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Research Article

6A3-5/Osa2 is an Early Activated Gene Implicated in the Control of Vascular Smooth Muscle Cell Functions

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Vascular smooth muscle cells (VSMC) growth plays a key role in the pathophysiology of vascular diseases. However, the molecular mechanisms controlling gene transcription in VSMC remain poorly understood. We previously identified, by differential display, a new gene (6A3-5) overexpressed in proliferating rat VSMC. In this study, we have cloned the full-length cDNA by screening a rat foetal brain cDNA library and investigated its functions. The 6A3-5 protein shows 4 putative conserved functional motifs: a DNA binding domain called ARID (AT-rich interaction domain), two recently described motifs (Osa Homology Domain), and a nuclear localization signal. The deduced protein sequence was observed to be 85% identical to the recently described human Osa2 gene. Immunolabelling, using an anti-6A3-5/Osa2 monoclonal antibody, showed a nuclear localization of the 6A3-5/Osa2 protein. In addition, PDGF upregulated 6A3-5/Osa2 expression at both the transcript and protein levels in a dose and time-dependent fashion. The pattern of upregulation by PDGF was reminiscent of the early responsive gene c-fos. The PDGF-induced upregulation of 6A3-5/Osa2 and proliferation of VSMC were significantly inhibited in a dose and sequence-dependent fashion by an antisense, but not by sense, scrambled or mismatched oligonucleotides directed against 6A3-5/Osa2. In VSMC of aortas derived from hypertensive (LH) rats, 6A3-5/Osa2 is overexpressed as compared to that in normotensive (LL) rats. The 6A3-5/Osa2-gene expression is downregulated by an ACE inhibitor and upregulated by exogenous AngiotensinII in LH rats. In summary, these results indicate that 6A3-5/Osa2 is an early activated gene that belongs to a new family of proteins involved in the control of VSMC growth.

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INTRODUCTION

Vascular smooth muscle cell (VSMC) growth plays a critical role in different pathological conditions such as atherosclerosis [1] and its clinical complications. Indeed, development of these vascular diseases is associated with a loss of vascular contractility counterbalanced by an increase of VSMC migration, proliferation, matrix secretion, and, in some cases, hypertrophy [2]. Different agonists modulate VSMC phenotype and activities in the vessel wall. For example, platelet-derived-growth factor (PDGF), particularly PDGF-BB, stimulates both proliferation and migration [3]. AngiotensinII (AngII), the active biological peptide of the renin-angiotensin system, has potent vasoconstrictor actions and is directly involved in the development of hypertension. AngII induces a multitude of signalling pathways which, depending on the VSMC phenotype, can lead to contraction, hyperplasia, or hypertrophy [4, 5]. Many transcription factors (such as c-fos [6], Ets-1 [7], NFκB [8]) and the subsequent expression of a large number of genes (eg, alpha-actin, Collagen IV, MCP-1, Endothelin-1, PDGF-A, TSP-1, bFGF, and PDGF A-chain [9]) are stimulated by AngII. However, the molecular mechanisms controlling gene transcription during these processes remain at this stage poorly understood.

A new gene (6A3-5/Osa2), which is overexpressed in proliferating, rat aortic VSMC, was initially identified by differential display [10]. This partially cloned gene of 1.2 kb, not referenced in Genbank, shares sequences homologies with the ARID (AT-rich interaction domain) transcription modulator family. ARID-containing proteins are involved in the

The first two authors are considered joint first authors of this work.

control of transcription during cell growth and embryonic development [11, 12]. However, their precise functions are not fully understood. In the current study, we have cloned the full-length rat 6A3-5/Osa2 cDNA and characterized its deduced protein sequence as a member of the ARID family. Moreover, the knock-down of 6A3-5/Osa2 expression, which is overexpressed in PDGF-dose and time-dependant manner, resulted in a significant reduction of VSMC proliferation. In vivo work showed that 6A3-5/Osa2 is overexpressed in SMC of aortas derived from hypertensive (Lyon hypertensive, LH) but not normotensive (Lyon low-blood pressure, LL) rats. The 6A3-5/Osa2-gene expression is downregulated by an ACE inhibitor and upregulated by exogenous AngII in hypertensive rats.

MATERIALS AND METHODS

Isolation of a full-length 6A3-5 rat cDNA

A 6A3-5 full-length cDNA was cloned by screening a rat foetal brain cDNA library (Origene Technologies, Inc) using primers generated from a previously derived partial sequence (Genbank accession number: AJ005202) [10], combined with in silico analysis of genome databases. BlastA (NCBI) and multiple alignments performed using ClustalW (EBI) were used for assessing sequence homologies.

Cell culture

Primary human and rat VSMC were cultured as previously described [13]. VSMC, at 80% of confluence, were serum starved for 48 h and stimulated by PDGF-BB. Dose-effect (0 to 20 ng/ml) and time-response (0, 2, 4, 8, or 24 h) experiments were performed on human VSMC. Following treatment, VSMC are harvested in Trizol or in lysis buffer (1% of 10 mM aprotinin, 10 mM leupeptine, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, 25 mM Tris pH 7.6, 150 mM NaCl, and 1% Triton X100).

Immunofluorescence

After fixation and permeabilization (100% methanol at −20°C during 5 min), nonspecific sites were blocked (PBS/3%BSA) for 1 hour at 25°C. The primary antibody (6H3 anti-Osa2 hybridoma supernatant (1 : 5) [14], mouse anti-α-actin monoclonal antibody (1 : 100) or a rabbit anti-NFKB polyclonal antibody (1 : 100), Dako) was incubated for 2 hours at 25°C. After 3 washing steps, VSMC were incubated in the blocking solution with an appropriate secondary antibody-FITC-conjugated (Dako) for 1 hour at 37°C. After 4 washing steps, coverslips were mounted and analysed by fluorescence microscopy.

Northern blot

Total RNA was isolated according to the Trizol procedure. Northerns were performed as previously described [15]. The abundance of 6A3-5/Osa2 mRNA was normalized with respect to 18 S rRNA and the ratio expressed in arbitrary units (au).

Western blot

Nitrocellulose membrane bearing electrotransfered proteins (30 μg), separated on 7% SDS-polyacrylamide gels, were blocked for 4 hours at 37°C with TBS/ 0.05% Tween20/3% gelatine, and incubated overnight at 4°C with an anti-Osa2 antibody (6H3, 1 : 5 [14]). A swine anti-mouse antibody, conjugated to horseradish peroxidase (Bio-Rad), was then used with a chemiluminescent technique (ECL kit^{TM}, *Amersham*). Expression level of 6A3-5/Osa2 protein was estimated by Quantity One tool (Bio-Rad) and normalized with Coomassie blue staining.

Gene knock-down by antisense oligonucleotides

The sequences and locations of the generated oligonucleotides targeted against human Osa2 cDNA AF468300 are summarized in Table 1. For transfection experiments, VSMC at 60–70% of confluence were serum-starved for 48 h and then incubated with 25–200 nM ODN at concentration in serum- and antibiotic-free MEM medium in the presence of oligofectamine (Invitrogen). After 4 hours, VSMC were stimulated by PDGF (20 ng/ml) for different periods of time (0, 2, 4, 6, and 24 h) and then harvested in a cell lysis buffer or Trizol. Alternatively, after transfection, VSMC were stimulated by PDGF for 24 hours and used for Bromodeoxyuridine incorporation test (Roche) to estimate cell proliferation.

Animal studies

Protocols for animals' (Lyon hypertensive (LH) and Lyon low-blood pressure (LL) strains) housing and treatment have been previously detailed [16]. Three groups were used: the first group (controls, $n = 8$) was untreated and used as controls. The second group (Ace I, $n = 8$) was treated with an ACE inhibitor, perindopril (3 mg/kg/d), for 4 weeks. The third group (Ace I+ANGII, $n = 8$) was treated with an ACE inhibitor, perindopril (3 mg/kg/d) and perfused subcutaneously with AngII (200 ng/kg/min) for 4 weeks.

Quantification of 6A3-5/Osa2 mRNA by quantitative-PCR

Frozen rat aortas were homogenized at 0°C in 500 μl Trizol and total RNA isolated. Reverse transcription product (Superscript II, *Invitrogen*) was used for quantitative real-time PCR (Q-PCR) on an ABIPrism 7900. Q-PCR assay was carried out using the Assay-on-Demand for 6A3-5, calponin, SM22-alpha, and 18 S mRNA levels were using the comparative Ct method.

Immunohistochemistry

Immunohistochemistry was performed on frozen aorta sections (5 μm) fixed in acetone as previously described [15] with an anti-Osa2 hybridoma supernatant (6H3) [14] or an anti-α-actin (Dako) monoclonal antibody. Primary antibody binding was detected using a secondary antibody

Table 1: Homologies of rat 6A3-5 sequences with ARID proteins. Homologues are divided into two subgroups. The first subgroup, which would define the subfamily called Osa, has members bearing an ARID motif and two OHD domains. The second subgroup indicates different ARID proteins.

	Species	Yeast	Drosophila	Mouse	Human
Subgroup 1	Names	Swi1	Osa, *eyelid*	Osa1	Osa1, *P270, B120, BAF250*
	Accesion no	M84390	AF053091	AF268912	AF521670
	Chromosome no	—	3	4	1p35-p36
	cDNA Length	3027 bp	10601 bp	7041 bp	6418 bp
	Protein Length	825 aa	2715 aa	1902 aa	1999 aa
	Functions	– Member of yeast SWI/SNF	– Member of Brahma complex – Antagonize wingless pathways	– Interaction with Brahma chromatin remodelling complex	– Member of human swi/snf – Co-factor of transcriptional activation by the steroid hormone receptors
	Name	nd	nd	nd	Osa2, held/Osa1, KIAA1235
	Accesion no	—	—	—	AF521671 and AF468300
	Chromosome no	—	—	—	6q25.1–q25.3
	cDNA Length	—	—	—	5482 pb
	Protein Length	—	—	—	1740 aa
	Functions	—	—	—	– Member of human swi/snf – Promotes transcriptional activation by the steroid hormone receptors
Subgroup 2	Name	nd	Dead Ringer (Dri)	Bright	DRIL-1
	Accesion no	—	U62542	U60335	U88047
	Chromosome no	—	—	10	19p13.3
	cDNA Length	—	3696 bp	4842 bp	2725 pb
	Protein Length	—	901 aa	601 aa	593 aa
	Functions	—	– Embryo patterning – Target sequence: AGATT/ATAA	– B-Cell activator – Target sequence: AGATTAA	– Binds the pRb controlled transcription – Target sequence: A/GATT/ATAA
	Name	nd	nd	Mrf2	Mrf2
	Accesion no	—	—	AF280065	M733837 (partial sequence)
	Chromosome no	—	—	10	10
	cDNA Length	—	—	3647 bp	—
	Protein Length	—	—	1188 aa	—
	Functions	—	—	– Accumulation of lipids in postnatal life – Target sequence: AATA(C/T)	—
	Name	nd	nd	Jumonji	Jumonji
	Accesion no	—	—	BC05244	U57592
	Chromosome no	—	—	—	6q24.p23
	cDNA Length	—	—	4939 bp	—
	Protein Length	—	—	1324 aa	1266 aa
	Functions	—	—	– Neural embryogenesis	Highly expressed by neuron cells during development

TABLE 2: *List of antisens oligonucleotides directed against 6A3-5*. Sequence and position (on human homologous held/Osa2) of different ODN directed against 6A3-5. Only ODNAS3 showed significant effects on 6A3-5 expression. Scrambled ODN3 (ODN Scr3) and mismatched (ODN Mis3) as used to test sequence specificity of ODNAS3.

