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

Kazem Zibara,[1, 2] Gwenaële Garin,[1, 2] and John L. McGregor[1, 2, 3]

[1]INSERM XR331, Faculty of Medicine RTH Laënnec, 69372 Lyon, France
[2]Genomics and Atherothrombosis Laboratory, Thrombosis Research Institute, London SW3 6LR, UK
[3]Center for Cardiovascular Biology and Medicine, King's College, University of London, London WC2R 2LS, UK

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.

Correspondence and reprint requests to Kazem Zibara, Medical Oncology Laboratory, Queen Mary & St. Bartholomew's Medical School, Cancer Research UK, Charterhouse Square, London EC1M 6BQ, UK, E-mail: kazem.zibara@cancer.org.uk

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 dT$_{30}$ 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-T$_{11}$M (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 μM of the H-T$_{11}$M primer (same as RT), 2 μM of a specific arbitrary primer, 25 μM dNTPs with 0.25 μL of α-^{33}P dATP (2000 Ci/mmole, 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% bromophenol 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 fingerprinting of the amplified fragments was common between the two cell types under study. (i) Differentially expressed bands (up- or downregulated) were recovered under sterile conditions by excising the gel slice from the dried gel using a razor blade. Each gel slice was placed in 100 μL sterile water, boiled for 15 minutes to solubilize the DNA, and then ethanol precipitated in the presence of glycogen. The pellet was resuspended in sterile water after ethanol washing. (ii) The reamplification is done with 4 μL of purified fragment using the same primer pair and PCR parameters that gave rise to the band. (iii) Reamplified DNA fragments were run on a 1.5% agarose gel. Bands that succeeded to be reamplified were cloned into PCR II vector (TA cloning kit, Invitrogen, The Netherlands). (iv) For DNA sequencing, minipreps of plasmidic DNA were carried out [19], followed by the dideoxy sequencing method [20] (T7 Sequencing Kit, Pharmacia, France).

Bioinformatics

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 Omiga 2.0 Software (Oxford molecular, UK).

Probes and northern blot

Total RNA was extracted as above, denatured, separated by electrophoresis in formaldehyde-MOPS-agarose gel and then transferred to a nylon membrane (Hybond, Amersham, UK). 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 cdk2α probe by RT-PCR (see below): cdk2α up: ACGGAGTGGTGTACAAAGCC, cdk2 down: GAGTCTCCAGGGAATAGGGC.

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 $1\,\mu$g of V8 poly(A+) RNA, using $10\,\mu$M 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 $\alpha - {}^{32}$P dCTP ($1\,\mu$Ci/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 $10\,\mu$M dNTP, $10\,\mu$M of the adapter primer (complementary to the cDNA adapter), $5\,\mu$L of the 50x KlenTaq polymerase, and $10\,\mu$M of gene-specific primer (GSP) complementary to the 3′ differentially expressed fragment (6A3-5 GSP: 5′-GTATTACAGTTTTAGGGAAGTGAATTC-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: TTGGGGATCGCAAAAACC, 6A3-5 L21: TAGTGAATGGGGCAGAGAAGC. 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 RcDNA 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, IU Taq polymerase, 100 ng of plasmid cDNA, and $10\,\mu$M 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 $20\,\mu$L reaction containing 100 mM DTT, $4\,\mu$L 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 $10\,\mu$L total volume with 10 mM dNTP, 10 mM DTT, $10\,\mu$M dN6 primers (Boehringer), 10 ng of total RNA of cultured SMCs (P9 or P200), 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, 0.04% 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: TTGGGGATCGCAAAAACC,

6A3-5 L21: TAGTGAATGGGGCAGAGAAGC,

6A3-5 U38: TAATACGACTCACTATAGGATTGG-GGATCGCAAAAACC,

6A3-5 L60: $(dT)_{18}$TAGTGAATGGGGCAGAGAA-GCAGACGCAACCAGAGAGTTCAG,

Actin U21: TCGTACCACTGGCATTGTGAT,

Actin L17: GGGCCGGACTCATCGTA,

Actin U41: TAATACGACTCACTATAGGATCGTA-CCACCACTGGCATTGTGAT,

Actin L57: $(dT)_{18}$GGGCCGGACTCATCGTAAGA-CAGCACTGTGTTGGCATAG.

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 ε-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 (2 μg/mL). Following 3 washing steps of 10 minutes in 0.05% TBS-Tween, a swine anti-rabbit-horse radish peroxidase-conjugated secondary antibody (DAKO) is incubated during 45 minutes at RT. The membrane is then washed (4×10 minutes) in 0.05% TBS-Tween and revealed by a chemiluminescent technique (ECL Kit, Amersham, UK) according to the manufacture's instructions. The ECL films are scanned with Sharp JX-330 scanner (Amersham) and 6A3-5 bands were quantified with appropriate software. In parallel, SDS-PAGE gels, containing identical sample volumes to those used for the western blot were coomassie stained. An electrophoretic band (220 kd) was scanned and used as a quantitative control to check for sample loading and 6A3-5 normalization.

FACScan analysis

Confluent SMCs are trypsinized, centrifuged at 1500 rpm for 5 minutes at 4°C, and washed with 1% PBS/BSA. Cells are then fixed and permeabilized by 100% methanol at −20°C during 10 minutes. After washing
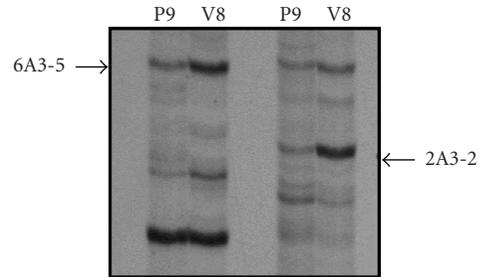
FIGURE 1. A representative differential display (DD) analysis showing up- and downregulated genes. Total RNA was extracted from synthetic (P9) and proliferating (V8) SMC, then subjected to DD analysis. Migration of PCR products on a denaturing 6% polyacrylamide gel for 2 different primer pairs is shown. Each primer pair gives similar RNA fingerprinting between the 2 cell types. The differentially expressed bands, having an altered expression, were cut from the gel and reamplified. Lanes correspond respectively to synthetic (P9) and rapidly proliferating cells (V8).

with 1% PBS/BSA, 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 dT$_{11}$A. P9* and V8* indicate synthetic and proliferating cells, respectively.

Similarity %	Band	Cell type overexpression	Similarity with
80–100%	4G3-2	V8*	Rat glia-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
			Mouse embryonal carcinoma
50–80%	3A1-2	P9	G protein coupled receptor
	4A1-7	P9	Human protooncogene tyrosine kinase
			Mouse ICAM 1, c-myc
	6A1-3	P9	Metalloprotease
			Homo sapiens 3′ EST
	2A3-5	V8	Mouse 3′ nerve growth factor
30–50%	2A1-2	V8	Thermo potent virus gene
			TSP-4 mRNA
	6A2-4	P9	Mouse mammary tumor virus
	2A2-1	V8	Protooncogene tyrosine kinase, c-myc
			human breast cancer susceptibility virus
No similarity	2A3-2	V8	—
	6A3-5	V8	—
	5C1	V8	—
	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 mRNA's 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.

