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Supplemental Information

The *miR-199a/214* Cluster Controls Nephrogenesis and Vascularization in a Human Embryonic Stem Cell Model

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Figure S1

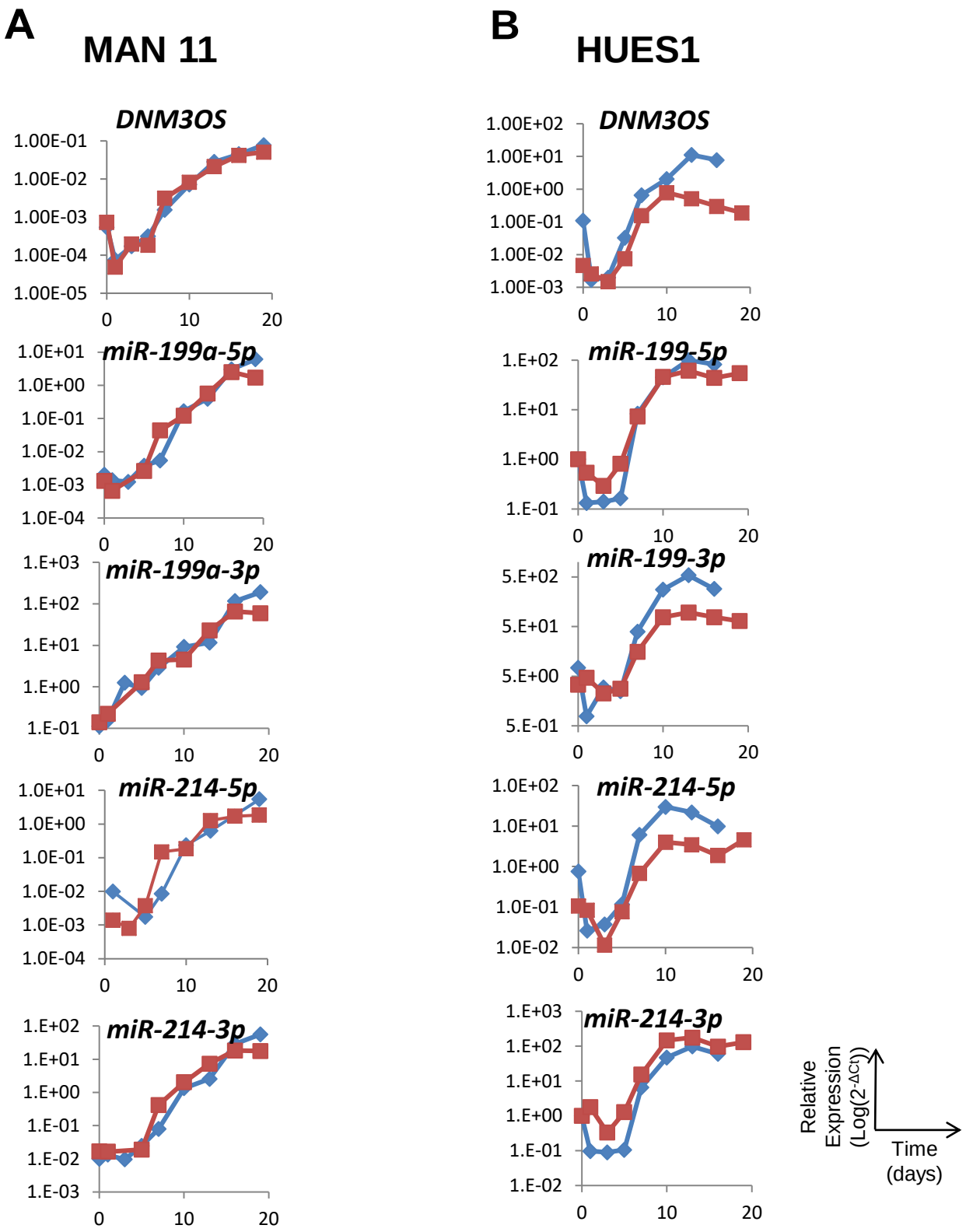

Figure S1. qPCR analysis of *miR-199a/214* cluster expression during 2D kidney differentiation

Figure S2

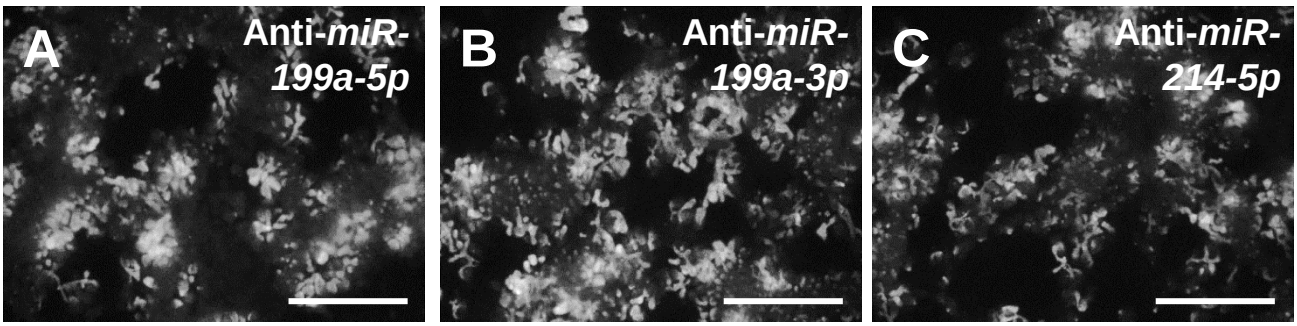

Figure S2. Inhibition of *miR-199a-5p*, *miR-199a-3p* or *miR-214-5p* by miRNA sponges in MAN13 differentiating 2D cultures

