Mutual epithelium-macrophage dependency in liver carcinogenesis mediated by ST18

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SUPPORTING INFORMATION

Supporting Materials and Methods

Doxycycline treatment

CD1-nude or Nod/Scid Hsd mice, injected with hepatoblasts (shp53, Myc, Ras^{V12}) containing the conditional shRNA vector (TtRMPVIR shST18) to silence ST18, were treated with Doxycycline to activate the knockdown of *ST18*. Mice were fed with 625mg/kg Doxycycline containing food (Mucedola). Additionally, we administrated the first dose of 200mg/ml Doxycycline (Sigma) in 300µl of water by oral gavage.

LPS treatment

C57/JHsd mice were injected intraperitoneally with 100 µg of lipopolysaccharide (LPS) and sacrificed 24h after treatment. Liver biopsies were dissected for pathological analysis.

Pathological and immunohistochemical analysis

Tumor or liver biopsies assigned to histological assessment were fixed in 4% formaldehyde overnight. The next day the samples were washed in 70% ethanol and subjected to paraffin embedding. 5 µm thick sections were stained with hematoxylin/eosin, and inspected by a mouse pathologist.

Human samples used in the study were obtained from formalin-fixed paraffin-embedded (FFPE) tissue from children diagnosed with PFIC1, PFIC2 or PFIC3. All specimens were obtained from native-liver hepatectomy performed during transplant surgery, carried out at the Hospital Papa Giovanni XXIII, Bergamo (Italy). Informed consent for the surgical procedure was obtained from the parents/carers. Formal approval for the use of the samples in the present work was provided by the local ethics committee (*Comitato Etico della Provincia di Bergamo*). All samples were kept strictly anonymous.

For Immunohistochemical analysis, 5 µm thick sections were de-waxed and re-hydrated through an ethanol scale, heated in EDTA (0.25mM, pH9, Dako #S2368) or citra solution

(BioGenex #HK086-9K) in a water bath at 99°C for 30 minutes for antigen unmasking, then left to cool down for 20 min, washed once in water and after 5 minutes, treated with 3% H_2O_2 for quenching of endogenous peroxidases. Slides were incubated with antibodies against Albumin (#106582, Abcam, 1:400), a-fetoprotein (#0008, Dako, 1:800), cytokeratin 19 (#901-242-012811, Biocare medical, 1:100), ST18 (#86563, Abcam, 1:200), Ki67 (#M7249, Dako, 1:500), Cleaved Caspase 3 (#9661, Cell Signaling, 1:200), Ve-Cadherin (#6458, Santa Cruz, 1:200), IBA1 (#019-19741, Wako, 1:1000), P65 (#86299, Abcam, 1:100) and P50 (#32360, Abcam, 1:200) in blocking solution (TBS containing 2% BSA, 2% goat serum, 0.02% Tween20) for 3h at RT. Slides were washed twice with TBS and incubated with the secondary antibody (DAKO Cytomation Envision System Labelled Polymer-HRP) for 45 minutes. After two additional washes in TBS, sections were developed with peroxidase substrate solution with DAB (DAKO) for 2-10 minutes. Slides were finally counterstained with hematoxylin, de-hydrated through alcoholic scale and mounted with Eukitt.

RNA extraction and analysis

Frozen tissue samples were homogenized with a dounce homogenizer or with the GentleMACS Dissociator (Miltenyi Biotec), depending on the tissue volume, prior to column extraction. RNA was extracted in Trizol (Invitrogen) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quantification was performed on Nanodrop, and quality was assessed on Bioanalyzer (Agilent). 0.5 µg of total RNA was used for cDNA synthesis (using the ImProm-II Reverse Transcriptase, Promega), and 1 µl of the obtained cDNA was generally used as template for qPCR expression analyses. qRT-PCR (SYBR-green, Life Technologies) analysis was performed on an Applied Biosystems 7500 Real-time PCR system. Gene expression analyses by qRT-PCR were accomplished using the mouse/human *ST18* primers (F' GAAAACGGCACATTGGACTT; R' GGTGAGGAAGTTGGGGGTAT). Values were normalized to *RPLP0 (F'* TTCATTGTGGGAGCAGAC; R' CAGCAGTTTCTCCAGAGC). For RNA-seq, 5 µg of total RNA were depleted of ribosomal RNA with the Ribo-Zero^{TM} rRNA Removal Kit from Epicentre®. Successful removal of ribosomal RNA was assessed using the BioAnalyser 2100. RNAseq libraries were prepared with the Illumina TruSeq RNA sample preparation kit v2 following the manufacturer's protocol. Briefly, RNA was fragmented and cDNA was synthesized, end-repaired and 3'-end-adenylated. Following adapter ligation, libraries were amplified by PCR for 15 cycles. Libraries with distinct TruSeq adapter indices were multiplexed (3 libraries per lane) on a HiSeq 2000 and sequenced for 50 bases in the paired-

