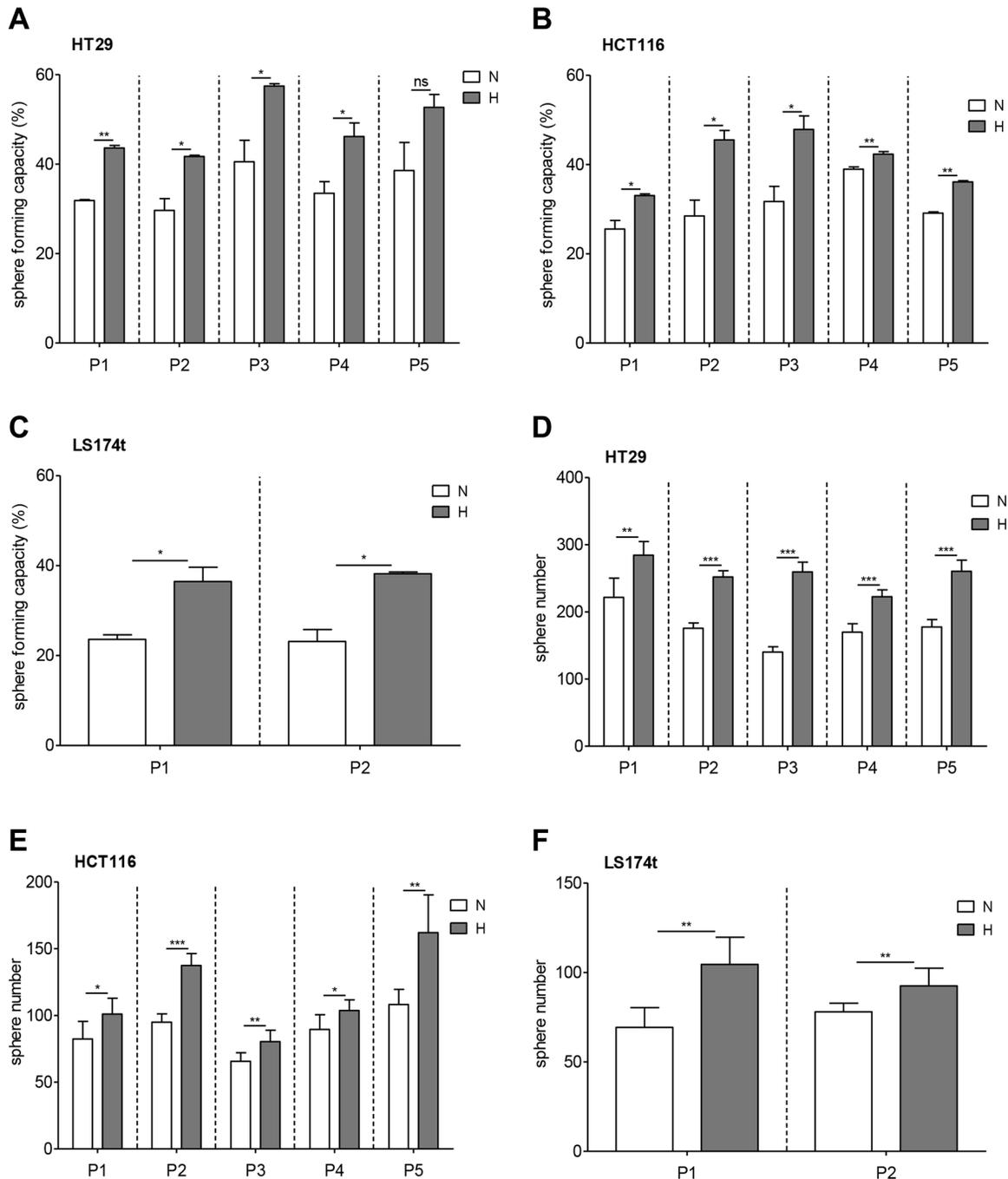
