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SUPPLEMENTAL MATERIAL

Lack of primary cilia primes shear-induced Endothelial-to-Mesenchymal Transition

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Short title: Role of primary cilia in shear induced EndoMT

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METHODS

Cell culture and supplements

Generation of mouse embryonic wild type EC (WT) and mouse embryonic EC with a mutation in the Tg737 gene (IFT88Tg737RPW or Tg737$^{orpk/orpk}$) from the Oak Ridge Polycystic Kidney mouse was previously described[1]. Cells were passaged twice a week and maintained on 1% w/v gelatin (Merck, Darmstadt, Germany) in advanced DMEM medium (Invitrogen, Breda, the Netherlands) supplemented with 4.5 g/L D-glucose (Invitrogen), 110 mg/L sodium pyruvate (Invitrogen), non-essential amino acids (Invitrogen), 2% (v/v) heat inactivated Fetal Calf Serum (Sigma-Aldrich Chemie, Steinheim, Germany), 0.5% (v/v) antibiotic/ antimyotic solution (Invitrogen), 1% (v/v) insulin, transferin, selenium supplement (Invitrogen), and 2mM L-glutamine (Invitrogen). For some of the experiments, the culture medium was supplemented with Tgfβ3 (1ng/ml), Tgfβ neutralizing antibody (α-Tgfβ, 2G7 IgG2b, 10ug/ml)[2], Alk5 kinase inhibitor SB431542 (10μmol/L in DMSO; Tocris)[3], or DMSO (Sigma).

Shear stress exposure

For the shear stress experiments, WT and Tg737$^{orpk/orpk}$ EC were seeded on fixed 1% (w/v) gelatin coated coverslips and grown to confluence. EC were subjected to 0.5 and 2.5 Pa shear stress for 2 or 24 hours at 37°C and 5% CO_2 in a re-circulation parallel plate flow system as previously reported[4]. Responses of shear-exposed cells were compared to those of static cultures. To block Alk5 kinase activity during exposure to shear stress, cells were pre-incubated with SB431542 for 1 hour and then exposed to shear stress in medium supplemented with the compound at the same concentration. 0.1% (v/v) DMSO (Sigma) served as a sham for the SB431542 experiments. To neutralize functional Tgfβ either present in the medium or produced by the cells during exposure to shear stress, cells were pre-incubated with a pan-Tgfβ neutralizing antibody for 1 hour prior to shear exposure in the presence of antibody in equal concentration. Static controls were treated identically with the omission of flow. Directly following incubation or exposure to flow, cells were fixed for immunofluorescence analysis (n=3) or lysed for RNA (n=4) or protein (n=3) isolation. Hoffman modulation contrast images were taken using Nikon Eclipse Ti inverted microscope system (20x and 40x objectives).

Constructs and transfection

The Ift88*/Tg737 expression construct encodes the Ift88 protein (90 kDa) and a 28kDa tag (mCherry, denoted by an asterisk (*)). Tg737$^{orpk/orpk}$ EC were transfected with Lipofectamine (Invitrogen) according to the manufacturer's protocol. Stable cell lines were generated by drug selection using 500 μg/ml G418 (Invitrogen) to obtain Tg737$^{orpk/orpk}$-Ift88*. Expression from the Ift88* construct was evaluated using Q-PCR and Western Blot analysis and compared to Tg737$^{orpk/orpk}$ cells which were stably transfected with pEGFP-N1 (Clontech R&D, Palo Alto, USA), which served as control.