Figure S3

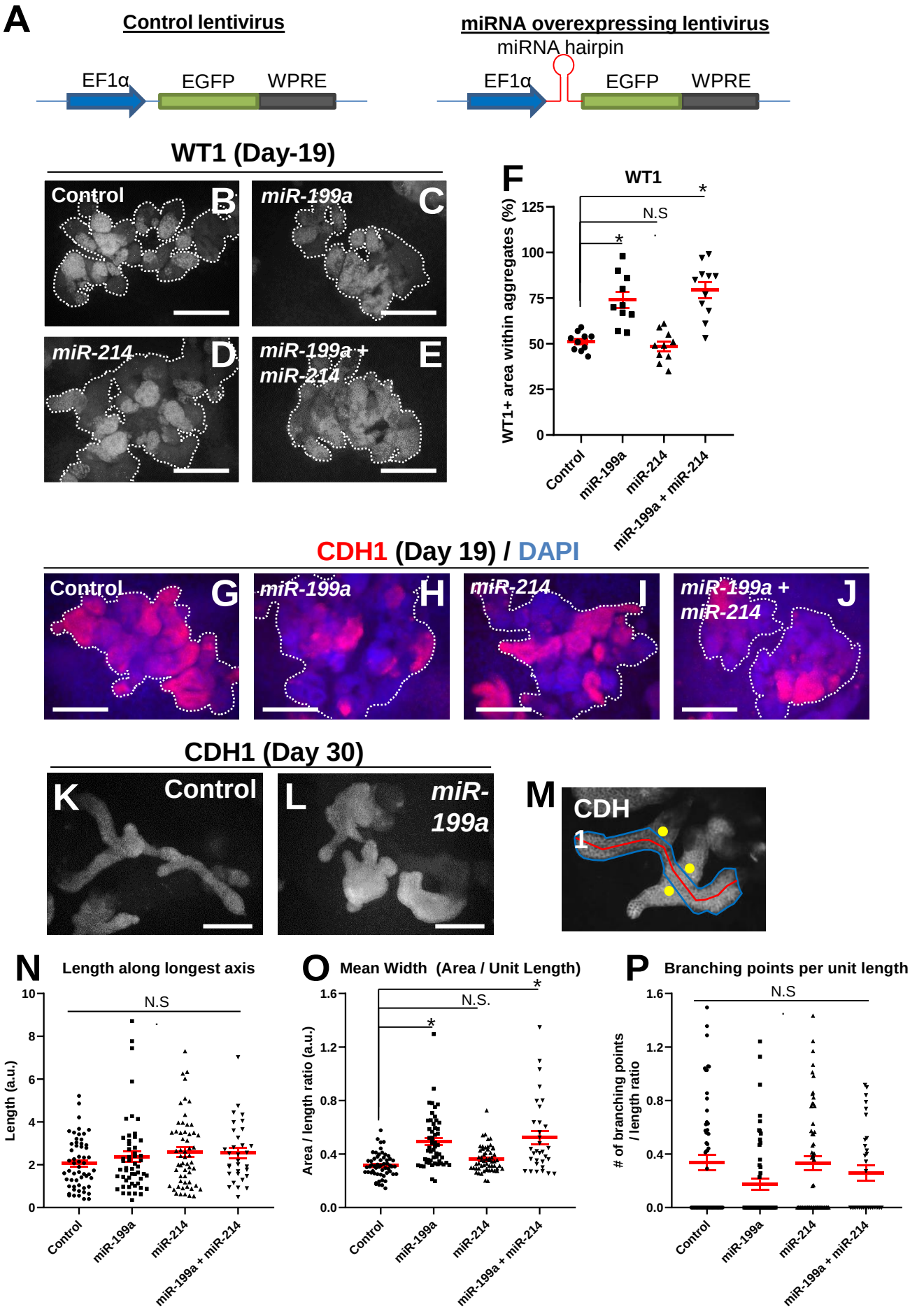

Figure S3. Overexpression of *miR-199a* and *miR-214* in differentiating 2D cultures.

Figure S4

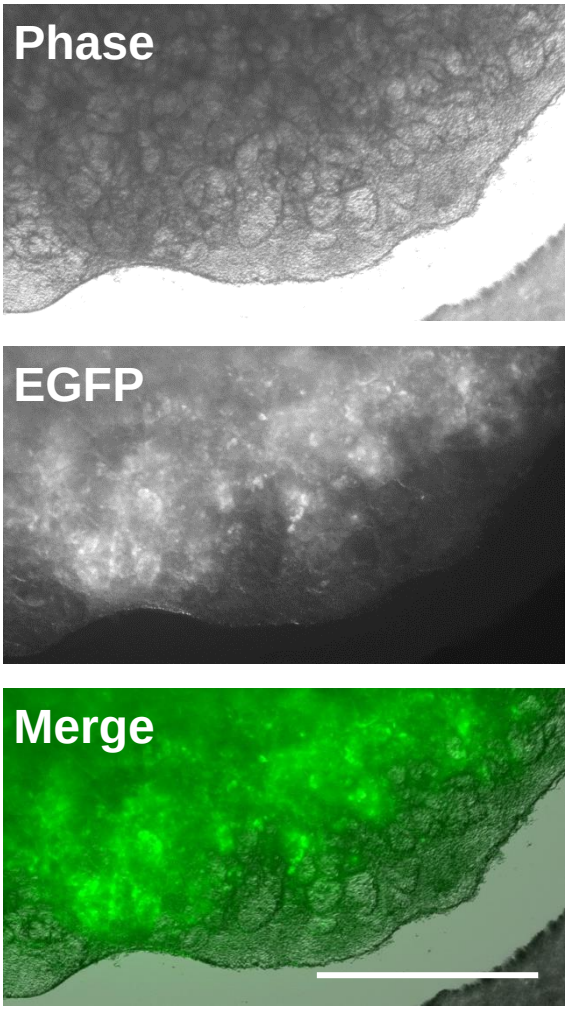

Figure S4. Lentiviral-mediated expression in 3D organoids.