Name	Sequence 5′–3′	Position/AF468300
ODNAs1	agcttgtcgaacttactggct	3870–3890
ODNAs2	cagcttgtcgaacttactggctt	3869–3889
ODNAs3	tgggatctgcccatg	57–71
ODN Scr3	agctcggttcacggt	—
ODN Mis3	agggagctaccc ctg	57–71
ODNAs4	tcacatctgagaatgg	2245–2260

conjugated to horseradish peroxidase followed by 3-amino-9-ethylcarbazole (Dako). The specific location of the α-actin in the media of aorta was used to define the medial boundaries. The media thickness was then measured at a magnification of X40 in slides counterstained with Haematoxylin (Dako).

RESULTS

Cloning of full-length rat 6A3-5 cDNA

The cloned gene has a 6569 bp cDNA sequence (GenBank accession number: AJ440711) and a deduced amino acid sequence corresponding to a 5276 bp open-reading frame (Figure 1(a)). The cDNA contained 1268 bp in the 3′-untranslated region; the 5′-untranslated sequence is not totally cloned. The putative 1758 amino acid 6A3-5 protein has an expected molecular weight of 180 kDa and bears four conserved motifs (Figure 1(b)). The first motif is a DNA binding domain, called AT-rich interaction domain or ARID, located in the N-terminal half (aa 568 to 672). Two other motifs comprising evolutionary conserved domains known as OHD (Osa Homology Domain)-1 (aa 1114 to 1200) and OHD2 (aa 1437 to 1758) are present within the *C*-terminal half of the protein. These three motifs are the signature of a novel family of transcription modulators called Osa family and indicate that 6A3-5 is the rat Osa2 homologue. Finally, a fourth motif represented by a nuclear localization signal is also present in the *C*-terminal of 6A3-5/Osa2 sequence suggesting a nuclear localization of this protein that was subsequently confirmed.

Multiple sequence alignment and homologies to rat 6A3-5/Osa2

Protein similarity searches revealed two subgroups with significant homologies to rat 6A3-5/Osa2 protein. The 1st subgroup comprises proteins bearing ARID, OHD1, and OHD2 functional domains. This group shows a remarkably high degree of conservation of amino acid sequences, and includes the recently cloned human Osa2 [14]. This protein appears to be the human orthologue of rat 6A3-5/Osa2, mouse, and human Osa1 [17], *Drosophila* Osa/eyelid [18] and yeast SWI1 protein [19] (Figure 2). The 2nd subgroup shows homologies that are limited to the ARID domain and include *Drosophila* dead-ringer protein [20], its homologues in mouse (bright) [21] and human (DRIL-1), mouse Mrf2 [22], and the murine and human jumonji proteins (Table 2).

Cellular localization

VSMC characterized with anti-α-actin antibody showed its nucleus to be equally labelled with an anti 6A3-5/Osa2 [14] or an anti-NFKB antibody. Negative controls showed no labelling (Figure 3).

6A3-5 expression in different phenotype of vascular SMC

Transcription levels of 6A3-5/Osa2 and α-actin markers were measured after dedifferentiation of ex vivo SMCs from a contractile (passage 0, P0) to an in vitro synthetic phenotype (passage 9, P9). Northern-blots showed that 6A3-5 is upregulated by 3-fold ($n = 3$) in the synthetic phenotype in comparison to the contractile quiescent phenotype. In contrast, α-actin expression is present in the contractile SMCs phenotype and lost on differentiation to a synthetic phenotype (Figure 4(a) and data not shown) [23]. The 6A3-5/Osa2 gene was significantly upregulated in a smooth muscle cell line (V8) that was observed to be highly proliferating [24] compared with secretory/ synthetic cells (results not shown).

Time course and dose effect of PDGF on 6A3-5/Osa2 in VSMC

Human and rat (data not shown) VSMC were serum starved, inducing a down-regulation of 6A3-5/Osa2 mRNA expression levels, and then treated with 20 ng/ml of PDGF-BB for 0, 2, 4, 8, and 24 hours. Northern blot analysis showed that the levels of 6A3-5/Osa2 mRNA reached a peak at 2 hours and remained above the control level for at least 24 hours after PDGF treatment (Figure 5(a)). In addition, a PDGF dose-dependant effect was also observed with a maximal increase achieved at 20 ng/ml (Figure 5(b)). Similar results were observed at 4 hours, by Western blot, for 6A3-5/Osa2-protein expression (Figures 5(c), 5(d)).

Antisense ODN inhibition of 6A3-5/Osa2 expression and VSMC proliferation

A series of 20-base phosphorothioate antisense ODN (Table 1, $\text{ODN}_{\text{AS1-4}}$) was screened for its ability to selectively inhibit 6A3-5/Osa2 protein expression in human VSMC. After transfection, VSMC were stimulated by PDGF-BB for 4 hours. The ODN_{AS3}, which hybridizes to the 6A3-5/Osa2 ATG translation initiation site, showed a significant inhibition of 6A3-5/Osa2 mRNA and protein expression in comparison to its sense, scrambled, and mismatched controls (Figures 6(a), 6(b), 6(c)). Moreover, treatment of human VSMC with increasing concentrations of ODN_{AS3}

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   1 agccccggca cccctggacc gaccatgggc agatcccagg gcagtccgat ggacccaatg
  61 gtgatgaaga gacctcagtt gtatgggatg ggcactcacc cccattcgca gccgcagcag
 121 agcagcccat acccaggagg tgcctacggc cccccaggcg cacagcggta tccccttggc
 181 atgcagggcc gggctccagg ggccctggga ggcttgcagt acccacagca gcagatgcca
 241 cctcagtatg gacagcaagg tgtgagtggt tactgccagc agggccaaca gccatattac
 301 agccagcagc cgcagccccc gcacctccca ccccaggcgc agtatctgcc gtcccagtcc
 361 cagcagaggt accagccgca gcaggacatg tctcaggaag gctatggaac tagatctcaa
 421 cctcctctgg cccccggaaa acctaaccat gaagacttga acttaataca gcaagaaaga
 481 ccatcaagtt taccagatct gtctggctcc attgatgacc tccccacggg aacggaagca
 541 actttgagct cagcagtcag tgcatccggg tccacgagca gccaagggga tcagagcaac
 601 ccggcgcagt cgcctttctc cccacatgca tcccctcatc tctccagcat cccgggggc
 661 ccatctccct ctcctgttgg ctctcctgta ggaagcaacc agtctcgatc tggcccaatc
 721 tctcctgcaa gtatcccagg ttttatggca ggcacacaaa gaaaccctca gatggctcag
 781 tatggacctc aacagacagg accatccatg tcgcctcatc cttctcctgg gggccagatg
 841 catgctggaa tcagtcgctt tcagcagagt aactcaagtg ggacttacgg tccacagatg
 901 agccagtatg gaccacaagg taactactcc agacccccag cgtatagtgg ggtgcccagt
 961 gcaagctaca gcggcccagg gcccggtatg ggtatcagtg ccaacaacca gatgcatgga
1021 caagggccaa gccagccatg tggtgctgtg cccctgggac gaatgccatc agctgggatg
1081 cagaacagac catttcctgg aaatatgcgc agcatgcccc ccagttctcc tggcatgtct
1141 cagcagggag ggccaggaat gggccgggca ccaggcccac caatgcccac tgtgaaccgc
1201 aaggcccagg aagctgccgc ggctgtgatg caggctgctg caaactcagc ccaaagcagg
1261 caaggcagct ttcctggcat gcaccagagt ggactcgtgg cctccagctc tccctacagc
1321 cagcccatga acaacaactc caacctaatg ggcacacagg cccagcccta cagcatcaca
1381 cccaccatgg tgaacagctc tacagcatct atgggtctta cagatatgat gtctcccagt
1441 gtgtccaaac tgtccgtgcc tctcaaagca gatggcaaag aagaaggtgt gccccagccc
1501 gagagcaagt caaaggatag ctacagctct cagggtattt ctcagcctcc aactccaggc
1561 aacctgccag tcccttcccc aatgtccccc agctctgcca gcatctcctc ctttcacgga
1621 gatgagagtg acagcattag cagcccaggc tggcccaaga ctccatcaag ccccaagtcc
1681 agctcctctt ccaccactgg ggagaagatc acaaaagtgt acgagctggg gactgcgccg
1741 gagaggaagc tgtgggtcga ccgctacctc acattcatgg aggagagggg gtcccccgtg
1801 tccagtctgc cggcagtggg caagaagccc ctggacctgt tccgactcta tgtgtgcgtc
1861 aaagagatcg gaggcttggc gcaggttcat acaaacaaga agtggcgcga gctggcaacc
1921 aacccgaacg ttggcacttc gagcagcgca gccagctccc cgaagaagca atatattcag
1981 tacctgttcg ccttcgagtg caaaatcgag cgtggggagg agcccccgcc ggaagtcttc
2041 agcacggggg atgcgaagaa gcagcccaag ctccagccgc catctcctgc caactcggga
2101 tccttacaag gtccacagac gccacagtca actggcagca gttccatggc agaggttccc
2161 ggcgacccga agccaccaac cccagcctcc accccctcacg gacaggggac ccccatgcaa
2221 agcggaagaa gcagtacagt cagtgtgcac gacccgttct cagacgtgag tgattcagcg
2281 tacccaaaac ggaactccac gactccaaac gccccatacc agcagggcat gggcatgcca
2341 gacatgttgg gcaggatgcc ctatgcgccc aacaaggacc ctttcagtgg aacgagaaaa
2401 gtgcctggaa gcagcgagcc ctttatgaca caaggacaga tgcccaacag cagtatgcag
2461 gacatgtaca accagagtcc ctcaggtgcc atgtccaatc tgggcatggg acagcggcag
2521 caatttccct atggaaccag ttacgaccga aggcacgagg cttacgggca gcagtaccca
2581 ggccaaggcc ctcccacagg acagccaccg tatggaggac accagcctgg cctgtaccca
2641 cagcagccga attacaaacg ccatatggat ggcatgtacg ggcctccagc caagcgccac
2701 gagggagaca tgtacaacat gcagtatggc agccaacagc aggagatgta caaccagtac
2761 ggaggctcct actctggccc ggacagaagg cccatccagg gacagtatcc ctacccctac
2821 aacagagaaa ggatgcaggg cccaggccag atgcaaacag atggaatccc acctcacatg
2881 atgggtggcc ccatgcagtc atcttccaat gaggggcctc agcagaatat gtgggctaca
2941 cgcaatgata tgccttatcc ctaccagaac aggcaaggcc caggtggccc tgcacaggca
3001 ccccccttacc caggcatgaa ccgcacagat catatgatgg tacctgatca gaggatcaat
3061 cacgagagcc agtggccttc tcatgtcagc cagcgccagc cttatatgtc atcatcggcc
3121 tccatgcaac ccatcacgcg cccacctcag tcatcctacc agacgccgcc gtcactgcca
3181 aaccacatct ccagggcacc cagccctgcc tccttcccgc gctccctgga gagccgcatg
3241 tctccaagca agtccccctt cctgcctgcc atgaagatgc agaaggtcat gcccacggtc
3301 cccacatccc aggtcaccgg accacccccca cagccaccgc caatcagaag ggagattacc
3361 tttcctcctg gctcagtaga agcatcgcag ccagtcccga aacaaaggcg gaagattacc
3421 tcaaaagata ttgttactcc cgaggcgtgg cgtgtgatga tgtcccttaa atccggtctg
3481 ttggctgaga gcacgtgggc tttggacacc atcaacattc tcctctatga tgacagcact
3541 gtcgccacct tcaatctttc ccagctgtcg ggttcctgg aactactagt ggagtacttc
3601 cgaaagtgcc taattgacat tttcggtatt cttatggaat atcaagtggg cgaccccagc
3661 caaagggcgc tcgatcaccg cacagggaag aaagacgaca gccagtcctc ggaggacgac
3721 tctgggaaag aagaagaaga tgccgagtgt ctggaggagg aggaggagga ggaggaagag
3781 gaggaggagg aagaggagca agtcagtaaa aagacagagt cagagggcaa gagcagctct
3841 gccctagctg ctccagacac cactgcggac cccaaggaga cgccgaggca ggccagtaag
3901 ttcgacaagc tgcccataaa gattgtcaag aagaacaacc tgtttgtggt ggaccggtcc
3961 gacaggctgg gtcgggttca agagttcaac agcgggctcc tccactggca gctgggtggt
4021 ggcgacacca ccgagcacat cctcactcac ttcgagagca agatggagat ccctcctcgc
4081 aggcgtccac ctgcgcctct cagctccacg ggtaagaaga aagagctggc aggcaagggc
4141 gattctgaag agcagccaga gaaaagcatc atagccacca tcgatgatgt cctgtctgcc
4201 cgaccagggg ccctgccgga agacagcaac cccggacccc aaaccgagag cggcaagttt
4261 ccctttggaa tccagcaggc caaaagccac cggaacatca ggctcctgga ggacgagccc
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(a)