(a)

(b)
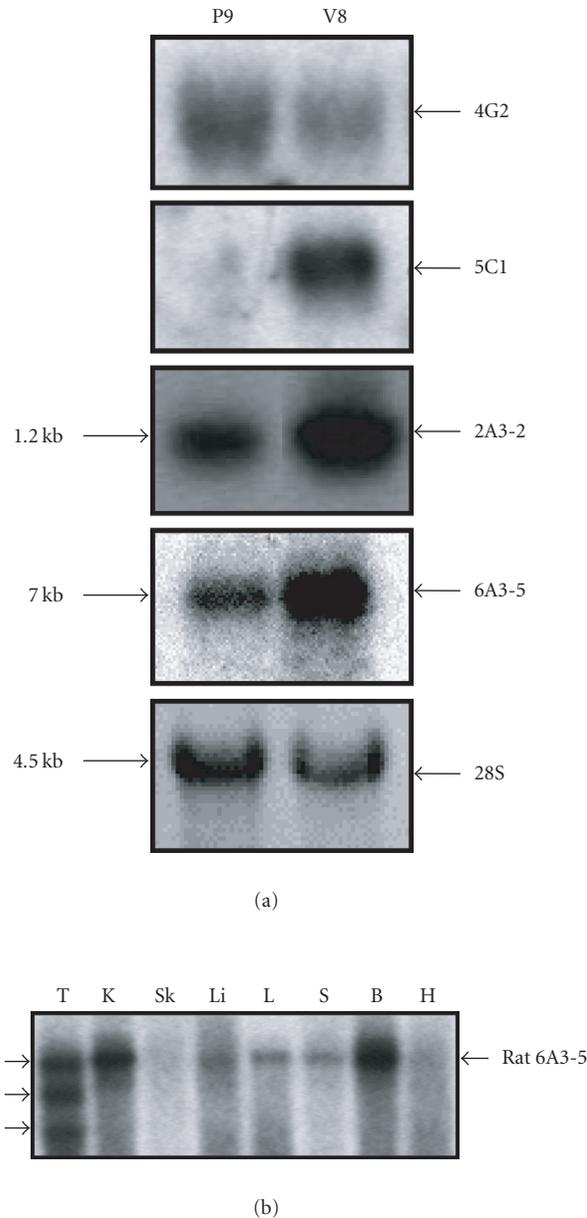
Figure 2. Northern blot analysis of 6A3-5 differentially displayed cDNA band. (a) Four genes (4G2, 5C1, 2A3-2, 6A3-5), showing no similarities in the databases, were confirmed by northern blots to be differentially expressed. The 6A3-5 gene is upregulated in proliferating (V8) but not synthetic cells (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

Figure 3. 5′ RACE-PCR amplification. A cDNA library (obtained from V8 proliferating cells) was used along with a primer coming from the 3′ end of the 6A3-5 band. The other primer in the PCR comes from the adapter that is already ligated to the cDNA library. The touchdown PCR technique was used during the 5′ RACE, which allowed us to obtain a part of the cDNA. The size of the 5′ RACE-amplified 6A3-5 fragment is 1.2 kb. ϕX174 is given on the left as a molecular weight marker. The lanes represent different dilutions of the cDNA library.

