# Supplementary Data

## Arterial gene transfer of the TGFβ signaling protein Smad3 induces adaptive remodeling following angioplasty: a role for CTGF.

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## EXPANDED MATERIALS AND METHODS

### General Materials

TGFß$_1$ was obtained from R & D Systems. Dulbecco's Modification of Eagle's Medium (DMEM) and cell culture reagents were from Gibco BRL Life Technologies. Chemicals, if not specified, were purchased from Sigma Chemical Co. Smad3 and smooth muscle specific α-actin antibodies were from Santa Cruz Biotechnology and Sigma, respectively. Tubulin and CTGF antibodies were from AbCam. Small interference RNA, both scrambled oligo and CTGF-specific sequences, were from Ambion. Lipofectamine was obtained from Invitrogen.

### Culture and characterization of aortic smooth muscle cells and fibroblasts

Aortic smooth muscle cells (SMCs) were isolated from the aortae of Sprague Dawley rats based on a protocol described by Clowes *et al.* and maintained in DMEM containing 10% FBS at 37°C with 5% $CO_2$.[1] In a manner similar to that described by Pagano *et al.*, the suprarenal abdominal aorta was removed in a sterile manner from Sprague-Dawley rats and placed in a 10% FBS DMEM medium containing 100 units/mL penicillin and 100 µg/ml streptomycin.[2] Using a dissecting microscope (0.7–4.2x magnification), aortas were cleared of periadventitial adipose tissue and cut longitudinally. Using forceps, the luminal surface of the aortas was gently denuded of endothelial cells. Medial smooth muscle cells were peeled from the adventitia. The adventitia, free of both endothelium and medial smooth muscle cells, was then digested in a DMEM containing 1 mg/mL collagenase and 0.125 mg/mL elastase for 4h at 37°C. This solution was spun at 675g for 5 minutes, the pellet resuspended in 10 mL 20% FBS DMEM and this suspension plated on 100mm uncoated dishes.

Confirmation of the identity of these adventitial cells as fibroblasts was provided by strong immunostaining for Thy-1.1 and the absence of staining for the smooth muscle cell marker MHC-1. (Supplementary Figure S6).

**In vitro viral infection**

Subconfluence SMCs were incubated in an infection medium that contained AdSmad3 or Adnull (20,000 viral particle/cell) and 2% serum (FBS) for 2 h. Following the removal of infection medium, cells were washed twice with PBS and allowed to recover for 24 h in 10% FBS medium. Cells were then washed with PBS and switched to a low serum media (0.5% FBS DMEM) containing 5 ng/ml TGFβ or solvent for another 48 h. The resulting media was used as conditioned media for the fibroblasts study. To ensure that is no residual infectious viral particles in the conditioned media, we conducted a parallel experiment by administering the conditioned media to SMCs for 24 h. Cells were then examined under fluorescent microscope for GFP expression, as AdSmad3 carries the GFP cDNA in its vector portion. No GFP positive cells were detected (Supplementary Figure S5).

**Semi-quantification of Immunohitological Staining**

The expression of Smad3 or CTGF was semi-quantified on immunohistochemical slides using Adobe Photoshop based on method described by Lehr et al[3]. Briefly, digital images of three representative sections (×200) from each artery were acquired using a 32–0062A—135 Retiga Exi color video camera (Qimaging, Burnaby, BC, Canada) and Olympus BX51 diagnostic microscope (Olympus America Inc., Melville, NY). Images were imported into a computer (Apple, Cupertino, CA) and opened in Photoshop (version 6.0, Adobe Systems, San Jose, CA).

The total image area in pixels was determined using the histogram command. A positively stained area within the image was selected and defined as “minimum positive signal”. Using the Select Similar function, all areas with a staining color above the defined “minimum” were determined and expressed as the number of pixels. The expression of Smad3 or CTGF was expressed as the number of positive pixels per image area.

**REFERENCES**


1. Clowes AW, Reidy MA, Clowes MM. Mechanisms of stenosis after arterial injury. *Lab Invest* 1983;**49**:208-215.

2. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc Natl Acad Sci U S A* 1997;**94**:14483-14488.

