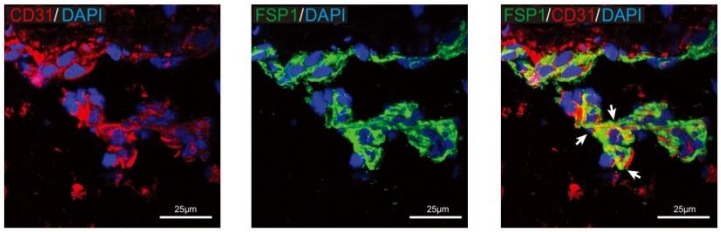
Supplemental Material

Online Figure I EndMT in EFE tissues

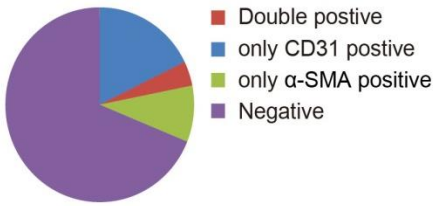
A



B

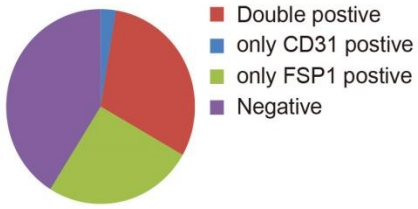
C

Sample	Total cells	CD31+	aSMA+	CD31+/aSMA
Nr.1	404	85	90	32
Nr.2	783	141	71	21
Nr.3	423	126	65	15
Nr.4	183	39	52	9
Nr.5	570	150	66	26
Nr.6	420	67	29	10
Mean	463,83	101,33	62,17	18,83



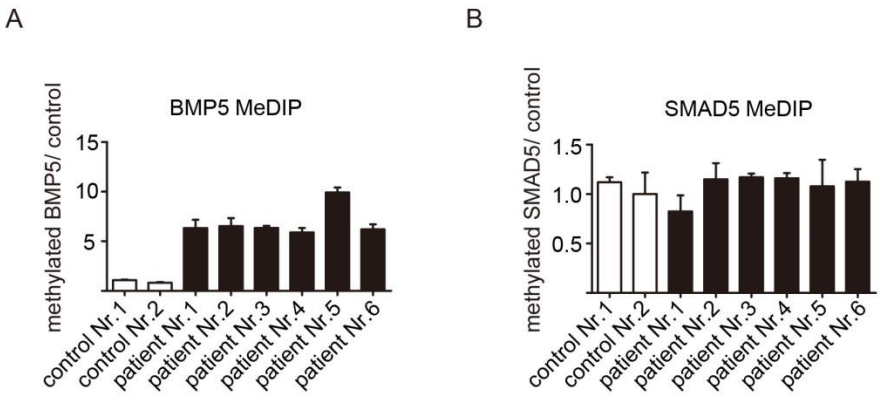
D

Sample	Total cells	CD31+	FSP1+	CD31+/FSP1
Nr.1	216	77	109	76
Nr.2	145	69	95	63
Nr.3	119	33	103	31
Nr.4	448	138	346	126
Nr.5	639	173	245	157
Nr.6	225	131	145	118
Nr.7	568	129	255	116
Nr.8	367	157	237	148
Mean	340,88	113,38	191,88	104,38



Online Figure I. (A) Representative confocal photomicrographs of surgically removed EFE tissue from HLHS patients double stained for endothelial (CD31) and fibroblast specific markers (FSP1). White arrows highlight representative double positive cells. **(B)** Cell counts of the percentage of aSMA/ FSP1 expressing cells and their co-expression with CD31. **(C, D)** Cell counts of the aSMA **(C)** and FSP1 **(D)** expressing cells and their co-expression with CD31 are presented as pie charts to demonstrate single-positive, double-positive as well as negative cells. White arrows denote representative double positive cells, the size of the scale bars represents as noted above.

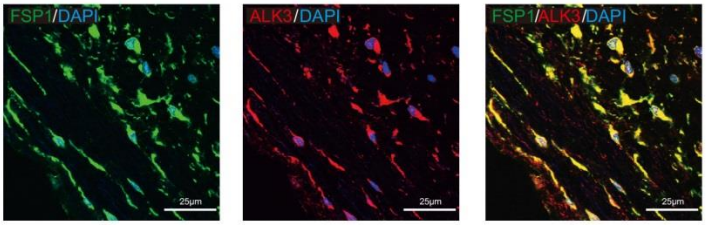
Online Figure II BMP5 and SMAD5 promoter methylation of EFE tissues detected by MeDIP assay



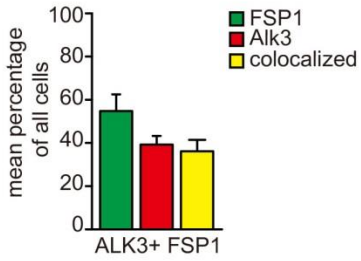
Online Figure II. Methylated DNA immunoprecipitation (MeDIP) to assess *BMP5* **(A)** *and SMAD5* **(B)** promoter methylation in healthy and EFE tissue samples. The immunoprecipited DNA was quantified by Real-time PCR analysis with the primers located in the methylated CpG promoter region.

