

BASIC SCIENCE ARTICLE



# Mechanical strain triggers endothelial-to-mesenchymal transition of the endocardium in the immature heart

Carina Vorisek<sup>1,3</sup>, Viktoria Weixler<sup>1,3</sup>, Massiel Dominguez<sup>1</sup>, Roland Axt-Fliedner<sup>2</sup>, Peter E. Hammer<sup>1</sup>, Rwei-Zeng Lin<sup>1</sup>, Juan M. Melero-Martin<sup>1</sup>, Pedro J. del Nido<sup>1</sup> and Ingeborg Friehs<sup>1</sup>✉

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**BACKGROUND:** Endothelial-to-mesenchymal-transition (EndMT) plays a major role in cardiac fibrosis, including endocardial fibroelastosis but the stimuli are still unknown. We developed an endothelial cell (EC) culture and a whole heart model to test whether mechanical strain triggers TGF- $\beta$ -mediated EndMT.

**METHODS:** Isolated ECs were exposed to 10% uniaxial static stretch for 8 h (stretch) and TGF- $\beta$ -mediated EndMT was determined using the TGF- $\beta$ -inhibitor SB431542 (stretch + TGF- $\beta$ -inhibitor), BMP-7 (stretch + BMP-7) or losartan (stretch + losartan), and isolated mature and immature rats were exposed to stretch through a weight on the apex of the left ventricle. Immunohistochemical staining for double-staining with endothelial markers (VE-cadherin, PECAM1) and mesenchymal markers ( $\alpha$ SMA) or transcription factors (SLUG/SNAIL) positive nuclei was indicative of EndMT.

**RESULTS:** Stretch-induced EndMT in ECs expressed as double-stained ECs/total ECs (cells:  $46 \pm 13\%$ ; heart:  $15.9 \pm 2\%$ ) compared to controls (cells:  $7 \pm 2\%$ ; heart:  $3.1 \pm 0.1$ ;  $p < 0.05$ ), but only immature hearts showed endocardial EndMT. Inhibition of TGF- $\beta$  decreased the number of double-stained cells significantly, comparable to controls (cells/heart: control:  $7 \pm 2\%/3.1 \pm 0.1\%$ , stretch:  $46 \pm 13\%/15 \pm 2\%$ , stretch + BMP-7:  $7 \pm 2\%/2.9 \pm 0.1\%$ , stretch + TGF- $\beta$ -inhibitor (heart only):  $5.2 \pm 1.3\%$ , stretch + losartan (heart only):  $0.89 \pm 0.1\%$ ;  $p < 0.001$  versus stretch).

**CONCLUSIONS:** Endocardial EndMT is an age-dependent consequence of increased strain triggered by TGF- $\beta$  activation. Local inhibition through either rebalancing TGF- $\beta$ /BMP or with losartan was effective to block EndMT.

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**IMPACT:**

- Mechanical strain imposed on the immature LV induces endocardial fibroelastosis (EFE) formation through TGF- $\beta$ -mediated activation of endothelial-to-mesenchymal transition (EndMT) in endocardial endothelial cells but has no effect in mature hearts.
- Local inhibition through either rebalancing the TGF- $\beta$ /BMP pathway or with losartan blocks EndMT.
- Inhibition of endocardial EndMT with clinically applicable treatments may lead to a better outcome for congenital heart defects associated with EFE.

**INTRODUCTION**

The endothelium is composed of a single layer of endothelial cells which allows for selective permeability of the vessel wall. This is also true for the endocardial endothelial layer covering the surface of the ventricular and atrial cavities. Endothelial cells react to physical and chemical stimuli in order to maintain metabolic and secretory function, and to preserve hemostatic balance.<sup>1</sup> Due to exposure to a large variety of stimuli, they are required to display high plasticity to adapt to physiological and pathological alterations which include adaptation to flow, stretch, or pathological events such as atherosclerosis.<sup>2</sup> Endothelial-to-mesenchymal-transition (EndMT) is an example of endothelial plasticity because it allows for endothelial cells to transform into mesenchymal cells when needed during fetal cardiac development but also contributes to organ fibrosis or atherosclerosis.<sup>3–5</sup>

EndMT is known as the phenotypical change of endothelial cells to mesenchymal cells.<sup>6</sup> For this phenotypical change to occur, intracellular molecular and architectural rearrangements are necessary. Endothelial cells are connected through vascular endothelial cadherin (VE-cadherin) which is a junctional protein for cell-cell interaction. Another constituent of the endothelial intercellular junction is CD31 (cluster of differentiation 31), also called platelet endothelial cell adhesion molecule 1 (PECAM1), which is a member of the immunoglobulin superfamily. This transmembraneous glycoprotein plays a major role in the adhesion process between endothelial cells and inflammatory cells during inflammation and angiogenesis.<sup>7</sup> During the process of EndMT, endothelial cells lose their endothelial phenotype which is defined through the expression of endothelial markers such as PECAM1 or VE-cadherin, and develop a mesenchymal phenotype

<sup>1</sup>Department of Cardiac Surgery, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA. <sup>2</sup>Center for Prenatal Medicine and Fetal Therapy, Justus-Liebig-University of Giessen, Giessen, Germany. <sup>3</sup>These authors contributed equally: Carina Vorisek, Viktoria Weixler. ✉email: [ingeborg.friehs@childrens.harvard.edu](mailto:ingeborg.friehs@childrens.harvard.edu)

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by expressing markers such as fibroblast-specific protein 1 (FSP1) and/or  $\alpha$ -smooth muscle antibody ( $\alpha$ -SMA).<sup>8</sup> Double-staining of individual cells with an endothelial marker and a fibroblast marker at the same time indicates active EndMT.

As we and others have previously reported in mature hearts, this process of EndMT leads to myocardial fibrosis as seen in heart failure following pressure-overload, or in organ rejection following heart transplantation.<sup>2,9</sup> Furthermore, we have shown that in immature hearts, EndMT occurs as a unique form of fibrosis within the subendocardial space. This type of fibrosis is called endocardial fibroelastosis (EFE) and is found as early as the fetal stage of cardiac development in humans when the heart is exposed to altered flow and pressure conditions due to an underlying congenital heart lesion such as hypoplastic left heart syndrome (HLHS).<sup>10,11</sup> Clinical observation indicates that EFE is associated with left ventricular dilation in some cases of developing HLHS.<sup>12</sup> Common features of EFE formation involve young age (e.g., developing heart) and hemodynamic alterations which are an inherent feature of HLHS.<sup>12</sup> It is, however, unclear whether mechanical changes imposed on the immature LV have an impact on the underlying mechanism of EFE formation through activation of EndMT.