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 SWI1) 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 transactivation 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 dedifferentiation 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

```
GGCACCAGGC CCACCAATGC CCACTGTGAA CCGCAAGGCC CAGGAAGCTG CCGCGGCTGT  60
GATGCAGGCT GCTGCAAACT CAGCCCAAAG CAGGCAAGGC AGCTTTCCTG GCATGAACCA 120
GAGTGGGACTC GTGGCCTCCA GCTCTCCCTA CAGCCAGCCC ATGAACAATA ACTCCAACCT 180
AATGGGGACA CAGGCCCAGC CCTACAGCAT GACACCCACC ATGGTGAACA GCTCTACAGC 240
ATCTATGGGT CTTACAGATA TGATGTCTCC CAGTGAGTCC AAACTGTCCG TGCCTCTTAA 300
AGCAGATGGC AAAGAAGAAG GTGTGCCCCA GCCTGAGAGC AAGTCAAAGG ATAGCTACAG 360
CTCTCAGGGT ATTTCTCAGC CTCCAACTCC AGGCAACCTG CCAGTCCCTT CCCCAATGTC 420
CCCCAGCTCT GCCAGCATCT CCTCCTTTCA CGGAGATGAG AGTGATAGCA TTAGCAGCCC 480
AGGCTGGCCC AAGACTCCAT CAAGCCCTAA GTCCAGCTCC TCTTCCACCA CTGGGGAGAA 540
GATCACAAAA GTGTATGAGC TGGGGACTGA GCCGGAGAGG AAGCTGTGGG TCGACCGCTA 600
CCTGACATTC ATGGAGGAGA GGGGGTCCCC CGTGTCCAGT CTGCCGGCAG TGGGCAAGAA 660
GCCCCTGGAC CTGTTCCGAC TCTATGTGTG CGTCAAAGAG ATCGGAGGCT TGGCGCAGGT 720
TAATAAAAAC AAGAAGTGGC GTGAGCTGGC AACCAACCTG AACGTTGGCA CTTCGAGCAG 780
CGCAGCCAGC TCCCTGAAGA AGCAATATAT TCAGTACCTG TTCGCCTTCG AGTGCAAAAT 840
TGAGCGTGGG GAGGAGCCCC CGCCTGAAGT CTTCAGCACG GGGGATGCGA AGAAGCAGCC 900
CAAGCTCCAG CCGCCATCTC CTGCTAACTC GGGATCCTTA CAAGGTCCAC AGACGCCACA 960
GTCAACTGGC AGCAGTTCCA TGGCAGAGGT TCCCGGTGAC CTGAAGCCAC CAACCCCAGC 1020
CTCCACCCCT CACGGACAGG GGACCCCCAT GCAAAGCGGA AGAAGCAGTA CAGTCAGTGT 1080
GCATGACCCG TTCTCAGACG TGAGTGATTC AGCGTACCCA AAACGGAACT CCATGACTCC 1140
AAACGCCCCA TACCAGCAGG GCATGGGCAT GCCAGACATG ATGGGCAGGA TGCCCTATGA 1200
GCCCAACAAG GACCCTTTCA GTGGAATGAG AAAAGTGCCT GGAAGCAGCG AGCCCTTTAT 1260
GACACAAGGA CAGATGCCCA ACAGCAGTAT GCAGGACATG TACAACCAGA GTCCCTCAGG 1320
TGCCATGTCC AATCTGGGCA TGGGACAGCG GCAGCAATTT CCCTATGGAA CCAGTTATGA 1380
CCGAAGGCAT GAGGGCTTACG GGCAGCAGTA CCCAGGCCAA GGCCCTCCCA CAGGACAGCC 1440
ACCGTATGGA GGACACCAGC CTGGCCTGTA CCCACAGCAG CCGAATTACA AACGCCATAT 1500
GGATGGCATG TACGGGCCTC CAGCCAAGCG CCACGAGGGA GACATGTACA ACATGCAGTA 1560
TGGCAGCCAA CAGCAGGAGA TGTATAACCA GTACGGAGGC TCCTACTCTG GCCCGGACAG 1620
AAGGCCCATC CAGGGACAGT ATCCCTACCC CTACAATAGA GAAAGGATGC AGGGCCCAGC 1680
CCAGATGCAA ACACATGGAA TCCCACTCAC AATGATGGGT GGCCCCATGC AGTCATCTTC 1740
CAATGAGGGG CCTCAGCAGA ATATGTGGGC TACACGCAAT GATATGCCTT ATCCCTACCA 1800
GAACAGGCAA GGCCCAGGTG GCCCTGCACA GGCACCCCCT TACCCAGGCA TGAACCGCAC 1860
AGATGATATG ATGGTACCTG ATCAGAGGAT CAATCACGAG AGCCAGTGGC CTTCTCATGT 1920
CAGCCAGCGC CAGCCTTATA TGTCATCATC GGCCTCCATG CAACCCATCA CGCGCCCACC 1980
TCAGTCATCC TACCAGACGC CGCCGTCACT GCCAAACCAC ATCTCCAGGG CACCCAGCCC 2040
TGCCTCCTTC CCGCGCTCCC TGGAGAGCCG CATGTCTCCA AGCCAAGTCCC CCTTCCTGCC 2100
TGCCATGAAG ATGCAGAAGG TCATGCCCAC GGTCCCCACA TCCCAGGTCA CCGGACCACC 2160
CCCACAGCCA CCGCCAATCA GAAGGGAGAT TACCTTTCCT CCTGGCTCAG TAGAAGCATC 2220
GCAGCCAGTC CTGAAACAAA GGCGGAAGAT TACCTCAAAA GATATTGTTA CTCCCGAGGC 2280
GTGGCGTGTG ATGATGTCCC TTAAATCCGG TCTGTTGGCT GAGAGCACGT GGGCTTTGGA 2340
CACCATCAAC ATTCTCCTCT ATGATGACAG CACTGTCGCC ACCTTCAATC TTTCCCAGCT 2400
GTCGGGGTTC CTGGAACTAC TAGTGGAGTA CTTCCGAAAG TGCCTAATTG ACATTTTCGG 2460
TATTCTGATG GAATATGAAG TGGGTGACCC CAGCCAAAGG GCGCTTGATC ACCGCACAGG 2520
GAAGAAAGAC GACAGCCAGT CCTCGGAGGA CGACTCTGGG AAAGAAGAAG ATGATGCCGA 2580
GTGTCTGGAG GAGGAGGAGG AGGAGGAGGA GAGGAAGAGG AGCAAGTCAG 2640
TAAAAAGACA GAGTCAGAGG GCAAGAGCAG CTCTGCCCTA GCTGCTCCAG ACACCACTGC 2700
GGACCCCAAG GAGACGCCGA GGCAGGCCAG TAAGTTTGAC AAGCTGCCCA TAAAGATTGT 2760
CAAGAAGAAC AACCTGTTTG TGGTGGACCG GTCCGACAGG CTGGGTCGGG TTCAAGAGTT 2820
CAACAGCGGG CTCCTCCACT GGCAGCTGGG TGGTGGCGAC ACCACTGAGC ACATCCTCAC 2880
TCACTTCGAG AGCAAGATGG AGATCCCTCC TCGCAGGCGT CCACCTGCGC CTCTCAGCTC 2940
CCACGGGAAG AAGAAAGAGC TGGCAGGCAA GGGCGATTCT GAAGAGCAGC CAGAGAAAAG 3000
CATCATAGCC ACCATTGATG ATGTCCTGTC TGCCCGACCA GGGGCCCTGC CGGAAGACAG 3060
CAACCCCGGA CCCCAAACCG AGAGCGGCAA GTTTCCCTTT GGAATCCAGC AGGCCAAAAG 3120
CCACCGGAAC ATCAGGCTCC TGGAGGACGA GCCCAGGAGC CGAGACGAGA CTCCCCTGTG 3180
CACCATTGCG CACTGGCAGG ACTCCCTGGC CAAACGCTGC ATCTGTGTGT CTAACATCGT 3240
GCGGGAGCTTG TCTTTCGTGC CTGGCAACGA CGCAGAGATG TCCAAACATC CGGGCTTGGT 3300
GCTGATCCTA GGAAAGTTGA TTCTGCTGCA TCATGAGCAT CCGGAGAGAA AACGGGCGCC 3360
ACAGACCTAT GAGAAGGAGG AGGATGAGGA CAAGGGGGTG GCCTGCAGCA AAGATGAGTG 3420
GTGGTGGGAC TGCCTCGAGG TCTTGCGGGA TAACACACTG GTCACATTGG CTAACATTTC 3480
CGGGCAGCTA GACTTGTCTG CTTACACAGA GAGCATCTGC TTGCCGATCC TGGACGGCTT 3540
GCTGCACTGG ATGGTGTGCC CGTCTGCAGA GGCACAGGAC CCCTTTCCCA CTGTGGGGCC 3600
CAACTCAGTT CTATCGCCAC AAAGACTTGT GCTGGAGACA CTGTGTAAAC TCAGTATCCA 3660
GGACAACAAT GTGGACCTGA TCTTGGCCAC ACCTCCATTT AGTCGTCAGG AGAAATTTTA 3720
TGCTACATTA GTTAGGTACG TTGGGGATCG CAAAAACCCA GTCTGCCGAG AAATGTCCAT 3780
GGCGCTTTTA TCGAACCTTG CCCAGGGGGA TACACTGGCA GCGAGGGCAA TAGCTGTGCA 3840
GAAAGGAAGC ATTGGGAACT TGATAGGCTT CCTGGAGGAC GGGGTCACGA TGGCGCAGTA 3900
TCAGCAGAGC CAGCATACCC TCATGCACAT GCAGCCCCCG CCTCTGGAAC CCCCCAGTGT 3960
GGACATGATG TGCAGGGCGG CCAAAGCTTT GCTGGCTATG GCTAGAGTGG ATGAGAACCG 4020
CTCGGAGTTC CTTTTGCACG AGGGTCGGTT GCTGGATATC TCGATATCCG CTGTCCTGAA 4080
CTCTCTGGTT GCGTCTGTCA TCTGTGATGT ACTGTTTCAG ATTGGGCAGT TAATGACACC 4140
CGTGAGGGCA CACATGTGTG AGGGAACATT AGAGGGTCAC ATATTGACTG GCTGTTTTCT 4200
GTTCTCGTTT ATCCAATGTA GGAAGAAGGA AAAGAAAAAT CTTTGCTTCT CTGCCCCATT 4260
CACTATTTAC CAATTGGGAA TTAAAGAAAT CATTAATTTG AACAGTTATA AATTAATATT 4320
TGCTGTCTGT GTGTATAAGT ACATCCGTTG GGGGATTTCT GTTTCTTTCT CTTTTTTTAA 4380
CCAAAGTTGC CGTCTAGTGC ATTCACAGGT CACATGTTTT TTTGTTTTTT TCATAATTTT 4440
TTTCATGTTG TATTACAGTT TTAGGGAAGT GAATTCACTT TATAAAGTAA AAAGGTTTGG 4500
CAAAAAAATG CTGATAGGAA AATTTCACCA CACTGAGTCA AAAAAGGTGA AAGGAAAATT 4560
GATCCTTAAA TTGATTTCCT ATGAATTTTA TTCTTCGCAG AATGAAAAAA GCGAAAGTGC 4620
ATCCCATTGC CCAAAGCTCT GTGCAATAGA AACTTCTAGA GATGTAGGTG TAGGGGCTCG 4680
AGGTATGGCA GTCAGCAGTC TGGCCCAGTG ATGCTGTTCT CTCCACAGGA AAGCGGTTGC 4740
ATTAGGCCTC GAGCAAAAAA CCGCACTCTC AGTTAGGGGT GAAAATCCAC TCCTAACCGC 4800
CAACAGCAGG ATTGCTTCCT CACCACGACC GCCATGTCTG CTGCGACTCA GCCTCCACCT 4860
CACAGATCTT CGTGATTCCT TTCATCATTT TTTAAATATT TTTTTTTTAC TGCCTATGGG 4920
CTGTGATGTA TATAGAAGTT GTACATTAAA CATACCCTCA TATTTTTTTC TTTTCTTTTT 4980
TTTTTTTTAG TACAAAGTTT TTAGTTTCTT TTTCATGATG TGGTAACTAC GAAGTGATGG 5040
TAGATTTAAA TAATTTTTTA TTTTTATTTT ATATATTTTT TCATTAGGAC CATATCTCCA 5100
AAAAACAAGA AAAAGAAACA AAAAATTCAA AAAATTAAAA CAAACAAAAA AAGGGGGTAA 5160
TGTACAAGTT TCTGTATGTA TAAAGTCATG CTCTCGTTGGG AGGGCAGCTG GTCCCAATTT 5220
GCTTCATGAA TCAAGGTGTG GAAATGGTTG CATACGGATT GATTTAGAAA ATGAATACCA 5280
GTACATACAA AAAAAAAGAA AAAAGAAAAA CCAATAAACT GAAGAAACAC AACTTCAAAG 5340
ATTTTTCTGT GACAAGAATC CGCATTTGTA TTTCAAGATA ATGTAGTTTA AGAAAAAAAA 5400
AAAAAAAAAA 5410
```

