

SUPPLEMENTAL MATERIALS

Title: A role for matrix metalloproteinase-10 in calcific aortic valve stenosis

Running title: MMP-10 in calcific aortic stenosis

Lara Matilla^{1,*}, Carmen Roncal^{2,3*}, Jaime Ibarrola¹, Vanessa Arrieta¹, Amaia García-Peña¹, Amaya Fernández-Celis¹, Adela Navarro¹, Virginia Álvarez¹, Alicia Gainza¹, Josune Orbe^{2,3}, Victoria Cachafeiro^{3,4}, Guillermo Zalba⁵, Rafael Sádaba¹, José A. Rodríguez^{2,3†}, Natalia López-Andrés^{1,6†}

¹ Cardiovascular Translational Research, Navarrabiomed, Complejo Hospitalario de Navarra (CHN), Universidad Pública de Navarra (UPNA), IdiSNA, Pamplona, Spain

² Laboratory of Atherothrombosis, Program of Cardiovascular Diseases, Cima Universidad de Navarra, IdiSNA, Pamplona, Spain.

³CIBERCV, Instituto de Salud Carlos III, Madrid, Spain.

⁴Departamento de Fisiología, Facultad Medicina, Universidad Complutense, Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain.

⁵Department of Biochemistry and Genetics, University of Navarra, IdiSNA, Pamplona. Spain.

⁶ Université de Lorraine, INSERM, Centre d'Investigations Cliniques-Plurithématique 1433, UMR 1116, CHRU de Nancy, Nancy, France.

*Contributed equally to this work.

†Share senior authorship.

Corresponding authors

Natalia López-Andrés, PhD

Cardiovascular Translational Research, Navarrabiomed (Miguel Servet Foundation)

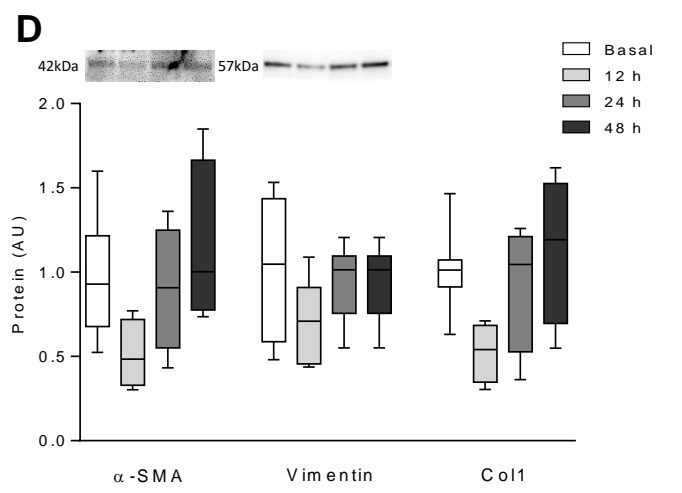
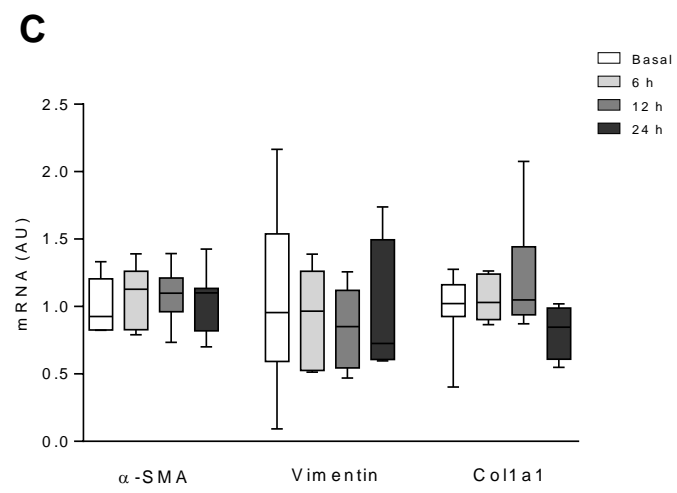
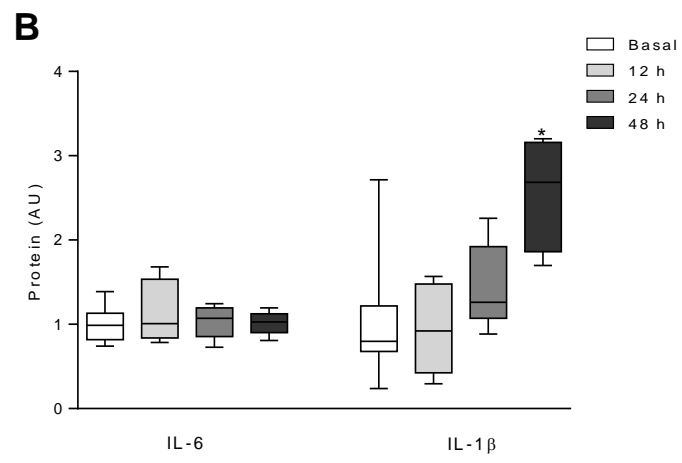
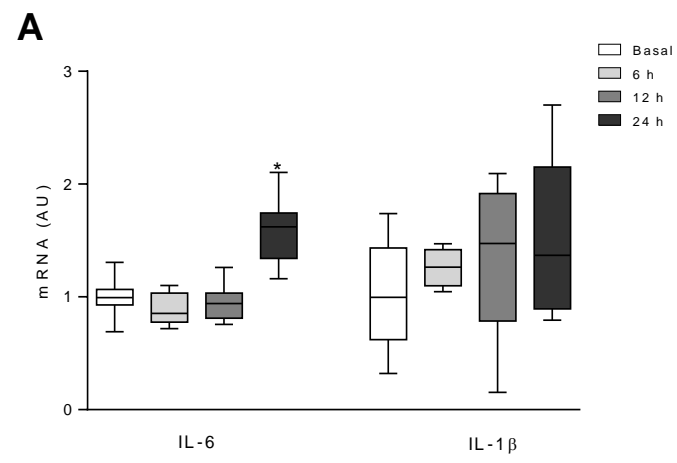
C/Irunlarrea 3. 31008 Pamplona, Spain. E-mail: natalia.lopez.andres@navarra.es