Figure 1: *Full-length ORF sequence of rat 6A3-5, 6569 pb.* (a) The full-length rat 6A3-5 cDNA has a first methionine that corresponds to an ATG codon (position 25 underlined) and a stop codon (TGA, position 5301) followed by polyadenylation signal (position 6299). (b) ORF translation sequence with 1758 aa. Conserved motifs are indicated: ARID motif (568–672) is underlined, OHD motifs (OHD1: 1114-1200; OHD2: 1437–1758) are boxed, and NLS (nuclear localization signal) (1352–1369) in bold.

4321 aggagccgag acgagactcc cctgtgcacc attgcgcact ggcaggactc cctggccaaa
4381 cgctgcatct gtgtgtccaa catcgtgcgg agcttgtctt tcgtgcctgg caacgacgca
4441 gagatgtcca aacatccggg cttggtgccg atcctaggaa agtcgattct gctgcatcat
4501 gagcatccgg agagaaaacg ggcgcccacag acctatgaga aggaggagga tgaggacaag
4561 ggggtggcct gcagcaaaga tgagtggtgg tgggactgcc tcgaggtctt gcgggacaac
4621 acactggtca cattggctaa catttccggg cagctagact tgtctgctta cacagagagc
4681 atctgcttgc cgatcctgga cggcttgctg cactggatgg tgtgcccgtc tgcagaggca
4741 caggacccct ttcccactgt ggggcccaac tcagttctat cgccacaaag acttgtgctg
4801 gagacactgt gtaaactcag tatccaggac aacaatgtgg acctgatctt ggccacacct
4861 ccatttagtc gtcaggagaa attttatgct acattagtta ggtacgttgg ggatcgcaaa
4921 aacccagtct gccgagaaat gtccatggcg cttttatcga accttgccca gggggataca
4981 ctggcagcga gggcaatagc tgtgcagaaa ggaagcattg ggaacttgat aggcttcctg
5041 gaggacgggg tcacgatggc gcagtatcag cagagccagc ataccctcat gcacatgcag
5101 cccccgcctc tggaaccccc cagtgtggac atgatgtgca gggcggccaa agctttgctg
5161 gctatggcca gagtggatca gaaccgctcg gagttccttt tgcacgaggg tcggttgctg
5221 gatatctcga tatccgctgt cctgaactct ctggttgcgt ctgtcatctg tgatgtactg
5281 tttcagattg ggcagttatg acacccgtga gggcacacat gtgtgaggga acattagagg
5341 gtcacatatg actggctgtt ttctgttctc gtttatccaa tgtaggaaga aggaaaagaa
5401 aaatctttgc ttctctgccc cattcactat ttaccaattg ggaattaaag aaatcattaa
5461 tttgaacagt tataaattaa tatttgctgt ctgtgtgtat aagtacatcc gttgggggat
5521 ttctgtttct ttctcttttt tttaaccaaa gttgccgtct agtgcattca caggtcacat
5581 gtttttttgt ttttttcata atttttttca tgttgtatta cagttttagg gaagtgaatt
5641 cactttataa agtaaaaagg tttggcaaaa aatgctgata ggaaaatttc accacactga
5701 gtcaaaaagg tgaaaggaaa aattgatcct taaattgatt tcctatgaat tttattcttc
5761 gcagaatgaa aaaagcgaaa gtgcatccca ttgcccaaag ctctgtgcaa tagaaacttc
5821 tagagatgta ggtgtagggg ctcgaggtat ggcagtcagc agtctggccc agtgatgctg
5881 ttctctccac aggaaagcgg ttgcattagg cctcgagcaa aaaaccgcac tctcagttag
5941 gggtgaaaat ccactcctaa ccgccaacag caggattgct tcctcaccac gaccgccatg
6001 tctgctgcga ctcagcctcc acctcacaga tcttcgtgat tcctttcatc attttttaaa
6061 tatttttttt ttactgccta tgggctgtga tgtatataga agttgtacat taaacatacc
6121 ctcatatttt tttcttttct tttttttttt ttagtacaaa gtttttagtt tctttttcat
6181 gatgtggtaa ctacgaagtg atggtagatt taaataattt tttattttta ttttatatat
6241 tttttcatta ggaccatatc tccaaaaaac aagaaaaaga aacaaaaaat tacaaaaaat
6301 taaacaaaca aaaaaagggg gtaatgtaca agtttctgta tgtataaagt catgctctgt
6361 tgggagggca gctggtccca atttgcttca tgaatcaagg tgtggaaatg gttgcatacg
6421 gattgattta gaaaatgaat accagtacat acaaaaaaaa agaaaaaaga aaaaccaact
6481 aaatgaagaa acacaacttc aaagattttt ctgtgacaag aatccgcatt tgtatttcaa
6541 gataatgtag tttaagaaaa aaaaaaaaa

(a)

1 MGRSQGSPMD PMVMKRPQLY GMGTHPHSQP QQSSPYPGGA YGPPGAQRYP LGMQGRAPGA
61 LGGLQYPQQQ MPPQYGQQGV SGYCQQGQQP YYSQQPQPPH LPPQAQYLPS QSQQRYQPQQ
121 DMSQEGYGTR SQPPLAPGKP NHEDLNLIQQ ERPSSLPDLS GSIDDLPTGT EATLSSAVSA
181 SGSTSSQGDQ SNPAQSPFSP HASPHLSSIP GGPSPSPVGS PVGSNQSRSG PISPASIPGF
241 MAGTQRNPQM AQYGPQQTGP SMSPHPSPGG QMHAGISRFQ QSNSSGTYGP QMSQYGPQGN
301 YSRPPAYSGV PSASYSGPGP GMGISANNQM HGQGPSQPCG AVPLGRMPSA GMQNRPFPGN
361 MRSMPPSSPG MSQQGGPGMG RAPGPPMPTV NRKAQEAAAA VMQAAANSAQ SRQGSFPGMH
421 QSGLVASSSP YSQPMNNNSN LMGTQAQPYS ITPTMVNSST ASMGLTDMMS PSVSKLSVPL
481 KADGKEEGVP QPESKSKDSY SSQGISQPPT PGNLPVPSPM SPSSASISSF HGDESDSISS
541 PGWPKTPSSP KSSSSSTTGE KITKVYELGT APERKLWVDR YLTFMEERGS PVSSLPAVGK
601 KPLDLFRLYV CVKEIGGLAQ VHTNKKWREL ATNPNVGTSS SAASSPKKQY IQYLFAFECK ARID
661 IERGEEPPPE VFSTGDAKKQ PKLQPPSPAN SGSLQGPQTP QSTGSSSMAE VPGDPKPPTP
721 ASTPHGQGTP MQSGRSSTVS VHDPFSDVSD SAYPKRNSTT PNAPYQQGMG MPDMLGRMPY
781 APNKDPFSGT RKVPGSSEPF MTQGQMPNSS MQDMYNQSPS GAMSNLGMGQ RQQFPYGTSY
841 DRRHEAYGQQ YPGQGPPTGQ PPYGGHQPGL YPQQPNYKRH MDGMYGPPAK RHEGDMYNMQ
901 YGSQQQEMYN QYGGSYSGPD RRPIQGQYPY PYNRERMQGP GQMQTDGIPP HMMGGPMQSS
961 SNEGPQQNMW ATRNDMPYPY QNRQGPGGPA QAPPYPGMNR TDHMMVPDQR INHESQWPSH
1021 VSQRQ**PY**MSS SASMQPITRP PQSSYQTPPS LPNHISRAPS PASFPRSLES RMSPSKSPFL
1081 PAMKMQKVMP TVPTSQVTGP PPQPPPIRRE ITFPPGSVEA SQPVPKQRRK ITSKDIVTPE
1141 AWRVMMSLKS GLLAESTWAL DTINILLYDD STVATFNLSQ LSGFLELLVE YFRKCLIDIF OHD1
1201 GILMEYQVGD PSQRALDHRT GKKDDSQSSE DDSGKEEEDA ECLEEEEEEE EEEEEEEEQV
1261 SKKTESEGKS SSALAAPDTT ADPKETPRQA SKFDKLPIKI VKKNNLFVVD RSDRLGRVQE
1321 FNSGLLHWQL GGGDTTEHIL THFESKMEIP P***RRRPPAPLS STGKKKELA***G KGDSEEQPEK
1381 SIIATIDDVL SARPGALPED SNPGPQTESG KFPFGIQQAK SHRNIRLLED EPRSRDETPL
1441 CTIAHWQDSL AKRCICVSNI VRSLSFVPGN DAEMSKHPGL VPILGKSILL HHEHPERKRA
1501 PQTYEKEEDE DKGVACSKDE WWWDCLEVLR DNTLVTLANI SGQLDLSAYT ESICLPILDG OHD2
1561 LLHWMVCPSA EAQDPFPTVG PNSVLSPQRL VLETLCKLSI QDNNVDLILA TPPFSRQEKF
1621 YATLVRYVGD RKNPVCREMS MALLSNLAQG DTLAARAIAV QKGSIGNLIG FLEDGVTMAQ
1681 YQQSQHTLMH MQPPPLEPPS VDMMCRAAKA LLAMARVDQN RSEFLLHEGR LLDISISAVL
1741 NSLVASVICD VLFQIGQL

(b)

Figure 1: Continued.

