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Novel α_1-Adrenergic Receptor Signaling Pathways: Secreted Factors and Interactions with the Extracellular Matrix

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Running Title: Novel α_1-adrenoceptor signaling

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Abbreviations: AR, adrenergic receptor; Ccnb1, cyclin B1; DAG, diacylglycerol; ECM, extracellular matrix; FBS, fetal bovine serum; Fst, follistatin; FGF7, fibroblast growth factor 7; GPCR, G-protein couple receptor; GRK, G-protein receptor kinase; HMG2, high mobility group box 2; IL, interleukin; Itpr1, inositol 1,4,5-triphosphate receptor 1; HA, hyaluronan; HBBS, Hanks' balanced salt solution; Nr4a3, nuclear receptor subfamily 4, group A, member 3; PBS, phosphates-buffered saline; PLC, phospholipase C; PKC, protein kinase C; RT-PCR, reverse transcriptase polymerase chain reaction; Sarc, sarcomeric muscle protein; SEM, standard error of the mean; Tub, tubulin;

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Abstract

α_1-adrenergic receptor (α_1-ARs) subtypes (α_{1A}, α_{1B} and α_{1D}) regulate multiple signal pathways such as PLC, PKC, and MAPKs. We employed oligonucleotide microarray technology to explore the effects of both short (1h) and long-term (18h) activation of the α_{1A}-AR to enable RNA changes to occur downstream of earlier well-characterized signaling pathways, promoting novel couplings. PCR studies confirmed that PKC was a critical regulator of α_{1A}-AR mediated gene expression with secreted IL-6 also contributing to gene expression alterations. We next focused on two novel signaling pathways that may be mediated through α_{1A}-AR stimulation, due to the clustering of gene expression changes for cell adhesion/motility (syndecan-4 and tenascin-C) and hyaluronan (HA) signaling. We confirmed that α_1-ARs induced adhesion in three cell types to vitronectin, an interaction that was also integrin, FGF7, and PKC-dependent. α_1-AR activation also inhibited cell migration, which was integrin and PKC-independent but still required secretion of FGF7. α_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. Since long cable structures of HA can bind leukocytes, this suggests that α_1-ARs may be involved in pro-inflammatory responses. Our results indicate α_1-ARs induce the secretion of factors that interact with the extracellular matrix to regulate cell adhesion, motility and pro-inflammatory responses through novel signaling pathways.

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Introduction

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 characteristics, ARs have been classified into α_1-ARs, α_2-ARs and β-ARs and three subtypes of α_1-ARs (α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR) have been cloned and characterized pharmacologically (Cotecchia et al., 1988; Perez et al., 1991; Perez et al., 1994).

Some of the common α_1-AR signaling pathways have been elucidated (reviewed in Graham et al. 1996). Upon ligand binding of α_1-AR, a signaling cascade occurs. First, there is the activation of Gq that leads to the activation of phospholipase C (PLC). PLC hydrolyzes phosphotidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptor and mobilizes intracellular calcium and DAG 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, α_1-ARs have also been shown to couple to MAPKs , STATs ,and small GTPases, (reviewed in Koshimizu et al., 2003; Gonzalez-Cabrera et al., 2003). Although we know PLC and MAPK pathways are involved in α_1-AR signaling, more detailed signaling mechanisms and gene programs that are subsequently activated downstream of PLC are not well understood.

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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 lab have shown that short-term activation of all three α_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 α_{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 α_1-AR activation.

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Material and Methods

Cell culture and treatment. Rat-1 fibroblasts stably transfected with human α_{1A}-AR cDNA (α_{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 Typical Culture Collection (ATCC) (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 100 μg/ml streptomycin in a humidified incubator at 37˚C in the presence of 5% CO_2. For Rat-1 fibroblasts, 500 μg/ml of the selection antibiotic G418 (Invitrogen, 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 β-AR blocker, propranolol (Sigma, MO) and α_2-AR blocker rauwolscine (Sigma, MO) were added into the cell culture medium at final concentrations of 1μM and 0.1 μM respectively, and incubated for 30 minutes before the addition of other agents. For α_1-AR agonist treated cells, epinephrine (Epi) (Sigma, MO) was added into the culture medium at a final concentration of 10μM and incubation continued for either 1 or 18 hours. For IL-6 treatment, IL-6 (R & D System, MN) was added into the cell culture medium at a final concentration of 1ng/ml and cells were incubated for 18 hours. For treatment with kinase inhibitors, cells were preincubated with either chelerythrine (CH) (Sigma, MO) at final concentration of 10μM or staurosporine (ST) (Sigma, MO) at final concentration 50 μM for 40 min before the addition of epinephrine.

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RNA extraction and microarray experiments. Media was removed from cell monolayers by aspiration and total RNAs were immediately isolated using TRIzol reagent (Invitrogen, CA) following the manufacturer's procedures. Total RNAs were purified using RNeasy Mini kit (Qiagen, CA). Double-stranded cDNAs were synthesized from 10 μg of total RNA using SuperScript Choice double-stranded cDNA synthesis kit from Invitrogen following 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, 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, CA) were carried out following 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-Parkard GeneArray scanner (Hewlett-Parkard, 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 to control.

Pattern analysis. To analyze the gene expression alteration patterns in different biological

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and functional categories 3,119 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 μg total RNA using oligo $(dT)_{12-18}$ and SuperScript II RNA H^- reverse transcriptase (Invitrogen) following 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 α-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, CA).

Real-time RT-PCR was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA). All assays were done in a total volume of 25 μl which included 12.5 μl 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 μl ddH2O, 1.0 μl template cDNA and 1.0 μl pre-designed 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 sec; 55˚C, 30 sec; and 72˚C, 30 sec), and an extension for 5

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minutes at 72˚C. The fluorescent signal from SYRB Green is detected immediately after the extension step of each cycle and the cycle at which product is first detectable Ct is recorded. Data were imported into Microsoft Excel for analysis.

Immunoblot analysis. Proteins from cultured cells were extracted after homogenizing whole cells in a lysis buffer (0.5% SDS, 25mM Tris, 2.5mM $MgCl_2$) in the presence of a protease inhibitor cocktail (Calbiochem, CA) and incubated at 100^0C for 10 min. Protein extracts were centrifuged for 10 minutes at 14,000rpm to remove cell debris. Secreted proteins in the medium were directly concentrated from medium in the presence of protease inhibitor cocktail using a centriplus filter (Millipore, MA) with a cut-off size of 5 Kd. Protein concentrations were determined using the Bio-Rad protein assay kit according to manufacture's protocol (Bio-Rad, CA).

Protein samples (~50 µg/lane) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore). Rabbit polyclonal anti-mouse IL-6 antibody (Chemicon International, CA) and Rabbit polyclonal anti-mouse sarcomeric muscle protein (Krp1) antibody (kindly provided by Dr. B Ozanne, The Beatson Institute for Cancer Research, UK) were used at 1:1000 dilution for immunoblotting. 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 (Amersham Bioscience, NJ). Protein bands were detected on X-mat film (Eastman Kodak, NY) using ECL chemiluminescence reagents (Amersham Bioscience) and scanned using an HP scanjet 3670 scanner (Hewlett-Parkard, CA). Bands were quantitated using Kodak ID Image Analysis software (Eastman Kodak Company, Rochester, NY).

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Immunohistochemistry. For syndecan 4 and tenascin C immunohistochemistry, 100,000 cells of Rat-1 α_{1A} fibroblast, A-10, or DDT1-MF-2 in DMEM medium 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, α_2-AR and β-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 continued for an additional 18 hours. For the control group, only α_2-AR and β-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% Trition X-100) for at least 1 hour at room temperature on a shaker. Primary antibody against syndecan 4 (Santa Cruz, CA) and Tenascin C (a gift from Josephine Adams, Cleveland Clinic Foundation) were then added to the blocking solution at 1:100 and 1:600 respectively, and the incubation continued for 1 day at 4ºC. The coverslips were then washed three times with PBS and incubated with goat anti-rabbit secondary antibody conjugated with Fluor 488 (Molecular Probe, OR) at 1:3000 for 1 hour at room temperature followed by washes in PBS. Coverslips were mounted in VectaShield medium with DAPI (Vector Laboratories, CA) and sealed with nail polish. Sections were analyzed on a confocal laser-scanning microscope (Leica, Inc. IL) representing optical sections of 2-3 micron axial resolution and an average of 3 line-scans.

Hyaluronan and CD44 immunohistochemistry was performed as previously described (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 -20ºC for 10 min and the coverslips were air-dried and preincubated with Hanks' balanced salt solution (HBBS) containing 2% FBS for 30 min at room

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temperature. The coverslips were incubated with a solution containing biotinylated hyaluronan-binding protein (Seikagaku, Japan) at 5 μg/ml and anti-CD44 antibody at 5ug/ml in HBSS containing 2% FBS for approximately 16 hours at 4ºC. 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 (H+L) directed against the anti-CD44 primary antibody (1:500) in HBSS containing 2% FBS for 1 hour 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 (version 5.1.2) from MediaCybernetics, Silverspring, 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 β-AR blocker propranolol (1 uM) and the α_2-AR blocker rauwolscine (0.1 uM). For the cell adhesion assay, non-treated Falcon 96 well plate (Becton Dickinson, NJ) was pre-coated with fibronectin at 10 μg/ml in PBS or vitronectin (VN) (Chemicon International, CA) at 1μg/ml in PBS overnight at 4°C. Coating solutions were removed and the wells were coated with 0.05% polyvinylpyrolidone (Sigma, MO) for 1 hour at 37 ºC, which is effective in reducing non-specific binding (Zhang and Plow, 1996), then washed twice with PBS. Cells in DMEM were treated with epinephrine, epinephrine plus RGD peptide (200μM), epinephrine plus anti-FGF-7 antibody (10μg/ml), or epinephrine plus chelerythrine (10uM) for 1 hr. About 100,000 treated cells were then added to each well and incubated at 37ºC for 30 min. Non-adherent cells were removed, and the wells were washed twice with PBS. The plate was frozen at -80ºC overnight. Adherent cells were quantified by determining the total DNA content on the bottom of the membrane using CytoFluor II software (Long Island Scientific, NY) after staining

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the cells with a CyQUANT Reagent kit, following manufacture's protocols (Molecular Probes, OR). Cell migration activity was determined using Transwell plates (Costar, Corning, NY) with 8μM pore size membrane, as previously described (Solovjov et al., 2005). Cells (5×10^5 in 150 μl DMEM) were added to the top chamber in the presence or absence of 5μM epinephrine, or epinephrine plus 1μM prazosin (Praz) (Sigma), epinephrine plus anti-FGF-7 antibody (10μg/ml), or epinephrine plus chelerythrine (10uM). Poly-L-lysine (Poly-L) (10 μM) (Sigma), fibronectin (FN) (10 μM) (Sigma) or 10%FBS in 600 μL DMEM were added to the bottom chamber. After 18 hour incubation, media in both top and bottom chambers were aspired. Cells on the top of the membrane were removed using cotton swaps and the Transwell was frozen at -80ºC overnight and quantified using CyQUANT Reagent kit as described above.

