase protein. In a previous study, we have shown that the mRNA level of *SERPINA3* was induced in placentas from pathological pregnancies concomitantly with a hypomethylation of the 5′ region of the gene. Such epigenetic modifications are now well documented in pathological placentas (31–36). However, most studies provide lists of disturbed profiles without further analysis of the mechanistic consequences of these anomalies on gene expression or physiopathology (37). Some authors have previously reported that a G/T polymorphism (rs1884082) located in the *SERPINA3* promoter influences its expression (21) and is associated with physiopathological processes, such as Alzheimer disease or cancer. Herein, we demonstrate for the first time the existence of a combinatorial influence of genetic and epigenetic factors in determining *SERPINA3* gene expression. DNA methylation appears to precondition the responsiveness of the promoter towards specific TFs. More specifically, the transactivation of the *SERPINA3* promoter by the ill-known ZBTB7B zinc-finger TF (also called c-KROX, and essentially known for its function in T cell commitment) follows a complex pattern that is both methylation- and allele-dependent which might impact physiology and physiopathology. In future studies, it would be interesting to develop a ZBTB7B-specific antibody suitable for supershift assays or chromatin IP that would permit to check whether there is a direct interaction of this factor with the SERPINA3 promoter element. The bioinformatics, as well as the short time necessary for triggering the luciferase response, suggests nevertheless that the interaction may be probably direct.

By transfection of a SERPINA3-encoding vector in JEG-3 cells, we could observe that the cells are protected from apoptosis. Although this result is totally novel for this specific protein, it has been shown that other SERPINS have an anti-apoptotic activity. For instance, PAI-1 (SERPINE1) has been shown in a fibrosarcoma cell model to induce resistance to chemotherapy-induced apoptosis (38). In the SERPINA clade itself, SERPINA1 has been shown to protect pancreatic β-cells from apoptosis, by blocking apoptosis mediated by activation of CASPASE-3 (39). Recently, it has been shown that SERPINA4 (aka Kallistatin) is able to moderate endothelial apoptosis by limiting the amount of reactive oxygen species, and suppressing tumour necrosis factor-α-induced apoptosis (40). Most interestingly, we have shown by proteomic analysis using the iTRAQ technology that SERPINA4 is increased in the plasma of women affected by PE accompanied with IUGR, but not in isolated PE.

Considering our results, we propose a multistep scenario that recapitulates *SERPINA3* regulation in placental diseases (Fig. 7). We assume that in normal placenta, the *SERPINA3* promoter is methylated (26). At early stages of placental development (corresponding approximately to the first trimester of gestation in humans), the placenta is normally exposed to low ppO_2 (hypoxia), which probably induces the expression of TFs among which SP1 and ZBTB7B, that in turn can up-regulate *SERPINA3* expression at a specific time. Some TFs will transactivate both variants of the promoter (such as SP1 or MZF1) while ZBTB7B (and maybe others) will transactivate the G allele, but poorly transactivate the T allele. This lower responsiveness of the T variant may have functional effects such as inadequate apoptosis or inappropriate maintenance of cell–cell and cell–ECM adhesion which may delay acquisition of migratory and invasive properties of trophoblasts (a process constituting a cornerstone of placental diseases). While in the non-pathological cases, the ppO_2 will increase and set back *SERPINA3* expression to basal level, in the pathological placentas hypoxia is abnormally maintained. Abnormal maintenance of low oxygen pressure