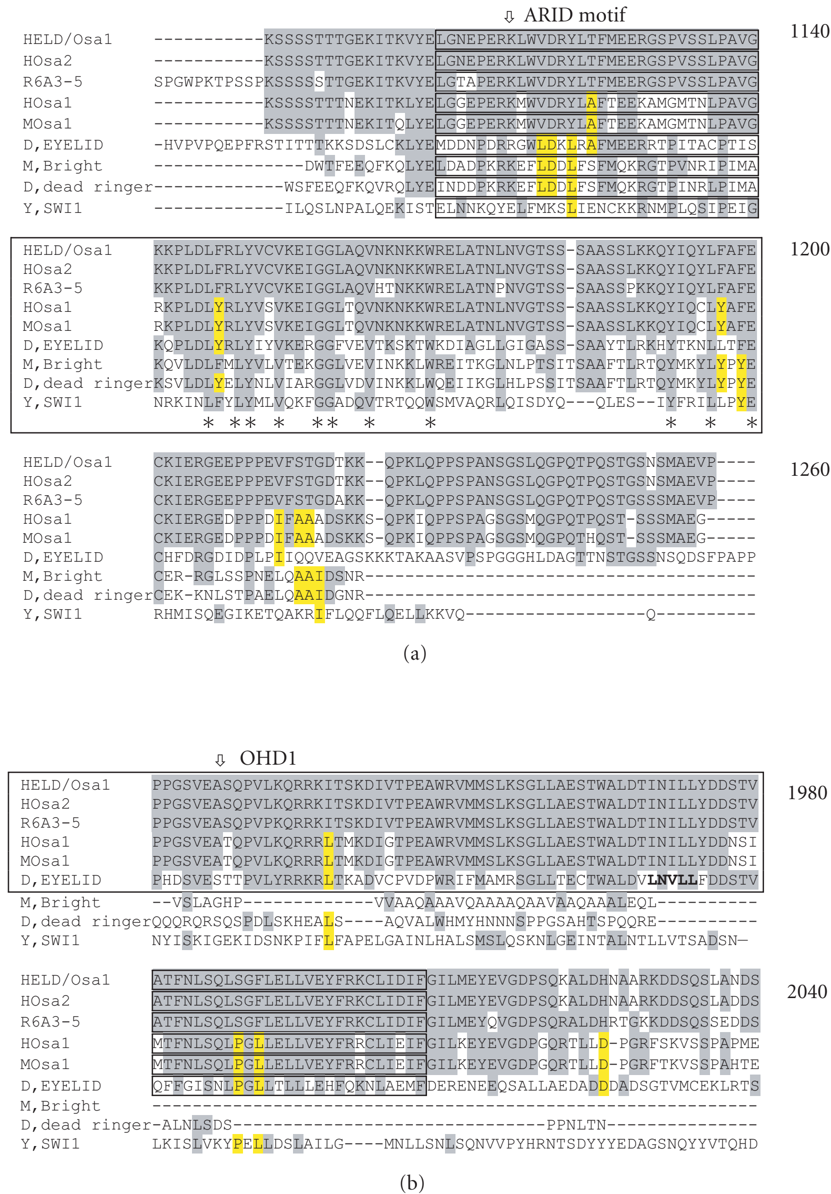

Figure 2: *Alignments of conserved domains of ARID proteins by CLUSTALW program.* (a) ARID motif alignment among different species is shown. (b) OHD-1 (Osa hoomology domain-1) motif alignment. (c) OHD-2 (Osa hoomology domain-1) motif alignment. Conserved aa residues are shown by underlining. Identical residues are indicated in grey. H: human, r: rat, M: Mouse, d: Drosophila, Y: yeast (hELD/OSA1 or hOsa2: human homolog of 6A3-5/Osa2).

(25, 100, 200 nM) resulted in a dose-dependent reduction in 6A3-5/Osa2 protein level (Figure 6(d)) but had no effect on P53 expression. Indeed, 6A3-5/Osa2 expression is reduced by 60–70% in presence of 200 nM of antisense ODN_{AS3}.

To investigate whether reduction of 6A3-5/Osa2 expression affected PDGF-induced proliferation, serum starved human VSMC were exposed to ODN_{AS3} and then stimulated by PDGF-BB for 24 hours. ODN_{AS3} reduced by 50–60% PDGF-induced proliferation in human VSMC (Figure 7(a)) while sense, scrambled, or mismatched oligonucleotides derived from ODN_{AS3} had no effect. Moreover, increasing the concentration of ODN_{AS3} significantly reduced PDGF-induced proliferation of VSMC in a dose-dependent manner (Figure 7(b)).

Expression of 6A3-5/Osa2 and vascular Phenotype in LH versus LL rats

Quantitative PCR performed on aorta excised from hypertensive (LH) rats exhibited significantly increased 6A3-5/Osa2 gene expression levels compared to those present in normotensive (LL) rats (Figure 8(a)). A significant decrease in VSMC contractile markers, calponin and

```
                                                                                        ⇩ OHD2
HELD/Osa1        KFPFGIQQAKS-------------------HRNIKLLEDEPRSRDETPLCTIAHWQDSLA        2340
HOsa2            KFPFGIQQAKS-------------------HRNIKLLEDEPRSRDETPLCTIAHWQDSLA
R6A3-5           KFPFGIQQAKS-------------------HRNIRLLEDEPRSRDETPLCTIAHWQDSLA
HOsa1            KFPFGISPAQS-------------------HRNIKILEDEPHSKDETPLCTLLDWQDSLA
MOsa1            KFPFGISPAQS-------------------HRNIKILEDDPHSKDETPLCTLLDWQDPLA
D,EYELID         RLTNGVAPCSSTPAIFDPRTTAKDEARVLQRRRDSSFEDECYTRDEASLHLVSESQDSLA
M,Bright         ----------------------------------------------EKKMALVADEQQRLM
D,dead ringerDYAHG----------------------------------EHNTTGNSSSMHDDSEPQQMNG
Y,SWI1           ILNFKKDLVIVLS------------------NISHLLEIASSIDCLLILILVISFGQPKL

HELD/Osa1        KRCICVSNIVRSLSFVPGNDAEMSKHPGL-VLILGKLILLHHEHPERKRAPQTYEKEEDE        2400
HOsa2            KRCICVSNIVRSLSFVPGNDAEMSKHPGL-VLILGKLILLHHEHPERKRAPQTYEKEEDE
R6A3-5           KRCICVSNIVRSLSFVPGNDAEMSKHPGL-VPILGKSILLHHEHPERKRAPQTYEKEEDE
HOsa1            KRCVCVSNTIRSLSFVPGNDFEMSKHPGL-LLILGKLILLHHKHPERKQAPLTYEKEEEQ
MOsa1            KRCVCVSNTIRSLSFVPGNDFEMSKHPGL-LLILGKLILLHHKHPERKQAPLTYEKEEEQ
D,EYELID         RRCIALSNIFRNLTFVPGNETVLAKSTRF-LAVLGRLLLLNHEHLRRTPKTRNYDREEDT
M,Bright         QRAVQQS-FLAMTAQLPMN--------------IR----INSQASESRQDSAVSLTSANG
D,dead ringerHHHHQTHHLDKSDDSAIENSPTTSTTTG------GSVGHRHSSPVSTKKKGGAKPQSGGK
Y,SWI1           NPMASSSSFGSESLTFNEFQLQWGKYQTFGVDILAKLFSLEKPNLNYFKSILLNKNTGNN

HELD/Osa1        DKGVACS----KDEWWWDCLEVLRDNTLVTLANISGQLDLSAYTESICLPILDGLLHWMV        2460
HOsa2            DKGVACS----KDEWWWDCLEVLRDNTLVTLANISGQLDLSAYTESICLPILDGLLHWMV
R6A3-5           DKGVACS----KDEWWWDCLEVLRDNTLVTLANISGQLDLSAYTESICLPILDGLLHWMV
HOsa1            DQGVSCN----KVEWWWDCLEMLRENTLVTLANISGQLDLSPYPESICLPVLDGLLHWAV
MOsa1            DQGVSCD----KVEWWWDCLEMLRENTLVTLANISGQLDLSPYPESICLPVLDGLLHWAV
D,EYELID         DFSDSCSSLQGEREWWWDYLITIRENMLVAMANIAGHLELSRYDELIARPLIDGLLHWAV
M,Bright         SNSISMS-----------VEMNGIVYTGVLFAQPP----------------------PPT
D,dead ringerDLPTEDK----------DASSSGKLNPLETLSLLSG-------------------MQFQV
Y,SWI1           LYDRNSN------NNHKDKKLLRRLLNLYNDNNKNNNNRHNLLNDVVSFLFSAIPLQQVL

HELD/Osa1        CPSAEAQDPFPTVGPNSVLSPQRLVLETLCKLSIQDNNVDLILATPPFSRQEKFYATLVR        2520
HOsa2            CPSAEAQDPFPTVGPNSVLSPQRLVLETLCKLSIQDNNVDLILATPPFSRQEKFYATLVR
R6A3-5           CPSAEAQDPFPTVGPNSVLSPQRLVLETLCKLSIQDNNVDLILATPPFSRQEKFYATLVR
HOsa1            CPSAEAQDPFSTLGPNAVLSPQRLVLETLSKLSIQDNDVDLILATPPFSHLEKLYSTMVR
MOsa1            CPSAEAQDPFSTLGPNAVLSPQRLVLETLSKLSIQDNNVDLILATPPFSRLEKLYSTMVR
D,EYELID         CPSAHGQDPFPSCGPNSVLSPQRLALEALCKLCVTDANVDLVIATPPFSRLEKLCAVLTR
M,Bright         APSAPGKGGVSSIGTN--------------------------TTTG--SR----------
D,dead ringerARNGTGDNGEPQLIVNLELNGVKYS----------G----VLVANVPLSQ----------
Y,SWI1           SQSADPSLLIDQFSPVISQSLTSILVIVQKILPLSNEVFEISENNSDSNSNNN-------

HELD/Osa1        YVGDRKNPVCREMSMALLSNLAQGDALAARAIAVQKGSIGNLISFLEDGVTMAQYQQSQH        2580
HOsa2            YVGDRKNPVCREMSMALLSNLAQGDALAARAIAVQKGSIGNLISFLEDGVTMAQYQQSQH
R6A3-5           YVGDRKNPVCREMSMALLSNLAQGDTLAARAIAVQKGSIGNLIGFLEDGVTMAQYQQSQH
HOsa1            FLSDRENPVCREMAVVLLANLAQGDSLAARAIAVQKGSIGNLLGFLEDSLAATQFQQSQA
MOsa1            FLSDRKNPVCREMAVVLLANLAQGDSLAARAIAVQKGSIGNLLGFLEDSLAATQFQQSQA
D,EYELID         HLCRNEDQVLREFSVNLLHYLAAADSAMARTVALQSPCISYLVAFIEQAEQTALGVANQH
M,Bright         -------TG------------ASGSTVSGGQVGLPGVSTPTMSSTSNNSLP---------
D,dead ringer------------------------SETRTSSPCHAEAPTVEEEKDEEEEEEEPKAAEEES
Y,SWI1           ---GNKDSSFNFNKNLPFVWLSSEENIGSGLLKLSEIILNINNSTSKNTLLQQQNYSKVL

HELD/Osa1        NLMHMQ-PPPLEPPSVDMMCRAAKALLAMARVDENRSEFLLHEGRLLDISISAVLNSLVA        2640
HOsa2            NLMHMQ-PPPLEPPSVDMMCRAAKALLAMARVDENRSEFLLHEGRLLDISISAVLNSLVA
R6A3-5           TLMHMQ-PPPLEPPSVDMMCRAAKALLAMARVDQNRSEFLLHEGRLLDISISAVLNSLVA
HOsa1            SLLHMQ-NPPFEPTSVDMMRRAARALLALAKVDENHSEFTLYESRLLDISVSPLMNSLVS
MOsa1            SLLHMQ-NPPFEPTSVDMMRRAPRALLALAKVDENHSEFTLYESRLLDISVSPLMNSLVS
D,EYELID         GINYLRENPDSMGTSLDMLRRAAGTLLHLAKHPDNRSLFMQQEQRLLGLVMSHILDQQVA
M,Bright         ------------------------------------------------------------
D,dead ringerHRSPVK----QENEDADQDMEGSEVLLNGGASAVGG--AGAGVG--VGVGVPLLKDAVVS
Y,SWI1           LPSINISCVQLIKCLVEKSICFENCLNNDPEILKKIASIPNLFPTDLEIFQLFTNPSVDI

HELD/Osa1        SVICDVLFQIGQL-----------------------------------------------        2700
HOsa2            SVICDVLFQIGQL-----------------------------------------------
R6A3-5           SVICDVLFQIGQL-----------------------------------------------
HOsa1            QVICDVLFLIGQS-----------------------------------------------
MOsa1            QVICDVLFLIGQS-----------------------------------------------
D,EYELID         LIISRVLYQVSRGTGPIHSVEFRLLQQRQQQQLRPGPAGKQAASAGGSATVKAETASTET
M,Bright         ------------------------------------------------------------
D,dead ringer------------------------------------------------------------
Y,SWI1           QIINQYQLLYNLKNDILTNLE---------------------------------------
```

