Supplemental Information

The miR-17-92 cluster counteracts quiescence and chemoresistance in a distinct subpopulation of pancreatic cancer stem cells

Michele Cioffi[1], Sara M. Trabulo[1], Yolanda Sanchez-Ripoll[1], Irene Miranda-Lorenzo[1], Enza Lonardo[1], Jorge Dorado[1], Catarina Reis Vieira[1,3], Juan Carlos Ramirez[3], Manuel Hidalgo[2], Alexandra Aicher[1], Stephan Hahn[4], Bruno Sainz, Jr.[1], Christopher Heeschen[1,5]

1. SUPPLEMENTAL DATA

- **Figure S1: Gemcitabine resistant cells are enriched for CSCs,** related to Figure 1
- **Figure S2: Slow cycling cells possess CSC phenotype,** related to Figure 2.
- **Figure S3: miR-17-92 downregulation in spheres and in gemcitabine resistant cells,** related to Figure 3.
- **Figure S4: miR-17-92 overexpression impairs CSC phenotype,** related to Figure 4.
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- **Table S1: Table of utilized primer sequences for real-time RT-qPCR**
- **Table S2: Oligonucleotides used for the production of 5 shRNAs against p21**

2. SUPPLEMENTAL EXPERIMENTAL PROCEDURES

1. SUPLEMENTAL FIGURE LEGENDS

Figure S1 – Gemcitabine resistant cells are enriched for CSCs. (**A**) Flow cytometry for surface expression of CD133, SSEA1, and CXCR4 in Gemcitabine resistant cells versus control cells. (**B**) QPCR analysis of pluripotency-associated genes (**left panel**) and genes related to EMT (**right panel**). (**C**) QPCR analysis for expression of TGFBR2, ALK4, SMAD2, SMAD4, and TBX3 in gemcitabine resistant cells versus control cells. (**D**) QPCR analysis for the expression of ABC transporters (ABCC1 and ABCG2) and Gemcitabine transporters (hENT1, hENT2, hCNT1, and hCNT3) in gemcitabine resistant cells versus control cells. Data are normalized for ß-Actin expression. (**E**) Flow cytometry analysis of gemcitabine treated tumors depleted by MACS for mouse CD45 and mouse CD146**.**

Figure S2 – Slow cycling cells possess CSC phenotype. (**A**) Flow cytometry for surface expression of CD133, SSEA1, and CXCR4 in PKH26+ and PKH26– cells, respectively (**B**) Sphere formation capacity of PKH26+ versus PKH26– cells isolated from A6l, 253, and 354 PDAC tumors (n=3; *P<0.05).

Figure S3 – miR-17-92 downregulation in spheres and in gemcitabine resistant cells. (**A**) QPCR analysis for the expression of miR-17-92 family members in Gemcitabine resistant cells and spheres versus respective control cells. (**B**) QPCR analysis for the expression of miR-17-92 family members in PKH26+ and PKH26– cells isolated from 185 and 354 PDAC tumors. (**C**) Western blot analysis for the expression of the cell cycle regulator p21 in adherent cells treated with antagomir-17-92 versus antagomir-Ctrl. (**D**) *In vivo* tumorigenicity of A6L and 185 treated with antagomir-17-92 versus antagomir-Ctrl.

Figure S4 – miR-17-92 overexpression impairs CSC phenotype. (**A**) Lentiviral overexpression of miR-17-92 in CSCs. Representative images of GFP expression post infection (**upper panel**), illustration of the utilized sorting strategy (**middle panel**), and qPCR analysis for the expression of miR-17-92 family members in GFP sorted cells (**lower panel**). (**B**) QPCR analysis for the expression of pluripotency-associated genes in miR-17-92 cells versus miR-Ctrl

cells. (**C**) Representative images for ß-galactosidase staining in miR-17-92 cells versus miR-Ctrl cells. (**D**) Percentage of PKH26+ cells over a period of 4 weeks in miR-17-92 cells versus miR-Ctrl. (**E**) Sphere counts during serial *in vivo* passaging of miR-17-92 cells versus miR-Ctrl cells. (**F**) Cell cycle analysis using Ki67 and DAPI of cell freshly isolated cells after serial *in vivo* passaging. (**G**) Quantification of migratory activity in the wound-healing assay with stimulation of TGF-ß1 or NODAL in the presence or absence of the ALK4,5,7 inhibitor SB 431502.