Table 1. Case-Control stuty of two SNPs of SERPINA3 in different populations

		All subjects, matched on site[a]	French PE (±IUGR)			Los Angeles PE			PE + HELLP		
		Odds ratio (95% CI)	Controls	Cases	Odds ratio (95% CI)	Controls	Cases	Odds ratio (95% CI)	Controls	Cases	Odds ratio (95% CI)
Promoter rs1884082	TT	1.00 (ref)	9 (20%)	17 (31%)	1.00 (ref)	97 (20%)	17 (31%)	1.00 (ref)	11 (20%)	47 (25%)	1.00 (ref)
	GT	1.05 (0.71; 1.57)	21 (48%)	24 (44%)	0.61 (0.22; 1.64)	55 (48%)	24 (44%)	0.99 (0.61; 1.63)	11 (48%)	97 (52%)	1.06 (0.83; 5.10)
	GG	0.71 (0.41; 1.22)	14 (32%)	14 (25%)	0.53 (0.18; 1.58)	14 (32%)	14 (25%)	0.71 (0.28; 1.18)	10 (32%)	41 (22%)	0.96 (0.37; 2.49)
	GG vs. TT/TG	0.69 (0.42; 1.13)			0.73 (0.30; 1.76)			0.71 (0.29; 1.75)			0.63 (0.27; 1.43)
	MAF		56%	47%		25%	23%		48%	48%	
	Trend		$P = 0.34$		$P = 0.26$			$P = 0.26$			$P = 0.99$
Exon2 rs4934	AA	1.00 (ref)	20 (46%)	18 (32%)	1.00 (ref)	97 (20%)	17 (31%)	1.00 (ref)	10 (30%)	38 (21%)	1.00 (ref)
	AG	1.80 (1.06; 3.06)	16 (36%)	26 (46%)	1.81 (0.74; 4.40)	55 (48%)	24 (44%)	1.54 (0.53; 4.48)	11 (33%)	100 (54%)	2.39 (0.94; 6.09)
	GG	1.49 (0.86; 2.59)	8 (18%)	12 (21%)	1.67 (0.56; 5.00)	14 (32%)	14 (25%)	1.52 (0.54; 4.28)	12 (36%)	47 (25%)	1.03 (0.40; 2.64)
	AG/GG vs. AA	1.66 (1.01; 2.76)			1.76 (0.78; 0.98)			1.52 (0.55; 4.23)			1.68 (0.74; 3.83)
	MAF		36%	45%		76%	79%		53%	52%	
	Trend	$P = 0.40$			$P = 0.27$			$P = 0.64$			

[a]Conditional logistic regression with site as grouping variable.

probably disturbs the cell differentiation program and thus can lead to modifications of the methylation landscape. Moreover, it has been shown that oxidative stress can trigger methylation changes. Therefore, anomalies of the ppO_2 probably lead to a progressive loss of methylation of the *SERPINA3* promoter. From then on, *SERPINA3* remains abnormally induced all along the pathological pregnancies. Contrary to the methylated promoter, the unmethylated T allele becomes accessible to TFs and reaches an even higher level of induction compared with the G allele especially in IUGR cases where *SERPINA3* inducers (SP1, MZF1 and ZBTB7B) are all significantly over-expressed. At mid-stage of placentation, when the second wave of invasion starts and the differentiated invasive extravillous cytotrophoblasts begins to secrete large amount of proteases to invade the maternal decidua, excessive amounts of SERPINA3 can have deleterious effects and might contribute to shallow invasion.

The genetic association study performed here has to be considered preliminary, given the limited number of placental samples that were available. Nevertheless, they have been checked on two different human populations and overall, the results, sustained by the *in vitro* experiments, comfort the idea that the T allele somehow increases the risk to develop placental diseases, especially IUGR. We did not found clear association of this SNP with PE. However, this may be explained by the co-existence of detrimental and advantageous effects of a strong SERPINA3 expression. SERPINA3 has anti-inflammatory and anti-apoptotic functions. Since maternal symptoms of PE are mainly triggered by the release of apoptotic syncitial debris and pro-inflammatory molecules in the blood flow, the abnormal up-regulation of SERPINA3 [also materialized by its increase in the maternal serum (25)] could induce compensatory effects that counterbalance PE symptoms. On the other hand, the G/A polymorphism (rs4934) located in the Exon2 of the gene was also found associated with placental diseases. The functional impact of this SNP remains to be investigated but it might influence protease targeting. The identification of detrimental and protective haplotypes using both SNP reinforces the idea that several *SERPINA3* SNPs intervene in the predisposition to the diseases and should be considered to evaluate the risk. The question of the high frequency of the T allele of the promoter in the Caucasian population (~50%) has to be envisaged in the context of balancing selection, since it can be supposed that in contexts outside placentation, a high level of expression of *SERPINA3* could constitute a significant advantage.