To examine whether mechanical forces induce stimulation of EndMT, we decided to use model systems where fluid forces from blood flow could be excluded as a cause for EndMT stimulation. Thus, we established an endothelial cell culture model where cells were exposed to uniaxial stretch. Secondly, we designed an isolated perfused whole heart preparation of excess strain on the LV which allowed for evaluation of endocardial endothelial cells in their natural environment and for maturation effects on strain-induced EndMT.

Based on these observations, we sought to determine whether a mechanical stimulus of excess strain triggers EndMT in isolated endothelial cells and in the whole heart ultimately leading to fibroelastic transformation of the endocardium and/or myocardium. To further our understanding of the development of potential therapeutic interventions, we evaluated whether the TGF- $\beta$  signaling pathway was involved in the regulation of EndMT induced by mechanical strain and tested potential strategies suitable for future treatment in humans.

## MATERIALS AND METHODS

### Ethical statement

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital.

### In vitro cell culture model

Human coronary endothelial cells (HCAEC) were purchased from Lonza and maintained according to the manufacturer's instructions in endothelial growth media (EGM-2 MV). All cells were used between passages 3 to 6 and maintained under 37 °C and 5% CO<sub>2</sub> humidified atmosphere. To expose HCAEC to increased stretch, cells were seeded on 10  $\mu$ g/ml fibronectin-coated silicone stretch chambers (B-Bridge International Inc., Santa Clara, CA). After cells reached 80% of confluency, the chambers were exposed to a uniaxial strain of 10% of their initial length. For these experiments, a mechanical strain instrument (STREX from B-Bridge International Inc., Santa Clara, CA) was used which was maintained in an incubator 5% CO<sub>2</sub> at 37 °C. After eight hours, the experiment was terminated, and cells were stained for analysis. HCAEC were seeded on stretch chambers but not exposed to stretch served as control (group 1). Four groups of cells were exposed to 10% uniaxial static stretch for 8 h whereof group 2 was stretched but remained in regular culture media but groups 3–5 were exposed to 10% uniaxial static stretch and inhibitors of the TGF- $\beta$  pathway were administered. All experiments were performed in

triplicates. Rebalancing of the TGF- $\beta$ /BMP pathway was addressed through the addition of recombinant human BMP-7 (ThermoFisher, Waltham, MA; group 3), direct inhibition with the TGF- $\beta$  inhibitor SB431542 (Tocris Bioscience, Bristol, UK; group 4), and Losartan potassium as clinically relevant TGF- $\beta$  inhibitor (Santa Cruz Biotechnology, Dallas, TX; group 5), were added to the culture media on a gelatin sponge as drug carrier. To ascertain that the drug carrier did not have any adverse effects, the unloaded drug carrier and all inhibitor drugs directly dissolved in culture media were examined in separate experiments also (data not shown).

To confirm that endothelial cells were still functional according to their phenotype, we used Dil complex acetylated low-density lipoprotein from human plasma (ThermoFisher, Waltham, MA), a compound that is only internalized by viable endothelial cells and fluoresces upon uptake. Mesenchymal cells do not internalize this compound and thus, it allows for endothelial cells with respective characteristics to be distinguished from mesenchymal cells.

### Ex vivo whole-heart model

A total number of 60 isolated rat hearts ( $n = 4$ –7/group) were used. Rats received sublethal anesthesia with isoflurane for excision of hearts which were rapidly transferred onto a non-working Langendorff perfusion system. Modified Krebs-Henseleit solution served as perfusion medium. Details were described in more detail previously by our group.<sup>13</sup> Since we wanted to determine whether immaturity directly affects stretch-induced EndMT, we used either immature rat hearts (heart weight <1 g) or mature (heart weight >1 g) rat hearts for these experiments.

Following stabilization, hearts were exposed to 3 h of static stretch by attaching a weight of 10 times the heart weight to the apex of the LV with a single suture (group 2). Unstretched hearts served as control (group 1). After constant perfusion at 37 °C, the hearts were harvested, and conserved in 4% formalin until paraffin sections were obtained. Equivalent to the cell culture experiments, the same three TGF- $\beta$  pathway inhibitors were used (groups 3–5), recombinant human BMP-7 (ThermoFisher, Waltham, MA), SB431542 (Tocris Bioscience, Bristol, UK), and Losartan potassium (Santa Cruz Biotechnology, Dallas, TX). Pharmacological compounds were inserted through the left appendage using a Gelfoam® absorbable gelatin sponge (Pfizer, New York, NY) as a drug carrier for localized administration at the site of EndMT, in concentrations which had been established in the in vitro cell culture experiments (10 ng/ml for BMP-7, 10  $\mu$ M for SB431542 and 10  $\mu$ M for Losartan). Sponges were replaced every hour in order to maintain the same localized drug concentrations. Mature and immature rat hearts were perfused and exposed to uniaxial stretch and inhibitor protocol as described.