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

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Results

Gene expression alterations by 1h and 18h α_{1A}-AR stimulation. RNA samples were prepared from two separate experiments for each control (un-stimulated) and stimulated conditions and hybridized to separate gene chips (i.e. 6 chips total). Data were analyzed by comparing the gene expression profiles from the un-stimulated control versus 1h or 18 h of epinephrine stimulation. After one hour of epinephrine treatment, 499 of 8799 genes (5.7%) are differentially expressed, with 204 genes (2.3%) increasing expression and 295 genes (3.4%) decreasing expression, compared to un-stimulated controls. After 18 hour epinephrine treatment, 1823 of the 8799 genes (20.7%) are differentially expressed, with 765 genes (8.7%) up-regulated (supplemental data file 1) and 1058 genes (12.0%) down-regulated (supplemental data file 2). Ninety-six (47%) of the 204 up-regulated genes after 1h treatment remained up-regulated after 18h treatment; 25 (12%) of the 204 genes remained down-regulated and 83 (41%) returned to un-stimulated levels. For the 295 down-regulated genes after 1h treatment, 79 (27%) of them remained down-regulated after 18h treatment; 38 (13%) increased expression and 178 (60%) returned to un-stimulated levels. It was apparent that more genes were down-regulated during both 1h (3.4 % out of 5.7%) and 18h (8.7% out of 20.7%) epinephrine treatment.

Pattern assessment after 18h α_{1A}-AR stimulation. Since 1h gene expression patterns have already been published (Gonzalez-Cabrera et al., 2003), we made a gene ontology tree for differentially expressed genes induced after 18h α_{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

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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 are three numerical numbers following 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, only a few categories such as cell death, necrosis, and integrin receptor signaling were the majority of differentially expressed genes up-regulated. Notably, large repertoires of genes were changing in cell cycle (54 out of 164 (33%)) and mitotic cycle control (12 out of 18 (67%)) categories, again with down-regulation being the dominant change.

PLC-associated gene expression. According to the microarray results, several gene expressions were altered after 18h of epinephrine stimulation that were associated with the Gq/PLC pathway (Fig 2). After 1 h stimulation and as previously reported (Gonzalez-Cabrera et al., 2003), c-fos mRNA levels are high, consistent with the expression pattern commonly seen in immediate early genes. However, after 18h stimulation, c-fos as well as several PLC-associated genes such as the IP3 receptor and PKC zeta became downregulated compared with the 1 hr timepoint. In contrast, GRK5, a GPCR receptor kinase known to be involved in the downregulation of ARs and is itself, modified by PKC (Pronin and Benovic, 1997), increased in expression. This data confirms the accuracy of our microarray since it is consistent with an expected down-regulation paradigm due to extensive α_1-AR stimulation.

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Potential novel α_1-AR signaling pathways. Some of the more interesting or significant genes whose expression levels were altered following α_{1A}-AR activation were identified and are shown in Table 2. IL-6, a previously characterized secreted protein produced after α_1-AR activation for 1h (Gonzalez-Cabrera et al., 2003) deceases 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), syndecan 4 (+2.5 fold) increased expression, suggesting α_{1A}-AR involvement in cell adhesion, which 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 α_{1A}-AR involvement in HA signaling. The breast cancer genes, BRCA1 and 2 were both decreased after 18h α_{1A}-AR stimulation (-9.8 and -4.2 fold, respectively).

RT-PCR and protein confirmation of gene expression changes. Rat-1 cells exposed to 10μM epinephrine for 1 or 18h 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 (Nr4a3), tenascin C (TenC), Syndecan 4 (Syn4), 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, CyclinB1 (Ccnb1), Gro, High mobility group box 2 (HMG2), Inositol 1, 4, 5-triphosphate receptor 1 (Itpr1), Cell cycle protein p55CDC (Cdc20), and α-Tubulin (α-Tub), were chosen and primers synthesized (Table 1). PCR of α–tubulin were used as an internal control for the RT-PCR. As shown in Figure 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.

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Protein levels of IL-6 secreted into the medium and Sarc in whole cell lysates were determined. Epinephrine treatment for 18h 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 α_1-AR endogenously expressing smooth muscle cell lines, A-10 and DDT1-MF2. DDT1-MF2 has been characterized to express only the α_{1B}-AR by ligand binding (Han et al., 1992), while the A10, a rat smooth muscle cell line from aorta is thought to express higher levels of the α_{1D}-AR (Faber et al., 2001), although the binding and signaling characteristics in this particular cell line is not extensive. After 18h of epinephrine stimulation, syndecan 4, which is expressed ubiquitously, is enriched in focal adhesions (arrows, Fig 5) as well as the cytoplasm. After 18h of epinephrine stimulation, tenascin C, a large protein, increased deposition in the extracellular matrix (ECM) in fibroblasts and the A-10 cells (arrows, Fig 6 A-D). In DDT1-MF2 cells, mildly increased tenascin C expression was evident in the cytoplasm, but not in the extracellular matrix (arrows, Fig 6 E-F). In all cases, cell morphology appeared to be changing in regards to cell spreading, being more evident in the A-10 cells.

Hyaluronan secretion and signaling may be activated by α_1-AR stimulation due to increases in the mRNA for its synthesizing enzyme (HAS2) and the dominate HA receptor, CD44. Since antibodies are unavailable for this glycosaminoglycan, a biotinylated hyaluronan-binding protein was used for analysis as well as using antibodies against CD44. After 18h 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 (green, Fig 7).

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CD44 was also up-regulated and at times co-localized with HA (red and yellow, Fig 7). All of the protein data appeared 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 18h epinephrine stimulation whereas other genes such as IL-6 and Nr4a3 were already differentially expressed only after 1h stimulation. We hypothesized that some of these α_{1A}-AR early gene expression changes and the resulting proteins may actually contribute to prolonged α_{1A}-AR mediated gene expression changes due to secretion of growth factors. IL-6 is a very good candidate to test the hypothesis because mRNA level of IL-6 is highly up-regulated at 1hr stimulation by epinephrine and the protein is secreted into the medium. Therefore, Rat-1 α_{1A} fibroblasts were treated with exogenous IL-6 (1ng/ml) or FGF7 (100ng/ml) and incubated for 18 hours. 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 while down-regulating Crem and Itpr1 (Fig 8A) but not affecting 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 upregulation of Crem, which was consistent with the microarray data (Fig 8B), but also caused the upregulation of Itpr1. To confirm that either IL-6 or FGF7 were contributing to α_1-AR mediated RNA changes, neutralizing antibodies to IL-6 and FGF7 were used in addition to epinephrine treatment. Only α_1-AR mediated RNA changes in

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Sarc and Fst were neutralized with anti-IL-6 but not anti-FGF7 (Fig 8C). Either neutralizing antibody did not affect all of the other genes tested.

PKC mediation of gene expression induced by α_{1A}-AR activation. While α_1-ARs have been previously shown to regulate the transcription of a number of genes (Hwa et al., 1996), the intermediate signaling pathways responsible for these gene expression alterations are not known, especially for the large repertoire reported by our microarray data. To identify signaling mechanism(s) leading to the altered expressions, we targeted four genes whose expression changed the greatest: IL-6, Crem, Fst and Sarc; and targeted kinases, such as PKC, a pivotal kinase in the signaling of α_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 18h 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, while staurosporine was better than chelerythrine 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 while staurosporine produced no notable changes. Results from real-time PCR (Fig 9) agreed with those from RT-PCR.

Cell adhesion and motility. Since 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 α_1-AR subtypes. Epinephrine enhanced adhesion of fibroblasts and the A-10 cell line to vitronectin (Fig 10), while 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

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binding to many integrins (Fig 10). FGF7, a growth factor, which is involved in integrin-mediated cell adhesion and whose gene expression increased after epinephrine stimulation (+3.5 fold, Table 2), also appeared to regulate cell adhesion since the addition of a neutralizing antibody against FGF7 partly inhibited the α_1-AR mediated cell adhesion to vitronectin (Fig 10). Finally, cell adhesion was also inhibited by chelerythrine indicating 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 hours in the upper chamber and either poly-lysine, fibronectin or 10%FBS in the bottom chamber. Epinephrine inhibited cell migration in most cases (Fig 11) but the effect was substrate-independent. The addition of prazosin, an α_1-AR antagonist, reversed the inhibitory effect of epinephrine, confirming involvement of α_1-ARs (Fig 11). Interestingly, similar to cell adhesion, FGF7 was also involved in α_1-ARs inhibiting cell migration, but this response was PKC-independent as chelerythrine could not block the effect (Fig 11).

