

# Novel $\alpha_1$ -Adrenergic Receptor Signaling Pathways: Secreted Factors and Interactions with the Extracellular Matrix<sup>[S]</sup>

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## ABSTRACT

$\alpha_1$ -Adrenergic receptor ( $\alpha_1$ -ARs) subtypes ( $\alpha_{1A}$ ,  $\alpha_{1B}$ , and  $\alpha_{1D}$ ) regulate multiple signal pathways, such as phospholipase C, protein kinase C (PKC), and mitogen-activated protein kinases. We employed oligonucleotide microarray technology to explore the effects of both short- (1 h) and long-term (18 h) activation of the  $\alpha_{1A}$ -AR to enable RNA changes to occur downstream of earlier well characterized signaling pathways, promoting novel couplings. Polymerase chain reaction (PCR) studies confirmed that PKC was a critical regulator of  $\alpha_{1A}$ -AR-mediated gene expression, and secreted interleukin (IL)-6 also contributed to gene expression alterations. We next focused on two novel signaling pathways that might be mediated through  $\alpha_{1A}$ -AR stimulation because of the clustering of gene expression changes for cell adhesion/motility (syndecan-4 and tenascin-C) and hyaluronan (HA) signaling. We confirmed that  $\alpha_1$ -ARs in-

duced adhesion in three cell types to vitronectin, an interaction that was also integrin-, FGF7-, and PKC-dependent.  $\alpha_1$ -AR activation also inhibited cell migration, which was integrin- and PKC-independent but still required secretion of FGF7.  $\alpha_1$ -AR activation also increased the expression and deposition of HA, a glycosaminoglycan, which displayed two distinct structures: pericellular coats and long cable structures, as well as increasing expression of the HA receptor, CD44. Long cable structures of HA can bind leukocytes, which this suggests that  $\alpha_1$ -ARs may be involved in proinflammatory responses. Our results indicate  $\alpha_1$ -ARs induce the secretion of factors that interact with the extracellular matrix to regulate cell adhesion, motility and proinflammatory responses through novel signaling pathways.

Adrenergic receptors (ARs) belong to the superfamily of G-protein-coupled receptors (GPCRs). They share a common structural motif consisting of seven transmembrane domains. ARs mediate the actions of the endogenous catecholamines norepinephrine and epinephrine and play very important roles in the regulation of different physiological and pathophysiological functions in various sympathetically targeted tissues and cells (Piascik and Perez, 2001). Based on their primary structures and pharmacological characteris-

tics, ARs have been classified into  $\alpha_1$ -ARs,  $\alpha_2$ -ARs, and  $\beta$ -ARs and three subtypes of  $\alpha_1$ -ARs ( $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR, and  $\alpha_{1D}$ -AR) have been cloned and characterized pharmacologically (Cotecchia et al., 1988; Perez et al., 1991, 1994).

Some of the common  $\alpha_1$ -AR signaling pathways have been elucidated (for review, see Graham et al., 1996). Upon ligand binding of  $\alpha_1$ -AR, a signaling cascade occurs. First, there is the activation of Gq that leads to the activation of phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 binds to its receptor and mobilizes intracellular calcium, and diacylglycerol activates protein kinase C (PKC). Both calcium and activated PKC will eventually regulate related gene programs and respective physiological functions, such as smooth muscle contraction. In addition to the well known PLC signaling pathways,  $\alpha_1$ -ARs

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**ABBREVIATIONS:** AR, adrenergic receptor; GPCR, G-protein-coupled receptor; PLC, phospholipase C; IP3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; Epi, epinephrine; RT-PCR, reverse-transcriptase polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; GRK, G protein-coupled receptor kinase; FGF, fibroblast growth factor; HA, hyaluronan; Crem, cAMP responsive element modulator; Fst, follistatin; Sarc, sarcomeric muscle protein; Ccnb1, cyclin B1; Itpr1, inositol 1,4,5-trisphosphate receptor 1;  $\alpha$ -Tub,  $\alpha$ -tubulin; ECM, extracellular matrix.

have also been shown to couple to MAPKs, signal transducer and activator of transcription, and small GTPases (for review, see Gonzalez-Cabrera et al., 2003; Koshimizu et al., 2003). Although we know PLC and mitogen-activated protein kinase pathways are involved in  $\alpha_1$ -AR signaling, more detailed signaling mechanisms and gene programs that are subsequently activated downstream of PLC are not well understood.

The emergence of DNA microarray technology has made it possible to investigate the expression of thousands of genes simultaneously (Lockhart et al., 1996; Schena et al., 1996; King and Sinha, 2001) and thus greatly facilitate the dissection of complex signaling pathways and networks (DeRisi et al., 1996; Ross et al., 2000). Previous gene expression studies performed in our laboratory have shown that short-term activation of all three  $\alpha_1$ -AR subtypes stimulate or repress the gene expression of a surprisingly large number of proteins, most notably the up-regulation of interleukin 6 (IL-6) gene transcription and its related signaling pathways (Gonzalez-Cabrera et al., 2003). This work has focused on characterizing large-scale gene expression alterations induced by both short- and long-term activation of the  $\alpha_{1A}$ -AR subtype in transfected fibroblasts. Long-term activation of the receptor may couple to further downstream pathways from PLC, allowing more RNA regulation to occur. Our goal was to provide some new insight into novel signaling mechanisms and pathways due to  $\alpha_1$ -AR activation.

## Materials and Methods

**Cell Culture and Treatment.** Rat-1 fibroblasts stably transfected with human  $\alpha_{1A}$ -AR cDNA ( $\alpha_{1A}$  fibroblasts) was a gift from GlaxoSmithKline (Research Triangle Park, NC). A-10 is a smooth muscle cell line derived from rat thoracic aorta and DDT1-MF2 is a hamster smooth muscle cell line. Both A-10 and DDT1-MF2 were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified incubator at 37°C in the presence of 5% CO<sub>2</sub>. For Rat-1 fibroblasts, 500  $\mu$ g/ml of the selection antibiotic G418 (Invitrogen, Carlsbad, CA) was additionally added to complete media.

Confluent cell monolayers in culture plates were detached by trypsinization and subcultured at a ratio of 1:3. For all experiments, when cells are at 80% confluence, the  $\beta$ -AR blocker propranolol (Sigma, St. Louis, MO) and the  $\alpha_2$ -AR blocker rauwolscine (Sigma) were added into the cell culture medium at final concentrations of 1  $\mu$ M and 0.1  $\mu$ M, respectively, and incubated for 30 min before the addition of other agents. For  $\alpha_1$ -AR agonist-treated cells, epinephrine (Epi) (Sigma) was added into the culture medium at a final concentration of 10  $\mu$ M and incubation continued for either 1 or 18 h. For IL-6 treatment, IL-6 (R&D System, Minneapolis, MN) was added into the cell culture medium at a final concentration of 1 ng/ml and cells were incubated for 18 h. For treatment with kinase inhibitors, cells were preincubated with either chelerythrine (Sigma) at final concentration of 10  $\mu$ M or staurosporine (Sigma) at final concentration 50  $\mu$ M for 40 min before the addition of epinephrine.

**RNA Extraction and Microarray Experiments.** Media was removed from cell monolayers by aspiration and total RNAs were immediately isolated using TRIzol reagent (Invitrogen) following the manufacturer's procedures. Total RNAs were purified using RNeasy Mini kit (QIAGEN, Valencia, CA). Double-stranded cDNAs were synthesized from 10  $\mu$ g of total RNA using SuperScript Choice double-stranded cDNA synthesis kit from Invitrogen according to the manufacturer's protocol. cDNAs were purified by phenol/chloroform

extraction and ethanol precipitation. Biotin-labeled cRNAs were synthesized by an in vitro transcription reaction using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, New York, NY). cRNAs were purified from the in vitro transcription reaction using RNeasy Mini kit (QIAGEN). The fragmentation of biotin-labeled cRNAs and hybridization of the fragments to the rat genome chip RG-U34A oligonucleotide array (Affymetrix, Santa Clara, CA) were carried out after standard Affymetrix protocols by the Case Western Reserve Affymetrix Core. The hybridization signal was amplified by the Antibody Amplification Protocol as described in the Affymetrix GeneChip Expression Analysis Manual and scanned using a Hewlett Packard GeneArray scanner (Hewlett Packard, Palo Alto, CA).

**Data Preprocessing.** Raw data were acquired using GCOS software of Affymetrix and normalized following a standard practice of scaling the trimmed mean of all gene signal intensities to a common arbitrary value, defaulted to 1000. The default parameters for rat genome chip RG-U34A were used throughout the analyses. Only genes that were not labeled as "Absent" in all experimental conditions using the default parameter were considered for further analysis. Significant changes were defined as having at least 1.7-fold change in gene expression compared with control.

**Pattern Analysis.** To analyze the gene expression alteration patterns in different biological and functional categories, 3119 genes that have available annotations for biological processes from the Gene Ontology Consortium (Ashburner et al., 2000) were annotated and categorized into functional groups using the software GeneSpring 7.2 from Silicon Genetics (Redwood City, CA). The categorized genes were further analyzed based on the gene expression levels in treated and untreated cells to find differentially expressed genes and their alteration patterns across different gene function categories.

**RT-PCR and Real-Time RT-PCR.** cDNAs were synthesized by reverse transcription of 2  $\mu$ g of total RNA using oligo(dT)<sub>12-18</sub> and SuperScript II RNA H-reverse transcriptase (Invitrogen) according to the company's protocol. Using cDNA preparations as templates, DNA fragments of selected genes from the microarray analysis were chosen for further study. Samples were amplified by 25 to 30 PCR cycles using Tag polymerase (Invitrogen) for end-product analysis. The PCR product from  $\alpha$ -tubulin served as a control for normalization. Sequences of the primers used for the end-product PCR amplifications are given in Table 1. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and quantified using the Fluor-Chem 800 imaging system (version 2.0; Alpha Innotech, San Leandro, CA).

Real-time RT-PCR was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). All assays were done in a total volume of 25  $\mu$ l, which included 12.5  $\mu$ l of RT Real-Time SYBR Green/ROX PCR Master Mix from SuperArray Bioscience (Frederick, MD) (containing real-time PCR buffer, a high-fidelity HotStart DNA tag polymerase, nucleotides, SYBR Green dye, and the ROX reference dye), 10.5  $\mu$ l of double-distilled H<sub>2</sub>O, 1.0  $\mu$ l of template cDNA, and 1.0  $\mu$ l of predesigned PCR Primer Set from SuperArray Bioscience. PCR amplification was performed with an initial 15-min step at 95°C to activate the HotStart DNA tag polymerase, followed by 40 cycles of (95°C, 30 s; 55°C, 30 s; and 72°C, 30 s), and an extension for 5 min at 72°C. The fluorescent signal from SYBR Green is detected immediately after the extension step of each cycle and the cycle at which product is first detectable cycle threshold is recorded. Data were imported into Microsoft Excel (Microsoft Corp., Redmond, WA) for analysis.

**Immunoblot Analysis.** Proteins from cultured cells were extracted after homogenizing whole cells in a lysis buffer (0.5% SDS, 25 mM Tris, and 2.5 mM MgCl<sub>2</sub>) in the presence of a protease inhibitor cocktail (Calbiochem, San Diego, CA) and incubated at 100°C for 10 min. Protein extracts were centrifuged for 10 min at 14,000 rpm to remove cell debris. Secreted proteins in the medium were directly concentrated from medium in the presence of protease inhibitor

cocktail using a Centrplus filter (Millipore Corporation, Billerica, MA) with a cut-off size of 5 kDa. Protein concentrations were determined using a protein assay kit according to manufacture's protocol (Bio-Rad, Hercules, CA).

Protein samples (~50  $\mu$ g/lane) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore). Rabbit polyclonal anti-mouse IL-6 antibody (Chemicon International, Temecula, CA) and rabbit polyclonal anti-mouse sarcomeric muscle protein (Krp1) antibody (kindly provided by Dr. B. Ozanne, The Beatson Institute for Cancer Research, Glasgow, UK) were used at 1:1000 dilution for immunoblotting. The protein level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control for protein loading, was determined by using a mouse monoclonal anti-GAPDH antibody (Chemicon International) at 1:5000 dilution. Secondary antibodies used are horseradish peroxidase-linked anti-IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein bands were detected on X-mat film (Eastman Kodak, Rochester, NY) using ECL chemiluminescence reagents (Amersham Biosciences) and scanned using an HP scanjet 3670 scanner (Hewlett Packard). Bands were quantitated using Kodak ID Image Analysis software (Eastman Kodak).

