Identification, Structural, and Functional Characterization of a New Early Gene (6A3-5, 7 kb): Implication in the Proliferation and Differentiation of Smooth Muscle Cells

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Arterial smooth muscle cells (SMCs) play a major role in atherosclerosis and restenosis. Differential display was used to compare transcription profiles of synthetic SMCs to proliferating rat cultured SMC line. An isolated cDNA band (6A3-5) was shown by northern (7 kb) to be upregulated in the proliferating cell line. A rat tissue northern showed differential expression of this gene in different tissues. Using 5‘ RACE and screening of a rat brain library, part of the cDNA was cloned and sequenced (5.4 kb). Sequence searches showed important similarities with a new family of transcription factors, bearing ARID motifs. A polyclonal antibody was raised and showed a protein band of 175 kd, which is localized intracellularly. We also showed that 6A3-5 is upregulated in dedifferentiated SMC (P9) in comparison to contractile SMC ex vivo (P0). This work describes cloning, structural, and functional characterization of a new early gene involved in SMC phenotype modulation.

INTRODUCTION

Migration and proliferation of smooth muscle cells (SMCs) into the intima plays a key role in the initiation and perpetuation of atherosclerotic lesions [1, 2, 3]. Indeed, arterial SMCs are a major component of atherosclerotic plaques and restenotic vessels. According to Ross [4], proliferation of SMCs in atherosclerotic lesions is the result of an excessive inflammatory fibroproliferative response to various forms of insult to the endothelium. In these diseased vessel walls, SMCs undergo a phenotypic modulation [5, 6] where they change from a highly contractile, fully differentiated, state to a synthetic and/or proliferating dedifferentiated phenotype [4, 7, 8]. Subsequently, SMCs are transformed into foam cells by accumulating lipids [9, 10, 11]. Harvested SMCs, under in vitro conditions, progressively lose their highly contractile phenotype to another phenotype that mimics synthetic SMCs present in diffuse intimal thickening [11, 12]. In long-term cultures, aortic SMCs generate a proliferating transformed phenotype [13, 14] with similarities to proliferating cells [15].

Differences have been observed, at the gene and protein level, between the contractile and the synthetic/proliferating phenotypes. However, at this stage, a greater understanding of the genes implicated in SMC phenotypic differentiation is vital to further understand the pathogenesis of atherosclerosis [16]. In the present study, rat SMCs showing synthetic (subcultures at passage 9) or highly proliferating (spontaneously growing V8 cells) phenotypes were compared with regards to their gene expression by differential display [17]. The rationale for comparing these cell cultures relies on the similar changes in SMC phenotypes that occur in the formation and progression of vascular lesions. Results obtained allowed the identification of a new transcription factor gene, bearing an ARID motif (AT-rich interaction domain), present at high levels in proliferating cultured SMCs. This gene may play an important role in SMC differentiation and proliferation.
MATERIALS AND METHODS

Surgical procedures and animal care strictly conformed to the Guidelines of the National Institute of Health and Medical Research (decree No 87-848 of 19th October 1987). Sprague-Dawley rats (species: Rattus rattus, strain: OFA, Iffa Credo, France) used in this study were anesthetized with an intraperitoneal injection of pentobarbital (0.11 mL/100 mg body weight).

Cell culture

Primary aortic SMCs were obtained from explants of medial thoracic aortas from 7 to 8 week-old male Sprague-Dawley rats (250 g) and cultured as previously described [12, 15]. Cell samples were preserved in liquid nitrogen at passages 2–10 and then every 10 passages. SMCs at passage 10 were shown to be in a synthetic state. A spontaneously highly proliferating rat smooth muscle cell line, V8, has been used in this study. This cell line was established from aortic media of adult rat and passaged for over 200 times [15]. In stimulation experiments, PMA was given at 50 ng/mL.

Total and poly A+ RNA preparation

After cell culturing, cells were washed with Hanks medium (Sigma, France), and used for RNA preparation. Total RNA was extracted using the guanidium thiocyanate [18] method. For differential display analysis, genomic DNA contamination was removed by DNase I (Message-Clean, GenHunter, Mass, USA). For cDNA library construction and rapid amplification of 5′ cDNA ends (5′ RACE), poly(A+) RNA was isolated from total RNA using oligo dT30 primers (Oligotex mRNA Kit, Qiagen, France). Differential display analysis

Differential display was performed as previously described [17] (RNaimage, GenHunter). Briefly, (i) reverse transcription (RT) reaction: 0.2 µg of total RNA from each sample was reverse transcribed with 100 U MMLV reverse transcriptase in the presence of 250 µM dNTPs and 2 µM H-T11M (M can be either dA, dG, dC, and H is the Hind III restriction site). The 20-µL RT reaction was reverse transcribed for 1 hour at 37°C, then the enzyme is denatured by heating at 75°C for 5 minutes. (ii) PCR amplification: 2 µL of the single-strand (ss) cDNA mixture thus obtained was used for 8 different PCR reactions, each containing a different arbitrary primer from the 5′ end. The 18-µL PCR mix included 2 µL of the H-T11M primer (same as RT), 2 µM of a specific arbitrary primer, 25 µM dNTPs with 0.25 µL of α-33P dATP (200 Ci/m mole, Amersham, UK) and 1 U of Taq DNA polymerase (Perkin Elmer Mass, USA). Thermal cycling amplification parameters (40 cycles) using GeneAmp PCR System 9600 (Perkin Elmer) were as follows: 94°C (15 seconds), 40°C (2 minutes), 72°C (30 seconds), and a final 5 minutes extension step at 72°C. (iii) Separation by electrophoresis: only 3.5 µL of the PCR products was separated on a 6% denaturing polyacrylamide gel in TBE buffer after addition of 2 µL loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.09% xylene cyanole, and 0.09% bromphenol blue). The gels were run for 4 hours at 1400 V, dried without fixation for 2 hours at 80°C, exposed for 72 hours, and then visualized by autoradiography.

Band recovery, cloning, and sequencing

The sequences obtained were compared with known sequences by similarity searching in the different databases (GenBank, EMBL, EST, STS, etc.) using the BLAST [21] and FASTA [22] programs. The multiple-sequence alignment was carried out using the Omega 2.0 Software (Oxford molecular, UK).