Full length mouse Klf4 cDNA was amplified by PCR using F:5'-CCCACATTAATGAGGCAGC-3' and R:5'-GGTCACATCCACTACGTGG-3' primers and cloned under the regulatory control of the human cytomegalovirus (CMV) immediate early promoter/enhancer into pcDNA3.3 (Invitrogen). Cells were transfected 24 hours prior to the start of experiments using Lipofectamine to obtain Tg737$^{orpk/orpk}$-Klf4. All experiments were performed in quadruplicate (n=4). pcDNA3.3-LacZ (Invitrogen) served as control.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 0.1mol/L phosphate buffer (pH7.4) for 10 min at room temperature. Permeabilized cells were incubated with antibody against acetylated α-tubulin (6-11B-1, 1:2000, Sigma) and αSMA (1A4, 1:1000, Sigma) for 3 hours at 37ºC, followed by incubation with FITC-labeled rabbit-anti-mouse antiserum (1:100, DAKO). For the CD31

staining, cells were incubated overnight with PECAM-1 antibody (M-20, 1:100, Santa Cruz), followed by incubation with biotin-labeled swine-anti-rabbit antiserum (1:100, DAKO) and FITC conjugated avidin (1:100, Vector labs). DAPI (1:1000, Invitrogen) was used for nuclear counterstaining and cells were mounted in ProLong Gold (Molecular Probes). Confocal scanning was performed using a Leica SP5 confocal scanning laser microscope (63x and 100x oil immersion objectives). ImageJ and Photoshop 10.0 were used for processing the data.

Q-PCR

Total RNA was isolated (RNeasy, Qiagen) and was treated with DNAse-I (Qiagen) according to the manufacturer's protocol. IScript cDNA synthesis kit (Bio-Rad) was used to reverse transcribe 500ng of RNA into cDNA. Real-time Q-PCR was performed using iQ SYBR Green Supermix (Bio-rad) in a Mx3000 real-time thermocycler (Stratagene) as described[5]. The reaction mixture consisted of the following: 1x PCR Master Mix, 1µl cDNA template, and 10 pmol of each specific primer. Gene specific primers are listed in Online Table I. The PCR program consisted of a hot start activation step, followed by 50 cycles of 30 seconds at 95°C, 60 seconds annealing at 58°C, and 30 seconds extension at 72°C. Dissociation analysis was performed in all reactions to exclude the presence of primer-dimers and confirm the amplification of unique targets. No-template controls were used as negative controls. Relative expression levels were normalized to the housekeeping gene *GAPDH* to compensate for the differences in RNA input.

Western Blotting

Western blot analysis was performed as described[6]. Protein samples were separated on a 8% SDS polyacrylamide gel, transferred onto a nitrocellulose membrane (Hybond P; Amersham Pharmacia Biotech), and incubated overnight at 4ºC with antibody against CD31 (M-20, 1:500, Santa Cruz), Klf4 (ab34814, 1:700, Abcam), Ift88 (Polaris B1700, 1:5000)[7], phospho-Smad2 (138D4, 1:1000, Cell Signaling) and GAPDH (6C5, 1:5000, Millipore). The blots were then incubated with peroxidase-conjugated anti-rabbit (CD31, Klf4, Ift88, phospho-Smad2) and anti-mouse (GAPDH) secondary antibody (1:1000; GE Healthcare), and developed using the ECL Plus Western blotting detection system (Thermo Scientific). Signals were quantified using densitometry and normalized to GAPDH. Western Blots shown in the Figures are representative of 3 independent experiments.

Mice

The Oak Ridge Polycystic Kidney ($Tg737^{orpk/orpk}$) animals were generated by a transgene insertion mutagenesis as described previously[8] and lines were maintained as heterozygous crosses on an inbred FVB/N genetic background. Animals were treated and maintained in accordance with the Institutional Animal Care and Use Committee regulations at the University of Alabama at Birmingham. Genotyping was performed as described previously[9]. E11.5 embryos were fixed overnight in 4% parafolmaldehyde in 0.1 mol/L phosphate buffer (pH7.4) and routinely processed for paraffin immunohistochemical analysis. Specimens were sectioned transversely through the heart at 10µm.

