

MCP/2019/001492-R1

NOTCH activation promotes valve formation by regulating the endocardial secretome

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Supplemental Data

Supplemental Figures and legends

Supplemental Tables legends

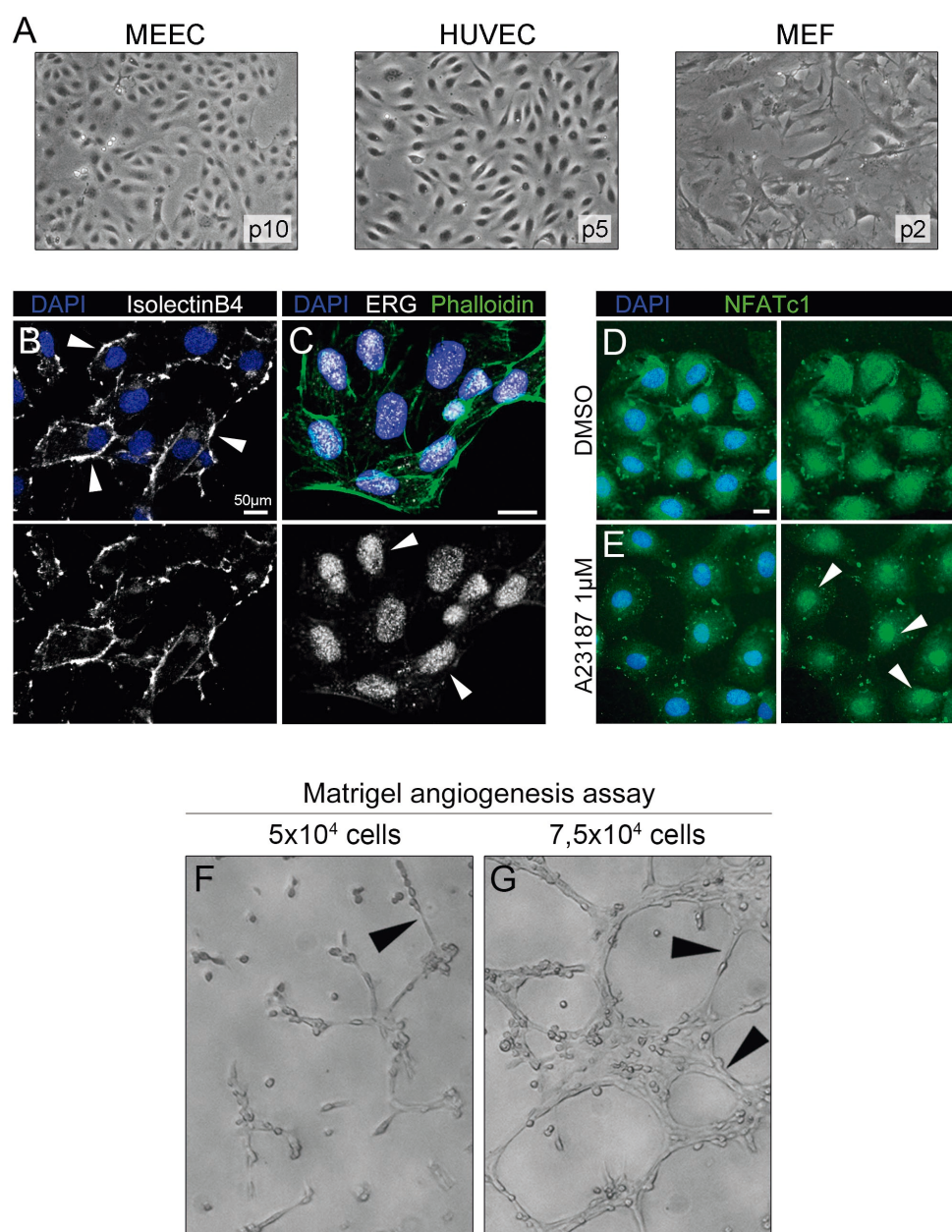


Figure S1 _ Torregrosa et al.