Our results add to the idea of the importance of SERPINA3 in the homeostasis of placental development and should encourage other teams to further investigate the predictive power of *SERPINA3* polymorphisms in placental diseases but also other pathologies in the double spectra of genetic haplotypes and DNA methylation of the promoter region.

MATERIALS AND METHODS

Patients and ethics

The French collection has already been described elsewhere (26,41). Placentas from normal and pathological pregnancies

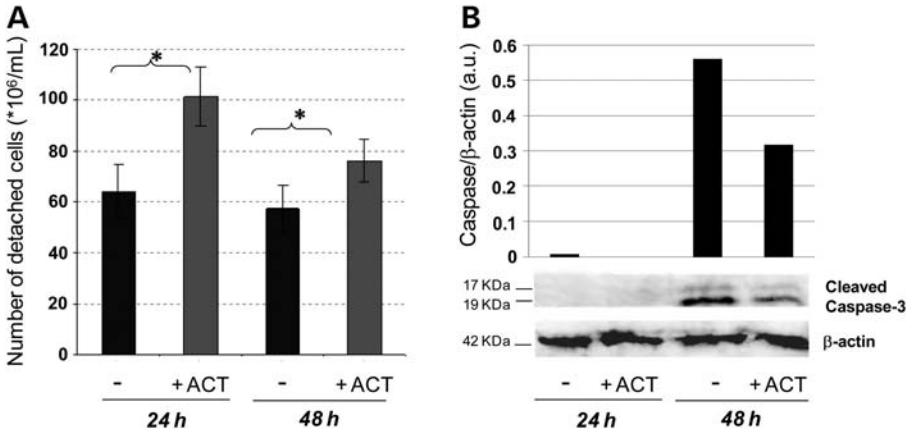

Figure 6. SERPINA3 overexpression results in a loss of adhesion of the JEG-3 cells and delay of apoptosis. Cells were collected in the supernatant of a JEG-3 cells culture, after transfection either by an expression vector encoding SERPINA3 (+ACT) or alternatively by an empty expression vector (−). (**A**) Cells found in the supernatant were counted in a Malassez cytometer. (**B**) Apoptotic status of detached cells was evaluated by western blot analysis of the protein extracts using anti-cleaved caspase-3 (monitoring apoptosis) and anti-β-actin antibodies (to check for the amount of protein in each extract).