Immunohistochemistry

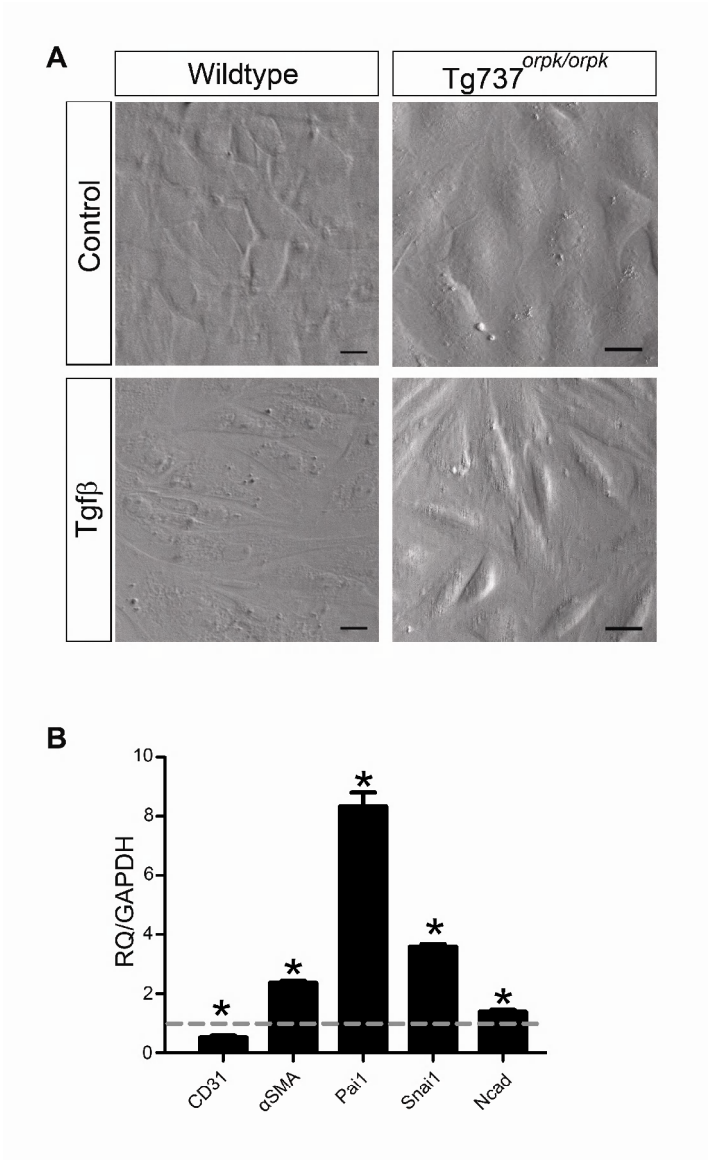
After deparaffination and rehydration of the sections, microwave antigen retrieval was applied by heating the slides for 12 min to 98ºC in citric acid buffer (0.01M in aqua-dest, pH6.0). Inhibition of endogenous peroxidase was performed after which the sections were incubated overnight with anti-phospho-Smad2 antibodies (138D4, 1:100 in PBS, Cell Signaling) and anti-αSMA antibodies (1A4, 1:3000 in 1%BSA/PBST, Sigma-Aldrich Chemie). Slides were rinsed in PBS and incubated with the secondary antibodies for 1 hour at room temperature: for phospho-Smad2 with biotin conjugated goat-anti-rabbit secondary antibodies (1:200 in PBS/1.5% NGS, Vector Laboratories) and for αSMA with horseradish peroxidase conjugated rabbit-anti-mouse antibodies

(1:250 in 1%BSA/PBST, DAKO). Subsequently phospho-Smad2 stained slides were incubated with ABC-reagent (Vector Laboratories) for 45 min and all the slides were visualized with 400ug/ml 3-3'di-aminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich Chemie). The slides were then dehydrated and mounted with Entellan (Merck). All the slides were processed simultaneously and Tg737$^{orpk/orpk}$ embryos (n=3) were compared with wild type littermates (n=4).

