Phenotypic Modulation of Vascular Smooth Muscle Cells:
Evidence for a Role of the G Protein-coupled P2Y$_2$ Receptor

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And hereby certify that in their opinion it is worthy of acceptance.

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ABBREVIATIONS

1°………………....Primary

2-MeSADP………… 2-methyl-thio-adenosine 5'-diphosphate

$[Ca^{2+}]_i$……………… Intracellular calcium ion concentration

ANOVA……….... Analysis of variance

ATP………………… Adenosine 5'-triphosphate

BSA………………Bovine serum albumin

CCD………………. Charge-coupled device

cDNA………………Complimentary deoxyribonucleic acid

CRBP-1……………. Cellular retinol binding protein-1

DIC………………… Differential Interference Contrast

DMEM………………Dulbecco's modified Eagle's medium

DNA…………………Deoxyribonucleic acid

EDTA………………Ethylenediaminetetraacetic acid

ERK…………………Extracellular signal regulated kinase

FBS………………… Fetal bovine serum

Fura-2 AM…………. Fura-2-acetoxymethyl ester

GAPDH……………. Glyceraldehyde-3-phosphate dehydrogenase

HBS…………………HEPES buffered saline

HBSS……………… HEPES buffered saline solution

HEPES………………N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IgG………………… Immunoglobulin gamma

LCI…………………Live cell imaging

mRNA………………Messenger ribonucleic acid

NM-MHC…………...Non-muscle myosin heavy chain

P1……………………Passage 1

P2……………………Passage 2

P3……………………Passage 3

P4……………………Passage 4

PBS………………… Phosphate buffered saline

PCR…………………Polymerase chain reaction

POC-R……………... Perfusion, Open, Closed – cell cultivation system

QICAM…………… QImaging camera

RNA……………… Ribonucleic acid

rRNA……………… Ribosomal ribonucleic acid

RT-PCR…………… Reverse transcription–polymerase chain reaction

SDS-PAGE………… Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

S.E.M……………… Standard error of the mean

SMC……………….. Smooth muscle cell

SM-MHC………….. Smooth muscle-myosin heavy chain

TBST………………..Tris buffered saline with Tween 20

UDP……………… Uridine 5'-diphosphate

UTP………………… Uridine 5'-triphosphate

PHENOTYPIC MODULATION OF VASCULAR SMOOTH MUSCLE CELLS:

EVIDENCE FOR A ROLE OF THE G PROTEIN-COUPLED P2Y$_2$ RECEPTOR

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ABSTRACT

G protein-coupled receptors regulate two major functions of vascular smooth muscle cells (SMCs) - the vasomotor contractile response and the proliferative response. The contractile phenotype of SMCs has mainly been studied *in situ* within intact blood vessels, whereas the synthetic or proliferative SMC phenotypes can be studied in culture and represent a model of the SMC phenotype seen in vascular proliferative diseases. G protein-coupled P2Y receptors for extracellular nucleotides are known to regulate vascular SMC functions, although there is little information on P2Y receptor expression, distribution or function in SMCs of different phenotypes. Previous studies indicate that the P2Y$_2$ receptor subtype for ATP or UTP regulates SMC proliferation and migration, and we postulate that its expression may be up-regulated in the proliferative phenotype of SMCs.

To investigate the distribution of the P2Y$_2$ receptor subtype in SMCs of different phenotypes in culture, SMC cultures were established from rat aorta that displayed different morphological and biological features. We analyzed the expression of P2Y$_2$ receptor mRNA in SMC cultures having contractile or synthetic phenotypes using real-time PCR and characterized receptor activity by monitoring agonist-induced intracellular calcium mobilization. Our data indicate a selective dominance in the distribution of P2Y$_2$ receptors in the contractile SMC phenotype, consistent with its role in the regulation of

SMC growth in culture. These data suggest that up-regulation of the $P2Y_2$ receptor subtype in SMCs may contribute to increased cell proliferation and migration associated with the development of atherosclerotic lesions.

CHAPTER I: INTRODUCTION

Development of atherosclerotic lesions involves the recruitment of smooth muscle cells (SMCs) from media to the arterial intima [Ross R, 1993]. Several studies have identified the presence of morphologically distinct SMC populations in many species including rat, pig and human [Walker LN *et al.,* 1986; Christen T *et al.,* 1999; Li S *et al.,* 2001]. The presence of at least two distinct populations of SMC has been described in the rat carotid artery injury model [Walker LN *et al.,* 1986; Bochaton-Piallat ML *et al.,* 1996]: 1) spindle-shaped phenotype with elongated cells that exhibit a "hill & valley" growth pattern, and 2) epithelioid phenotype with cobblestone morphology that grow in a monolayer. The functional descriptions attributed to these phenotypes are contractile phenotype and synthetic phenotype, respectively [Hao H *et al.,* 2003; Chamley-Campbell J *et al.,* 1979]. The presence of these distinct SMC subpopulations was also shown in pig [Christen T *et al.,* 1999], and human [Li S *et al.,* 2001], suggesting that the nature and purpose of SMC heterogeneity in blood vessels is the regular maintenance of proper vessel function during development and disease. Unlike terminally differentiating skeletal or cardiac muscle cells, SMCs retain high levels of plasticity and can undergo phenotypic switching in response to local environmental changes [Owens GK, 1995], a process called phenotypic modulation. Though the term phenotypic modulation was originally established based on morphological criteria [Chamley-Campbell J *et al.,* 1979], today its definition has expanded to include the full spectrum of changes in structural and functional properties of SMCs in response to local environmental changes. The long-standing dogma for the origin of intimal SMCs in atherosclerotic lesions has been that

majority of them are derived from pre-existing medial SMCs that migrate into the intima and undergo phenotypic modulation [Ross R and Glomset JA, 1976]. A recent study has demonstrated the capacity of SMC clones from porcine coronary artery to reversibly modulate to both ends of the phenotypic spectrum [Campbell GR and Chamley-Campbell JH 1981; Hao H *et al.,* 2002]. Though the terms contractile and synthetic phenotype have proven useful to describe the SMC phenotypes, this two-state model based on morphology falls short in explaining the full range of SMC diversity exhibited under various physiological and pathological conditions. To compliment the morphological states of SMC phenotypes, several markers indicative of their relative state of differentiation were identified, but no single marker was found to exclusively identify a SMC phenotype to the exclusion of all other cell types [Rensen SS *et al.,* 2007]. To date there is no evidence of exclusive marker proteins for SMC with different phenotypes, but they express varying levels of the same marker proteins [Rensen SS *et al.,* 2007]. Among the myriad of marker proteins, the marker protein that best defines a contractile phenotype is smooth muscle-myosin heavy chain (SM-MHC) [Miano JM *et al.,* 1994] and the marker protein that best defines a synthetic SMC phenotype is cellular retinol binding protein-1 (CRBP-1) [Neuville P *et al.,* 1997].

During arterial injury, endothelial cells, SMCs, and other blood cells can release chemotactic factors and mitogens, including ATP and other nucleotides [Di Virgilio F and Solini A, 2002]. Activation of specific P2 purinoceptors on SMCs by purine nucleotides can directly affect vasoconstriction or vasodilation [Burnstock G and Kennedy C, 1986; Olsson RA and Pearson JD, 1990; Ralevic V and Burnstock G, 1991]. The two major classes of P2 purinoceptors: P2X and P2Y are known to elicit contraction

and relaxation of blood vessels, respectively, based on the order of agonist potencies [Abbracchio MP *et al.,* 2006]. Extracellular nucleotides such as adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) exert potent and diverse effects in the cardiovascular system, including vasoconstriction and dilatation by activating P2Y nucleotide receptors [Ralevic V and Burnstock G, 1991]. Receptor regulation orchestrates two major functions of SMCs - the vasomotor contractility and proliferation attributed to contractile and synthetic phenotypes, respectively. In the context of atherosclerosis, activation of purinergic nucleotide receptors induces not only the proliferation and migration of vascular SMCs, but also apoptosis, a process involved in the evolution of an atherosclerotic plaque [Isner JM *et al.,* 1995]. P2Y receptors, the G protein-coupled receptor family for extracellular nucleotides, are known to participate in the control of afore mentioned functions of SMCs [Boarder MR and Hourani SMO, 1998; Burnstock G, 2002; Di Virgilio F and Solini A, 2002]. $P2Y_2$ receptors are the predominant functional P2Y receptor that responds to ATP and UTP in cultured rat aortic SMCs [Kumari R *et al.,* 2003]; however $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors are also present in rat aortic SMCs [Erlinge D *et al.,* 1998; Chang K *et al.,* 1995]. Consistent with these findings, a recent mRNA quantification study in human SMCs also shows that the P2Y receptor with the highest level of expression is the $P2Y_2$ receptor [Wang L *et al.,* 2002]. Seye *et al.* have demonstrated the upregulation of $P2Y_2$ receptor mRNA in rat aorta after balloon injury [Seye CI *et al.,* 1997], reinforcing the pathological importance of the $P2Y_2$ receptor in disease and injury.

In light of the diversity of SMCs present within the arterial wall, we sought to determine whether: (i) $P2Y_2R$ expression is associated with a specific SMC phenotype

and (ii) upregulation of the $P2Y_2R$ in cultured SMCs is the result of dominance of a specific SMC phenotype over others. Using real-time PCR, we showed that $P2Y_2Rs$ are upregulated in tissue culture. We next isolated, and biochemically characterized two phenotypically distinct populations of SMC from rat aorta. We showed that spindle-shaped SMCs expressing high-levels of SM-myosin heavy chain, a specific marker of contractile SMCs, exhibited higher levels of $P2Y_2R$ mRNA and activity as compared to epithelioid SMCs which express CRBP-1, a selective marker of synthetic SMCs. Furthermore, we determined that epithelioid SMCs displayed a remarkable ability to shift towards a spindle-shaped phenotype accompanied by increased levels of $P2Y_2R$ mRNA and activity. Our data demonstrate for the first time that upregulation of $P2Y_2Rs$ in culture is a result of a selective dominance of spindle-shaped, contractile SMCs. Therefore, the diversity of SMC phenotypes must be taken into account when analyzing nucleotide receptor function in the vasculature. We speculate that the $P2Y_2R$ may play a role in the control of vasomotor tonus in vascular proliferative diseases.

