

SUPPLEMENTAL METHODS

Patient population

This prospective, observational study included a total of 103 patients with severe mitral regurgitation due to MVP, referred to our center for mitral valve replacement from June 2015 to November 2018. MVP was defined by transthoracic echocardiography if there was systolic displacement of the mitral leaflet into the left atrium at least 2 mm from the mitral annular plane in the parasternal long axis window, following current guidelines.¹ Exclusion criteria were other etiologies of mitral valve disease other than myxomatous degeneration, such as rheumatic, senile degenerative, infectious, ischemic, etc. All patients were evaluated by transthoracic echocardiography and in 63% transesophageal echocardiography was performed, based on clinician's criterion. Informed consent was obtained from each patient and control and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Cell isolation and culture

Human VICs and VECs were isolated from 7-9 human mitral valves obtained from mitral valve replacement surgery. Mitral valves were incubated with Collagenase type 2 (240 U/mg) (Worthington Biochemical Product) for 10 minutes four times for VECs isolation and for 1 hour one time for VICs isolation. For VECs isolation, supernatants were re-collected and centrifuge 5 minutes at 10000g. VECs were grown on 2% gelatin-coated dishes in EBM-2 medium supplemented with GA1000, Hydrocortisone solution, Fetal Bovine Serum, Human Fibroblast Growth Factor basic (hFGFb), Human Vascular Endothelial Growth Factor (hVEGF), Analog of Human Insulin-Like Growth Factor-1, Long R3-IGF-1, Ascorbic Acid Solution and Human Epidermal (Lonza). For VICs, after 1 hour in Collagenase type 2 (240 U/mg), supernatant was centrifuged 5 minutes at

10000g VICs were cultured on DMEM F-12 (Gibco) medium supplemented with 10% FBS. All assays were done at 37°C, 95% sterile air and 5% CO₂ in a saturation humidified incubator. The VIC phenotype of isolated cells was confirmed through immunocytochemistry by probing for the VIC markers vimentin and alpha-smooth muscle actin (α -SMA), whereas the VEC phenotype was confirmed by the expression of the endothelial markers CD31 and VE-cadherin (data not shown). Only cell cultures characterized by purity greater than 90% were used for subsequent studies. Cells were used between passages 3-4 for VECs and 3-6 for VICs.

Single culture cell assays

VICs were treated with Aldo (10^{-8} , Sigma Aldrich) or CT-1 (10^{-7} , Vitro) for 6 hours for mRNA studies and for 24 hours for protein studies. VECs were treated with Aldo (10^{-8} , Sigma Aldrich) or CT-1 (10^{-7} , Vitro) for 24 hours for mRNA studies and for 96 hours for protein studies. In VECs cultures, the duration of the treatment was chosen based on previous studies that analyze EndMT². Cells were treated with Spironolactone (10^{-6} , Sigma Aldrich) for MR blockade 30 minutes before Aldo stimulation.

Conditioned media assays

Cells were treated with conditioned media that had been produced by the specified cell type for 24 hours (in VICs) or 96 hours (in VECs), at which point it was sterile filtered and added to target cells. Media was replaced with fresh conditioned media obtained in VICs every 2 days for VECs treatment.

Real-time reverse transcription PCR

Total RNA was extracted with Trizol Reagent (Qiagen), according to the manufacturer's instructions. First strand cDNA was synthesized according to the manufacturer's instructions (Bio-Rad). Quantitative PCR analysis was performed with SYBR green PCR technology (Bio-Rad) (Supplemental Table 1). Relative quantification was achieved with MyiQ software. Data were normalized by HPRT, GADPH and β -actin levels and expressed as percentage relative to controls. All PCRs were performed at least in triplicate for each experimental condition.

Western blot analysis

Aliquots of 20 μ g of total proteins were prepared from cells and mitral valves and electrophoresed on SDS polyacrylamide gels and transferred to Hybond-c Extra nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies for: Mineralocorticoid Receptor (MR; from Dr. Gomez-Sanchez), α -Smooth Muscle Actin (α -SMA; Sigma), Vimentin (Santa Cruz), Metalloproteinase 2 (MMP-2; Santa Cruz), Slug (Santa Cruz), cluster of differentiation 31 (CD31; Santa Cruz), VE-Cadherin (Abcam), Von Willebrand Factor (vWF; Santa Cruz), Decorin (Santa Cruz), Biglycan (Santa Cruz), Lumican (Abcam), cluster of differentiation 14 (CD14; Abcam) and Cardiostrophin 1 (CT-1; Abcam). Stain free detection was used as loading control. After washing, detection was made through incubation with peroxidase-conjugated secondary antibody, and developed using an ECL chemiluminescence kit (Amersham). After densitometric analyses, optical density values were expressed as arbitrary units. All Western Blots were performed at least in triplicate for each experimental condition.