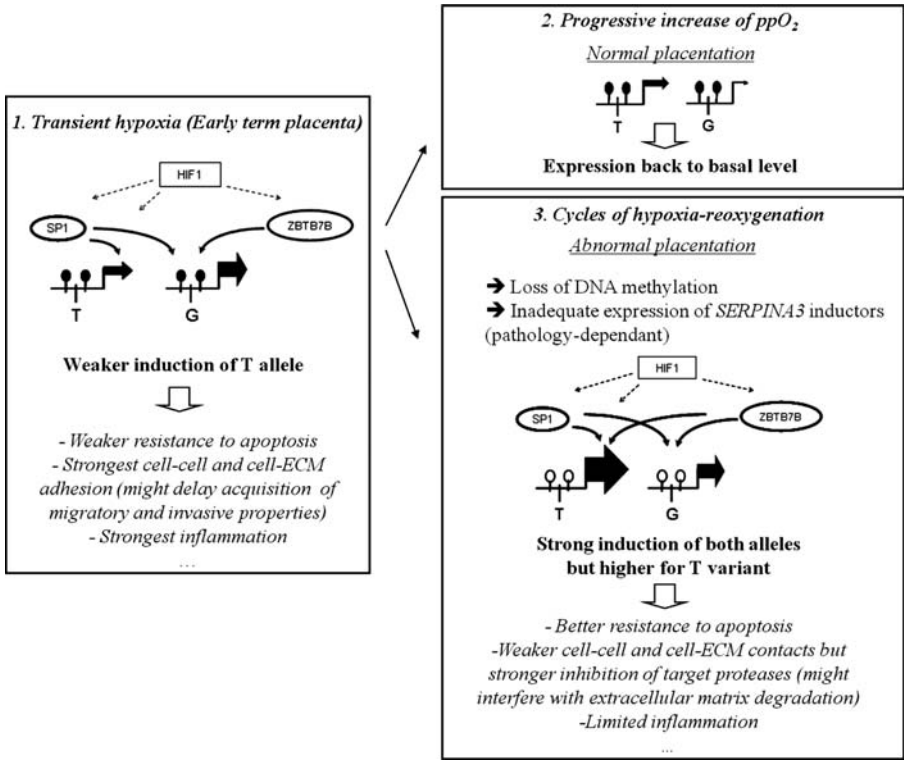

Figure 7. Putative scenario recapitulating the link between regulation of *SERPINA3* promoter and placental diseases.

were collected after Caesarean delivery, before labor, from three maternities (Paris, France), following the indications of the Hôpital Cochin ethical committee CCPPRB (Comité Consultatif pour la Protection des Personnes participant à la Recherche Biomédicale). The samples of patients and controls included in the study were collected between 27–39 weeks and 37–39 weeks of gestation, respectively. We obtained signed informed consent with access to complete clinical data for each individual. The inclusion criteria used for PE patients were: systolic pressure >140 mmHg, diastolic pressure >90 mmHg and proteinuria ≥0.3 g/24 h. The inclusion criteria used for IUGR were reduction in fetal growth during gestation with a birth weight below the 10th percentile. We classified the patients into three groups: (i) isolated PE (PE), (ii) PE associated with IUGR (PE + IUGR), and (iii) vascular IUGR (IUGR) when a notch was observed by Echo-Doppler in at least one uterine artery. Women who underwent caesarean surgery without suffering any disease during pregnancy formed the control group. Exclusion criteria included obesity, chronic hypertension, diabetes, renal diseases, multiple pregnancies and smoking habits.

Human material samples

Fragments from the placenta subchorial zone were dissected as previously described, systematically from caesarean sections, in order to avoid expression modifications induced by the labor (27,42). Most generally, we obtained the tissue fragments from two to three locations corresponding to different cotyledons, in order to correct for possible heterogeneities. Since RNA processing time is the most crucial factor to prepare placental RNA, we processed the samples in less than half an hour after the cesarean. A first fraction of chorionic villi was kept in TRIzol® Reagent (Invitrogen) and frozen before RNA extraction. Total RNA was then extracted according to the manufacturer's protocol. RNA quality was controlled (precise integrity checks and sample quantitation) by Agilent bioanalyser 2100. Only samples with RNA integrity number >8 were kept, which was the case for $>90\%$. Genomic DNA was extracted from the second fraction after mechanical grinding using an electric Ultra-turrax homogenizer, using a classical protocol (Phenol/Chloroform extraction followed by ethanol precipitation).

Genotyping

The study performed on French samples was based on PCR–restriction fragment length polymorphism assays. Specific PCRs on the promoter region or Exon2 were performed in a final volume of 10 μl containing 50 ng of DNA template, 0.1 units of Platinium Taq DNA polymerase (Invitrogen), 2 mM of $MgCl_2$ and 0.4 pmol of each primer. The amplification was started with an initial denaturation at 95°C for 2 min, followed by 35 cycles of (95°C for 15 s, 55°C for 20 s and 72°C for 30 s). Then, the amplicons (121 and 96 bp for promoter and Exon2, respectively) were digested at 37°C overnight following direct addition of 30 μl of digestion mix containing either *Van*91I (Fermentas) for the promoter analysis or *Bsu*RI (Fermentas) for the Exon2 analysis. For the promoter, the primers used were 5′-GGTCATTTCCAGTCC GAGAA-3′ and 5′-GGATTTTCATGAATGCTGAGG-3′. For the Exon2, the primers were 5′-TCTGGCCCTCTGAGA CTTAAA-3′ and 5′- AGCCGCCAAGAGCCCCAGgG-3′. Note that in this case, the reverse primer is mutated to introduce a conditional restriction site for the restriction enzyme *Bsu*RI. Digestion patterns were analyzed by electrophoresis in agarose gel.

