miR-27a-3p regulates expression of intercellular junctions at the brain endothelium and controls the endothelial barrier permeability

Short Title: Regulation of inter-endothelial junctions by miR-27a-3p

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Figures

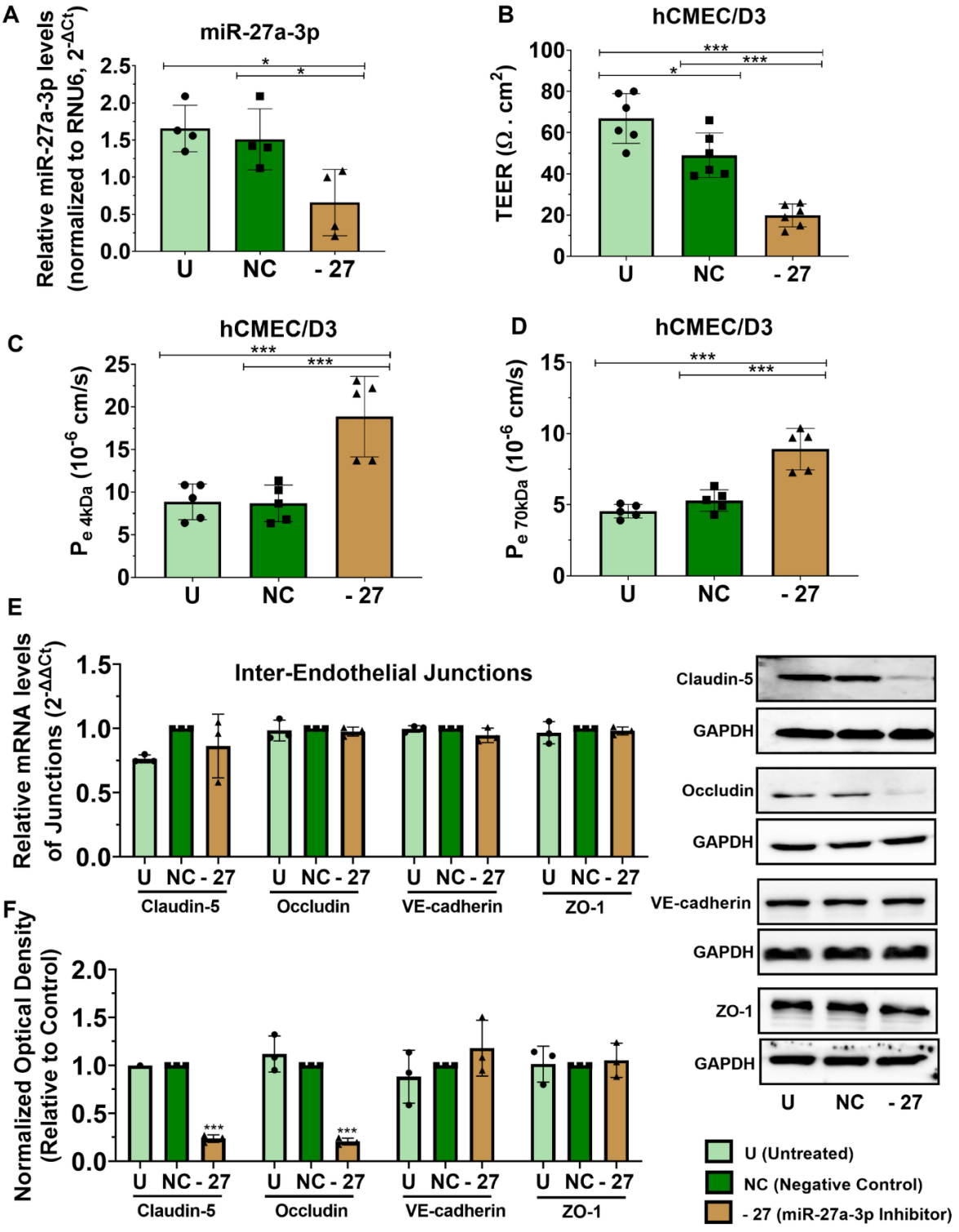
A
miR-27a-3p
Relative miR-27a-3p levels (normalized to RNU6, $2^{-\Delta Ct}$)
U
NC
- 27
B
hCMEC/D3
TEER (Ω . cm^2)
C
hCMEC/D3
$P_{e\ 4kDa}$ (10^{-6} cm/s)
D
hCMEC/D3
$P_{e\ 70kDa}$ (10^{-6} cm/s)
E
Inter-Endothelial Junctions
Relative mRNA levels of Junctions ($2^{-\Delta\Delta Ct}$)
Claudin-5
Occludin
VE-cadherin
ZO-1
GAPDH
F
Normalized Optical Density (Relative to Control)
U (Untreated)
NC (Negative Control)
- 27 (miR-27a-3p Inhibitor)