Note: The terms epithelioid and spindle-shaped phenotypes will be used interchangeably with the terms synthetic and contractile phenotypes, respectively.

CHAPTER II: MATERIALS AND METHODS

Material. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Taq DNA polymerase were obtained from Invitrogen (Carlsbad, CA). Chromatographically purified collagenase was purchased from Worthington Biochemical (Lakewood, NJ). Fura 2-AM was purchased from Calbiochem (Gibbson, NJ). Cell culture media were from Invitrogen (Carlsbad, CA). Oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). RNeasy kit was purchased from Qiagen (Chatsworth, CA). Taq DNA polymerase was obtained from Invitrogen (Carlsbad, CA). The cDNA synthesis kit was obtained from Invitrogen (Carlsbad, CA). Reagents for quantification of mRNA were purchased from Applied Biosystems (Foster City, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Animals. For the purpose of our experiments, 8 – 12 week old, healthy, outbred Sprague Dawley rats purchased from Harlan, Inc. (Indianapolis, IN) were used. Protocols conformed to Animal Care and Use guidelines of the University of Missouri-Columbia.

Isolation and culture of rat aortic smooth muscle cells. Aortas were dissected from 10-12 week old rats and gently denuded of endothelium and adventitia. Denuded aortas were subjected to enzymatic digestion in filter sterilized DMEM containing 0.1% (w/v) collagenase (Worthington Biochemical Corp, Lakewood, NJ) and 1% (w/v) bovine serum albumin (BSA) at 37°C with gentle rocking. Cells dissociated in the first 30 min were discarded to enrich the smooth muscle cell population. The remaining tissue was digested

for an additional 60 min in fresh DMEM containing collagenase to release SMCs from the media. Dispersed cells were recovered and cultured at 37°C with 5% CO_2 in DMEM/high-glucose medium containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). SMCs were initially characterized by closely monitoring the morphology and growth pattern under the microscope and later by checking for the presence of NM-MHC by immunoblotting.

Cell spreading assay by time-lapse microscopy. SMCs were rinsed with sterile phosphate buffered saline (PBS) and replaced with 0.25% (w/v) trypsin, and incubated at 37°C. The cells in the culture flask were examined under the microscope every two to three minutes until the cells began to round up and detach from the bottom of culture flask and suspended in DMEM under appropriate experimental conditions and plated on collagen-coated glass cover-slips. Cells were visualized using differential interference contrast (DIC) on an Axiovert 200M inverted microscope equipped with a 25×/0.8 NA LCI (Live Cell Imaging) Plan-Neofluar objective immersed in water (LSM 510 META system, Zeiss, Thronwood, NY). Cells were maintained at 37ºC on a coverslip inside a POC-R chamber on a heated microscope stage (LaCon, Staig, Germany). The stage was pre-heated before beginning each time course. Three or four fields of view from each coverslip were chosen by visual inspection and the position of the stage at each field was marked with the microscope control software (Zeiss LSM 5 version 4.0). At the different time points, each marked position was automatically moved into the field of view, manually focused, and scanned with a 488 nm laser while detecting transmitted light. In between time points, the water-immersion objective was removed to minimize

heat sinking from the culture. The cells were considered spread when they first flatten and the rounded edges deform extensively occupying a larger surface area.

Identification of SMC phenotypic morphology. Cultured SMC phenotypes were visualized by brightfield microscopy on a DMI400B automated inverted microscope equipped with a 10x/0.25 NA objective lens (Leica, Wetzlar, Germany). Images were captured with a QICAM cooled CCD camera (QImaging, Surrey, Canada) and QCapture Pro software (ver. 5.1.1.14, QImaging).

Cloning of SMC subtypes. Freshly isolated SMCs were seeded on a 150 mm culture dish and grown until well-isolated viable colonies were identifiable under an inverted microscope. Once colonies have been located, cloning cylinders with smooth silicone high vacuum grease were set over colonies and the cylinder was gently pressed down evenly with the forceps to avoid leakage of the grease seal. The content of the cylinder was rinsed with sterile phosphate buffered saline (PBS), replaced with 0.2 ml of 0.25% (w/v) trypsin, and incubated at 37°C. The cells in the cylinder were examined under the microscope every two to three minutes until the cells began to round up and detach from the dish bottom. A few drops of growth medium were added into the cylinder to neutralize the trypsin and the cells were gently transferred to a six-well culture dish with a micro-pipettor and 2.5 ml of growth medium was added. Cells were incubated and cultured at 37°C with 5% CO_2.

Intracellular free [Ca^{2+}] measurement. SMCs were washed with phosphate buffered saline (PBS) (50 mM KH$_2$PO$_4$; 150 mM NaCl; pH 7.2) and detached from flasks by incubation with 0.05% (w/v) Trypsin-EDTA. Cells were pelleted and resuspended in HBS containing 10.0 mM glucose, 0.1% (w/v) BSA, and 1.0 mM CaCl$_2$ (HBSS). Two µM fura-2 acetoxymethyl ester (fura-2AM) was added for 30 min at 37°C, and the cells were pelleted, resuspended in HBSS at a density of 0.5–1.0 x 10^6 cells/ml and equilibrated for 20 min at 37°C. Two ml aliquots of cell suspension were sampled, pelleted, and resuspended in 2.0 ml of HBSS. Changes in the concentration of intracellular free calcium ion, [Ca^{2+}]$_i$, in response to nucleotides were determined, as previously described [Garrad RC *et al.,* 1998; Gonzalez FA *et al.,* 1989]. The fluorescence of fura-2 was detected by an ICCD camera (SIM Security and Electronic Systems, Neustadt, Germany) coupled to an inverted Nikon Diaphot microscope (Nikon, Melville, NY, USA) and a Deltascan illumination system (Photon Technologies International, Princeton, NJ, USA) using dual excitation at 340 and 380 nm (selected by 2 monochromators and directed to the microscope with a quartz fiber-optics device), a 400 nm dichroic mirror, and a band-pass interference filter centered at 510 nm for emission. The cells were maintained at 37°C with a Nikon air curtain. Images (collected with a Nikon 40X Fluor immersion objective, NA 1.3) were the average of 8 frames acquired at 10 sec intervals and stored on a hard disk for off-line analysis. The ratio of absorbances collected was analyzed using ImageMaster software (Photon Technologies International, Princeton, NJ, USA) to calculate the [Ca^{2+}]$_i$, as previously described [Gonzalez FA *et al.,* 1988].

Single cell $[Ca^{2+}]_i$ measurement. The $[Ca^{2+}]_i$ was quantified in single smooth muscle cells with the Ca^{2+}-sensitive fluorescent dye fura-2, using an InCyt Dual-Wavelength Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH). Suspended smooth muscle cells in assay buffer (HBSS) were incubated with 2.0 μM fura 2-acetoxymethyl ester (fura-2 AM) for 30 min at 37°C, washed, transferred to coverslips treated with Cell-Tak (Becton Dickinson Labware, Bedford, MA), according to the manufacturer's instructions, and incubated for 20 min at 37°C. Coverslips with adherent cells, were positioned on the stage of a fluorescence microscope and stimulated with agonists at 37°C, as described in the figure legends. Cells were exposed to 340/380 nm light and fluorescence emission at 505 nm was converted to $[Ca^{2+}]_i$ using a standard curve created with solutions containing known concentrations of Ca^{2+}. Increases in $[Ca^{2+}]_i$ were measured by subtracting basal $[Ca^{2+}]_i$ from the peak $[Ca^{2+}]_i$ measurement. The percentage of cells that responded to nucleotides was also determined. Viable cells were identified by responsiveness to carbachol and non-responding cells were eliminated.

RNA extraction and relative quantification of mRNA. Total RNA was isolated from cultured SMCs using the GenElute™ Mammalian Total RNA Miniprep Kit, according to the manufacturer's instructions. cDNA synthesis was performed using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Ten percent of the cDNA was used as template in the PCR. mRNAs for the $P2Y_2$ receptor, 18s rRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified using an Applied Biosystems (Foster City, CA) 7500 Real Time PCR system with TaqMan methods, using 18s rRNA and GAPDH as internal standards. This technique uses the 5'

nuclease activity of Taq DNA polymerase to produce a real-time quantitative DNA assay. mRNA-specific oligonucleotide probes (TaqMan probe) with 5'-fluorescent reporter and 3'-quencher dyes designed by the manufacturer (Applied Biosystems, Foster City, CA) were used for the extension phase of the PCR. The reporter dye, FAM, gets degraded and released resulting in fluorescence at 518 nm, which is monitored during the whole amplification process. cDNA (n = 3) from control and $P2Y_2$ receptor groups were analyzed simultaneously by real-time PCR, with each sample run in triplicate. The single-reporter real-time PCR protocol was used to prepare the PCR mixture, according to the manufacturer's instructions (Applied Biosystems PRISM 7500), and the PCR was run using the 7500 system software.

Western blot analysis. Cell lysates were sonicated for 5 seconds with a Branson Sonifier 250 (microtip; output level 5; duty cycle 50%) and boiled for 5 min. The lysates were subjected to 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline (0.137 M NaCl, 0.025 M Tris (hydroxymethyl)-aminomethane, pH 7.4) containing 0.1% (v/v) Tween-20 (TBST) and immunoblotted overnight at 4°C in TBST containing 3% (w/v) BSA and 0.02% (w/v) sodium azide and rabbit anti-rat non-muscle myosin II heavy chain B polyclonal antibody (1:1000 dilution; Covance, Emeryville, CA), rabbit anti-human CRBP-1 polyclonal antibody (1:1000 dilution; Santa Cruz Biotechnology) that recognizes an epitope corresponding to amino acids 1–135 of full length CRBP-1, or mouse anti-human smooth-muscle myosin monoclonal antibody (1:1000 dilution; Sigma, St. Louis, MO)

that recognizes the myosin heavy chain polypeptides. Membranes were washed three times with TBST during a 45 min period and incubated with peroxidase-linked goat anti-rabbit IgG antibody (1:2000 dilution; Santa Cruz Biotechnology) or goat anti-mouse IgG antibody (1:2000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. After three more washes with TBST, the membrane was subjected to chemiluminescence detection and the protein bands were visualized on X-ray film and quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA). For signal normalization, membranes were treated with stripping buffer (0.1 M glycine, pH 2.9, and 0.02% (w/v) sodium azide) and re-probed with goat anti-rabbit (total) extracellular signal regulated kinase (ERK) antibody (1:1000 dilution; Santa Cruz Biotechnology. All experiments were performed in duplicate and repeated at least three times.