Immunohistological evaluation

Histological determinations in mitral valve tissue were performed in 5 µm-thick sections. Slides were treated with H₂O₂ for 10 min to block peroxidase activity. All samples were blocked with 5% normal goat serum in PBS for 1 h and incubated for 1h with Vascular Cell Adhesion Protein 1 (VCAM-1; BD System), CD31 (Santa Cruz), VE-Cadherin (Abcam), α-SMA (Sigma), Decorin (Santa Cruz), Biglycan (Santa Cruz), Lumican (Abcam), Aggrecan (Abcam), Versican (Abcam), cluster of differentiation 14 (CD14; Abcam) and Cardiotrophin 1 (CT-1; Abcam). After incubation, tissues were washed three times, and then incubated for 30 min with the horseradish peroxidase-labeled polymer conjugated to secondary antibodies (Dako Cytomation). The signal was revealed by using DAB Substrate Kit (BD Pharmingen). As negative controls, samples followed the same procedure described above but in the absence of primary antibodies. For Alcian blue-Sirius red staining, slides were hydrated and incubated with Alcian-blue (Sigma-Aldrich) for 20 minutes, after washing slides were incubated with 1% Sirius red in picric acid for and 30 minutes. Elastin fibers were visualized by Elastin stain kit according to the manufacturer's instructions (Sigma-Aldrich). All measurements and quantifications were performed blind in an automated image analysis system (Nikon).

Cytokine array

After incubation time as indicated (24 hours) cell supernatants were collected and analyzed using a human cytokine array kit following manufacturer's instructions (Abcam (ab169816)). The results were normalized to the control condition in each cell type. Data were expressed as a fold change relative to controls.

ELISA

Decorin, Lumican, Aggrecan, Syndecan-1, Hyaluronan, CT-1 and CD14 were measured in valve extracts, serum and cells supernatants by ELISA according to the manufacturer's instructions (R&D Systems). CT-1 was measured in valve extracts, serum and cells supernatants by ELISA according to the manufacturer's instructions (Abcam).

Transfection of cells with siRNA

Cells were seeded into twelve-well plates at 100% confluence and transfected with a pool of five siRNAs (GeneCust) for CT-1 and for CD14 target-specific using MATra-si (Promokine-Promocell) according to the manufacturer's recommendations. Cells were allowed to recover for 24 h before stimulation. Scramble siRNAs were used as a control.

In vivo studies

Wild Type 129S2/Sv mice were obtained from Charles River Laboratories. Osmotic minipumps (Alzet) delivering Nordexfenfluramine (NDF) (1 mg/kg/day; Sigma-Aldrich) were implanted subcutaneously. The MRA Spironolactone (1 mg/kg per day) was administered as an additive in the food for 28 days. Mice with MR inactivation in endothelial cells (Vecadh-MR-KO) were generated by crossing MRf/f floxed mice ³ (kindly provided by Dr. Berger, Heidelberg, Germany) with transgenic mice expressing the Cre recombinase under the control of the VE-Cadherin promoter ⁴ (The Jackson Laboratory, Bar Harbor, ME). Mice with MR knocked out in smooth muscle cells (SMA-MR-KO) were generated by crossing MRf/f floxed mice with transgenic mice expressing the inducible CreERT2 recombinase under the control of the SMA promoter (kindly provided by Dr. Metzger, Strasbourg, France) ⁵ MRf/f littermates lacking the

Cre transgene were used as controls. All mice were generated in the C57Bl/6 genetic background (The Jackson Laboratory). All mice were sacrificed 28 days later. Animals were housed in a climate-controlled facility with a 12- hours/12-hours light/dark cycle. Experiments were approved by the Darwin ethics committee of Pierre et Marie Curie University, and conducted according to the INSERM animal care and use committee guidelines (APAFIS#4488-20 1 6010614517136 v3).