**Immunohistochemistry.** For syndecan 4 and tenascin C immunohistochemistry,  $1 \times 10^5$  cells of Rat-1  $\alpha_1$  fibroblast, A-10, or DDT1-MF-2 in DMEM were seeded in 12-well plates containing a sterile circular coverslip coated with poly-L-lysine. Cells were grown to 90% confluence. For the experimental group,  $\alpha_2$ -AR and  $\beta$ -AR blockers were added to each well and the plate was incubated for 30

min. Epinephrine was then added to the wells, and the incubation was continued for an additional 18 h. For the control group, only  $\alpha_2$ -AR and  $\beta$ -AR blockers were added to each well. After incubation, coverslips were washed three times with PBS and the cells on the coverslip were fixed overnight in 4% formaldehyde. After fixation, formaldehyde was removed by washing the coverslip twice with PBS. The coverslips were first incubated with blocking buffer (6% BSA and 0.3% Triton X-100) for at least 1 h at room temperature on a shaker. Primary antibody against syndecan 4 (Santa Cruz Biotechnology, Santa Cruz, CA) and Tenascin C (a gift from Josephine Adams, Cleveland Clinic Foundation, Cleveland, OH) were then added to the blocking solution at 1:100 and 1:600, respectively, and the incubation continued for 1 day at 4°C. The coverslips were then washed three times with PBS and incubated with goat anti-rabbit secondary antibody conjugated with Fluor 488 (Invitrogen) at 1:3000 for 1 h at room temperature followed by washes in PBS. Coverslips were mounted in VectaShield medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and sealed with nail polish. Sections were analyzed on a confocal laser-scanning microscope (Leica Microsystems, Inc., Deerfield, IL) representing optical sections of 2 to 3  $\mu$ m axial resolution and an average of three line-scans.

Hyaluronan and CD44 immunohistochemistry was performed as described previously (de la Motte et al., 2003). Cells were prepared and treated in same way as described above. Cells on coverslips were fixed with methanol at -20°C for 10 min, and the coverslips were air-dried and preincubated with Hanks' balanced salt solution (HBSS) containing 2% FBS for 30 min at room temperature. The coverslips were incubated with a solution containing biotinylated hyaluronan-binding protein (Seikagaku America, Rockville, MD) at 5  $\mu$ g/ml and anti-CD44 antibody at 5  $\mu$ g/ml in HBSS containing 2% FBS for approximately 16 h at 4°C. The coverslips were washed three times with HBSS and incubated with a solution containing fluorescein-tagged streptavidin (1:500) and Texas-red conjugated anti-IgG directed against the anti-CD44 primary antibody (1:500) in HBSS containing 2% FBS for 1 h at room temperature. After incubation, coverslips were washed three times with HBSS. Coverslips were mounted and analyzed as described above. Images from the immunohistochemistry were quantitated for reactivity to an antibody by using the software Image Pro Plus (ver. 5.1.2; Media Cybernetics, Silver Spring, MD).

**Cell Adhesion and Migration Assays.** Cells were detached from plate with Hanks'-based enzyme-free cell dissociation buffer (Invitrogen) and washed three times with serum-free DMEM containing the  $\beta$ -AR blocker propranolol (1  $\mu$ M) and the  $\alpha_2$ -AR blocker rauwolfscine (0.1  $\mu$ M). For the cell adhesion assay, a nontreated Falcon 96-well plate (BD Biosciences, San Jose, CA) was precoated with fibronectin at 10  $\mu$ g/ml in PBS or vitronectin (Chemicon International) at 1  $\mu$ g/ml in PBS overnight at 4°C. Coating solutions were removed, and the wells were coated with 0.05% polyvinylpyrrolidone (Sigma) for 1 h at 37°C, which is effective in reducing nonspecific binding (Zhang and Plow, 1996), then washed twice with PBS. Cells in DMEM were treated with epinephrine, epinephrine plus RGD peptide (200  $\mu$ M), epinephrine plus anti-FGF-7 antibody (10  $\mu$ g/ml), or epinephrine plus chelerythrine (10  $\mu$ M) for 1 h. Approximately 100,000 treated cells were then added to each well and incubated at 37°C for 30 min. Nonadherent cells were removed, and the wells were washed twice with PBS. The plate was frozen at -80°C overnight. Adherent cells were quantified by determining the total DNA content on the bottom of the membrane using CytoFluor II software (Long Island Scientific, Setauket, NY) after staining the cells with a CyQUANT Reagent kit, following manufacture's protocols (Invitrogen). Cell migration activity was determined using Transwell plates (Corning Life Sciences, Acton, MA) with 8- $\mu$ m pore size membrane, as described previously (Solovjov et al., 2005). Cells ( $5 \times 10^5$  in 150  $\mu$ l of DMEM) were added to the top chamber in the presence or absence of 5  $\mu$ M epinephrine, or epinephrine plus 1  $\mu$ M prazosin (Sigma), epinephrine plus anti-FGF-7 antibody (10  $\mu$ g/ml), or epi-

TABLE 1  
Primers for RT-PCR

Primer Name	Primer Sequence
CD44	
Sense	5'-GCAAGGATACAGACTCCAGT-3
Antisense	5'-GTTGGAGTCAGTAGCAAGAG-3
Crem	
Sense	5'-ACTAGCAGAAGAAGCAACTCGAAAG-3
Antisense	5'-TTAAGAGACCCATCTACAAGTCCAT-3
CyclinB1	
Sense	5'-CTTGGAGCTGTCAACTTTCAGATCTT-3
Antisense	5'-TTAAACAGCGTTAAGCAGCATTGTG-3
Fst	
Sense	5'-AGTGCCTGCCACCTGAGAAAGGCCA-3
Antisense	5'-CGAGATGGAGTTGCAAGATCCGGAG-3
Gro	
Sense	5'-GCGTTTCATCGATGGTCGTTCAATT-3
Antisense	5'-AAAGGCATGTGCCCCACAACTAG-3
HMG2	
Sense	5'-GGTAGGCCAACAGGCTCAAAGAAGA-3
Antisense	5'-TACGTTGTAGCCCCGAAAGGCTA-3
Il6	
Sense	5'-ACTCGGCAACCTAGTGTGTATGC-3
Antisense	5'-TTGCAAGAAACCATCTGGCTAGGTA-3
Itpr1	
Sense	5'-TCTGAACCAACATGATGCAGTAGGT-3
Antisense	5'-AACTTTTGTGCAGAGATGGTCTAGT-3
Nr4a1	
Sense	5'-CTCCTCATTCCAGAAGATGGACAGA-3
Antisense	5'-CTGTCAATCACCTTCTTGAGTGACA-3
p55CDC	
Sense	5'-CAGAACCAAGCTGGTTATTTGGAAGT-3
Antisense	5'-TTGGTGGATGAGGCTACTTTTAGAT-3
Sarc	
Sense	5'-TGTTGGGACAAGAGGGCTTAATGT-3
Antisense	5'-ATGCACAAGATTCTGTCGTAGTCTC-3
Syn4	
Sense	5'-TTCTCGAGATCGAGCGGAG-3
Antisense	5'-CTCATCACCTTCTCTCTAAG-3
Ten-c	
Sense	5'-AGGCCACTGAGTACGAAATT-3
Antisense	5'-GACCATCGAGAGCTGTGATT-3
$\alpha$ -Tubulin	
Sense	5'-AGAGAGCTGTGTGTATGCTGAGCAA-3
Antisense	5'-GGACCAGAATAAACATCCCTGTGAA-3



nephrine plus chelerythrine (10  $\mu$ M). Poly-L-lysine (10  $\mu$ M; Sigma), fibronectin (10  $\mu$ M; Sigma), or 10% FBS in 600  $\mu$ l of DMEM was added to the bottom chamber. After 18 h of incubation, media in both chambers were aspirated. Cells on the top of the membrane were removed using cotton swabs and the Transwell was frozen at  $-80^{\circ}\text{C}$  overnight and quantified using CyQUANT Reagent kit as described above.

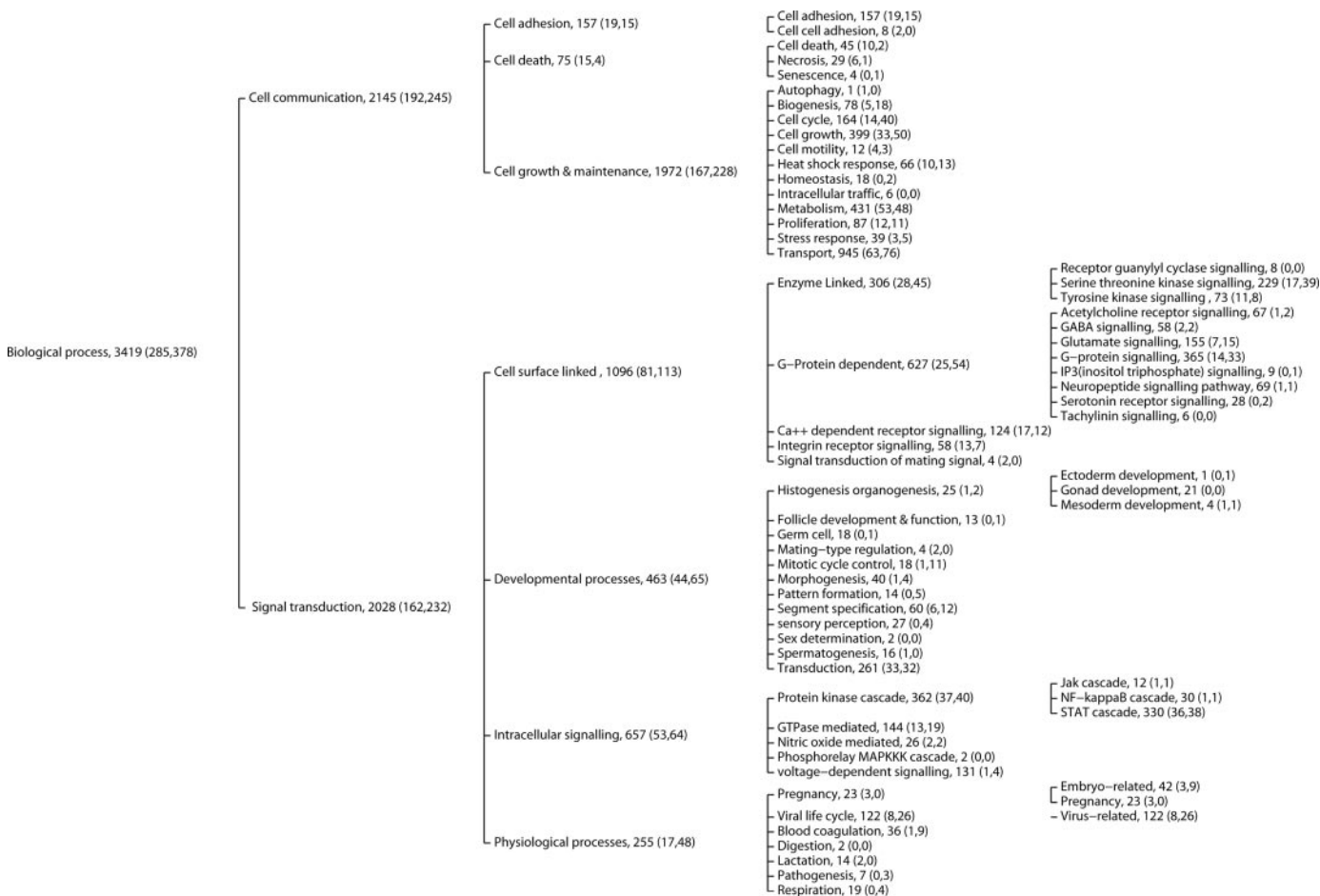
**Statistics.** Significance of cell-based assays was determined by analysis of variance followed by a Neuman-Keuls post test where  $p < 0.05$  was considered significant.