Fig 1. miR-27a-3p inhibitor increases the brain endothelial barrier permeability and downregulates claudin-5 and occludin protein expression.

hCMEC/D3 cells were transfected with miR-27a-3p inhibitor or negative control for 72 hours. **(A)** miR-27a-3p relative expression measured by real-time PCR. The small nuclear RNA (RNU6-2) was used as an internal standard. Data are represented as $2^{\wedge-\Delta Ct}$. Experiments were carried out four times with PCR performed in duplicates for each experiment. **(B)** Transendothelial electrical resistance (TEER) of hCMEC/D3 cells treated with miR-27a-3p inhibitor or negative control. Experiments were carried out six times with monolayer cultures performed in triplicates. **(C,D)** The permeability coefficient (P_e, cm/s) of the endothelial monolayer assessed by the 4 (C) and 70 kDa FITX-dextran flux assay (D). Experiments were carried out five times with monolayer cultures performed in triplicates. **(E)** Relative mRNA expression of inter-endothelial junctions (claudin-5, Occludin, VE-cadherin and ZO-1) measured by real-time PCR. GAPDH was used as an internal standard. Data are represented as ($2^{\wedge-\Delta\Delta Ct}$). Experiments were carried out three times with PCR performed in duplicates for each experiment. **(F)** Claudin-5, Occludin, VE-cadherin and ZO-1 protein expression measured by western-blot in hCMEC/D3. Optical densities of three independent images were analyzed with Image Lab 6.0.1 software (Bio-Rad) and normalized to GAPDH. Results are represented as normalized optical densities. Experiments were carried out three times with each preparation representing pooled protein lysates from monolayer cultures performed in triplicates. Data represent mean ± SD from the independent experiments (biological replicates). **p<0.05, **p<0.01, ***p<0.001.*