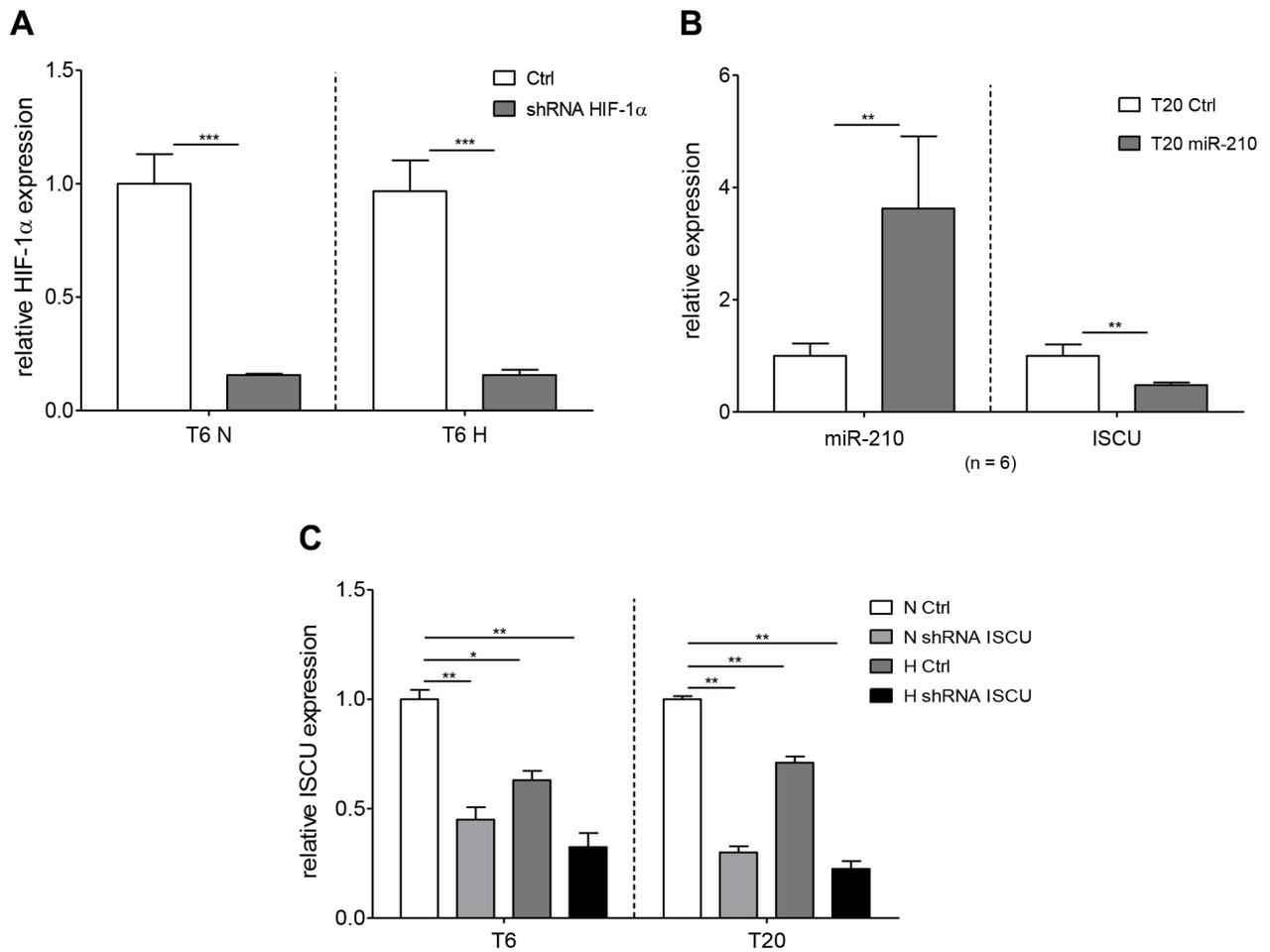