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].

```
APGPPMPTVN  RKAQEAAAAV  MQAAANSAQS  RQGSFPGMNQ  SGLVASSSPY  SQPMNNNSNL  60
MGTQAQPYSM  TPTMVNSSTA  SMGLTDMMSP  SESKLSVPLK  ADGKEEGVPQ  PESKSKDSYS  120
SQGISQPPTP  GNLPVPSPMS  PSSASISSFH  GDESDSISSP  GWPKTPSSPK  SSSSSTTGEK  180
ITKVYELGTE  PERKLWVDRY  LTFMEERGSP  VSSLPAVGKK  PLDLFRLYVC  VKEIGGLAQV  240
NKNKKWRELA  TNLNVGTSSS  AASSLKKQYI  QYLFAFECKI  ERGEEPPPEV  FSTGDAKKQP  300
KLQPPSPANS  GSLQGPQTPQ  STGGSSMAEV  PGDLKPPTPA  STPHGQGTPM  QSGRSSTVSV  360
HDPFSDVSDS  AYPKRNSMTP  NAPYQQGMGM  PDMMGRMPYE  PNKDPFSGMR  KVPGSSEPFM  420
TQGQMPNSSM  QDMYNQSPSG  AMSNLGMGQR  QQFPYGTSYD  RRHEAYGQQY  PGQGPPTGQP  480
PYGGHQPGLY  PQQPNYKRHM  DGMYGPPAKR  HEGDMYNMQY  GSQQQEMYNQ  YGGSYSGPDR  540
RPIQGQYPYP  YNRERMQGPA  QMQTHGIPLT  MMGGPMQSSS  NEGPQQNMWA  TRNDMPYPYQ  600
NRQGPGGPAQ  APPYPGMNRT  DDMMVPDQRI  NHESQWPSHV  SQRQPYMSSS  ASMQPITRPP  660
QSSYQTPPSL  PNHISRAPSP  ASFPRSLESR  MSPSKSPFLP  AMKMQKVMPT  VPTSQVTGPP  720
PQPPPIRREI  TFPPGSVEAS  QPVLKQRRKI  TSKDIVTPEA  WRVMMSLKSG  LLAESTWALD  780
TINILLYDDS  TVATFNLSQL  SGFLELLVEY  FRKCLIDIFG  ILMEYEVGDP  SQRALDHRTG  840
KKDDSQSSED  DSGKEEDDAE  CLEEEEEEEE  EEEEEEQVS  KKTESEGKSS  SALAAPDTTA  900
DPKETPRQAS  KFDKLPIKIV  KKNNLFVVDR  SDRLGRVQEF  NSGLLHWQLG  GGDTTEHILT  960
HFESKMEIPP  RRRPPAPLSS  HGKKKELAGK  GDSEEQPEKS  IIATIDDVLS  ARPGALPEDS  1020
NPGPQTESGK  FPFGIQQAKS  HRNIRLLEDE  PRSRDETPLC  TIAHWQDSLA  KRCICVSNIV  1080
RSLSFVPGND  AEMSKHPGLV  LILGKLILLH  HEHPERKRAP  QTYEKEEDED  KGVACSKDEW  1140
WWDCLEVLRD  NTLVTLANIS  GQLDLSAYTE  SICLPILDGL  LHWMVCPSAE  AQDPFPTVGP  1200
NSVLSPQRLV  LETLCKLSIQ  DNNVDLILAT  PPFSRQEKFY  ATLVRYVGDR  KNPVCREMSM  1260
ALLSNLAQGD  TLAARAIAVQ  KGSIGNLIGF  LEDGVTMAQY  QQSQHTLMHM  QPPPLEPPSV  1320
DMMCRAAKAL  LAMARVDENR  SEFLLHEGRL  LDISISAVLN  SLVASVICDV  LFQIGQLMTP  1380
VRAHMCEGTL  EGHILTGCFL  FSFIQCRKKE  KKNLCFSAPF  TIYQLGIKEI  INLNSYKLIF  1440
AVCVYKYIRW  GISVSFSFFN  QSCRLVHSQV  TCFFVFFIIF  FMLYYSFREV  NSLYKVKRFG  1500
KKMLIGKFHH  TESKKVKGKL  ILKLISYEFY  SSQNEKSESA  SHCPKLCAIE  TSRDVGVGAR  1560
GMAVSSLAQ   1569
```

FIGURE 5. Analysis of the different parts of the rat 6A3-5 cDNA. Predicted 6A3-5 rat protein sequence (1569 aa). The ARID motif (187–296) is shown in bold whereas LXXLL motif (1177–1181), serine-rich region (112–175), Q-rich region (1298–1304), and phenyl-rich region (1472–1481) are underlined.