Bioinformatics

After capillary blotting performed overnight, the membrane was baked for 2 hours at 80°C. Probes for northern blots were prepared following the random priming method (High Prime, Boehringer, Germany), using the PCR amplified inserts in the PCR II vector described above, and then purified using G-sephadex (Quick Spin Columns, Boehringer). Prehybridization and hybridization were done according to standard protocols [23]. Blots were exposed, at −70°C, with intensifying screens against a Kodak film for one week. Similar loading of RNA was assessed by using the actin probe. The following primers (Eurogentec, Belgium) were used for the preparation of cdk2a probe by RT-PCR (see below): cdk2a up: ACGGAGTGGTGTAACGAGCC, cdk2 down: GAGCTCAGGGAAAGGC.
**5′ rapid amplification of c-DNA ends (5′ RACE)**

To obtain the upstream 5′ region of the new gene, the 5′ RACE technique was carried out basically by applying the touchdown PCR principle [24] and by using Marathon cDNA amplification and Advantage KlenTaq polymerase kits (Clontech Calif, USA). (i) In the first step, ss cDNA is synthesized with 1 μg of V8 poly(A+) RNA, using 10 μM of the cDNA synthesis primer and MMLV-RT for 1 hour at 42°C. DNA synthesis was verified by the addition of dNTPs among which one was radiolabeled α - 32P dCTP (1 μCi/μL, NEN, France). (ii) The second step was the synthesis of ds DNA carried out at 16°C for 3 hours in an enzyme mixture containing E coli DNA polymerase I, Rnase H, and E coli DNA ligase. These enzymes allow the synthesis of ds cDNA, RNA degradation, and the formation of blunt ends, respectively. A 1% agarose gel electrophoresis is done to estimate the quantity and quality of the ds cDNA synthesized. The gel is then dried and put in contact with a Kodak film at -70°C in order to visualize the DNA smear. (iii) The third step allows us to obtain a library of ds cDNA, from V8 cells, by ligating an adapter to both ends of the ds cDNA, using a T4 DNA ligase at 16°C overnight. (iv) In the last step, an aliquot of the library is subjected to PCR. The 50-μL PCR reaction contains 10 μM dNTP, 10 μM of the adapter primer (complementary to the cDNA adapter), 5 μL of the 50x KlenTaq polymerase, and 10 μM of gene-specific primer (GSP) complementary to the 3′ differentially expressed fragment (6A3-5 GSP: 5′-GTATTACAGTTTTAAGGGAATGGAATTCT-3′). The mixture was subjected to a PCR step at 94°C (1 minute); followed by 33 cycles of 94°C (30 seconds), 60°C (30 seconds), and 68°C (2 minutes and 15 seconds); and a 5 minutes extension step at 72°C. The amplified DNA fragments were cloned into the PCR II vector and purified using Qiagen Plasmid Midi Kit (Qiagen). The insert DNA is then sequenced commercially (Genome Express, France).

**Screening of a rat brain cDNA library**

A cDNA library originating from the rat brain and containing hard-to-clone 5′ end of long cDNAs was purchased from OriGene Technologies, Md, USA. Screening was done according to manufacturer’s guidelines. Briefly, the 96-well master plate was screened by PCR using gene-specific primers that were constructed from the previously cloned 1.2 kb. The following primers were used: 6A3-5 U18: TTGGGATCGAAAAACC, 6A3-5 L21: TAGTGAATGGGGCAGAGAAGC, 6A3-5 U38: TAATACGACTCACTATAGGATCGTTGGGAATGGAAGAC, 6A3-5 L60: (dT)15TAGTGAATGGGGCAGAGAAGCAGACGCAAACCAGAGAGTTCAG, 6A3-5 U41: TAATACGACTCACTATAGGATCGTTGGGAATGGAAGATATTACAGTTTTAGGGAAGTGAATTC-3′). The cycling conditions (40 cycles) were as follows: 94°C (30 seconds); 94°C (15 seconds), 60°C (45 seconds), 72°C (1 minute), and a final extension step of 5 minutes at 72°C. After identification of a positive well, a 96-well subplate containing dilutions of the master positive well is then screened. The same gene-specific primers are then used on the subplate and positive wells identified. Bacteria are then plated and a clone of interest is isolated by filter hybridization. The positive clone is then inoculated, purified, and sequenced after midiprep plasmid preparation.

**Quantitative competitive RT-PCR**

The quantitative competitive RT-PCR was performed as described [25, 26]. Briefly, this technique is based on the addition of a known quantity of a serial dilution of an exogenous internal recombinant RNA (RcRNA) standard to a constant quantity of total RNA target RNA sample. Target and internal standard transcripts are reverse transcribed and amplified simultaneously with the same primers. These primers give rise to 2 bands of different molecular weights but of equal intensities when identical number of initial RNA molecules are present. (1) In the first step, synthesis of the RcRNA is done in a 4-step procedure: (i) amplification of the RdNA using 2 Rc primers constructed by Oligo 5.0 Primer Analysis Software (MedProde, Norway). This is done in a 50-μL PCR reaction containing 10 mM dNTP, 1U Taq polymerase, 100 ng of plasmid cDNA, and 10 μM of each Rc primer. (ii) The PCR product is run on a 1.5% agarose gel in order to purify the band by Jetsorb (Bioprobe Miss, USA). (iii) Transcription of the RcRNA (Riboprobe in vitro Transcription, Promega) is done in a 20 μL reaction containing 100 mM DTT, 4 μL rNTP, 20 U RNasine, and 10 U of T7 RNA polymerase for 2 hours at 37°C. (iv) The product is treated with 0.5 U RQ1 RNase-free DNase for 30 minutes at 37°C, to eliminate plasmidic DNA, and then the RcRNA concentration is measured by spectrophotometry. (2) In the second step, the RT reaction is carried out in a 10 μL total volume with 10 mM DTT, 10 mM DTT, 10 μM dN6 primers (Boehringer), 10 ng of total RNA of cultured SMCs (P9 or P20), 20 U RNasine (Promega, Wis, USA), 200 U MMLV-RT (Gibco, France), and a serial dilution of the RcRNA (10 pg/μL, 5 pg/μL, 2.5 pg/μL, 1 pg/μL) for 1 hour at 42°C. (3) In the third step, the competitive PCR is performed by using the quantitative primers with 4xPCR buffer (50 mM Kcl, 0.4% gelatin) and 1 U of Taq Polymerase. The PCR conditions (30 cycles) are as follows: 95°C (30 seconds); 94°C (10 seconds), 60°C (30 seconds), 72°C (2 minutes), and a final extension step of 5 minutes at 72°C. (4) In the last step, PCR products were resolved by gel electrophoresis and relative density of the signals was determined. The following primers (Eurogentec) were used:

6A3-5 U18: TTGGGATCGAAAAACC,
6A3-5 L21: TAGTGAATGGGGCAGAGAAGC,
6A3-5 U38: TAATACGACTCACTATAGGATCGTTGGGAATGGAAGAC,
6A3-5 L60: (dT)15TAGTGAATGGGGCAGAGAAGCAGACGCAAACCAGAGAGTTCAG,
Actin U21: TCGTACCACTGGCATTGTGAT,
Actin L17: GGGCCGGACTCATCGTA,
Actin U41: TAATACGACTCACTATAGGATCGTCACCACATGGCATTGTGAT,
Actin L57: (dT)15GGCGCGACTCATCTAGGTAGCCAGACGCAAACCAGAGAGTTCAG.
Western blot

(i) Protein extraction: cultured cells are washed with Hanks, trypsinized, and centrifuged at 1200 g during 5 minutes. The cell pellet is then lysed in a lysis buffer containing 1% of 10 mM aprotinin, 10 mM leupeptin, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (inhibitor cocktail, ICN), 25 mM Tris pH 7.6, 150 mM NaCl, and 1% Triton X100. Cell lysate is then incubated during 40 minutes at 4°C under agitation. When rat tissues are used, they were maintained at −180°C in liquid nitrogen and pound in a mortar, then homogenized with a Polytron at 0°C (two 10 seconds burst) in 50 mM Tris-buffered saline pH 7.6 containing 1% aprotinin, 2 mM e-aminocaproic acid, and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate is then centrifuged at 3000 rpm for 5 minutes to remove the unhomogenized fragment. Cell- or tissue lysate is then centrifuged at 14000 rpm for 5 minutes to remove cell debris and unlysed fragments, and the supernatant is retained. Quantification of proteins in the supernatant is realized by colorimetry (BCA kit, Pierce, France). (ii) SDS-PAGE: proteins in the supernatant are then diluted in Laemmli buffer, denatured for 5 minutes at 100°C, and separated on 7% acrylamide SDS-PAGE gels. Migration is done under a constant voltage (100 mV) in a migration buffer (200 mM glycine, 25 mM Tris, 1% SDS). The gel is then equilibrated in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 minutes. (iii) Transfer of proteins and revelation: nitrocellulose membranes (immobilon P, Millipore) are incubated in methanol for 30 seconds then rinsed in water and equilibrated in the transfer buffer for 15 minutes. The proteins are transferred to the membrane (100 V for 1 hour) then blocked for 2 hours with blocking solution containing 3% gelatin and 0.05% Tween 20 in Tris buffered saline pH 7.5 (TBS). After washing, the membrane is incubated overnight at 4°C with the rabbit anti-rat 6A3-5 polyclonal antibody (5 µg/mL) is incubated for 1 hour at 4°C with agitation every 15 minutes. Cells are then washed and centrifuged for 10 minutes at 1200 g. They are then incubated with the secondary antibody (Goat anti-rabbit FITC-conjugated, DAKO) during 45 minutes at 4°C. Following washing steps, cells are centrifuged during 10 minutes at 1200 g. The cell pellet is then suspended, fixed in 1% PBS/formaldehyde, and stocked away from light at 4°C before analysis by the FACScan apparatus (Becton Dickinson, France).

RESULTS

Differential display

Fifty bands were differentially expressed between synthetic (P9) and highly proliferating cells (V8, Figure 1). Thirty-six, out of the fifty bands, were selected because of their high molecular weight. Only 22 bands, out of the 36, were successfully reamplified and cloned into PCR II plasmid. Reproducible sequence information, containing flanking sequences corresponding to the particular poly dT and arbitrary primers used for PCR, was obtained for only 16 bands.

Sequencing and similarity searches

Sequences from different clones were then sent to GenBank, for identity and similarity search. Some sequences (3A1-1, 3A2-7, 4C1-4, 4G3-2, 4A1-4) had 80–100% similarity with known genes such as: rat glucose-regulated protein, rat assembly protein, rat glia-derived nexin, and 2C9 gene. Others (3A1-2, 4A1-7, 6A1-3, 2A3-5) showed 50–80% similarity with protooncogenes, kinases, and proteases. Four other genes (4G2, 5C1, 2A3-2, 6A3-5) had no similarities in the databases. Results of similarity searches in databases are summarized in Table 1.
Table 1. Summary of similarities (%) with identified genes in databases. Results were classified in different categories (80–100%, 50–80%, 30–50%, or no similarity). The band 3A2–4 means that the 5′ primer used was arbitrary primer 3 (AP3), and the 3′ primer was dT11A. P9* and V8* indicate synthetic and proliferating cells, respectively.

| Similarity % | Band | Cell type overexpression | Similarity with                                      |
|--------------|------|--------------------------|-----------------------------------------------------|
| 80–100%      | 4G3-2 V8* | Rat gliá-derived nexin |                                                    |
|              | 3A2-7 V8   | Bombyx nuclear polyhedrosis virus |                                                |
|              | 3A1-1 P9*  | Rat glucose-regulated protein |                                                  |
|              | 4A1-4 V8   | 2C9 gene                 |                                                    |
|              | 4C1-4 V8   | Rat assembly protein      |                                                    |
|              | 5C1 V8     | Mouse embryonal carcinoma |                                                    |
| 50–80%       | 3A1-2 P9   | G protein coupled receptor |                                                    |
|              | 4A1-7 P9   | Human protooncogene tyrosine kinase |                                              |
|              | 6A1-3 P9   | Mouse ICAM 1, c-myc      |                                                    |
|              | 2A3-5 V8   | Metalloprotease           |                                                    |
|              | 6A3-5 V8   | Homo sapiens 3′ EST       |                                                    |
| 30–50%       | 6A2-4 P9   | Thermo potent virus gene  |                                                    |
|              | 2A2-1 V8   | TSP-4 mRNA                |                                                    |
|              | 2A3-2 V8   | Mouse mammary tumor virus |                                                    |
|              | 6A3-5 V8   | Protooncogene tyrosine kinase, c-myc |                                   |
|              | 5C1 V8     | human breast cancer susceptibility virus |                                 |
|              | 4G2 P9     | —                        |                                                    |

**Northern blot analysis and tissue distribution of 6A3-5 gene**

Four genes (4G2, 5C1, 2A3-2, 6A3-5), showing no similarities in the databases, were confirmed by northern blots to be differentially expressed (Figure 2a). Three of these genes (5C1, 2A3-2, 6A3-5) were upregulated in the highly proliferating cell line, compared with synthetic cells (Figure 2a). One of these genes (2A3-2) was cloned and characterized in our lab in a previous study [27]. In this work, another gene (6A3-5, 7 kb) upregulated in the highly proliferating cell line, compared with synthetic cells, was further analyzed. Indeed, a rat multiple-tissue northern blot, probed by the 6A3-5 cDNA band, showed this gene (7 kb) to be present in different organs (Figure 2b). Some tissues such as brain, kidney, and testis showed a very high expression of the gene. Other tissues such as skeletal muscles and heart expressed the gene to a lesser extent. Testis had 3 independent mRNAs that might come from different polyadenylation sites [28]. The multiple northern blot shows that 6A3-5 gene is not an artifact induced by cell culturing but is present in vivo in different tissues.