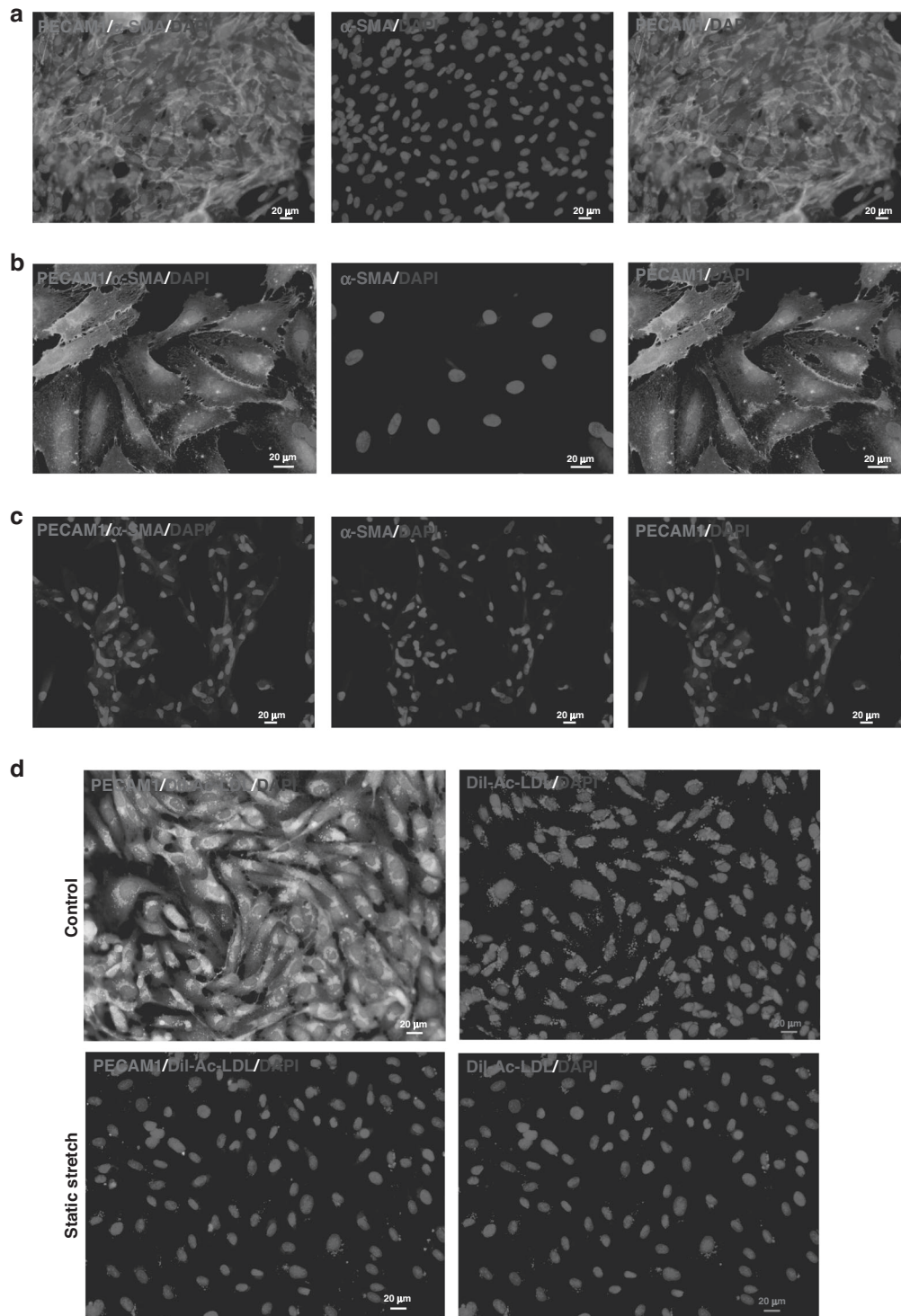
### Evaluation of EndMT

Immunofluorescent staining was performed for detection of the endothelial marker PECAM-1 (Santa Cruz, Dallas, TX) and the mesenchymal marker monoclonal anti-actin  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; Sigma Aldrich, St. Louis, MO). Whole hearts cross-sections were also stained for the transcription factor SLUG/SNAIL (Santa Cruz, Dallas, TX). EndMT was defined as cells staining positive for both PECAM-1 and  $\alpha$ -SMA, or EC nuclei staining positive for SLUG/SNAIL, respectively.

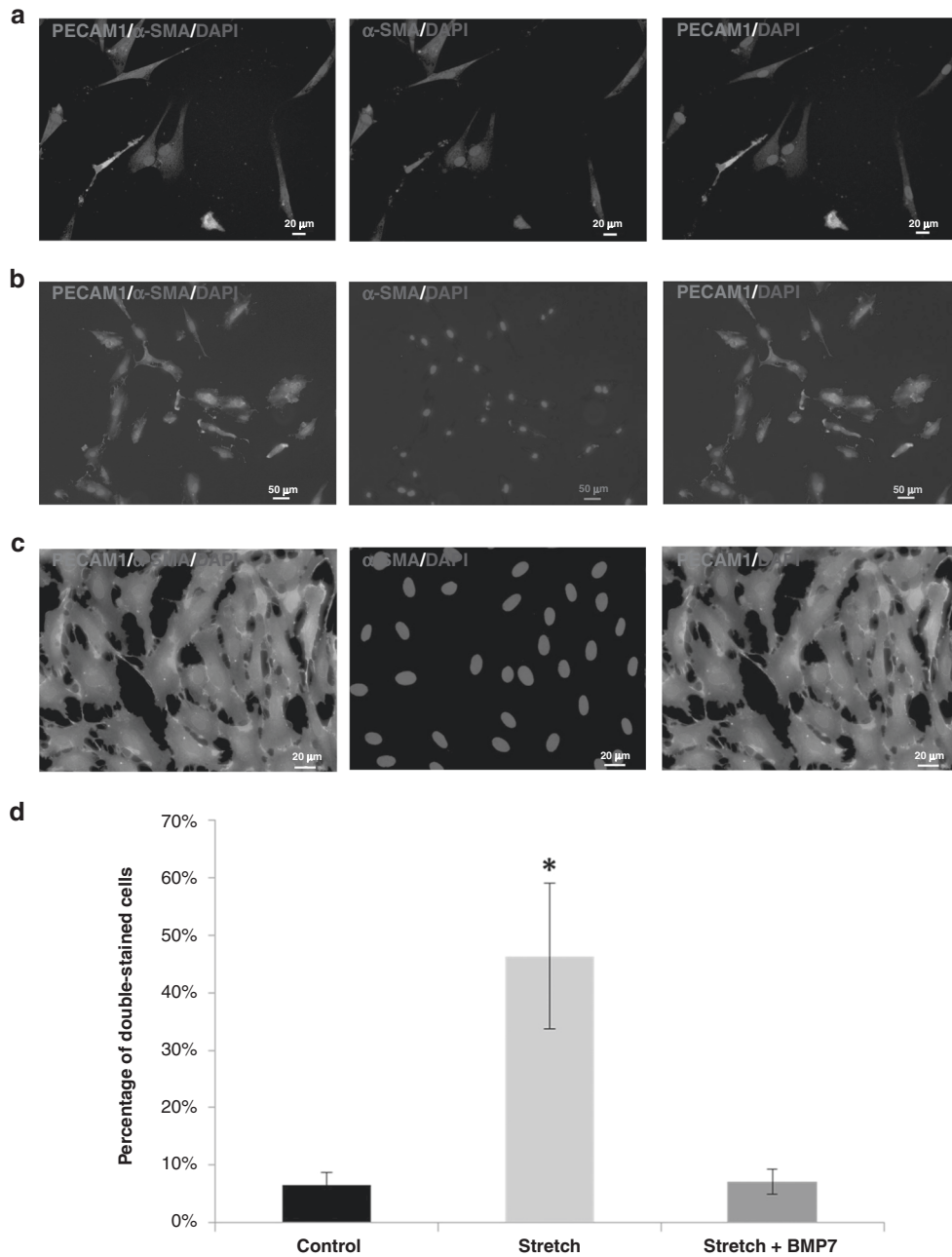
In order to label endothelial cells within the myocardium, FITC Lectin (Sigma Aldrich, St. Louis, MO) from *Lycopersicon esculentum* (tomato) was perfused through the aortic root into the coronaries at a concentration of 20  $\mu$ l/ml at the end of the 3 h perfusion period, which labels endothelial cells as we have previously reported in more detail.<sup>14</sup> All slides were visualized using a Zeiss Observer.Z1 fluorescent microscope with a Nikon 20x objective (NA =  $\times 20/0.45$ ). Ten randomly selected fields from each slide were taken and quantification was performed with ImageJ (version 2.0.0-rc-43, obtained from the National Institute of Health, Bethesda, MD).