## Hypoxia-responsive miR-210 promotes self-renewal capacity of colon tumor-initiating cells by repressing ISCU and by inducing lactate production

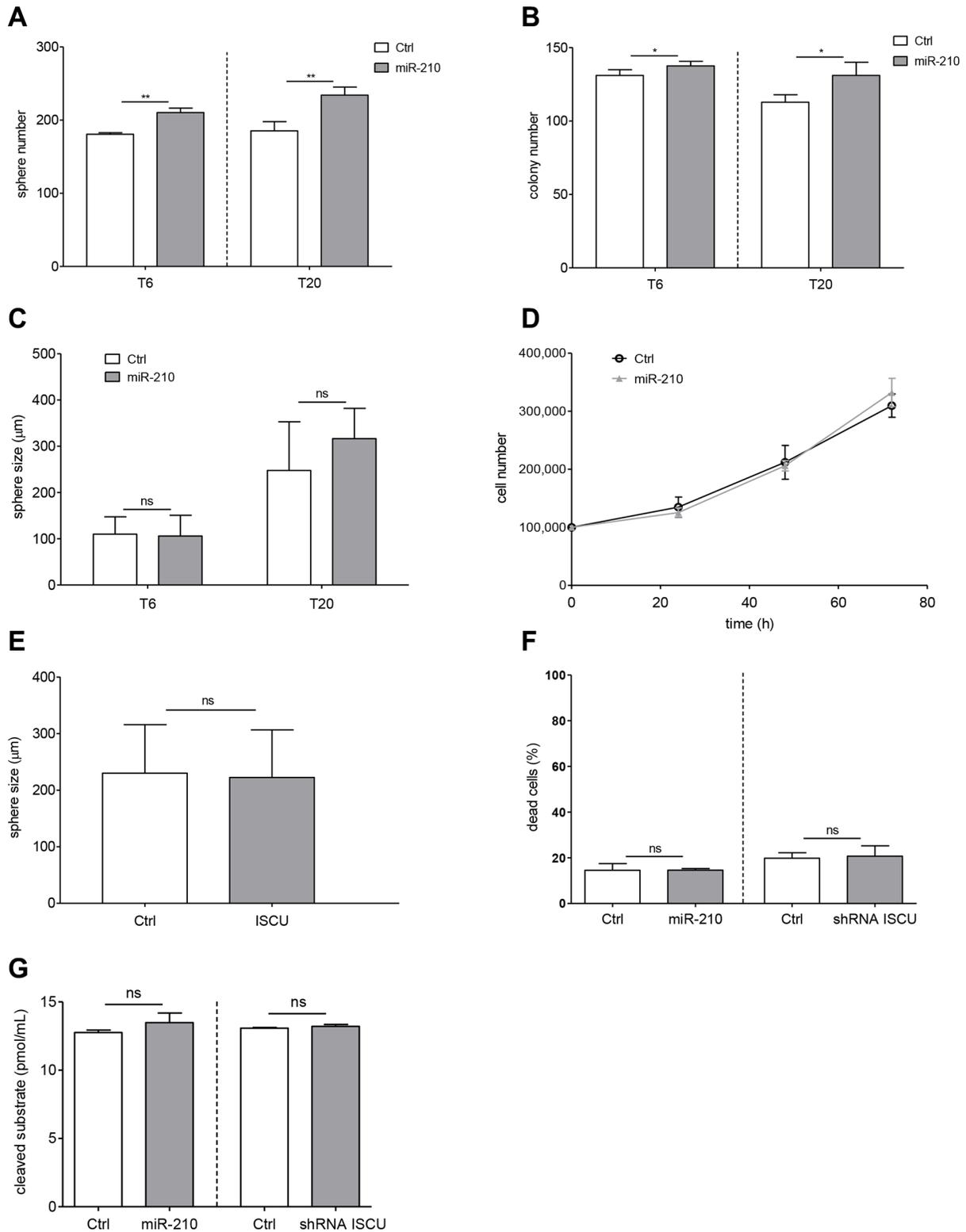
### Supplementary Materials



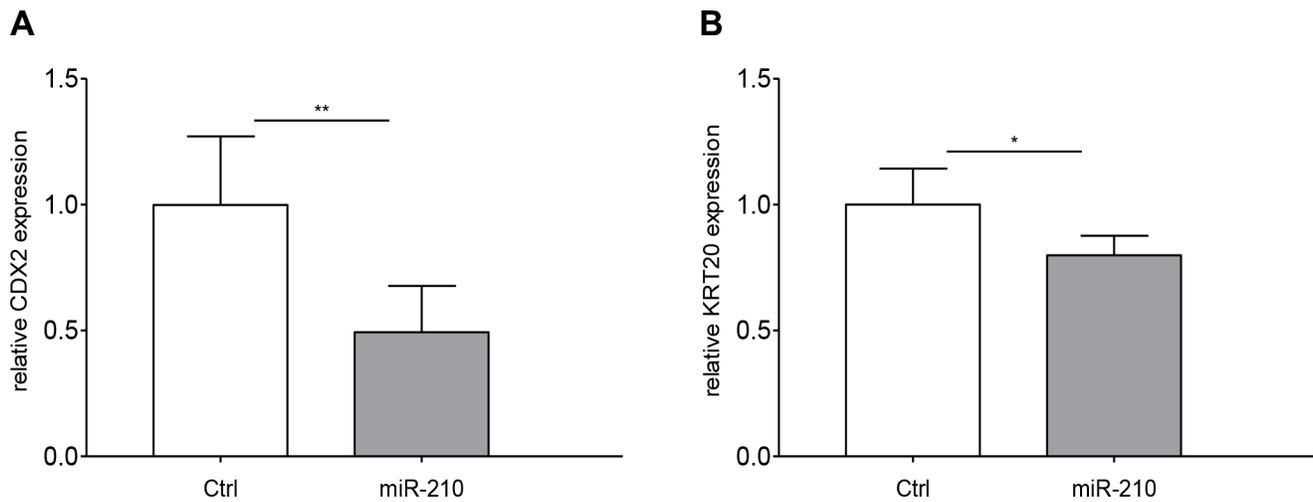
**Supplementary Figure S1: Hypoxia promotes colon TIC self-renewal over consecutive passages.** Single cell assays were performed for (A) HT29, (B) HCT116, and (C) LS174t SCs over consecutive passages under normoxic and hypoxic conditions, respectively. 1,000 cell assays were performed for (D) HT29, (E) HCT116, and (F) LS174t SCs over consecutive passages under normoxic and hypoxic conditions, respectively. Data are presented as mean  $\pm$  SD; unpaired Student's *t*-test was used to compare normoxia and hypoxia; ns = not significant, \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001; N–normoxia, H–hypoxia.