Statistical analysis. Data are expressed as means ± S.E.M. with 95% confidence intervals. All graphs were plotted using GraphPad Prism Ver. 5.0 (San Diego, CA). Means of two groups were compared using Student's *t*-test (unpaired, two tailed) and two-way ANOVA, while one-way ANOVA was used for comparison of more than two groups with $p < 0.05$ considered to be statistically significant.

CHAPTER III: RESULTS

Cells isolated from normal media of rat aorta exhibit unique phenotypic properties

In order to determine whether SMC subpopulations with distinct phenotypes similar to those reported *in vivo* are present within the arterial vessel, a rat aorta was subjected to an initial 30 min collagenase digestion to strip away endothelium, tunica intima and tunica adventitia, then the aorta was subjected to an additional 60 min collagenase digestion to release SMCs from the medial layer. The primary cells were seeded at low density in 100 mm culture dishes and were incubated in culture medium with 10% (v/v) fetal bovine serum. Five days later, two morphologically distinct cell colonies present in the primary cultures were isolated using cloning cylinders. Cells in one colony appeared cobblestone or epithelioid in shape and, at confluence, formed a monolayer (Fig. 1a). Cells in the other colony appeared elongated and spindle-shaped and, at confluence, formed the "hill & valley" pattern traditionally described for SMCs (Fig. 1b).

These observations agree with previous studies reported by Walker LN *et al.,* which demonstrate the presence of spindle-shaped and epithelioid phenotypes within rat aorta [Walker LN *et al., 1986*].

Epithelioid and spindle-shaped SMCs express different biochemical markers

To further characterize and confirm the existence of two different cell phenotypes in the rat aorta, we next used biochemical markers that have been used to distinguish between epithelioid and spindle-shaped SMCs [Miano JM *et al.,* 1994; Neuville P *et al.,*

1997]. Western blot analysis showed that CRBP-1 is highly expressed in the epithelioid SMC phenotype, but is barely present in spindle-shaped SMCs (Fig. 2). In contrast, we found that SM-MHC, a protein characteristic of highly differentiated SMCs, is expressed at higher levels in cells cultured from SMC colonies with spindle-shaped phenotype than epithelioid (Fig. 2).

These data demonstrate that morphological differences observed between epithelioid and spindle-shaped SMCs are accompanied with the expression of distinct biochemical markers in these SMC colonies.

Modulation of P2Y$_2$ receptors in cultured SMCs

Previous reports, though not specifically about tissue culture, employed cultured SMCs for their studies, have shown that the P2Y$_2$R is upregulated in response to tissue culture [Seye et al., 1997; Hou M et al., 1999]. To confirm these findings we used real-time PCR to measure the P2Y$_2$R mRNA levels in primary cultured SMCs and in SMCs after subsequent secondary passages. As shown in Fig. 3, there is a steady increase in P2Y$_2$R mRNA levels as a function of passage number after tissue culture of freshly isolated rat aortic SMCs. The P2Y$_2$R mRNA levels increased more than three-fold by passage 3 and almost fourteen-fold by passage 4.

To test for the presence of functional P2Y$_2$Rs and other P2Y receptors expressed in SMCs [Chang K et al., 1995; Erlinge D et al., 1998], cultured SMCs from the aortic media (containing both phenotypes) were monitored for increases in $[Ca^{2+}]_i$ in response to agonists of different P2Y receptors. Cultured medial SMCs (primary through passage 4) exhibited very potent increases in $[Ca^{2+}]_i$ in response to the P2Y$_2$ receptor-selective

agonists, ATP (10 μM) and UTP (10 μM), but only moderate increases in $[Ca^{2+}]_i$ in response to UDP (10 μM)), a potent agonist of the $P2Y_6$ receptor and 2-MeSADP (10 μM), a selective agonist of the $P2Y_1$ receptor (Fig. 4). The increases in $[Ca^{2+}]_i$ in response to ATP and UTP were greater in passages P3 and P4, but the increases in $[Ca^{2+}]_i$ in response to UDP and 2-MeSADP remained unchanged between primary culture and passage 4 (Fig. 4). These data demonstrate that the $P2Y_2R$ is functionally upregulated in response to tissue culture. However, several questions remain unanswered:

1) How are $P2Y_2Rs$ distributed among the different SMC subpopulations within the artery?

2) How do these subpopulations evolve when grown together during the tissue culture process?

3) Does $P2Y_2$ receptor upregulation result from dominance of a specific cell phenotype during the culture process?

$P2Y_2$ receptor expression in contractile (spindle-shaped) and synthetic (epithelioid) SMCs)

We used real-time PCR to determine if there was a difference in the levels of expression of $P2Y_2$ receptor mRNA between the two phenotypes. As shown in Fig. 5, $P2Y_2R$ mRNA expression in contractile SMCs was about four-fold higher, as compared to synthetic SMCs (Fig. 5).

We next used fura-2 digital imaging of single SMCs to measure the increase in $[Ca^{2+}]_i$ in response to ATP and UTP, two equipotent agonists of the $P2Y_2R$, 2-MeSADP,

a potent agonist of the $P2Y_1$ receptor, and UDP, a preferential agonist of the $P2Y_6$ receptor.

SMCs of both phenotypes exhibited increases in $[Ca^{2+}]_i$ in the presence of ATP) (Fig. 6). ATP-induced increases in $[Ca^{2+}]_i$ were greater in spindle-shaped SMCs (293±27 nM), as compared to epithelioid SMCs (179±14 nM) (Fig. 6). Similar increases in $[Ca^{2+}]_i$ were observed in the presence of UTP in spindle-shaped SMCs (264±17 nM), as compared to epithelioid SMCs (219±13 nM). More than 90% of SMCs from both phenotypes responded to ATP and UTP (Fig. 7).

The $P2Y_1$ receptor-selective agonist, 2-MeSADP, significantly increased $[Ca^{2+}]_i$ in epithelioid SMCs (114±19 nM), but not in spindle-shaped SMCs (11±4 nM) (Fig. 6). There were about 70% epithelioid SMC responders, as compared to 30% spindle-shaped SMC responders.

However, when tested for the presence of functional $P2Y_6$ receptors in SMC phenotypes, none of the cells of spindle-shaped phenotype showed increases in $[Ca^{2+}]_i$ in the presence of the $P2Y_6$ receptor-selective agonist, UDP, but cells of epithelioid phenotype showed an increase in $[Ca^{2+}]_i$ (131±9 nM) (Fig. 6). Only 25% of spindle-shaped SMCs responded to UDP, as compared to 65% of epithelioid SMC responders.

Spindle-shaped SMCs are the main subtype found in subsequent passages of primary SMCs.

As described above, primary cultures of SMCs from aortic media display two different SMC phenotypes (Fig. 1). Upon monitoring the morphology of primary SMCs after a few passages, we found that secondary cultures predominantly exhibit a spindle-

shaped phenotype by passage 4 with cells displaying a "hill and valley" pattern, characteristic of contractile SMCs (Fig. 8).

These data suggest that culturing primary SMCs promotes the propagation of spindle-shaped over epithelioid SMCs.

Synthetic SMCs appear to revert back to a contractile phenotype.

Several investigators have shown that SMCs with a contractile phenotype modulate towards a synthetic phenotype in culture [Chamley-Campbell JH *et al.,* 1979, Thyberg J *et al.,* 1990], but there has been much debate about the possibility of modulation from a synthetic phenotype towards a contractile phenotype.

To test whether the synthetic phenotype can become contractile, we isolated a single cell colony of 1° rat aortic SMCs that exhibited epithelioid marker expression. The colony was seeded in a 35 mm culture dish and grown until confluent. At confluence, the colony showed phenotypic and biochemical characteristics consistent with a synthetic phenotype, as described above. Cells were then trypsinized and cultured for another passage (P2). At confluence, 40% of cells at passage 2 displayed a phenotype reminiscent of contractile (spindle-shaped) SMCs (Fig. 9b). At passage 3, the percentage of spindle-shaped SMCs reached approximately 85% (Fig. 9c). By passage 4, all the cells completely reverted to a spindle-shaped phenotype and at confluence displayed the "hill and valley" pattern characteristic of contractile SMCs (Fig. 9d). Consistent with a transition from epithelioid to spindle-shaped phenotype, Western blot analysis showed an increase in SM-MHC expression from P2 to P4 that was inversely correlated with the expression of CRBP-1 (Fig. 10).

We also found that epithelioid SMCs showed a gradual increase with passage number in $[Ca^{2+}]_i$ in response to stimulation with the P2Y$_2$R agonists, ATP or UTP (Fig. 11). UDP, a preferential agonist of P2Y$_6$R caused a significant increase in $[Ca^{2+}]_i$ in cultured cells at P2 (118±04 nM). However, this response was significantly decreased by P3 (64±09 nM) and P4 (42±05 nM). Similarly, cells at P2 (161±09 nM) exhibited a strong increase in $[Ca^{2+}]_i$ in response to the P2Y$_1$R agonist, 2-MeSADP, but this response was significantly decreased by P3 (92±06 nM) and P4 (51±05 nM).

These observations clearly demonstrate the gradual shift in morphology, biochemical marker and P2Y receptor distribution in epithelioid SMCs during phenotypic modulation towards a spindle-shaped SMC phenotype.

Phenotypically distinct cell subpopulations exhibit markedly different spreading kinetics under identical conditions.

To determine whether morphologically distinct SMC phenotypes exhibit different cell spreading capabilities, serum-starved epithelioid and spindle-shaped SMCs were seeded on collagen-coated glass coverslips and their attachment and spreading rates were measured for 2 h 30 min intervals after stimulation with serum, the P2Y$_2$R agonist UTP, or the P2Y$_1$R agonist 2-MeSADP. SMCs were considered to have spread or starting to spread when adherent cells on the coverslip exhibited initial deformation of cell boundaries as compared to cells that were round and spherical without any deformation of cell boundaries.