3. Lehr HA, Mankoff DA, Corwin D, Santeusanio G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* 1997;**45**:1559-1565.

**SUPPLEMENTARY FIGURE LEGENDS**

**SUPPLEMENTARY FIGURE S1. Intraluminal adenoviral delivery of LacZ** Distribution of gene transfer following treatment of injured artery with AdLacZ. Rat carotid arteries were subjected to balloon angioplasty with immediate infection with adenoviral LacZ and were harvested 3, 5, and 14 days later. The injured arteries were sectioned and X-gal stained. Representative sections from five experiments are shown. Top: 40X. Bottom: 200X. Arrows: medial boundaries. (n=5)

**SUPPLEMENTARY FIGURE S2. Intraluminal adenoviral delivery and expression of Smad3 (A)**Representative samples showing expression of Smad3 on days 0, 3, 7, 14 and 28 after arterial injury. Arrows: medial boundaries. Top: AdNull-infected control. 200X. Bottom: AdSmad3-infected samples. 200X. **(B)** Quantification of medial Smad3 expression as determined by immunohistochemistry on days 0, 3, 7, 14 or 28 after arterial injury. (n=6, *$p<0.05$). **(C)** Quantification of neointimal Smad3 expression, as determined by immunohistochemistry at 0, 3, 7, 14 or 28 days after arterial injury. No detectable neointima was present at 0 and 3 days. (n=6, *$p<0.05$).

**SUPPLEMENTARY FIGURE S3. Experimental design of *in vitro* cell-cell communication studies.** Cultured rat aortic vascular smooth muscle cells were infected with AdSmad3 and then stimulated with TGFß$_1$ (5ng/mL) for 48 hours, after which their culture medium was aspirated, filtered and administered to rat aortic adventitial fibroblasts for 72 hours. The fibroblasts were

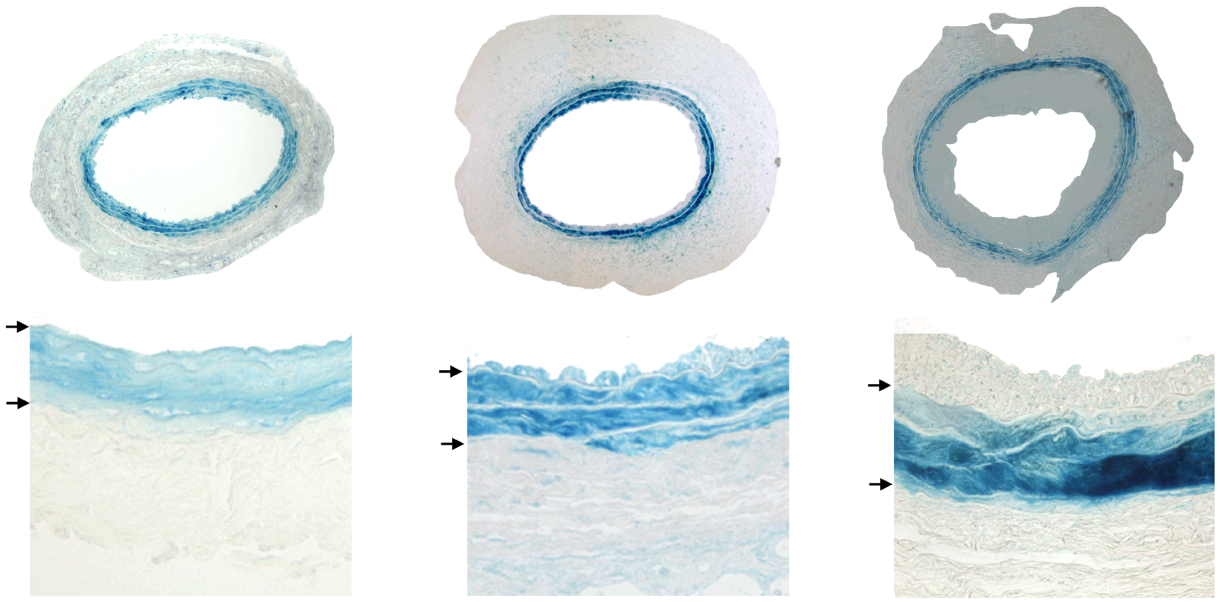
assessed for smooth muscle specific α-actin (SMA) expression, proliferation, migration and collagen synthesis as described in Methods.