(c)

Figure 2: Continued.

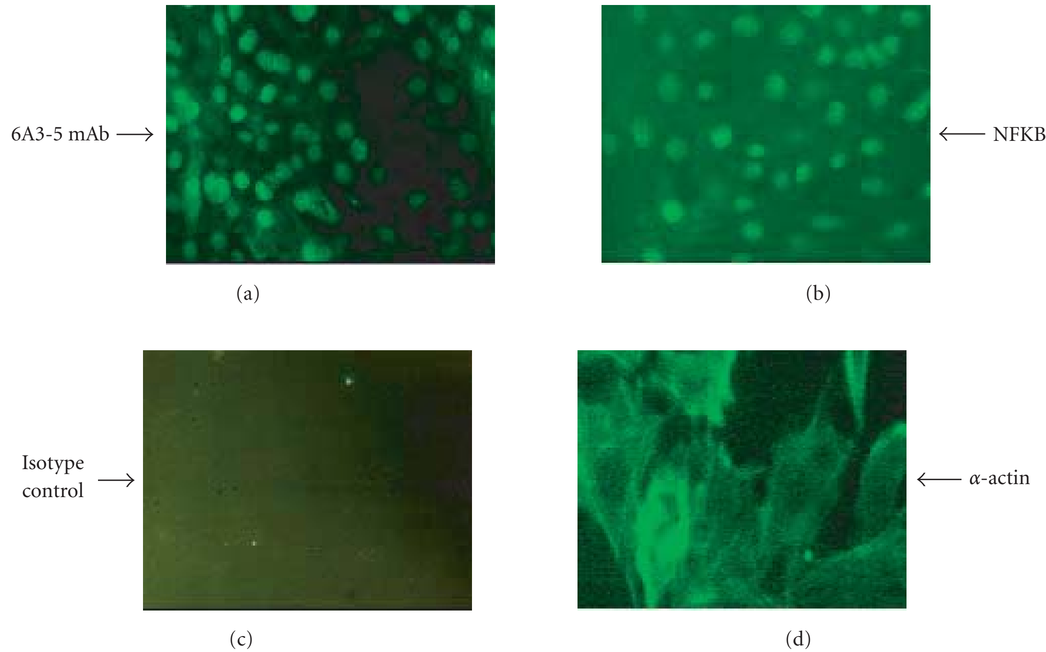

Figure 3: *6A3-5/Osa2 cellular localization on VSMC.* Actin was used as a cytoplasmic control of smooth muscle cells marker, while NFKB served as a nuclear control. Cell nucleus was labelled with an anti-6A3-5/Osa2 or an anti-NFKB antibody in comparison to isotype control (mouse IgG).

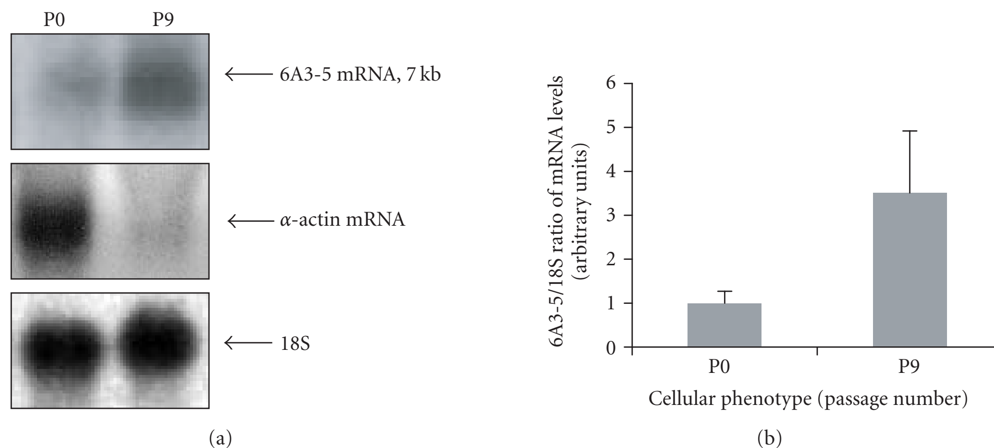

Figure 4: *6A3-5 and α-actin SMC expression in different vascular SMC.* (a) Levels of 6A3-5 gene transcription were compared, by northern blot, between the contractile (passage 0, P0) and the secretory/synthetic phenotype (9th passage, P9). Phenotypes were characterized by the α-actin SMC marker. The 18 S served as a control for loading and quantification. (b) Quantification of 6A3-5 signals, done on 3 independent northern blots, reported to the 18 S levels. Results show 6A3-5 mRNA levels to be increased by 3 folds in synthetic cells compared to contractile cells.

SM22-alpha, was observed in LH but not LL rats (Figures 8(b), 8(c)).

Immunolabelling indicated the presence of 6A3-5/Osa2 in VSMC of LH and LL aortas (Figures 9(a), 9(b)), but no labelling was observed in negative controls (Figure 9(e)). Interestingly, the 6A3-5/Osa2 antibody shows similar labelling to those observed with proto-oncogene c-fos (Figures 9(c), 9(d)). Such an increased level of 6A3-5/Osa2 was associated with a state of hypertension. Indeed, work by Aguilar et al [16] has shown that LH rats have a systolic blood pressure (SBP) of 166 ± 3.59 compared to 131 ± 2.78 mmHg for LL.