Kinetics of SMC spreading in serum-free media: The synthetic SMCs demonstrate much higher rates of spreading, as compared to contractile SMCs. After 60 min of plating, ~

50% of synthetic SMCs exhibited spreading. In contrast, very little or no spreading was observed with contractile SMCs (Fig. 12-1).

Kinetics of SMC spreading in response to serum: SMCs of synthetic phenotype exhibited a markedly different spreading response, as compared to the cells of contractile phenotype in response to 10% (v/v) serum stimulation (Fig. 12-2). SMCs of synthetic phenotype spread more rapidly than did cells of contractile phenotype (Fig. 12-1). Whereas more than 75% of synthetic SMCs flattened and deformed extensively by 60 min of plating, only ~60% of contractile SMCs showed similar levels of spreading even after 120 min.

Kinetics of SMC spreading in response to UTP: When SMCs were allowed to adhere and spread in response to 10 μM UTP, contractile SMCs exhibited faster spreading kinetics than synthetic SMCs (Fig. 12-3). In contrast to 7% of synthetic SMCs that exhibited spreading in response to UTP, more than 25% of contractile SMCs displayed a similar extent of spreading within 30 min. Kinetics of spreading in response to UTP at other time points was similar for both phenotypes with the contractile phenotype exhibiting slightly higher rates.

Kinetics of SMC spreading in response to 2-MeSADP: The spreading properties of SMCs of different phenotypes were very different in response to 10 μM 2-MeSADP (Fig. 12-4). By 90 min, there was very little or no spreading observed for the contractile phenotype, but about 50% of cells with the synthetic phenotype exhibited extensive flattening. Fig. 13 represents a comparison of cell spreading capabilities as a percentage of total cells for both SMC phenotypes.

The observations of phenotypic differences in spreading kinetics suggest the possible involvement of distinct SMC subtypes in the initial stages of lesion development before the cells migrate to the intima in response to local environmental cues.

CHAPTER IV: DISCUSSION

The findings of the present study support and extend the experimental *in vitro* [Bochaton-Piallat ML *et al.,* 1996] and *in vivo* [Walker LN *et al.,* 1986] data showing that distinct SMC subpopulations exist in the same artery in rat. The present study demonstrates that at least two SMC subpopulations can be identified in the medial layer of mature rat aorta (Fig. 1), each carrying distinct properties in terms of their growth, protein marker expression, and spreading capabilities. The expression of marker proteins for the identification of SMC phenotypes was complicated in that no single marker protein was exclusive for SMCs with different phenotypes, but each phenotype expressed different levels of specific marker proteins. Contractile proteins important for the differentiated function of mature SMCs make obvious candidates for distinguishing contractile from synthetic phenotypes. One of the prominent contractile proteins specific to SMCs is smooth muscle myosin heavy chain (SM-MHC), whereas cellular retinol binding protein (CRBP-1) is an effective candidate to identify synthetic SMCs, as this protein is markedly upregulated in proliferating SMCs [Neuville P *et al.,* 1997]. Cultured vascular SMCs express smooth muscle as well as non-muscle variants of myosin heavy chain [Rovner AS *et al.,* 1986]. SM-MHC makes a very effective marker for contractile SMCs [Miano JM *et al.,* 1994]. In agreement with previously reported findings summarized in a recent review [Rensen SS *et al.,* 2007], we show that the expression levels of CRBP-1 are higher in epithelioid than in spindle-shaped SMCs, whereas SM-MHC expression is higher in spindle-shaped than in epithelioid SMCs (Fig. 2).

A major finding of the present study is the potential of the spindle-shaped SMC phenotype to retain its morphology for over ten passages, whereas epithelioid SMCs lose its phenotype after a few passages. This distinction points out the underlying differences in factors that could influence the direction of phenotypic modulation.

Thyberg J. has described the importance of the transition from contractile to synthetic phenotype as a prerequisite for involvement of SMCs in the formation of atherosclerotic and restenotic lesions [Thyberg J, 1996]. Several other studies in rat models have shown that the spindle-shaped phenotype modulates to assume an epithelioid phenotype [Chamley-Campbell JH *et al.,* 1979; Thyberg J *et al.,* 1990; Owens GK *et al.,* 2004], but little has been shown about the epithelioid phenotype modulating towards a spindle phenotype. In the present study, we demonstrate the ability of an epithelioid phenotype from the medial layer to modulate to a spindle-shaped phenotype (Fig. 9). We confirm the observed morphological changes by using biochemical markers at different stages of phenotypic modulation (Fig. 10).

Many events occur during arterial injury including the release of chemotactic factors and mitogens as well as extracellular nucleotides by endothelial cells, SMCs, and other blood cells [Di Virgilio F and Solini A, 2002]. The potent effects of extracellular nucleotides such as ATP and UTP on the cardiovascular system have gained considerable attention [Ralevic V and Burnstock G, 1991].

$P2Y_2Rs$ are upregulated in tissue culture and in response to tissue injury [Seye CI *et al.,* 1997; Erlinge D *et al.,* 1998; Seye CI *et al.,* 2002]. However, it is not clear from these studies whether phenotypic differences observed for SMCs in the media play a role in $P2Y_2R$ upregulation. Here, we show for the first time that spindle-shaped SMCs

express higher levels of P2Y$_2$R mRNA, as compared to epithelioid SMCs. Our data also indicate that when primary SMCs are cultured for additional passages, only the spindle-shaped phenotype persists after four passages. Moreover, we observed that epithelioid SMCs reverted to a spindle-shaped phenotype after a few passages. These morphological changes were correlated with the expression of protein markers that characterize SMC phenotypes. Taken together, our data demonstrate that upregulation of the P2Y$_2$R in tissue culture is due to a phenotypic transition of epithelioid SMCs into spindle-shaped SMCs that express high level of P2Y$_2$Rs. Therefore, the diversity of SMC phenotypes must be taken into account when analyzing nucleotide receptor function in the vasculature.

It would be interesting to examine in further studies whether the phenotypic modulation of SMCs can be accelerated by P2Y$_2$ nucleotide agonists *in vitro*. Such studies will help to better understand the role of P2Y$_2$Rs in the mechanism of plaque stabilization.

UTP has been shown to induce SMC migration [Chaulet H *et al.,* 2001], however, prior to migration the cells need to attach and spread on the extracellular matrix. In the present study, we explore the differences in the spreading properties of the two SMC phenotypes on a collagen-coated surface in response to agonists of selective P2Y receptors and serum. Among the G protein-coupled P2Y receptors, the P2Y$_2$ receptor subtype has been shown to be the predominant functional P2Y receptor that responds to ATP and UTP in cultured rat aortic SMCs [Kumari R *et al.,* 2003]. From our data, we show that the main difference between the phenotypes with respect to cell spreading in response to growth stimulus or agonists of P2Y receptors is that the contractile phenotype

is highly responsive to UTP, a potent agonist of the $P2Y_2$ receptor. On the other hand, synthetic SMCs revealed a stronger spreading response to culture medium without FBS, culture medium with FBS, and 2-MeSADP than contractile SMCs, suggesting that discrimination occurs in the expression of functional P2Y receptors between the phenotypes (Figs. 12 & 13). During vascular injury, when extracellular nucleotides and other inflammatory cytokines are released by endothelial cells, it is possible that contractile SMCs, the dominant phenotype in the normal media, migrate to the intima. Our data support the hypothesis that the early stages of attachment and spreading during the migration of SMCs to the intima are essentially mediated by $P2Y_2R$ activation without excluding the contribution of other P2Y receptor subtypes.

In conclusion, our findings clearly indicate that phenotypic modulation of SMCs plays an important role in the regulation of $P2Y_2R$ expression. Since $P2Y_2R$ upregulation accompanies the development of vascular diseases, further studies are needed to better understand the role of this receptor in the phenotypic modulation of SMCs during atherosclerosis and restenosis after angioplasty.

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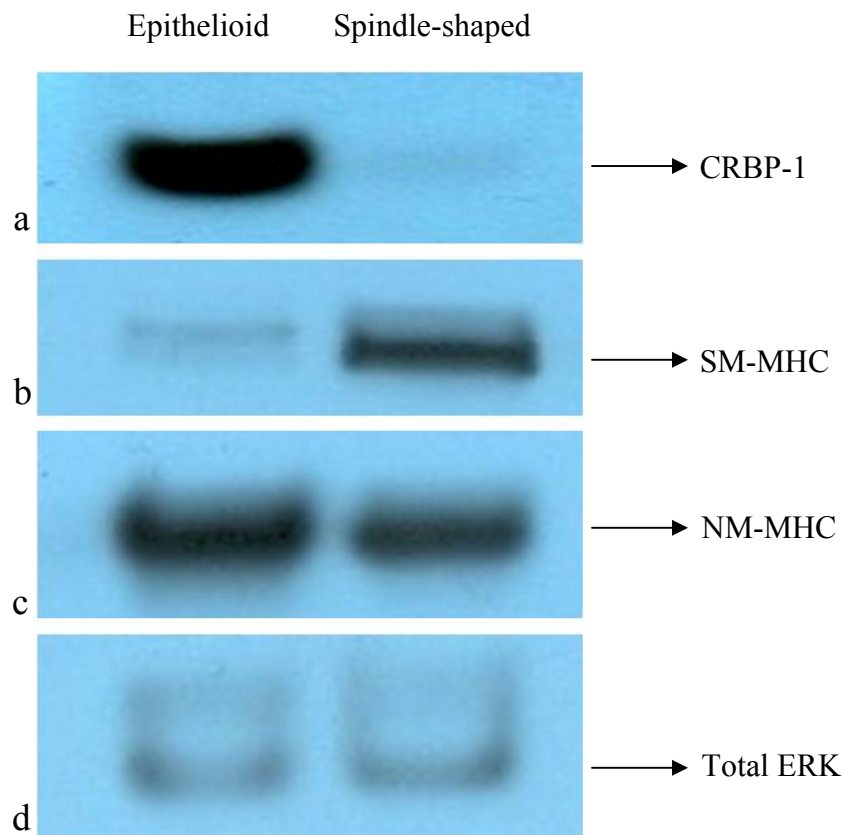
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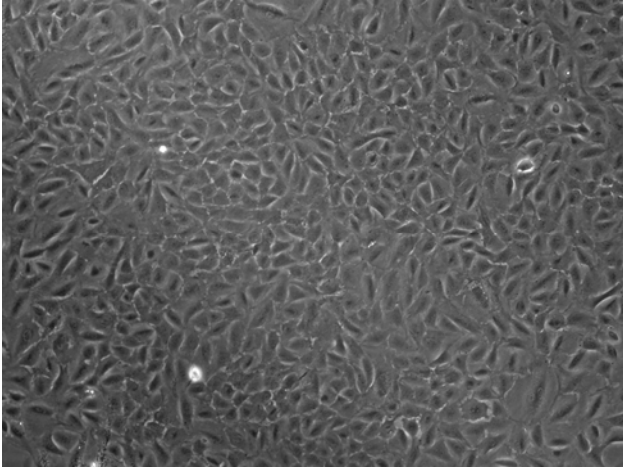
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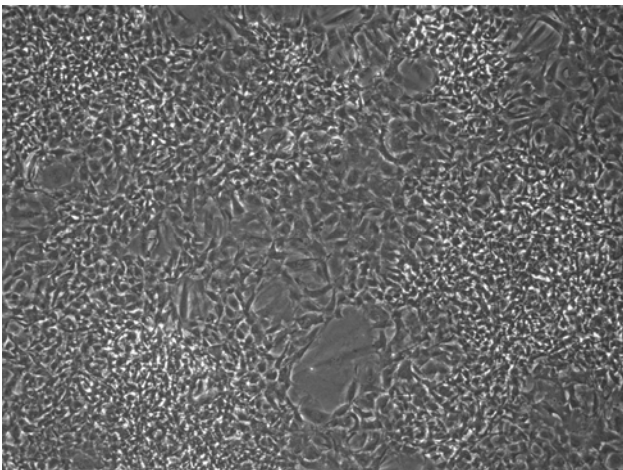
Figure 1. SMCs isolated from rat aorta show two morphologically distinct