### Statistical analysis

All experiments were triplicates for the cell culture model. The ratio of PECAM-1/ $\alpha$ -SMA double-stained endothelial cells/total endothelial cells count was calculated in ten randomly selected fields of vision in each sample. Cell counts were performed by two blinded investigators and are reported as mean  $\pm$  standard error of the mean (SEM). Observer variability was determined by calculating intra- and inter-rater reliability (RStudio, version 1.4.1106, package irrICC). After confirmation of normal distribution of data, student *t*-test (GraphPad Prism 5.1, SPSS 23, IBM Corporation, Armonk, NY) or ANOVA for multiple group comparisons and Bonferroni's post-hoc analyses were performed to obtain calculations of statistical significance. Probability values of  $\leq 0.05$  were regarded as statistically significant.



**Fig. 1** Static stretch triggers EndMT in isolated endothelial cells. **a** HCAEC present with cobblestone morphology typical for endothelial cells. **b, c** Representative histological sections of control endothelial cells and stretched endothelial cells are shown. Both slides were stained with the EC marker, PECAM1 (green), the mesenchymal marker,  $\alpha$ SMA (red), and nuclei in blue (DAPI). Endothelial cells in the control picture only stained for the EC marker PECAM1 indicative of the endothelial phenotype. In comparison, stretched endothelial cells were positive for both markers. Double-staining with an EC and a mesenchymal marker is indicative of active EndMT. **d** To confirm the results of stretch-induced EndMT in HCAEC, we added the functional endothelial marker Dil complex acetylated Low Density Lipoprotein (Dil-Ac-LDL) to the media of the cell culture dish. Dil-Ac-LDL (red) is only internalized by endothelial cells and fluoresces upon uptake. As indicated by the representative pictures, stretched cells had no functional endothelial cells compared to the endothelial cells (green) in the control group.



**Fig. 2** Stretch-induced EndMT is mediated through activation of the TGF- $\beta$  pathway. **a** Endothelial cells exposed to TGF- $\beta$  served as a positive control for active EndMT. **b** Endothelial cells exposed to 8 h of uniaxial stretch underwent EndMT. **c** When BMP-7 was added to endothelial cells exposed to uniaxial stretch, cells retained their endothelial morphology and only stained positive for the EC marker PECAM1. **d** Following BMP-7 treatment, significantly less cells stained for both the EC marker PECAM1 and the mesenchymal marker  $\alpha$ SMA compared to the cells in the stretch chamber not containing BMP-7 as indicated by this graph.

## RESULTS

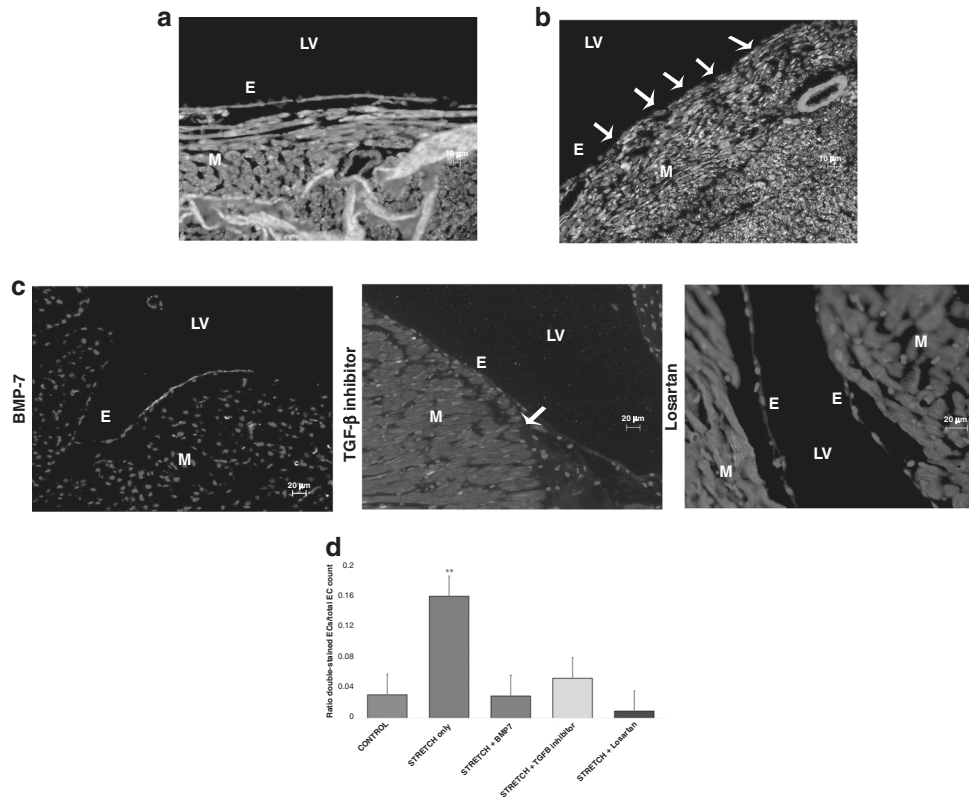
### Static stretch triggers EndMT in isolated cells

After exposure to 10% uniaxial static stretch for 8 h, HCAEC stained positive for both, PECAM-1 and  $\alpha$ -SMA, and additionally lost their cobblestone morphology typical for endothelial cells, indicating that static stretch triggers EndMT (Fig. 1a–c). Unstretched control cells showed  $7 \pm 2\%$  double-stained cells per total cell count compared to stretched cells which displayed active EndMT in  $46 \pm 13\%$  of total cell count ( $p = 0.05$ ). To confirm endothelial cells, we used Dil complex acetylated low-density lipoprotein from the human plasma which is only internalized by viable and phenotypical endothelial cells and fluoresces upon uptake (Fig. 1d).