Figure S5

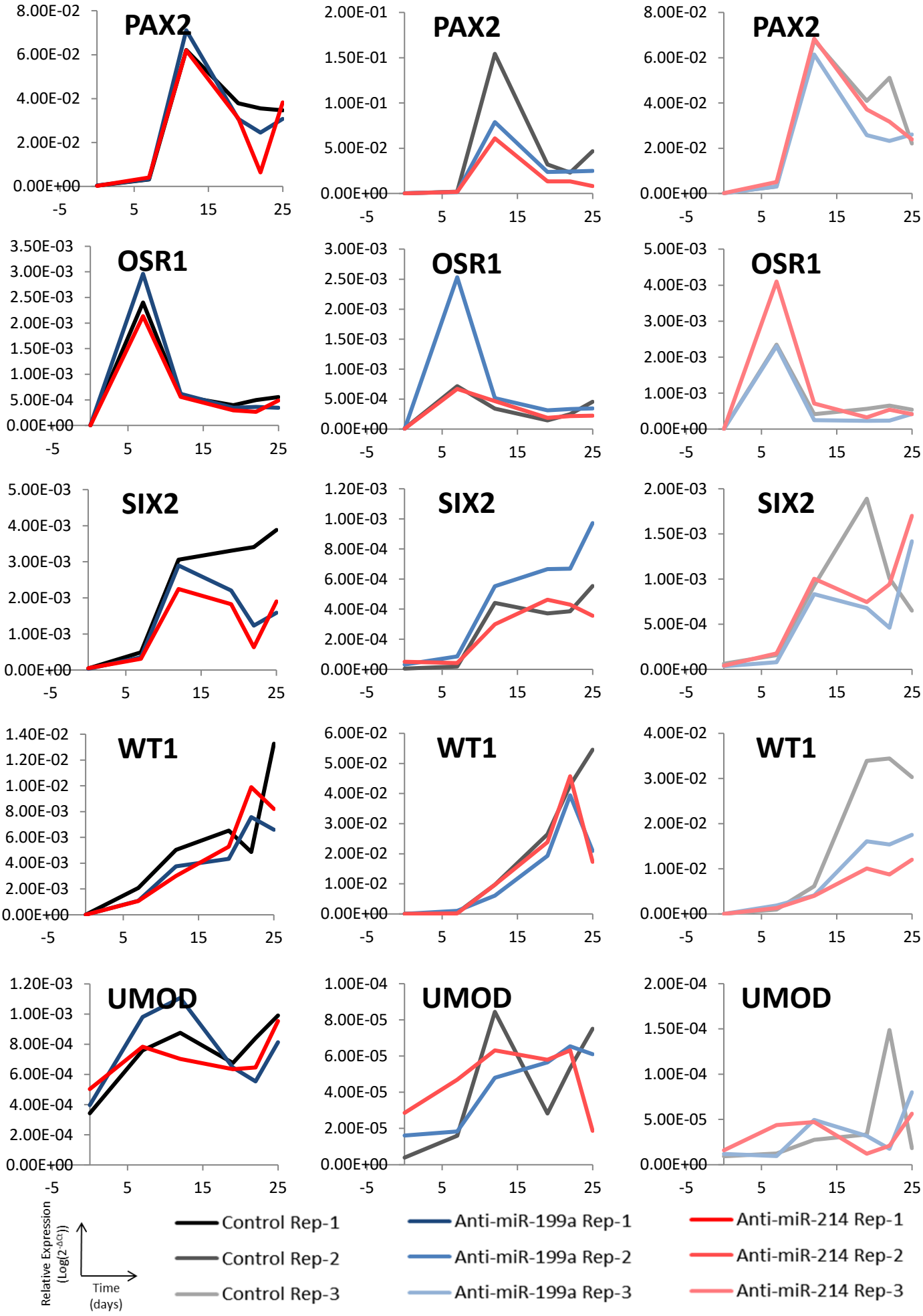
PAX2
OSR1
SIX2
WT1
UMOD
Relative Expression (Log(2^-ΔCt))
Time (days)
Control Rep-1
Control Rep-2
Control Rep-3
Anti-miR-199a Rep-1
Anti-miR-199a Rep-2
Anti-miR-199a Rep-3
Anti-miR-214 Rep-1
Anti-miR-214 Rep-2
Anti-miR-214 Rep-3

Figure S5 (Continued)

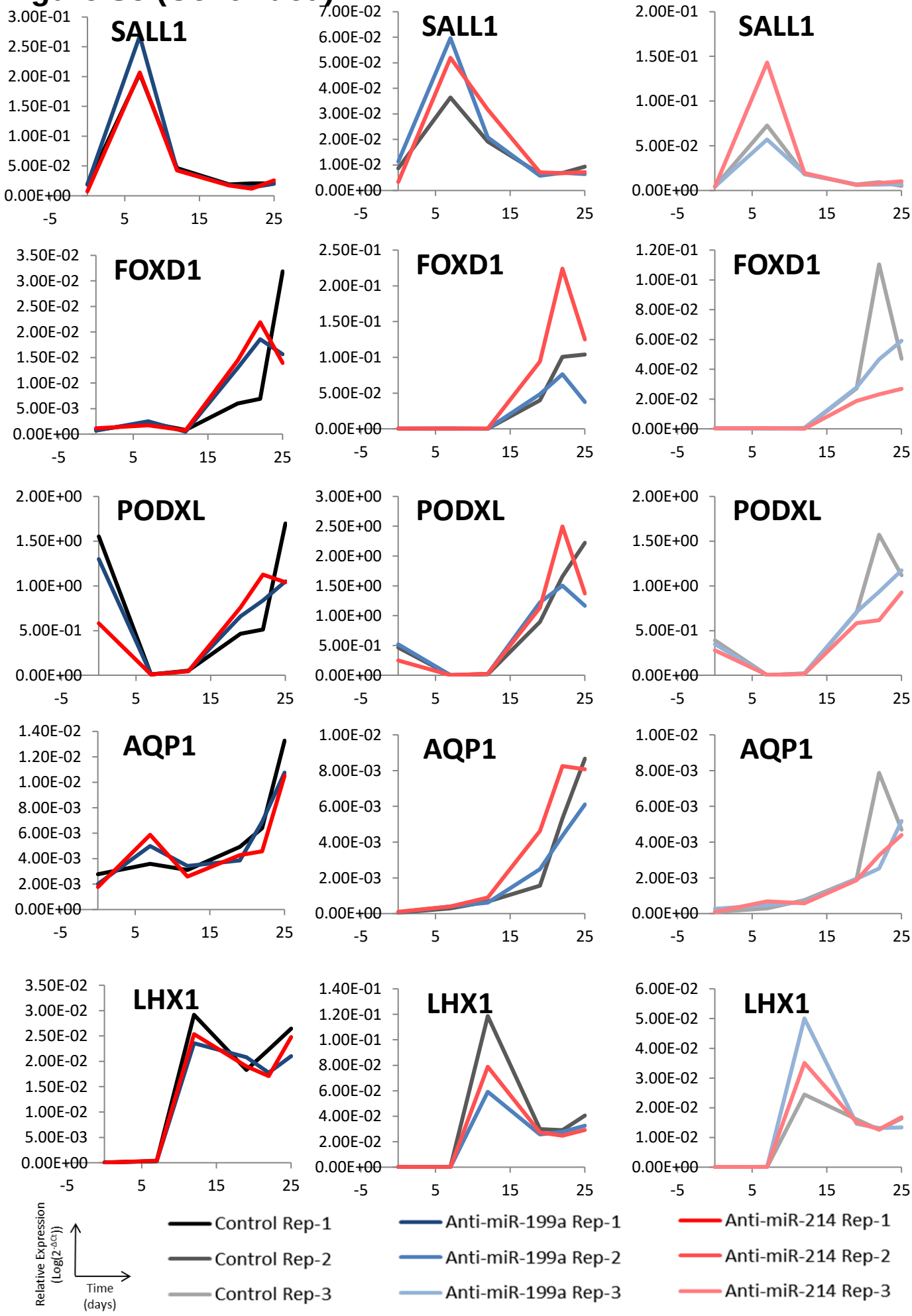

Figure S5 (Continued)