Online Figure III BMPR1A (ALK3) receptor in EFE

A

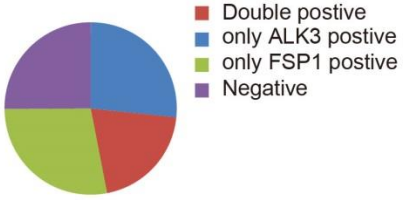


B

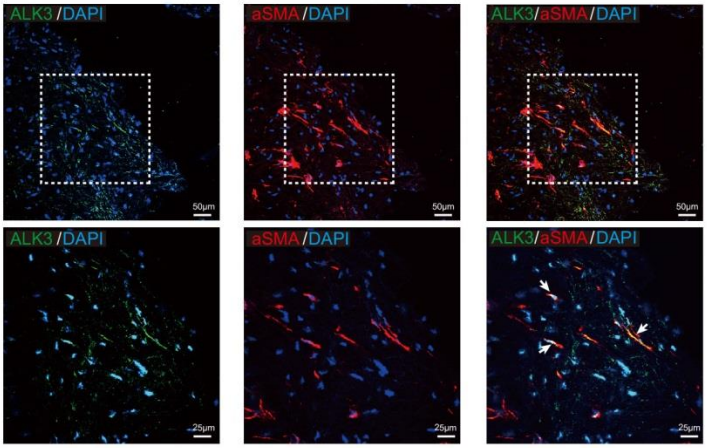


C

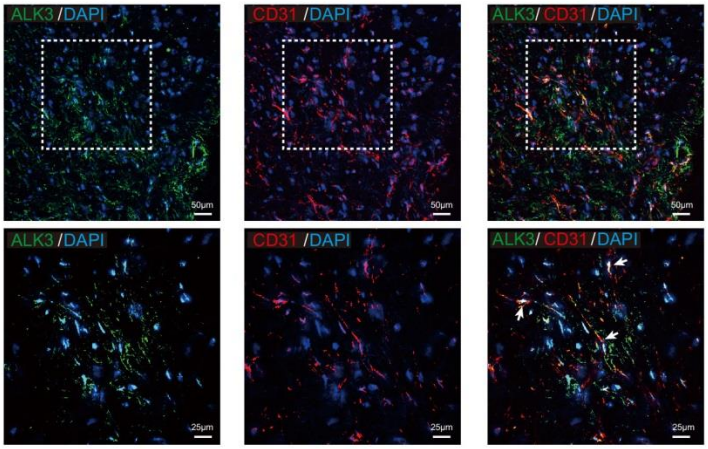
Sample	Total cells	ALK3+	FSP1+	ALK3+/FSP1+
Nr.1	154	68	96	65
Nr.2	158	49	58	46
Nr.3	186	102	73	68
Nr.4	92	44	66	44
Nr.5	221	130	85	76
Nr.6	275	94	133	71
Mean	181,00	81,17	85,17	61,67