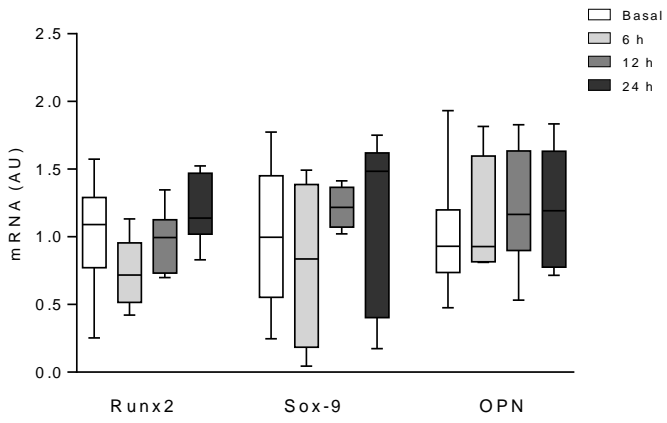
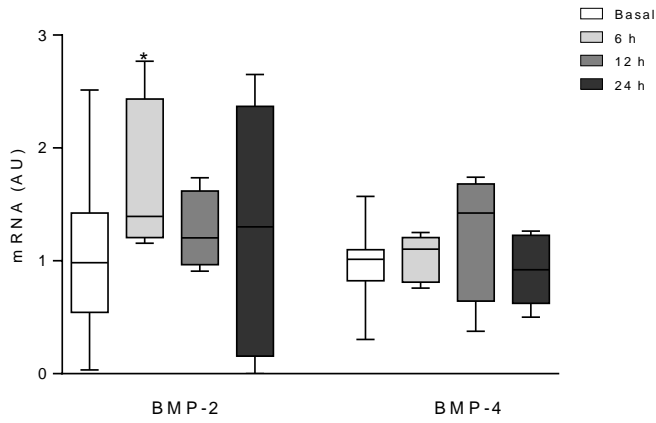
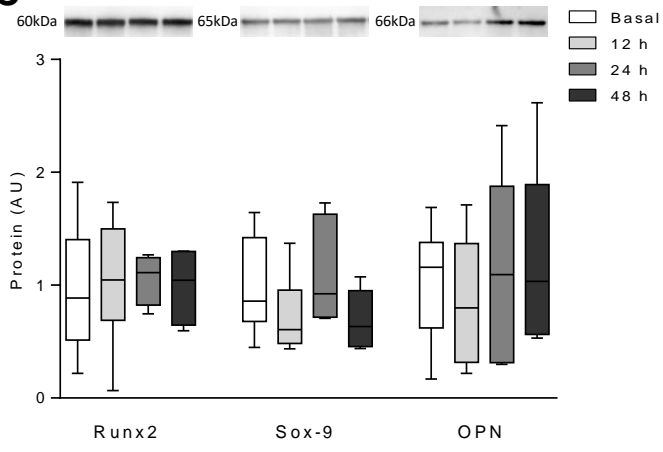
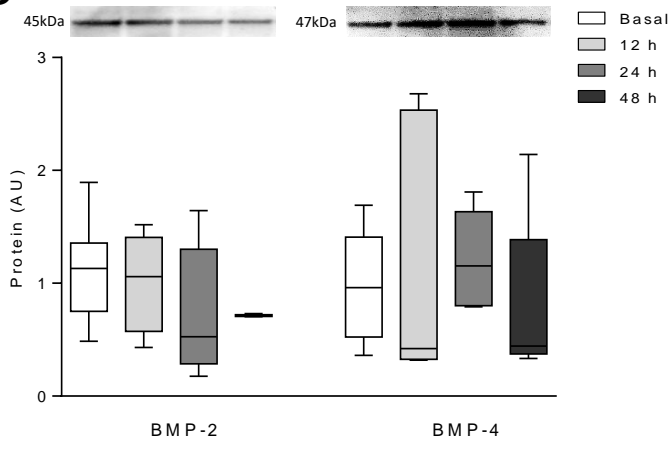
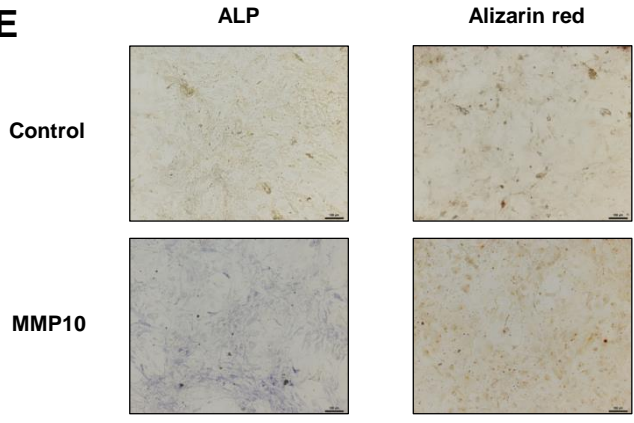