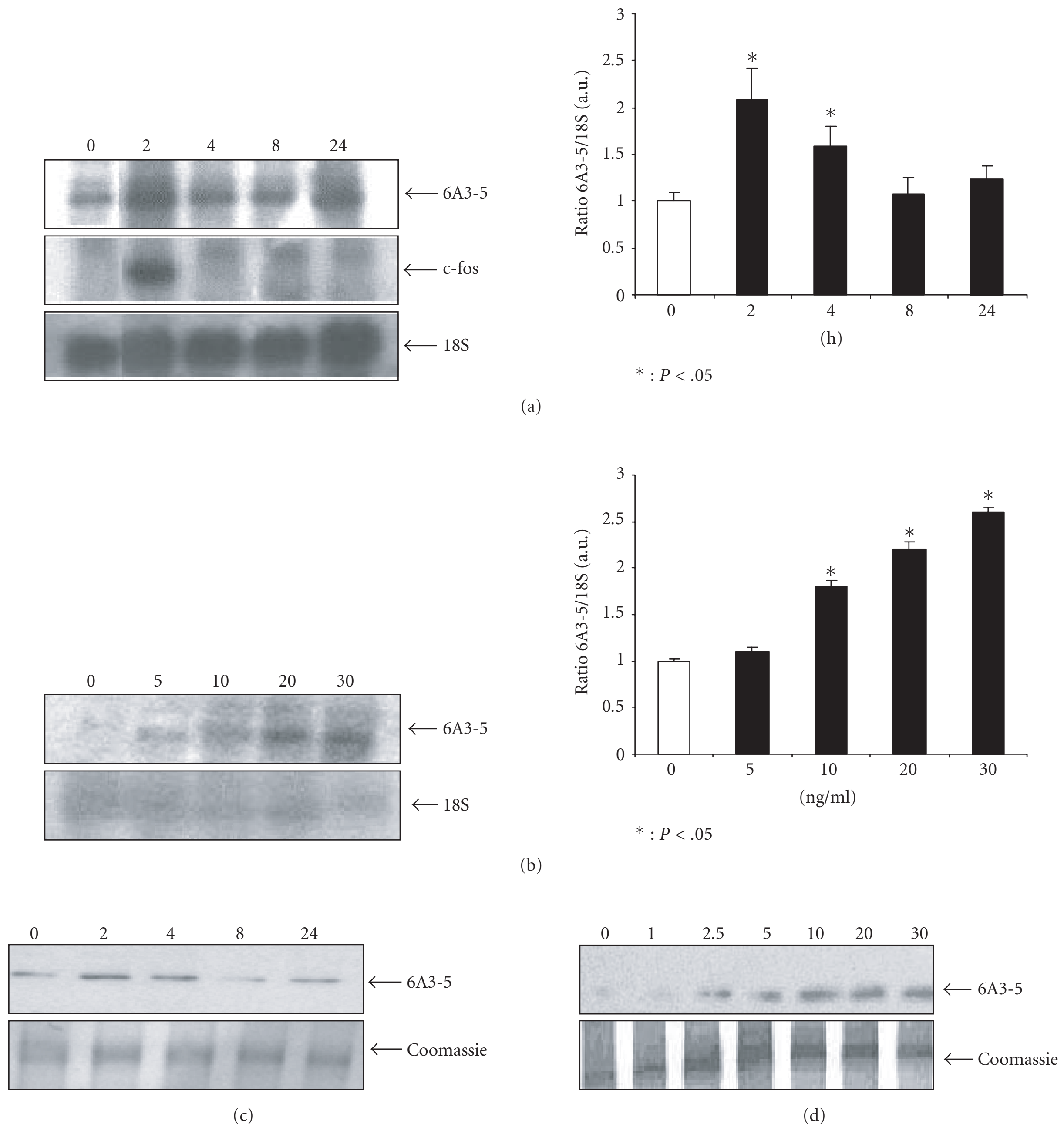

FIGURE 5: *Time course and dose effect of PDGF on 6A3-5/Osa2 expression*. (a) Time course of 6A3-5/Osa2 mRNA level analysed by Northern-blot, following treatment of human proliferating VSMC (9th passage) with 20 ng/ml PDGF. VSMC were serum starved for 48 hours before analysis. (b) Dose effect of 6A3-5/Osa2 mRNA level analysed by Northern-blot, following treatment of VSMC with increasing concentrations of PDGF for 2 hours. (c) Time course of 6A3-5/Osa2 protein levels investigated by Western blot. (d) Dose effect of 6A3-5/Osa2 protein levels investigated by Western blot, following VSMC treatment with increasing concentrations of PDGF for 4 hours. The results are representative of three independent experiments. Northern-blots were quantified by Quantity One tool (Bio-Rad) and normalized by 18 S rRNA level. Data are presented as means ± SEM.* : $P < .05$ versus nonstimulated control cells. The Coomassie blue-stained gel indicates equal protein loading.

Expression of 6A3-5/Osa2 and vascular phenotype in ACE inhibitor treated LH and LL rats

Four-week treatment with Perindopril (an ACE inhibitor) significantly reduced SBP in both LH (from 166 ± 3.59 to 134 ± 1.84 mmHg) and LL (from 131 ± 2.78 to 104 ± 2.39 mmHg) compared to untreated animals [16]. Interestingly, the 6A3-5/Osa2-gene expression level decreased in treated LH, but not LL, rats (Figure 10(a)). Moreover, VSMC contractile markers showed, by Q-PCR, a decrease

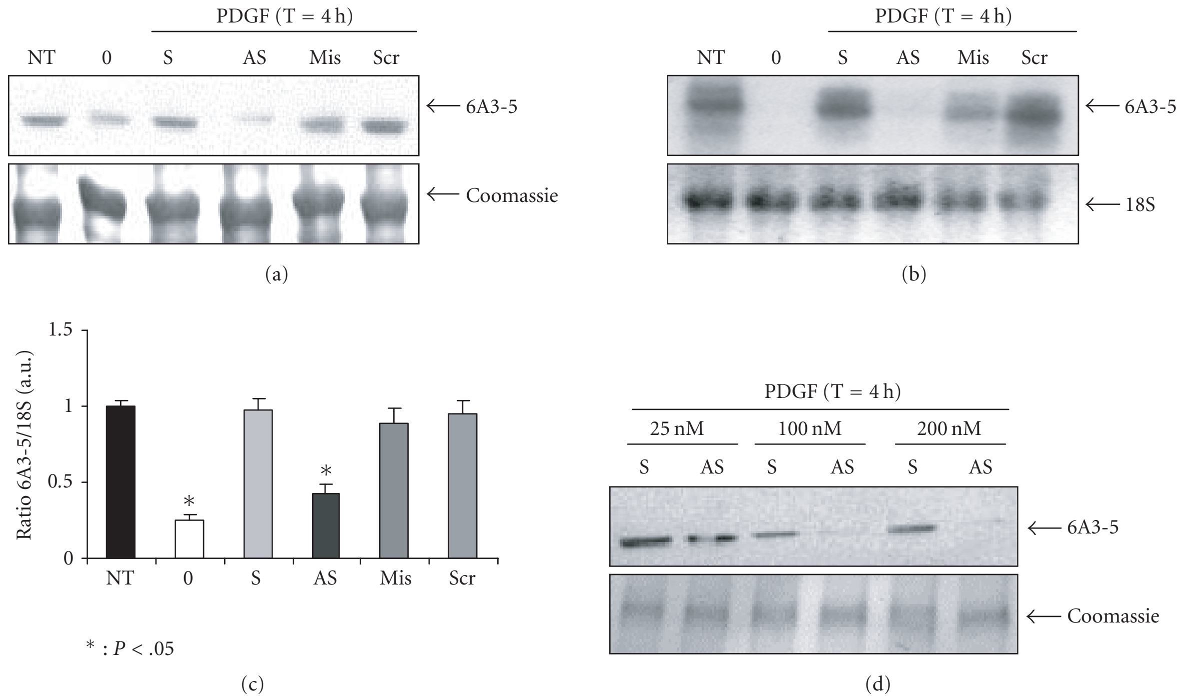

Figure 6: Inhibition of 6A3-5/Osa2 expression by ODN3 antisense. (a) Western blot of 6A3-5/Osa2 protein expression, following 4 hours of PDGF-BB stimulation. Serum starved VSMC (0) were treated, first, by 6A3-5/Osa2-ODN3 sense (S), antisense (AS), mismatched (Mis), scrambled (Scr), or vehicle (NT) at 200 nM. (b) Northern blot of 6A3-5/Osa2 mRNA expression, following 4 hours of PDGF-BB stimulation. (c) Quantification of Northern blot results, which are representative of three independent experiments. (d) Western blot of 6A3-5/Osa2 protein expression, following 4 hours of PDGF-BB stimulation. Serum starved VSMC (0) were treated, first, by 6A3-5/Osa2-ODN3 sense or antisense at different concentrations (25, 100, and 200 nM).

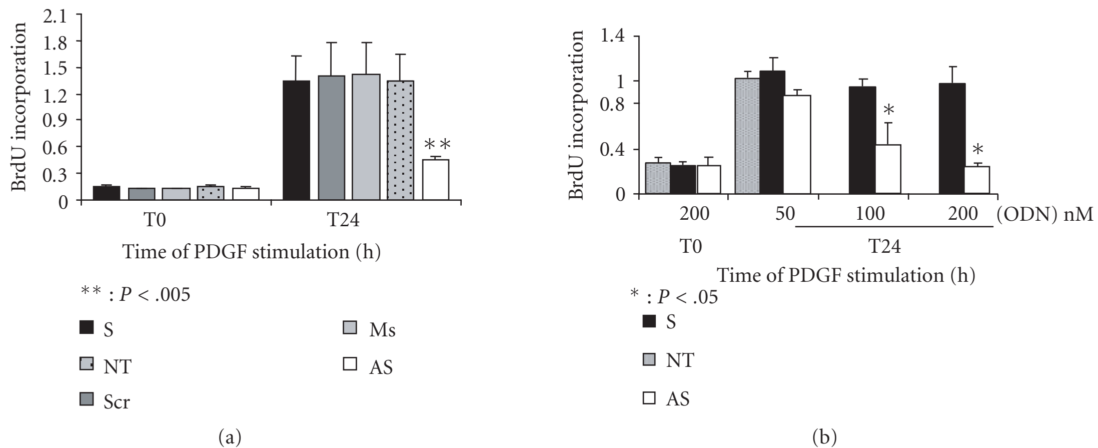

Figure 7: *Inhibition of PDGF-stimulated VSMC proliferation by antisense ODN3.* (a) Serum starved VSMC were treated by ODN3 sense, antisense, mismatched, and scrambled oligos at 200 nM followed by PDGF-BB (20 ng/ml) stimulation for 0 or 24 hours in presence of BrdU. (b) Serum starved VSMC were treated by ODN3 sense and antisense (at 50, 100, or 200 nM) following 0 and 24 hours of PDGF-BB (20 ng/ml) stimulation. Untreated VSMC are used as controls of proliferation rate. The results are representative of four independent experiments. Data are presented as means ± SEM.* : $P < .05$ versus nontransfected cells (NT).

in calponin and SM22-alpha in both LH and LL animals (Figures 10(b), 10(c)). However, Vessel wall media thickness in LH and LL was not affected by such a treatment (Figure 11(c)).

Expression of 6A3-5/Osa2 and vascular phenotype in AngiotensinII-perfused LH and LL rats

Perindopril treatment, of the 2 strains, was followed by chronic perfusion of AngII which showed, over a period