Cell culture

JEG-3 cells were grown in Dulbecco's modified Eagle's medium Glutamax (Gibco-Invitrogen, CA, USA) supplemented with 10% of heat inactivated fetal calf serum (Gibco-Invitrogen) and 1% penicillin/streptomycin, at 37°C in the presence of 5% CO_2. To analyze the effect of hypoxia on gene expression, cells were seeded in 60 mm diameter glass dishes, placed in a Lwoff chamber at 37°C and exposed to an oxygen-depleted atmosphere (2% O_2, 5% CO_2) or maintained as control at 37°C in humidified normal atmosphere (21% O_2–5% CO_2). Cells were then harvested for RNA extraction using TRIzol® Reagent (Invitrogen) at 3, 6, 12, 24, 36 and 48 h. The kinetics was done twice independently.

Quantitative RT–PCR conditions

Four micrograms of total DNAse-treated RNA were reverse transcribed using the moloney murine leukemia virus cDNA synthesis kit (Invitrogen) according to a standardized protocol (42). Quantitative RT–PCR was carried out using 2 μl of a 10-fold dilution of the cDNA and 15 μl of mix containing amplification kit Platinum© SYBR® Green qPCR SuperMix-UDG (Invitrogen), $MgCl_2$ (4 mmol/l), BSA (0.05 g/l) and primers (10^{-3} mmol/l). The reactions were performed in a Light Cycler Thermocycler (Roche). Primers were designed from coding sequences (GenBANK) using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3). The different couples were chosen to amplify all described isoforms and aligned with BLAST software to avoid non-specific annealing. For SERPINA3, the primers were A3F: AGCAGTGGGGCTC TCAGTAA and A3R: ATAAGCAGACAGGGCCACAC. According to Ensembl, these primers are able to amplify robustly most of the protein-coding alternative transcript described in the Ensembl database (seven out of eight). The analysis of isoform-specific regulation was beyond the scope of our study, although it is an interesting question that could be raised in the future. The PCR program was 50°C for 120 s; 95°C for 120 s, followed by 35 cycles of three temperature steps (94°C for 5 s; 58°C for 10 s; 72°C for 15 s). Finally, samples were submitted to a progressive temperature elevation (from 65 to 99°C at 0.1°C/s), resulting in a melting curve, enabling to check the homogeneity of the PCR products. The products were then controlled by agarose gel electrophoresis. The Ct (threshold cycle number) values were collected with the LightCycler software (Roche) in the exponential phase of the PCR reaction. These Ct values were normalized to the Ct values obtained for expression of Succinate Dehydrogenase subunit A (*SDHA*) as a reference gene [previously shown to be stable and highly expressed in the placenta (43)] applying the ΔΔCt method. Quantitative RT–PCR was carried out in duplicates for each point.

The primers used were: SDHA.F: 5′-TACAAGGTGCG GATTGATGA-3′, SDHA.R: 5′-CAAAGGGCTTCTTCTGTT GC-3′, SERPINA3.F: 5′-AGCAGTGGGGCTCTCAGTAA-3′, SERPINA3.R: 5′-ATAAGCAGACAGGGCCACAC-3′; MZ F1.F: 5′-GAGGCTGCTGCCCTAGTAGA-3′; MZF1.R: 5′-G AGGGCTCCATCTTCTCTGA-3′; ZBTB7B.F: 5′-AGGTCT GCGGTGTTCGATT-3′; ZBTB7B.R: 5′-GGTCGTAGCTG TGCAGGAAG-3′; SP1. F: GCACCTGCCCCTACTGTAAA; SP1. R: 5′-TTGCCATACACTTTCCCACA-3′.