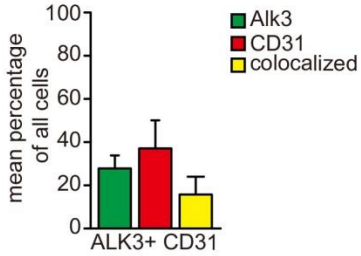
D



E

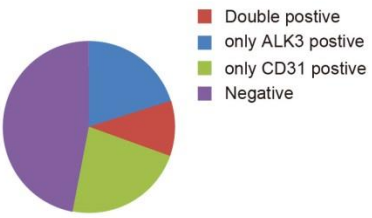


F



G

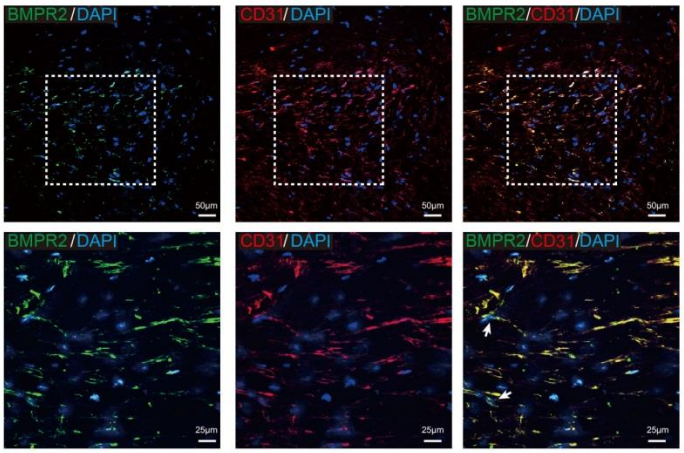
Sample	Total cells	ALK3+	CD31+	ALK3+/CD31+
Nr.1	82	56	36	33
Nr.2	188	42	54	20
Nr.3	216	60	83	39
Nr.4	107	51	25	7
Nr.5	425	44	66	25
Nr.6	368	99	130	58
Mean	231,00	58,67	65,67	30,33



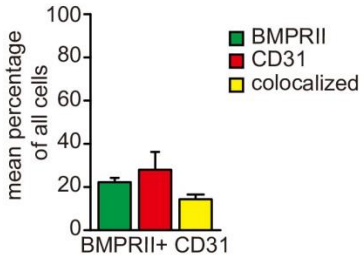
Online Figure III. Representative confocal photomicrographs of surgically removed EFE tissue from HLHS patients double stained for Bone morphogenetic protein receptor BMPR1A (ALK3) with fibroblast specific marker FSP1 **(A)** and aSMA **(D)** or with endothelial specific marker CD31 **(E)**. White arrows denote representative double positive cells; dotted areas denote the regions were magnified in the lower panel. The size of the scale bars represents as noted above. **(B, F)** The bar graphs display cell counts of the percentage of Alk3 expressing cells and their co-expression with FSP1 **(B)** or with CD31 **(F)**. Cell counts of the number of Alk3 expressing cells and their co-expression with FSP1 **(C)** or with CD31 (**G**) and representation as table and pie chart.