Figure S5 – miR-17-92 target multiple members of NODAL/TGF-ß1 signaling. (**A**) Sequence alignment of miR-17-92 family members' seed sequence in *TGFBR2* 3′ UTR, *ALK4* 3′ UTR, *SMAD2* 3′ UTR, *SMAD4* 3′ UTR, *p21* 3′ UTR, *p57* 3′ UTR, and *TBX3* 3′ UTR. (**B**) QPCR analysis for the expression of miR-17-92 family members 96h after induction with Doxycycline. (**C**) RTqPCR analysis of miR-17-92 target genes after 24h of treatment with single antagomirs against each of the six members of the miR-17-92 cluster.

Figure S6 – Knockdown of p21 or TBX3 inhibits cancer stem cell phenotype. (**A**) Representative images of invaded cells **(left panel).** Percentage of invading cells (Ctrl versus sh-p21) through Matrigel^{TM} following stimulation with FBS, NODAL or TGF-ß1 **(right panel**). (**B**) RTqPCR analysis of TBX3 mRNA levels in multiple PDAC sphere cultures (s) compared to their respective adherent controls ($n = 3$; $*p < 0.05$). (**C**) Western blot analysis for the expression of TBX3 in cells sorted for CD133 surface expression (**upper panel**) and densitometric quantification of the western blot (**lower panel**). (**D**) QPCR analysis for the expression of TBX3 in cells sorted for ALK4 surface expression. (**E**) Percentage of invading cells through Matrigel^{TM} following stimulation with NODAL, ACTIVIN or TGF-ß1 ($n = 3$; $*p < 0.05$).

Figure S7: *In vivo* overexpression of miR-17-92 reverses quiescence and chemoresistance. Treatment effects of gemcitabine and abraxaen in miR-17-92 overexpressing cells in established tumors derived from **(A)** A6L and (**B**) 185 cells, respectively, in immunocompromised mice. The mean tumor volume is given; n 6 tumors per group. P value was determined by Student t test.

SUPPLEMENTARY MATERIALS & METHODS

Sphere formation assay. Spheres were generated by culturing ~2×10^4 pancreatic cancer cells in suspension in serum-free DMEM/F12 medium supplemented with B27 (1:50, Invitrogen, Alcobendas, Spain), 20ng/ml bFGF and 50units/ml pen/strep for a total of 7 days, allowing spheres to reach a size of >75μm. For serial passaging, 7-day-old spheres were harvested using 40μm cell strainers, dissociated into single cells, and then re-cultured for 7 additional days as previously described (Lonardo et al. 2011).

RNA preparation and quantitative real-time PCR. Total RNAs from human primary pancreatic cancer cells and spheres were extracted with TRIzol (Life Technologies Inc.) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies) and random hexamers. Quantitative real-time PCR was performed using SYBR Green PCR master mix (QIAGEN), according to the manufacturer's instructions. The list of utilized primers is depicted in **Table S1**.

Microarray analysis of miRNA. Total RNA concentrations extracted with Trizol were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The miRNA microarray experiments were performed using the Agilent Microarray Platform (Agilent Technology, Foster city, CA) G4471A-025987 miRNA array composed by 866 human miRNAs probes and the Human Gene Expression 4x44K v2 Microarray contain 34,127 Entrez Gene RNAs. 100ng total RNA was hybridized to the miRNA array and for each miRNA, multiple probes were spotted on the array. In addition, multiple spots were included as negative controls. The arrays were scanned using an Agilent Technology G2565BA scanner and the scanned images were processed using the Feature Extraction software package version 9.5 (Agilent Technology). For data processing, we performed a global normalization via quantile normalization for each set of sample pairs and subsequently a pair wise comparison via unpaired t-test and Benjamini Hochberg correction for false discovery rate.

Quantification of mature miRNA levels using quantitative real-time PCR. For miRNA analysis, one microgram of total RNA was reverse-transcribed using the NCode VILO miRNA cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). This step adds a polyadenylate tail to the miRNA population within the total RNA samples. The resulting cDNA was subjected to real-time PCR using SYBR Green ER qPCR Mix (Invitrogen). The Universal qPCR Primer was provided in the VILO kit and the forward primer for miR-17, 18a, 19a, 19b, 20a, 92, Snord95 and Snord44 were purchasing from Qiagen.