Statistical analyses

For cellular studies, data were expressed as mean \pm SEM. Normality of distributions was verified by means of the Kolmogorov–Smirnov test. Data were analyzed using a one-way analysis of variance, followed by a Newman-Keuls to assess specific differences among groups or conditions using GraphPad Software Inc. For animal studies, data were expressed as means \pm SEM. The normality of distributions was verified by means of the Kolmogorov–Smirnov test. Data were analysed using a one-way ANOVA, followed by a Newman–Keuls test to assess specific differences among groups or conditions, using GraphPad Software Inc. For human studies, data were expressed as mean \pm SEM. Spearman’s correlation coefficients were calculated to determine correlations. Categorical variables were expressed as percentages and compared using χ^2 -test, or Fisher exact test when the expected value in any cell of the contingency table was below 5. Quantitative variables were analyzed by T student test or Mann-Whitney U test if the normality was not met. Clinical data of the patients enrolled were analyzed with IBM SPSS statistics 25 software.

Supplemental bibliography

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SUPPLEMENTAL TABLE

Table S1: Primers used in humans in real time PCR analysis

Gene	Primer	Sequence (5' to 3')
VE-Cadherin	Forward	CAG CCC AAA GTG TGT GAG AA
	Reverse	CGG TCA AAC TGC CCA TAC TT
α-SMA	Forward	ACT GCC TTG GTG TGT GAC AAT GG
	Reverse	TGG TGC CAG ATC TTT TCC ATG
MMP-2	Forward	CGA CCA CAG CCA ACT ACG AT
	Reverse	GTC AGG AGA GGC CCC ATA GA
Decorin	Forward	CCT GAT GAC CGC GAC TTC GAG
	Reverse	TTT GGC ACT TTG TCC AGA CCC
Versican	Forward	ACG GGA TTG AAG ACA CAC AAG
	Reverse	AGC CTC AAA ATT CAG TGT GTA
Biglycan	Forward	TCT GTC ACA CCC ACC TAC AGC
	Reverse	AGG GGA GAT CTC TTT GGG CAC
Lumican	Forward	TGA GCT GGA TCT GTC CTA TAA
	Reverse	ATC TTG CAG AAG CTC TTT ATG
Chm-1	Forward	CAT CGG CCT TCT ACT TCT
	Reverse	GGC ATG ATC TTG CCT TCC AG
Vimentin	Forward	GTT TCC CCT AAA CCG CTA GG
	Reverse	AGC GAG AGT GGC AGA GGA
CD31	Forward	TTC CCA CGC CAA AAT GTT A
	Reverse	CAC AGC ACA TTG CAG CAC A
Slug	Forward	GGA CGC ACA CCT TAC CTT GT
	Reverse	CGA GAA GGT TTT GGA GCA AC
VWF	Forward	TAA GTC TGA AGT AGA GGT GG
	Reverse	AGA GCA GCA GGA GCA CTG GT
Syndecan-1	Forward	CCC CTG AAG ATC AAG ATG GC
	Reverse	GGT TCT GGA GAC GTG GGA AT

Hyaluronan	Forward	TGT TGT GGT AGC ACT GGA CT
	Reverse	TCT GGG CTT TGT GAT GGG AT
CT-1	Forward	CTG GAC CCC CAG ACT GAT TC
	Reverse	CCT GGA GCT GCA CAT ATT CCT
CD14	Forward	CTG CAA CTT CTC CGA ACC TC
	Reverse	CCA GTA GCT GAG CAG GAA CC
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

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. MR expression in mitral valve tissue and cells. Western blot of MR in mitral valve tissue, VICs and VECs.

Supplemental Figure 2. Effects of Aldo and Spironolactone in human mitral VICs and VECs. Quantification of qVICs maker (Chm-1) and VICs activation markers (α -SMA, vimentin and MMP-2) at mRNA levels in VICs (**A**). Quantification of proteoglycans (decorin, biglycan, lumican, syndecan-1, hyaluronan and versican) at mRNA levels (**B**) in VICs. Quantification of VECs markers (CD31, VE-cadherin and vWF) at mRNA levels (**C**) in VECs. Quantification of VICs activation markers (α -SMA, vimentin, MMP-2 and slug) at mRNA levels (**D**) in VECs. Quantification of proteoglycans (decorin, biglycan, lumican, syndecan-1, hyaluronan and versican) at mRNA levels (**E**) in VECs. The bars in the histograms represent the mean \pm SEM of each group in arbitrary units normalized to HPRT, GADPH and β -actin for cDNA. * $p < 0.05$ vs. Control. \$ $p < 0.05$ vs Spiro.