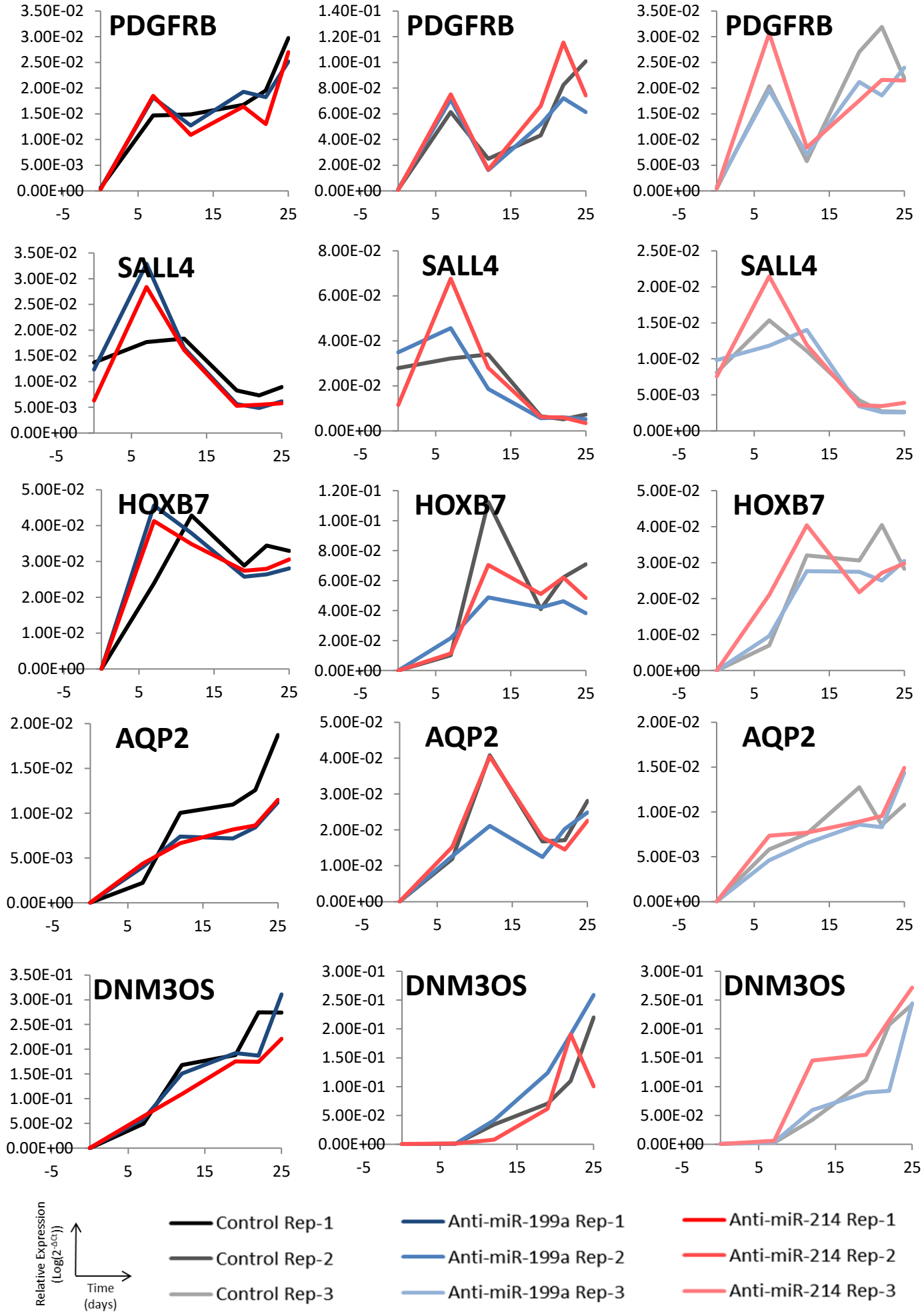

Figure S5 (Continued)

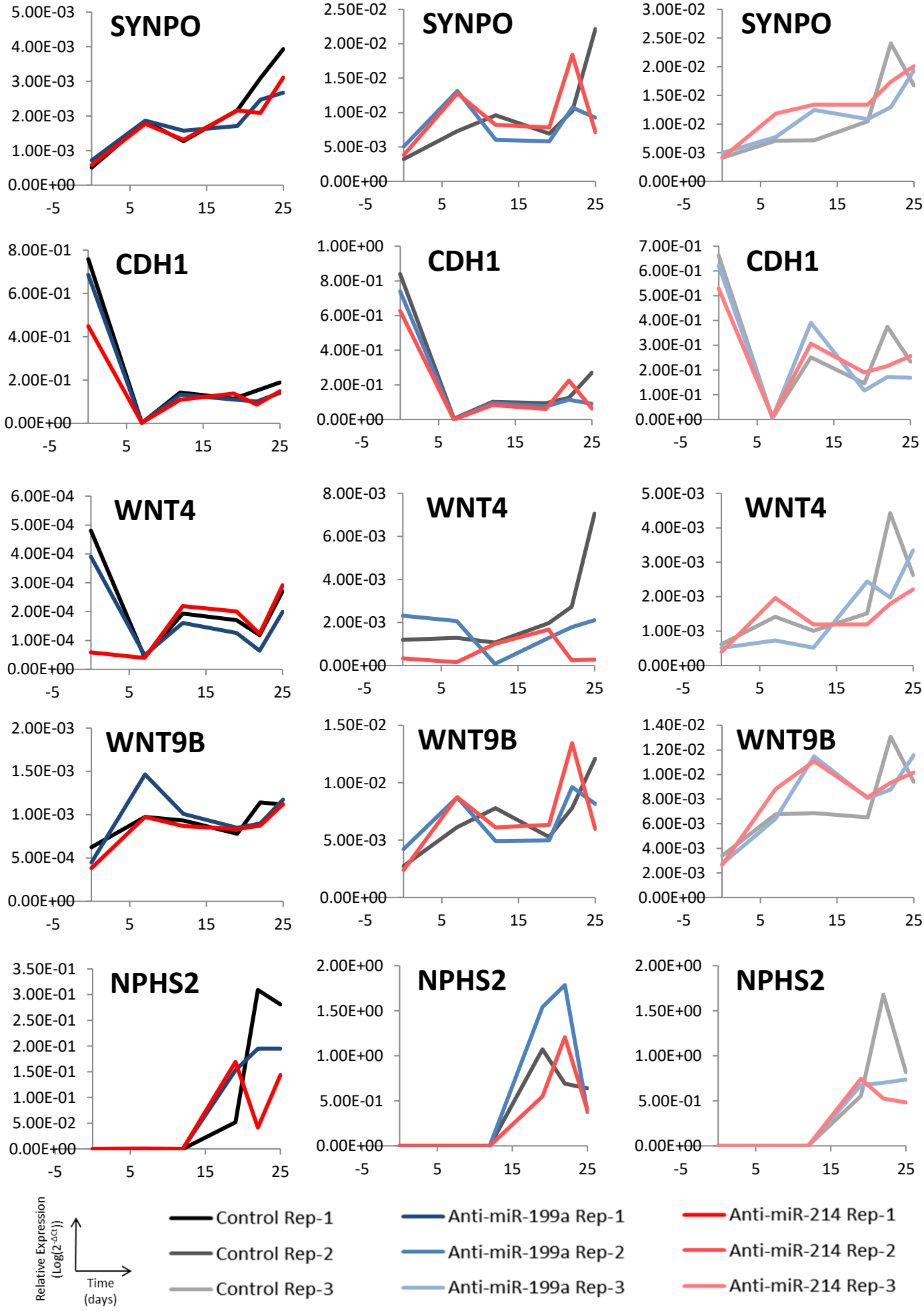

Figure S5 (Continued)