**5′ RACE, screening of a rat brain library, cloning, and sequencing of the 6A3-5 cDNA**

Part of the 5′ coding region of 6A3-5 was obtained by 5′ RACE using a cDNA library that we constructed from the V8 highly proliferating cells. The size of the 5′ RACE-PCR product was 1.2 kb while the full length mRNA size was determined, by northern, to be 7 kb. The 5′ RACE-PCR product (Figure 3) was amplified, purified, cloned, and sequenced. This original 6A3-5 nucleotide sequence was then sent to GenBank and to the European Molecular Biology Laboratory (EMBL) to get an accession number (AJ005202). Gene-specific primers were then constructed and used on a cDNA library originating from the rat brain and containing long cDNAs. Screening of the brain library allowed the isolation of a specific clone that was fully sequenced (5.4 kb). This clone contained the previously identified 1.2 kb.

**Characteristics of the 6A3-5 cDNA and protein**

The open reading frame of the sequenced part of the gene (5410 bp) was identified and showed to contain 4708 bp running to a TGA stop codon (Figure 4). This sequence contained poly-CAG repeats between nucleotides 3896 and 3913. The 5′ untranslated region, as well as the uppermost 5′ coding region, has not yet been cloned. The cDNA contained 681 bp in the 3′-untranslated region with a typical poly-A signal (AATAAA) that was determined 73 bp upstream of the poly-A tail [29]. On the protein level, 6A3-5 had an ARID domain (187–296), LXXLL motif (1177–1181), a Q-rich region (1298–1304), a serine-rich region (112–175), and a phenyl-rich region (1472–1481) (Figure 5). Analysis of the 6A3-5 protein fragment revealed the presence of multiple glycosylation sites, phosphorylation sites, myristyl sites, and amidation sites. The hydropathy analysis data indicated that there were no significant hydrophobic transmembrane domains.
Figure 2. Northern blot analysis of 6A3-5 differentially displayed cDNA band. (a) Four genes (4G2, 5C1, 2A3-2, 6A3-5), showing no similarities in the databases, were confirmed by northern blots to be differentially expressed. The 6A3-5 gene is upregulated in proliferating (V8) but not synthetic cells (P9). Quantification of 6A3-5 signals (n = 3), reported to 28S levels, showed a 3-fold increase in the V8 compared to the P9 cells. The 6A3-5 mRNA has a size of 7 kb as given by northern blot. The internal deposition control of the same RNA quantity is given by 28S. Lanes P9 and V8 correspond respectively to synthetic and rapidly proliferating cells. (b) Multiple-tissue northern blot analysis with the 6A3-5 cDNA band in the rat. The blot contained 20 µg of total RNA from various rat tissues and was probed with the 6A3-5 cDNA fragment isolated by DD. Transcripts of ∼ 7 kb could be observed in all rat tissues analyzed, but at different levels of expression. Indeed, brain, kidney, and testis tissues expressed this gene at very high levels. Two lower transcripts of ∼ 6 and ∼ 5 kb were also observed for testis. Lane T, testis; lane K, kidney; lane Sk, skeletal muscle; lane Li, liver; lane L, lung; lane S, spleen; lane B, brain; lane H, heart.

Nucleotide similarity search

DNA FASTA search program was used to search for sequences showing relationships to rat 6A3-5 (see Table 2). Similarity searches revealed important similarities mainly with mouse, rat, and human ESTs. The highest similarities with 6A3-5 were with the following. (1) An EST (99% identity) coming from rat PC12 cells [30]. This EST clone could not be reproduced by the TIGR
institute due to contamination problems. (2) A newly identified human clone (92% identity, KIAA1235) originating from a brain library. This partially sequenced clone (5.3 kb) contains an ARID domain (AT-rich interaction domain). It is known that genes of the ARID family are important for binding to DNA [31, 32]. (3) A cDNA product (72% identity, b120) whose coding sequence was cloned as part of a search for genes containing CAG repeats [33]. (4) p270 cDNA (72% identity) which is also a transcription factor of the ARID family. It is interesting to note that b120 sequence appears to be a portion of p270, but whose coding sequence contains a frame-shift that gives rise to a truncated p270.

Protein similarity search

FASTA program identified several proteins with statistically significant degree of relationship to rat 6A3-5 (Table 3). Proteins with significant similarity to r6A3-5 include the following. (1) A translated human brain KIAA1235 clone (99% identity). (2) p270, an ARID transcription factor (78% identity) which was first identified through its shared antigenic specificity with p300 and CREB binding protein (CBP). This protein (p270 or SW11) is member of the SWI-SNF complex which is implicated in the regulation of the transcription by modifying the conformation of nucleosomes [34, 35, 36]. (3) b120 protein (78% identity) which is highly expressed in skeletal muscles and the brain. It was suggested to be implicated in lipid metabolism and could be responsible for Schnyder crystalline corneal dystrophy [37]. (4) Eyelid protein (eld, also referred to as OSA) which is another transcription factor of the ARID family. Our protein sequence had 52% identity with the eld protein. Eyelid is an ubiquitous expressed protein involved in embryonic growth, development, and differentiation of the eye in the drosophila (segmentation and photoreceptor differentiation) [38, 39]. p270 and eyelid are large proteins with high degree of identity. (5) Finally, there were also interesting similarities to other transcription factors such as Ikb epsilon, human BAT2, and APETALA-1. Sequences of KIAA1235, p270, b120, Eld, and Osa genes reveal shared motifs that are potentially functional. They bear a Q-rich region that might be implicated in transcriptional functions [40]. They also contain the amino acid motif LXXLL, which has been shown to be critical for the binding of a variety of nuclear proteins to nuclear hormone receptors [41]. Finally, they contain an ARID domain that is implicated in the binding to the DNA. This ARID domain on 6A3-5 sequence runs over 105 aa and has 86% similarity with the other members of the ARID family.