**SUPPLEMENTARY FIGURE S4. Effect of recombinant CTGF on fibroblasts *in vitro*. (A)** A representative Western blot of media conditioned by smooth muscle cells infected with AdSmad3 or AdNull and stimulated by TGFß$_1$ using an anti-CTGF antibody. **(B)** A representative Western blot for smooth muscle specific α-actin in cell lysate of fibroblasts in the presence or absence of recombinant CTGF (100 ng/mL, 72 hours) **(C)** Proliferation of primary fibroblasts in the presence or absence of recombinant CTGF (100 ng/mL, 72 hours) as measured by $^3$H-thymidine incorporation assay. **(D)** Collagen production in primary aortic adventitial fibroblasts in the absence or presence of recombinant CTGF (100 ng/mL, 72 hours) as measured by $^3$H-proline incorporation assay **(E)** Activity of COL1A2 promoter luciferase reporter construct in a fibroblast cell line in the absence or presence of recombinant CTGF (100 ng/mL, 72 hours), as measured with a luciferase reporter construct. (n=4, *$p<0.05$)

**SUPPLEMENTARY FIGURE S5. Characterization of cultured primary fibroblasts *in vitro*.** Isolation and culture of primary aortic adventitial fibroblasts from Sprague-Dawley rats was conducted as described in Methods. The identity of the cultured cells as adventitial fibroblasts was confirmed by the presence of Thy-1.1 and the absence of smooth muscle cell marker myosin heavy chain (MHC). **(A)** Negative control for both anti-Thy-1.1 and anti-MHC, treated with murine IgG in place of primary antibody. **(B)** Immunostaining for MHC **(C)** Immunostaining for Thy-1.1.

**SUPPREMENTARY FIGURE S6. Confirmation of the inability of conditioned media in gene transfer.**

SMCs were treated with AdSmad3 or conditioned media (CM) as described in methods. The cells were photographed under fluorescence microscope. Upper panels show SMCs infected with AdSmad3, and lower panels show SMCs treated with CM. There is no GFP signal detected in SMCs treated with CM, although there were abundant GFP expression in AdSmad3 infected SMCs.