**Supplementary Figure S2: Knockdown and overexpression controls.** (A) Evaluation of HIF-1 $\alpha$  knockdown after stable transduction of lentiviral particles with HIF-1 $\alpha$  short hairpin RNA and after 72 h under normoxic or hypoxic conditions, respectively. Representative figure of 2 independent experiments with technical triplicates. (B) Relative expression of miR-210-3p and ISCU in extracted T20 tumors, 1 month after subcutaneous injection of 10,000 miR-210-overexpressing or control cells, respectively;  $n = 6$  mice/group. (C) Relative expression of ISCU in T6 and T20 SCs after lentiviral transduction of ISCU short hairpin RNA or control vector, respectively. SCs were exposed to 72 h normoxic or hypoxic conditions prior to assessing ISCU expression levels. Representative figure of 2 independent experiments with technical duplicates; data represented as mean  $\pm$  SD; unpaired Student's  $t$ -tests were used for (A) and (C); paired Student's  $t$ -test was used for (B); \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ; N–normoxia, H–hypoxia, shRNA–short hairpin RNA.



**Supplementary Figure S3: miR-210 promotes sphere and colony formation without affecting proliferation, viability, or apoptotic rates of colon TICs.** (A) Sphere numbers in T6 and T20 1,000 cell assays after stable overexpression of miR-210. (B) Colony formation assays were used to determine the clonogenic capacity of T6 and T20 SCs after stable overexpression of miR-210. (C) Size of single-cell-derived miR-210-overexpressing or control T6 and T20 spheroids, after 10 days in culture. (D) Growth curve of miR-210-overexpressing and control T20 SCs over 3 days. (E) Size of single-cell-derived T20 control spheroids and T20 SCs after lentiviral transduction of ISCU short hairpin RNA, after 10 days in culture. (F) Assessment of dead T20 cells by flow cytometry after stable overexpression of miR-210 or knockdown of ISCU, respectively. (G) Apoptotic rates, assessed by caspase 3 activity assay, after stable overexpression of miR-210 or knockdown of ISCU in T20 SCs. Results are representative of at least 2 independent experiments with 3 technical replicates for (A), 4 for (B), 8 for (C), 3 for (D), 8 for (E), 2 for (F) and 3 for (G). Data are presented as mean  $\pm$  SD; unpaired Student's *t*-tests were used to compare two groups; ns = not significant, \* $p < 0.05$ , and \*\* $p < 0.01$ .



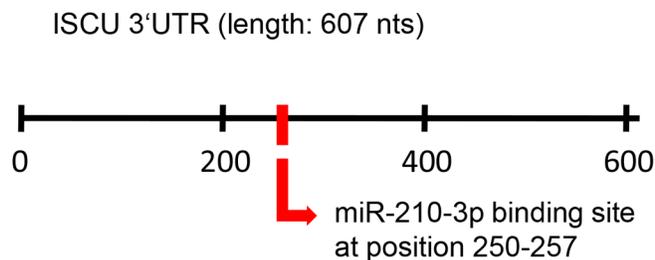
**Supplementary Figure S4: Decreased expression of differentiation markers in xenografts after overexpression of miR-210.** Relative expression of (A) CDX2 and (B) KRT20 (cytokeratin 20) in extracted T20 tumors, following subcutaneous injection of 10,000 cells with stable overexpression of miR-210 or respective control vector. Data are represented as mean ± SD;  $n = 6$  mice/group; unpaired Student's  $t$ -test was used to assess statistical significance;  $*p < 0.05$  and  $**p < 0.01$ .