Generation of Lentivirus. To stably over-express the miR-17-92 cluster we used lentiviral particles carrying the pre-miR-17-92 cluster in the pcopGFP lentivector (System Biosciences, Mountain View, CA) and pPACK packaging mixture. To produce lentiviral particles carrying a doxycycline-inducible pre-miR-17-92 cluster, a custom lentiviral vector was constructed, with constitutively expressed mCherry and M2rtTA (a reverse doxycycline transactivator), and a custom-designed promoter with the doxycycline operator and a minimal CMV promoter, under which the miR-17-92, amplified by PCR from pLVX-eG2N-1792 (construct previously described), was cloned. Replication-incompetent lentiviral particles were then produced by calcium-phosphate transfection of HEK293T cells using the packaging plasmids pMD.2G (VSV-G) and pPAX2 and the shuttle vector pInd-miR-17-92-mCherry. Six hours post transfection, the medium was refreshed and 48 h later, the medium was collected, cleared by low-speed centrifugation, filtered through 0.45μm pore-size PVDF filters, and stored in aliquots at –80ºC. To construct the lentiviral vectors carrying different shRNAs targeting p21, a forward and a reverse oligonucleotide for each shRNA was designed, in order to reconstitute the shRNAs through a hybridization PCR and to introduce restriction sites at the 5’ and 3’ ends of the shRNA for subsequent cloning. The shRNA oligonucleotides are listed in **Table S2**. The shRNAs were cloned into the lentiviral vector pLVX-shRNA2 from Clontech, under the control of the U6 promoter and with a zsGreen fluorescent protein reporter. Replication-incompetent lentiviral

particles were produced as described above. Tbx3 lentiviral vector and empty vector control were obtained from Origene.

Western Blot Analysis. Cells were harvested in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). 50µg of protein was resolved by SDS-PAGE and transferred to PVDF membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 1X TBS containing 5% BSA (w/v), 1% chicken albumin (w/v) and 0.1% Tween20 (v/v), incubated with a 1:1,000 dilution of antibodies against p21 (2947), p27 (3688), p57 (2557), Cyclin D1 (2926), pSMAD2 (3108) from Cell Signaling or GAPDH (ab8245), Tbx3 (ab58264), Nanog (ab14959) from Abcam overnight at 4ºC, washed 3 times with 1X PBS containing 0.05% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rat or goat anti-mouse antibody (Sigma), and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Amersham, Barcelona, Spain).

Flow cytometry. To identify pancreatic CSCs, the following antibodies were used: anti-CD133/1-APC or PE; (Miltenyi, Bergisch-Gladbach, Germany), anti-CXCR4-APC, SSEA-1-APC (both from Beckton Dickinson [BD], Heidelberg, Germany), anti-Alk4, (Cell Signaling Technology, Inc.), or appropriate isotype-matched control antibodies. DAPI was used for exclusion of dead cells (eBiosciences, San Diego, CA). Samples were analyzed by flow cytometry using a FACS Canto II (BD) and data were analyzed with FlowJo 9.2 software (Ashland, OR).

Wound-healing assay. Confluent cultures of primary cancer cells seeded in a 6-well plates were scratched using a 1 ml pipette tip after overnight starvation. Cells were washed twice with PBS to remove cell debris and then incubated at 37°C with serum-free media in the presence or absence of 300ng/ml recombinant human NODAL, 100ng/ml ACTIVIN, or 10 ng/ml TGF-β1. Migration was evaluated 24h later by calculating the average size of the wound determined by measuring the size of the wound at three locations (n = 3 wounds per cell/treatment).

Invasion assay. Invasion assays were performed using modified Boyden chambers filled with Matrigel™ (BioCoat, BD Biosciences, Heidelberg, Germany). Adherent and sphere culture derived cells were used for these experiments. Cells were added to the Matrigel™-coated inserts and 750µL of serum-free medium with 300ng/ml recombinant human NODAL, 100ng/ml ACTIVN, and 10ng/ml TGF-β1 was added to the lower chamber. The assay chambers were incubated for 24h at 37°C. Invasive cells were fixed in 4% PFA and stained with DAPI using Prolong Gold (Invitrogen). The number of invaded cells was compared to control.