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Discussion

Gene expression alterations in rat-1 fibroblasts induced by short-term (1h) epinephrine-stimulation of all three α_1-AR subtypes were previously reported (Gonzalez-Cabrera et al. 2003). We repeated these studies for consistency and for direct comparison to gene expression changes that occur during prolonged stimulation (18h) focusing on the α_{1A}-AR subtype. We rationalized that prolonged stimulation would enable RNA changes to occur downstream of earlier well-characterized signaling pathways, such as PLC, MAPK, cfos, and this might lead to the discovery of novel signaling and physiological pathways coupled to α_{1A}-AR stimulation. Pattern analysis of gene expression alterations induced by prolonged α_{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 α_{1A}-AR activation and provide new directions for future investigation. The most notable 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 α_1-ARs are involved in cell cycle control and that α_{1A}-AR activation leads to a G1-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 α_1-AR signal transduction, consistent with the α_{1A}-AR becoming downregulated after 18h stimulation, were differentially expressed in the microarray (Fig 2). Gene expression changes in the microarray at 18h for the IP3 receptor (Itpr1), PKC zeta, and cFos decreased

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compared to the microarray results from 1h epinephrine-stimulation, consistent with the downregulation of these components of the α_{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). As 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 α_{1A}-AR stimulation, due to 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 play 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 α_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 following α_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 α_{1A}-AR stimulation (Fig 4). While the mechanism of how IL-6 is secreted after α_1-AR stimulation is not clear, our results suggest that IL-6 itself may affect some of the same gene expression alterations induced by α_{1A}-AR activation (Fig 8A). This was confirmed for Sarc and Fst gene expression since a neutralizing antibody to IL-6 blocked α_1-AR mediated RNA changes (Fig 8C). While 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, while FGF7 also affected the gene

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transcription of Crem (Fig 8B), a neutralizing antibody to FGF7 could not block the α_1-AR mediated changes in RNA, suggesting that FGF7 does not contribute to α_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, still needs to be determined, as inhibition by staurosporin, a broad base Ser/Thr kinase inhibitor, was better than 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 α_{1A}-AR stimulation is largely through PKC activation but secreted factors may also contribute as a result of their secondary effects on gene transcription.

Immunocytochemistry results (Fig 5 & 6) confirmed the α_{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 multicomponent signaling complex (Burridge & Chrzanowska-Wodnicka, 1996). Syndecan-4 is a transmembrane heparan sulphate 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 α_1-AR signaling pathway. Indeed, α_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 CD), functionally

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linking all three α_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. α_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 α_{1B}-AR cell line, there was increased expression of tenascin-C in the cytoplasm (Fig 6EF). These results imply potential functional associations between α_1-AR activation and the ECM, including the regulation of cell adhesion.

In direct cell adhesion functional assays, α_{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 as 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 α_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 (Buensuceso et al., 2001), thus establishing a mechanistic link between α_1-AR signals and ECM activation. Indeed, using chelerythrine, PKC was shown to be critically involved in α_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 α_1-AR-mediated cell adhesion to vitronectin.

Our results suggest that α_1-AR activation regulates the expression and function of three key players in integrin-mediated cell adhesion activity: syndecan-4, tenascin-C and secreted

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FGF7. Syndecan 4 likely interacts with both the FGF7 receptor and integrins, to mediate the effects of α_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, signaling, and regulate events such as matrix contraction that are necessary for tissue remodeling (Midwood et al., 2004). Since tenascin-C is classified as an anti-adhesive to fibronectin (Chiquet-Ehrismann & Chiquet, 2003), and is heavily secreted upon α_1-AR activation (Fig 6), this may explain the lack of an α_1-AR-mediated adhesive response to fibronectin.

Since α_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 ranget 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 α_1-AR activation inhibited cell migration in the presence of three substrates, poly-lysine, fibronectin, and 10% FBS in all three cell lines (Fig 11). As opposed to cell migration in response to a particular substrate, α_1-ARs effects on cell motility are substrate-independent. Since migration inhibition also occurred with poly-lysine, a nonspecific and non integrin-mediated adhesion polypeptide (Chen et al., 1994), α_1-AR motility effects are integrin-independent, in contrast to α_1-ARs recruiting integrin involvement in adhesion. Since integrins are not involved in α_1-AR mediated motility, PKC might be predicted to have different effects on motility. Indeed, the PKC inhibitor chelerythrine could not block α_1-ARs effects on motility (Fig 11). On the other hand, similar to adhesion, α_1-AR mediated secretion of FGF7 was also involved in regulating motility by utilizing the anti-FGF7 antibody

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(Fig 11). Our results are confirmatory to those of Kang et al., 2004, in which the α_{1B}-AR was found 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 α_{1A}- and α_{1B}-ARs enhance cell migration. In these same studies, the α_{1D}-AR was found to inhibit migration in primary rat vascular smooth muscle cells (Zhang et al., 2002). These conflicting results are likely due to cell-type differences in the α_1-AR mediated responses.

Hyaluronan (HA) secretion and signaling may also be activated by α_1-AR stimulation due to changes in the mRNA for its synthesizing enzyme (HAS2), its surface receptor, CD44, and its intracellular receptor, Hmmr 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 18h 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 co-localized with HA (Fig 7). It has been reported that CD44 is important for the retention of the coat-like structures while an IαI-related proteoglycan is critical for cable formation (de la Motte et al., 2003). Evidence suggests that not only the quantity, but 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 ulcerative colitis and Crohn's patients, confirming a hypothesis that HA-associating proteins confer pro-inflammatory properties (de la Motte et al.,

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2003). HA has also been shown to activate PLC/PKC/Raf1/ERK/Src and NF-kB, leading to cell proliferation responses and wound-healing, again linking the ECM to α_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 α_1-AR induced pathway.

In other novel gene discoveries, long-term α_{1A}-AR activation caused the downregulation 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 BRCAs transcriptionally regulate some genes involved in cell cycle and apoptosis (Yoshida and Miki, 2004).

A model of the novel α_1-AR signaling pathways suggested by our studies is shown in Figure 12. PKC was shown to regulate the transcription of several of the genes altered in the microarray. α_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 α_1-AR activation while secreted FGF7 was involved in regulating adhesion and motility. α_1-AR mediated integrin recruitment and PKC activation may be key regulators of these processes. While α_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 likely interacts with both the FGF7 receptor and integrins, to mediate the effects of α_1-AR enhanced FGF7 secretion after becoming activated or primed by PKC via "inside-out" signaling. The mechanism for the non-involvement of integrins in migration is not known but could involve the differential activation of PKC

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isozymes**.** This work is the first *in vitro* characterization of large-scale gene expression alterations induced after long-term (18h) 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 due to α_1-AR activation.

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Figure Legends

Figure 1. Biological process ontology tree of 3419 genes showing patterns of gene expression alterations in α_{1A}-AR fibroblasts induced after 18h epinephrine-stimulation. There are three numerical numbers following 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 the parentheses represents the percentage of genes that are down-regulated. Percentages are based upon the average of two independent experiments and compared to control (non-stimulated).

Figure 2. Microarray gene expression changes after 1h vs. 18h epinephrine-stimulation for common early signal transduction genes associated with α_1-AR activation. While c-fos expression was elevated after 1h epinephrine-stimulation, at 18h stimulation expression was decreased as well as for genes for the IP3 receptor and PKC zeta, while GRK5 increased expression, consistent with downregulation of α_1-AR signaling. Data are taken directly from the microarray results and fold-changes are the average of two independent experiments, compared to control (non-stimulated).

Figure 3. RT-PCR analysis on the effect of α_{1A}-AR activation on gene expression alterations in transfected fibroblasts. Cells were exposed to 10 uM epinephrine for 1 hour (1h) or 18 hour (18h) in the presence of β-AR and α_2-AR blockers. RT-PCR of α-tubulin was used as an internal normalization control for the PCR reactions. Cells exposed to β-AR and α_2-AR blockers only

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served as control (C). (**A)** Up-regulated gene expressions. (**B)** Down-regulated gene expressions. Numbers below indicate fold-changes resulting from the microarray analysis for comparison.

Figure 4. (A) Immunoblot analysis of increased protein expression of IL-6 and Sarc induced after 18h α_{1A}-AR activation in transfected fibroblasts versus untreated controls (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 n=3 western blots. Bands were quantitated using Kodak ID Image Analysis software (Eastman Kodak Company, Rochester, NY).

Figure 5. Confocal images of Syndecan-4 expression before (Basal) and after (Epi) 18h epinephrine-stimulation. Panel **A**, **C** and **E**: Basal Syndecan-4 expression in transfected α_{1A}-AR fibroblasts, A-10 and DDT1-MF2 cells exposed to α_2-AR and β-AR blockers only. Panel **B**, **D** and **F**: Syndecan-4 expression levels after treatment with epinephrine (10 uM) for 18h. α_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 α_1-AR stimulation (Panel **D**). Scale bar=10 microns. **(G)** Quantitation of syndecan 4 using the software Image pro Plus (version 5.1.2) from MediaCybernetics, Silverspring, MD.

Figure 6. Confocal images of Tenascin-C expression before (Basal) and after (Epi) 18h epinephrine-stimulation. Panel **A**, **C** and **E**: Basal Tenascin-C expression in transfected α_{1A}-AR fibroblasts, A-10 and DDT1-MF2 cells exposed to α_2-AR and β-AR blockers only. Panel **B**, **D** and **F**: Tenascin-C expression after treatment with epinephrine (10 uM) for 18h. α_1-AR

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activation caused increased deposition of Tenascin-C into the extracellular matrix (arrows). DDT1-MF2 cells have increased expression of Tenascin-C in the cytoplasm (Panel **F**). Scale bar=10 microns. **(G)** Quantitation of Tenascin C using the software Image pro Plus (version 5.1.2) from MediaCybernetics, Silverspring, MD.