6A3-5 gene expression of synthetic SMCs following stimulation by PMA or FCS

Functional characterization of 6A3-5 gene and its implication in SMC proliferation was then studied. Northern blots showed that 6A3-5 gene expression is reduced to a minimum in quiescent and synchronic SMCs after serum depletion (0 minutes), in comparison to levels of expression in standard cell culture conditions (Figure 9). In contrast, 10% FCS induced 6A3-5 gene transcription to a maximum after 1 hour of stimulation (12-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 go back 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 and quantitative competitive RT-PCR. Moreover, rat multiple-tissue northern showed the presence of this 7 kb mRNA in different tissues. (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 the rat glia-derived nexin (GDN) or protease nexin I. This gene is implicated in neurogenesis and neurite growth [42]. In addition, a study showed that a nexin-derived serine protease (GdNPF) is implicated in the migration of the neuronal cells [43]. Moreover, the GDN has similarities [44] with plasminogen activator inhibitor (PAI), antithrombin III (ATIII), and α-1 proteinase inhibitor. The second interesting sequence, 3A2-7, had 97% similarity with autographa calcifornica nuclear polyhedrosis virus. This viral tyrosine/serine phosphatase gene [45] is used for the overexpression of eukaryotic genes [46]. The third sequence, 3A1-1, had a 98% similarity with the glucose-regulated protein GRP78 and rat immunoglobulin heavy chain binding protein. It was demonstrated that GRP78 is upregulated in cells in case of energy restrains [47]. Since this gene is overexpressed

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	196 947
85%	2796	EM_HUM:AL591545 human DNA sequence	41211
85%	2796	EM_HTG:BX284653 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 AI673781 NCI_CGAP GAS4 HOMO (TO73A09.X1)	533
91%	469	EM_EST AI439094 NCI_CGAP KID11 HOMO (TI87A06.X1)	467
91%	384	EM_EST human hela cell 5′ cDNA EST clone Aa180335	384
91%	251	EM_EST human testis I 5′ 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

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 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%	1471	SW:SMF1_HUMAN O14497 SWI/SNF-related, p270	1902
30%	1734	SW:OSA_DROME Q8IN94 trithorax group protein OSA	2716
27%	454	SW:DRI1_MOUSE Q62431 dead ringer like-1 protein	601
22%	670	SW:DRI_DROME Q24573 dead ringer protein	911
26%	527	SW:DRI1_HUMAN Q99856 dead ringer like-1 protein	593
99%	228	Q9ULI5 KIAA1235 protein	1485
99%	228	Q9NWF5 cDNA FLJ10059 FIS, clone HEMBA 1001	412
78%	238	Q9NUD8 DJ50O24.6 (B120, C1ORF4)	1644
50%	221	O61603 drosophila melanogaster eyelid protein	2715
50%	221	Q9VEG7 OSA Protein	2703
23%	839	SW:GLT5_WHEAT P10388 glutenin, high molecular weight	839
29%	441	SW:W146_HUMAN Q9C0J8 WD-repeat protein WDC146	1336
24%	514	SW:CA1A_BOVIN P23206 collagen alpha 1(X) chain protein	674
19%	774	SW:FP1_MYTED Q25460 adhesive plaque matrix protein	875
29%	432	SW:SSXT_HUMAN Q15532 SSXT protein	418
28%	433	SW:SSXT_MOUSE Q62280 SSXT protein (SYT protein)	418
27%	435	SW:CBPA_DICDI P35085 Calcium-binding protein	467
26%	437	SW:CBP1_CAEEL P34545 protein cbp-1	2056
34%	119	SW:OSA_DROYA Q9NGB4 trithorax group protein OSA	324
22%	524	SW:SN24_HUMAN P51532 possible global transcription activator	1647
21%	1083	SW:PCLO_RAT Q9JKS6 Piccolo protein	5085
24%	503	SW:FP1_MYTCO Q25434 adhesive plaque matrix protein	872
22%	829	SW:NCO6_HUMAN Q14686 nuclear receptor coactivator	2063
23%	503	SW:K10_DROME P13468 DNA-binding protein K10	463
27%	284	SW:PRPL_HUMAN P10162 salivary proline-rich protein	276
24%	777	SW:CA14_CAEEL P17139 collagen alpha 1(IV) chain p	1758
24%	399	SW:CA1C_MOUSE Q60847 collagen alpha 1(XII) chain	3119
23%	923	SW:DRPL_HUMAN P54259 atrophin-1	1185
24%	459	SW:BRM_DROME P25439 homeotic gene regulator	1638
24%	503	SW:CBP_HUMAN Q92793 CREB-binding protein	2442
25%	263	SW:SSB3_CHICK Q98948 single-stranded DNA-binding	368
24%	424	SW:FYB_HUMAN O15117 FYN-binding protein	783
26%	447	SW:BCL9_MOUSE Q9D219 B-cell lymphoma 9 protein	449
21%	742	SW:NCO6_MOUSE Q9JL19 nuclear receptor coactivator	2067
29%	311	SW:SM41_HEMPU Q26264 41 kd spicule matrix protein	407
21%	556	SW:HRS_DROME Q960X8 hepatocyte growth factor regulator	760
32%	210	P91018 human BAT2 protein	1357
30%	182	G2739158 IkB Epsilon	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

(a)

(b)
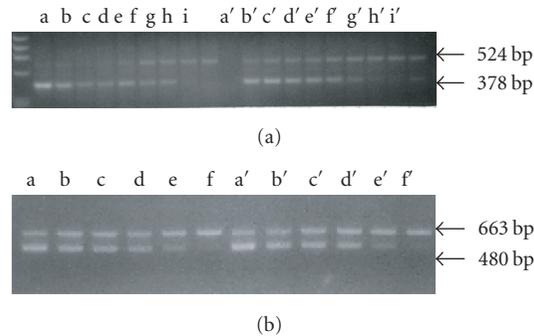
FIGURE 6. Quantitative competitive PCR on cultured cells. (a) 6A3-5 RT-PCR done on P9 (a, b, c, d, e, f) and V8 cells (a′, b′, c′, d′, e′, f′). We have, in P9 cells, 1–2 pg of 6A3-5 in 50 ng of total RNA whereas it is 7.5–10 pg, in V8 cells. Molecular weight difference between normal and recombinant 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.

(a)

(b)

(A)　　　　　　(B)　　　　　　(C)

(c)
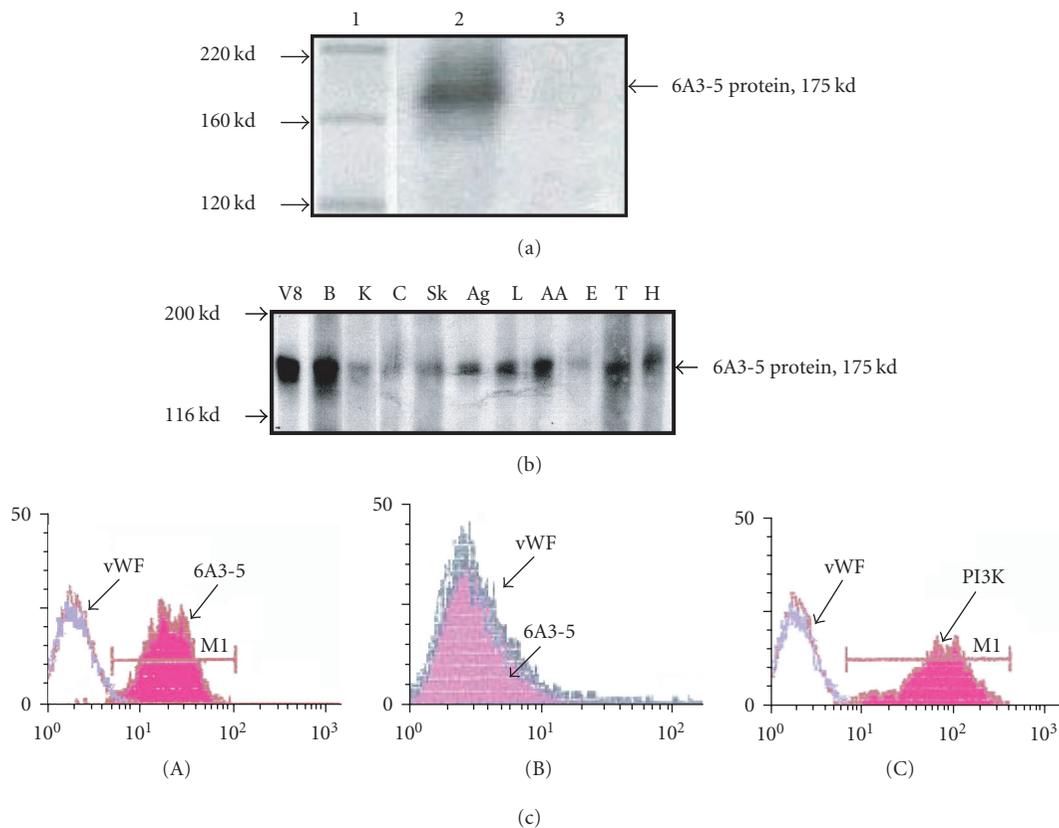
FIGURE 7. Structural characterization of 6A3-5 protein by western blot and FACScan. (a) Polyclonal antibodies directed against 6A3-5 bound, by western blot, to a unique band migrating with an apparent molecular weight of 175 kd. Lane 1, molecular weight markers; lane 2, V8 SMCs with anti-6A3-5 Ab; lane 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.