## Results

**Gene Expression Alterations by 1-h and 18-h  $\alpha_{1A}$ -AR Stimulation.** RNA samples were prepared from two separate experiments for each control (unstimulated) and stimulated conditions and hybridized to separate gene chips (i.e., six chips total). Data were analyzed by comparing the gene expression profiles from the unstimulated control samples versus 1 or 18 h of epinephrine stimulation. After 1 h of epinephrine treatment, 499 of 8799 genes (5.7%) were differentially expressed, with 204 genes (2.3%) increasing expression and 295 genes (3.4%) decreasing expression, compared with unstimulated control samples. After 18 h of epinephrine treatment, 1823 of the 8799 genes (20.7%) are differentially expressed, with 765 genes (8.7%) up-regulated (Supplemen-

tal Data file 1) and 1058 genes (12.0%) down-regulated (Supplemental Data file 2). Ninety-six (47%) of the 204 genes up-regulated after 1-h treatment remained up-regulated after 18-h treatment; 25 (12%) of the 204 genes remained down-regulated and 83 (41%) returned to unstimulated levels. For the 295 genes down-regulated after 1-h treatment, 79 (27%) of them remained down-regulated after 18-h treatment, 38 (13%) increased expression, and 178 (60%) returned to unstimulated levels. It was apparent that more genes were down-regulated during both 1-h (3.4% of 5.7%) and 18-h (8.7% of 20.7%) epinephrine treatment.

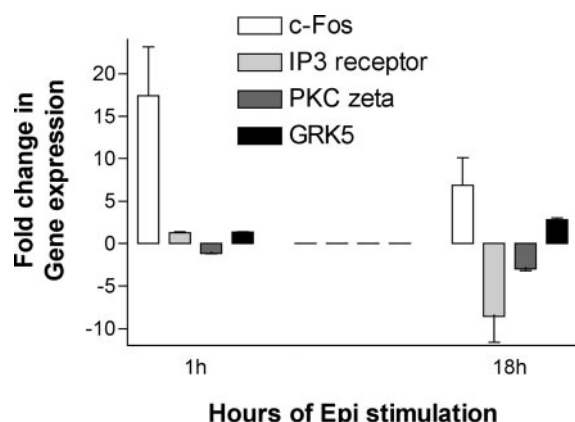
**Pattern Assessment after 18-h  $\alpha_{1A}$ -AR Stimulation.** Because 1-h gene expression patterns have already been published (Gonzalez-Cabrera et al., 2003), we made a gene ontology tree for differentially expressed genes induced after 18 h  $\alpha_{1A}$ -AR stimulation (Fig. 1). Of the 8799 genes on rat genome chip RG-U34A, 3419 genes have available annotations under the heading of biological processes from the Gene Ontology Consortium (Ashburner et al., 2000). These annotated genes were then categorized and analyzed based on their gene expression levels in treated and untreated cells. Overall, the annotated genes were categorized into more than 70 different functional groups suggesting diverse gene expression alterations induced by epinephrine (Fig. 1). There



**Fig. 1.** Biological process ontology tree of 3419 genes showing patterns of gene expression alterations in  $\alpha_{1A}$ -AR fibroblasts induced after 18-h epinephrine stimulation. There are three numbers after the name of each gene category. The first integer represents the number of genes in the category. The first number in parentheses stands for the percentage of genes in that category that are up-regulated after epinephrine treatment. The second number in parentheses represents the percentage of genes that are down-regulated. Percentages are based upon the average of two independent experiments and compared with control (nonstimulated).

are three numerical numbers after the name of each gene category. The first integer represents the number of genes listed in the category as available from the Gene Ontology Consortium. The first number in parentheses stands for the number of genes in the category that are significantly up-regulated, and the second number in parentheses represents the number of genes that were down-regulated after epinephrine stimulation.

In many functional categories, the majority of the differentially expressed genes are down-regulated. In contrast, in only a few categories, such as cell death, necrosis, and integrin receptor signaling, were the majority of differentially expressed genes up-regulated. It is noteworthy that large repertoires of genes were changing in cell cycle [54 of 164 (33%)] and mitotic cycle control [12 of 18 (67%)] categories, again with down-regulation being the dominant change.



**Fig. 2.** Microarray gene expression changes after 1- versus 18-h epinephrine stimulation for common early signal transduction genes associated with  $\alpha_1$ -AR activation. Although c-fos expression was elevated after 1-h epinephrine stimulation, at 18-h stimulation, expression was decreased as well as for genes for the IP3 receptor and PKC- $\zeta$ , whereas GRK5 increased expression, consistent with down-regulation of  $\alpha_1$ -AR signaling. Data are taken directly from the microarray results and -fold changes are the average of two independent experiments, compared with control (nonstimulated).

**TABLE 2**  
Notable differentially expressed genes

Gene Names	Accession No.	-Fold Change		Function
		1 h	18 h	
Sarcomeric muscle protein	AI639444	N.C.	+110.7	Cell elongation
Nuclear receptor subfamily 4, group A	AI176710	+7.0	+68.8	Transcriptional activator
cAMP responsive element modulator	S66024	+10.5	+41.4	CREM; transcription
Follistatin	AA858520	N.C.	+8.9	Growth factor
Interleukin 6	M26744	+74	+10.1	Cytokine
Hyaluronan synthase 2	AI008741	N.C.	+4.8	Inflammation
Fibroblast growth factor 7	X56551	N.C.	+3.5	Proliferation/adhesion
Tenascin C	U09401	N.C.	+3.4	Integrin-dependent cell adhesion/motility
CD44 antigen (Hyaluronan receptor)	AF014365	N.C.	+2.6	Inflammation/cell movement/adhesion
Syndecan 4	S61868	N.C.	+2.5	Integrin-dependent cell adhesion/motility
Jun-B	X54686	+3.0	+2.8	Transcription factor
BRCA1	AA800265	N.C.	-9.8	Breast cancer gene
Lamin B1	AA957201	N.C.	-8.1	Structural protein
High mobility group box 2 (Hmgb2)	AI008836	N.C.	5.5	Transcription regulator
IL-15	AF015719	N.C.	-5.4	Cytokine
Cell cycle protein p53CDC	AF052695	N.C.	-4.8	Cell Cycle
BRCA2	U89653	N.C.	-4.2	Breast cancer gene
Pleiotrophin	AI102795	N.C.	-4.1	Growth factor
Gro	D11445	+2.5	-2.7	Chemokine
Interleukin 1 receptor-like 1	U04319	N.C.	-2.5	DNA binding
Hyaluronan motility receptor (Hmhr or RHAMM)	U87983	N.C.	-5.15	HA-mediated motility

N.C., no change.

**PLC-Associated Gene Expression.** According to the microarray results, several gene expressions were altered after 18 h of epinephrine stimulation that were associated with the Gq/PLC pathway (Fig. 2). After 1-h stimulation and as reported previously (Gonzalez-Cabrera et al., 2003), c-fos mRNA levels are high, consistent with the expression pattern commonly seen in immediate early genes. However, after 18-h stimulation, c-fos as well as several PLC-associated genes, such as the IP3 receptor and PKC- $\zeta$  became down-regulated compared with the 1-h timepoint. In contrast, GRK5, a GPCR receptor kinase known to be involved in the down-regulation of ARs and is itself, modified by PKC (Pronin and Benovic, 1997), increased in expression. This data confirms the accuracy of our microarray because it is consistent with an expected down-regulation paradigm as a result of extensive  $\alpha_1$ -AR stimulation.

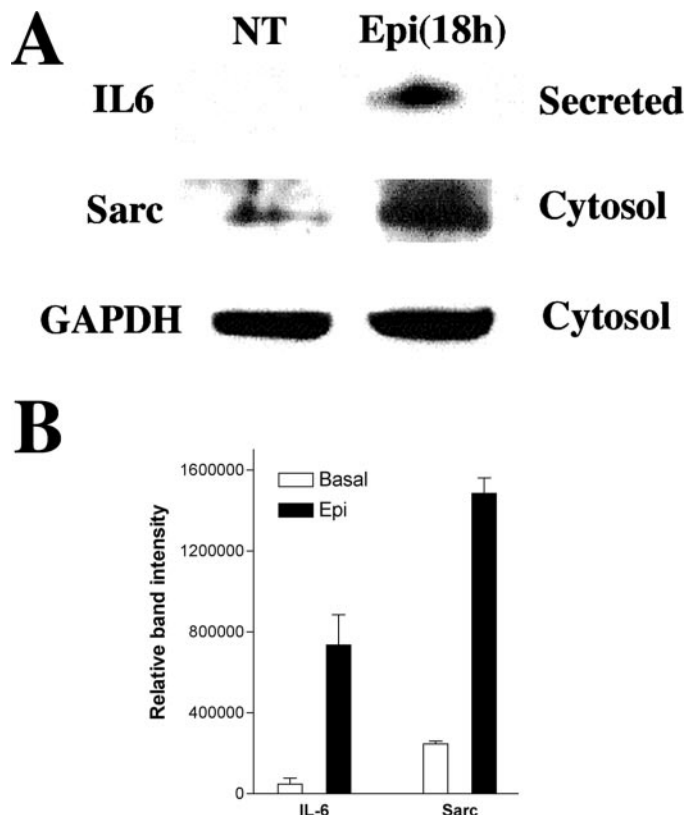
**Potential Novel  $\alpha_1$ -AR Signaling Pathways.** Some of the more interesting or significant genes whose expression levels were altered after  $\alpha_{1A}$ -AR activation were identified and are shown in Table 2. IL-6, a previously characterized secreted protein produced after  $\alpha_1$ -AR activation for 1 h (Gonzalez-Cabrera et al., 2003) decreases its level of expression after 18 h. Genes involved in cell adhesion, motility, and growth, such as FGF7 (+3.5-fold), tenascin C (+3.4-fold), and syndecan 4 (+2.5-fold), increased expression, suggesting  $\alpha_{1A}$ -AR involvement in cell adhesion factors that may serve as ligands and/or modulators of integrin and adhesion receptors. Genes involved with hyaluronan (HA), such as hyaluronan synthase 2 (+4.8-fold), CD44, a receptor for HA (+2.6-fold), and the HA motility receptor (-5.15-fold), suggests  $\alpha_{1A}$ -AR involvement in HA signaling. The breast cancer genes BRCA1 and -2 were both decreased after 18 h of  $\alpha_{1A}$ -AR stimulation (-9.8- and -4.2-fold, respectively).

**RT-PCR and Protein Confirmation of Gene Expression Changes.** Rat-1 cells exposed to 10  $\mu$ M epinephrine for 1 or 18 h were analyzed to confirm differential mRNA expression via PCR. To confirm up-regulated genes, cAMP-responsive element modulator (Crem), follistatin (Fst), IL-6,

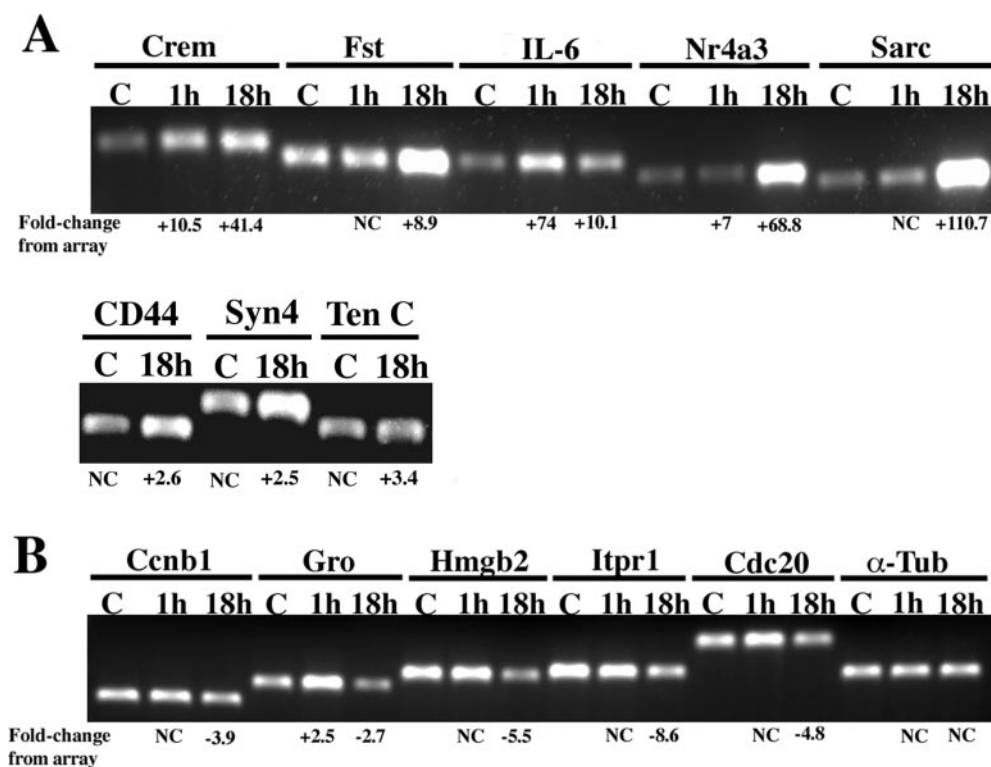
nuclear receptor subfamily 4, group A, member 3, tenascin C, syndecan 4, CD44, and sarcomeric muscle protein (Sarc), were chosen for analysis and primers synthesized as shown in Table 1. For the analysis of down-regulated genes, cyclin B1 (Ccnb1), Gro, high mobility group box 2, inositol 1,4,5-triphosphate receptor 1 (Itpr1), cell cycle protein p53CDC, and  $\alpha$ -tubulin ( $\alpha$ -Tub), were chosen and primers were synthesized (Table 1). PCR of  $\alpha$ -tubulin was used as an internal control for the RT-PCR. As shown in Fig. 3, up- or down-regulation of mRNA was confirmed.