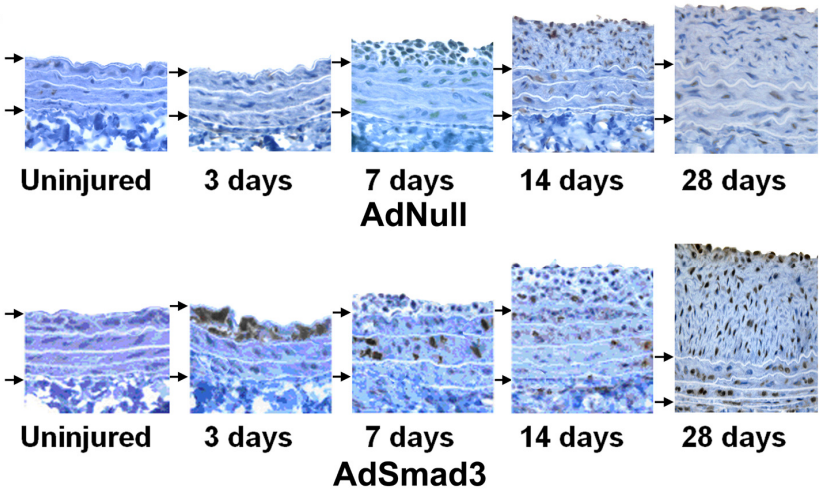


**3 Days After Injury**

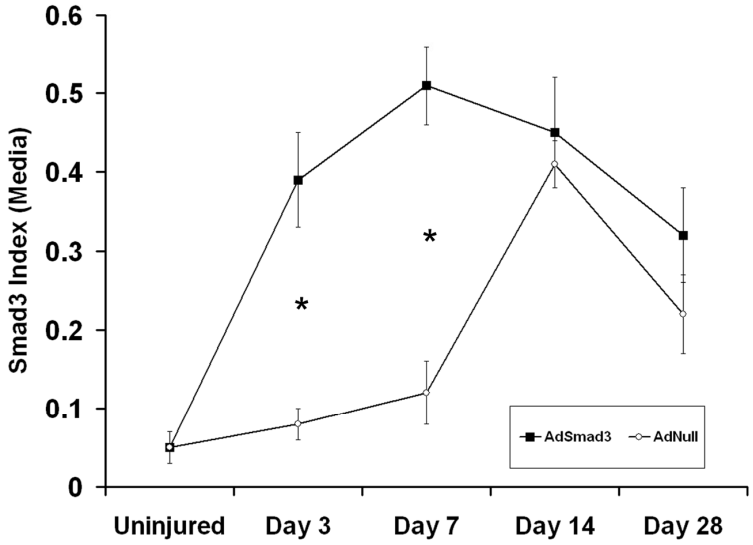
**5 Days After Injury**

**14 Days After Injury**

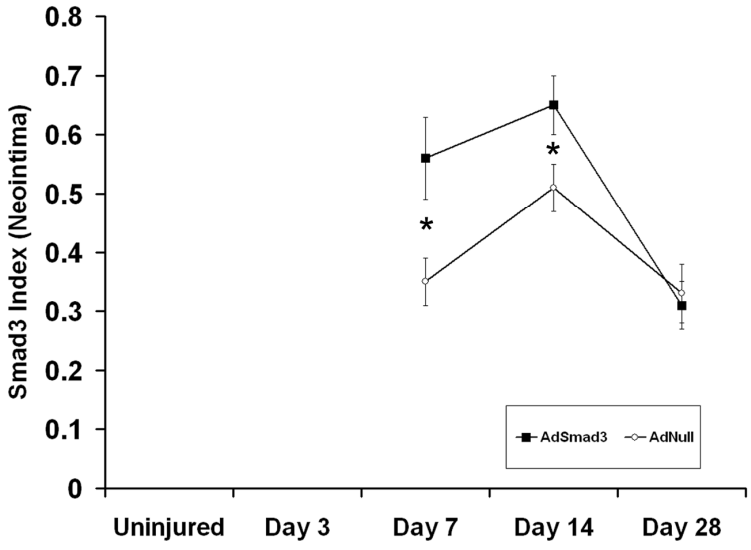
A.