(a)

(b)
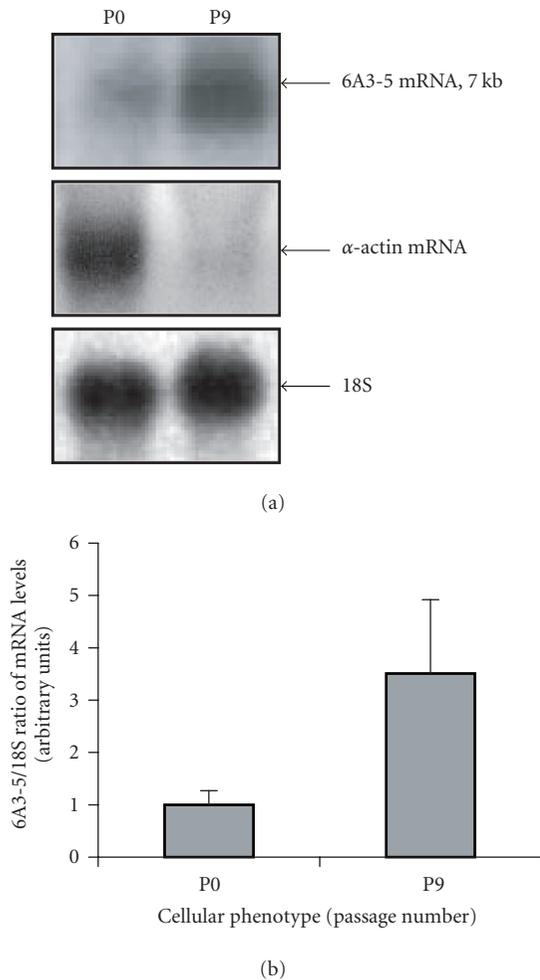
FIGURE 8. *Functional characterization of 6A3-5 in vitro*. (a) Levels of 6A3-5 gene transcription were compared, by northern blot, between the contractile (passage 0, P0) and the secretory/synthetic phenotype (9th passage, P9). Northern blots showed that 6A3-5 mRNA transcription levels are very low in the contractile phenotype, in comparison to the synthetic one. The contractile phenotype (P0) and its loss (P9) were characterized by the α-actin marker as a positive internal control of the experiment. The 18S served as a control for loading and quantification. (b) Quantification of 6A3-5 signals, done on 3 independent northern blots, reported to the 18S levels. Results show that 6A3-5 mRNA levels are increased by 300% (3-fold increase) in synthetic cells compared to contractile cells. Results are represented in a quantitative way with the mean $+/-$ SEM.

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

(a)

(b)
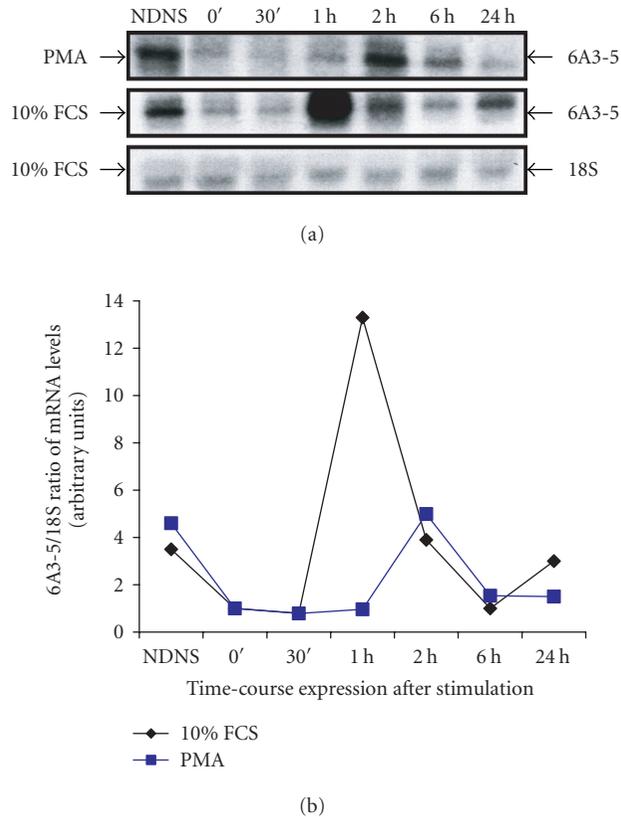
FIGURE 9. Effects of mitogenic agents on transcriptional expression of 6A3-5 and its possible implication in the proliferation of SMCs. (a) Synthetic SMCs (9th passage) were depleted from serum during 48 hours, then stimulated by mitogens such as 10% FCS or PMA (50 ng/mL). Northern blots showed that 6A3-5 gene is downregulated in depleted SMCs (0 minute) in comparison to nondepleted and nonstimulated SMCs (NDNS). Moreover, the potent mitogen PMA induced a peak of 6A3-5 transcription levels after 2 hours of stimulation. However, northern blots showed that 10% FCS induced 6A3-5 gene transcription to a maximum after 1 hour of stimulation. The 18S served as a control for loading and quantification. (b) Quantification of 6A3-5 mRNA signals reported to the 18S levels. Results show that 6A3-5 gene expression is overexpressed by at least 400% (4-fold increase) in PMA-stimulated cells, 1–2 hours of stimulation. Stimulation with 10% FCS results in a 12-fold increase of 6A3-5 gene transcription 1 hour only after induction.

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.

REFERENCES

[1] Schwartz SM, Reidy MA, O'Brien ER. Assessment of factors important in atherosclerotic occlusion and restenosis. *Thromb Haemost*. 1995;74(1):541–551.

[2] Campbell JH, Kocher O, Skalli O, Gabbiani G, Campbell GR. Cytodifferentiation and expression of alpha-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells: correlation with cell density and proliferative state. *Arteriosclerosis*. 1989;9(5):633–643.