Quantitative RT-PCR analysis of the 6A3-5 gene

Levels of mRNA expression, in synthetic and highly proliferating SMCs, were also measured using quantitative competitive RT-PCR. Recombinant and quantification primers used are given in the methods section. The quantitative competitive RT-PCR on the 6A3-5 gene (Figure 6a) showed its expression to be increased by at least five times in the proliferating (7.5–10 pg) compared to synthetic cells (1–2 pg). RT-PCR was also done using an actin control, on P9 and V8 cells. This was considered as an internal control in order to verify that the same amounts of RNA would give rise to the same number of actin molecules in both cell types (Figure 6b). This control gene was expressed at the same level in both cell types. These results further confirm those observed by northern.

Structural characterization of the 6A3-5 protein in vitro and in vivo

Polyclonal antibodies were raised by rabbit immunization of specific peptides from the predicted rat protein sequence. Antibodies revealed specifically, by western blot, a unique band of 175 kd in V8 SMCs (Figure 7a). Moreover, the 175 kd protein band was also observed in different rat tissues, but at different levels of expression (Figure 7b). It is worth noting that brain tissues expressed the protein at very high levels. Furthermore, FAC-Scan analysis revealed the presence of this protein only when SMCs were permeabilized, but not in intact cells (Figure 7c). This suggests that 6A3-5 protein is not present on the cell membrane but has an intracellular localization.

6A3-5 expression in contractile versus synthetic SMCs

The implication of 6A3-5 in the phenotypic modulation of SMCs was then investigated. Transcription and protein expression levels of 6A3-5 were measured after de-differentiation of ex-vivo SMCs from a contractile (passage 0, P0) to an in vitro synthetic phenotype (9th passage, P9). Northern blots showed that 6A3-5 is downregulated in the contractile quiescent phenotype and upregulated in
Figure 4. Analysis of the different parts of the rat 6A3-5 cDNA. The nucleotide sequence (5410 bp) with the poly-A signal (AATAAA) underlined. The poly-A tail is given at the end of the sequence whereas the stop codon (TGA) is shown in bold (at position 4708). An example of poly-CAG repeats is given between 3896–3913. The 3'-untranslated region (3' UTR) is 681 bp. The uppermost 5' coding region, with the initiation codon and the 5' UTR, has not been cloned yet.

the synthetic phenotype. Indeed, results show that 6A3-5 mRNA levels are increased by 300% (3-fold increase, $n = 3$) in synthetic SMCs (Figures 8a and 8b). One should note the presence of α-actin marker in northern blots of contractile SMCs (P0) and its loss in the synthetic phenotype (P9) [16].
The above statement: (1) diATF is overexpressed in proliferating, but not synthetic, rat aortic SMCs. These results were confirmed by northern blot analysis.

Moreover, these results were confirmed by northern blot analysis. (2) 5′ RACE technique, followed by screening of a rat brain library, allowed us to clone and sequence 5.4 kb of the cDNA. (3) This new gene shows, on Northern analysis, the presence of this 7 kb mRNA transcript. Quantitative competitive RT-PCR showed that 6A3-5 gene expression is reduced to a minimum in quiescent and synchronized SMCs after serum depletion (0 minutes), in comparison to levels of expression in standard cell culture conditions (Figure 9). In contrast, 10% serum induced a peak of 6A3-5 after 2 hours of stimulation (4-fold increase). On the other hand, PMA induced a peak of 6A3-5 after 2 hours of stimulation (4-fold increase). These data indicate that 6A3-5 gene is induced at a very early stage in response to stimuli. Moreover, 6A3-5 mRNA levels decrease after 1–2 hours then increase, after 24 hours, to its normal level observed prior to serum depletion and stimulation.

**DISCUSSION**

Using differential display, we have identified for the first time a new 7 kb transcription factor gene (6A3-5) that is overexpressed in proliferating, but not synthetic, rat smooth muscle cells. Several lines of evidence back the above statement: (1) differential display shows an upregulation of 6A3-5 in proliferating but not synthetic SMCs. These results were confirmed by northern blot analysis. (2) 5′ RACE technique, followed by screening of a rat brain library, allowed us to clone and sequence 5.4 kb of the cDNA. (3) This new gene shows, on database search, important similarities to different human EST clones. Strong similarities were observed with transcription factors of the ARID family (AT-rich interaction domain). The ARID motif, which runs over 105 aa and which had 86% similarity with other ARID family members, has been identified, sequenced, and localized on our protein sequence. 6A3-5 also had similarities with functional domains such as the LXXLL motif and a Q-rich region.

(4) A polyclonal antibody, raised against a 6A3-5 peptide, showed a 175 kd unique protein band under in vitro and in vivo conditions. (5) FACScan analysis showed that the protein was only accessible after cell permeabilization. (6) 6A3-5 was upregulated, using northern and western blots, in dedifferentiated secretory SMCs in comparison to contractile quiescent phenotype. (7) This new gene was significantly upregulated, in synthetic P9 cells, 1–2 hours following stimulation by PMA or FCS.

Using differential display, we have identified a number of sequences (12) that showed either 80 to 100%, 50 to 80%, or no similarities to known genes. Five genes, in the 80–100% cluster, showed interesting similarities in databases. Indeed, the 4G3-2 sequence had a 93% similarity with other ARID family members, has been identified, sequenced, and localized on our protein sequence. 6A3-5 also had similarities with functional domains such as the LXXLL motif and a Q-rich region. (4) A polyclonal antibody, raised against a 6A3-5 peptide, showed a 175 kd unique protein band under in vitro and in vivo conditions. (5) FACScan analysis showed that the protein was only accessible after cell permeabilization. (6) 6A3-5 was upregulated, using northern and western blots, in dedifferentiated secretory SMCs in comparison to contractile quiescent phenotype. (7) This new gene was significantly upregulated, in synthetic P9 cells, 1–2 hours following stimulation by PMA or FCS.