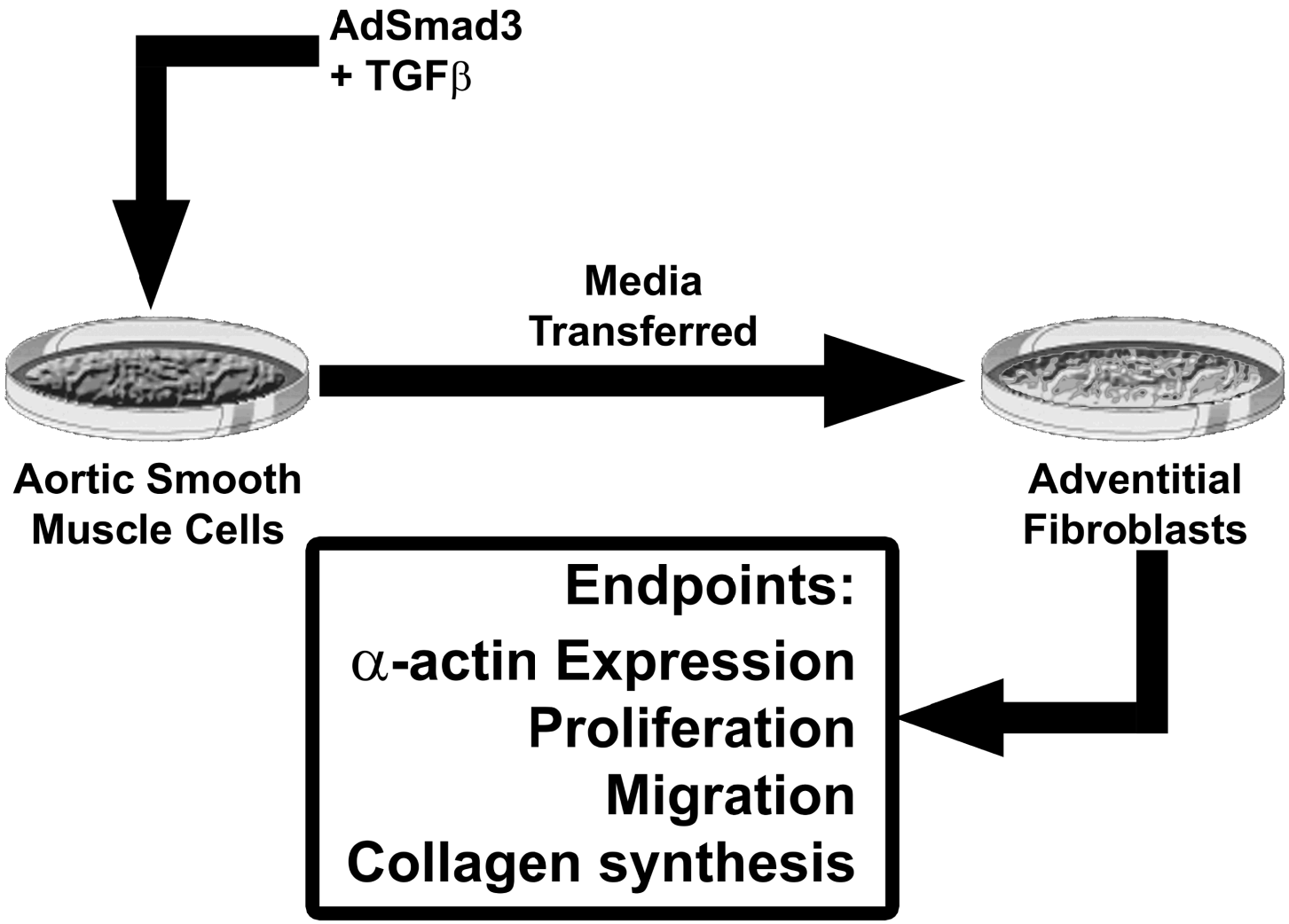

Uninjured
3 days
7 days
14 days
28 days
AdNull
Uninjured
3 days
7 days
14 days
28 days
AdSmad3