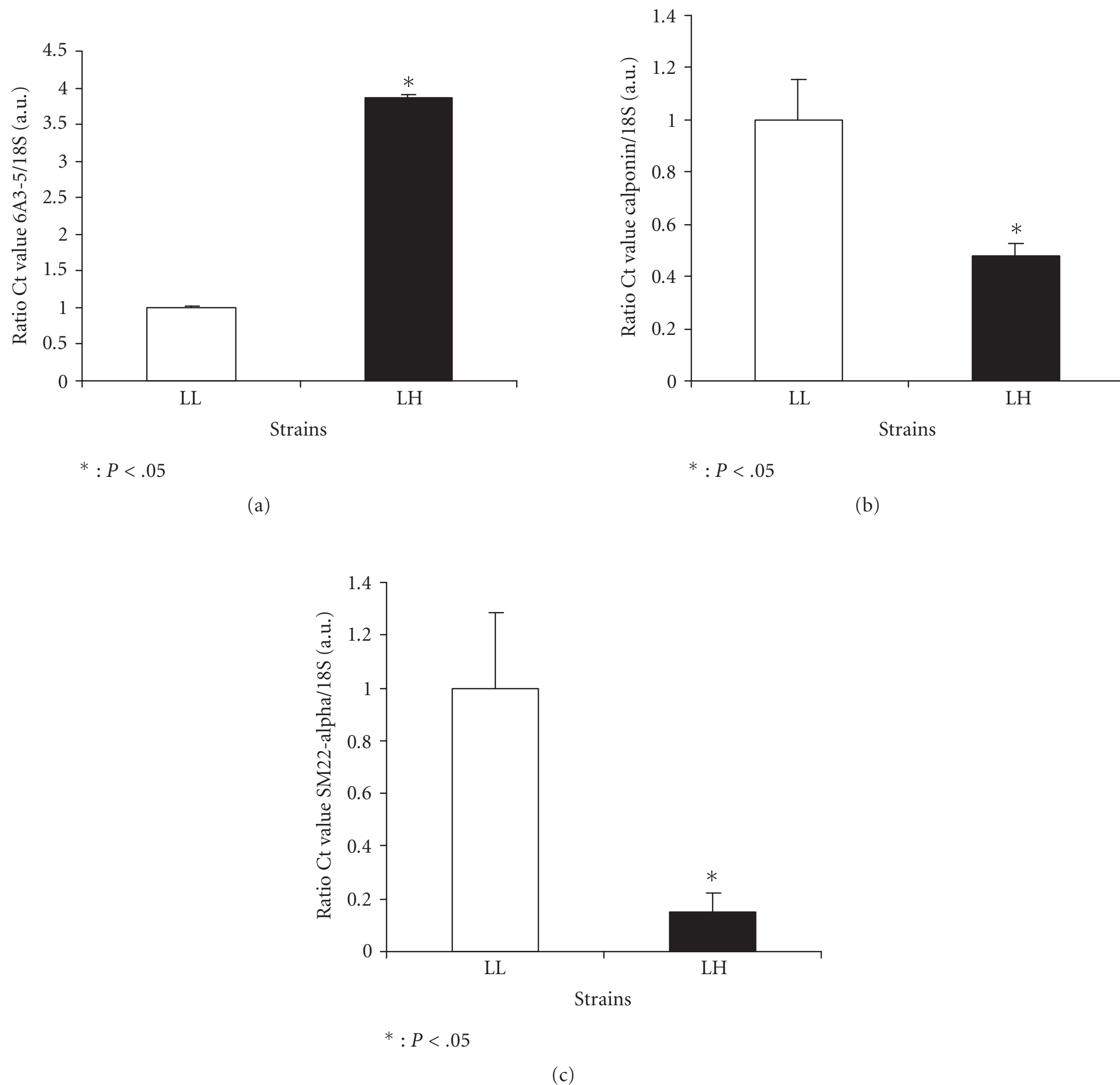

Figure 8: *Expression of 6A3-5/Osa2 and vascular phenotype in LH versus LL rats*. (a) 6A3-5/Osa2 aortic mRNA expression is significantly higher in hypertensive (LH) compared to normotensive (LL) rats. (b) Calponin (VSMC contractile phenotype marker) aortic mRNA gene expression is significantly reduced in LH versus LL rats. (c) SM22 alpha (VSMC contractile phenotype marker) aortic mRNA gene expression is significantly reduced in LH versus LL rats. Results are indicated as a ratio of mRNA expression in comparison to 18 S expression. Data are presented as means ± SEM.* : $P < .05$ versus LL normotensive rats.

of 4 weeks, an increase of SBP in LH (from 134 ± 1.84 to 231 ± 5.67 mmHg) and a steady SBP (from 104 ± 2.39 to 192 ± 5.46 mmHg) in LL rats [16]. AngII induces a significant upregulation of aortic 6A3-5/Osa2 excised from hypertensive (LH) rats in comparison to their unperfused controls (Figure 10(a)). Moreover, decrease in VSMC contractile markers, closely followed the hypertrophy state of the vessel wall in these two strains (Figures 10(b), 10(c)). In contrast, aortic 6A3-5/Osa2-gene expression was not modified in normotensive (LL) rats. One should note that AngII perfusion induced a significant aortic media hypertrophy in LH (Figure 11(c)) and to a much lesser extent in LL rats in comparison to their unperfused controls (Figure 11(c)).

DISCUSSION

This study reports the cloning and the characterization of a new gene (6A3-5/Osa2) overexpressed in proliferating rat vascular smooth muscle cells. Several lines of evidence show that this new gene is an early-gene activator that may be implicated in the control of VSMC activities.

6A3-5/Osa2 protein bears a DNA binding motif called ARID and two recently described conserved motifs, OHD1 and OHD2. These functional domains define the recently described Osa family of transcription modulator. Recently, Hurlstone et al [14] cloned the human homologue of 6A3-5 and showed that the OHD2 motif is necessary for binding

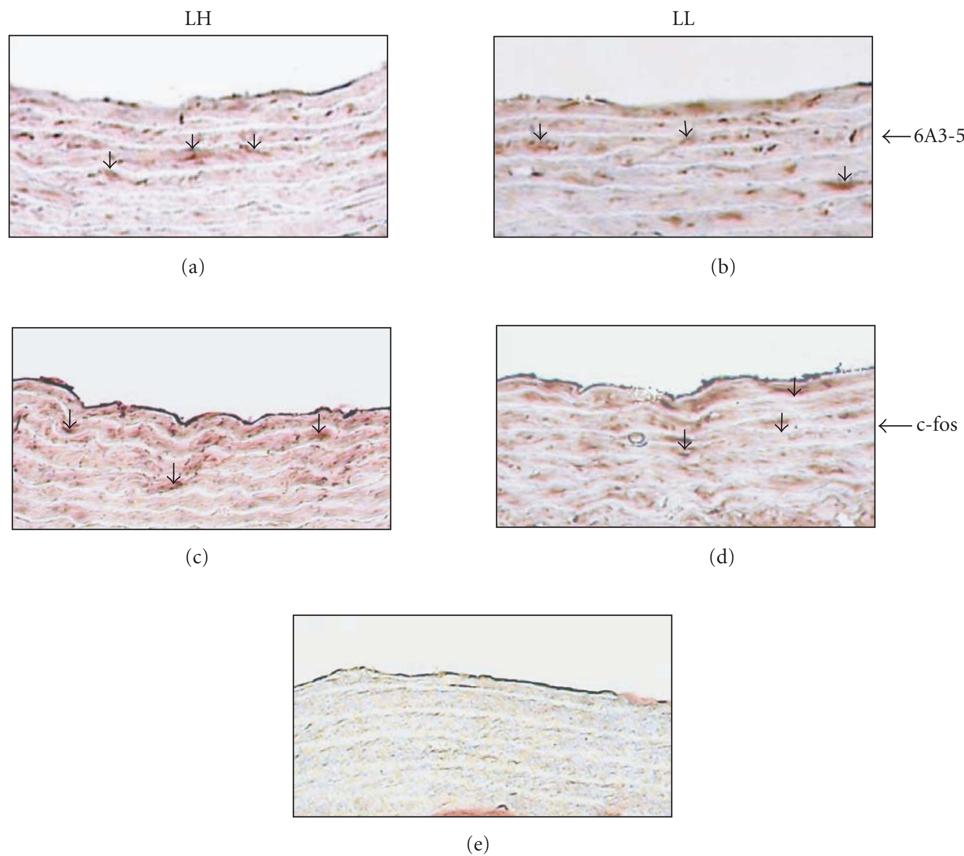

Figure 9: *Localization of 6A3-5/Osa2 in aortas from LH and LL rats.* (a) 6A3-5/Osa2 labelling is observed in SMC of the inner media from AngII-perfused LH rats (X40). (b) 6A3-5/Osa2 labelling is observed in SMC of the inner media from AngII-perfused LL rats. (c) c-fos antibody showed a similar localization to 6A3-5/Osa2 in LH rats. (d) c-fos antibody showed a similar localization to 6A3-5/Osa2 in LL rats. (e) Negative control showed no labelling. Similar localization and labelling was observed for 6A3-5/Osa2 for all tested aortas (data not shown).

BRG-1 (Brahma-related gene-1), a key catalytic component of the SWI/SNF-A chromatin remodelling complex. In contrast to other ARID proteins, Osa proteins show no sequence preference for AT rich sites. Nonetheless, work using *Drosophila* suggest that Osa proteins may participate in targeting SWI/SNF to a subset of promoters in vivo and induce the activation or repression of target gene expression. Prior to our study, no Osa protein had been described in vascular cells, and very little is known about the function of these proteins in mammals.

In this study, we have observed, in a similar way to c-fos, an early upregulation of 6A3-5/Osa2 soon after mitogenic stimulation of human or rat VSMC by PDGF-BB. Increased activity of the PDGF signalling pathway has been implicated as a contributing factor in the progression of atherosclerosis or restenosis. PDGF induces activation and phosphorylation of several cytosolic signalling molecules and nuclear transcription factors, including Egr-1 (early growth response-1), Ets-1, *c-fos*, and *c-jun*, which stimulate expression of their target genes. These data indicate that 6A3-5/Osa2 is an early PDGF-responding gene potentially implicated in VSMC proliferation. To validate this hypothesis, we generated four specific sets of ODN antisense directed against 6A3-5/Osa2. Only one of these, ODN3, is able to inhibit 6A3-5/Osa2 expression at the mRNA and the protein level in dose and sequence-dependant manner. It is interesting to note that ODN3 targets the ATG initiation site. Previous studies have demonstrated that such targeting is very effective in inhibiting gene expression by antisense phosphorothioate oligonucleotides. Indeed, ODN controls used in the present study indicated that 6A3-5/Osa2 RNA and protein depletion was due to a sequence-specific antisense effect, as neither the sense nor the scrambled or mismatched control ODNs caused 6A3-5/Osa2 depletion. Moreover, we observed no effect on p53 gene expression following Osa2 inhibition, suggesting that ODN_{AS} inhibit selectively 6A3-5 expression. We then used ODN3 in association with BrdU incorporation assays to assess the role of 6A3-5/Osa2 in VSMC proliferation. ODN3 antisense was able to significantly reduce proliferation of PDGF-stimulated VSMC in a dose and sequence-dependent manner. Recently, Watanabe et al [22] produced the first evidence that an ARID protein family member is implicated in differentiation and control of VSMC proliferation. Their study showed that overexpression of Mrf2 induces expression