**A**

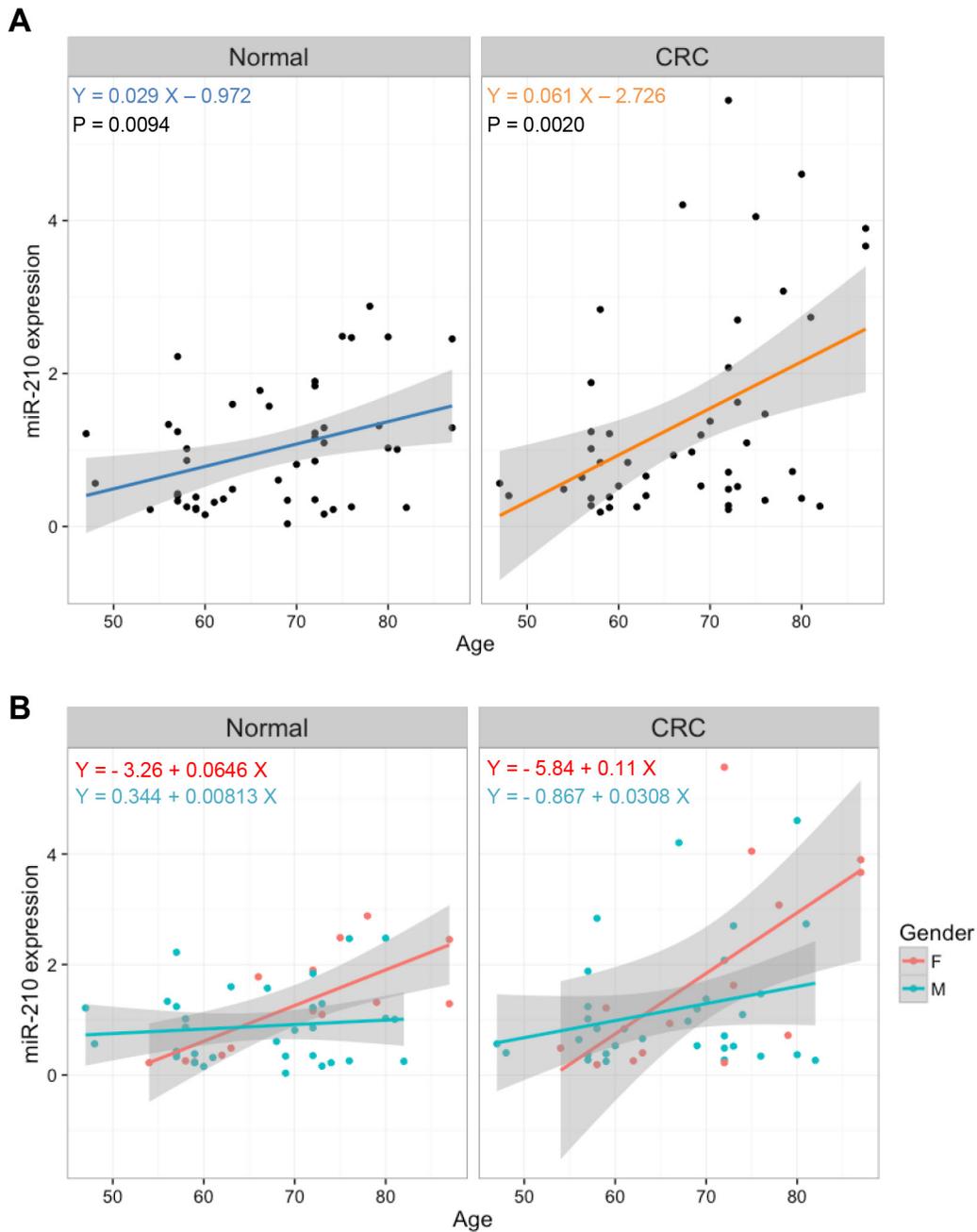
Gene	log <sub>2</sub> fold change	adj. P-value	miR-210 binding site
<i>BAAT</i>	-0,82	0,0019	no
<i>VAMP7</i>	-0,66	0,0046	yes
<i>MAP2K6</i>	-0,63	0,0475	no
<i>PLXND1</i>	-0,62	0,0015	no
<i>DIMT1</i>	-0,54	0,0055	yes
<i>ISCU</i>	-0,54	0,0497	yes
<i>SLC2A2</i>	-0,53	0,0133	no
<i>QPRT</i>	-0,50	0,0319	no
<i>NUPR1</i>	-0,47	0,0319	no
<i>NPC1L1</i>	-0,43	0,0319	no
<i>TEX9</i>	-0,42	0,0319	no
<i>RAB8B</i>	-0,39	0,0392	no