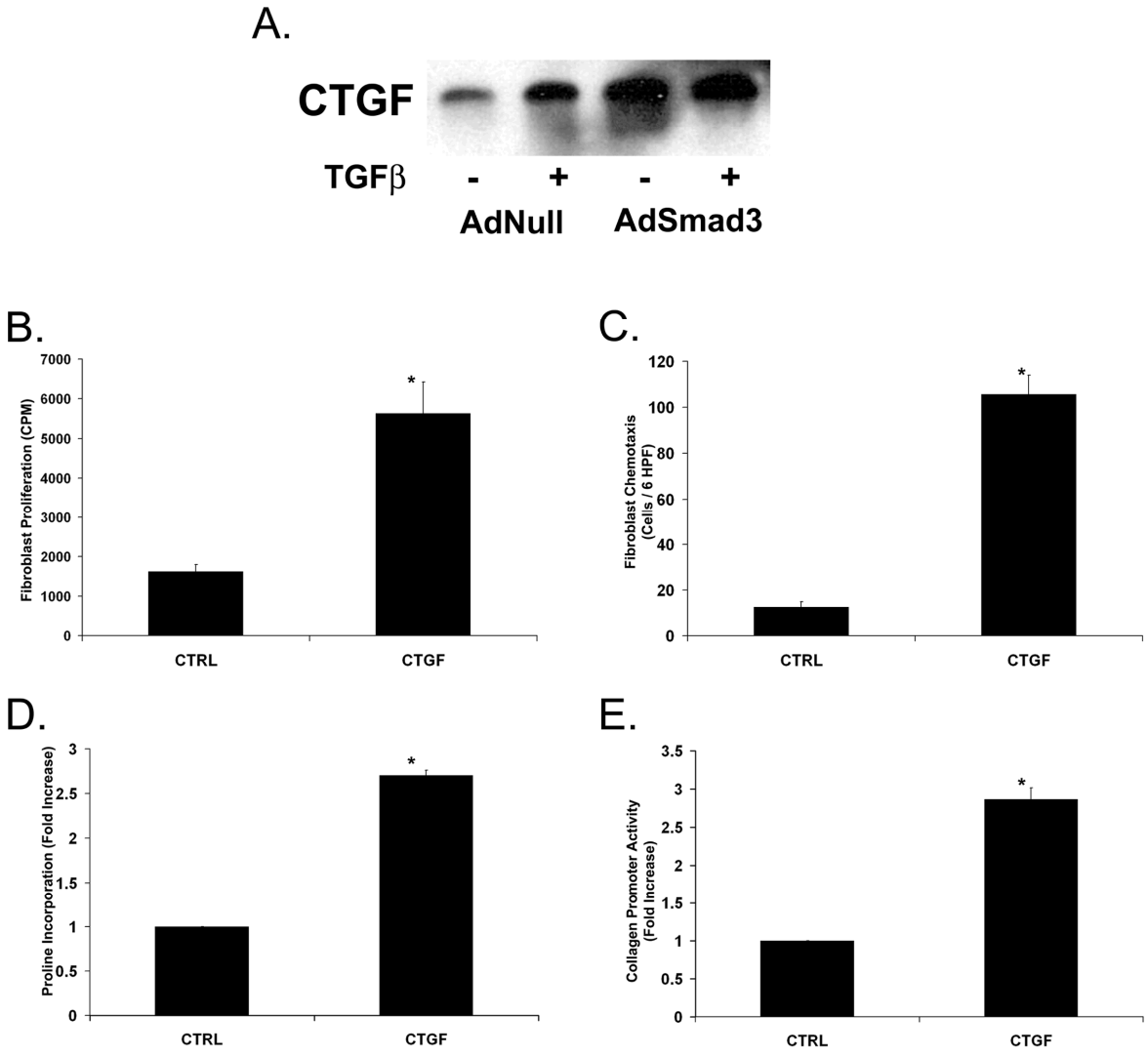
B.


Smad3 Index (Media)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
AdSmad3
AdNull
Uninjured
Day 3
Day 7
Day 14
Day 28

C.

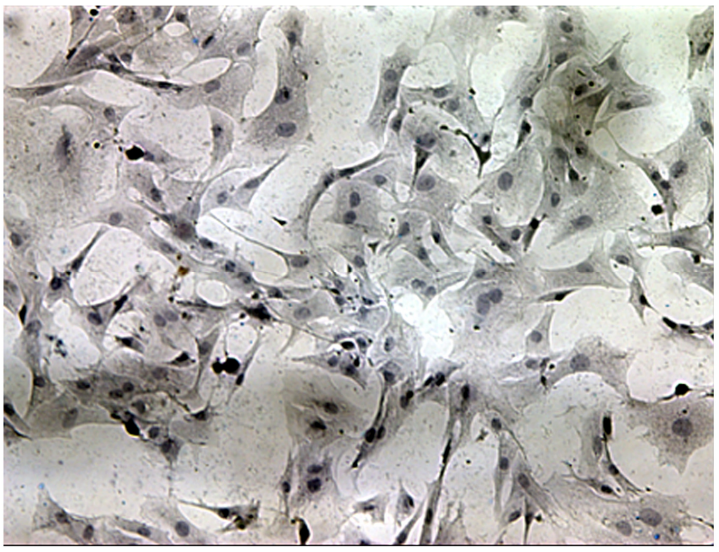

Smad3 Index (Neointima)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
AdSmad3
AdNull
Uninjured
Day 3
Day 7
Day 14
Day 28