phenotypes. (**a**) Epithelioid in shape and form a monolayer at confluence (**b**) elongated

and spindle-shaped and exhibit "hills & valleys" pattern at confluence. The images are

representations of distinct SMC phenotypes under a brightfield microscope at 10X

magnification.

Figure 2. Biochemical Characterization of SMC phenotypes. Postconfluent cells from epithelioid or spindle-shaped SMC colonies were harvested and cell extracts were separated in 7.5% (w/v) SDS-PAGE and immunoblotted with antibodies against CRBP-1, a marker of synthetic phenotype (a), SM-MHC, a marker of contractile phenotype (b), and NM-MHC, a marker of cultured SMCs (c). Blots were stripped and reprobed with anti-ERK antibody to control for equal loading (d).
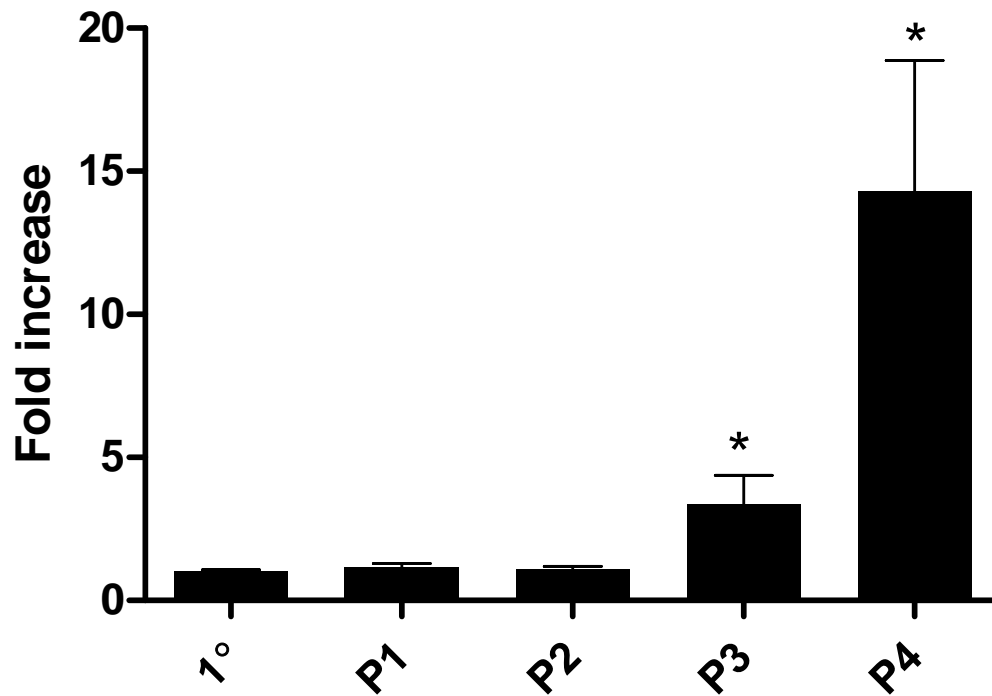
Figure 3. Relative quantification of P2Y$_2$ receptor mRNA expression in a cultured SMC population. The bar graph represents the levels of expression of P2Y$_2$ receptor mRNA in a population of cultured rat aortic SMCs normalized to 18S internal control. Data were obtained using the $\Delta\Delta C_T$ method to calculate the fold increase in mRNA levels, expressed at 95% confidence intervals of results from three independent experiments. The $^*p < 0.02$ indicates a significant difference from mRNA levels at the indicated passage (P) number, as compared to primary culture.
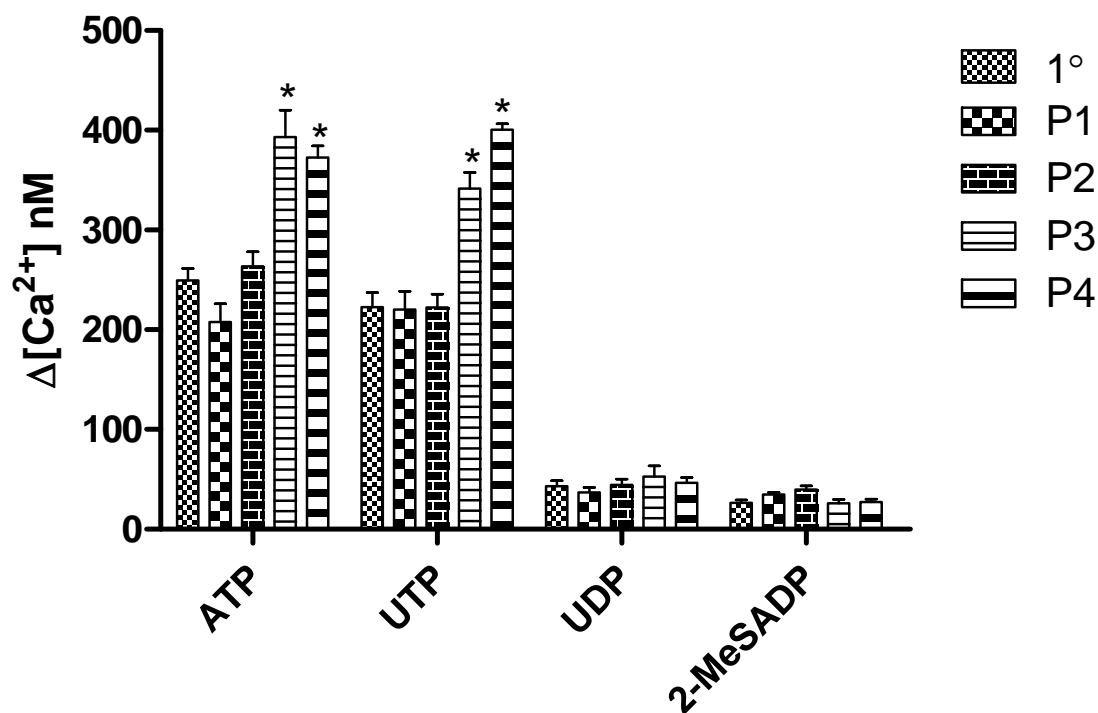
Figure 4. P2Y receptor activity profile in cultured smooth muscle cells. SMCs were dispersed into single cell suspensions. Changes in $[Ca^{2+}]_i$ in response to ATP (10 μM), UTP (10 μM), UDP (10 μM), or 2-MeSADP (10 μM) were measured in primary culture and subsequent passages (P) of SMCs. Agonist-induced changes in the $[Ca^{2+}]_i$ in responding cells are shown. Increases in $[Ca^{2+}]_i$ were expressed by subtracting basal $[Ca^{2+}]_i$ (prior to agonist addition) from the peak $[Ca^{2+}]_i$ responses to agonist. Basal levels of $[Ca^{2+}]_i$ in all cells were approximately 100 nM. One-way analysis of variance shows that the means of the data for specific passages are significantly different from responses to corresponding nucleotides in primary cultures of SMCs ($^*p < 0.0001$), however the data were not significantly different when analyzed by unpaired Student's *t*-test ($p > 0.05$).