Figure S1: Characterization of MEEC as an embryonic endocardial cell line. (A) Phase-contrast microscopy images showing morphology of immortalized MEEC at passage 10 (left panel), primary HUVEC at passage 5 (middle panel) and primary MEF at passage 2 (right panel). (B,C) Fluorescence staining of MEEC monolayer with the endocardial markers isolectin B4 and anti-ERG. Isolectin B4 (white) stains the cellular membrane (B, arrowheads), ERG (white) is detected in the nucleus (C, arrowheads), and phalloidin immunolabeling (green) stains filamentous actin to provide an outline of general MEEC morphology (C). (D,E) A23187 calcium ionophore-induced nuclear translocation of NFATc1 in MEEC. NFATc1 (green) is broadly detected in the cytosol and nuclei in control cells (D), and A23187 treatment for 6 hours triggers its translocation to the nucleus (E, arrowheads). Nuclei are counterstained with DAPI (blue). Scale bars, 50 μm . (F-G) MEEC tube formation assay on Matrigel. Either 5×10^4 (F) or 7.5×10^4 cells (G) were seeded per well on growth factor-reduced Matrigel after a 7-day-long treatment with (20ng/mL) of the pro-angiogenic cytokine VEGF₁₆₅. Phase-contrast microscopy images were taken after 6 hours of incubation. Arrowheads mark capillary-like tubes.

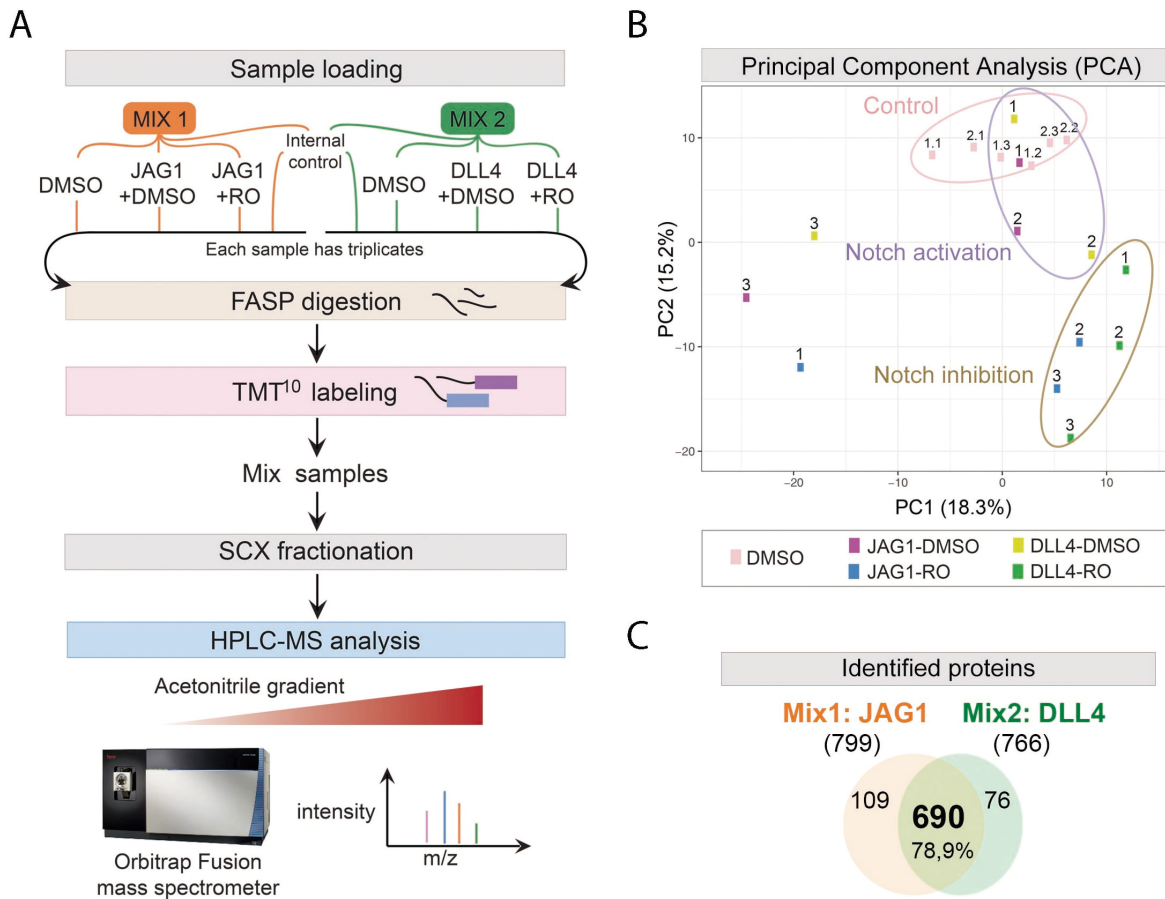


Figure S2 _ Torregrosa et al.