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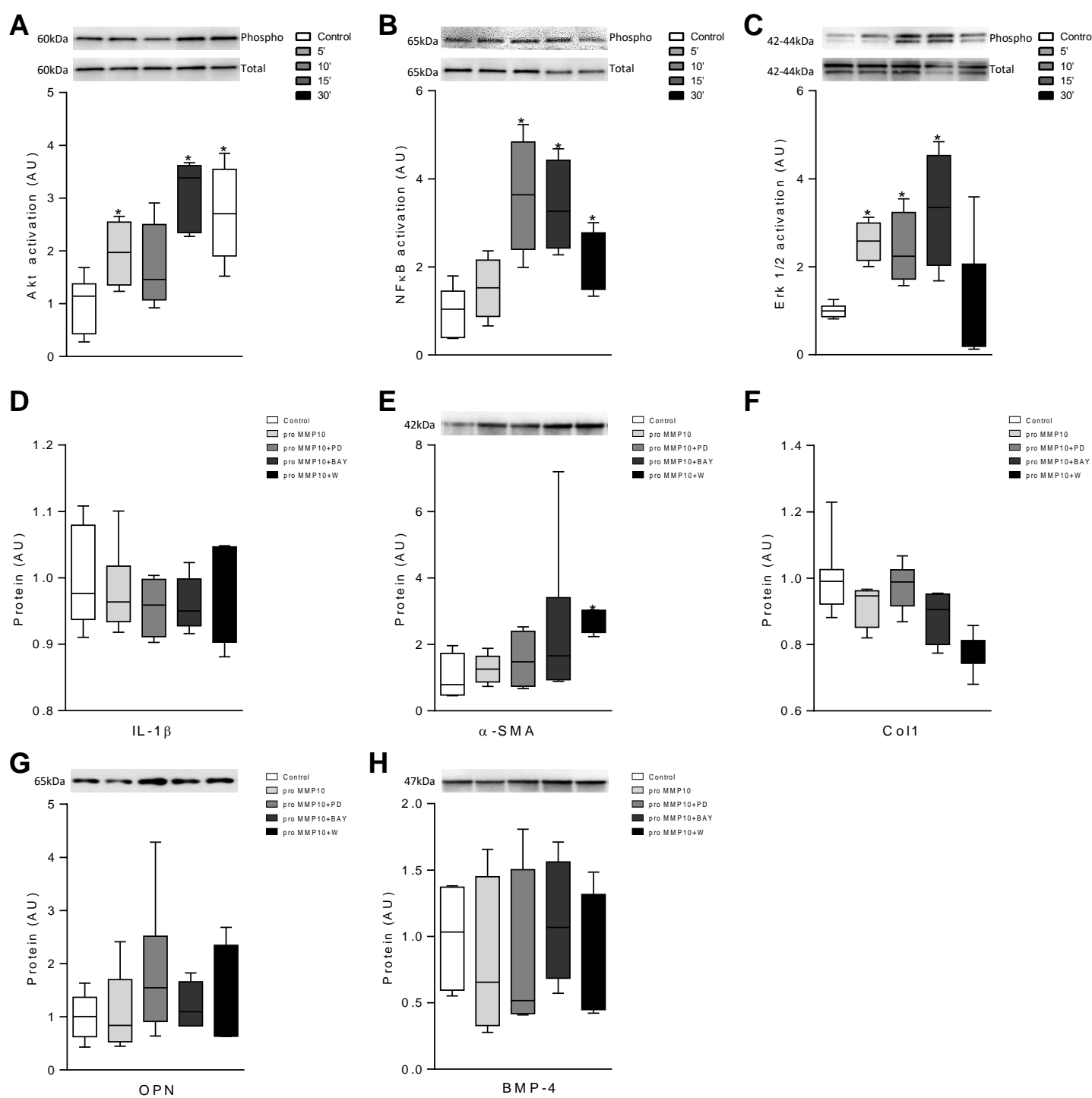
Laboratory of Atherothrombosis, Program of Cardiovascular Diseases, Cima Universidad de Navarra, Avda Pío XII 55. 31008 Pamplona, Spain. E-mail: josean@unav.es