Online Figure IV BMPRII receptor in EFE

A

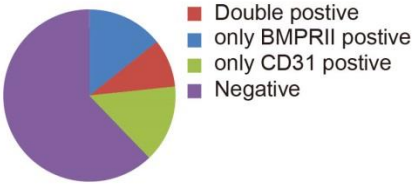


B



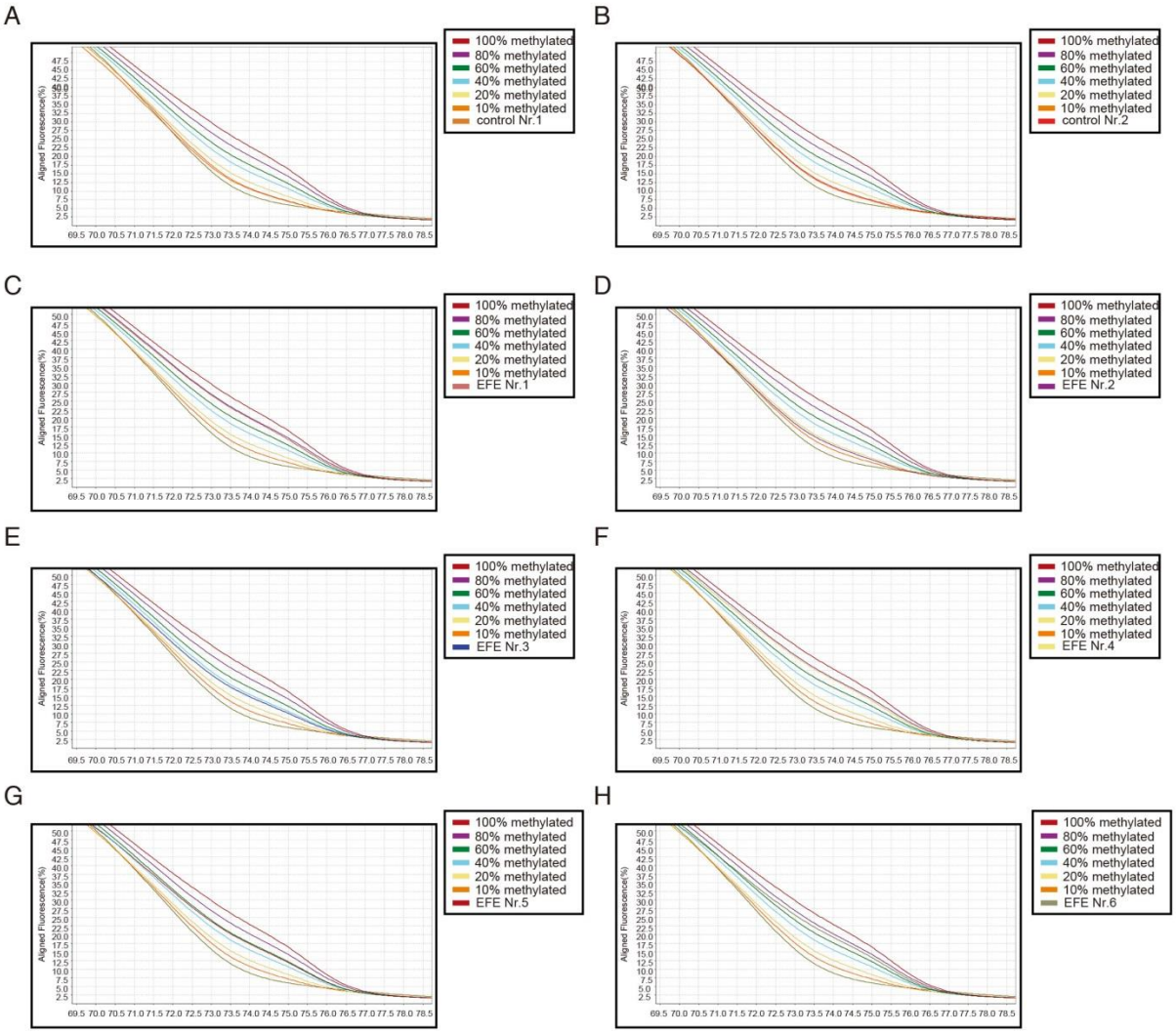
C

Sample	Total cells	BMPRII+	CD31+	BMPRII+/CD31+
Nr.1	90	41	21	19
Nr.2	226	53	61	38
Nr.3	316	94	82	57
Nr.4	98	48	19	14
Nr.5	526	52	87	46
Nr.6	421	94	117	67
Mean	279,50	63,67	64,50	40,17



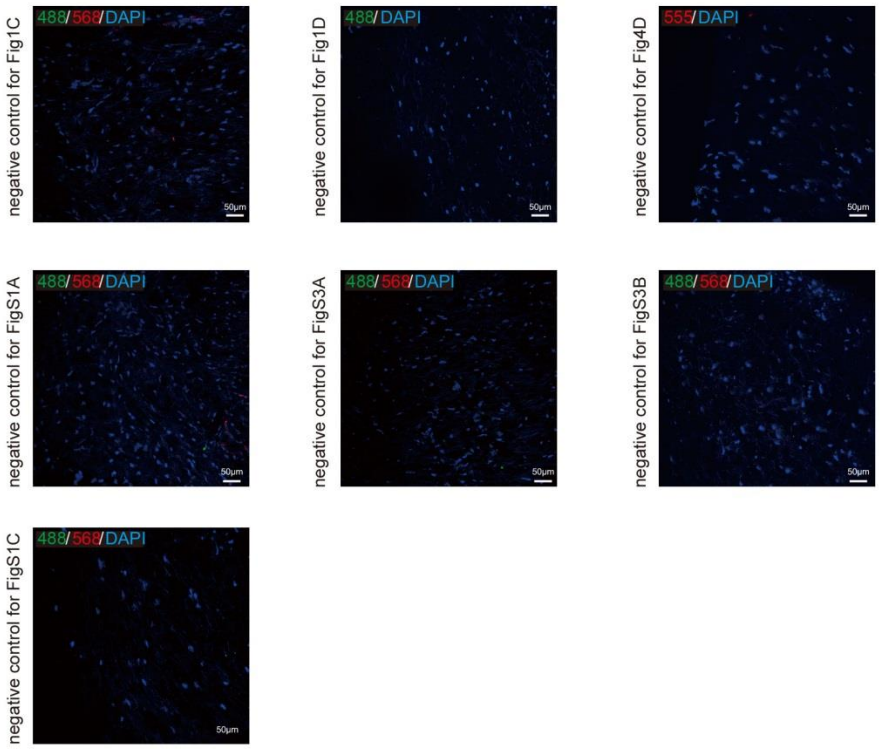
Online Figure IV. Representative confocal photomicrographs of surgically removed EFE tissue from HLHS patients double stained for **(A)** Bone morphogenetic protein receptor BMPRII with endothelial specific marker (CD31). White arrows denote representative double positive cells; dotted areas denote the regions were magnified in the lower panel. The size of the scale bars represents as noted above. **(B)** The bar graphs display cell counts of the percentage of BMPRII expressing cells and their co-expression with CD31. **(C)** Cell counts of the number of BMPRII expressing cells and their co-expression with CD31 and represented as table and pie chart.