Cell-cycle analysis. Cells were trypsinized, washed in PBS, centrifuged, and pellets were fixed in 200 µl of 70% ethanol and stored at -20°C until use. Cells were centrifuged and pellets resuspended in 200 µl of PBS with 10 µg/mL of RNAse A. Cells were incubated for 1 hour at 37°C prior to resuspension in DAPI. For the identification of G0 quiescent population, cells were fixed in 100% ethanol at -20°C overnight, washed with PBS twice and stained with Ki67 (BD, Heidelberg, Germany) for 30min at room temperature, followed by an additional wash with PBS and staining with DAPI. Cell-cycle analysis was carried out by flow cytometry (CANTO II). Data were analyzed by FlowJO software.

PKH26 assay. Human primary pancreatic cancer cells were labeled with PKH26 red fluorescent cell membrane dye (Sigma) according to the manufacturer's instructions. Every 7 days for a total of 4 weeks, cells were harvested and PKH26+ cells were determined by flow cytometry.

Senescence studies. Expression of pH-dependent senescence associated beta-galactosidase (SA-beta-gal) activity was analyzed in primary pancreatic cancer cells using the SA-beta-gal staining kit (Cell Signaling Technology, Boston, MA).

Apoptosis assay. Cancer cells and CSCs were plated at $3x10^5$ cells/well in a 6-well tissue culture plate and cultured in presence of gemcitabine (100ng/ml) for 7 days. Attached and floating cells were collected, resuspended and stained with Annexin V (550474; BD Bioscience) and after incubation with Annexin V binding buffer (556454; BD Pharmingen) cells were

incubated with DAPI. Annexin V+ cells were determined by cytometry.

Plasmid construct and transfection. The pCAGA12-luc SMAD4 reporter plasmid is a synthetic SMAD responsive luciferase reporter vector that was generated by cloning 12xCAGA (consensus SMAD binding element) into the pGL3 basic plasmid (Promega) (Dennler et al., 1998; Savary et al., 2013). Human primary pancreatic cells were plated in 24-well tissue culture plates and co-transfected with pCAGA12-luc SMAD4 reporter and control Renilla plasmid using Lipofectamine 2000. Forty-eight hours post transfection, Gaussian luciferase and Renilla luciferase were measured using a Dual Luciferase assay kit (Promega). Luciferase activity is plotted as a percentage of the activity measured in control transfected cultures.

3' UTR luciferase reporter assays. The GLuc-ALK4-3'-UTR clone and GLuc-Tbx3-3'-UTR clone (GeneCopoeia, Labomics, Nivells, Belgium) and a control 3'UTR-reporter construct were transfected in human embryonic kidney (HEK) 293T cells seeded in presence of 50 nmol/L of miR-17-92 precursor or negative control. Gaussian luciferase and alkaline phosphatase activities were measured by luminescence in conditioned medium 48 hours after transfection using Dual-Light® detection system (Genecopoeia). Luciferase activity was plotted as a percentage of the activity measured in control-transfected cultures.

Antagomirs. Knockdown of miR-17-92 was achieved *in vitro* and *in vivo* by administering a miR-17, -18a, -19a, -19b, -20a, -92 antagomir mix or scrambled control, which were chemically synthesized as 2□-O-methyloligoribonucleotides by BioSpring (Frankfurt, Germany). The antagomirs are labeled with Cy3 and contain cholesterol, which facilitates both their tracking and entry into cells, respectively.

Immunohistochemistry. For histopathological analysis, FFPE blocks were serially sectioned (3μm thick) and stained with hematoxylin and eosin (H&E). Additional serial sections were used for *in situ* hybridization (ISH) with ALU probe or immunohistochemical (IHC) analysis for CYTOKERATIN-19. For ISH, antigens were visualized using a DAKO ISH

Detection systems, and for IHC, antigens were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+). Counterstaining was performed with hematoxylin. Histological quantification of digitalized slides was performed using Pannoramic Viewer (3DHistech, Budapest, Hungary).

Statistical analyses. Results for continuous variables are presented as means ± standard deviation (SD) unless stated otherwise. Treatment groups were compared with the independent samples t test. Pair-wise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. P values <0.05 were considered statistically significant. All analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, Illinois).

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