Downregulated genes after overexpression of miR-210 in T20 SCs. Differentially expressed genes (DEGs) were determined by setting a false discovery rate  $< 0.05$ . DEGs are ranked according to the absolute value of log<sub>2</sub> fold change (expression of miR-210 vs. Ctrl).

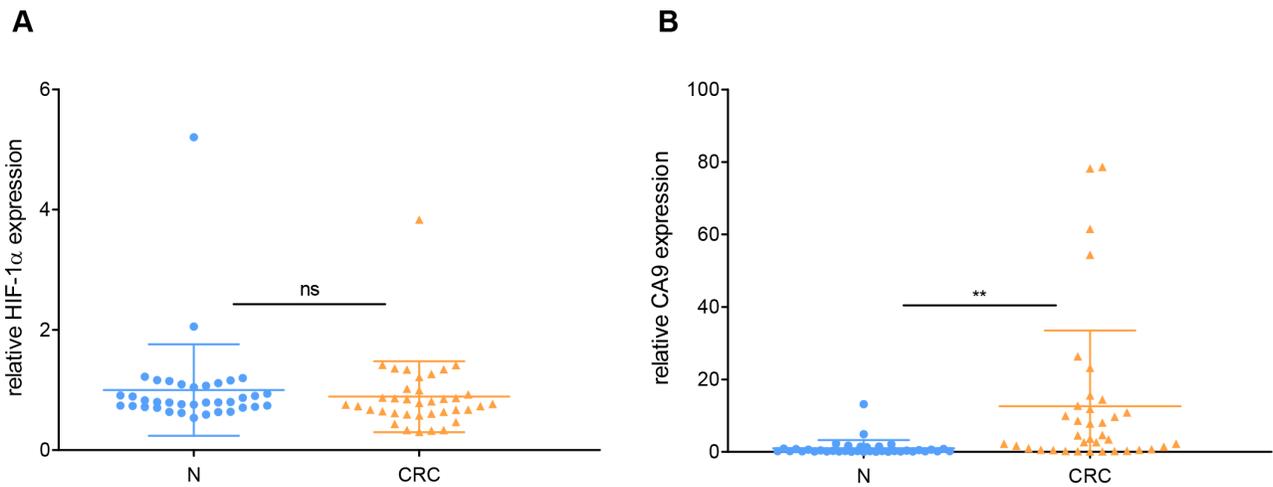
**B**



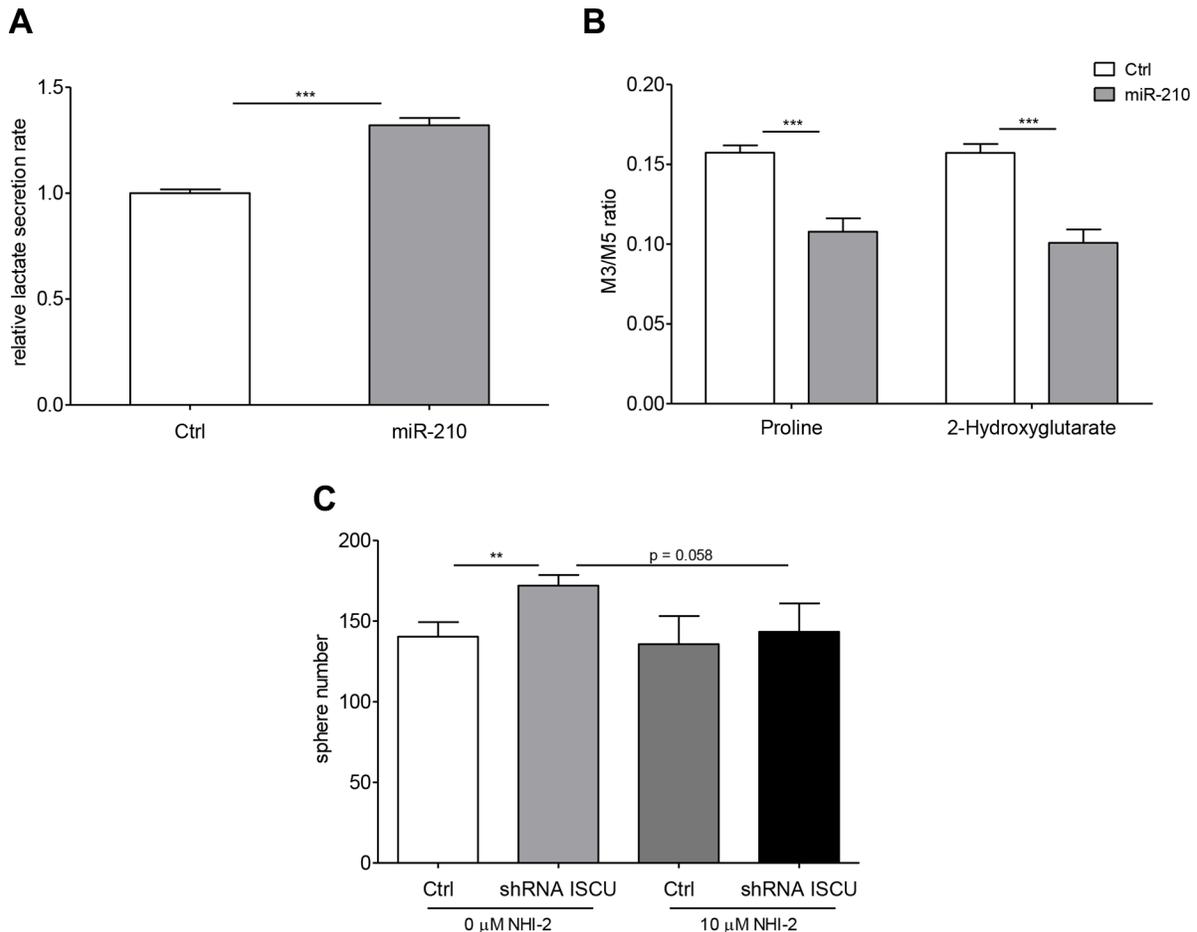
**Supplementary Figure S5: *ISCU* is a potential miR-210 target gene.** (A) List of 12 miR-210-responsive down-regulated genes, identified with microarray experiments for T20 SCs after lentiviral transduction of either miR-210 or respective control vectors; significance cutoff was set at FDR  $< 0.05$ . (B) Schematic representation of the miR-210-3p binding site in the 3'UTR of its potential target gene *ISCU*.



**Supplementary Figure S6: miR-210-3p expression correlates with patients' age and gender.** (A) Linear regression of the expression of miR-210-3p explained by patients' age in normal counterparts (left panel) and corresponding CRC samples (right panel);  $n = 47$ ; grey areas indicate 95% confidence interval. (B) Multiple linear regressions of the expression of miR-210-3p explained by patients' age and gender. Models built for both normal colon (left) and paired CRC samples (right);  $*p < 0.05$  for gender vs. age interaction in both cases; grey areas indicate 95% confidence interval.