To ascertain that the drug carrier did not have any adverse effects, the unloaded drug carrier and all inhibitor drugs directly dissolved in culture media were examined in separate experiments and did not show any results different from when the drug carrier was used (data not shown).

### Stretch-induced EndMT is mediated through activation of the TGF- $\beta$ pathway

After 8 h of 10% static uniaxial stretch, endothelial cells underwent EndMT. In order to determine whether the TGF- $\beta$  pathway was involved in this process, we used either BMP-7, or a specific TGF- $\beta$  inhibitor, or losartan, respectively added to the culture media when



**Fig. 3 Excess strain induces EndMT in immature hearts.** Heart tissue was stained for the endothelial marker PECAM1 (green), the mesenchymal marker  $\alpha$ SMA (red), and DAPI (blue). Double staining for both PECAM1 and  $\alpha$ SMA indicated EndMT (white arrows point out EndMT positive endothelial cells). Representative immunohistochemical stains for double-labeling of endothelial cells with PECAM1 and  $\alpha$ SMA are shown for **a** control hearts, **b** immature rat hearts stretched for 3 h, **c** immature hearts stretched with an inhibitor present (BMP-7 on the left, TGF- $\beta$ -inhibitor in the middle, Losartan on the right). **d** A summary of the results is shown in this graph. Ratio of double-stained (PECAM1/ $\alpha$ SMA) endothelial cells/total endothelial cell count in controls, stretched and stretch with inhibitors are shown. There were significantly more double-stained cells in stretched hearts compared to all other groups (\*\* $p < 0.05$ ) but there was no significant difference between the other groups.

exposing the cells to uniaxial stretch. Stretch stimulated EndMT ( $46 \pm 13\%$  of total cell count) which could be inhibited to baseline levels by the addition of BMP-7 ( $7 \pm 2\%$ ;  $p < 0.001$ ) leading to cells retaining their endothelial morphology (Fig. 2a–c). These results were confirmed by using a specific TGF- $\beta$  inhibitor and losartan (data not shown). These results suggest that static stretch-induced EndMT is regulated through activation of the TGF- $\beta$  pathway.

#### Excess strain induces EndMT in immature whole hearts

After exposing immature hearts to uniaxial stretch (10 times their heart weight amounting to  $27 \pm 10\%$  static stretch from baseline) with constant perfusion on a Langendorff apparatus for 3 h, significantly more double-stained ( $\alpha$ -SMA /PECAM1) endothelial cells/total endothelial cells were found than in control hearts which were only perfused ( $15.9\% \pm 2$  vs.  $3.1\% \pm 0.8$ ,  $p < 0.01$ ; Fig. 3). These results were confirmed by staining for the transcription factors SLUG/SNAIL downstream of the TGF- $\beta$  pathway (control:  $15.2\% \pm 3.3$  versus stretch:  $46.8\% \pm 3.7$ ), which was significantly different ( $p < 0.01$ ) (Fig. 5).

To establish that inhibition of the TGF- $\beta$  pathway can block EndMT, we locally applied BMP-7, the TGF- $\beta$  inhibitor SB431542 or losartan, respectively in uniaxial-stretched immature ex vivo hearts. Following administration of either one of the three TGF- $\beta$  inhibitors, the ratio of  $\alpha$ -SMA /PECAM1 double-stained endothelial cells was significantly lower (stretch + BMP-7:  $2.9\% \pm 0.1$ , stretch + SB431542:  $5.2\% \pm 1.3$  and in stretch + losartan:  $0.09\% \pm 0.1$ ) compared to hearts exposed to uniaxial stretch ( $p < 0.01$ ) (Fig. 3c). Compared to controls, stretched hearts with one of the three TGF- $\beta$

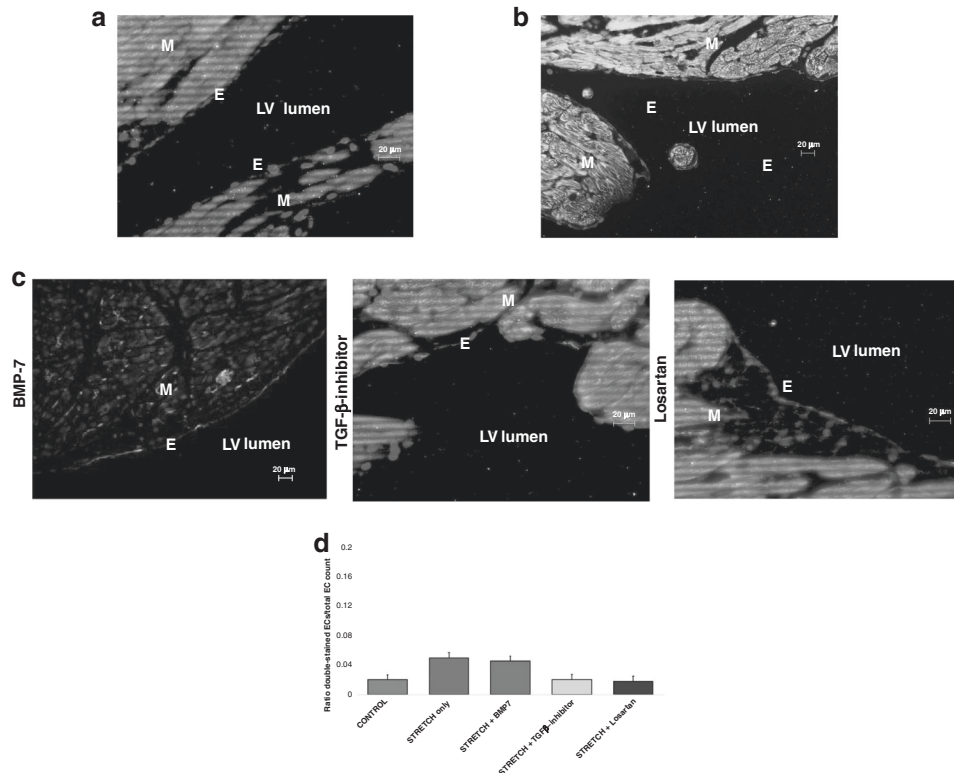
inhibitors was not significantly different ( $p > 0.05$ ) as indicated by the data summary in Fig. 3d. These data were confirmed by staining of nuclear colocalization of the transcription factors SLUG/SNAIL, which is shown as representative slides in Fig. 5a and data summary in Fig. 5c.