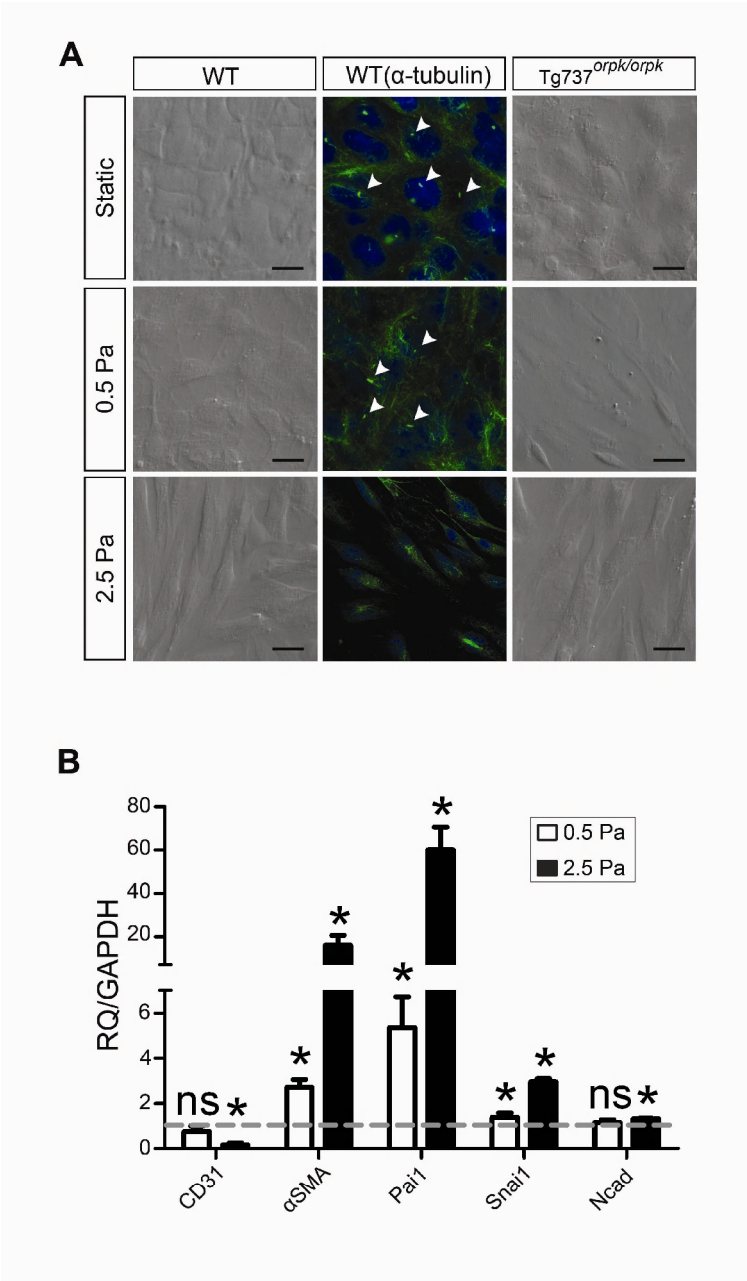
Statistical Analysis

For comparison of the means, independent experiments were performed and analyzed using SPSS 14.0 (SPSS Inc.). All results are expressed as mean ± SEM. Independent t-tests, including Levene's analyses for equality of variances, were used to analyze differences between groups. Values of $P < 0.05$ and a power ≥ 0.80 were considered statistically significant and are marked by an asterisk (*) in the figures.