**Supplementary Figure S7: HIF-1 $\alpha$  and CA9 expression in CRC patient samples.** Relative expression of (A) HIF-1 $\alpha$  and (B) CA9 in 47 CRC patient tumor samples, compared to their matching normal colon tissue. Paired Student's *t*-test was used to compare both groups; ns = not significant; \*\* $p < 0.01$ .



**Supplementary Figure S8: Overexpression of miR-210 and knockdown of ISCU result in altered TCA cycle activity.** (A) Relative extracellular lactate levels in T20 miR-210-overexpressing and respective control SCs, normalized to the corresponding cell numbers after 72 h. Data presented as mean  $\pm$  SD; representative experiment of 5 biological replicates with technical triplicates. (B) M3/M5 isotopologue ratios of proline and 2-hydroxyglutarate, for T20 miR-210-overexpressing or control SCs, after 72 h of incubation with U-13C gln. Data presented as mean  $\pm$  SD; representative figure of 2 independent experiments, each with technical triplicates. (C) Sphere formation of T20 SCs after stable knockdown of ISCU and inhibition of LDHA with 10  $\mu$ M NHI-2. Representative figure of 3 independent 1,000 cell assays with technical triplicates; data presented as mean  $\pm$  SD. Unpaired Student's *t*-tests were used to compare two groups; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