Plasmid constructs

The two promoter vectors (T and G alleles) were a kind gift from Drs Kalsheker and Morgan. MZF1, SP1 and ZBTB7B expression vectors were obtained from Origene. The SERPINA3 expressing vector was obtained from Invitrogen. The expression vector expressing the stabilized form of HIF1α was a kind gift from Dr M. Celeste Simon. All plasmid preparations were made using the NucleoBond® PC 500 kit (Masherey Nagel). Methylated plasmids were obtained

by incubation of 5 μg of plasmid with the CpG methyltransferase *Sss*I (New England Biolabs) during 4–6 h at 37°C according to the manufacturer's instructions, followed by DNA clean up (NucleoSpin Extract II kit, Masherey Nagel).

Luciferase assays

JEG-3 cells were seeded 24 h before transfection in 24-well plates at 30% of confluence. The cells were then transfected using the calcium phosphate method and rinsed the next day. A Renilla luciferase vector (pRL-RSV, Promega) was systematically co-transfected in all experiments to monitor transfection efficiency. Quantities of plasmid DNA used for transfections were total 1 μg per well, i.e. 590 ng of reporter luciferase, 400 ng of TF-expressing vector and 10 ng of pRL-RSV. Cells were harvested after 72 h. Transfection-induced cell mortality was taken into consideration. The day following transfection, the wells were rinsed in order to remove the dead cells and fresh medium was added. The cells were also rinsed before the cell lysis that preceded the luciferase measurement. The viability of the cells was checked under light microscope, without clear differences between the different transfections. In addition, the amount of proteins was quantified and not strongly altered by the transfections. By all means, the use of pRL-RSV as an internal control made it possible to take into consideration exclusively the viable transfected cells.

Transcriptional activity was assessed by the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Berthold Lumat LB9507 luminometer (EG&G Berthold). The experiments were performed at least twice independently with six replicates per experiment for each condition. The observed firefly activity was divided by the activity recorded from the Renilla luciferase vector, and the mean values of the replicates were calculated. These mean values were then divided by the mean values of the corresponding condition with the empty pGL3-Luc to obtain the fold induction compared with the empty vector. In the case where the constructs were methylated, each condition was compared with the one with empty methylated vector.

Western blot

JEG-3 cells were seeded 24 h prior to transfection on flasks of 25 cm^2. The cells were transfected in triplicate using either SERPINA3 expressing vector or empty vector using the calcium phosphate method and rinsed 24 h after transfection. Single transfections were performed using 5 μg of plasmid DNA/flask. Forty-eight hours after transfection, cells were scrapped directly in Laemmli buffer, sonicated for 20 s (three times), diluted in Laemmli buffer and then denatured for 10 min at 90°C. The protein concentrations were determined by the method of Bradford, using bovine serum albumin as standard. Equal amounts of proteins (25 μg) were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis on 8% gels and electrotransfered onto nitrocellulose membranes (Amersham). Molecular weight markers were run in parallel. Membranes were blocked with 5% non-fat milk in PBS/0.05% Tween-20 for 1 h, incubated overnight at 4°C with anti-cleaved caspase-3 (Asp 173) primary antibodies together with a polyclonal antibody raised against human b-actin (ACTB) both diluted 1/1000 (Cell Signaling Technology) and 45 min with secondary antibody anti-rabbit IgG-peroxydase diluted 1/5000 (Amersham Life Science). The blots were developed with ECL reagents according to the manufacturer's guidelines (Amersham Biosciences) and visualized on Kodak X-ray films. Densitometric analysis was performed using the National Institutes of Health (NIH) Image J software (http://rsb.info.nih.gov/ij) and allowed to normalize the intensity of the signal to β-actin in the samples studied.

Statistical analysis

Statistics were systematically performed using the StatistiXL add-in, and by full factorial analysis of variance, followed by *post hoc* Student–Neumann–Keuls *t*-test. Values of $P < 0.05$ were considered significant.

TF analysis

Putative TF binding sites inside the promoter were identified using the Matinspector function of the Genomatix software (http://genomatix.de).

Conflict of Interest statement. None declared.

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