Online Figure V MS-HRM melting curves in EFE samples



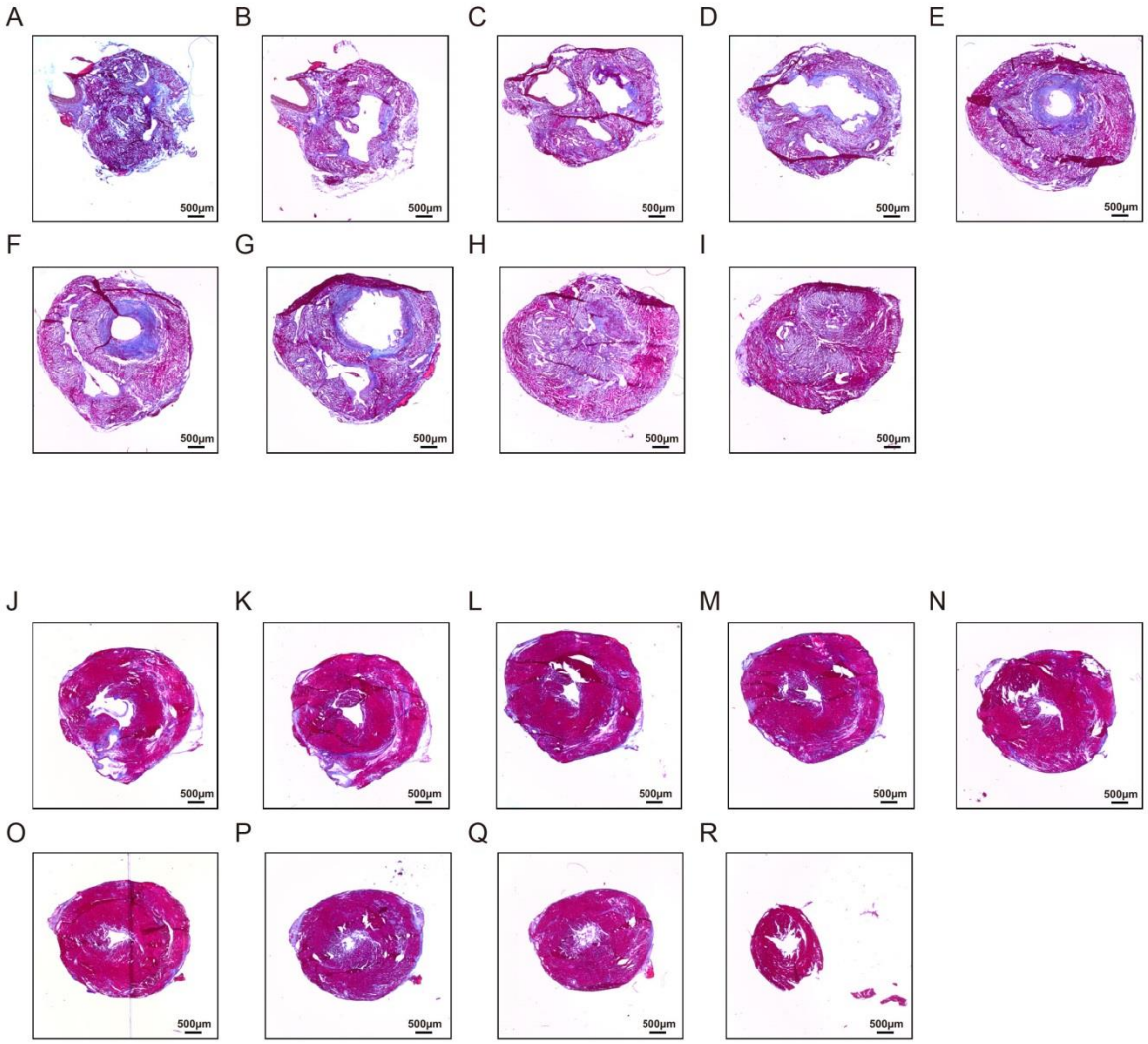
Online Figure V. Normalized HRM curve-based estimation of methylation levels for human *BMP7* promoter. HRM curves derived from control samples **(A, B)** and EFE samples **(C-H)** were plotted against HRM profiles of PCR product derived from standard with known concentration of methylated to unmethylated template. The results show that the methylation of the control samples are in the range of 1-10%, in contrast, the EFE samples vary between 20-80%.

Online Figure VI Negative controls for staining (secondary antibodies only)



Online Figure VI. The confocal photomicrographs of surgically removed EFE tissue from HLHS patients stained only with secondary antibodies to rule out the false positive effect from unspecific binding. Single or double staining with different combinations are indicated on the images corresponding to the immunofluorescence staining with primary antibodies.

Online Figure VII EFE area of slices located from base to apex of neonatal rat heart



Online Figure VII. Representative photomicrographs of Masson's Trichrome–stained serial sections located from base to apex of neonatal rat hearts two weeks after heterotopic transplantation without (A-I) and after rhBMP7 treatment (J-R).