**Supplementary Table S1: Hypoxia-responsive miRNAs in HT29 and HCT116 SCs**

<b>HT29</b>			
<b>name</b>	<b>log fold change</b>	<b>fold change</b>	<b>p-value</b>
miR-210-3p	0.57	3.70	1.82E-03
miR-600	-0.42	0.39	1.42E-03
miR-885-5P	0.41	2.55	2.84E-03
miR-1539	0.33	2.12	1.57E-02
miR-708-3p	-0.32	0.48	1.96E-03
miR-95-3p	-0.31	0.49	2.13E-02
miR-491-5P	0.31	2.04	1.61E-03
miR-558	0.29	1.94	7.05E-03
miR-218-5p	0.29	1.94	4.61E-03
miR-3142	0.28	1.88	4.12E-03
miR-380-3p	0.25	1.79	1.48E-02
miR-875-3P	-0.25	0.56	5.40E-03
miR-148a-3p	0.22	1.66	3.55E-02
miR-155-5p	0.22	1.66	1.87E-02
miR-576-5P	0.18	1.53	3.16E-02
miR-574-3P	-0.18	0.66	1.59E-02
miR-520g-3p	-0.18	0.66	2.40E-02
miR-582-5P	-0.18	0.66	1.60E-02
miR-671-3P	0.18	1.50	4.73E-02
miR-935	-0.18	0.67	4.11E-02
miR-21-3p	0.17	1.48	2.68E-02
miR-200a-5p	0.16	1.46	3.17E-02
miR-3922	-0.13	0.74	4.47E-02
<b>HCT116</b>			
<b>name</b>	<b>log fold change</b>	<b>fold change</b>	<b>p-value</b>
miR-210-3p	0.61	4.07	3.90E-05
miR-301b	0.38	2.41	2.73E-02
miR-30c-2-3p	-0.34	0.45	3.01E-03
miR-148b-5p	0.33	2.16	2.75E-03
miR-520e	-0.33	0.46	3.30E-03
miR-34c-3p	-0.31	0.49	1.53E-02
miR-3157-5p	-0.30	0.50	2.42E-02
miR-520f-3p	-0.29	0.52	4.47E-02
miR-24-1-5p	0.29	1.94	2.85E-02
miR-449c-5p	0.26	1.81	1.65E-02
miR-4318	0.25	1.80	2.36E-02
miR-875-3p	-0.23	0.58	1.28E-02
miR-181d-5p	0.23	1.71	1.35E-02
miR-558	-0.20	0.62	3.42E-02
miR-891A	-0.19	0.64	4.59E-02
miR-181b-5p	0.19	1.55	2.75E-02

Differentially expressed miRNAs (DEMs) were determined by setting a  $p$ -value  $< 0.05$  as significance threshold. DEMs are ranked according to the absolute value of  $\log_{10}$  fold change (expression under hypoxia vs. normoxia).

**Supplementary Table S2: RT-qPCR primer pairs used in this study**

<b>Gene symbol</b>	<b>Forward</b>	<b>Reverse</b>
<i>EEF1A1</i>	5'-TTG-TCG-TCA-TTG-GAC-ACG-TAG-3'	5'-TGC-CAC-CGC-ATT-TAT-AGA-TCA-G-3'
<i>ACTB</i>	5'-GCA-AAG-ACC-TGT-ACG-CCA-ACA-3'	5'-ACA-CGG-AGT-ACT-TGC-GCT-CAG-3'
<i>YWHAZ</i>	5'-ACT-TTT-GGT-ACA-TTG-TGG-CTT-CAA-3'	5'-CCG-CCA-GGA-CAA-ACC-AGT-AT-3'
<i>ISCU</i>	5'-TCT-GCC-TTC-CTC-CCG-TGA-AAC-T-3'	5'-GAG-GGC-TCA-TTT-CTT-CTC-TGC-C-3'
<i>HIF1A</i>	5'-CGT-TCC-TTC-GAT-CAG-TTG-TC-3'	5'-TCA-GTG-GTG-GCA-GTG-GTA-GT-3'
<i>CDX2</i>	5'-ACA-GTC-GCT-ACA-TCA-CCA-TCC-G-3'	5'-CCT-CTC-CTT-TGC-TCT-GCG-GTT-C-3'
<i>KRT20</i>	5'-TGT-CCT-GCA-AAT-TGA-TAA-TGC-T-3'	5'-AGA-CGT-ATT-CCT-CTC-TCA-CTC-TCA-TA-3'
<i>CA9</i>	commercial RT <sup>2</sup> qPCR primer assays for human Ca9 (Qiagen)	