AdSmad3
+ TGFβ
Media
Transferred
Aortic Smooth
Muscle Cells
Adventitial
Fibroblasts
Endpoints:
α-actin Expression
Proliferation
Migration
Collagen synthesis

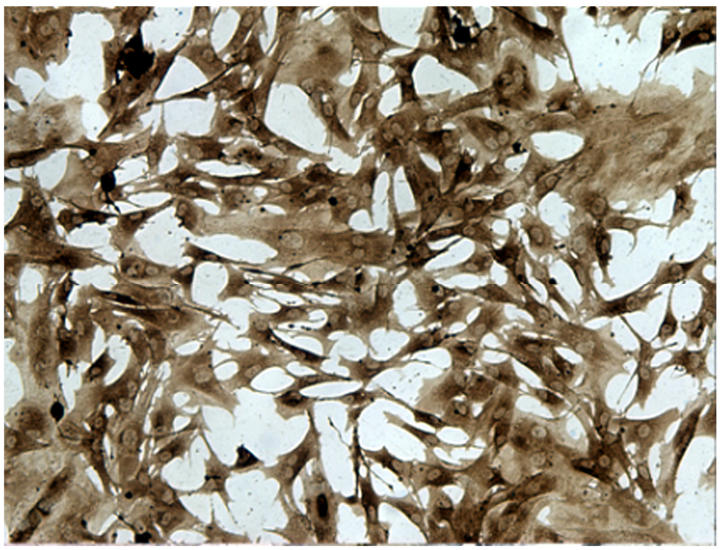

A.
CTGF
TGFβ - + - +
AdNull AdSmad3
B.
Fibroblast Proliferation (CPM)
7000
6000
5000
4000
3000
2000
1000
0
*
CTRL
CTGF
C.
Fibroblast Chemotaxis (Cells / 6 HPF)
120
100
80
60
40
20
0
*
CTRL
CTGF
D.
Proline Incorporation (Fold Increase)
3
2.5
2
1.5
1
0.5
0
*
CTRL
CTGF
E.
Collagen Promoter Activity (Fold Increase)
3.5
3
2.5
2
1.5
1
0.5
0
*
CTRL
CTGF

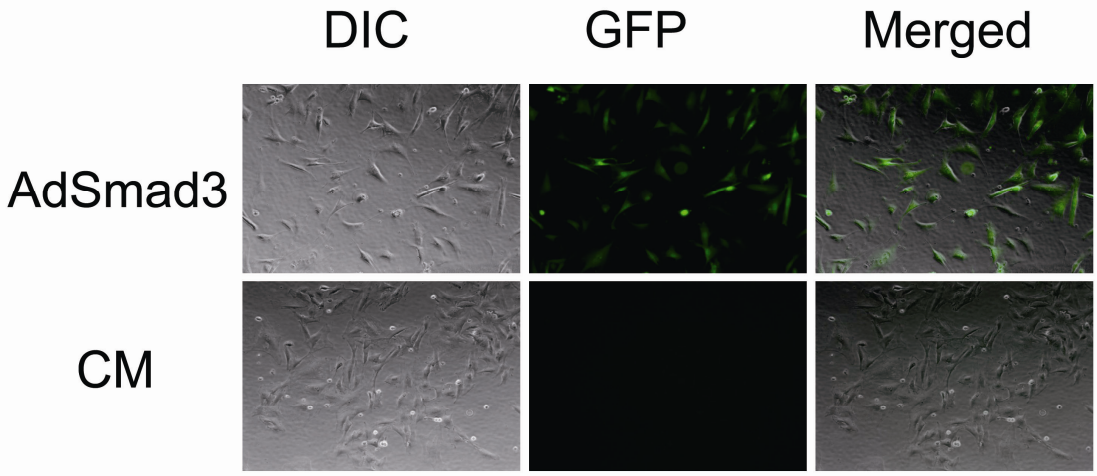
A.

B.



C.




DIC
GFP
Merged
AdSmad3
CM