To confirm the correlation between the gene expression alterations at the protein level, immunoblot assays were performed on two of the most highly up-regulated genes, IL-6 and Sarc. Protein levels of IL-6 secreted into the medium and Sarc in whole cell lysates were determined. Epinephrine treatment for 18 h in the fibroblasts increased IL-6 production and the protein levels of Sarc (Fig. 4), consistent with the microarray data.

We also detected increased protein expression of syndecan 4 and tenascin C, two interactive proteins involved in cell adhesion, via immunohistochemistry in Rat 1 fibroblasts as well as two  $\alpha_1$ -AR endogenously expressing smooth muscle cell lines, A-10 and DDT1-MF2. DDT1-MF2 has been characterized to express only the  $\alpha_{1B}$ -AR by ligand binding (Han et al., 1992), whereas the A10, a rat smooth muscle cell line from aorta is believed to express higher levels of the  $\alpha_{1D}$ -AR (Faber et al., 2001), although the binding and signaling characteristics in this particular cell line are not extensive. After 18 h of epinephrine stimulation, syndecan 4, which is expressed ubiquitously, is enriched in focal adhesions (Fig. 5, arrows) as well as the cytoplasm. After 18 h of epinephrine stimulation, tenascin C, a large protein, increased deposition in the extracellular matrix (ECM) in fibroblasts and the A-10 cells (Fig. 6, A–D, arrows). In DDT1-MF2 cells, mildly increased tenascin C expression was evident in the cytoplasm

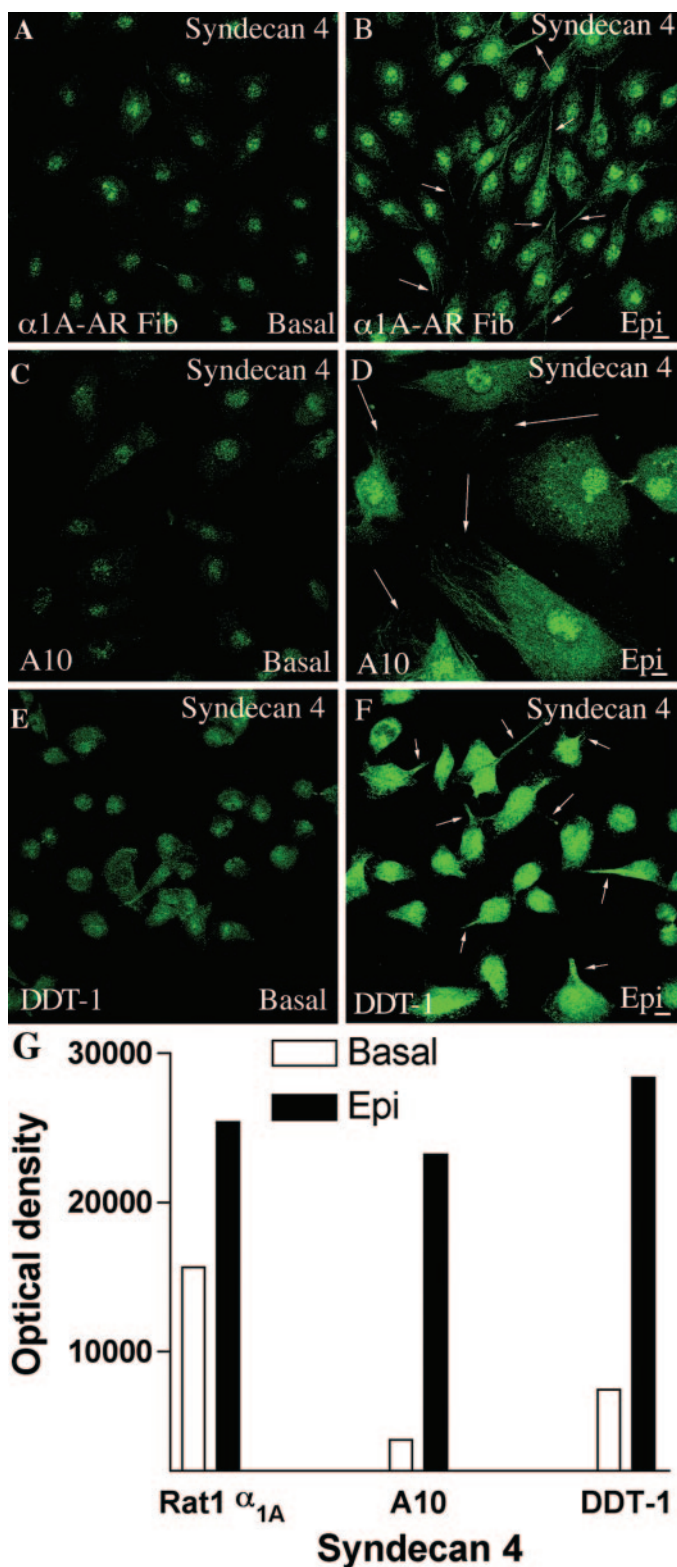


**Fig. 4.** A, immunoblot analysis of increased protein expression of IL-6 and Sarc induced after 18-h  $\alpha_1$ -AR activation in transfected fibroblasts versus untreated control samples (NT). Protein levels of IL-6 were secreted into medium and protein levels of Sarc were expressed in the cytosol. GAPDH serves as a protein loading control. B, quantitation of IL-6 and Sarc expression from three Western blots. Bands were quantitated using Kodak ID Image Analysis software.



**Fig. 3.** RT-PCR analysis on the effect of  $\alpha_1$ -AR activation on gene expression alterations in transfected fibroblasts. Cells were exposed to 10  $\mu$ M epinephrine for 1 or 18 h in the presence of  $\beta$ -AR and  $\alpha_2$ -AR blockers. RT-PCR of  $\alpha$ -tubulin was used as an internal normalization control for the PCR reactions. Cells exposed to  $\beta$ -AR and  $\alpha_2$ -AR blockers only served as control (C). A, up-regulated gene expressions. B, down-regulated gene expressions. Numbers at bottom indicate -fold changes resulting from the microarray analysis for comparison.





**Fig. 5.** Confocal images of Syndecan-4 expression before (Basal) and after (Epi) 18-h epinephrinestimulation. A, C, and E, basal Syndecan-4 expression in transfected  $\alpha_1$ -AR fibroblasts, A-10, and DDT1-MF2 cells, respectively, exposed to  $\alpha_1$ -AR and  $\beta$ -AR blockers only. B, D, and F, syndecan-4 expression levels after treatment with epinephrine (10  $\mu$ M) for 18 h.  $\alpha_1$ -AR activation caused all of the cell lines to form focal adhesions (arrows) expressing syndecan-4. The A-10 cell line also spread in response to  $\alpha_1$ -AR stimulation (D). Scale bar, 10  $\mu$ m. G, quantitation of syndecan 4 using the software Image Pro Plus (MediaCybernetics).

but not in the extracellular matrix (Fig. 6, E and F, arrows). In all cases, cell morphology seemed to be changing with regard to cell spreading, being more evident in the A-10 cells.

Hyaluronan secretion and signaling may be activated by  $\alpha_1$ -AR stimulation as a result of increases in the mRNA for its synthesizing enzyme (HAS2) and the dominant HA receptor, CD44. Because antibodies are unavailable for this glycosaminoglycan, a biotinylated hyaluronan-binding protein was used for analysis as well as using antibodies against CD44. After 18 h of epinephrine stimulation, HA expression and deposition increased and displayed two distinct structures: pericellular coats and cables of HA that can span several cell lengths (Fig. 7, green). CD44 was also up-regulated and at times colocalized with HA (Fig. 7, red and yellow). All of the protein data seemed consistent with the microarray results.

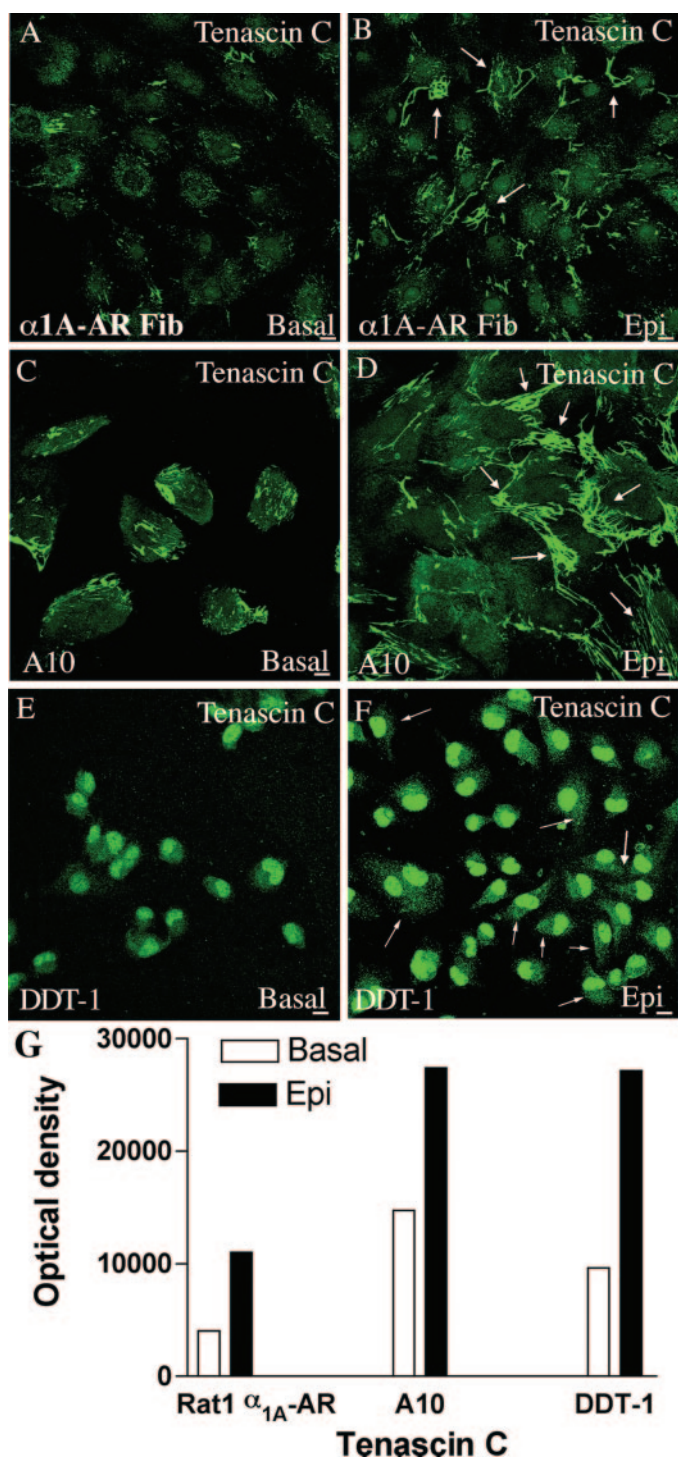
**Gene Expression Alterations Mediated by IL-6 and FGF7.** Gene expression results indicate that many genes such as Sarc and Fst are differentially expressed only after 18 h epinephrine stimulation whereas other genes, such as IL-6 and nuclear receptor subfamily 4, group A, member 3, were already differentially expressed only after 1-h stimulation. We hypothesized that some of these  $\alpha_1$ -AR early gene expression changes and the resulting proteins may actually contribute to prolonged  $\alpha_1$ -AR mediated gene expression changes as a result of secretion of growth factors. IL-6 is a very good candidate to test the hypothesis because the mRNA level of IL-6 is highly up-regulated at 1-h stimulation by epinephrine, and the protein is secreted into the medium. Therefore, Rat-1  $\alpha_1$  fibroblasts were treated with exogenous IL-6 (1 ng/ml) or FGF7 (100 ng/ml) and incubated for 18 h. Total RNAs were extracted from treated and untreated cells, and RT-PCR experiments were performed to determine the relative expression levels of selected genes. The results indicate that the addition of IL-6 indeed up-regulated the gene expression of Fst, Sarc, and Ccnb1 and down-regulated Crem and Itpr1 (Fig. 8A) but did not affect the expression of the other genes tested. Up-regulation of Fst and Sarc and the down-regulation of Itpr1 by IL-6 were consistent with the microarray data, suggesting that the secondary secretion of IL-6 after epinephrine-stimulation may contribute to the mechanism of their gene expression regulation. FGF7-mediated gene expression caused the up-regulation of Crem, which was consistent with the microarray data (Fig. 8B) but also caused the up-regulation of Itpr1. To confirm that either IL-6 or FGF7 was contributing to  $\alpha_1$ -AR mediated RNA changes, neutralizing antibodies to IL-6 and FGF7 were used in addition to epinephrine treatment. Only  $\alpha_1$ -AR-mediated RNA changes in Sarc and Fst were neutralized with anti-IL-6 but not anti-FGF7 (Fig. 8C). Neither neutralizing antibody affected any of the other genes tested.