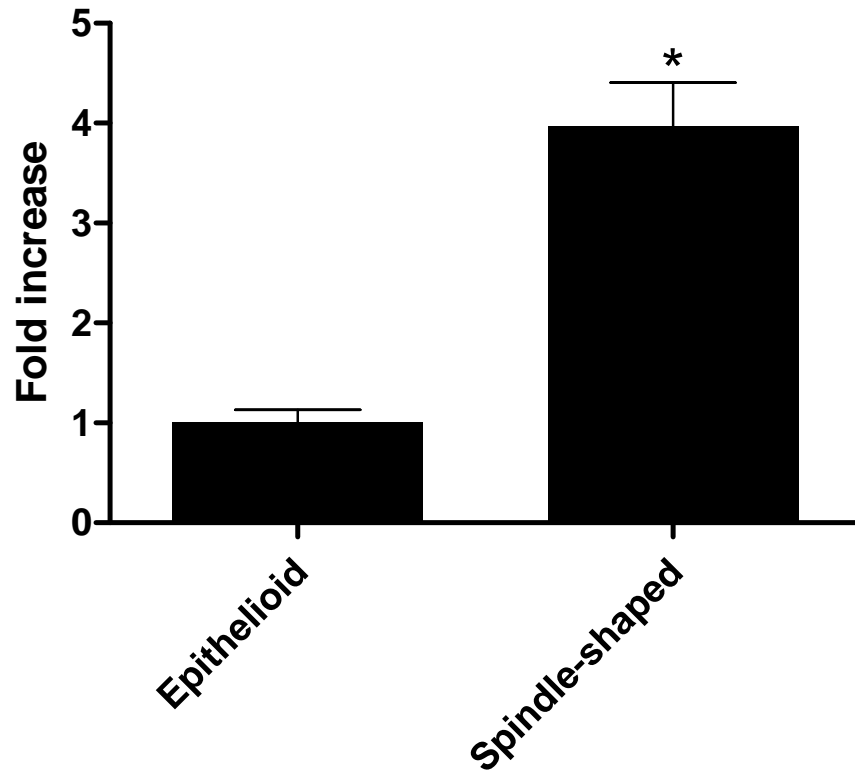
Figure 5. Relative quantification of P2Y$_2$ receptor mRNA expression in SMC phenotypes. The bar graph represents the levels of expression of P2Y$_2$ receptor mRNA in cultured SMC phenotypes normalized to 18S internal control. The $^*p < 0.006$ indicating a significant difference from P2Y$_2$R mRNA levels in cells obtained from SMC cultures with epithelioid phenotype.
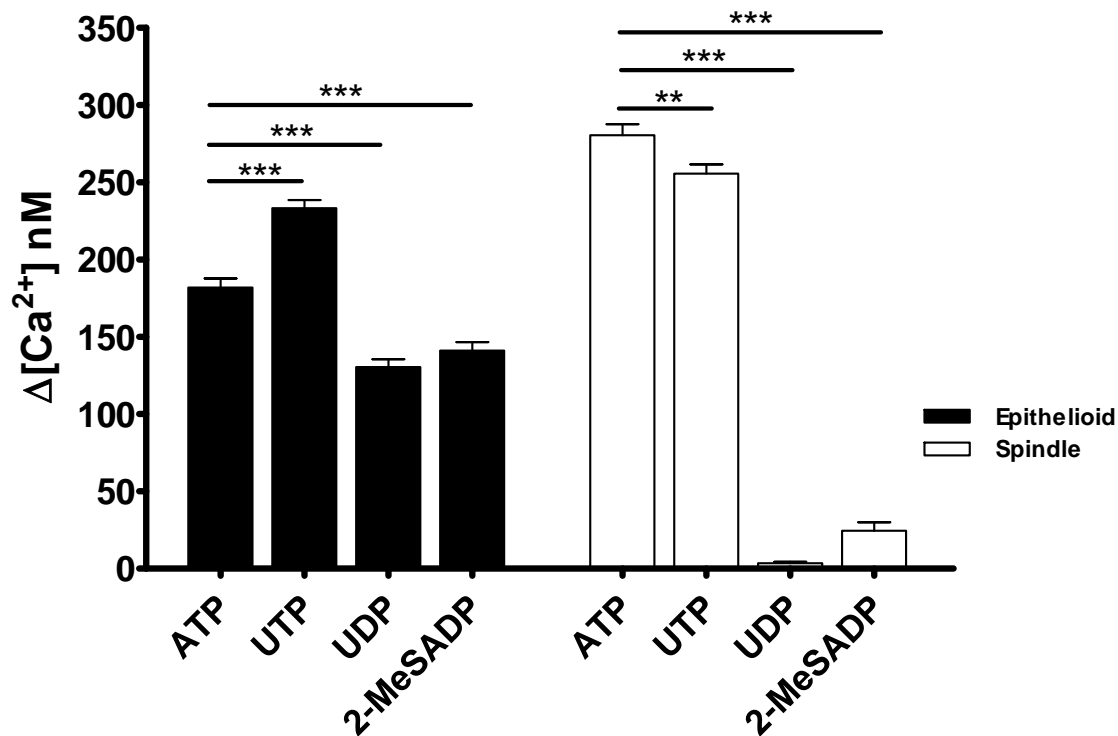
Figure 6. P2Y receptor activity profile in SMC phenotypes. SMCs of epithelioid or spindle-shaped phenotypes were dispersed into single cells. Changes in $[Ca^{2+}]_i$ in response to ATP, UTP, UDP, or 2-MeSADP were measured in synthetic SMCs (black bars) and contractile SMCs (white bars). Agonist-induced changes in the $[Ca^{2+}]_i$ in responding cells are shown. Increases in $[Ca^{2+}]_i$ were expressed by subtracting basal $[Ca^{2+}]_i$ (prior to agonist addition) from the peak $[Ca^{2+}]_i$ responses to agonist. Basal levels of $[Ca^{2+}]_i$ in all cells were approximately 100 nM. Data are expressed as the mean ± S.E.M. ("n" represents number of cells; $n = 50$ for all nucleotides in epithelioid SMCs and $n = 50$ for ATP; $n = 50$ for UTP; $n = 14$ for UDP; $n = 20$ for 2-MeSADP in spindle-shaped SMCs). **$p < 0.001$, ***$p < 0.0001$ indicate significant differences from responses to ATP. The results are representative of four independent experiments.
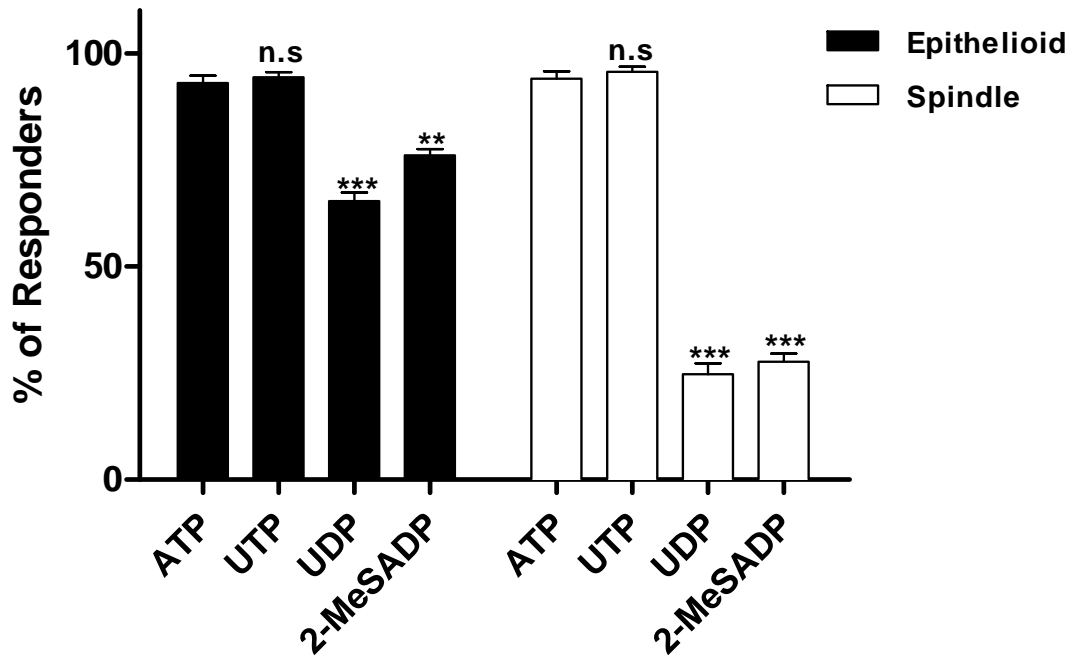
Figure 7. Percentage of P2Y agonist-responsive SMCs of distinct phenotypes. Bar graph represents the percentage of cells that responded to agonist stimulation in the experiment represented in figure 3. Data represent the means ± S.E.M. of results from four experiments, where [**]$p < 0.002$ and [***]$p < 0.0005$ indicates a significant difference from cells responding to ATP, while n.s indicates that data is not significantly different.
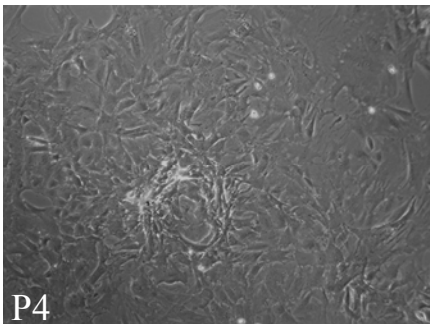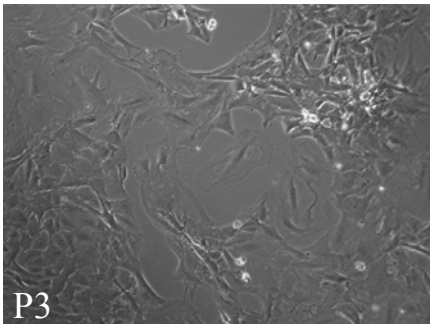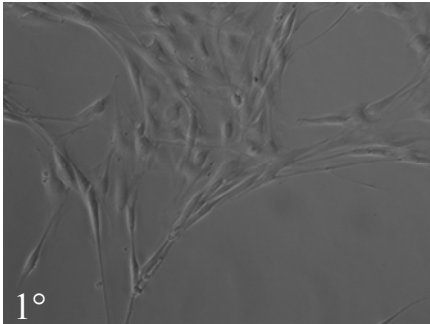