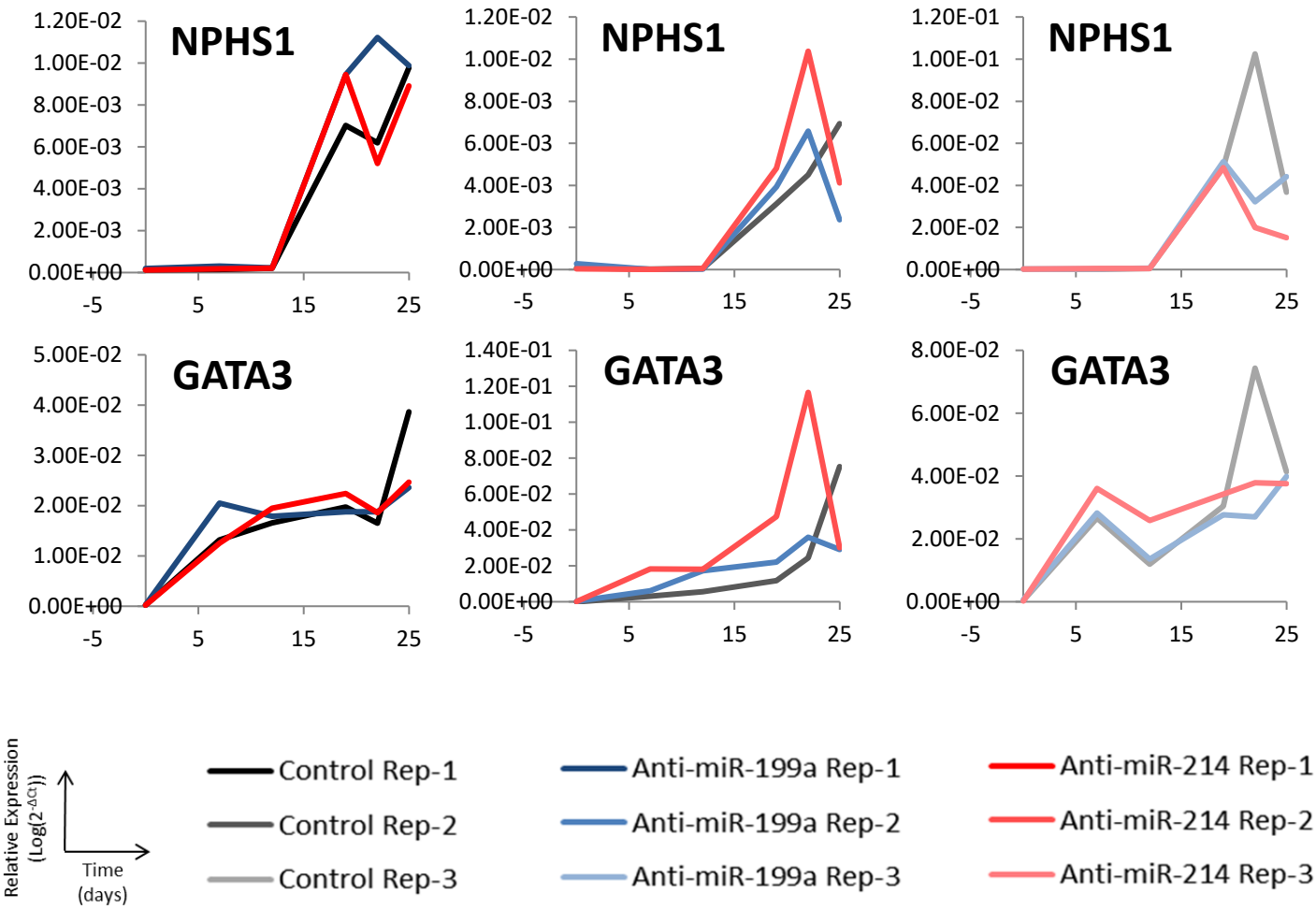

Figure S5. QPCR analysis of key marker gene expression in developing 3D kidney organoids derived from MAN13 hESCs transduced with either control or anti-miRNA sponge-expressing lentiviruses.