#### Excess strain does not induce EndMT in mature whole hearts

In all mature hearts (10 times their heart weight amounting to  $25 \pm 7\%$  static stretch from baseline), excess strain on the LV did not induce endocardial EndMT. Stretch alone and addition of TGF- $\beta$  pathway inhibitors did not show any significant difference in the number of double-stained endocardial cells compared to unstretched controls (control:  $2 \pm 0.6\%$ , stretch:  $5 \pm 1.2\%$ , stretch + BMP-7:  $4.6 \pm 0.1\%$ , stretch + TGF- $\beta$  inhibitor:  $2.1 \pm 0.06\%$ ; stretch+losartan:  $1.8 \pm 0.5\%$  of total cell count;  $p > 0.05$ ) (Fig. 4a–d). These results were confirmed by staining for SLUG/SNAIL which showed equivalent result with no significant difference between the groups (Fig. 5b). To determine the accuracy of our measurements within and between blinded observers' assessments, inter- and intra-rater reliability were determined which showed substantial agreement with 0.7234946 and almost perfect agreement with 0.8194278, respectively.

#### DISCUSSION

In this study, we showed that endocardial EndMT is an age-dependent process that results as a consequence of increased



**Fig. 4 Excess strain does not induce EndMT in mature hearts.** Heart tissue was stained for the endothelial marker PECAM1 (green), the mesenchymal marker  $\alpha$ SMA (red), and DAPI (blue). Double staining for both PECAM1 and  $\alpha$ SMA indicated EndMT. Representative immunohistochemical stains for double-labeling of endothelial cells with PECAM1 and  $\alpha$ SMA are shown for **a** control hearts, **b** mature rat hearts stretched for 3 h, **c** mature hearts stretched with an inhibitor present (BMP-7 on the left, TGF- $\beta$ -inhibitor in the middle, Losartan on the right). **d** A summary of the results are shown in this graph. Ratio of double-stained (PECAM1/ $\alpha$ SMA) endothelial cells/total endothelial cell count in controls, stretched and stretch with inhibitors are shown. There were no significant differences between the groups. Uniaxial stretch in mature hearts did not induce endocardial EndMT.

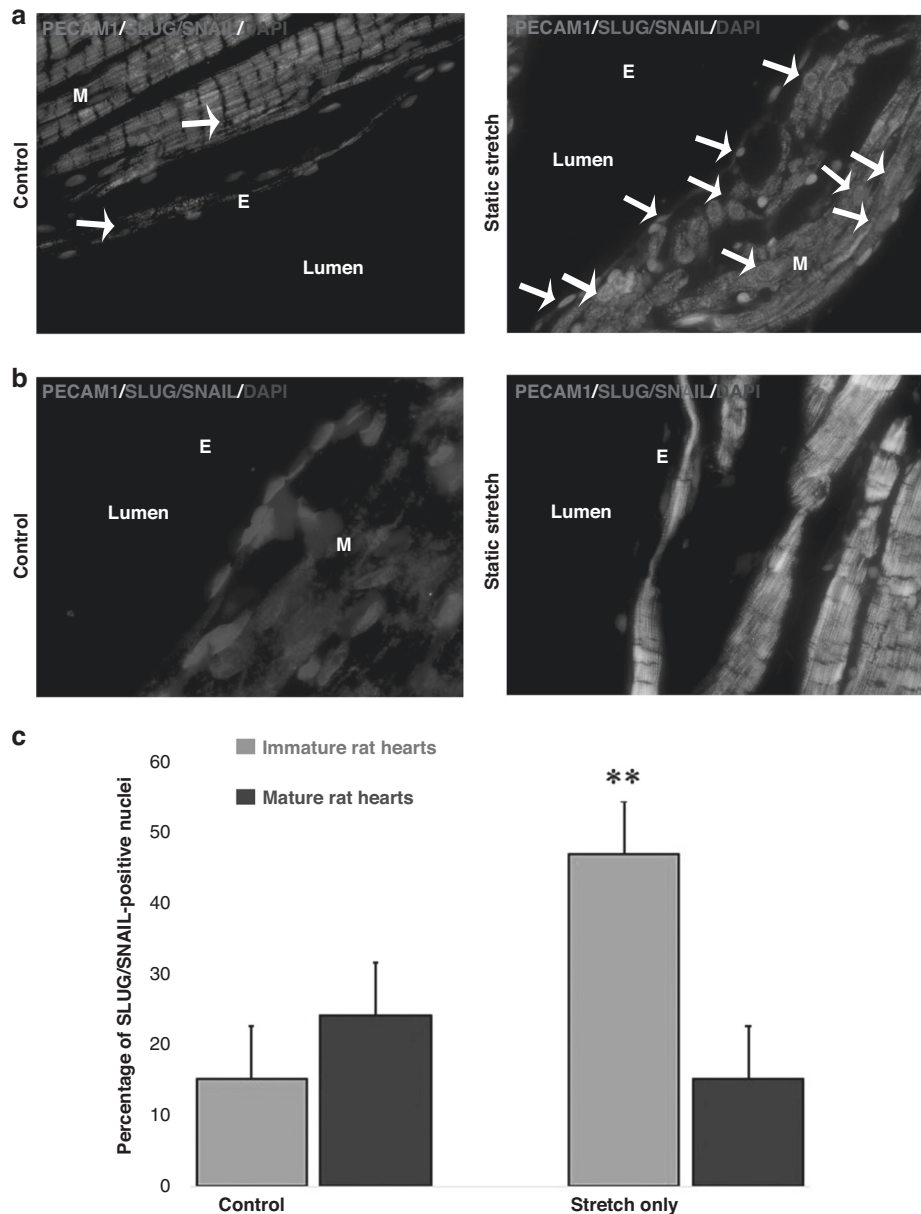
strain on the left ventricle. Increased mechanical forces mediate activation of the TGF- $\beta$  pathway as shown by the use of a TGF- $\beta$  inhibitor, and lead to EndMT of endocardial endothelial cells. Rebalancing the TGF- $\beta$ /BMP pathway through the addition of BMP-7 or localized application of losartan at the site of active EndMT, respectively, showed a significant reduction in EndMT of endocardial endothelial cells. These results correspond with our *in vitro* cell culture experiments when exposing endothelial cells to uniaxial stretch.