Methods in detail

Animals Model of Unloaded Heart Transplantation

Heterotopic heart transplantation was performed in C57/BL6 mice and Lewis rats as well as ubiquitous EGFP rats (strain F344-Tg(UBC-EGFP)F455Rrrc) from the Rat Resource & Research Center (RRRC-P40OD011062) following a method previously described[1]. Young adult Lewis rats (100-120g) or C57/BL6 mice (20-25g) served as recipients and donor hearts were obtained from neonatal rats (2-4 days of age) or Tie2Cre;Rosa-Stop-YFP mice (7-10 days of age) respectively. Tie2Cre and Rosa-Stop-YFP mice have been described previously. Animals were anesthetized with ketamine (40-60mg/kg i.p.)/xylazine (10mg/kg i.p.) and isoflurane via endotracheal tube. The donor rats and mice received 300IU and 60IU heparin respectively, and the heart was explanted through a midline thoracic incision followed by storage in cold high potassium Krebs-Henseleit solution as previously described[1]. Implantation of the heart was performed as a heterotopic infra-renal graft unloaded with aorta to aorta and pulmonary artery (PA) to inferior vena cava (IVC) anastomoses. Animals were survived for 2 weeks. The recipient rats received either 30 µg/kg rhBMP7 or vehicle buffer i.p. every other day, starting on the day of surgery.

DNA isolation

Tissues were first lysed in DNA lysis buffer (Qiagen, Hilden, Germany) then precipitated and isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) followed by manufacturer's protocol.

Histological Analysis of Transplanted Hearts and EFE tissue

Snap frozen human EFE tissue embedded in OCT was sectioned and stained with Hematoxylin & Eosin and Masson Trichrome staining. The same staining was applied to the paraffin-embedded rat and mouse hearts, which were sectioned, de-paraffinized and rehydrated with xylene and graded alcohol series prior to staining. Mouse hearts from Tie2Cre; Rosa-Stop-YFP mice and EGFP rats were additionally equilibrated into 30% sucrose prior to embedding to preserve endogenous YFP signal.

Fibrosis in rat hearts was quantified using the Image Pro Plus Software.

Immunofluorescence

Frozen human EFE tissue sections were fixed with ice cold acetone for 10min at -20°C, after 3 times washing with PBS, and then blocked with 1%BSA in PBS for 30min at room temperature. To examine the marker gene expression in the tissue, samples were incubated with primary antibody overnight at 4°C, washed three times in PBS and incubated with the secondary antibody for one hour at room temperature. The primary and secondary antibodies used in this study with dilution factors are listed below. Microscopic images were acquired using an Olympus Confocal Microscope FV-1000 and Fluoview program. The acquired images were processed using Photoshop CS3 software.

Antibody	**Product code**	**Dilution**	**Company**
Rabbit Anti-Human αSMA	ab32575	1:100	Abcam
Mouse Anti-Human CD31	M0823	1:100	Dako
Rabbit Anti-Human Twist1	ABD29	1:100	Millipore
Rabbit Anti-Human FSP1	A5114	1:50	Dako
Goat Anti-Human BMPR-1A	sc-5676	1:50	Santa Cruz
Rabbit Anti-Human BMPR-1A	sc-20736	1:50	Santa Cruz
Rabbit Anti-Human BMPR2	ab106266	1:100	Abcam
Alexa Fluor 488 donkey anti rabbit	A21206	1:200	Life technologies
Alexa Fluor 568 donkey anti mouse	A10037	1:200	Life technologies
Alexa Fluor 488 donkey anti goat	A11055	1:200	Life technologies
Alexa Fluor 555 donkey anti rabbit	A31572	1:200	Life technologies

In vitro EndMT assay

HCAEC (6 x 10^5 cells per plate) were plated in 10cm cell culture plates and cultured for 24 hours. Then the cells were starved with endothelial cell basal medium (Genlantis) overnight. EndMT induction was performed by 1:10 dilution of HCAEC growth medium by endothelial basic medium (Genlantis, San Diego, USA) with 10 ng/ml TGFβ1 (R&D Systems, USA). Medium with TGFß1 was changed every other day. BMP7 was added together with TGFß1 using 100 ng/ml for EndMT rescue.

Methylated DNA immunoprecipitation (MeDIP)

Methylated DNA was captured using Methylamp Methylated DNA capture Kit (Epigentek, Farmingdale, USA). 1.0µg of sonicated DNA was used in each antibody coated well and incubated at room temperature for 2 hours on a horizontal shaker. The captured DNA was released with proteinase K at 65 °C for 1 hour. DNA was eluted from the column and adjusted to a final volume of 100µl with nuclease-free water. For each sample, an input vial was performed using total sonicated DNA as loading control. To visualize the

immunoprecipitated products, PCR products were loaded on the Bioanalyzer 2100 electrophoresis system (Agilent Technologies). Electrophoresis results are shown as a virtual gel as previously described[2].