end mode. Duplicated reads were eliminated using rmdup function from the suite samtools (http://samtools.sourceforge.net/). Differentially expressed genes were determined using the DESeq2 tool (15) available from Bioconductor with default parameters. Functional annotation was performed using the Gene Ontology categories of the bioinformatics tool Gene Set Enrichment Analysis (GSEA) based on Molecular Signatures Database (MSigDB). The dataset was deposited in the NIH GEO database under accession number GSE72403 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=kjgnciyupdkdbib&acc=GSE72403)

Flow cytometry and cell sorting

Primary hepatoblasts transduced with TtRMPVIR shRNA plasmids were monitored by flow cytometry for expression of the associated Venus marker without prior fixation, as single cell suspension in PBS. Cells were analyzed using a FACSCalibur (Becton-Dickinson; Mountain View, CA) flow cytometer. Macrophages were detected in peripheral blood with anti-mouse CD115 APC (#17-1152, eBioscience, 1:100). Neutrophils were detected with Anti-Mouse Ly-6G (#551459, BD Pharmingen 1:300). Liver progenitor cells, derived from E18.5 p53-null mice, transduced with retroviruses expressing c-*myc*, oncogenic Ras (H-Ras^{V12}) and shST18 (expressed from the vector TtRMPVIR) (12), were sorted for expression of the associated Venus marker using a Moflo Astrios (Beckman Coulter). All FACS data were analyzed using the FlowJo software (TreeStar).

Supporting Figure Legends

Supporting Fig. 1. Subcutaneous tumors derived from liver progenitor cells are of hepatic origin. **A,** Hematoxylin and eosin (H&E) stained sections show close resemblance of hepatoblast-derived subcutaneous tumors with different histological subtypes of human HCC (Solid, Pseudoglandular, Trabecular), as indicated above each panel. **B,** Tumor sections were stained for the liver progenitor markers albumin, α-fetoprotein, or cytokeratin 19. A representative subcutaneous tumor generated with shp53, Myc and Ras^{V12}-transduced E18.5 hepatoblasts is shown here as an example. All tumors examined in this study were positive for at least two of the three markers, confirming their liver origin. **C,** Staining for the pan-macrophage marker IBA1 (32) reveals abundant macrophage infiltration in subcutaneous tumors. All scale bars in A-D are 100 μm. **D.** *ST18* induction in hepatoblasts upon co-culture for 12h with normal bone marrow-derived macrophages (*p = 0.0020) or RAW 264.7 (**** p = 0.0008). Pre-treatment of the macrophages with LPS for 1h further augmented their *ST18*-inducing potential (**p = 0.0011 and ****p = 0.028 both relative to co-culture with untreated macrophages). Hepatoblasts were purified by sorting based on the Venus marker before RNA extraction and qRT-PCR. **E.** *ST18* induction in hepatoblasts by treatment with macrophage supernatant (1h). * p = 0.0367. Scale bars: 100 μm.

Supporting Fig. 2. ST18 expression is required for tumor development *in vivo*. A, Mice transplanted with c-*myc*- and H-Ras^{V12}-transformed $p53^{-/-}$ hepatoblasts expressing a doxycycline-inducible *ST18* shRNA hairpin (shST18-6) show reduced tumor development, compared with either non-induced shST18-6, or the control hairpin shREN.713. The photographs show three different mice per experimental condition, with Doxycycline used to induce either shST18-6 or shREN.713 from the day of seeding (day 0). **B,** Tumor volumes, measured at day 21, with (+) of without (-) induction of either shST18-6, or shREN.713 from day 0.