Endothelial cells are stabilized by their environment but display high plasticity to adapt to different pathological processes which include organ fibrosis or cancer.<sup>15</sup> This adaptive alteration of endothelial cells by phenotypical transformation to mesenchymal cells is known as EndMT which is regulated mainly by the TGF- $\beta$  pathway. It has been shown that in heart failure due to chronic pressure or volume loading, loss of endothelial cells to EndMT leads to myocardial fibrosis,<sup>9</sup> an indication that endothelial cells respond to mechanical forces adapting by changing their behavior. In isolated human umbilical vein endothelial cells, it has been reported that cyclic stretch induces EndMT through an integrin  $\beta$ 1 pathway.<sup>16</sup> Thus, EndMT has been identified as a new therapeutic target for fibrotic disorders such as pulmonary, intestinal, cardiac, and kidney fibrosis<sup>3,5,17</sup> but also has achieved interest in the cancer community for normal cells to take on the ability to migrate and transform into malignant cells.<sup>15</sup>

In this study, we sought to further investigate mechanical triggers for EndMT while controlling for flow and thus, an *in vitro* cell culture model was developed. HCAEC were used since they are known for their overlapping embryogenic origin with endocardial cells.<sup>18</sup> Cells were exposed to uniaxial static stretch-

inducing EndMT, which was confirmed by immunohistochemical assessment for double-stained cells.<sup>19</sup> Isolated cells in culture have limitations due to lack of environmental cues from other myocardial cells which is an important regulator for endothelial cell behavior. Thus, our next step was to develop a whole heart preparation of excess strain on the LV in a no-flow environment to eliminate flow alterations as a potential trigger for EndMT. We could show that endocardial endothelial cells but not endothelial cells of the myocardial microvasculature underwent EndMT when exposed to mechanical strain. However, only immature hearts responded through induction of EndMT of endocardial endothelial cells but mature hearts did neither develop endocardial nor myocardial EndMT in the short time frame of 3 h of increased stretch. Unlike myocardial fibrosis as a result of EndMT in hypertrophy and heart failure, endocardial fibroelastosis also a result of EndMT in humans, is predominantly present in young, immature hearts which these *in vitro* results shed more light on.<sup>6</sup> Furthermore, an imbalance of the BMP/TGF- $\beta$  pathway due to hypermethylation of the BMP-7 promoter was identified as a trigger for TGF- $\beta$  mediated EndMT in human endocardial fibroelastosis.<sup>2,6</sup> Clinical observation indicates that hemodynamic alterations imposed on the growing heart likely stimulate EndMT of endocardial endothelial cells and mechanical strain such as distention of the LV negatively impacts the progression of this fibrotic process especially in utero but could also be replicated in an animal model of EndMT-induced endocardial fibroelastosis.<sup>12,20</sup>

Confirmation of TGF- $\beta$ 's main regulatory role in endocardial EndMT in immature hearts, was supported by staining for the transcription factors SLUG/SNAIL which is downstream of TGF- $\beta$ -regulated expression of VE-cadherin.<sup>21</sup> Immature hearts exposed



**Fig. 5 EndMT-regulating transcription factors (SLUG/SNAIL) in immature versus mature hearts.** Immature (a) and mature hearts (b) were perfused in a non-working Langendorff set-up and exposed to static uniaxial stretch with 10x their heart weight for 3 h. Heart tissue was stained for the endothelial marker PECAM1 (green), SLUG/SNAIL (red), and DAPI (blue). Immature hearts showed supporting evidence by staining for the transcription factors SLUG/SNAIL (red), which indicated active EndMT by double-staining of nuclei with DAPI in blue. c Summary of the results are shown in this graph. Ratio of double-stained (SLUG/SNAIL/alpha-SMA) endothelial cells/total endothelial cell count in controls and stretched for immature and mature hearts. There were no significant differences between the groups in the mature rat hearts. Uniaxial stretch in mature hearts did not induce endocardial EndMT. In contrast, immature rats showed significantly more cells undergoing EndMT in stretched hearts compared to controls (\*\* $p < 0.05$ ).

to myocardial strain had significantly more nuclei staining positive for SLUG/SNAIL corresponding to the number of double-stained endocardial endothelial cells with endothelial markers (VE-cadherin, PECAM1) and mesenchymal markers ( $\alpha$ -SMA). To determine whether the TGF- $\beta$  pathway regulated stretch-induced EndMT in our two models, we exposed stretched endothelial cells and stretched hearts to local inhibitors of the TGF- $\beta$  pathway. The choice of inhibitors used in this study was based on our previous findings where we unraveled an imbalance of the TGF- $\beta$ /BMP-7 pathway in EndMT-induced fibrosis of the endocardium. BMP-7 has already been shown to inhibit EndMT in vitro in cell culture and in vivo in a rat model of endocardial fibroelastosis and we could now show, was also beneficial in

stretched hearts.<sup>6</sup> Secondly, we used a specific TGF- $\beta$  inhibitor that directly blocks TGF- $\beta$  binding to the ALK5-kinase receptor, which has previously been shown to upregulate claudin-5, a component of the endothelial tight junction responsible for cell-cell contacts in cell culture.<sup>22</sup> Furthermore, from a clinical perspective, our goal was to identify a clinically applicable inhibitor, and thus, we tested losartan which as an angiotensin receptor inhibitor blocks the TGF- $\beta$  pathway indirectly.<sup>23</sup>