RNA extraction and quantitative real-time PCR

RNA was extracted from cells by direct lysis with Trizol regent (Ambion) and subsequent isolation using the PureLink RNA Mini Kit (Ambion) following the manufacturers protocol. Tissues were homogenized using the TissueLyserLT (Qiagen) and RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was synthesized using the SuperScript II Reverse Transcriptase (Life technologies) according to manufacturer's instructions. *Quantitative* Real-Time PCR was carried out using the Fast SYBR Green Master Mix (Life Technologies) in the StepOne Plus realtime PCR system (Life Technologies) with the real-time PCR primers (sequence listed below). Measurements were standardized to GAPDH using delta delta Ct methods.

Gene	Sequence	Supplier
a-SMA	F: AAGCACAGAGCAAAAGAGGAAT R: ATGTCGTCCCAGTTGGTGAT	Primer design Southampton, UK
BMP2	F: GGGCATCCTCTCCACAAAAG R: CCACGTCACTGAAGTCCAC	Primer design Southampton, UK
BMP5	F: ATCCTCGTCGCATACAGTTATC R: TGTCAGCATCATTCAGAAAGTTG	Primer design Southampton, UK
BMP7	F: CCTCCATTGCTCGCCTTG R: TATGCTGCTCATGTTTCCTAATAC	Primer design Southampton, UK
CD31	F: AAGGAACAGGAGGGAGAGTATTA R: GTATTTTGCTTCTGGGGACACT	Primer design Southampton, UK
COL1A1	F: AGACAGTGATTGAATACAAAACCA R: GGAGTTTACAGGAAGCAGACA	Primer design Southampton, UK
FSP-1	F: TCTTTCTTGGTTTGATCCTGACT R: AGTTCTGACTTGTTGAGCTTGA	Primer design Southampton, UK
GAPDH	undisclosed	Primer design Southampton, UK
ID1	F: CTGCTCTACGACATGAACGG R: GAAGGTCCCTGATGTAGTCGAT	Eurofins MWG Operon
ID2	F: AGTCCCGTGAGGTCCGTTAG R: AGTCGTTCATGTTGTATAGCAGG	Eurofins MWG Operon
ID3	F: GAGAGGCACTCAGCTTAGCC R: TCCTTTTGTCGTTGGAGATGAC	Eurofins MWG Operon
NOGGIN	F: GCCAGCACTATCTCCACATCC R: GCAGCAGCGTCTCGTTCA	Primer design Southampton, UK
SLUG	F: ACTCCGAAGCCAAATGACAA R: CTCTCTCTGTGGGTGTGTGT	Primer design Southampton, UK
SMAD5	F: TCTCCAAACAGCCCTTATCCC	Eurofins MWG

	R: GCAGGAGGAGGCGTATCAG	Operon
SNAIL	F:GGCAATTTAACAATGTCTGAAAAGG R:GAATAGTTCTGGGAGACACATCG	Primer design Southampton, UK
TGFß1	F: CACTCCCACTCCCTCTCTC R: GTCCCCTGTGCCTTGATG	Primer design Southampton, UK
TGFß2	F: TACGCCAAGGAGGTTTACAAA R: TGAAGTAGGGTCTGTAGAAAGTG	Primer design Southampton, UK
TGFß3	F: ACTATGCCAACTTCTGCTCAG R: CAGATGCTTCAGGGTTCAGA	Primer design Southampton, UK
TWIST	F: CTCAAGAGGTCGTGCCAATC R: CCCAGTATTTTTATTTCTAAAGGTGTT	Primer design Southampton, UK
VE-Cadherin	F: CGTGAGCATCCAGGCAGTGGTAGC R: GAGCCGCCGCCGCAGGAAG	Eurofins MWG Operon

SYBR-based amplification of methylated BMP7 promoter

For quantification of methylated DNA amplification, 5µl of eluted DNA was used for each PCR reaction, with a ROX passive reference dye (Bio-Rad, Hercules, USA) and diluted 2x SYBR green Supermix (Bio-Rad, Hercules, USA) in a final volume of 25 µl for each PCR reaction. The real-time PCR reactions were performed in a 96-well plate using the Mx3000P qPCR system (Stratagene, Santa Clara, USA). PCR reaction was manually terminated when the fluorescent signal increased over the threshold. The electrophoresis of PCR products was performed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) according to the manufacturer's protocol. Electrophoresis results are shown as virtual gel images. Oligonucleotide sequences for methylated BMP7, BMP5, SMAD5 are listed below.