Figure S2: High-throughput LC-MS/MS secretome analysis workflow. (A) Cartoon showing the steps followed to reliably identify and quantify the secretome repertoire of unstimulated (control), DLL4-stimulated and JAG1-stimulated MEEC. Two mixes were prepared and analyzed independently. Mix1 includes triplicates from 3 conditions: MEEC treated with DMSO (vehicle), with JAG1 plus DMSO, and with JAG1 plus RO (γ -secretase inhibitor). Mix2 includes triplicates from the same conditions, but with DLL4 as the NOTCH-signaling ligand. (B) Two-dimensional principal components analysis (PCA) of the secretome from non-stimulated MEEC (pink), NOTCH-stimulated MEEC (JAG1 plus DMSO in magenta and DLL4 plus DMSO in light green) and NOTCH-inhibited MEEC (JAG1 plus RO in blue and DLL4 plus RO in dark green). Each condition was carried out in triplicate. (C) Venn diagram showing overlapping of 690 proteins identified in Mix1 and Mix2.

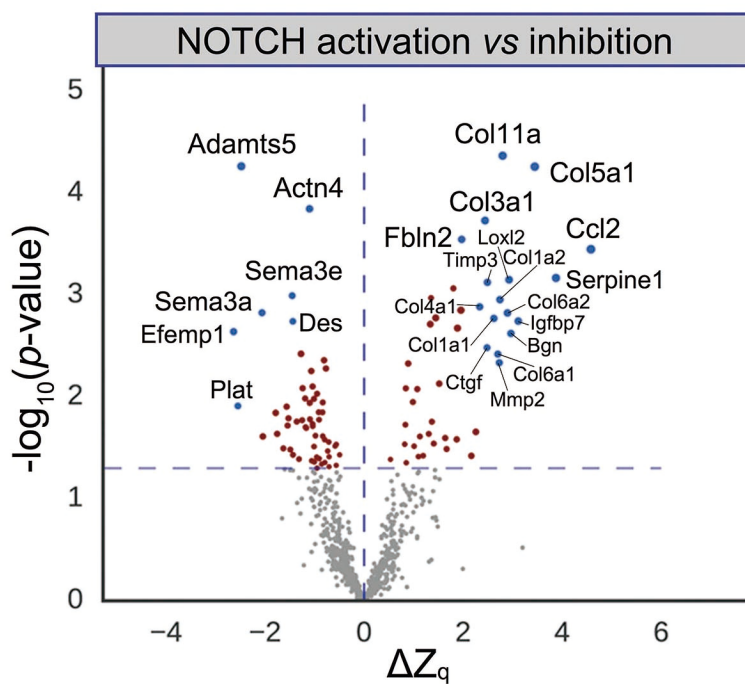
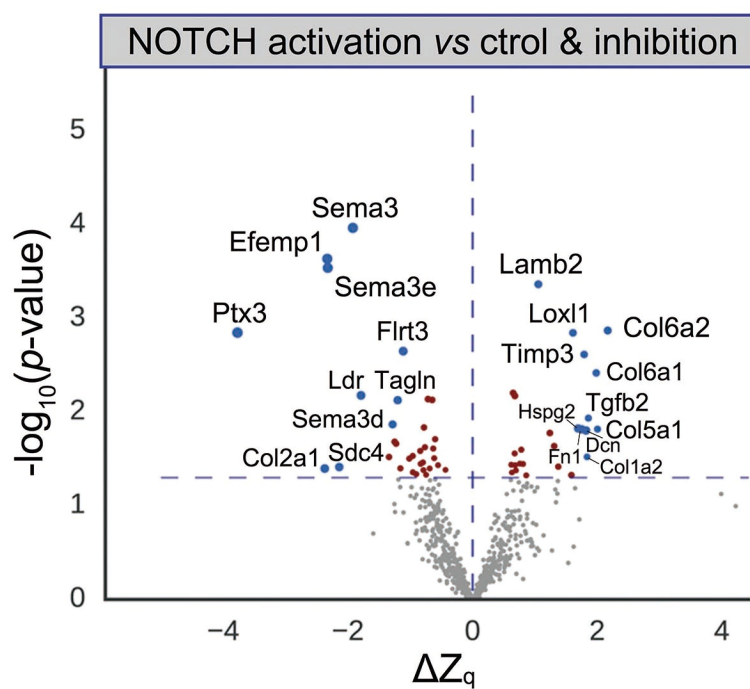
A**B**

Figure S3_Torregrosa et al.