Figure S6

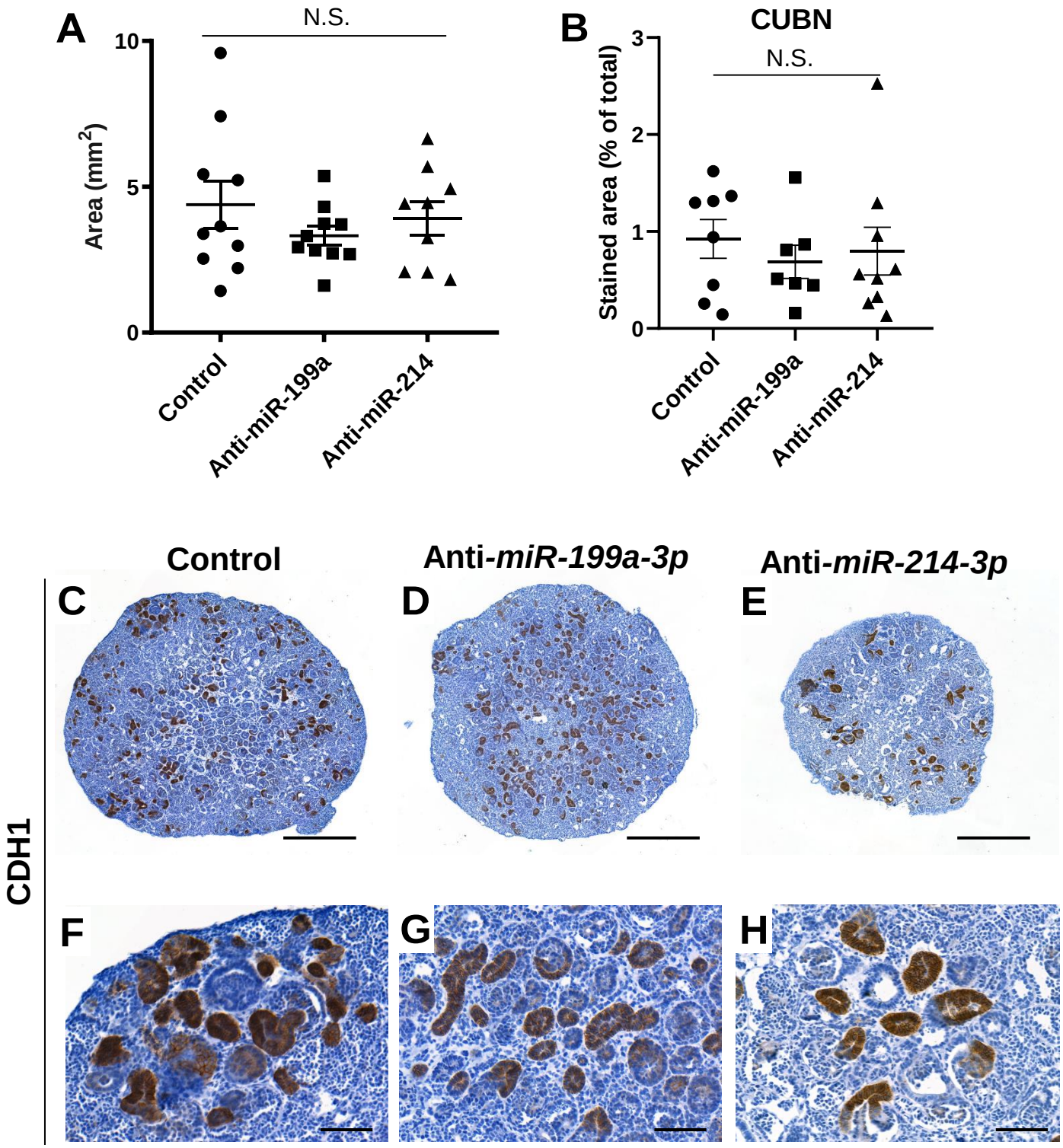

Figure S6. Size, total CUBN expression and CDH1 expression of anti-miRNA sponge-treated organoids.

Figure S7

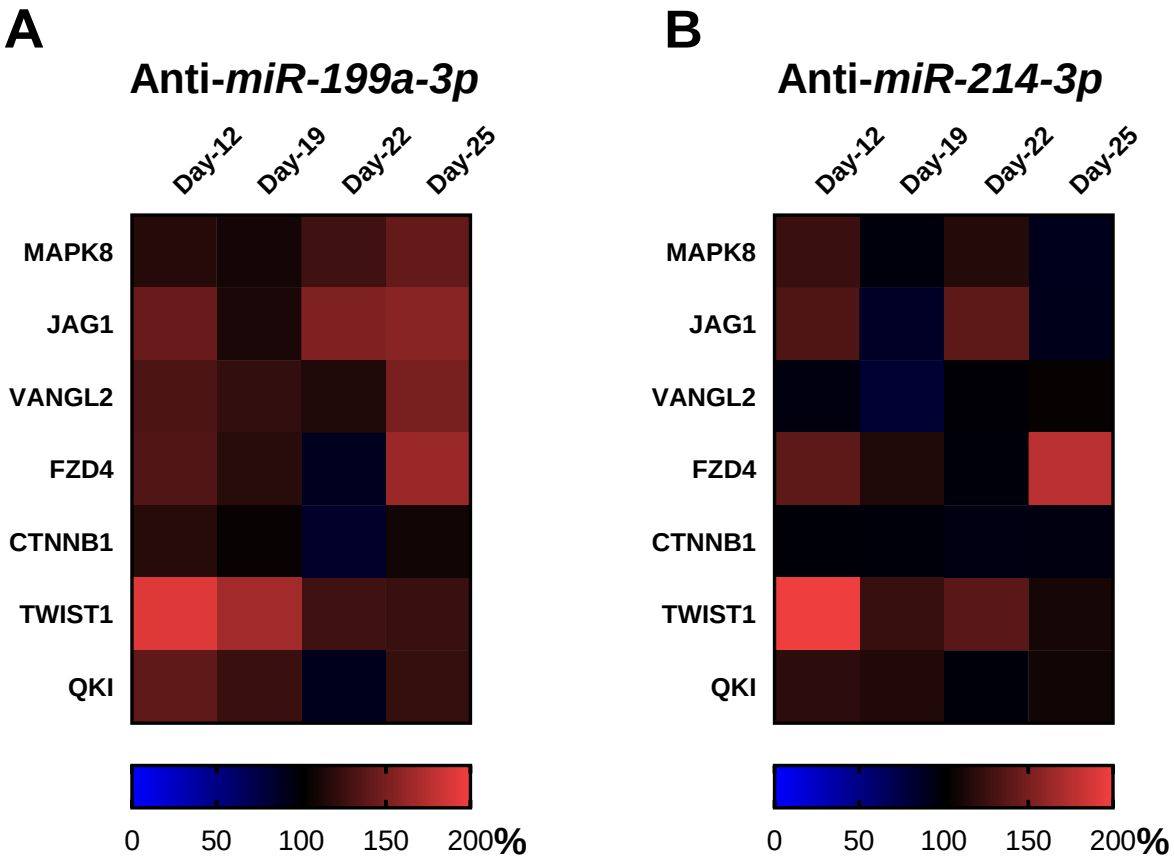

Figure S7. Derepression of targets of the *miR-199a/214* cluster, in the presence of anti-miRNA sponges..

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (Related to Figure 3). qPCR analysis of *miR-199a/214* cluster expression during 2D kidney differentiation in **(A)** MAN11 and **(B)** HUES1 hESCs. Different colours denote separate runs of the experiment.

Figure S2 (Related to Figure 4)**.** Inhibition of *miR-199a-5p*, *miR-199a-3p* or *miR-214-5p* by miRNA sponges in MAN13 differentiating 2D cultures did not alter the morphology of the developing epithelium, as shown by immunostaining with an anti-CDH1 antibody (compare to control in Figure 4).

Figure S3. (Related to Figure 4). Overexpression of *miR-199a* and *miR-214* in differentiating 2D cultures. MAN11 hESCs were transduced with either control or miRNA-overexpressing lentiviral vectors and differentiated to kidney in 2D (schematically shown in **(A)**). **(B-E)** Cultures were fixed at day 19 of differentiation and stained with antibodies against WT1. Overexpression of *miR-199a* or *miR-199a and miR-214 together* resulted in an increase of the $WT1^+$ cell population (MM) in emanating cell aggregates. Scale bars: 120μm. **(F)** Quantification of $WT1^+$ in kidney cell aggregates (one differentiation experiment; dots represent separate images taken from the same run of the experiment; mean±S.E.M. *P<0.001, ANOVA followed by post-hoc t-tests). **(G-J)** Day 19 cultures were also immunostained for CDH1. Overexpression of *miR-199a* or *miR-199a and miR-214 together* resulted in a decrease of the $CDH1^+$ epithelia in emanating cell aggregates. **(K-L)** Cultures were also fixed at day 30 (end of protocol) and immunostained for CDH1, revealing a change in morphology of the epithelial structures, towards a more rounded, compact shape after *miR-199a overexpression*. Scale bar: 60μm. **(M)** Example cell aggregate with schematic overlay explaining the metrics used to quantify its shape, following overexpression of the miRNAs. Parameters measured were: The length of the aggregate along its longest $CDH1^+$ axis (red), quantified in **(N)**; the ratio of the area of the main axis (blue) divided by the length (red), as a measure of how wide or elongated the aggregate was, quantified in **(O)**; the number of branching points (yellow) per unit length of the main axis, quantified in **(P)**. Overall, *miR-199a* overexpression (or overexpression of both miRNAs together) resulted in more rounded aggregates. Results in (N-P) are from one differentiation experiment, with between 32 and 57 aggregates measured per experimental group. Mean±S.E.M; *P<0.001, Kruskal-Wallis followed by post-hoc Dunn's tests.