Subject Terms: Valvular Heart Disease; Pathophysiology; Mechanisms; Gene Expression and Regulation; Inflammation

Key words: aortic valve stenosis, calcification, MMP-10, inflammation, valvular interstitial cells.



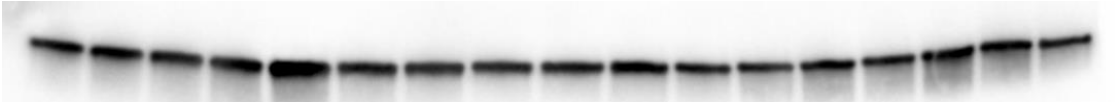
A**B****C****D****E**



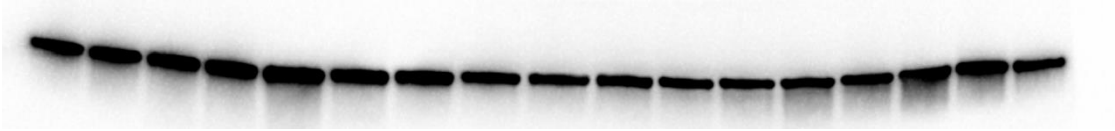
α -SMA



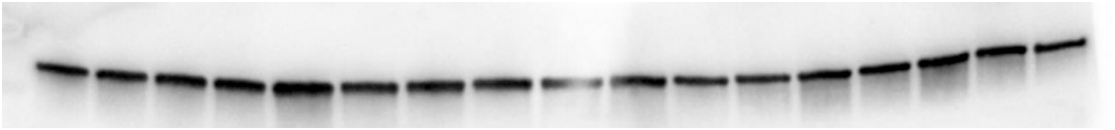
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Vimentin



Opn



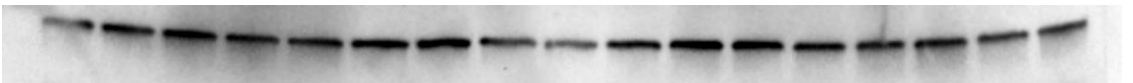
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Periostin