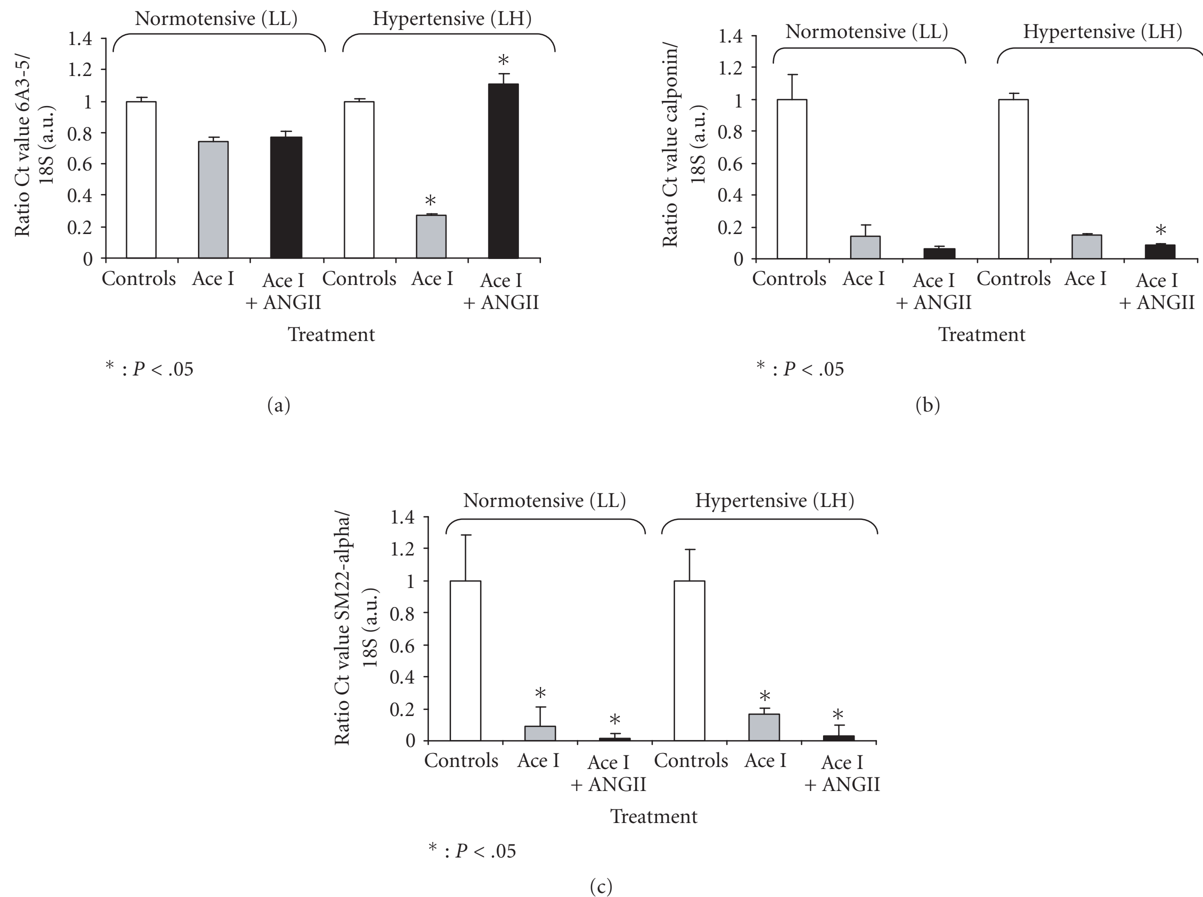

Figure 10: *Expression of 6A3-5/Osa2 and vascular phenotype in ACE inhibitor treated LH and LL rats.* (a) 6A3-5/Osa2 aortic mRNA expression in untreated LH and LL rats (controls) was compared to ACE inhibitor treated rats in the absence (Ace I) or presence of perfused ANGII (Ace I + ANGII). 6A3-5/Osa2 gene expression is downregulated by the ACE inhibitor and upregulated by exogenous AngiotensinII in LH rats. (b) Calponin gene expressions were quantified in these same animals. (c) SM22 alpha gene expressions were also quantified in these same animals. Results are indicated as a ratio of 6A3-5/Osa2 mRNA expression in comparison to 18 S expression. Data are presented as means ±SEM.* : $P < .05$ versus controls untreated rats, for each strain.

of specific smooth muscle marker, such as alpha-actin and SM-22alpha. Interestingly, in contrast to 6A3-5/Osa2, Mrf2 retarded cellular proliferation. It is interesting to note that Mrf-2 binds a specific DNA sequence (AATA(C/T)) in contrast to Osa proteins. The apparent functional divergence in regard to cellular proliferation between the two ARID-bearing proteins could be linked to different properties of their DNA binding activities. The mechanism by which 6A3-5/Osa2 influences cell proliferation is unknown. However, human Osa2 was recently shown, to stimulate transcription as a cofactor of glucocorticoid receptor-dependent transcriptional activation in cultured mammalian cells [25]. Interestingly, glucocorticoids are known to modulate proliferation and expression of some target genes in VSMC (such as IκB, NaKATPase, adrenomedullin). Further investigation will be necessary to investigate by which molecular mechanisms, that is, by which target genes 6A3-5/Osa2 influences VSMC proliferation.

In a similar way to PDGF, we have previously observed an early upregulation of 6A3-5/Osa2 in cultured rat VSMC in response to AngII [15]. Several signalling responses are shared between PDGF and AngII activation. Indeed, AngII stimulation of VSMC is associated, in a similar manner to PDGF, with an upregulation of early activated genes such as c-fos and c-myc and growth factors such as PDGF and bFGF [9]. ACE inhibition by perindopril induces a reduction of c-fos and c-jun expression in response to balloon injury [26]. In vitro study has shown a link between AngII receptor and PDGFβ receptor in cultured VSMC [27]. Moreover, AngII has recently been reported to transactivate the PDGFβ receptor by cross-talk in stroke-prone SHR rats by comparison, Wistar-Kyoto rats their normotensive controls, did not show this effect [28]. In this study, hypertensive rats (LH) had significantly higher aortic 6A3-5/Osa2 gene expression levels in comparison to normotensive rats (LL). Moreover, while perindopril treatment reduced blood pressure in these 2 strains, it only affected 6A3-5/Osa2-gene expression in LH but not LL. Finally, exogenous AngII perfusion in the presence of ACE inhibitor increased blood pressure levels in both strains but increased 6A3-5/Osa2-gene expression only in LH

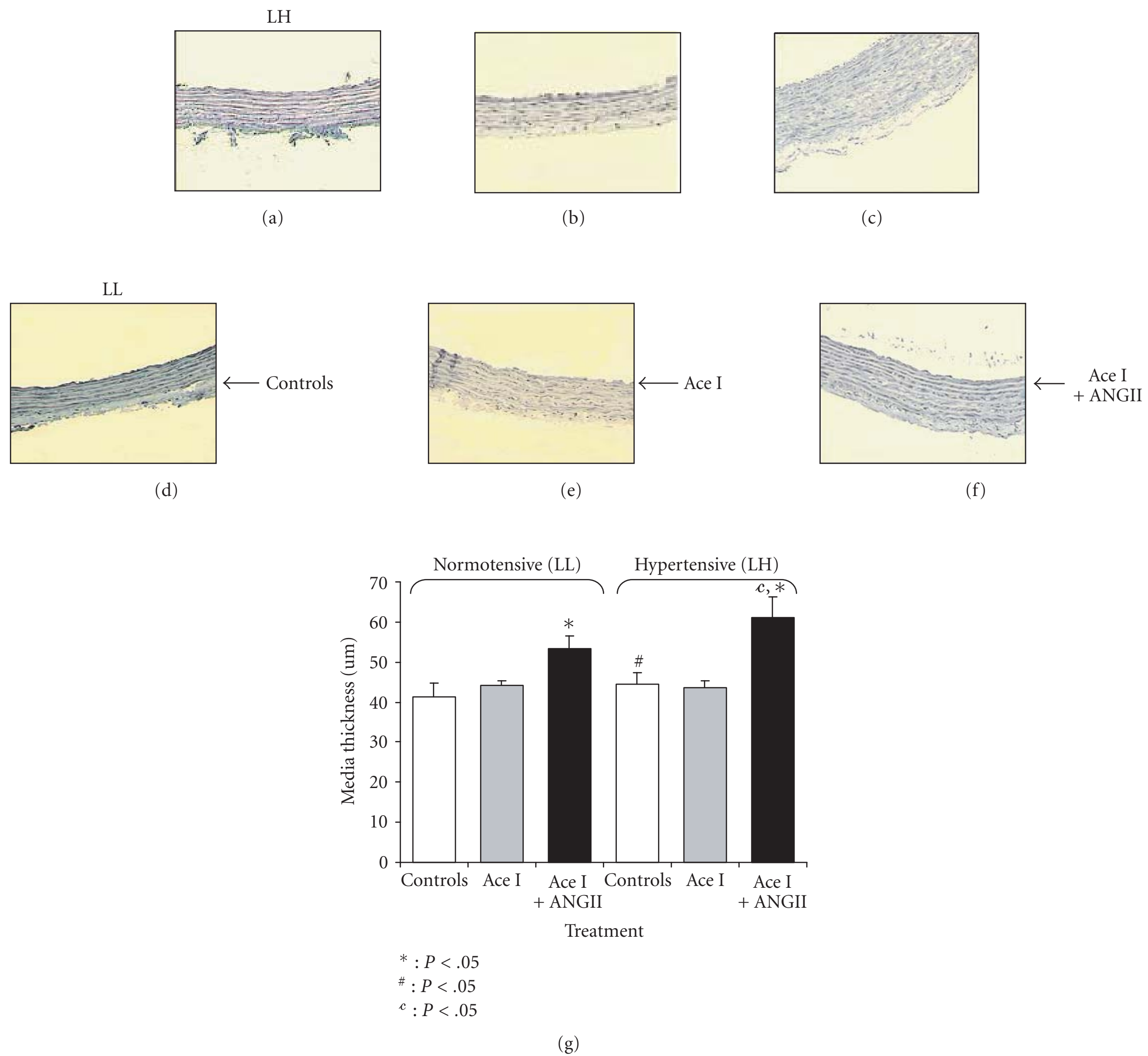

Figure 11: *Analysis of media hypertrophy*. Media thickness was determined, following haematoxylin/eosin staining of aorta sections. (a) Control LH rats were studied for their media thickness. (b) AceI treated LH rats were also analysed. (c) AceI and ANGII treated LH rats. (d) Control LL rats. (e) AceI treated LL rats. (f) AceI + ANGII treated LL rats. (g) Quantification of the above data is presented as means ± SEM* : $P < .05$ versus controls for each strain. # : $P < 0.05$ versus normotensive controls rats, c : $P < .05$ versus AngII-perfused normotensive rats.

but not LL. Interestingly, Kim et al [28] have reported that treatment of SHR rats with perindopril significantly reduced aortic PDGF-β receptor phosphorylation and ERKinase activity which is restored by chronic (but not acute) infusion of AngII. It is known that PDGFβ receptor is chronically activated in SHR compared to Wistar-Kyoto rats.

While LH rats present a higher blood pressure than LL rats, similar levels of plasma AngII were reported [29]. Interestingly, results by Lantelme et al [30], have shown that inhibition of the renin-angiotensinII system in newborn LH rats prevents the development of hypertension. It is conceivable that VSMC of LH rats are very much more sensitive to AngII compared to LL. Such hypersensitivity has been reported for VSMC, isolated from SHR rats, which show abnormal growth in vitro with accelerated entry into S phase of cell cycle and increased cdk2 activity in comparison to VSMC from Wistar-Kyoto rats [31]. Aortic gene expression of 6A3-5/Osa2 is significantly increased in LH compared to LL rats. Such enhanced expression of 6A3-5/Osa2 gene in LH rats may be linked to the potential hypersensitivity of the VSMC that not only results in increased blood pressure but modified phenotype gene markers and media hypertrophy. On treatment with an ACE inhibitor, LH rats show a significant reduction in aortic 6A3-5/Osa2 expression, not

observed in LL rats, that is presumably due to the hypersensitivity of the VSMC to AngII. Chronic perfusion of AngII, in the presence of an ACE inhibitor, induces a significant increase in 6A3-5/Osa2 expression in LH but not in LL rats. Sabri et al [32], have shown that AngII perfusion induces a reversion of VSMC to an immature phenotype. Similarily to AngII, PDGF under in vitro conditions induces suppression of smooth muscle-specific gene (□-actin and SM22alpha) through activation of Pi3K/Akt signalling pathways and subcellular redistribution of serum response factor [33]. One should also note that higher glucocorticoid plasma levels are observed in LH strains in response to AngII [16]. As previously indicated, 6A3-5/Osa2 has been implicated as a cofactor of glucocorticoid receptor-dependent transcription [25]. The overall data in this study strongly suggests that the potential hypersensitivity of VSMC, in LH rats, not only controls blood pressure levels but also 6A3-5/Osa2 expression, gene markers of VSMC phenotype, and media hypertrophy.

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