**PKC Mediation of Gene Expression Induced by  $\alpha_1$ -AR Activation.** Although  $\alpha_1$ -ARs have previously been shown to regulate the transcription of a number of genes (Hwa et al., 1996), the intermediate signaling pathways are not known, especially for the large repertoire reported by our microarray data. To identify signaling mechanism(s) that lead to the altered expressions, we targeted four genes whose expression changed the most (IL-6, Crem, Fst, and Sarc) and kinases, such as PKC, a pivotal kinase in the signaling of  $\alpha_1$ -ARs. We performed both end-product RT-PCR and real-

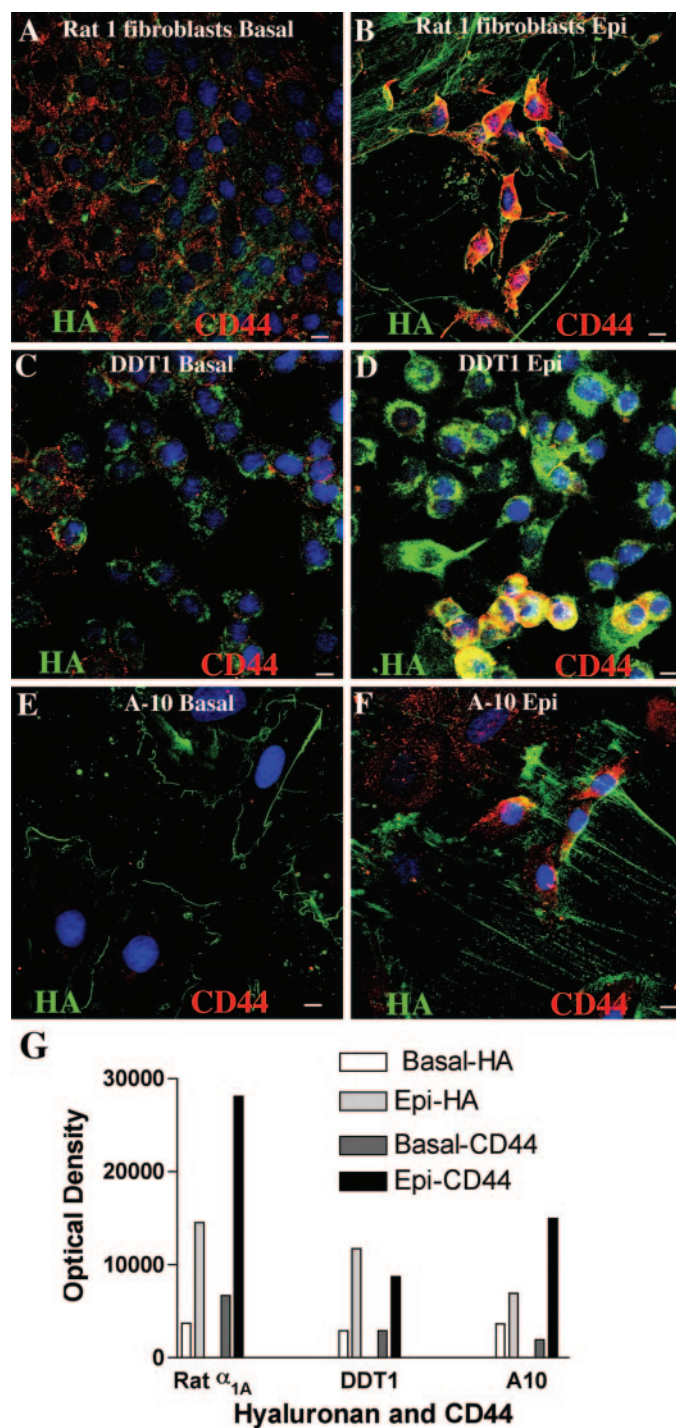


time RT-PCR and analyzed the effects of staurosporine, a broad-based kinase inhibitor, and chelerythrine, a PKC-specific inhibitor, on mRNA expression after 18-h epinephrine stimulation. The results from end product RT-PCR (Fig. 9)

indicated that both chelerythrine and staurosporine completely blocked the up-regulation of Crem induced by epinephrine, whereas staurosporine was better than cheleryth-



**Fig. 6.** Confocal images of Tenascin-C expression before (Basal) and after (Epi) 18-h epinephrine stimulation. A, C, and E, basal tenascin-C expression in transfected  $\alpha_1A$ -AR fibroblasts, A-10, and DDT1-MF2 cells, respectively, exposed to  $\alpha_2$ -AR and  $\beta$ -AR blockers only. B, D, and F, tenascin-C expression after treatment with epinephrine (10  $\mu$ M) for 18 h.  $\alpha_1$ -AR activation caused increased deposition of tenascin-C into the extracellular matrix (arrows). DDT1-MF2 cells have increased expression of tenascin-C in the cytoplasm (F). Scale bar, 10  $\mu$ m. G, quantitation of tenascin C using Image Pro Plus software.



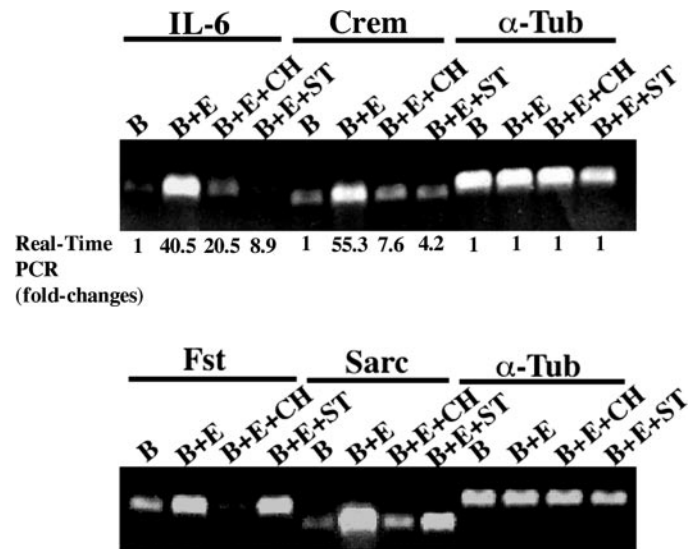
**Fig. 7.** Confocal images of hyaluronan (HA) and CD44 expression in cells before (Basal) and after (Epi) 18-h epinephrine stimulation. A, C, and E, basal HA (green) and CD44 (red) expression in transfected  $\alpha_1A$ -AR fibroblasts, DDT1-MF2, and A-10 cells, respectively, exposed to  $\alpha_2$ -AR and  $\beta$ -AR blockers only. B, D, and F, HA (green) and CD44 (red) expression after treatment with epinephrine (10  $\mu$ M) for 18 h.  $\alpha_1$ -AR activation caused increased deposition of HA into the extracellular matrix resulting in long cable structures (B and F) or increased pericellular expression (D). Some colocalization of CD44 with HA is evident (yellow). Nuclei are blue. Scale bar, 10  $\mu$ m. G, quantitation of CD44 and HA using Image Pro Plus software.



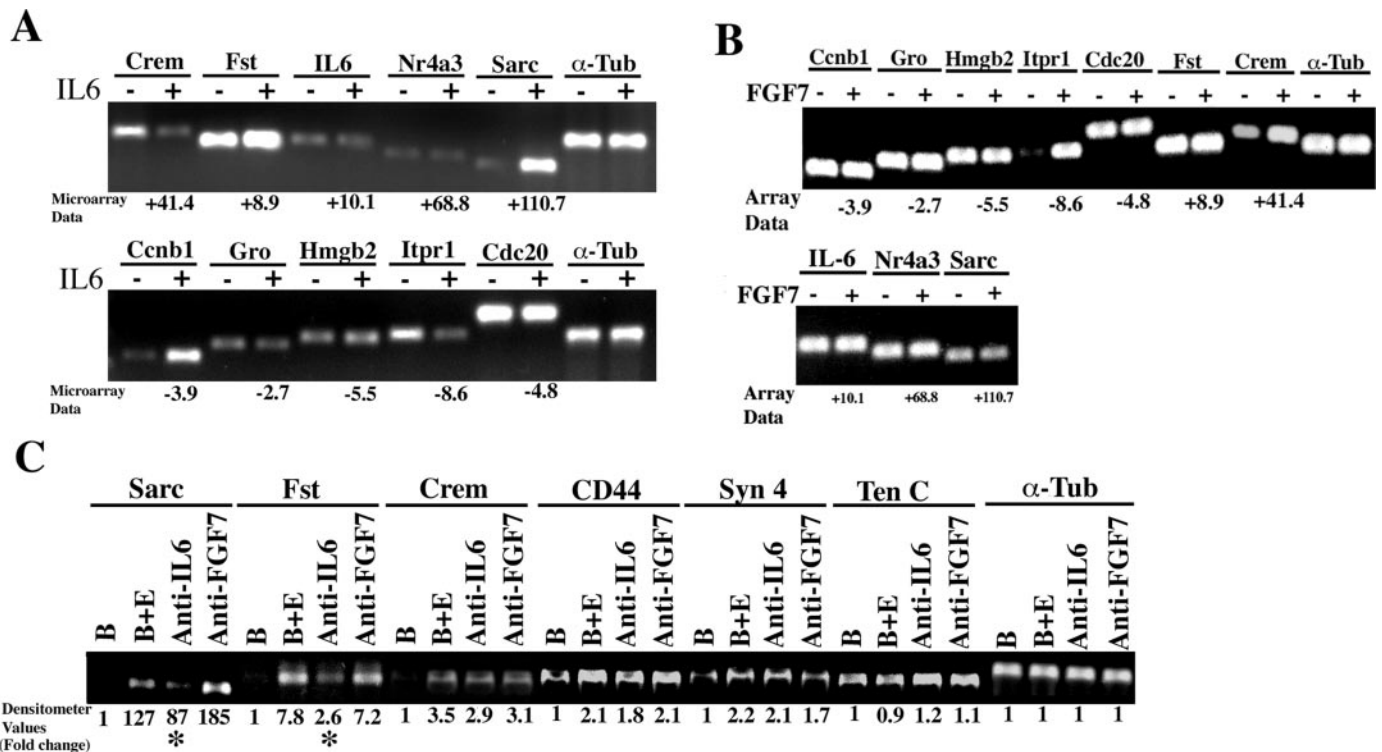
rine in blocking up-regulation of IL-6. For Sarc, chelerythrine completely blocked and staurosporine partially blocked its up-regulation. For Fst, chelerythrine completely blocked its up-regulation; staurosporine produced no notable changes. Results from real-time PCR (Fig. 9) agreed with those from RT-PCR.

**Cell Adhesion and Motility.** Because products of several up-regulated genes in Table 2 are involved in cell adhesion and migration, we performed cell adhesion assays using the three cell lines to show effects can be regulated by all three  $\alpha_1$ -AR subtypes. Epinephrine enhanced adhesion of fibroblasts and the A-10 cell line to vitronectin (Fig. 10), although the binding of all cell lines to fibronectin was unaffected. The vitronectin-mediated cell adhesion was integrin-dependent, as it was blocked by the Arg-Gly-Asp (RGD) peptide, which is an inhibitor of ligand binding to many integrins (Fig. 10). FGF7, a growth factor that is involved in integrin-mediated cell adhesion and whose gene expression increased after epinephrine stimulation (+3.5-fold; Table 2), also seemed to regulate cell adhesion, because the addition of a neutralizing antibody against FGF7 partly inhibited the  $\alpha_1$ -AR mediated cell adhesion to vitronectin (Fig. 10). Finally, cell adhesion was also inhibited by chelerythrine, indicating that PKC was also mechanistically involved.