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in the synthetic cells, it can act as a suppressor of the migration and proliferation of synthetic cells. The fourth sequence, 4A1-4, had 93% homology with 2C9 gene which is activated after overexpression of c-fos and is implicated in cellular invasion [48] or metastasis. It is interesting to note that c-myc and c-fos are upregulated during restenosis, so it is possible that this gene is implicated in cellular proliferation. The fifth gene, 4C1-4, had a 92% similarity with mouse embryonal carcinoma F9 clone [49] and with rat assembly protein associated with clathrin vesicles [50]. It is difficult at this stage to identify the relationship between these genes. However, some of them may act in concert following SMC stimulation.

The 6A3-5 cDNA band, following identification by differential display and confirmation by northern blot, was selected for further study as it was observed to be upregulated in the rapidly proliferating SMCs. This gene did not show, at the initial stage of the study, any significant similarity to known genes. DNA database search showed that 6A3-5 has significant similarities to human, rodent, and fruit fly ESTs. Interestingly, 6A3-5 shares important homologies (90–100%) with ESTs originating from human fetal brain, testis, neuronal cells, and numerous cancerous cell line libraries. One of these similarities (99%) was with an EST present in a rat pheochromocytoma PC12 cell line [30] that differentiates into a neuronal

### Table 2. Similarities of rat 6A3-5 gene with nucleotide sequences in the databases. After comparison to all databases, 6A3-5 had similarities mainly to human EST clones (established sequence tags). One interesting EST sequence was the rat PC-12 EST clone. This rat EST sequence could not be obtained and amplified. Another interesting EST clone was KIAA1235 which is thought to be a transcription factor expressed in the brain. The corresponding gene was estimated to be of 6.5 kb.

| Identity % | Similarity over (bp) | Similarity with | Total length (bp) |
|------------|----------------------|----------------|------------------|
| 99%        | 358                  | EM_EST RS6253 rat PC-12 NGF-treated cDNA (H34625) | 358               |
| 99%        | 2843                 | EM_HTG:AC094993 Rattus norvegicus clone | 178 015          |
| 89%        | 4557                 | EM_HUM:AB033061 Homo sapiens mRNA for KIAA123 | 5834             |
| 92%        | 1542                 | EM_HUM:AK000921 Homo sapiens cDNA for FLJ10059 | 1539             |
| 89%        | 2992                 | EM_HTG:AK033272 Mus musculus 15 days embryo | 2684             |
| 87%        | 4056                 | EM_HUM:AF521671 Homo sapiens SWI/SNF | 7757             |
| 88%        | 3814                 | EM_HUM:AF468300 Homo sapiens BRG1-b | 5482             |
| 88%        | 4056                 | EM_HUM:AF259792 Homo sapiens p250R | 5123             |
| 87%        | 4077                 | EM_HUM:AF253515 Homo sapiens BAF250 | 6361             |
| 88%        | 2791                 | EM_MUS:AC084826 genomic sequence | 199 47           |
| 85%        | 2796                 | EM_HUM:AL591545 human DNA sequence | 41211            |
| 85%        | 2796                 | EM_HTG:HK284653 human DNA sequence | 520 331          |
| 94%        | 1387                 | EM_MUS:AK129314 Mus musculus mRNA | 1455             |
| 89%        | 1994                 | EM_NEW:AK030698 Mus musculus 10 day | 2243             |
| 67%        | 2680                 | EM_HUM:AF521670 Homo sapiens SWI/SNF | 6418             |
| 67%        | 2680                 | EM_HUM:AF265208 Homo sapiens SWI-SNF | 6251             |
| 67%        | 2681                 | EM_HUM:AF219114 Homo sapiens chromatin remodeling | 6042             |
| 67%        | 2679                 | EM_HUM:AF231056 Homo sapiens BRG1-a | 7696             |
| 68%        | 2413                 | EM_HUM:AK074940 Homo sapiens cDNA | 2693             |
| 68%        | 2234                 | EM_HUM:AF268913 Homo sapiens OSA1 | 5120             |
| 87%        | 957                  | EM_HUM:AK025945 Homo sapiens cDNA: FLJ22292 | 2220             |
| 68%        | 1983                 | EM_HUM:AK027655 Homo sapiens cDNA: FLJ14749 | 2701             |
| 68%        | 2413                 | EM_HUM:AK074940 Homo sapiens cDNA: FLJ90459 | 2693             |
| 85%        | 840                  | EM_EST:AU117440 Homo sapiens cDNA clone | 840              |
| 92%        | 742                  | EM_EST:CD630360 FLP Homo sapiens | 757              |
| 72%        | 833                  | EM_HUM:AF265208 Homo sapiens SWI SNF Complex | 6251             |
| 72%        | 834                  | EM_NEW:AF219114 Homo sapiens chromatin remodeling | 6042             |
| 69%        | 701                  | Bovine thigh muscle gene AF045073 | 1637             |
| 57%        | 729                  | EM_INV AF053091 drosophila melanogaster eyelid gene | 10 601           |
| 93%        | 504                  | EM_EST AA709949 mouse mammary gland (T92D01.R1) | 500              |
| 93%        | 504                  | EM_EST mouse mammary gland 5′cDNA EST Aa709949 | 504              |
| 92%        | 387                  | EM_EST human neuronal 5′cDNA EST clone Aa243104 | 387              |
| 91%        | 535                  | EM_EST A673781 NCI_CGAP GAS4 HOMO (T073A09.X1) | 533              |
| 91%        | 469                  | EM_EST A439094 NCI_CGAP KID11 HOMO (T187A06.X1) | 467              |
| 91%        | 384                  | EM_EST human hela cell 5′cDNA EST clone Aa180335 | 384              |
| 91%        | 251                  | EM_EST human testis t5′cDNA EST clone Aa383798 | 251              |
| 89%        | 517                  | EM_EST human fetal lung 5′cDNA EST clone w16714 | 551              |
| 67%        | 490                  | EM_EST human fetal liver 5′cDNA EST clone N73163 | 532              |
Table 3. Similarities of rat 6A3-5 to known proteins in the databases. After comparison to all protein databases (SWISSPROT, TREMBL, PIR, ...), we had only few similarities to known proteins. The best similarities were with a number of transcription factors. p270 and b120 had 78% identity (83% similarity matching) while eyelid had 52% identity (61% similarity matching) with 6A3–5.