Figure 7. Confocal images of hyaluronan (HA) and CD44 expression in cells before (Basal) and after (Epi) 18h epinephrine-stimulation. Panel **A**, **C** and **E**: basal HA (green) and CD44 (red) expression in transfected α_{1A}-AR fibroblasts, DDT1-MF2, and A-10 cells exposed to α_2-AR and β-AR blockers only. Panel **B**, **D** and **F**: HA (green) and CD44 (red) expression after treatment with epinephrine (10 uM) for 18h. α_1-AR activation caused increased deposition of HA into the extracellular matrix resulting in long cable structures (Panels **B**, **F**) or increased pericellular expression (Panel **D**). Some colocalization of CD44 with HA is evident (yellow). Nuclei are blue. Scale bar=10 microns. **(G)** Quantitation of CD44 and HA using the software Image pro Plus (version 5.1.2) from MediaCybernetics, Silverspring, MD.

Figure 8. **A.** RT-PCR analysis of IL-6-mediated regulation of gene expression in transfected α_{1A}-AR fibroblasts. Cells were treated with IL-6 at 1ng/ml for 18h and compared to non-treated cells as a control. IL-6 increased expression of Fst, Sarc, Ccnb1, and decreased expression of Crem, and Itpr1. **B.** RT-PCR analysis of FGF7-mediated regulation of gene expression in α_{1A}-AR fibroblasts. Cells were treated with FGF-7 at 100ng/ml for 18h and compared to non-treated 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

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expression. α-Tubulin (α-Tub) was used as an internal control for the RT-PCR. Numbers below the panels indicate fold-changes from the microarray analysis or densitometer values.

Figure 9. RT-PCR analysis on the effect of kinase inhibitors on gene expression alterations induced after α_{1A}-AR activation in transfected fibroblasts. For treatment involving kinase inhibitors and/or epinephrine, cells were preincubated with α_2-AR and β-AR blockers only (B), blockers and 10uM epinephrine (B+E), blockers, 10uM epinephrine, and 10uM chelerythrine (B+E+CH) or blockers, 10uM epinephrine, and 50uM staurosporine (B+E+ST). α-Tubulin (α-Tub) was used as an internal control for the RT-PCR. α_1-AR mediated gene expression (18h) was blocked by both chelerythrine and staurosporine but not all to the same extent.

Figure 10. Effect of α_1-AR activation on the cell adhesion activity of A-10, DDT-MF2, and transfected α_{1A}-AR fibroblasts. Cell adhesion assays were done using nontreated Falcon 96 well plates coated with either vitronectin (1ug/ml) or fibronectin (10ug/ml). Cells in DMEM were treated with epinephrine (Epi), epinephrine plus RGD peptide (200 uM), or epinephrine plus anti-FGF-7 antibody (10 μg/ml) or epinephrine plus chelerythrine (10uM) for 1 hr. Cells treated with α_2-AR and β-AR blockers only served as controls. α_1-AR activation enhanced vitronectin-dependent cell adhesion that was blocked by the addition of either the RDG peptide, a neutralizing antibody to FGF7 or chelerythrine. Data shown are the mean $\pm$ SEM from 3-5 independent experiments performed in duplicate. *$p<0.05$ versus control or epinephrine-treatment.

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Figure 11. Effect of α_1-AR activation on the migration of A-10, DDT-MF2, and transfected α_{1A}-AR fibroblasts. Cell migration assays were done using transwell plates with 8-uM-pore size membranes. Cells were added to the top chamber in the presence or absence (Control) of 5μM epinephrine (Epi), 5μM epinephrine plus 1μM prazosin (Epi+ Praz), epinephrine plus 10 uM chelerythrine (Epi + CH) or epinephrine plus anti-FGF7 antibody (10ug/ml) for 18h, while Poly-L-lysine (10 μM), Fibronectin (10 μM) or 10%FBS in 600 μL DMEM were added to the bottom chamber. α_1-AR activation inhibited cell migration that was substrate-independent indicating effects on cell motility that is integrin-independent and which could be blocked with anti-FGF7 but not with chelerythrine. Data shown are the mean $\pm$ SEM from 3-5 independent experiments performed in duplicate. *$p<0.05$ versus control**.**

Figure 12. Novel α_1-AR signaling pathways mediated through secreted factors (IL-6, FGF7, HA) and interactions with the extracellular matrix. Prolonged α_1-AR activation causes the release of IL-6 (green), FGF7 (brown), and hyaluronan (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 α_{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 α_1-AR stimulated cell motility. α_1-AR mediated deposition of HA into cable structures (gold line) that may bind inflammatory cells (turquoise circles). PKC was found central to α_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 α_1-AR activation.

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Table 1. Primers for RT-PCR

Primer name	Primer sequence
CD44-sense	5'-GCAAGGATACAGACTCCAGT-'3
CD44-antisense	5'-GTTGGAGTCAGTAGCAAGAG-'3
Crem-sense	5'-ACTAGCAGAAGAAGCAACTCGAAAG -'3
Crem-antisense	5'-TTAAGAGACCCATCTACAAGTCCAT -'3
CyclinB1-sense	5'-CTTGAGCTGTCAACTTTCAGATCTT-'3
CyclinB1-antisense	5'-TTAAACAGCGTTAAGCAGCATTGTG-'3
Fst-sense	5'-AGTGCCTGCCACCTGAGAAAGGCCA-'3
Fst-antisense	5'-CGAGATGGAGTTGCAAGATCCGGAG-'3
Gro-sense	5'-GCGTTTCATCGATGGTCGTTCAATT-'3
Gro-antisense	5'-AAAGGCATTGTGCCCTACAAACTAG-'3
HMG2-sense	5'-GGTAGGCCAACAGGCTCAAAGAAGA -'3
HMG2-antisense	5'-TACGTTGTAGCCCCTGAAAAAGCTA -'3
Il6-sense	5'-ACTCGGCAAACCTAGTGTGCTATGC-'3
Il6-antisense	5'-TTGCAAGAAACCATCTGGCTAGGTA-'3
Itpr1-sense	5'-TCTGAACCAACATGATGCAGTAGGT-'3
Itpr1-antisense	5'-AACTTTTGTGCAGAGATGGTCTAGT-'3
Nr4a1-sense	5'-CTCCTCATTCCAGAAGATGGACAGA-'3
Nr4a1-antisense	5'-CTGTCAATCACCTTCTTGAGTGACA-'3
p55CDC-sense	5'-CAGAACCAGCTGGTTATTTGGAAGT -'3
p55CDC-antisense	5'-TTGGTGGATGAGGCTACTTTTAGAT -'3
Sarc-sense	5'-TGTTGGGACAAGAGGGCCTTAATGT-'3
Sarc-antisense	5'-ATGCACAAGATTCCTGTCGTAGCTC-'3
Syn4-sense	5'-TTCTCGAGATCGAGCGGAG-'3
Syn4-antisense	5'-CTCATCACCTTCCTCCTAAG-'3
Ten-c-sense	5'-AGGCCACTGAGTACGAAATT-'3
Ten-c-antisense	5'-GACCATCGAGAGCTGTGATT-'3
α-Tubulin-sense	5'-AGAGAGCTGTGTGTATGCTGAGCAA-'3
α-Tubulin-antisense	5'-GGACCAGAATAAACATCCCTGTGAA -'3

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Table 2. Notable differentially expressed genes

Gene Names	Acession #	Fold change		Function
		1 hour	18hour	
Sarcomeric muscle protein	AI639444	NC	+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	NC	+ 8.9	Growth factor
Interleukin 6	M26744	+74	+10.1	Cytokine
Hyaluronan synthase 2	AI008741	NC	+ 4.8	Inflammation
Fibroblast growth factor 7	X56551	NC	+3.5	Proliferation/adhesion
Tenascin C	U09401	NC	+3.4	Integrin-dependent cell adhesion/motility
CD44 antigen (Hyaluronan receptor)	AF014365	NC	+2.6	Inflammation/cell movement/adhesion
Syndecan 4	S61868	NC	+2.5	Integrin-dependent cell adhesion/motility
Jun-B	X54686	+3.0	+2.8	Transcription factor
BRCA1	AA800265	NC	-9.8	Breast cancer gene
Lamin B1	AA957201	NC	-8.1	Structural protein
High mobility group box 2 (Hmgb2)	AI008836	NC	-5.5	Transcription regulator
IL-15	AF015719	NC	-5.4	Cytokine
Cell cycle protein p55CDC	AF052695	NC	-4.8	Cell Cycle
BRCA2	U89653	NC	-4.2	Breast cancer gene
Pleiotrophin	AI102795	NC	-4.1	Growth factor
Gro	D11445	+2.5	-2.7	Chemokine
Interleukin 1 receptor-like 1	U04319	NC	-2.5	DNA binding
Hyaluronan motility receptor (Hmmr or RHAMM)	U87983	NC	-5.15	HA-mediated motility