Figure S4. (Related to Figures 6 and 7). Lentiviral-mediated expression in 3D organoids. Example of a day 25 organoid, derived from MAN13 hESCs that were transduced with a lentiviral vector expressing an anti-miR-199a-3p sponge and an EGFP tag (see schematic representation of the vector in figure 3A). The transgenes continue to be expressed through to the end of the differentiation protocol, as shown by the persistence of the EGFP signal. Scale bars: 600μm.

Figure S5. (Related to Figures 6 and 7). QPCR analysis of key marker gene expression in developing 3D kidney organoids derived from MAN13 hESCs transduced with either control or anti-miRNA sponge-expressing lentiviruses. The results of three independent differentiation experiments are shown in each case. At least three organoids were pooled for each RNA sample, at each time point. Expression shown as $2^{-\Delta Ct}$ relative to GAPDH against time.

Figure S6. (Related to Figures 6 and 7). Size, total CUBN expression and CDH1 expression of anti-miRNA sponge-treated organoids. **(A)** Quantification of the size of histological sections used in IHC experiments, showed that inhibition of *miRNA-199a-3p* or *miR-214-3p* activity had no effect on the overall mid-section area of organoids, reflecting their size. (Mean ± S.E.M.; each dot represents a separate organoid; organoids from three independent experiments were used; N.S.: Not significant, ANOVA). **(B)** Quantification of the extent of CUBN staining over entire histological sections showed that there was no significant change in the proportional area of $CUBN^+$ tubules (Mean ± S.E.M.; each dot represents a separate organoid; organoids from three independent experiments were used; N.S.: Not significant, ANOVA). **(C-E)** Low and **(F-H)** higher magnification representative images of sections from control and anti-miRNA sponge-treated organoids, immunostained for CDH1 (brown, over haematoxylin (blue) counterstain). Scale bars: A-C, 500μm; D-F, 100μm.

Figure S7. (Related to figures 6 and 7). Derepression of targets of the *miR-199a/214* cluster, in the presence of anti-miRNA sponges. **(A, B)** Heatmaps showing the derepression of key kidney developmental genes in anti-miRNA sponge treated organoids, during their differentiation. Gene expression was determined at different time points by qPCR, and each box of the map represents the change of expression relative to untreated controls (set at 100%, in each case). Each box on the map is the median of three independent differentiation experiments.

SUPPLEMENTAL TABLES

Table S1. Antibodies used in immunocytochemical (ICC) and immunohistochemical (IHC) analyses. (Related to Experimental Procedures).

Antibody	Host	Source	Catalogue #	Application	Dilution
CUBN	Goat	Santa Cruz	sc-20607	IHC	1:100
CDH1	Mouse	Abcam	76055	ICC	1:300
				IHC	1:1000
MEIS1/2/3	Mouse	Active Motif	39796	IHC	1:200
PODXL	Mouse	R&D Systems	MAB1658	IHC	1:200
PECAM1 (CD31)	Mouse	Cell Signalling	3528	IHC	1:100
SYNPO (H-140)	Rabbit	Santa Cruz	sc-50459	IHC	1:200
WT1	Rabbit	Santa Cruz	sc-192	ICC	1:100
WT1	Rabbit	Calbiochem	CA1026	IHC	1:1000

Table S2. Primers used for quantitative PCR. (Related to Experimental Procedures).

Gene Name	Forward or Reverse	Sequence
AQP1	Fwd	ATTAACCCTGCTCGGTCCTT
AQP1	Rev	ACCCTGGAGTTGATGTCGTC
AQP2	Fwd	GTGCGCCGAAAATTTCCA
AQP2	Rev	CCTCGACTTCTCCTTGAAGCA
CDH1	Fwd	CAATACATCTCCCTTCACAGCA
CDH1	Rev	AATGATAGATTCTTGGGTTGGGTC
CITED2	Fwd	CACCAATGGGCTGCACCATCAC
CITED2	Rev	GCCGCTCGTGGCATTCATGTTG
CTNNB1	Fwd	GCCACAAGATTACAAGAAACGG
CTNNB1	Rev	CAAGATCAGCAGTCTCATTCCA
DNM3OS	Fwd	CTTACAATGCTTCCACTTCTCTG
DNM3OS	Rev	TCTGCCTTCGTTTACAAATTCC
FZD4	Fwd	CTGACAACTTTCACACCGCT
FZD4	Rev	CATTGGCACATAAACAGAACAAAGG
GAPDH	Fwd	AGCCACATCGCTCAGACAC
GAPDH	Rev	GCCCAATACGACCAAATCC
GATA3	Fwd	GCCCCTCATTAAGCCCAAG
GATA3	Rev	TTGTGGTGGTCTGACAGTTCG
HOXB7	Fwd	GCCTACAAATCATCCGGCCA
HOXB7	Rev	GGTTGGAAGCAAACGCACAA
JAG1	Fwd	CTTCACCTTGTGCCTTTGGA
JAG1	Rev	GGTCTCCCTGAAACTTCCTG
LHX1	Fwd	ATGCAACCTGACCGAGAAGT

LHX1	Rev	CAGGTCGCTAGGGGAGATG
MAPK8	Fwd	CTCTCCTTTAGGTGCAGCAG
MAPK8	Rev	CGGATCTGTTGACATTGAAGAC
NPHS1	Fwd	AGTGTGGCTAAGGGATTACCC
NPHS1	Rev	TCACCGTGAATGTTCTGTTCC
NPHS2	Fwd	CAAAGTGCGGATGATTGCTG
NPHS2	Rev	GTGTGGAGGTATCGAAGCTG
OSR1	Fwd	CTCCTCGAGATCCGGATTGAG
OSR1	Rev	GTTCACTGCCTGAAGGAAGG
PAX2	Fwd	GCAACCCCGCCTTACTAAT
PAX2	Rev	AACTAGTGGCGGTCATAGGC
PDGFRB	Fwd	GCCGTCAAGATGCTTAAATCC
PDGFRB	Rev	TATAGATGGGTCCTCCTTTGGT
PODXL	Fwd	TCATCATCACCATCGTCTGC
PODXL	Rev	CCACCTTCTTCTCCTGCATC
QKI	Fwd	CTGATGAACGACAAGAAGCTC
QKI	Rev	CGTACTCTGCTAATTTCTTCGTC
SALL1	Fwd	AGCGAAGCCTCAACATTTCCAATCC
SALL1	Rev	AATTCAAAGAACTCGGCACAGCACC
SALL4	Fwd	CAGATCCACGAGCGGACTCA
SALL4	Rev	CCCCGTGTGTCATGTAGTGA
SIX2	Fwd	CGCCCATGTGGGTCAGTGGG
SIX2	Rev	AGCCGGGAGCGCTGTAGTCA
SYNPO	Fwd	CATGGTGGAAAGGAGGATGATGG
SYNPO	Rev	ACTTGGGGTCGGAGCTGGGATAC
TWIST1	Fwd	CTTCTCGGTCTGGAGGATGG
TWIST1	Rev	TTCTCCTTCTCTGGAAACAATGAC
UMOD	Fwd	AACATCACTGATATCTCCCTCCT
UMOD	Rev	TTGTCTCTGTCATTGAAGCCC
VANGL2	Fwd	GATGAGCGGGATGACAACTG
VAGNL2	Rev	GTGTGAGGTCATCATGGGAG
WNT4	Fwd	ACCTGGAAGTCATGGACTCG
WNT4	Rev	TCAGAGCATCCTGACCACTG
WNT9B	Fwd	AGTACAGCACCAAGTTTCTGAG
WNT9B	Rev	ACTCTTCACAGCCTTGATGC
WT1	Fwd	GGCAGCACAGTGTGTGAACT
WT1	Rev	CCAGGCACACCTGGTAGTTT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Quantitative PCR