Gene	Sequence	Supplier
BMP7	F: GCAGGTCTTGGAGGTCTCTG R: CCAGTGGGTTCATTCATTCC	Eurofins MWG Operon
BMP5	F: GTCCCATGCATCACTGTTTCA R: TGCTGGGAAAGAAGAGGCTT	Eurofins MWG Operon
SMAD5	F: GGATACACACCCTTGATAGGACTG R: TCTAACTACCAGCTGATTGCAGTAGCC	Eurofins MWG Operon

TGF-β/BMP qPCR Array

A human TGFβ/BMP Signaling Pathway PCR array (SABiosciences, PAHS-035Y) was used to analyze the expression levels of BMP signaling pathway components in healthy neonatal left ventricular control tissue and EFE tissue. Briefly, 5 μg of total RNA was used for first-strand cDNA synthesis with the RT2 first strand kit (Qiagen, Germany). The PCR array was carried out following the manufacturer's instructions using the ready-to-use RT^2-qPCR master mix (RT^2-SYBR® Green/Fluorescein qPCR master mix, SABiosciences,

Germany). Twenty microliters of the experimental cocktail were added into each well containing pre-dispensed, gene-specific primer pairs and run on a StepOne Plus realtime PCR system (Life technologies). Data analysis was performed using the web-based standard RT PCR array suite (SABiosciences, Germany).

Bisulfite modification and Bisulfite genomic sequencing

200 ng of DNA from each sample were treated with sodium bisulfite using the "Cells- to-CpG Bisulfite Conversion Kit" (Life technologies, UK) according to the manufacturer's protocol. Gene specific bisulfite sequencing analysis was performed as previously described[3]. In short, for sequencing of methylated BMP7 promoter region, the DNA samples were first bisulfite converted and amplified using primers, (forward: AAAAGGATATAGGGATTGAGGGGTAAG, reverse: CCGCCCTCCCCCCAACTATACCCAATAAATTC). Then the PCR product was gel-extracted, purified and then ligated into pGEM-T Easy vector system (Promega). The sequencing experiment was performed by the Seqlab using T7 primer. The result was analyzed with BDPC, a webpage-based program[4].

High resolution melting analysis (HRM)

Methylation sensitive high resolution melting experiments were performed as previously described[5]. Briefly, PCR amplification and high resolution melting analysis were carried out using StepOne Plus realtime PCR system (Life technologies). PCR was carried out with MeltDoctor HRM Master Mix (Life technologies) and 1 µl of bisulphite modified DNA template. The amplification consisted of 10 min at 95°C, followed by 40 cycles of 15 s 95°C, 1min of anneal/extension. The continuous ramp mode of melting curves was added at the end of PCR cycles. All reactions were performed in triplicate. The melting curves were normalized by calculation of the 'line of best fit' in between two normalization regions. 100% methylated DNA was mixed in different ratios with unmethylated DNA to generate MS-HRM standard curves with 120bp amplicon containing 5 CpG dinucleotides. The primer sequence used in MS-HRM analysis were forward: CACGGAGTAATTTAAACAGT, reverse: CCCAAAGACCTCCAAAACCT.

In vitro BMP7 promoter methylation and luciferase assay

For human BMP7 promoter methylation analyses, a 2.1 kb promoter fragment (2271 bp to 161 bp upstream of the BMP7 transcription start site) was cloned from BAC clone into

BamHI and HindIII sites of pCpG-Basic vector[6]. The construct was *in vitro* methylated using M.SssI, CpG methyltransferase (NEB) according to manufacturer's protocol, and luciferase activity was measured by Duo-Glo luciferase assay system (Promega) as suggested by the company's instruction.

Regulation of VE-Cadherin promoter activity by Smad-dependent pathways.

In order to achieve optimal transfection efficiency, we immortalized HCAEC cells with human telomerase reverse transcriptase (hTERT) delivered by lentivirus. The immortalized endothelial cells were transfected with Lipofectamine 2000 transfection reagent(Life Technologies) according to the manufacture's recommendations, with combinations of the following plasmids: human VE-Cadherin-pGL3, Renilla expression vector pGL4.73(Promega), Smad expression vector(pcDEF3-Flag(N)-hSmad3, pcDEF3-Flag(N)-hSmad4, pcDEF3-Flag(N)-hSmad5), constitutively active type I receptor expression vector pcDNA3-HASL-ALK3(QD) and pcDNA3-HASL-ALK5(TD) and empty control pcDNA3 vectors (Promega) as previously described[7-9]. The luciferase activity was determined by Duo-Glo luciferase assay system (Promega) and normalized luciferase activities to Renilla luciferase activity under the control of the CMV promoter.

References:

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