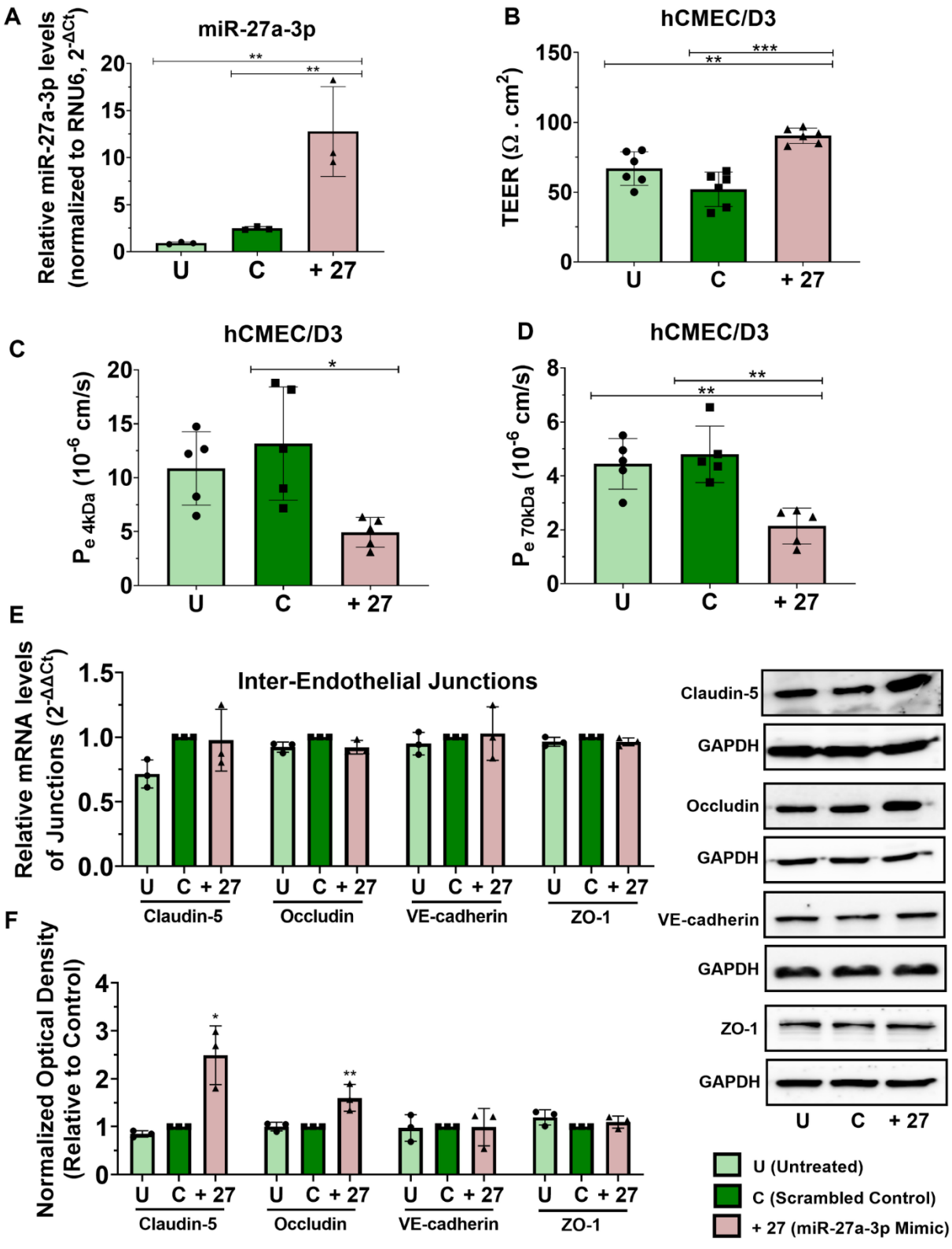
A
miR-27a-3p
Relative miR-27a-3p levels (normalized to RNU6, $2^{-\Delta Ct}$)
U
C
+ 27
B
hCMEC/D3
TEER (Ω . cm^2)
C
hCMEC/D3
$P_{e\ 4kDa}$ (10^{-6} cm/s)
D
hCMEC/D3
$P_{e\ 70kDa}$ (10^{-6} cm/s)
E
Inter-Endothelial Junctions
Relative mRNA levels of Junctions ($2^{-\Delta\Delta Ct}$)
Claudin-5
Occludin
VE-cadherin
ZO-1
F
Normalized Optical Density (Relative to Control)
Claudin-5
GAPDH
Occludin
GAPDH
VE-cadherin
GAPDH
ZO-1
GAPDH
U (Untreated)
C (Scrambled Control)
+ 27 (miR-27a-3p Mimic)

Fig 2. miR-27a-3p mimic reduces the brain endothelial barrier permeability and upregulates claudin-5 and occludin protein expression.

hCMEC/D3 cells were transfected with miR-27a-3p mimic or control for 72h. **(A)** miR-27a-3p relative expression measured by real-time PCR. The small nuclear RNA (RNU6-2) was used as an internal standard. Data are represented as $2^{\wedge -\Delta Ct}$. Experiments were carried out three times with PCR performed in duplicates for each experiment. **(B)** TEER of hCMEC/D3 cells treated with miR-27a-3p mimic or control. Experiments were carried out six times with monolayer cultures performed in triplicates. **(C,D)** The permeability coefficient (P_e, cm/s) of the endothelial monolayer assessed by the 4 (C) and 70 kDa FITX-dextran flux assay (D). Experiments were carried out five times with monolayer cultures performed in triplicates.**(E)** Relative mRNA expression of inter-endothelial junctions (claudin-5, Occludin, VE-cadherin and ZO-1) measured by real-time PCR. GAPDH was used as an internal standard. Data are represented as ($2^{\wedge -\Delta\Delta Ct}$). Experiments were carried out three times with PCR performed in duplicates for each experiment. **(F)** Claudin-5, Occludin, VE-cadherin and ZO-1 protein expression measured by western-blot in hCMEC/D3. Optical densities of three independent images were analyzed with Image Lab 6.0.1 software(Bio-Rad) and normalized to GAPDH. Results are represented as normalized optical densities. Experiments were carried out three times with each preparation representing pooled protein lysates from monolayer cultures performed in triplicates. Data represent mean ± SD from the independent experiments (biological replicates). **p<0.05, **p<0.01, ***p<0.001.*