Supplemental Figure 3. Effects in mitral VICs treated with conditioned media from the culture of mitral VECs treated with Aldo and effects in mitral VECs treated with conditioned media from the culture of VICs treated with Aldo. Quantification of VICs activation markers (α -SMA, vimentin and MMP-2) at protein levels in VICs (**A**). Quantification of intracellular and extracellular proteoglycans in VICs (decorin, biglycan, aggrecan, lumican, syndecan-1 and hyaluronan) at protein levels (**B**). Quantification of VECs markers (CD31, VE-cadherin and vWF) (**C**) and VICs activation markers (α -SMA, vimentin, MMP-2 and slug) (**D**) at protein levels. Quantification of intracellular and extracellular proteoglycans in VECs (decorin, biglycan, lumican, aggrecan, syndecan-1 and hyaluronan) at protein levels (**E**). The

bars in the histograms represent the mean \pm SEM of each group in arbitrary units (AU) normalized to stain free gel for protein. * $p < 0.05$ vs. control. \$ $p < 0.05$ vs. Aldo.

Supplemental Figure 4. Aldo effects in VICs is mediated by Cardiotrophin-1.

Representative images of human cytokine array blots probed with the cell supernatant samples. Each blot represents the immunoreactive staining with each cytokine. The lack of dots represented the negative and blank control. The blots marked inside the box are the CT-1 expression. The fold of change of CT-1 was determined by comparing the pixel intensity of the respective blots to that of the positive control on the same array (A). Quantification of extracellular and intracellular CT-1 (B). CT-1 effects on qVICs maker (Chm-1) and VICs activation markers (α -SMA, vimentin, MMP-2 and slug) at mRNA levels (C) and protein levels (D) in VICs. CT-1 effects on proteoglycans expression in VICs (decorin, biglycan, lumican and versican) at mRNA levels (E) and protein levels (F). siRNA for knock down CT-1 was used in VICs. Aldo effects on qVICs maker (Chm-1) and VICs activation markers (α -SMA and MMP-2) at mRNA levels (G) in CT-1 knockdown cells. Aldo effects on proteoglycans (decorin, biglycan and versican) at mRNA levels (H) in CT-1 knockdown cells. The bars in the histograms represent the mean \pm SEM of each group in arbitrary units (AU) normalized to HPRT, GADPH and β -actin or stain free gel for cDNA and protein respectively. * $p < 0.05$ vs. Control. \$ $p < 0.05$ vs Spiro, Aldo.

Supplemental Figure 5. Aldo effects in VECs is mediated by CD14. Representative images of human cytokine array blots probed with the supernatant samples. Each blot represents the immunoreactive staining with each cytokine. The lack of dots represented the negative and blank control. The blots marked inside the box are the CD14 expression. The fold of change of CD14 was determined by comparing the pixel intensity of the respective blots to that of the positive control on the same array (A).

Quantification of extracellular CD14 **(B)**. siRNA for knock down CD14 was used in VECs. Aldo effects on VECs markers (CD31 and VE-cadherin) at mRNA levels **(C)** in CD14 knockdown cells. Aldo effects on qVICs maker (Chm-1) and VICs activation markers (α -SMA, vimentin, MMP-2 and slug) at mRNA levels **(D)** in CD14 knockdown cells. Aldo effects on proteoglycans (decorin, biglycan and lumican) at mRNA levels **(E)** CD14 knockdown cells. The bars in the histograms represent the mean \pm SEM of each group in arbitrary units (AU) normalized to HPRT, GADPH for cDNA. * $p < 0.05$ vs. Control. \$ $p < 0.05$ vs Spiro, Aldo.

Supplemental Figure 6. Paracrine interactions between VICs and VECs. VECs have been treated with conditioned media obtained from VICs after CT-1 knock-down. Expression of (EndMT markers) and proteoglycans and secretion of proteoglycans in VECs **(A)**. VICs have been treated with conditioned media obtained from VECs after CD14 knock-down. Vimentin and proteoglycans expression and secretion in VICs **(B)**.

Supplemental Figure 7. Expression of CT-1 and CD14 in a MVP mouse model. Representative microphotographs of mouse mitral valve sections for CT-1 and CD14. Magnification 40x.

Supplemental Figure 8. Effects of NDF administration on proteoglycans content in mitral valve from SMA-MR-KO mice. Individual points are shown as circles (control group), squares (NDF group) and triangles (SMA-MR-KO + NDF group) to represent the mitral valve area **(A)** and thickness **(B)**. Representative microphotographs of mitral valve sections from SMA-MR-KO mice stained for Sirius red, Alcian blue, decorin, biglycan, lumican and aggrecan **(C)**. Box plots show the individual data and horizontal bars indicate mean and SEM bars in arbitrary units versus control group. Magnification 40x. * $p < 0.05$ vs. control.