QPCR for the *DNM3OS* and protein-coding transcripts was performed using the TaqMan® RNA-to-Ct™ 1-Step Kit (ThermoFisher, #4392653) according to the manufacturer's instructions, on a BioRad C1000™ Thermal Cycler fitted with a CFX384™ Real Time System, using 15ng of RNA per reaction. GAPDH was used as the housekeeping gene to normalise readings between samples. Primers used are listed in Table S2.

For mature miRNA detection, the following TaqMan™ miRNA Assays (ThermoFisher, #4366597) were used: *hsa-miR-199a* (assay number #000498), *hsa-miR-199a** (assay number #000499), *hsa-miR-214* (assay number #002306), *hsa-miR-214** (assay number #002293). All readings were normalised against the *RNU6b* (assay number #001093). Ten ng of RNA were reverse transcribed in a 15µl reaction, using the TaqMan™ MicroRNA Reverse Transcription kit (ThermoFisher #4366596) and the RT primer of the appropriate miRNA assay according to the manufacturer's instructions. For the PCR, 1µl of the RT reaction was amplified in a total volume of 15µl, using the appropriate miRNA Assay mix and TaqMan™ Universal Mastermix II, no UNG (ThermoFisher, #4440040) according to the manufacturer's instructions.

In situ *hybridisation of miRNA in kidney tissue and 3D organoid sections*

3D organoids were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 µm. Digoxigenin-labelled miRCURY LNA® probes against *miR-199a-3p* (#YD00615410) and *miR-214-3p* (#YD00611471) and a control scrambled probe (#YD00699004) were purchased from QIAGEN and used according to the manufacturer's instructions. Proteinase K (QIAGEN, miRCURY LNA miRNA ISH Buffer Set (FFPE), #339450) treatment was for 10min, at 0.33x strength, at 37ºC. Scrambled and anti-*miR-199a* probes were used at a final concentration of 40nM, whereas the anti-*miR-214-3p* probe was used at 1nM at a hybridisation temperature of 55ºC. An alkaline phosphatase-linked, sheep anti-digoxygenin antibody (Sigma-Aldrich, #11093274910) was then used at 1:400 dilution. The alkaline phosphatase catalysed reaction was allowed to proceed for 2h followed by counterstaining with Nuclear Fast Red. Images were acquired on a 3D-Histech Pannoramic-250 microscope slide-scanner using a 40x/0.95 Plan Apochromat objective (Zeiss). Snapshots of the slide-scans were taken using the Case Viewer software (3D-Histech).

Immunostaining of 2D cultures

Cells were washed twice in PBS and then fixed in 4% paraformaldehyde (PFA) for 20 minutes, followed by another two PBS washes. The fixed cells were blocked and permeabilised for 30 min with 3% bovine serum albumin (BSA)/0.3% Triton-X in PBS before overnight incubation at 4˚C with primary antibodies (Table S1) diluted in 3% BSA/PBS. They were then washed three times with PBS/0.1% Triton-X, followed by Alexa-Fluor™-488- or Alexa-Fluor™-594-labelled, anti-mouse or anti-rabbit (as needed) (Life Technologies; 1:300 dilution in 3% BSA/PBS). Images were captured on an Olympus IX-71 inverted fluorescence microscope and captured using a QImaging Retiga SRV camera. Images were then processed and analysed using ImageJ (http://imagej.net/Fiji/Downloads).

Imunohistochemistry on 3D organoid sections

Tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 µm. Sections were dewaxed and rehydrated. After rehydration, slides were boiled in an 800W microwave in 10 mM sodium citrate buffer (pH 6.0). After cooling to room temperature, endogenous peroxidase activity was blocked using 0.3% H_2O_2 in PBS for 10 minutes. Sections were permeabilized using 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes and blocked using 1% bovine serum albumin (BSA) with 10% serum from the species in which the secondary antibody was raised. Sections were incubated overnight at 4°C with the primary antibody + 1% BSA. Primary antibodies used are listed in Table S1. Biotin-conjugated species specific secondary antibodies with 1% BSA were incubated at room temperature for 2 hours. Following

PBS washes, slides were incubated in avidin-biotin enzyme complex (Vector Laboratories VECTASTAIN Elite ABC Reagent, PK-6100) for 1 hour at room temperature. Peroxidase activity was detected with the 3, 3'-diaminobenzidine (DAB) peroxidase substrate solution (Vector Laboratories, SK4100). Images were acquired on a 3D-Histech Pannoramic-250 microscope slide-scanner using a 40x/0.95 Plan Apochromat objective (Zeiss). Snapshots of the slide-scans were taken using the Case Viewer software (3D-Histech).

Lentiviral vector production and hESC transduction

Briefly, HEK-293T cells were transfected by calcium phosphate precipitation, in 15cm dishes, at 50% confluency, with 10µg shuttle plasmid, 10µg pMDLg-pRRE, 3.4µg pMD2.G and 2µg pRSV-Rev per dish. Cell medium was collected over two days and centrifuged at 6,000g overnight, the pellet resuspended in PBS and the new suspension centrifuged in a SW40-Ti rotor (Beckman Coulter Ltd, High Wycombe, UK) at 50,000g for 90min. Finally, the resulting pellet was resuspended in PBS, at 1:2,000 of the original medium volume. The viral titre was calculated by FACS (detecting EGFP fluorescence) on HEK-293T cells transduced with serial dilutions of the viral preparations.

Combined KEGG pathway / miRNA-target predicition analysis

Version 2 of the DIANA miRPath software was used (Vlachos et al., 2012). The four mature species of the human *miR-199a/214* cluster (*hsa-miR-199a-5p, hsa-miR-199a-3p, has-miR-214-5p, hsa-miR-214-3p)* were used as input.