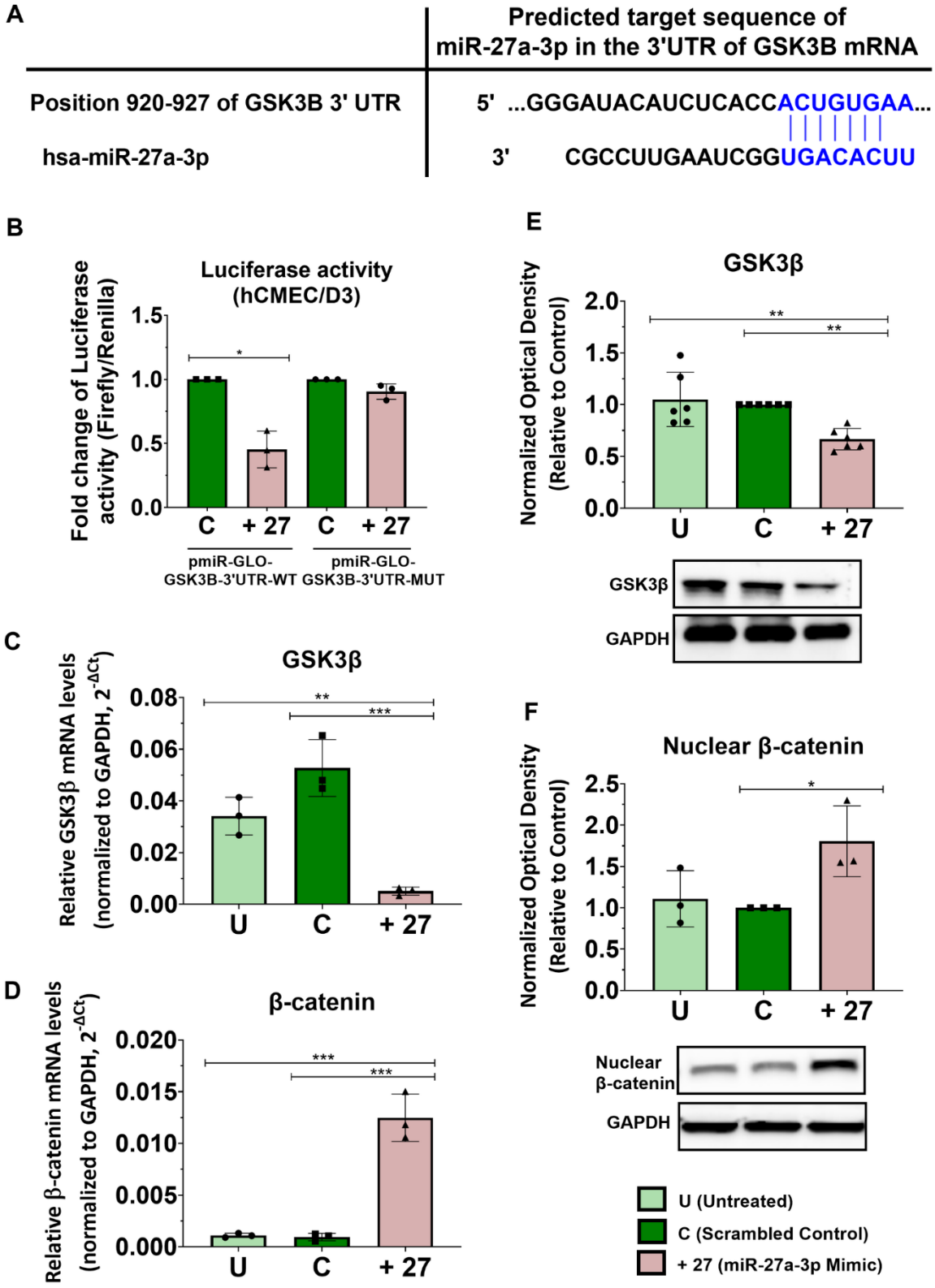
A
Predicted target sequence of miR-27a-3p in the 3'UTR of GSK3B mRNA
Position 920-927 of GSK3B 3' UTR
5' ...GGGAUACAUCUCACCACUGUGAA...
hsa-miR-27a-3p
3' CGCCUUGAAUCGGUGACACUU
B
Luciferase activity (hCMEC/D3)
Fold change of Luciferase activity (Firefly/Renilla)
C
+ 27
C
+ 27
pmiR-GLO-GSK3B-3'UTR-WT
pmiR-GLO-GSK3B-3'UTR-MUT
C
GSK3β
Relative GSK3β mRNA levels (normalized to GAPDH, 2-ΔCt)
U
C
+ 27
D
β-catenin
Relative β-catenin mRNA levels (normalized to GAPDH, 2-ΔCt)
U
C
+ 27
E
GSK3β
Normalized Optical Density (Relative to Control)
U
C
+ 27
GSK3β
GAPDH
F
Nuclear β-catenin
Normalized Optical Density (Relative to Control)
U
C
+ 27
Nuclear β-catenin
GAPDH
U (Untreated)
C (Scrambled Control)
+ 27 (miR-27a-3p Mimic)