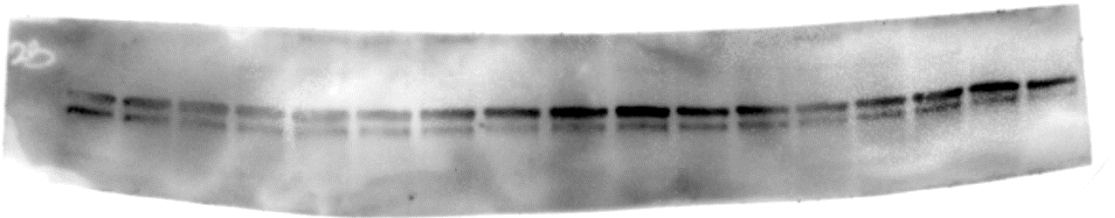


Sox9



BMP-4

BMP-2



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. proMMP10 induces inflammatory expression in AVICs. Effects of proMMP10 on the inflammatory markers IL-6 mRNA levels (A) and IL-6 and IL1 β secretion (B). α -SMA, vimentin and Col1a1 mRNA (C) and protein (D) levels in AVICs treated with 10^{-9} M proMMP10. All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) normalized to HPRT, β -actin and GADPH for cDNA and to stain free for protein. * $p < 0.05$ vs Control. proMMP10: Pro metalloproteinase 10; IL-6: interleukin 6; IL1 β : interleukin 1 beta; α -SMA: alpha smooth muscle actin; Col1a1: collagen Type I Alpha 1 Chain.

Supplemental Figure II. proMMP10 effects on AVICs osteogenesis. Runx2, Sox-9 and osteopontin mRNA expressions in AVICs treated with 10^{-9} M proMMP10 (A). BMP-2 and BMP-4 mRNA expressions in AVICs treated with proMMP10 (B). Protein expressions of Sox-9, osteopontin (C), BMP-2 and BMP-4 (D) in AVICs incubated with proMMP10. ALP staining of AVICs cultivated with proMMP10 at day 14 (E). Alizarin red staining of AVICs cultivated with 10^{-9} M proMMP10 at day 14 (F). All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) normalized to HPRT, β -actin and GADPH for cDNA and to stain free for protein. * $p < 0.05$ vs Control. proMMP10: Pro metalloproteinase 10; Runx2: runt-related transcription factor 2; Sox-9: SRY (sex-determining region Y)-box 9; OPN: osteopontin; BMP-2: bone morphogenetic protein-2; BMP-4: bone morphogenetic protein-4.

Supplemental Figure III. Effects of proMMP10 on phosphorylation of intracellular pathways in AVICs and effects of cell signaling chemical inhibitors on the inflammatory, fibrotic and osteogenic effects of proMMP10 in AVICs. Akt (A), NF κ B (B) and ERK1/2 (C) phosphorylation was measured by Western Blot in AVICs treated with 10^{-9} M proMMP10 at 5, 10, 15, 30 and 60 minutes. IL-1 β (D), α -SMA (E), Collagen type I (F), osteopontin (G) and BMP-4 (H) protein expressions were measured by Western Blot or ELISA in AVICs pre-treated with PD98059, BAY117082 or Wortmannin 30 minutes before the addition proMMP10 (10^{-9} M final) and incubated for 24 h. All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) normalized to stain free for protein and total Akt, total NF κ B, and total ERK1/2. * $p < 0.05$ vs Control. ProMMP10: pro-metalloproteinase 10; Akt: protein kinase B; NF κ B: nuclear factor kappa B; Erk1/2: extracellular signal-regulated kinase 1 and 2. IL-1 β : interleukin 1 beta; α -SMA: alpha smooth muscle actin; Col1: collagen Type I; OPN: osteopontin; BMP-4: bone morphogenetic protein-4.

Supplemental Figure IV. Representative images for Western blots.