Figure 8. Persistence of spindle-shaped over epithelioid phenotype in cultures with a mixed population. The images are representative of SMCs from cultures of mixed cell populations under a brightfield microscope at 10X or 20X magnification. Regions on a culture dish with primary (1°) cells where both phenotypes grow together, and after passage 3 (P3), or passage 4 (P4) with spindle-shaped phenotype.
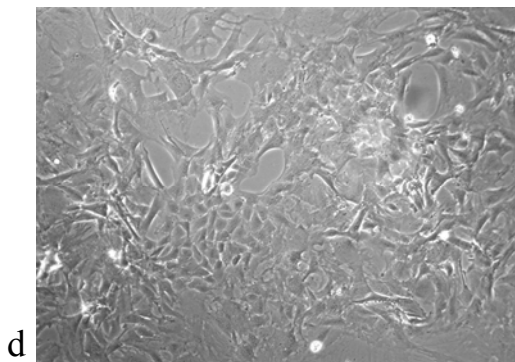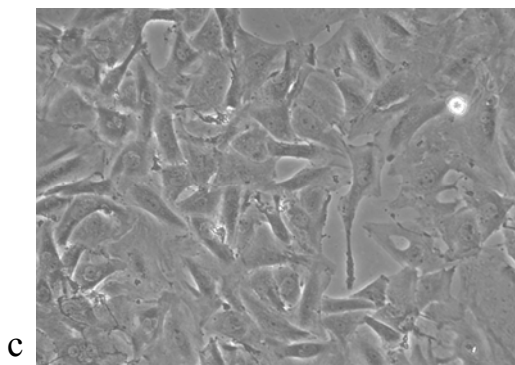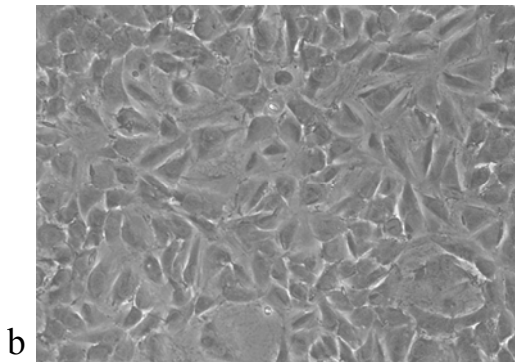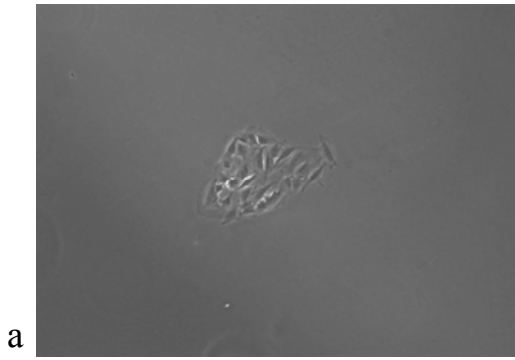
Figure 9. Phenotypic modulation of SMCs from epithelioid to spindle-shaped morphology. The images are representative of distinct SMC phenotypes under a brightfield microscope at 10X or 20X magnification. Single colonies of primary (1°) cells with epithelioid phenotype (a), and cells at P2 (b), P3 (c), or P4 (d).
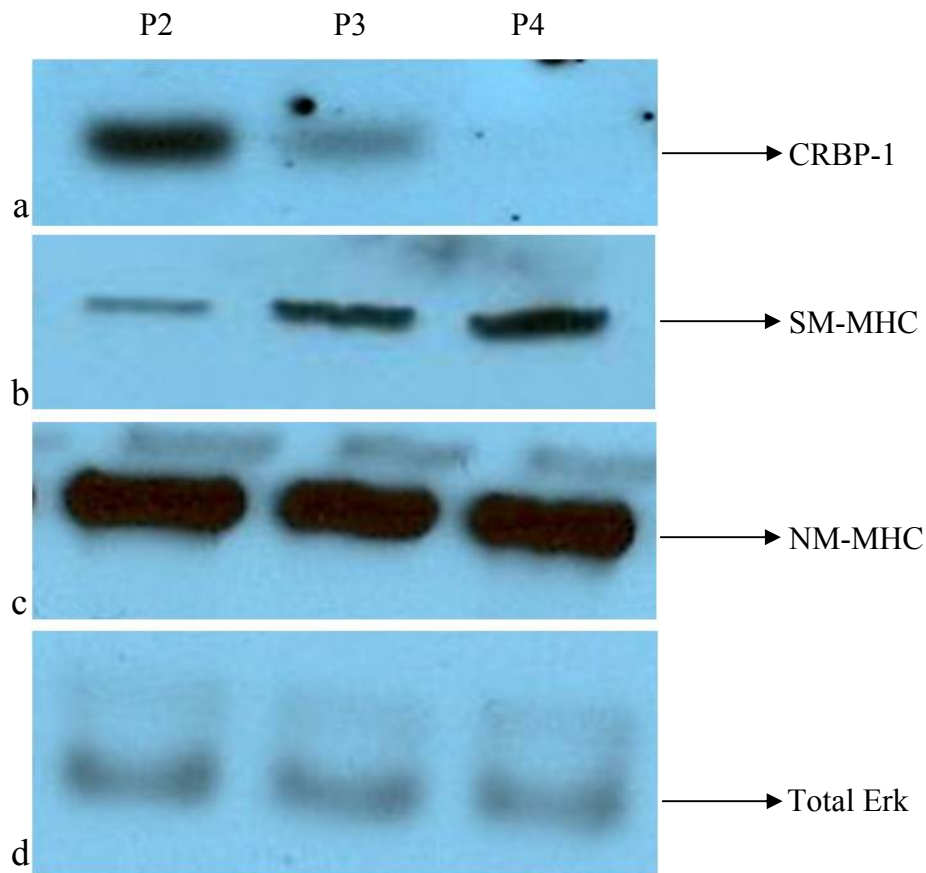
Figure 10. Immunoreactivities of SMC phenotypic markers during phenotypic modulation. Postconfluent cells from passages P2, P3, and P4 grown from 1° cells with epithelioid phenotype. Cells were harvested and cell extracts were separated in 7.5% or 12% (w/v) SDS-PAGE and immunoblotted for CRBP-1, a marker of the synthetic phenotype (a), SM-MHC, a marker of the contractile phenotype (b), and NM-MHC, a marker of cultured SMCs (c). Blots were stripped and reprobed with anti-ERK antibody to control for equal loading (d).
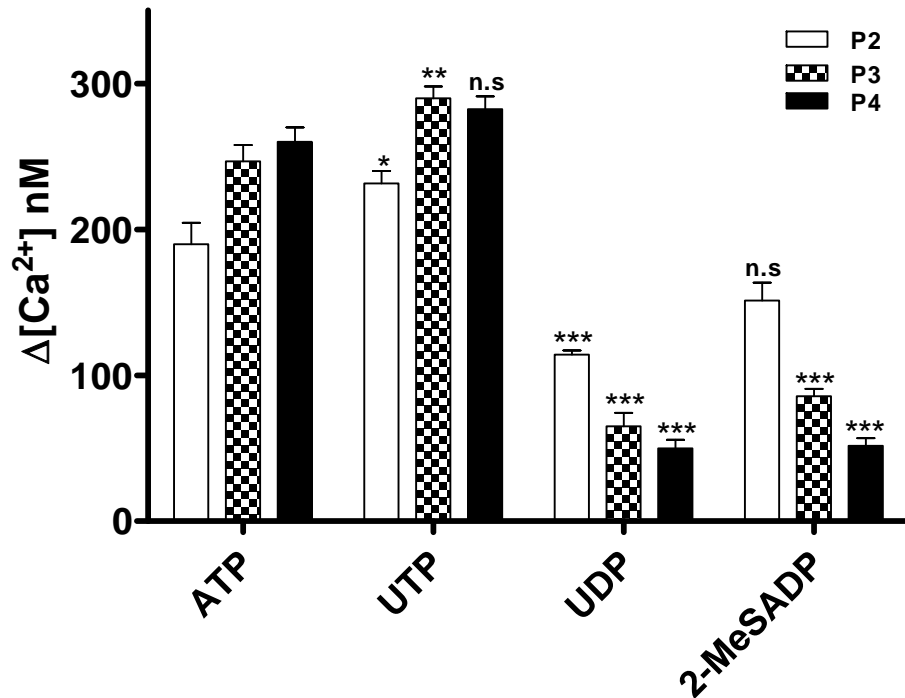
Figure 11. P2Y receptor activity profile during phenotypic modulation. SMCs with synthetic phenotype from passages P2, P3, and P4 were dispersed into single cells. Changes in $[Ca^{2+}]_i$ in response to ATP, UTP, UDP, or 2-MeSADP were measured in cells. Agonist-induced changes in $[Ca^{2+}]_i$ in responding cells are shown. Increases in $[Ca^{2+}]_i$ were expressed by subtracting basal $[Ca^{2+}]_i$ (prior to agonist addition) from the peak $[Ca^{2+}]_i$ responses to agonist. Basal levels of $[Ca^{2+}]_i$ in all cells were approximately 100 nM. Data represent the means ± S.E.M ($n = 8$) of results, where $^*p < 0.05$, $^{**}p < 0.007$, and $^{***}p < 0.0002$ indicate a significant difference from cells stimulated with ATP, whereas n.s. indicates that differences are not significant.
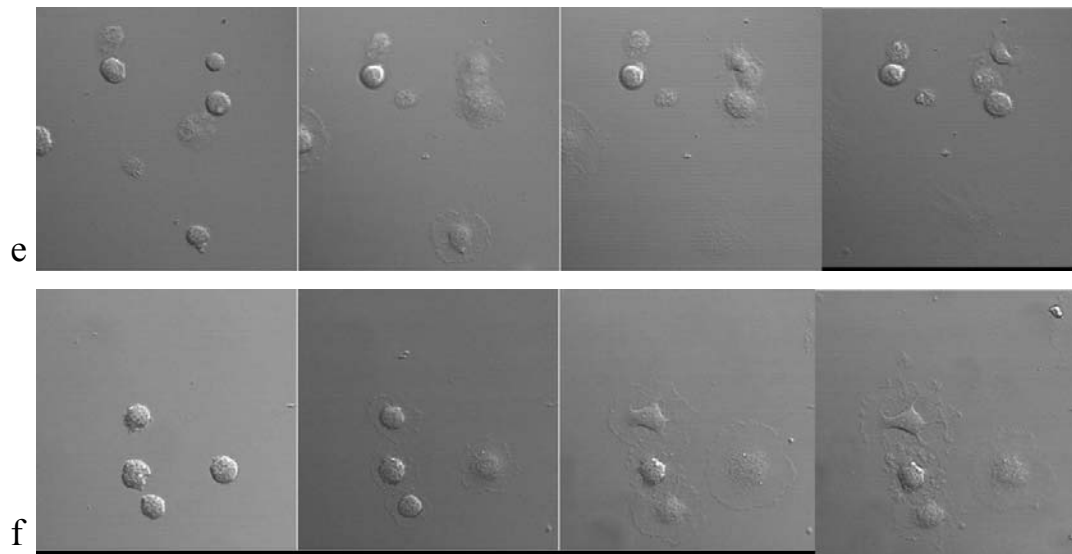
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a