Fig 3. miR-27a-3p regulates claudin-5 and occludin expression by targeting GSK3ß and upregulating Wnt/ß-catenin

(A) Schematic representation of the GSK3ß 3'-UTR with the miR-27a-3p binding site. Complementary sequences are represented in blue. **(B)** Effect of miR-27a-3p mimic on GSK3ß 3'UTR luciferase reporters (wild type and mutant). Results are expressed as the fold change of the ratio (firefly to Renilla luciferase activity). Experiments were carried out three times with tests performed in triplicates for each experiment. **(C,D)** Relative mRNA expression of GSK3ß (C) and ß-catenin (D) measured by real-time PCR in hCMEC/D3 cells transfected with miR-27a-3p mimic or control for 72h. GAPDH was used as an internal standard. Data are represented as ($2^{-\Delta Ct}$). Experiments were carried out three times with PCR performed in duplicates for each experiment. **(E,F)** Protein expression of the GSK3ß (E) and nuclear ß-catenin (F) measured by western-blot in hCMEC/D3. Optical densities of three independent images were analyzed with Image Lab 6.0.1 software(Bio-Rad) and normalized to GAPDH. Results are represented as normalized optical densities. Experiments were carried out three to six times with each preparation representing pooled protein lysates from monolayer cultures performed in triplicates. Data represent mean ± SD from the independent experiments (biological replicates). **p<0.05, **p<0.01, ***p<0.001*