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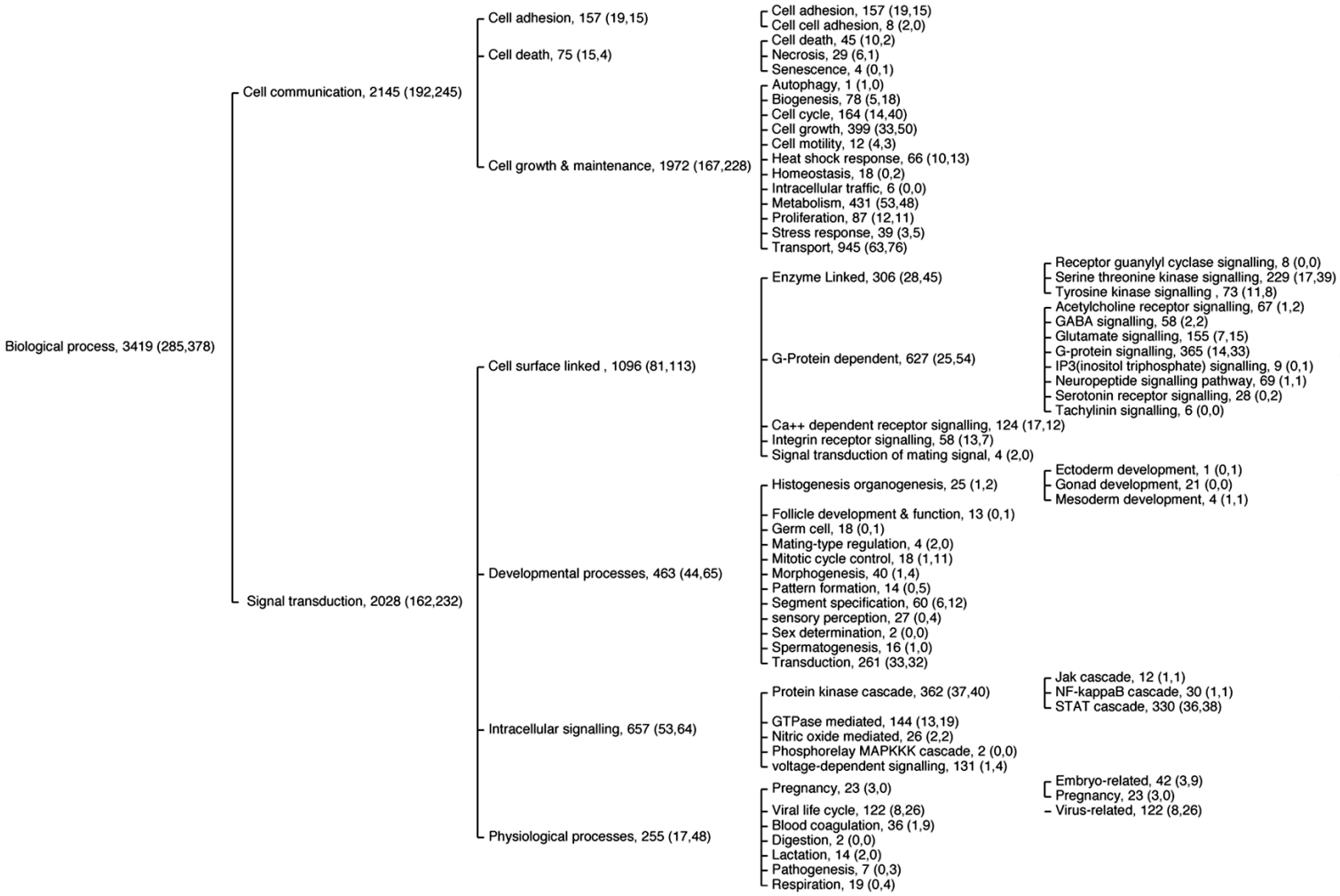

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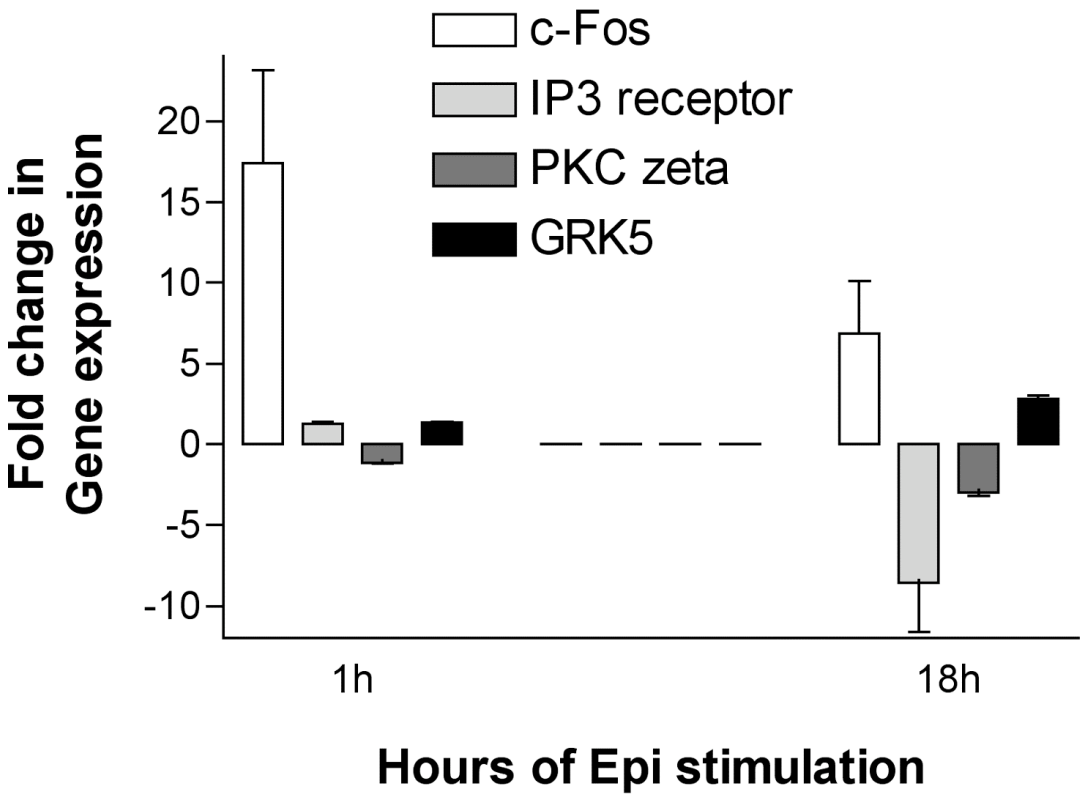

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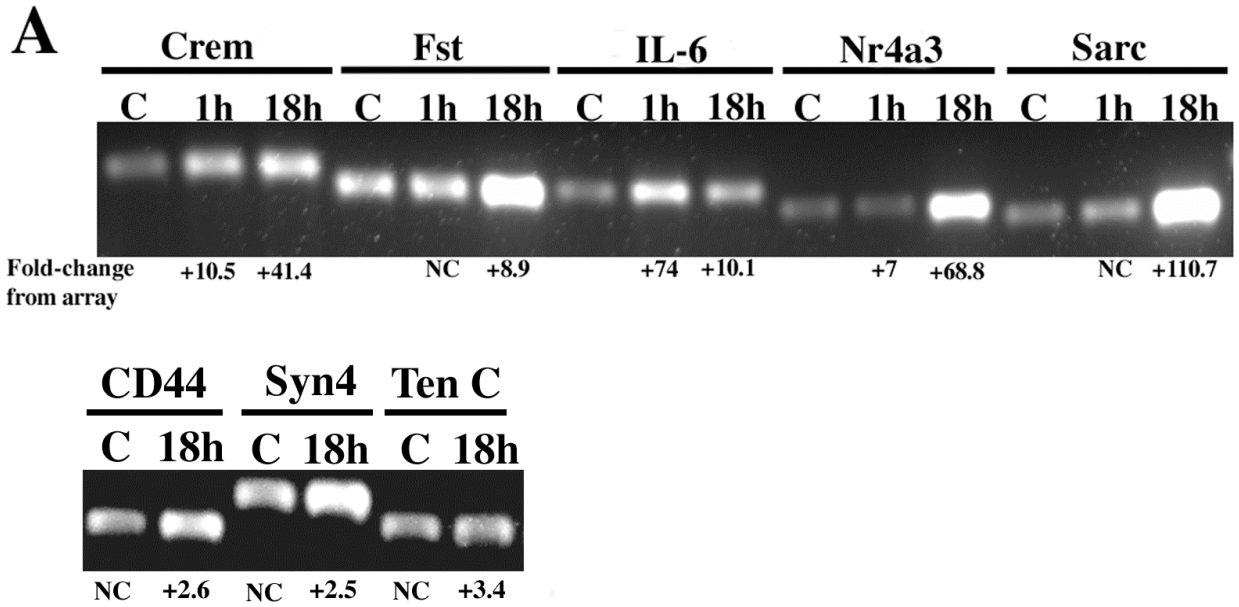
Figure 3