| Identity % | Similarity over (aa) | Similarity with Total length (aa) |
|------------|----------------------|----------------------------------|
| 57%        | SW:SMF1_HUMAN O14497 SWI/SNF-related, p270 | 1902 |
| 30%        | SW:OSA_DROME Q8IN94 trithorax group protein OSA | 2716 |
| 27%        | SW:DR11_MOUSE Q62431 dead ringer like-1 protein | 601 |
| 22%        | SW:DR1_DROME Q24573 dead ringer protein | 911 |
| 26%        | SW:DR11_HUMAN Q99856 dead ringer like-1 protein | 593 |
| 99%        | Q9UL5 KIAA1235 protein | 1485 |
| 99%        | Q9NWF5 cDNA FLJ10059 FIS, clone HEMBA 1001 | 412 |
| 78%        | Q9NUDE D50324.6 (B120, C1ORF4) | 1644 |
| 50%        | Q61603 drosophila melanogaster eyelid protein | 2715 |
| 50%        | Q9VEG7 OSA Protein | 2703 |
| 23%        | SW:GLT5_WHEAT P10388 glutenin, high molecular weight | 839 |
| 29%        | SW:W146_HUMAN Q9C0J8 WD-repeat protein WDC146 | 1336 |
| 24%        | SW:CA1A_BOVIN P23206 collagen alpha 1(X) chain protein | 674 |
| 19%        | SW:FP1_MYT TED Q25460 adhesive plaque matrix protein | 875 |
| 29%        | SW:SSXT_HUMAN Q15532 SSXT protein | 418 |
| 28%        | SW:SSXT_MOUSE Q62280 SSXT protein (SYT protein) | 418 |
| 27%        | SW:CBPA_DICDI P35085 Calcium-binding protein | 467 |
| 26%        | SW:CBP1_CAEL P34545 protein cbp-1 | 2056 |
| 34%        | SW:OSA_DROYA Q9NBG4 trithorax group protein OSA | 324 |
| 22%        | SW:SN24_HUMAN P51532 possible global transcription activator | 1647 |
| 21%        | SW:PCLO_RAT Q9JKS6 Piccolo protein | 5085 |
| 21%        | SW:FP1_MYT TC O25434 adhesive plaque matrix protein | 872 |
| 22%        | SW:NC06_HUMAN Q14686 nuclear receptor coactivator | 2063 |
| 23%        | SW:K10_DROME P13468 DNA-binding protein K10 | 463 |
| 27%        | SW:PRPL_HUMAN P10162 salivary proline-rich protein | 276 |
| 24%        | SW:CA14_CAEL P17139 collagen alpha 1(IV) chain p | 1758 |
| 24%        | SW:CA1C_MOUSE Q60847 collagen alpha 1(XII) chain | 3119 |
| 23%        | SW:DRPL_HUMAN P54259 atrophin-1 | 1185 |
| 24%        | SW:BRM_DROME P25439 homeotic gene regulator | 1638 |
| 24%        | SW:CBP_HUMAN Q92793 CREB-binding protein | 2442 |
| 25%        | SW:SSB3_CHICK Q98948 single-stranded DNA-binding | 368 |
| 24%        | SW:FYB_HUMAN O15117 FYN-binding protein | 783 |
| 26%        | SW:BCL9_MOUSE Q9D219 B-cell lymphoma 9 protein | 449 |
| 21%        | SW:NC06_MOUSE Q9J119 nuclear receptor coactivator | 2067 |
| 29%        | SW:SM41_HEMPU Q26264 41 kd spicule matrix protein | 407 |
| 21%        | SW:HR5_DROME Q960X8 hepatoocyte growth factor regulator | 760 |
| 32%        | SW:HR5_DROME Q960X8 hepatoocyte growth factor regulator | 1357 |
| 30%        | SW:HR5_DROME Q960X8 hepatoocyte growth factor regulator | 364 |

phenotype following stimulation by NGF. Another important similarity was with an ARID containing human brain clone called KIAA1235. In addition, ARID-motif bearing transcription factor genes (human p270, human b120, and drosophila eld), albeit with lower similarities to 6A3-5, have been obtained in similarity searches. It is of considerable interest that other transcription factors (IkB epsilon, human BAT2 and APETALA-1) share some similarities to 6A3-5.

On the other hand, when investigating the protein database for structural and functional relationships to 6A3-5, we come across a number of proteins having the new DNA binding motif termed ARID. It is important to note that ARID genes are transcription factors (activators, coactivators, or co repressors) strongly implicated in different physiologic processes such as the regulation of cell growth, development, and tissue-specific gene expression. The ARID domain, which runs over 105 aa and which had 86% similarity with other ARID family members, has been identified, sequenced, and localized on our protein sequence. The presence of an ARID motif on our protein significantly bolsters the role of 6A3-5 as a potential transcription factor since ARID domains are known to be implicated in the binding to DNA. We were particularly interested in the appearance of human p270, human eyelid, and drosophila eyelid in this list of proteins. p270 is part of the SWI-SNF complex, first identified in yeast cells, involved in the regulation of a multiple of inducible
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Figure 6. Quantitative competitive PCR on cultured cells. (a) 6A3-5 RT-PCR done on P9 (a, b, c, d, e, f) and V8 cells (a’ , b’, c’, d’, e’, f’). We have, in P9 cells, 1–2 pg of 6A3-5 in 50 ng of total RNA whereas it is 7.5–10 pg, in V8 cells. Molecular weight difference between normal and recombinant 6A3-5 RNAs is 146 bp (the competitor RNA is 378 bp whereas the normal RNA is 524 bp). aa’, bb’, cc’, dd’, ee’, ff’, gg’, hh’, ii’ = 25, 20, 15, 10, 5, 2, 1, 0.5, and 0.1 pg, respectively. (b) Actin RT-PCR. Both P9 and V8 cells have 40 pg of actin in 10 ng of total RNA. Molecular weight difference between normal and recombinant RNAs, used in the quantification, is 183 bp (the competitor RNA is 480 bp whereas the normal RNA is 663 bp). aa’, bb’, cc’, dd’, ee’, ff’ = 75, 50, 40, 35, 30, and 20 pg, respectively.