[3] Munro JM, Cotran RS. The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Lab Invest*. 1988;58(3):249–261.

[4] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362(6423):801–809.

[5] Campbell JH, Campbell GR. Phenotypic modulation of smooth muscle cells in primary culture. In: Campbell JH, Campbell GR, eds. *Vascular Smooth Muscle in Culture. Vol 1.* Boca Raton, Fla:CRC Press; 1987:39–55.

[6] Gabbiani G. Cytoskeletal features of aortic smooth muscle cells. In: Rousset BAF, ed. Vol 171. *Montrouge*. Collioure, France: Colloque INSERM/John Libbey Eurotext Ltd; 1988:179–182.

[7] Thyberg J, Hedin U, Sjolund M, Palmberg L, Bottger BA. Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis*. 1990;10(6):966–990.

[8] Jonasson L, Holm J, Skalli O, Gabbiani G, Hansson GK. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. *J Clin Invest*. 1985;76(1):125–131.

[9] Rosenfeld ME, Tsukada T, Chait A, Bierman EL, Gown AM, Ross R. Fatty streak expansion and maturation in Watanabe Heritable Hyperlipemic and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis*. 1987;7(1):24–34.

[10] Dartsch PC, Bauriedel G, Schinko I, Weiss HD, Hofling B, Betz E. Cell constitution and characteristics of human atherosclerotic plaques selectively removed by percutaneous atherectomy. *Atherosclerosis*. 1989;80(2):149–157.

[11] Fowler SD, Mayer EP, Greenspan P. Foam cells and atherogenesis. *Ann N Y Acad Sci*. 1985;454:79–90.

[12] Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. *Physiol Rev*. 1979;59(1):1–61.

[13] Absher M, Woodcock-Mitchell J, Mitchell J, Baldor L, Low R, Warshaw D. Characterization of vascular smooth muscle cell phenotype in long-term culture. *In Vitro Cell Dev Biol*. 1989;25(2):183–192.

[14] Perez-Reyes N, Halbert CL, Smith PP, Benditt EP, McDougall JK. Immortalization of primary human smooth muscle cells. *Proc Natl Acad Sci U S A*. 1992;89(4):1224–1228.

[15] Blaes N, Bourdillon MC, Daniel-Lamaziere JM, Michaille JJ, Andujar M, Covacho C. Isolation of two morphologically distinct cell lines from rat arterial smooth muscle expressing high tumorigenic potentials. *In Vitro Cell Dev Biol*. 1991;27A(9):725–734.

[16] Shanahan CM, Weissberg PL. Smooth muscle cell heterogeneity: patterns of gene expression in vascular smooth muscle cells in vitro and in vivo. *Arterioscler Thromb Vasc Biol*. 1998;18(3):333–338.

[17] Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*. 1992;257(5072):967–971.

[18] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162(1):156–159.

[19] Kraft R, Tardiff J, Krauter KS, Leinwand LA. Using miniprep plasmid DNA for sequencing double stranded templates with sequenase. *Biotechniques*. 1988;6(6):544–546.

[20] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977;74(12):5463–5467.

[21] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215(3):403–410.

[22] Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A*. 1988;85(8):2444–2448.

[23] Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manuel*. 2nd ed. Cold Spring Harbor, NY:Cold Spring Harbor Laboratory Press; 1989.

[24] Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res*. 1991;19(14):4008.

[25] Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci U S A*. 1990;87(7):2725–2729.

[26] Vanden Heuvel JP, Tyson FL, Bell DA. Construction of recombinant RNA templates for use as internal standards in quantitative RT-PCR. *Biotechniques*. 1993;14(3):395–398.

[27] Zibara K, Bourdillon MC, Chignier E, Covacho C, McGregor JL. Identification and cloning of a new gene (2A3-2), homologous to human translational elongation factor, upregulated in a proliferating rat smooth muscle cell line and in carotid hyperplasia. *Arterioscler Thromb Vasc Biol*. 1999;19(7):1650–1657.

[28] Wahle E, Keller W. The biochemistry of 3'-end cleavage and polyadenylation of messenger RNA precursors. *Annu Rev Biochem*. 1992;61:419–440.

[29] Proudfoot NJ, Brownlee GG. 3' non-coding region sequences in eukaryotic messenger RNA. *Nature*. 1976;263(5574):211–214.

[30] Lee NH, Weinstock KG, Kirkness EF, et al. Comparative expressed-sequence-tag analysis of differential gene expression profiles in PC-12 cells before and after nerve growth factor treatment. *Proc Natl Acad Sci U S A*. 1995;92(18):8303–8307.

[31] Kikuno R, Nagase T, Suyama M, Waki M, Hirosawa M, Ohara O. HUGE: a database for human large proteins identified in the Kazusa cDNA sequencing project. *Nucleic Acids Res*. 2000;28(1):331–332.

[32] Iwahara J, Clubb RT. Solution structure of the DNA binding domain from Dead ringer, a sequence-specific AT-rich interaction domain (ARID). *EMBO J*. 1999;18(21):6084–6094.

[33] Takeuchi T, Chen BK, Qiu Y, Sonobe H, Ohtsuki Y. Molecular cloning and expression of a novel human cDNA containing CAG repeats. *Gene*. 1997;204(1-2):71–77.

[34] Dallas PB, Yaciuk P, Moran E. Characterization of monoclonal antibodies raised against p300: both p300 and CBP are present in intracellular TBP complexes. *J Virol*. 1997;71(2):1726–1731.

[35] Dallas PB, Cheney IW, Liao DW, et al. p300/CREB binding protein-related protein p270 is a component of mammalian SWI/SNF complexes. *Mol Cell Biol*. 1998;18(6):3596–3603.

[36] Schnitzler G, Sif S, Kingston RE. Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state. *Cell*. 1998;94(1):17–27.

[37] Takeuchi T, Furihata M, Heng HH, Sonobe H, Ohtsuki Y. Chromosomal mapping and expression of the human B120 gene. *Gene*. 1998;213(1-2):189–193.

[38] Treisman JE, Luk A, Rubin GM, Heberlein U. Eyelid antagonizes wingless signaling during Drosophila development and has homology to the Bright family of DNA-binding proteins. *Genes Dev*. 1997;11(15):1949–1962.

[39] Vázquez M, Moore L, Kennison JA. The trithorax group gene osa encodes an ARID-domain protein that genetically interacts with the brahma chromatin-remodeling factor to regulate transcription. *Development*. 1999;126(4):733–742.

[40] Seipel K, Georgiev O, Schaffner W. Different activation domains stimulate transcription from remote ("enhancer") and proximal ("promoter") positions. *EMBO J*. 1992;11(13):4961–4968.

[41] Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature*. 1997;387(6634):733–736 (see comments).

[42] Meier R, Spreyer P, Ortmann R, Harel A, Monard D. Induction of glia-derived nexin after lesion of a peripheral nerve. *Nature*. 1989;342(6249):548–550.

[43] Gloor S, Odink K, Guenther J, Nick H, Monard D. A glia-derived neurite promoting factor with protease inhibitory activity belongs to the protease nexins. *Cell*. 1986;47(5):687–693.

[44] Sommer J, Gloor SM, Rovelli GF, et al. cDNA sequence coding for a rat glia-derived nexin and its homology to members of the serpin superfamily. *Biochemistry*. 1987;26(20):6407–6410.