Upon prolonged incubation, cells may migrate in response to stimuli or attractants. Cell migration was determined using transwell plates and incubating cells with or without epinephrine for 18 h in the upper chamber and either polyllysine, fibronectin, or 10% FBS in the bottom chamber. Epi-



**Fig. 9.** RT-PCR analysis on the effect of kinase inhibitors on gene expression alterations induced after  $\alpha_{1A}$ -AR activation in transfected fibroblasts. For treatment involving kinase inhibitors and/or epinephrine, cells were preincubated with  $\alpha_2$ -AR and  $\beta$ -AR blockers only (B), blockers and 10  $\mu$ M epinephrine (B+E), blockers, 10  $\mu$ M epinephrine, and 10  $\mu$ M chelerythrine (B+E+CH), or blockers, 10  $\mu$ M epinephrine, and 50  $\mu$ M staurosporine (B+E+ST).  $\alpha$ -Tubulin ( $\alpha$ -Tub) was used as an internal control for the RT-PCR.  $\alpha_1$ -AR-mediated gene expression (18 h) was blocked by both chelerythrine and staurosporine but not to the same extent.



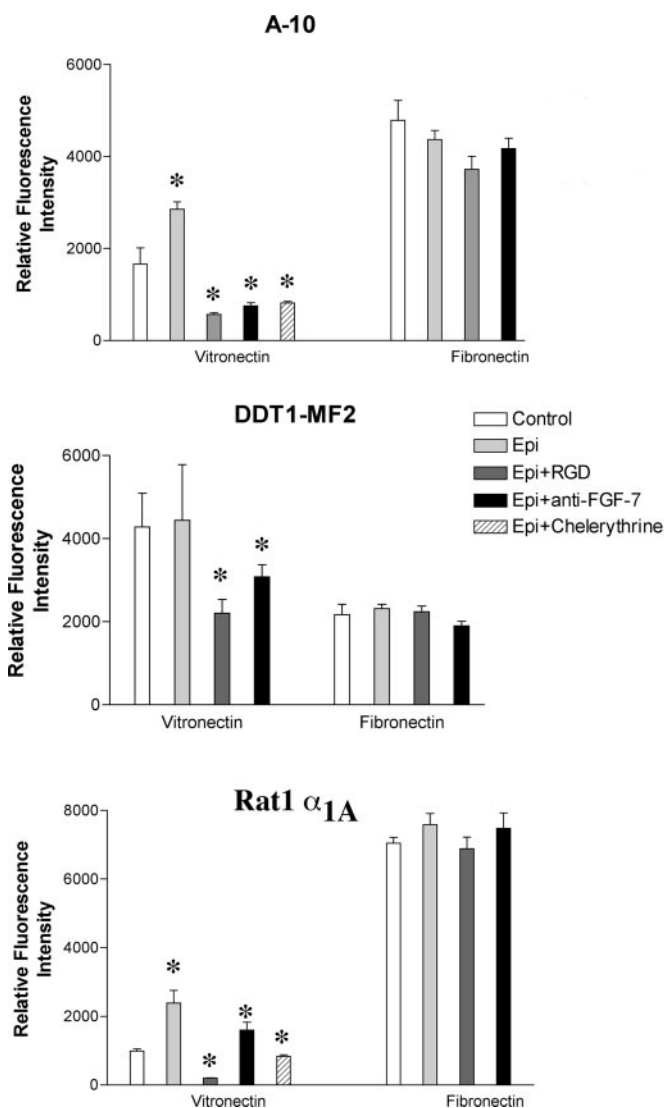
**Fig. 8.** A, RT-PCR analysis of IL-6-mediated regulation of gene expression in transfected  $\alpha_{1A}$ -AR fibroblasts. Cells were treated with IL-6 at 1 ng/ml for 18 h and compared with nontreated cells as a control. IL-6 increased expression of Fst, Sarc, and Ccnb1 and decreased expression of Crem and Itpr1. B, RT-PCR analysis of FGF7-mediated regulation of gene expression in  $\alpha_{1A}$ -AR fibroblasts. Cells were treated with FGF-7 at 100 ng/ml for 18 h and compared with nontreated cells as a control. FGF7 increased expression of Itpr1 and Crem. C, neutralizing antibodies to IL-6 inhibit epinephrine-mediated increases in Sarc (\*) and Fst (\*) gene expression without affecting other genes (Ccnb1 and Itpr1 not shown). Anti-FGF7 displayed no effects on gene expression.  $\alpha$ -Tubulin ( $\alpha$ -Tub) was used as an internal control for the RT-PCR. Numbers at bottom indicate -fold changes from the microarray analysis or densitometer values.

nephrine inhibited cell migration in most cases (Fig. 11), but the effect was substrate-independent. The addition of prazosin, an  $\alpha_1$ -AR antagonist, reversed the inhibitory effect of epinephrine, confirming involvement of  $\alpha_1$ -ARs (Fig. 11). It is noteworthy that, similar to cell adhesion, FGF7 was also involved in  $\alpha_1$ -ARs inhibiting cell migration, but this response was PKC-independent because chelerythrine could not block the effect (Fig. 11).

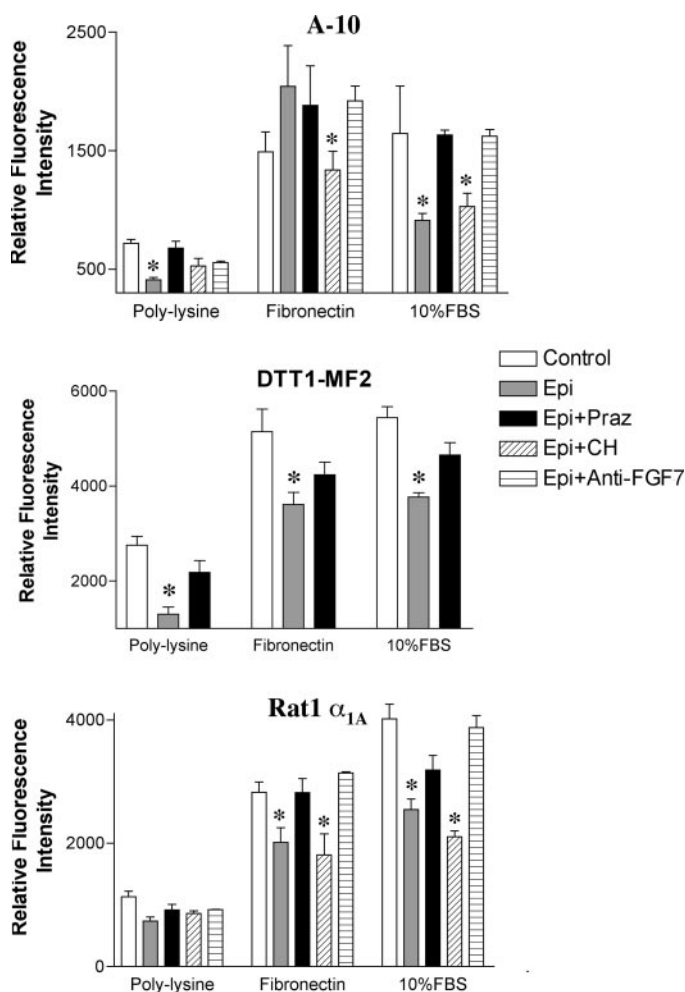
## Discussion

Gene expression alterations in rat-1 fibroblasts induced by short-term (1 h) epinephrine-stimulation of all three  $\alpha_1$ -AR subtypes were previously reported (Gonzalez-Cabrera et al.,

2003). We repeated these studies for consistency and for direct comparison with gene expression changes that occur during prolonged stimulation (18 h) focusing on the  $\alpha_{1A}$ -AR subtype. We rationalized that prolonged stimulation would enable RNA changes to occur downstream of earlier well characterized signaling pathways, such as PLC, mitogen-activated protein kinase, cfos, and this might lead to the discovery of novel signaling and physiological pathways coupled to  $\alpha_{1A}$ -AR stimulation. Pattern analysis of gene expression alterations induced by prolonged  $\alpha_{1A}$ -AR activation based on the gene annotations from the Gene Ontology Consortium (Ashburner et al., 2000) revealed diverse gene expression alterations across more than 70 gene function categories (Fig. 1). These interesting patterns of gene expression alterations imply important functional pathways that are regulated or altered by prolonged  $\alpha_{1A}$ -AR activation and provide new directions for future investigation. The most nota-



**Fig. 10.** Effect of  $\alpha_1$ -AR activation on the cell adhesion activity of A-10, DDT1-MF2, and transfected  $\alpha_{1A}$ -AR fibroblasts. Cell adhesion assays were done using nontreated Falcon 96-well plates coated with either vitronectin (1  $\mu$ g/ml) or fibronectin (10  $\mu$ g/ml). Cells in DMEM were treated with epinephrine (Epi), epinephrine plus RGD peptide (200  $\mu$ M), epinephrine plus anti-FGF-7 antibody (10  $\mu$ g/ml), or epinephrine plus chelerythrine (10  $\mu$ M) for 1 h. Cells treated with  $\alpha_2$ -AR and  $\beta$ -AR blockers only served as controls.  $\alpha_1$ -AR activation enhanced vitronectin-dependent cell adhesion that was blocked by the addition of either the RGD peptide, a neutralizing antibody to FGF7 or chelerythrine. Data shown are the mean  $\pm$  S.E.M. from three to five independent experiments performed in duplicate. \*,  $p < 0.05$  versus control or epinephrine-treatment.



**Fig. 11.** Effect of  $\alpha_1$ -AR activation on the migration of A-10, DDT1-MF2, and transfected  $\alpha_{1A}$ -AR fibroblasts. Cell migration assays were done using transwell plates with 8- $\mu$ m pore size membranes. Cells were added to the top chamber in the presence or absence (Control) of 5  $\mu$ M epinephrine (Epi), 5  $\mu$ M epinephrine plus 1  $\mu$ M prazosin (Epi+ Praz), epinephrine plus 10  $\mu$ M chelerythrine (Epi+CH), or epinephrine plus anti-FGF7 antibody (10  $\mu$ g/ml) for 18 h, whereas poly-L-lysine (10  $\mu$ M), fibronectin (10  $\mu$ M), or 10% FBS in 600  $\mu$ l of DMEM was added to the bottom chamber.  $\alpha_1$ -AR activation inhibited cell migration that was substrate-independent, indicating effects on cell motility that are integrin-independent and that could be blocked with anti-FGF7 but not with chelerythrine. Data shown are the mean  $\pm$  S.E.M. from three to five independent experiments performed in duplicate. \*,  $p < 0.05$  versus control.



ble pattern is the dramatic, down-regulated gene expression alterations in the cell cycle and mitotic cycle control category (Fig. 1). This pattern is consistent with our previous studies indicating that  $\alpha_1$ -ARs are involved in cell cycle control and that  $\alpha_{1A}$ -AR activation leads to a G<sub>1</sub>-S cell cycle arrest of rat-1 fibroblasts as well as other cell types (Gonzalez-Cabrera et al., 2004).

In confirmation of our hypothesis about downstream signal indicators, known genes involved in  $\alpha_1$ -AR signal transduction, consistent with the  $\alpha_{1A}$ -AR becoming down-regulated after 18-h stimulation, were differentially expressed in the microarray (Fig. 2). Gene expression changes in the microarray at 18 h for the IP3 receptor (Itpr1), PKC- $\zeta$ , and cFos decreased compared with the microarray results from 1-h epinephrine-stimulation, consistent with the down-regulation of these components of the  $\alpha_{1A}$ -AR signaling pathway. GRK5 increased gene expression, consistent with the increased function of this protein in phosphorylating the receptor. In addition, PCR studies confirmed the results of the microarray directly, by agreeing with directional changes in RNA expression of a selected group of genes (Fig. 3). Because RNA changes may not correlate to changes in the protein, we also confirmed the microarray by assessing changes in protein and function. However, we focused on three apparent novel pathways that may be mediated through  $\alpha_{1A}$ -AR stimulation because of the clustering of gene expression changes for secreted growth factors (IL-6, FGF7), cell adhesion/motility, and hyaluronan signaling.