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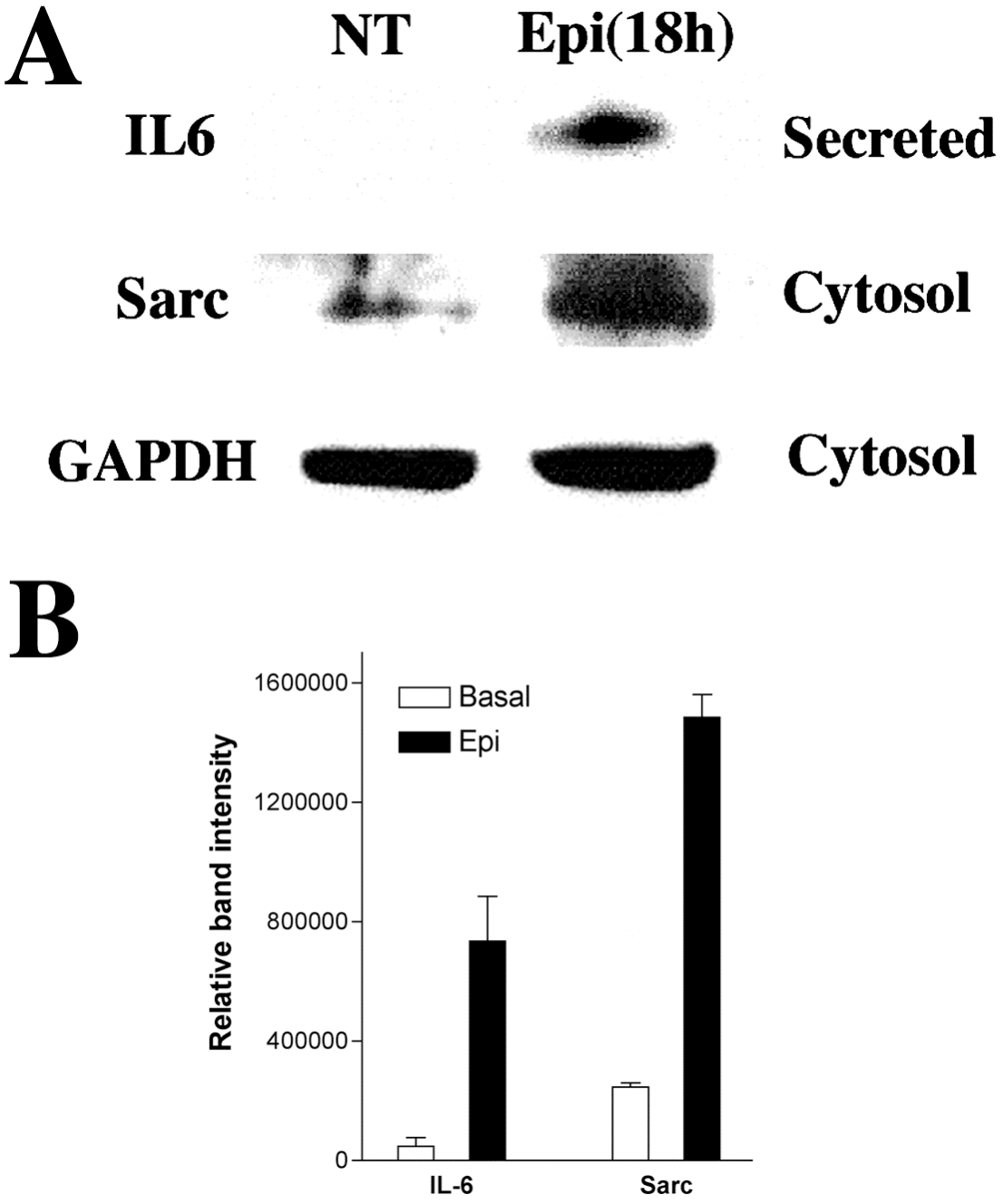

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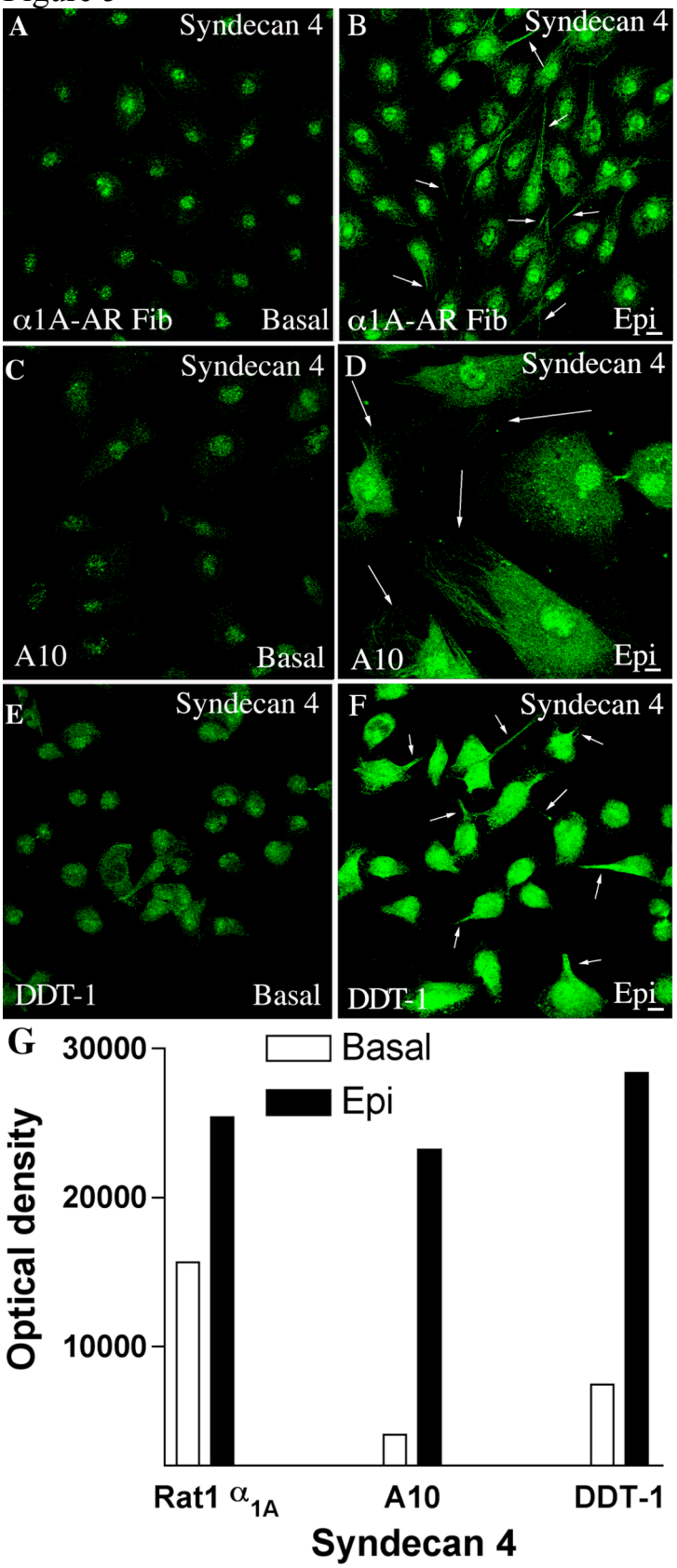

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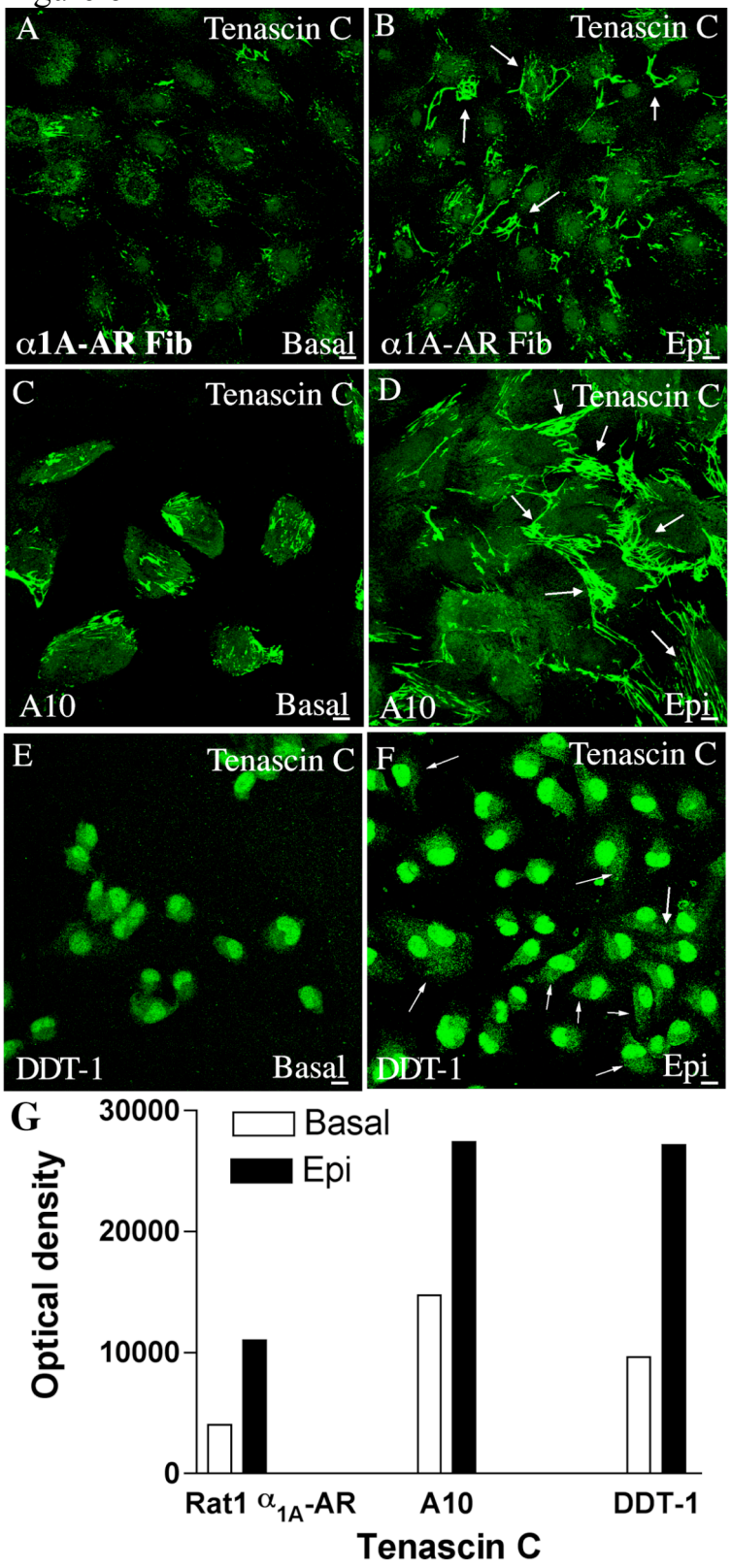
Figure 6