[45] Kim D, Weaver RF. Transcription mapping and functional analysis of the protein tyrosine/serine phosphatase (PTPase) gene of the *Autographa californica* nuclear polyhedrosis virus. *Virology*. 1993;195(2):587–595.

[46] Possee RD, Sun TP, Howard SC, Ayres MD, Hill-Perkins M, Gearing KL. Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis 9.4 kbp EcoRI-I and -R (polyhedrin gene) region. *Virology*. 1991;185(1):229–241.

[47] Tillman JB, Mote PL, Walford RL, Spindler SR. Structure and regulation of the mouse GRP78 (BiP) promoter by glucose and calcium ionophore. *Gene*. 1995;158(2):225–229.

[48] Hennigan RF, Hawker KL, Ozanne BW. Fos-transformation activates genes associated with invasion. *Oncogene*. 1994;9(12):3591–3600.

[49] Nishiguchi S, Joh T, Horie K, Zou Z, Yasunaga T, Shimada K. A survey of genes expressed in undifferentiated mouse embryonal carcinoma F9 cells: characterization of low-abundance mRNAs. *J Biochem (Tokyo)*. 1994;116(1):128–139.

[50] Thurieau C, Brosius J, Burne C, et al. Molecular cloning and complete amino acid sequence of AP50, an assembly protein associated with clathrin-coated vesicles. *DNA*. 1988;7(10):663–669.

[51] Kingston RE, Bunker CA, Imbalzano AN. Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev*. 1996;10(8):905–920.

[52] Peterson CL, Tamkun JW. The SWI-SNF complex: a chromatin remodeling machine? *Trends Biochem Sci*. 1995;20(4):143–146.

[53] Hurlstone AF, Olave IA, Barker N, van Noort M, Clevers H. Cloning and characterization of hELD/OSA1, a novel BRG1 interacting protein. *Biochem J*. 2002;364(pt 1):255–264.

[54] Chiba H, Muramatsu M, Nomoto A, Kato H. Two

human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucleic Acids Res.* 1994;22(10):1815–1820.

[55] Fryer CJ, Archer TK. Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature.* 1998;393(6680):88–91.

[56] Ostlund Farrants AK, Blomquist P, Kwon H, Wrange O. Glucocorticoid receptor-glucocorticoid response element binding stimulates nucleosome disruption by the SWI/SNF complex. *Mol Cell Biol.* 1997;17(2):895–905.

[57] Yoshinaga SK, Peterson CL, Herskowitz I, Yamamoto KR. Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. *Science.* 1992;258(5088):1598–604.

[58] Kadonaga JT. Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell.* 1998;92(3):307–313.

[59] Kingston RE, Narlikar GJ. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev.* 1999;13(18):2339–2352.

[60] Gregory SL, Kortschak RD, Kalionis B, Saint R. Characterization of the dead ringer gene identifies a novel, highly conserved family of sequence-specific DNA-binding proteins. *Mol Cell Biol.* 1996;16(3):792–799.

[61] Herrscher RF, Kaplan MH, Lelsz DL, Das C, Scheuermann R, Tucker PW. The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific trans-activator that describes a new DNA-binding protein family. *Genes Dev.* 1995;9(24):3067–3082.

[62] Kortschak RD, Reimann H, Zimmer M, Eyre HJ, Saint R, Jenne DE. The human dead ringer/bright homolog, DRIL1: cDNA cloning, gene structure, and mapping to D19S886, a marker on 19p13.3 that is strictly linked to the Peutz-Jeghers syndrome. *Genomics.* 1998;51(2):288–292.

[63] Lu PJ, Sundquist K, Baeckstrom D, et al. A novel gene (PLU-1) containing highly conserved putative DNA/chromatin binding motifs is specifically up-regulated in breast cancer. *J Biol Chem.* 1999;274(22):15633–15645.

[64] Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Control of gene expression. *Molecular Biology of the Cell.* 4th ed. New York, NY: Garland Science Publishing; 2002:375–468.

[65] Shi Y, Fard A, Galeo A, Hutchinson HG, et al. Transcatheter delivery of c-myc antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury. *Circulation.* 1994;90(2):944–951.

[66] Simons M, Edelman ER, DeKeyser JL, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature.* 1992;359(6390):67–70.

[67] Mills CJ, Northrup JL, Hullinger TG, Simmons CA, Shebuski RJ, Jones DA. Temporal expression of c-fos mRNA following balloon injury in the rat common carotid artery. *Cardiovasc Res.* 1996;32(5):954–961.

[68] Autieri MV, Yue TL, Ferstein GZ, Ohlstein E. Antisense oligonucleotides to the p65 subunit of NF-kB inhibit human vascular smooth muscle cell adherence and proliferation and prevent neointima formation in rat carotid arteries. *Biochem Biophys Res Commun.* 1995;213(3):827–836.

[69] Indolfi C, Avvedimento EV, Rapacciuolo A, et al. Inhibition of cellular ras prevents smooth muscle cell proliferation after vascular injury in vivo. *Nat Med.* 1995;1(6):541–545.

[70] Gadeau AP, Campan M, Millet D, Candresse T, Desgranges C. Osteopontin overexpression is associated with arterial smooth muscle cell proliferation in vitro. *Arterioscler Thromb.* 1993;13(1):120–125.

[71] Hu Y, Cheng L, Hochleitner BW, Xu Q. Activation of mitogen-activated protein kinases (ERK/JNK) and AP-1 transcription factor in rat carotid arteries after balloon injury. *Arterioscler Thromb Vasc Biol.* 1997;17(11):2808–2816.

[72] Rakugi H, Kim DK, Krieger JE, Wang DS, Dzau VJ, Pratt RE. Induction of angiotensin converting enzyme in the neointima after vascular injury. Possible role in restenosis. *J Clin Invest.* 1994;93(1):339–346.

[73] Morishita R, Gibbons GH, Ellison KE, et al. Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. *J Clin Invest.* 1994;93(4):1458–1464.

[74] Delmolino LM, Castellot JJ Jr. Heparin suppresses sgk, an early response gene in proliferating vascular smooth muscle cells. *J Cell Physiol.* 1997;173(3):371–379.

[75] Sibinga NE, Foster LC, Hsieh CM, et al. Collagen VIII is expressed by vascular smooth muscle cells in response to vascular injury. *Circ Res.* 1997;80(4):532–541.

[76] Bendeck MP, Regenass S, Tom WD, et al. Differential expression of alpha 1 type VIII collagen in injured platelet-derived growth factor-BB–stimulated rat carotid arteries. *Circ Res.* 1996;79(3):524–531.

[77] Koike H, Karas RH, Baur WE, O'Donnell TF Jr, Mendelsohn ME. Differential-display polymerase chain reaction identifies nucleophosmin as an estrogen-regulated gene in human vascular smooth muscle cells. *J Vasc Surg.* 1996;23(3):477–482.

[78] Wang X, Yue TL, Ohlstein EH, Sung CP, Feuerstein GZ. Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation, and inflammatory response. *J Biol Chem.* 1996;271(39):24286–24293.

[79] Sobue K, Hayashi K, Nishida W. Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. *Mol Cell Biochem.* 1999;190(1-2):105–118.