Figure S3. Global NOTCH-associated secretome analysis. (A,B) Volcano plots of Δz_q against the negative \log_{10} of the FDR-corrected p -value. Graphs show differentially secreted proteins considering two pairwise comparisons: (A) Δz_q of global NOTCH activation (mean of DLL4- and JAG1-DMSO combined) versus NOTCH inhibition (mean of DLL4- and JAG1-RO combined). (B) Δz_q of global NOTCH activation versus control (mean of DMSO) and NOTCH inhibition combined. Colored dots above the dashed lines represent significantly changed protein abundance. Red indicates higher abundance during NOTCH activation; blue indicates lower abundance.

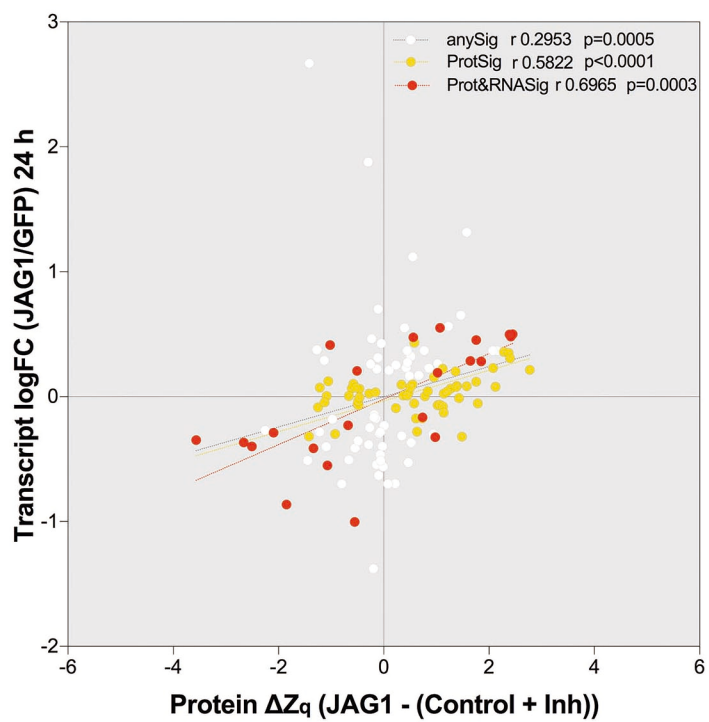
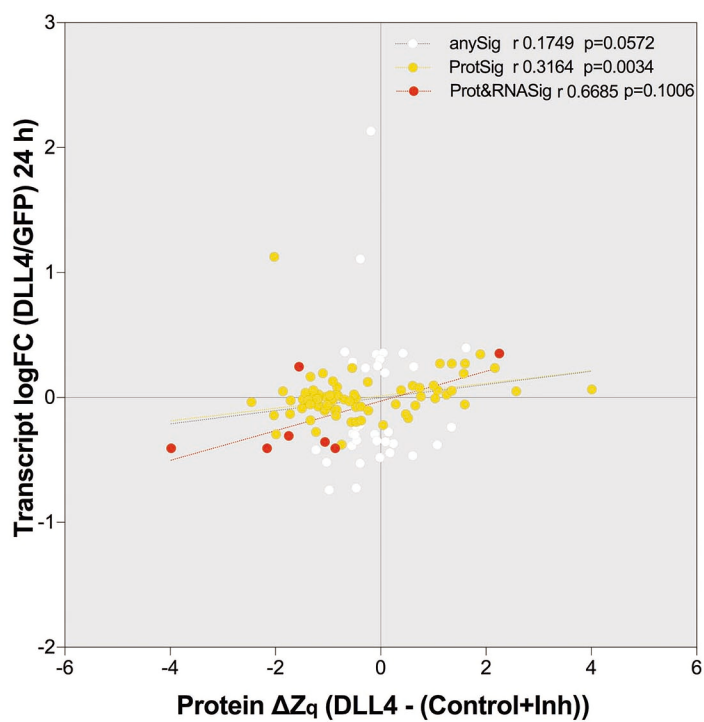
A**B**

Figure S4_Torregrosa et al.