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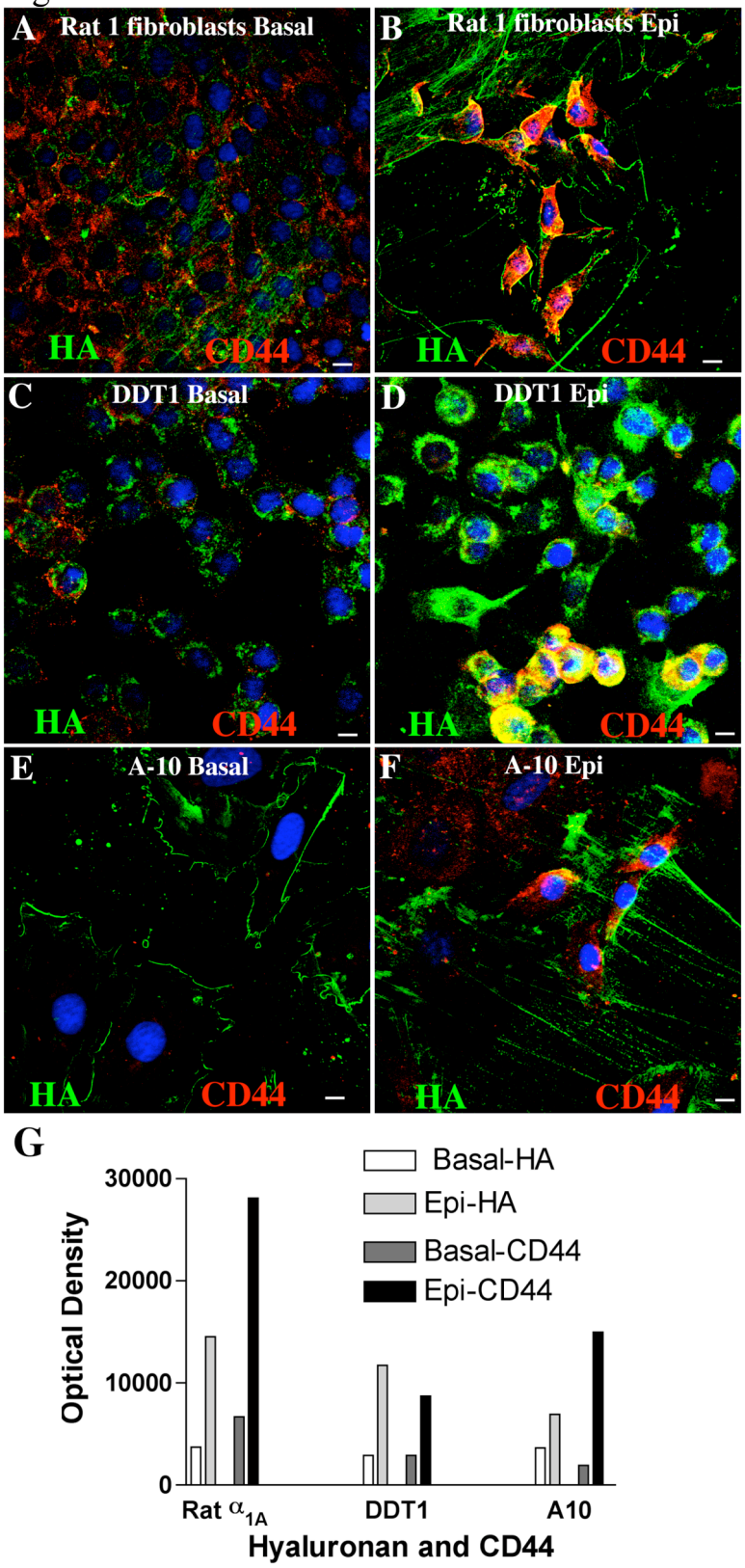

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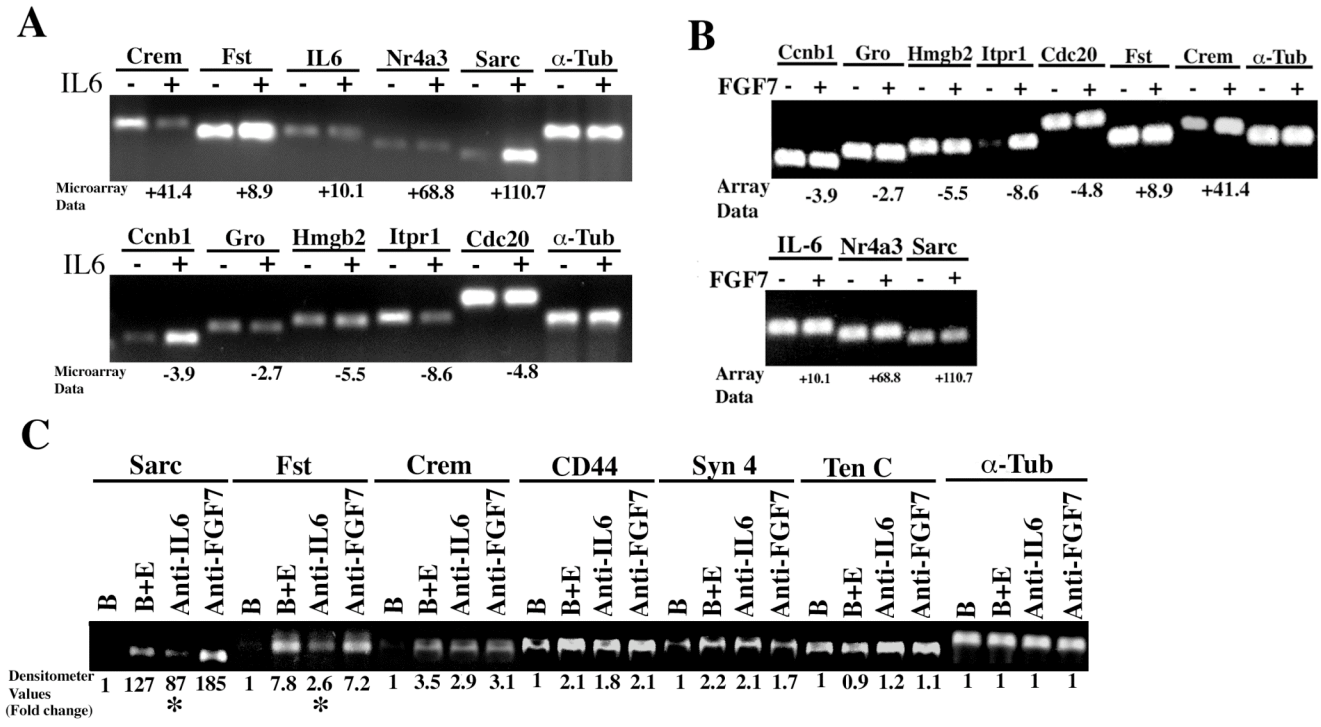

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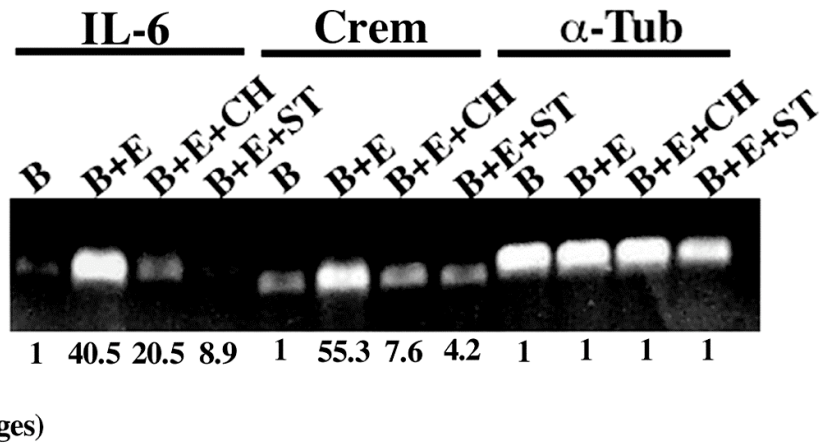