IL-6 is a secreted proinflammatory cytokine that plays key roles in cell growth, differentiation, inflammation, and in the pathogenesis of many diseases, such as cardiac hypertrophy and coronary artery disease (Kanda et al., 2000). In addition to our previous study, which demonstrated  $\alpha_1$ -AR subtype-dependent effects on IL-6/gp130/stat3 signaling (Gonzalez-Cabrera et al., 2003), the up-regulation of IL-6 mRNA and protein secretion was also reported in astrocytes and hepatocytes after  $\alpha_1$ -AR activation (Norris and Benveniste, 1993; Jung et al., 2000). We also confirmed that IL-6 secretion was maintained by Western blot after 18 h of  $\alpha_{1A}$ -AR stimulation (Fig. 4). Although the mechanism of IL-6 secretion after  $\alpha_1$ -AR stimulation is unclear, our results suggest that IL-6 itself may affect some of the same gene expression alterations induced by  $\alpha_{1A}$ -AR activation (Fig. 8A). This was confirmed for Sarc and Fst gene expression because a neutralizing antibody to IL-6 blocked  $\alpha_1$ -AR mediated RNA changes (Fig. 8C). Although the effects of other potentially secreted growth factors revealed through the microarray (IL-15, Pleiotrophin, Gro) (Table 2) have not been tested yet, it is possible that they also will have similar effects on gene transcription. However, although FGF7 also affected the gene transcription of Crem (Fig. 8B), a neutralizing antibody to FGF7 could not block the  $\alpha_1$ -AR mediated changes in RNA, suggesting that FGF7 does not contribute to  $\alpha_1$ -AR induced gene transcription. Effects of kinase inhibitors on mRNA expression of IL-6, Crem, Fst, and Sarc clearly indicate that PKC is a critical mediator of all of the long-term induced gene expression alterations tested (Fig. 9). Whether PKC activation is sufficient for all the long-term induced gene expression alterations remains to be determined; inhibition by staurosporine, a broad-based Ser/Thr kinase inhibitor, was better than that by chelerythrine, a more selective PKC inhibitor, in blocking the changes in gene expression for IL-6, suggesting

the involvement of other kinases than PKC. Our results indicate that the surprisingly large repertoire of gene expression alterations induced through  $\alpha_{1A}$ -AR stimulation occurs largely through PKC activation, but secreted factors may also contribute as a result of their secondary effects on gene transcription.

Immunocytochemistry results (Figs. 5 and 6) confirmed the  $\alpha_{1A}$ -AR-mediated gene expression changes of two cell adhesion-related genes at the protein and functional level, syndecan-4 and tenascin-C. Interaction of cells with the ECM can result in cell attachment, spreading, and the assembly of focal adhesions and stress fibers, which can lead to the aggregation of integrins and the resulting assembly of a multi-component signaling complex (Burrige and Chrzanowska-Wodnicka, 1996). Syndecan-4 is a transmembrane heparan sulfate proteoglycan that binds to ECM proteins. Syndecan-4 can interact with integrins in a Rho-dependent manner to assemble focal adhesions (Saoncella et al., 1999). It can also bind and activate PKC- $\alpha$  (Keum et al., 2004), after first being phosphorylated by PKC- $\delta$  (Murakami et al., 2002), linking syndecan 4 to the  $\alpha_1$ -AR signaling pathway. Indeed,  $\alpha_1$ -AR stimulation resulted in the assembly of focal adhesions (Fig. 5, arrows) in three different cell types and resulted in cell spreading in the A10 smooth muscle cell line (Fig. 5, C and D), functionally linking all three  $\alpha_1$ -AR subtype signals with cell adhesion activity. Tenascin-C is an ECM protein that regulates fibronectin-mediated responses within the matrix. Syndecan-4 is also required for tenascin-C action.  $\alpha_1$ -AR stimulation resulted in an increased deposition of tenascin-C into the ECM in rat 1 fibroblasts and the A10 cell line (Fig. 6, A–D). In DDT-1, an  $\alpha_{1B}$ -AR cell line, there was increased expression of tenascin-C in the cytoplasm (Fig. 6, E and F). These results imply potential functional associations between  $\alpha_1$ -AR activation and the ECM, including the regulation of cell adhesion.

In direct cell adhesion functional assays,  $\alpha_{1A}$ -AR activation resulted in statistically significant increase in adhesion of the rat-1 fibroblasts and A10 cell lines (Fig. 10). However, this response was substrate-dependent because cell adhesion occurred with vitronectin as the substrate and not fibronectin. The primary receptors involved in adhesion to both vitronectin and fibronectin are integrins (Ruoslahti and Pierschbacher, 1987). The differential recognition of these ECM proteins suggests involvement of integrins that recognize vitronectin preferentially, such as  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  (Jones et al., 1996). In support of integrin involvement, the  $\alpha_1$ -AR-mediated response was also inhibited by the RGD peptide. In addition to PKC activating syndecan 4, PKC can also activate integrins in an “inside-out” signaling mechanism (Buen-suceso et al., 2001), thus establishing a mechanistic link between  $\alpha_1$ -AR signals and ECM activation. Indeed, using chelerythrine, PKC was shown to be critically involved in  $\alpha_1$ -AR-mediated cell adhesion. Secreted FGF7 may also be involved in integrin-mediated cell adhesion (Bass and Humphries, 2002). This was confirmed by the application of a FGF7-neutralizing antibody, which inhibited  $\alpha_1$ -AR-mediated cell adhesion to vitronectin.

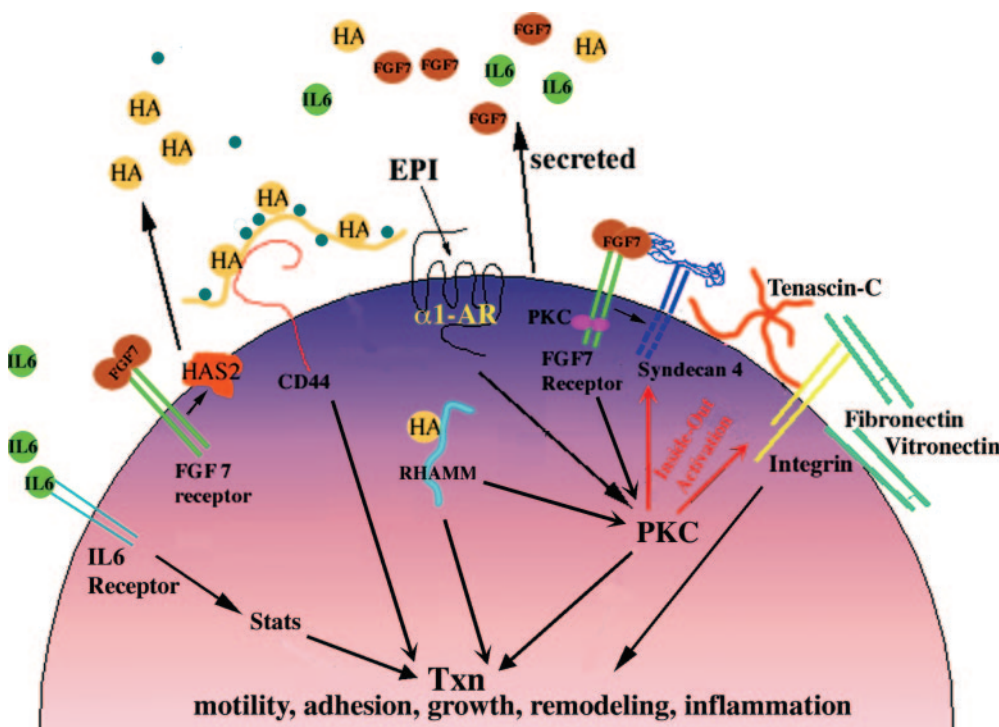
Our results suggest that  $\alpha_1$ -AR activation regulates the expression and function of three key players in integrin-mediated cell adhesion activity: syndecan-4, tenascin-C, and secreted FGF7. Syndecan 4 is likely to interact with both the FGF7 receptor and integrins and to mediate the effects of

$\alpha_1$ -AR-enhanced FGF7 secretion after becoming activated by PKC via “inside-out” signaling (Fig. 12). It is known that decreases in syndecan-4 function inhibit tenascin-C activity and increased expression of syndecan-4 circumvents the effects of tenascin-C. Hence, tenascin-C and syndecan-4 work together to control morphology and signaling and to regulate events, such as matrix contraction, that are necessary for tissue remodeling (Midwood et al., 2004). Because tenascin-C is classified as an anti-adhesive to fibronectin (Chiquet-Ehrismann and Chiquet, 2003) and is heavily secreted upon  $\alpha_1$ -AR activation (Fig. 6), this may explain the lack of an  $\alpha_1$ -AR-mediated adhesive response to fibronectin.

Because  $\alpha_1$ -ARs are involved in cell adhesion, they may also be involved in cell migration. Cells migrate in various ways, and this response is regulated by different mechanisms. Gradients of extracellular ligands establish a range of bound receptors on the cell surface that directs localized polymerization of actin and the formation of lamellipodia and filopodia, in the direction of eventual cell migration. We confirmed that  $\alpha_1$ -AR activation inhibited cell migration in the presence of three substrates, poly-L-lysine, fibronectin, and 10% FBS in all three cell lines (Fig. 11). As opposed to cell migration in response to a particular substrate,  $\alpha_1$ -ARs effects on cell motility are substrate-independent. Because migration inhibition also occurred with poly-L-lysine, a non-specific and non-integrin-mediated adhesion polypeptide (Chen et al., 1994),  $\alpha_1$ -AR motility effects are integrin-independent, in contrast to  $\alpha_1$ -ARs recruiting integrin involvement in adhesion. Because integrins are not involved in  $\alpha_1$ -AR-mediated motility, PKC might be predicted to have different effects on motility. Indeed, the PKC inhibitor chelerythrine could not block  $\alpha_1$ -ARs' effects on motility (Fig. 11). On the other hand, similar to adhesion,  $\alpha_1$ -AR-mediated secretion of FGF7 was also involved in regulating motility by using the anti-FGF7 antibody (Fig. 11). Our results confirm those of Kang et al. (2004), who found the  $\alpha_{1B}$ -AR to inhibit

cell migration in DDT1 and primary human aortic smooth muscle cells. However, our results are contradictory to studies in primary dendritic (Maestroni, 2000), primary rat vascular smooth muscle cells, and adventitial fibroblasts (Zhang et al., 2002), where  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs enhance cell migration. In these same studies, the  $\alpha_{1D}$ -AR was found to inhibit migration in primary rat vascular smooth muscle cells (Zhang et al., 2002). These conflicting results are probably due to cell-type differences in the  $\alpha_1$ -AR-mediated responses.

HA secretion and signaling may also be activated by  $\alpha_1$ -AR stimulation as a result of changes in the mRNA for its synthesizing enzyme (HAS2), its surface receptor (CD44), and its intracellular receptor, Hmmer or RHAMM. Hyaluronan is the major glycosaminoglycan in the ECM. The binding of HA to CD44 causes cells to adhere to the ECM and has been implicated in several biological activities such as inflammation, growth, tumorigenesis, and differentiation (Spicer and Tien, 2004). After 18 h of epinephrine stimulation, HA expression and deposition increased and displayed two distinct structures: pericellular coats and long cable structures that can span several cell lengths (Fig. 7). CD44 was also up-regulated and at times colocalized with HA (Fig. 7). It has been reported that CD44 is important for the retention of the coat-like structures, whereas an IaI-related proteoglycan is critical for cable formation (de la Motte et al., 2003). Evidence suggests that not only the quantity but also the structure of HA is crucial for its function. The cable but not the coat-like structures have been shown to bind mononuclear leukocytes and can be induced through viral infection. Intracellular HA has been associated with mitosis (Brecht et al., 1986; Evanko and Wight, 1999). In addition, HA and CD44 staining is increased in inflamed colon tissue from patients with ulcerative colitis and Crohn's disease, confirming a hypothesis that HA-associating proteins confer proinflammatory properties (de la Motte et al., 2003). HA has also been shown to activate PLC/PKC/Raf1/extracellular signal-regulated ki-



**Fig. 12.** Novel  $\alpha_1$ -AR signaling pathways mediated through secreted factors (IL-6, FGF7, HA) and interactions with the extracellular matrix. Prolonged  $\alpha_1$ -AR activation causes the release of IL-6 (green), FGF7 (brown), and HA (gold) into the media or deposited onto the extracellular matrix. Secreted IL-6 can regulate the transcription (Txn) of genes, some of which are similar to those regulated through  $\alpha_{1A}$ -AR activation. Secreted FGF7 can regulate HA synthesis (HAS2; orange) and activates syndecan-4 (dark blue) to regulate cell adhesion through integrin-mediated (yellow) pathways. Secreted FGF7 also mediated  $\alpha_1$ -AR stimulated cell motility.  $\alpha_1$ -AR mediated deposition of HA into cable structures (gold line) that may bind inflammatory cells (turquoise circles). PKC was found central to  $\alpha_1$ -AR mediated effects by regulating adhesion through priming syndecan 4 and integrins through inside-out signaling (red). PKC was also found to mediate Txn through  $\alpha_1$ -AR activation.



nase/Src and nuclear factor- $\kappa$ B, leading to cell proliferation responses and wound-healing, again linking the ECM to  $\alpha_1$ -AR signaling pathways (Slevin et al., 2002). In addition, FGF7 has been shown to stimulate the mRNA for HAS2, CD44, and the resulting HA accumulation (Karvinen et al., 2003), but we have yet to test whether FGF7 is secondarily mediating this  $\alpha_1$ -AR induced pathway.