Figure 7. Structural characterization of 6A3-5 protein by western blot and FACScan. (a) Polyclonal antibodies directed against 6A3-5 bound, by western blot, to a unique band migrating with an apparent molecular weight of 175 kd. Lane 1, molecular weight markers; lane 2, V8 SMCs with anti-6A3-5 Ab; lane 3, V8 SMCs with rabbit anti-vWF polyclonal antibody. (b) Western blot analysis of the 6A3-5 protein in different rat tissues. A unique band of 175 kd, corresponding to the 6A3-5 protein, was observed in all analyzed rat tissues, but at different levels of expression. Sizes of molecular weight markers are shown on the left (in kd). Lane V8, proliferating V8 cells; lane B, brain; lane K, kidney; lane C, cerebellum; lane Sk, skeletal muscles; lane Ag, adrenal gland; lane L, lung; lane AA, aorta and aortic arch; lane E, eyes; lane T, testis; lane H, heart. The blot contained the following amounts of total protein extracts: lane V8, 30 µg; lanes B to L, 50 µg; Lanes AA to H, 80 µg. (c) FACScan confirmed the specificity of polyclonal antibodies raised against 6A3-5 protein. In addition, the protein was only labeled by the antibody when SMCs were permeabilized. This suggests that the protein has an intracellular localization. (A) Permeabilized SMCs with anti-6A3-5 Ab, (B) nonpermeabilized SMCs with anti-6A3-5 Ab, (C) permeabilized SMCs with anti-PI3Kinase Ab as a positive control. Anti-vWF represents the negative control.
genes including those required for the mating-type switch and sucrose fermentation pathways [51, 52, 53]. More recent studies suggested that SWI-SNF complex, in response to control by multiple steroid hormone receptors [54, 55, 56, 57], also has a more general role in the regulation of gene expression during cell growth and development in all organisms [58, 59]. Moreover, the complex has a general nucleosome-remodelling activity that can be upregulated in response to various signals. It is of interest to note that the drosophila eyelid [38] protein is implicated in embryonic development and is thought to be a transcription factor acting as an antagonist to the wingless (Wg) pathway. In fact, target genes in this pathway are activated in the absence of eyelid and inhibited in the presence of an excess of the gene. One should note that the rat homolog to human p270 is not yet known. Moreover, human and rat homologs to drosophila eyelid have yet to be identified. However, 6A3-5 appears to be a homolog of the human brain clone (KIAA1235). It is conceivable that both 6A3-5 and KIAA1235 are the homologs of drosophila eyelid gene. Other proteins with ARID regions, but with no similarities to 6A3-5, include human and murine bright, drosophila DRI and its human homolog DRILI, the CMV enhancer binding proteins MRF-1 and MRF-2, retinoblastoma binding proteins (RBP) 1 and 2, PLU-1, and yeast SWI1 [60, 61, 62, 63]. None of the ARID genes have been reported to be implicated in differentiation and proliferation of SMCs. However, 6A3-5 and ARID nuclear proteins show similar high molecular weight (> 140 kd) and are differentially expressed in tissues [6, 11, 18, 61].

Northern blot analysis showed substantial levels of 6A3-5 mRNA in brain, kidney, and testis. Moreover, western blot of 6A3-5 showed a unique band of a molecular weight of 175 kd, present in multiple rat tissues, albeit at substantially high levels in brain and testis. It is of interest to note that the possible role of 6A3-5 in the brain is supported by a 99% similarity with a rat cell line (PC12) sequence that differentiates into a neuronal phenotype following stimulation by NGF. Moreover, a human clone (KIAA1235), bearing an ARID nuclear domain, was isolated in the brain and showed also an important similarity (99%) to 6A3-5. Experimental data indicate that 6A3-5 may be a transcription factor implicated in the dedifferentiation and proliferation of SMCs. Indeed, the antibody directed against 6A3-5 confirmed, by FACScan, that 6A3-5 protein is not localized on the membrane but has a cytoplasmic or nuclear localization. Transcription factors are either permanently present in an inactive form in the nucleus, or translocated from the cytoplasm to the nucleus in response to a specific stimulus [64].

We have observed that at every stage when SMCs change phenotype, this affects the expression of 6A3-5. Our data suggest that this protein may be a potential factor involved in the processes of differentiation and proliferation of cells. First, the P9-V8 dedifferentiation model (synthetic versus proliferating cells) demonstrates that 6A3-5 is upregulated in the dedifferentiated V8 cells in comparison to P9 cells. Second, the P0-P9 differentiation model (contractile versus synthetic cells) demonstrates that 6A3-5 is upregulated in the dedifferentiated P9 cells in comparison to differentiated contractile P0 cells. These contractile quiescent cells (passage 0), in comparison to dedifferentiated SMCs (passage 9), show substantially lower mRNAs and protein levels of 6A3-5. Third, P9 synthetic cells stimulated by FCS or PMA after cell arrest (an in vitro model of cell proliferation) demonstrates
that 6A3-5 is upregulated (1–2 hours after stimulation) in comparison to resting P9 cells. In fact, when dedifferentiated SMCs are synchronized in the quiescent G0 phase, 6A3-5 mRNA levels are significantly increased (within a period of 1–2 hours) following stimulation by PMA or FCS. Induction of SMC differentiation and proliferation by mitogenic agents results in a burst of 6A3-5 mRNA levels at a very early stage.

Modulation of SMC phenotypes are known to induce the upregulation of a number of genes such as c-myc, c-myb, c-fos, p65 subunit of NF-kB, ras proteins, Osteopontin, mitogen-activated protein (MAP) kinases, angiotensin II, and cdk2 kinase [65, 66, 67, 68, 69, 70, 71, 72, 73]. Moreover, some new genes [74, 75, 76, 77, 78] were recently found to be upregulated in activated proliferating SMCs such as sgk (serum and glucocorticoid-regulated kinase), type VIII collagen, nucleophosmin (a nuclear phosphoprotein implicated in the regulation of cell growth and protein synthesis), and Interferon inducible protein-10 (IP-10).

In conclusion, this work describes the structural and functional characterization of a new early gene. In essence, theses results, when taken together, suggest that the 6A3-5 gene may play a key role in genetic control of cellular differentiation and proliferation. The identification of 6A3-5 as a member of the emerging family of ARID proteins suggests that it might function as a coactivator or corepressor. Such activity may take place in combination with nuclear hormone receptors, as implied by the presence of the LXXLL motif. This takes place before activation complexes (including coactivators as p300 and CBP) are formed at specific promoter sites. Further work will be needed to delineate the role of this new gene in vascular lesions. Phenotypic modulation of SMCs from a contractile into a secretory and proliferate phenotypes is the result of changes in gene expression of multiple genes [79]. The 6A3-5 gene, identified in this study in SMCs, could conceivably be part of genes involved in modulating SMC phenotype. Carefully mapping the cascade of genes implicated in SMC migration and proliferation, in
Atherosclerosis and restenosis, may ultimately allow a better understanding of the SMC phenotypic modulation. It remains to be seen if the role of 6A3-5 in differentiation is limited to SMC or is implicated in other cellular or pathological models of differentiation.

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