In conclusion, with these two models, an in vitro cell culture and an ex vivo Langendorff model, we could successfully demonstrate that EndMT is induced by mechanical strain on the endocardium. Our data show for the first time that only immature hearts exposed to increased myocardial strain develop endocardial

endothelial changes but mature hearts do not respond by activation of EndMT at least in the short time frame of exposure to increased strain. These experimental data fit well with the clinical observation that endocardial EndMT is a disease affecting young patients and transforms into a more invasive growth pattern into the underlying myocardium with increasing age displaying the histological picture of myocardial fibrosis of adult heart failure.<sup>10</sup> By using specific inhibitors, we showed that this endocardial fibrotic process is TGF- $\beta$  pathway mediated and that clinically relevant drugs such as BMP-7 and losartan can successfully inhibit this process preserving the endocardium and subendocardium from EndMT-mediated fibrosis. From a clinical point of view, the information obtained from these in vitro studies is the basis to better understand the underlying mechanism which triggers the development of this unique endocardial fibrotic process. Despite progress with the treatment of EFE, currently only surgical resection is available which partially addresses the problem. As a next step, we will test pharmacological treatments identified by these experiments in an in vivo whole animal model which we have already developed with the ultimate goal to translate these findings into clinically applicable therapies for children.<sup>20</sup>

## DATA AVAILABILITY

The original data set used and analyzed during the current study is readily available from the corresponding author on reasonable request.

## REFERENCES

- Sumpio, B. E., Riley, J. T. & Dardik, A. Cells in focus: endothelial cell. *Int J. Biochem. Cell Biol.* **34**, 1508–1512 (2002).
- Traub, O. & Berk, B. C. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler. Thromb. Vasc. Biol.* **8**, 677–685 (1998).
- Zeisberg, E. M. et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat. Med.* **13**, 952–961 (2007).
- Arciniegas, E. et al. Endothelial-mesenchymal transition occurs during embryonic pulmonary artery development. *Endothelium* **12**, 193–200 (2005).
- Zeisberg, E. M. et al. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. *J. Am. Soc. Nephrol.* **19**, 2282–2287 (2008).
- Xu, X. et al. Endocardial fibroelastosis is caused by aberrant endothelial to mesenchymal transition. *Circ. Res.* **116**, 857–866 (2015).
- Pusztaszeri, M. P., Seelentag, W. & Bosman, F. T. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand Factor, and Fli-1 in normal human tissues. *J. Histochem. Cytochem.* **54**, 385–395 (2006).
- Yoshimatsu, Y. & Watabe, T. Roles of TGF- $\beta$  signals in endothelial-mesenchymal transition during cardiac fibrosis. *Int. J. Inflamm.* **2011**, 724080 (2011).
- Illigens, B. M. W. et al. Vascular endothelial growth factor prevents endothelial-to-mesenchymal transition in hypertrophy. *Ann. Thorac. Surg.* **104**, 932–939 (2017).
- Weixler, V. et al. Flow disturbances and the development of endocardial fibroelastosis. *J. Thorac. Cardiovasc. Surg.* **159**, 637–646 (2020).
- Emani, S. M. et al. Staged left ventricular recruitment after single-ventricle palliation in patients with borderline left heart hypoplasia. *J. Am. Coll. Cardiol.* **60**, 1966–1974 (2012).
- McElhinney, D. B. et al. Assessment of left ventricular endocardial fibroelastosis in fetuses with aortic stenosis and evolving hypoplastic left heart syndrome. *Am J Cardiol.* **106**, 1792–1797 (2010).
- Friehs, I. et al. Impaired glucose transporter activity in pressure-overload hypertrophy is an early indicator of progression to failure. *Circulation* **100**, II187–II193 (1999).
- Friehs, I. et al. Promoting angiogenesis protects severely hypertrophied hearts from ischemic injury. *Ann. Thorac. Surg.* **77**, 2004–2010 (2004).
- Kalluri, R. & Zeisberg, M. Fibroblasts in cancer. *Nat. Rev. Cancer* **6**, 392–401 (2006).
- Suzuki, M. et al. Up-regulation of integrin  $\beta 3$  expression by cyclic stretch in human umbilical endothelial cells. *Biochem. Biophys. Res. Commun.* **239**, 372–376 (1997).
- Rieder, F. et al. Inflammation-induced endothelial-to-mesenchymal transition: a novel mechanism of intestinal fibrosis. *Am. J. Pathol.* **179**, 2660–2673 (2011).
- Wu, B. et al. Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. *Cell* **151**, 1083–1096 (2012).
- Kong, P. et al. Lack of specificity of fibroblast-specific protein 1 in cardiac remodeling and fibrosis. *Am. J. Physiol.* **305**, H1363–H1372 (2013).
- Shimada, S. et al. Distention of the immature left ventricle triggers development of endocardial fibroelastosis: An animal model of endocardial fibroelastosis introducing morphopathological features of evolving fetal hypoplastic left heart syndrome. *Biomed. Res. Int.* **2015**, 46469 (2015).
- Lopez, D. et al. Tumor-induced upregulation of Twist, Snail, and Slug represses the activity of the human VE-cadherin promoter. *Arch. Biochem. Biophys.* **482**, 77–82 (2009).
- Watabe, T. et al. TGF- $\beta$  receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. *J. Cell Biol.* **163**, 1303–1311 (2003).
- Wu, M. et al. Losartan attenuates myocardial endothelial-to-mesenchymal transition in spontaneous hypertensive rats via inhibiting TGF- $\beta$ /Smad signaling. *PLoS ONE* **11**, e0155730 (2016).

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## AUTHOR CONTRIBUTIONS

C.V., V.W., and I.F. provided substantial contributions to conception and design, C.V., V.W., M.D., P.H., R.-Z.L., and I.F. contributed to the acquisition and analysis of data. C.V., V.W., M.D., P.H., J.M.M.-M., R.A.-F., P.J.d.N., and I.F. were involved in the interpretation of data. C.V., V.W., and I.F. drafted the article, and R.A.-F., P.H., R.-Z.L., J.M.M.-M., and P.J.d.N. commented for revision of the manuscript. All authors granted final approval of the version to be published.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

**Correspondence** and requests for materials should be addressed to Ingeborg Friehs.

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