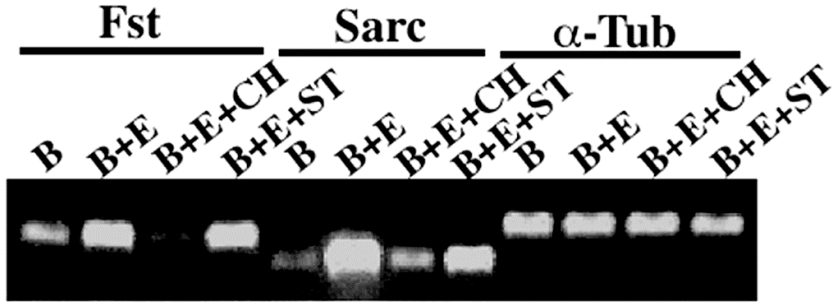

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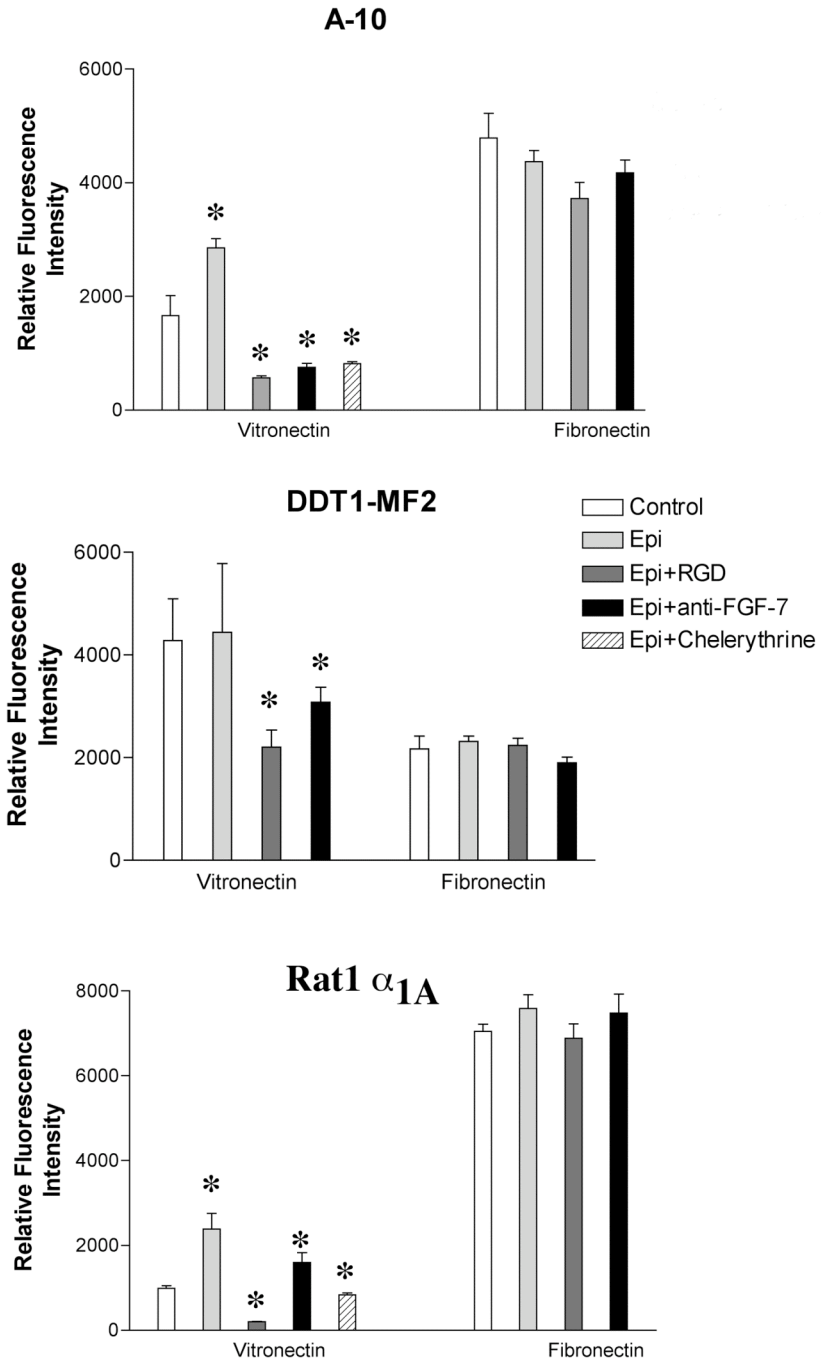
Figure 10



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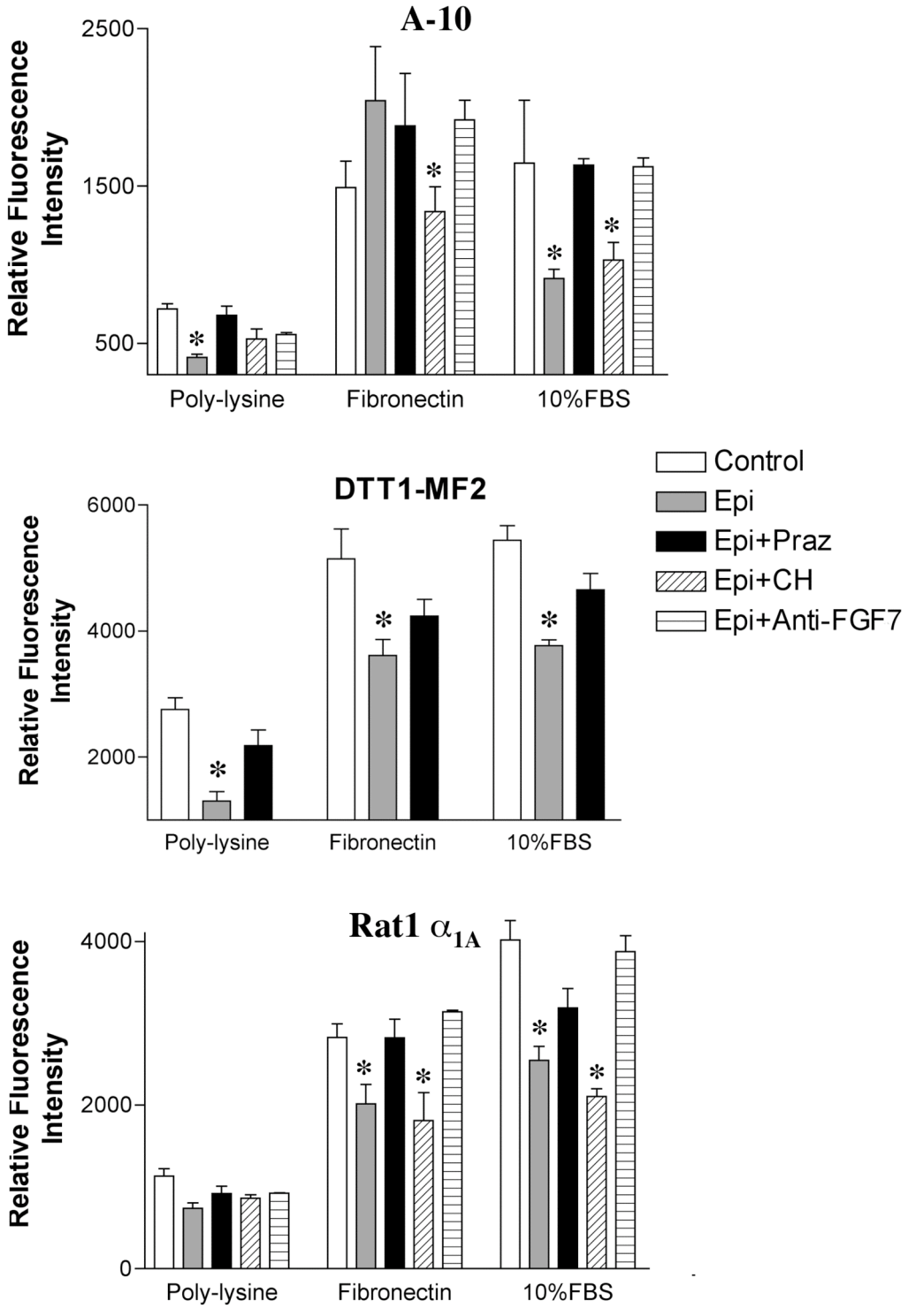

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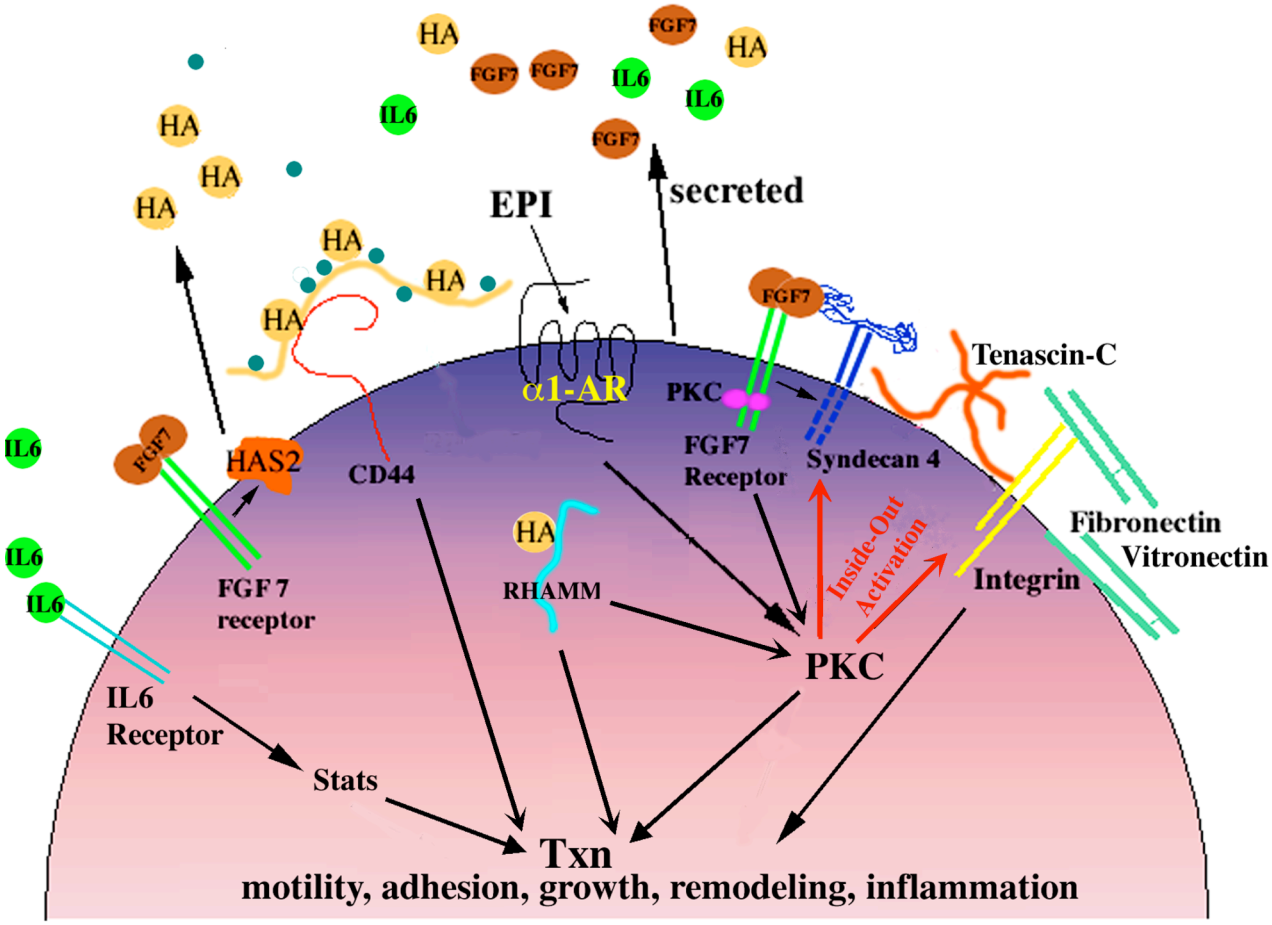

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