b

12-2

c

d

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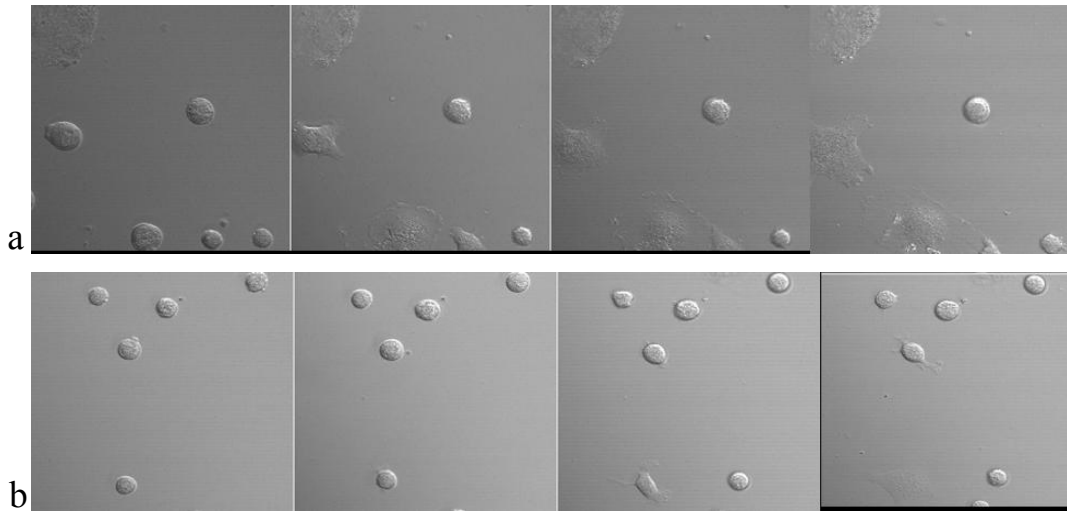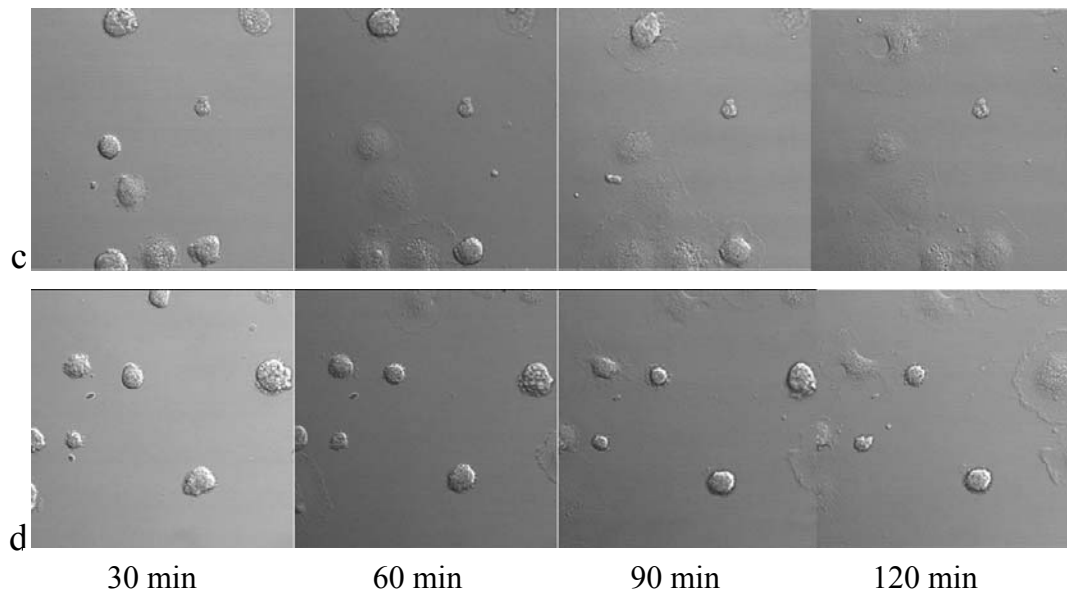
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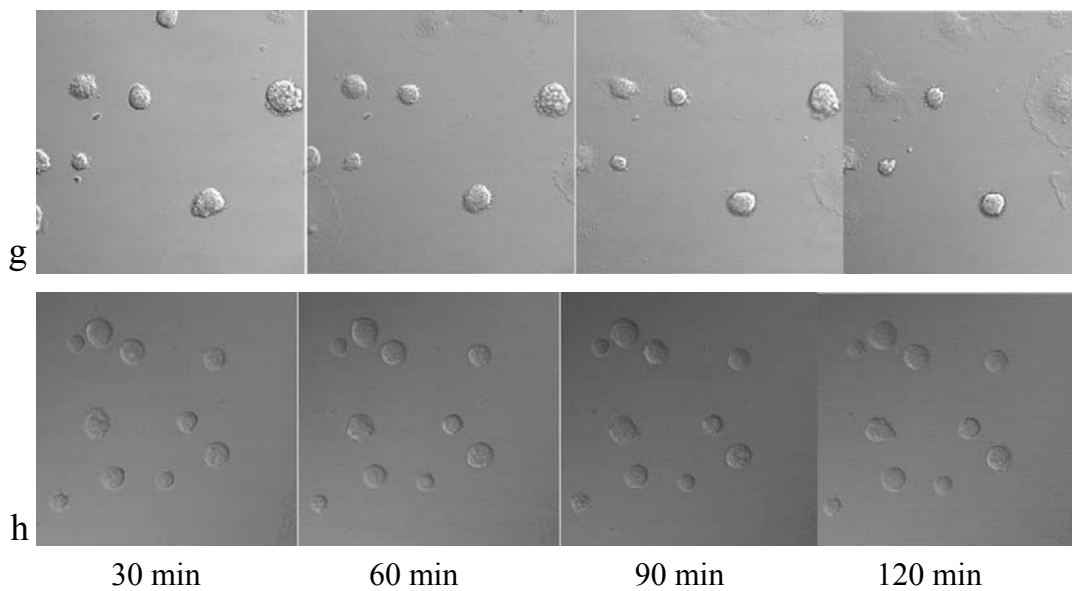
Figure 12. Effect of P2Y agonists on kinetics of SMC spreading. SMCs were suspended in culture media with or without receptor agonist or serum, seeded on a collagen-treated surface and monitored under a microscope with a motorized stage to track the cells at different time points. SMCs in serum-free DMEM (12-1) or DMEM with 10% FBS (12-2). Panels a and c represent the synthetic phenotype, whereas panels

b and d represent the contractile phenotype. SMCs in DMEM with 10 μM UTP (12-3) or DMEM with 10 μM 2-Me-SADP (12-4). Panels e and g represent the synthetic phenotype, whereas panels f and h represent the contractile phenotype.
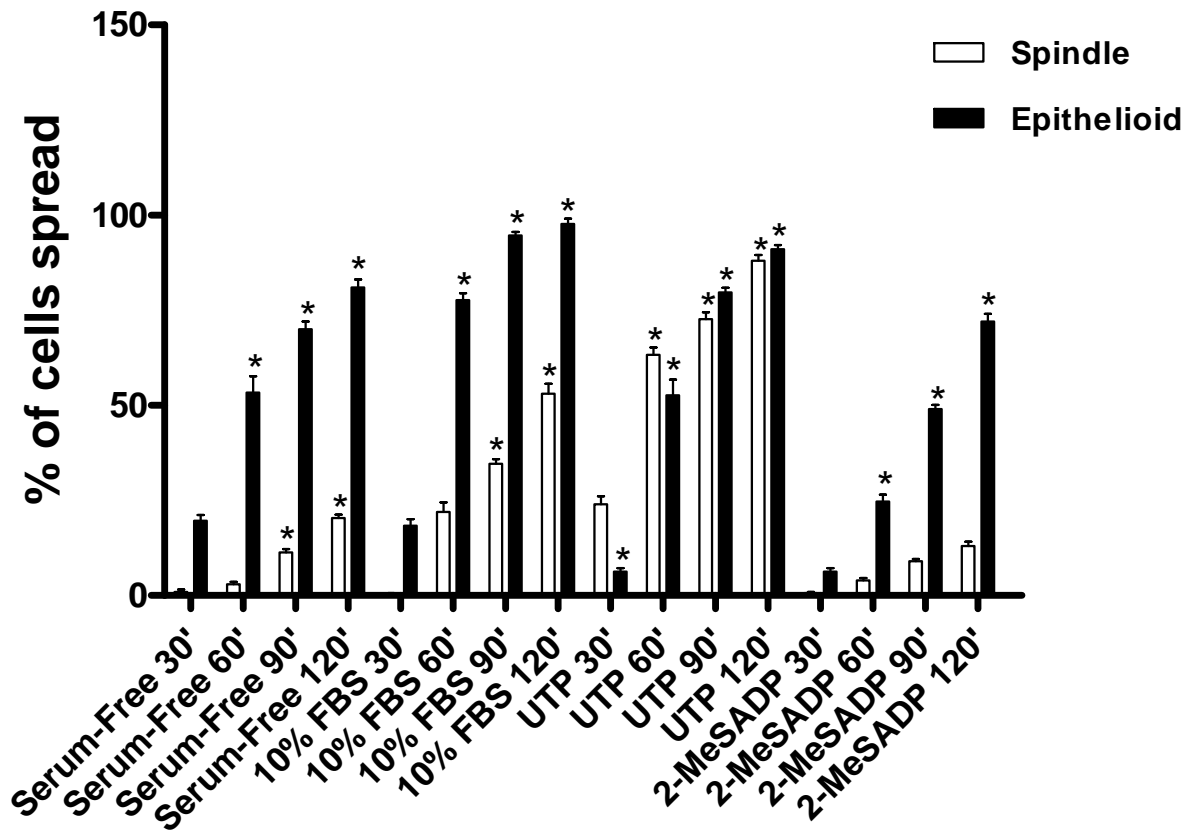
Figure 13. Percentage of SMC spreading in response to serum or nucleotide stimulation. Data are expressed as the mean ± S.E.M. ($n = 3$). *P values* obtained by two-way analysis of variance for interaction, row factor and column factor were [*]$p < 0.0001$ versus relevant control, indicating that the data are statistically significant. These data are representative of three independent experiments.