FIGURES AND FIGURE LEGENDS

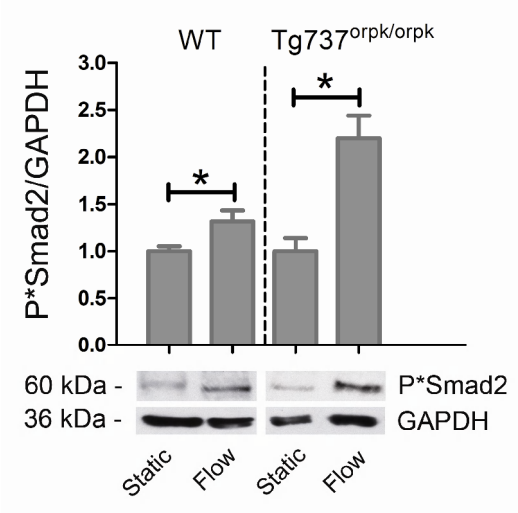


Online Figure I. Tgfβ induced EndoMT in ciliated WT and non-ciliated Tg737$^{orpk/orpk}$ EC. (A) Morphology of WT and Tg737$^{orpk/orpk}$ cells which were stimulated with Tgfβ ligand to induce EndoMT under static conditions. Note the transition from cobble-stone to fibroblast-like morphology upon stimulation with Tgfβ. Scale bars: 25 µm. (B) Relative mRNA expression of *CD31*, *αSMA*, *Pai1*, *Snai1*, and *Ncad* in WT EC upon stimulation with Tgfβ ligand. Expression is normalized to *GAPDH* and relative to shams, as represented by the dashed line.



Online Figure II. WT EC looses their cilia and undergo EndoMT after exposure to high flow.

(A) Morphology of WT (left and middle column) and Tg737$^{orpk/orpk}$ cells (right column) under static conditions (top row) and after exposure to 0.5 and 2.5 Pa shear stress (middle and lower rows, respectively). The central column shows immunostaining for acetylated α-tubulin. Arrowheads indicate cilia. Note the presence of primary cilia at 0.5 Pa shear and the non-ciliated phenotype of WT cells after exposure to 2.5 Pa shear stress. Scale bars: 25 μm. (B) Relative mRNA expression of *CD31*, *αSMA*, *Pai1*, *Snai1*, and *Ncad* in WT cells under 0.5 and 2.5 Pa shear. Expression is normalized to *GAPDH* and relative to static shams, as represented by the dashed line.



Online Figure III. Exposure to shear stress results in enhanced phospho-Smad2 expression in Tg737$^{orpk/orpk}$ cells.
Western Blot analysis and quantification of P*Smad2 protein levels in WT and Tg737$^{orpk/orpk}$ cells under static conditions and after exposure to 2 hours of 0.5 Pa shear stress. Western Blot shown is representative of 3 independent experiments.

TABLES

Online Table I. List of Q-PCR primers used.

Gene of interest	Gene ID	Oligonucleotide sequence
muActa2 (αSMA)	11475	F: 5'-CATCATGCGTCTGGACTTG-3' R: 5'-ATCTCACGCTCGGCAGTAG-3'
muCD31	18613	F: 5'-CTCCAACAGAGCCAGCAGTA-3' R: 5'-GACCACTCCAATGACAACCA-3'
muGAPDH	14433	F: 5'-TTGATGGCAACAATCTCCAC-3' R: 5'-CGTCCCGTAGACAAAATGGT-3'
muIft88	21821	F: 5'-GCAAATGGAACGTGAAAGG-3' R: 5'-AAGACGCTTCGATCACAGG-3'
muKlf2	16598	F: 5'-ATTGCAACTGGGAAGGATG-3' R: 5'-GTGGCACTGAAAGGGTCT G-3'
muKlf4	16600	F: 5'-CAGGCGAGAAACCTTACCA-3' R: 5'-TGTGTGTTTGCGGTAGTGC-3'
muCdh2 (Ncad)	12558	F: 5'-AATCCCACTTATGGCCTTTC-3' R: 5'-AGGATTTGGGGCAAAATAAG-3'
muSerpine1 (Pai1)	18787	F: 5'-GCCAACAAGAGCCAATCAC-3' R: 5'-ACCCTTTCCCAGAGACCAG-3'
muSnai1	20613	F: 5'-CTTGTGTCTGCACGACCTG-3' R: 5'-CAGTGGGAGCAGGAGAAT-3'

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