In other novel gene discoveries, long-term  $\alpha_{1A}$ -AR activation caused the down-regulation of the RNA for BRAC1 and 2.BRCA1 (breast-cancer susceptibility gene 1) and BRCA2 are tumor suppressor genes, the mutant phenotypes of which predispose women to breast and ovarian cancers. Both genes contribute to DNA repair and transcriptional regulation in response to DNA damage. Recent data also show that BRCA2 transcriptionally regulate some genes involved in cell cycle and apoptosis (Yoshida and Miki, 2004).

A model of the novel  $\alpha_1$ -AR signaling pathways suggested by our studies is shown in Fig. 12. PKC was shown to regulate the transcription of several of the genes altered in the microarray.  $\alpha_1$ -AR activation leads to the secretion of factors, such as IL-6, FGF7, and HA, which interact with the extracellular matrix to regulate cell adhesion, motility and inflammation. Secreted IL-6 was involved in some transcriptional changes mediated through  $\alpha_1$ -AR activation, whereas secreted FGF7 was involved in regulating adhesion and motility.  $\alpha_1$ -AR-mediated integrin recruitment and PKC activation may be key regulators of these processes. Although  $\alpha_1$ -AR mediated secretion of FGF7 is involved in both adhesion and motility, integrins and PKC were recruited for adhesion but not for migration. Syndecan 4 is likely to interact with both the FGF7 receptor and integrins, to mediate the effects of  $\alpha_1$ -AR-enhanced FGF7 secretion after becoming activated or primed by PKC via "inside-out" signaling. The mechanism for the noninvolvement of integrins in migration is not known but could involve the differential activation of PKC isozymes. This work is the first in vitro characterization of large-scale gene expression alterations induced after long-term (18 h) GPCR activation. Our work supports the hypothesis that microarray technology can lead to the discovery of novel signaling pathways. Our work supports the novel discovery that secreted growth factors and the extracellular matrix can play very important roles in the signaling and physiologically responses as a result of  $\alpha_1$ -AR activation.

## References

- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al. (2000) Gene ontology: tool for the unification of biology. *Nat Genet* **25**:25–29.
- Bass MD and Humphries MJ (2002) Cytoplasmic interactions of syndecan-4 orchestrate adhesion receptor and growth factor receptor signalling. *Biochem J* **368**:1–15.
- Brecht M, Mayer U, Schlosser E, and Prehm P (1986) Increased hyaluronate synthesis is required for fibroblast detachment and mitosis. *Biochem J* **239**:445–450.
- Buensuceso CS, Woodside D, Huff JL, Plopper GE, and O'Toole TE (2001) The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration. *J Cell Sci* **114**:1691–1698.
- Burridge K and Chrzanowska-Wodnicka M (1996) Focal adhesions, contractility and signaling. *Annu Rev Cell Dev Biol* **12**:463–518.
- Chen Q, Kinch MS, Lin TH, Burridge K, and Juliano RL (1994) Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J Biol Chem* **269**:26602–26605.
- Chiquet-Ehrismann R and Chiquet M (2003) Tenascins: regulation and putative functions during pathological stress. *J Pathol* **200**:488–499.
- Cotecchia S, Schwinn DA, Randall RR, Lefkowitz RJ, Caron MG, and Kobilka BK (1988) Molecular cloning and expression of the cDNA for the hamster  $\alpha_1$ -adrenoceptor. *Proc Natl Acad Sci USA* **85**:7159–7163.
- de la Motte CA, Hassall VC, Drazba J, Bandyopadhyay SK, and Strong SA (2003) Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. *Am J Pathol* **163**:121–133.
- DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, Chen Y, Su YA, and Trent JM (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat Genet* **14**:457–460.
- Evanko SP and Wight TN (1999) Intracellular localization of hyaluronan in proliferating cells. *J Histochem Cytochem* **47**:1331–1342.
- Faber JE, Yang N, and Xin X (2001) Expression of  $\alpha$ -adrenoceptor subtypes by smooth muscle cells and adventitial fibroblasts in rat aorta and in cell culture. *J Pharmacol Exp Ther* **298**:441–452.
- Gonzalez-Cabrera PJ, Gaivin RJ, Yun J, Ross SA, Papay RS, McCune DF, Rorabaugh BR, and Perez DM (2003) Genetic profiling of  $\alpha_1$ -adrenoceptor receptor subtypes by oligonucleotide microarrays: coupling to interleukin-6 secretion but differences in STAT3 phosphorylation and gp-130. *Mol Pharmacol* **63**:1104–1116.
- Gonzalez-Cabrera PJ, Shi T, Yun J, McCune DF, Rorabaugh BR, and Perez DM (2004) Differential regulation of the cell cycle by  $\alpha_1$ -adrenoceptor subtypes. *Endocrinology* **145**:5157–5167.
- Graham RM, Perez DM, Hwa J, and Piascik MT (1996)  $\alpha_1$ -Adrenoceptor receptor subtypes: Molecular structure, function and signaling. *Circ Res* **78**:737–749.
- Han C, Esbenshade TA, and Minneman KP (1992) Subtypes of alpha1-adrenoceptors in DDT1 MF-2 and BC3H-1 clonal cell lines. *Eur J Pharmacol* **226**:141–148.
- Hwa J, DeYoung M, Perez DM, and Graham RM (1996) Autonomic control of myocardium: alpha adrenoceptor mechanisms, in *The Nervous Control of the Heart* (Shepherd J and Vatner SF eds); *The Autonomic Nervous System* (Burnstock G ed), vol VIII, Harvard Academic Press, Cambridge, MA, 1996.
- Kang SK, Yi KS, Kwon NS, Park KH, Kim UH, Baek KJ, and Im MJ (2004)  $\alpha_{1B}$ -adrenoceptor signaling and cell motility: GTPase function of G $\alpha$ /transglutaminase 2 inhibits cell migration through interaction with cytoplasmic tail of integrin  $\alpha$  subunits. *J Biol Chem* **279**:36593–36600.
- Karvinen S, Pasonen-Seppanen S, Hyytiäinen JM, Pienimäki JP, Torronen K, Jokela TA, Tammi MI, and Tammi R (2003) Keratinocyte growth factor stimulates migration and hyaluronan synthesis in the epidermis by activation of keratinocyte hyaluronan synthases 2 and 3. *J Biol Chem* **278**:49495–49504.
- Keum E, Kim Y, Kim J, Kwon S, Lim Y, Han I, and Oh ES (2004) Syndecan-4 regulates localization, activity and stability of protein kinase C- $\alpha$ . *Biochem J* **378**:1007–1014.
- King HC and Sinha AA (2001) Gene expression profile analysis by DNA microarrays: promise and pitfalls. *J Am Med Assoc* **286**:2280–2288.
- Koshimizu TA, Tanoue A, Hirasawa A, Yamauchi J, and Tsujimoto G (2003) Recent advances in  $\alpha_1$ -adrenoceptor pharmacology. *Pharmacol Ther* **98**:235–244.
- Jones JJ, Prevett T, Gockerman A, and Clemmons DR (1996) Ligand occupancy of the alpha-V-beta3 integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor. *Proc Natl Acad Sci USA* **93**:2482–2487.
- Jung BD, Kimura K, Kitamura H, Makondo K, Okita K, Kawasaki M, and Saito M (2000) Norepinephrine stimulates interleukin-6 mRNA expression in primary cultured rat hepatocytes. *J Biochem (Tokyo)* **127**:205–209.
- Kanda T, Inoue M, Kotajima N, Fujimaki S, Hoshino Y, Kurabayashi M, Kobayashi I, and Tamura J (2000) Circulating interleukin-6 and interleukin-6 receptors in patients with acute and recent myocardial infarction. *Cardiology* **93**:191–196.
- Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, et al. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* **14**:1675–1680.
- Maestroni GJM (2000) Dendritic cell migration controlled by  $\alpha_{1B}$ -adrenoceptor receptors. *J Immunol* **165**:6743–6747.
- Midwood KS, Valenick LV, Hsia HC, and Schwarzbauer JE (2004) Coregulation of fibronectin signaling and matrix contraction by tenascin-C and syndecan-4. *Mol Biol Cell* **15**:5670–5677.
- Murakami M, Horowitz A, Tang S, Ware JA, and Simons M (2002) Protein kinase C (PKC)  $\delta$  regulates PKC $\alpha$  activity in a Syndecan-4-dependent manner. *J Biol Chem* **277**:20367–20371.
- Norris JG and Benveniste EN (1993) Interleukin-6 production by astrocytes: induction by the neurotransmitter norepinephrine. *J Neuroimmunol* **45**:137–145.
- Perez DM, Piascik MT, and Graham RM (1991) Solution-phase library screening for the identification of rare clones: isolation of an  $\alpha_{1A}$ -adrenoceptor cDNA. *Mol Pharmacol* **40**:876–883.
- Perez DM, Piascik MT, Malik N, Gaivin RJ, and Graham RM (1994) Cloning, expression and tissue distribution of the rat homolog of the bovine  $\alpha_{1C}$ -adrenoceptor provide evidence for its classification as the  $\alpha_{1A}$ -subtype. *Mol Pharmacol* **46**:823–831.
- Piascik MT and Perez DM (2001)  $\alpha_1$ -Adrenoceptor receptors: new insights and directions. *J Pharmacol Exp Ther* **298**:403–410.
- Pronin AN and Benovic JL (1997) Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J Biol Chem* **272**:3806–3812.
- Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, et al. (2000) Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* **24**:227–235.
- Ruoslahti E and Pierschbacher MD (1987) New perspectives in cell adhesion: RGD and integrins. *Science (Wash DC)* **238**:491–497.
- Saoncella S, Echtermeyer F, Denhez F, Nowlen JK, Mosher DF, Robinson SD, Hynes RO, and Goetinck PF (1999) Syndecan-4 signals cooperatively with integrins in a Rho-dependent manner in the assembly of focal adhesions and actin stress fibers. *Proc Natl Acad Sci USA* **96**:2805–2810.
- Schena M, Shalon D, Heller R, Chai A, Brown PO, and Davis RW (1996) Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc Natl Acad Sci USA* **93**:10614–10619.
- Slevin M, Kumar S, and Gaffney J (2002) Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. *J Biol Chem* **277**:41046–41059.
- Solovjov DA, Pluskota E, and Plow EF (2005) Distinct roles for the  $\alpha$  and  $\beta$  subunits in the functions of integrin  $\alpha$ 5 $\beta$ 2. *J Biol Chem* **280**:1336–1345.

- Spicer AP and Tien JYL (2004) Hyaluronan and morphogenesis. *Birth Defects Res* **72**:89–108.
- Yoshida K and Miki Y (2004) Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription and cell cycle in response to DNA damage. *Cancer Sci* **95**: 866–871.
- Zhang H, Facemire CS, Baner AJ, and Faber JE (2002) Different  $\alpha$ -adrenoceptors mediate migration of vascular smooth muscle cells and adventitial fibroblasts in vitro. *Am J Physiol* **82**:H2364–H2370.

Zhang L and Plow EF (1996) Overlapping, but not identical, sites are involved in the recognition of C3bi, neutrophil inhibitory factor and adhesive ligands by the  $\alpha$ M $\beta$ 2 integrin. *J Biol Chem* **271**:18211–18216.

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