Figure S4. Correlation of transcriptomics and proteomics data in NOTCH-stimulated MEEC. (A,B) Correlation of \log_2 fold expression changes (FC) by RNA-Seq analysis of JAG- (A) and DLL4-stimulated MEEC (B) (y axis) with proteomic changes given as Δz_q (x axis). Δz_q refers to the comparison of NOTCH activation (by DLL4 or JAG1) and the combination of control plus NOTCH inhibition. The Pearson correlation coefficient was calculated for all factors with significance at the transcript or protein level (anySig, white dots), at least at the protein level (ProtSig, yellow dots), or at both the transcript and the protein level (Prot&RNASig, red dots).

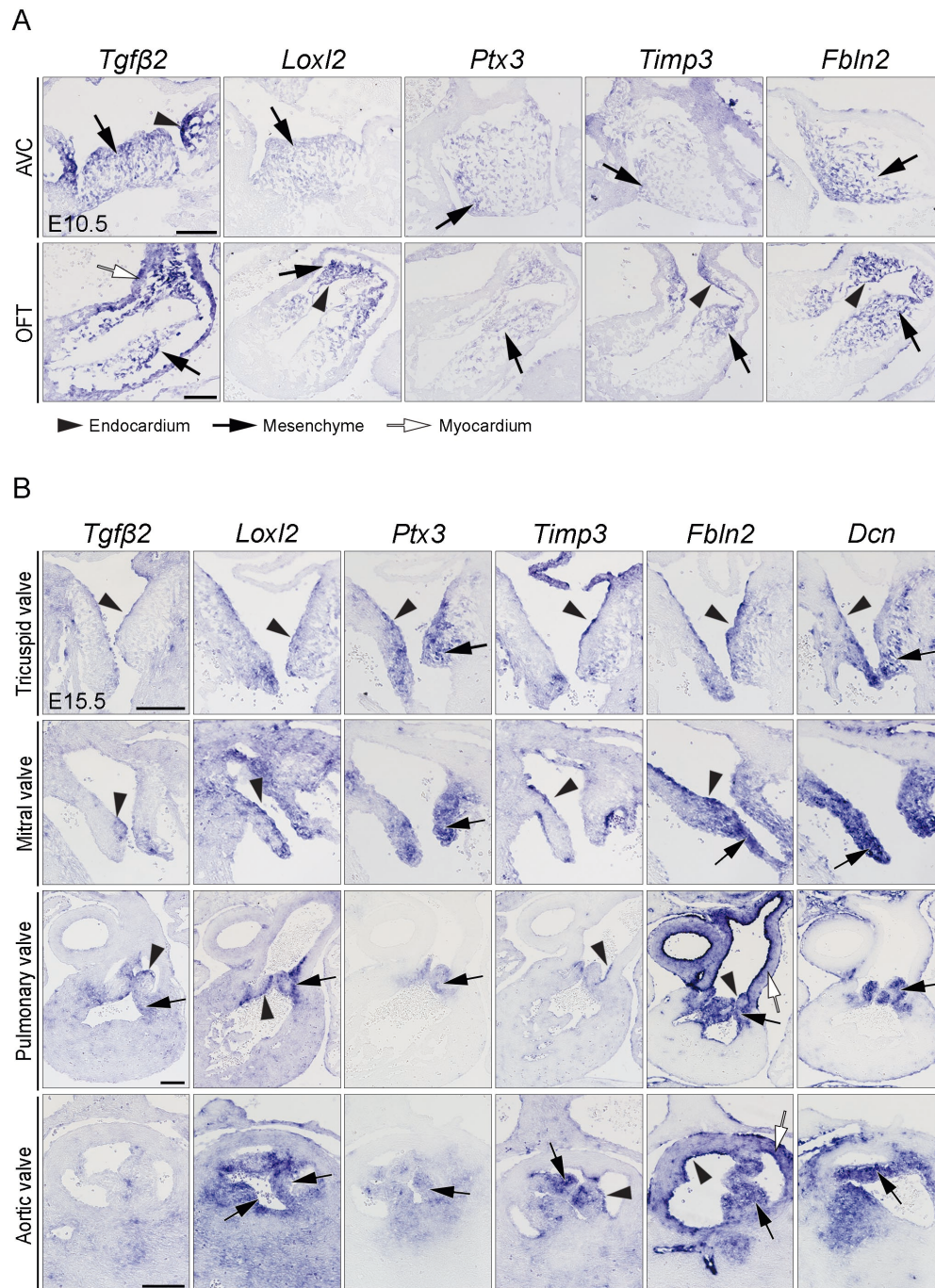


Figure S5 _ Torregrosa et al.

Figure S5: Candidate endocardium-derived NOTCH-dependent secreted factors identified *in vitro* are expressed in the heart during early EndMT and later in valve remodeling. (A) ISH analysis showing

Tgfb2, *Loxl2*, *Ptx3*, *Timp3*, and *Fbln2* expression in the AVC (upper panels) and OFT (lower panels) of E10.5 WT heart sections. (B) ISH analysis showing *Tgfb2*, *Loxl2*, *Ptx3*, *Timp3*, *Fbln2* and *Dcn* expression in the atrioventricular valves (tricuspid and mitral) and semilunar valves (pulmonary and aortic) of E15.5 WT heart sections. Scale bars, 100 μ m. avc (atrioventricular canal), oft (outflow tract).

Supplementary Table legends

Table S1: List of peptides and proteins. List of peptides and proteins identified (FDR<1%) and quantified in the secretome from MEEC cells stimulated with JAG1 (Mix1) and DLL4 (Mix2)..

Table S2: Protein classification. List of proteins identified by mass spectrometry analysis and annotated as “Extracellular” (**sheet A**), “Transmembrane” (**sheet B**), potentially secreted by “Classical secretion” (**sheet C**), potentially secreted by “Non-classical secretion” (**sheet D**) and “Intracellular” (**sheet E**). Related to Figure 1D.