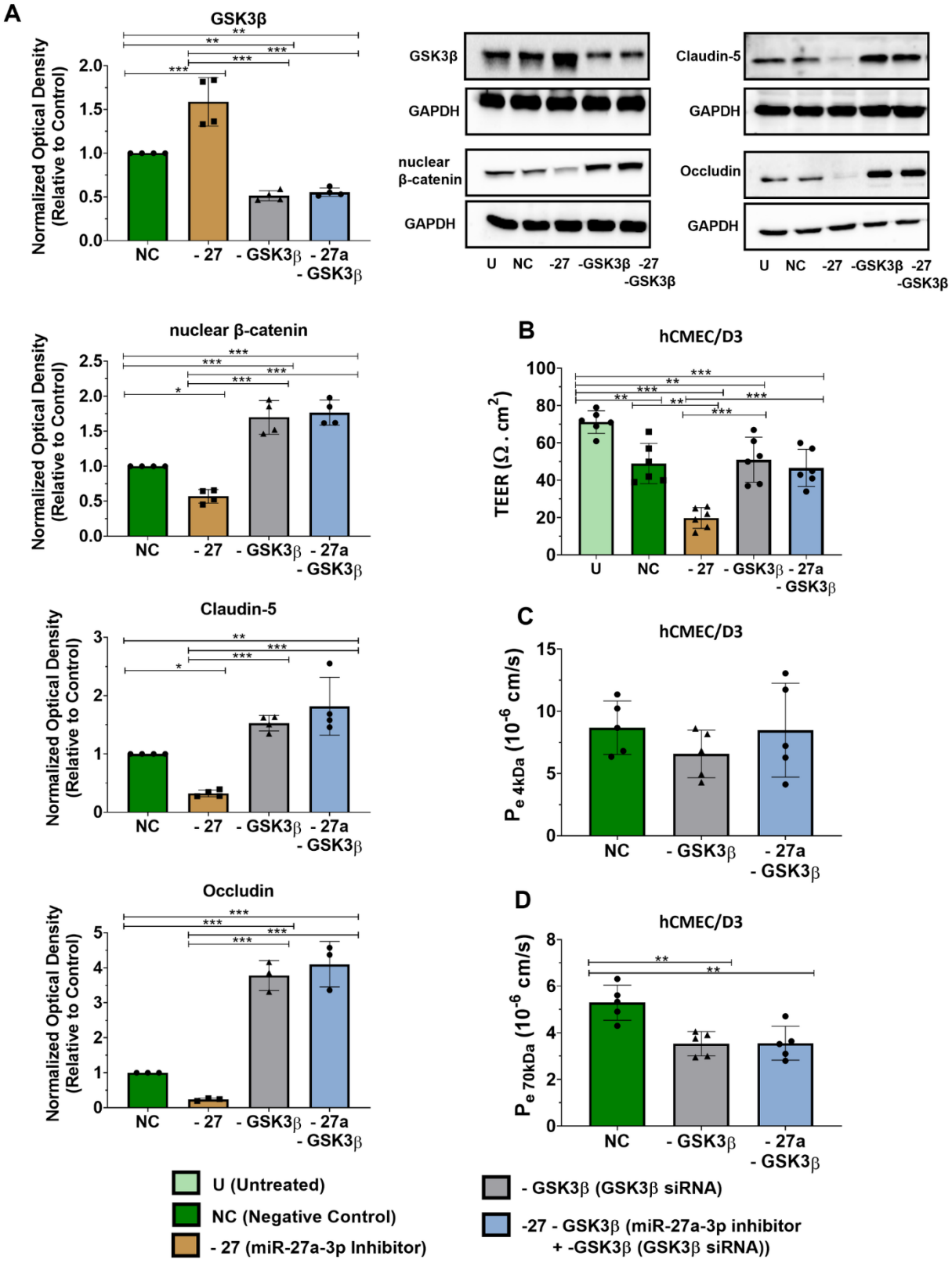
A
GSK3β
Normalized Optical Density (Relative to Control)
NC
- 27
- GSK3β
- 27a - GSK3β
GSK3β
GAPDH
nuclear β-catenin
Claudin-5
Occludin
U
NC
-27
-GSK3β
-27 -GSK3β
nuclear β-catenin
Claudin-5
Occludin
B
hCMEC/D3
TEER (Ω . cm²)
C
hCMEC/D3
P e 4kDa (10⁻⁶ cm/s)
D
hCMEC/D3
P e 70kDa (10⁻⁶ cm/s)
U (Untreated)
NC (Negative Control)
- 27 (miR-27a-3p Inhibitor)
- GSK3β (GSK3β siRNA)
-27 - GSK3β (miR-27a-3p inhibitor + -GSK3β (GSK3β siRNA))

Fig 4. GSK3ß inhibition rescues the activation of GSK3ß by miR-27a-3p inhibitor

hCMEC/D3 cells were transfected with miR-27a-3p inhibitor and/or GSK3B siRNA or negative control for 72h. **(A)** Protein expression of GSK3ß, nuclear ß-catenin and claudin-5 and occludin measured by western-blot in hCMEC/D3. Optical densities of three independent images were analyzed with Image Lab 6.0.1 software(Bio-Rad) and normalized to GAPDH. Results are represented as normalized optical densities. Experiments were carried out three to four times with each preparation representing pooled protein lysates from monolayer cultures performed in triplicates. **(B)** Transendothelial electrical resistance (TEER) of hCMEC/D3 cells treated with miR-27a-3p inhibitor and/or GSK3B siRNA or negative control after 72 hours of transfection. Experiments were carried out six times with monolayer cultures performed in triplicates. **(C,D)** The permeability coefficient (P_e, cm/s) of the endothelial monolayer assessed by the 4 (C) and 70 kDa FITX-dextran flux assay (D). Experiments were carried out five times with monolayer cultures performed in triplicates. Data represent mean ± SD from the independent experiments (biological replicates). *$p<0.05$, **$p<0.01$, ***$p<0.001$*