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BMP-4 and TGF-β1 Mechanisms in Acute Valvular Response to Supra-Physiologic Hemodynamic Stresses

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MATERIALS AND METHODS

Immunostaining

Frozen tissue sections were flash frozen in optimum cutting temperature compound. The slides were first thawed to room temperature and then blocked using 10% animal serum in PBS (Sigma-Aldrich), 0.2% Triton X-100 (Sigma-Aldrich) and 1% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific) in 1x PBS for 1 hour at room temperature. The slides were then incubated overnight at 4°C in primary antibody in 2 – 10% blocker at the following dilutions: VCAM-1 (1:50; Santa Cruz Biotechnology), ICAM-1 (1:50; Southern Biotech), TGF-β1 (1:25; Santa Cruz), BMP-4 (1:150; Abcam), cathepsin L (1:25; Santa Cruz Biotechnology), cathepsin S (1:25; Santa Cruz Biotechnology), MMP-2 (1:100; EMD Millipore) and MMP-9 (1:100; EMD Millipore). Following primary antibody incubation, sections were washed 3 times in 1x PBS and incubated with anti-rabbit or anti-mouse (all from Santa Cruz) secondary antibodies at 1:100 dilution for 2 hours at room temperature. The tissue sections were then washed 3 times in 1x PBS, counterstained with 1 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich), mounted with fluorescence mounting medium (Dako), coverslipped and stored at 4°C. Slides were subsequently imaged under

the mercury lamp of a Nikon E600 imaging microscope using a TR/FITC/DAPI filter. The semi-quantitative assessment of the stained images was carried out using ImageJ (NIH) following our previously published protocol[1–3]. Briefly, the intensities of positively stained regions were estimated and normalized by the number of cells visible in the images to yield a quantity consistent to a biomarker expression per cell.

Immunoblotting

The tissue specimens were pulverized using a mortar and pestle in liquid nitrogen, homogenized in ice-cold RIPA buffer (Santa Cruz Biotechnology) and centrifuged at 7,000g to pellet extracellular matrix debris for 8 minutes at 4°C. The supernatant was assayed for protein concentration using a bicinchoninic acid (BCA) protein assay (Pierce). Equal amounts of tissue lysates were resolved by reducing SDS-PAGE. After transfer to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore) using a mini trans-blot cell (Bio-Rad), the blots were blocked with 5% non-fat drymilk and probed with a primary antibody against BMP-4 (1:500, Abcam), TGF-β1 (1:200, Santa Cruz Biotechnology). Depending on the primary antibody, appropriate anti-rabbit or anti-mouse HRP secondary antibody (1:2000, Santa Cruz Biotechnology) was then used. The membranes were finally incubated in horseradish peroxidase-conjugated streptavidin. Immunopositive bands were detected using a luminol-based chemiluminescence reagent (Pierce) against standard radiography film in a darkroom. The films were analyzed by densitometry using the dedicated macro of ImageJ to plot the histogram of individual lanes in blots. These histograms were then integrated to obtain the mean intensity of each immune-positive band. These intensities for a particular protein were then normalized by the intensity values for β-actin (Santa Cruz Biotechnology), which was used as a housekeeping protein.

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