SUPPLEMENTAL TABLE I. Primers used in humans in real time PCR analysis

Gene	Primer	Sequence (5' to 3')
MMP-10	Forward	CCA CTC TAC AAC TCA TTC ACA
	Reverse	TGA ATG CCA TTC ACA TCA TCT TG
α-SMA	Forward	ACT GCC TTG GTG TGT GAC AAT GG
	Reverse	TGG TGC CAG ATC TTT TCC ATG
Vimentin	Forward	GTT TCC CCT AAA CCG CTA GG
	Reverse	AGC GAG AGT GGC AGA GGA
Colla1	Forward	GGA CAC AGA GGT TTC AGT GGT
	Reverse	CAC CAT CAT TTC CAC GAG CA
IL-6	Forward	AGT TCC TGC AGA AAA AGG CAA AG
	Reverse	CAT TTG CCG AAG AGC CCT CA
IL-1β	Forward	AGC TGA TGG CCC TAA ACA GA
	Reverse	GCA TCT TCC TCA GCT TGT CC
Runx2	Forward	GGT TAA TCT CCG CAG GTC ACT
	Reverse	CAC TGT GCT GAA GAG GCT GTT
Sox-9	Forward	GAG GAA GTC GGT GAA GAA CG
	Reverse	ATC GAA GGT CTC GAT GTT GG
OPN	Forward	CAA ACG CCG ACC AAG GAA AA
	Reverse	AGC TGC TTT TCC TCA GAA CT
BMP-2	Forward	TGC GGT CTC CTA AAG GTC G
	Reverse	GGG GTG GGT CTC TGT TTC AG
BMP-4	Forward	AGC TTC CAC CAC GAA GAA CAT
	Reverse	AAG CCC CTT TCC CAA TCA GG
HPRT	Forward	TTG CTT TCC TTG GTC AGG CA
	Reverse	ATC CAA CAC TTC GTG GGG TC
β-actin	Forward	GCC GCC AGC TCA CCA T
	Reverse	TCG ATG GGG TAC TTC AGG GT
GADPH	Forward	ACC AGC CCC AGC AAG AGC ACA AG
	Reverse	TTC AAG GGG TCT ACA TGG CAA CTG F

Major Resources Tables

ELISA

Target antigen	Vendor or Source	Catalog #	Lot # (preferred but not required)
IL-6	R&D Systems	DY206	
Col-1	R&D Systems	DY6220-05	
IL-1 β	R&D Systems	DY201	
MMP10	R&D Systems	DY910	
TNF- α	R&D Systems	DY210	
TIMP-1	R&D Systems	DY970	
BMP-4	R&D Systems	DY314	
OPN	R&D Systems	DY1433	

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
MMP10	R&D Systems	MAB9101	5 $\mu\text{g}/\text{mL}$	
α -SMA	Sigma Aldrich	A7607	10 $\mu\text{g}/\text{mL}$	
Vimentin	Santa Cruz	sc-373717	2 $\mu\text{g}/\text{mL}$	
Runx2	Sigma Aldrich	AMAb90591	10 $\mu\text{g}/\text{mL}$	
Sox-9	Sigma Aldrich	AMAb90795	10 $\mu\text{g}/\text{mL}$	
OPN	Santa Cruz	sc-21742	2 $\mu\text{g}/\text{mL}$	
Periostin	Santa Cruz	sc-67233	2 $\mu\text{g}/\text{mL}$	
BMP-2	Abcam	ab14933	7.3 $\mu\text{g}/\text{mL}$	
BMP-4	Abcam	ab39973	10 $\mu\text{g}/\text{mL}$	
AKT	Cell Signaling	#9272	0.34 $\mu\text{g}/\text{mL}$	
p-AKT	Cell Signaling	#9271	0.1 $\mu\text{g}/\text{mL}$	
ERK 1/2	Cell Signaling	#9102	0.19 $\mu\text{g}/\text{mL}$	
p-ERK 1/2	Cell Signaling	#4377	0.98 $\mu\text{g}/\text{mL}$	
NF κ B	Cell Signaling	#4764	0.83 $\mu\text{g}/\text{mL}$	
p-NF κ B	Cell Signaling	#3033	0.57 $\mu\text{g}/\text{mL}$	
BMP-9	Santa Cruz	sc-514211	2 $\mu\text{g}/\text{mL}$	
Smad 1/5/8	Sigma Aldrich	SAB2702532	10 $\mu\text{g}/\text{mL}$	

Immunochemistry

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
Alkaline phosphatase: Naphthol AS-MX N,N-Dymethylformamide MgCl ₂ Blue BB Salt Tris	Sigma Aldrich	855-20mL 227056-100mL M8266-100g F3378-5g RDD008	0.1 mg/mL 0.5% 2mM 0.6mg/mL 0.1M	
Alizarin red	Sigma Aldrich	A5533-25 g	2%	