Table S3: Global NOTCH-associated secretome analysis. Sheet A: z_q values of the 690 secreted proteins identified in both “Mix1:JAG1” (orange) and “Mix2:DLL4” (green) mixes (related to Figure S2C). **Sheet B:** z_q values of the 129 differentially secreted proteins (related to Figure 2A). Hypersecretion is shown in red and hyposecretion in blue. NP refers to the number of peptides used for protein identification.

Table S4: JAG1- and DLL4-specific secretome analysis. Sheet A: z_q values of the 82 JAG1-specific differentially secreted proteins (related to Figure 4B). **Sheet B:** z_q values of the 113 DLL4-specific differentially secreted proteins (related to Figure 4C). **Sheet C:** z_q values of the common 24 JAG1- and DLL4-specific differentially secreted proteins (related to Figure 4D).

Table S5: GO and KEGG pathway analysis of the NOTCH-associated secretory profile. Sheet A: Overrepresented GO terms and KEGG pathways for the global NOTCH-associated secretory profile (related to Figure 2B,C). **Sheet B:** Overrepresented GO terms and KEGG pathways for the specific JAG1- and DLL4-

associated secretome (related to Figure 4E). GO terms are listed by the Z-score value. Data are the standard output from GO Elite.

Table S6: STRING analysis. Sheet A: STRING database input from the NOTCH-associated secretory profile (related to Figure 3A). **Sheet B:** STRING database output from the NOTCH-associated secretory profile (related to Figure 3A).

Table S7: RNA-seq analysis of MEEC. Sheet A: List of the 624 proteins identified by RNA-Seq and secretome analysis. Binary code (1,0) allows selection of proteins significantly changed at RNA level, protein level or both for correlation analysis (related to Figure S4A,B). **Sheet B:** List of the DLL4-specific differentially expressed genes (DEG) identified by RNA-seq ($P<0.05$) overlapping with the DLL4-specific differentially secreted proteins. **Sheet C:** List of the JAG1-specific DEG identified by RNA-seq ($P<0.05$) overlapping with the JAG1-specific secreted proteins. **Sheet D:** Comparative of \log_2FC of DEG and z_q values of differentially secreted proteins (related to Figure 5C).

Table S8. qRT-PCR primers. List of the qRT-PCR primers used in this study.