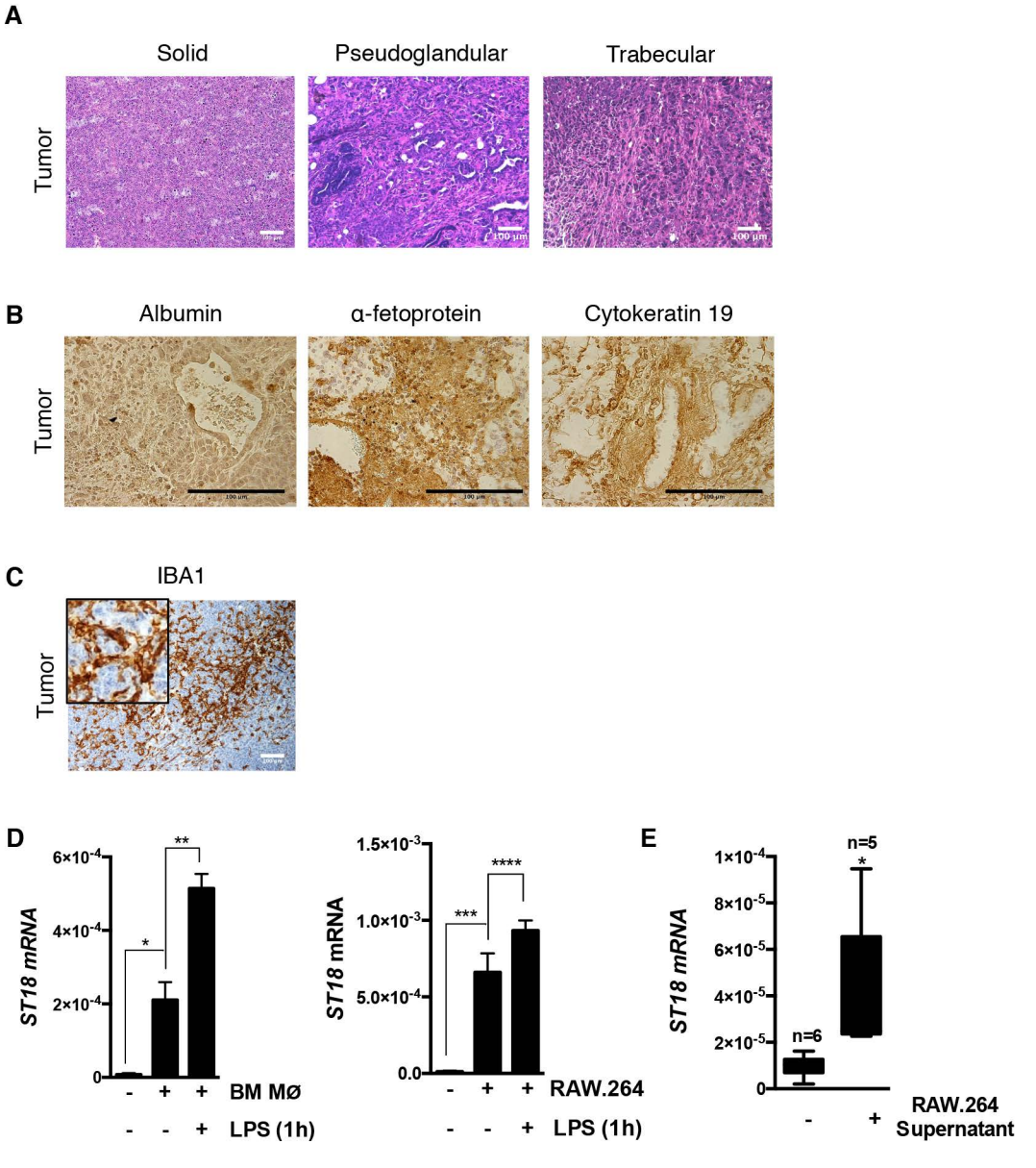
Supporting Fig. 3. ST18 depletion causes rapid hemorrhages *in vivo*. A, As Fig. 2A, and **B-D** as Fig. 2D, for two other ST18-specific shRNAs (shST18-1 and -7) and the shREN.713 control, as indicated. Scale bars: 100μm.

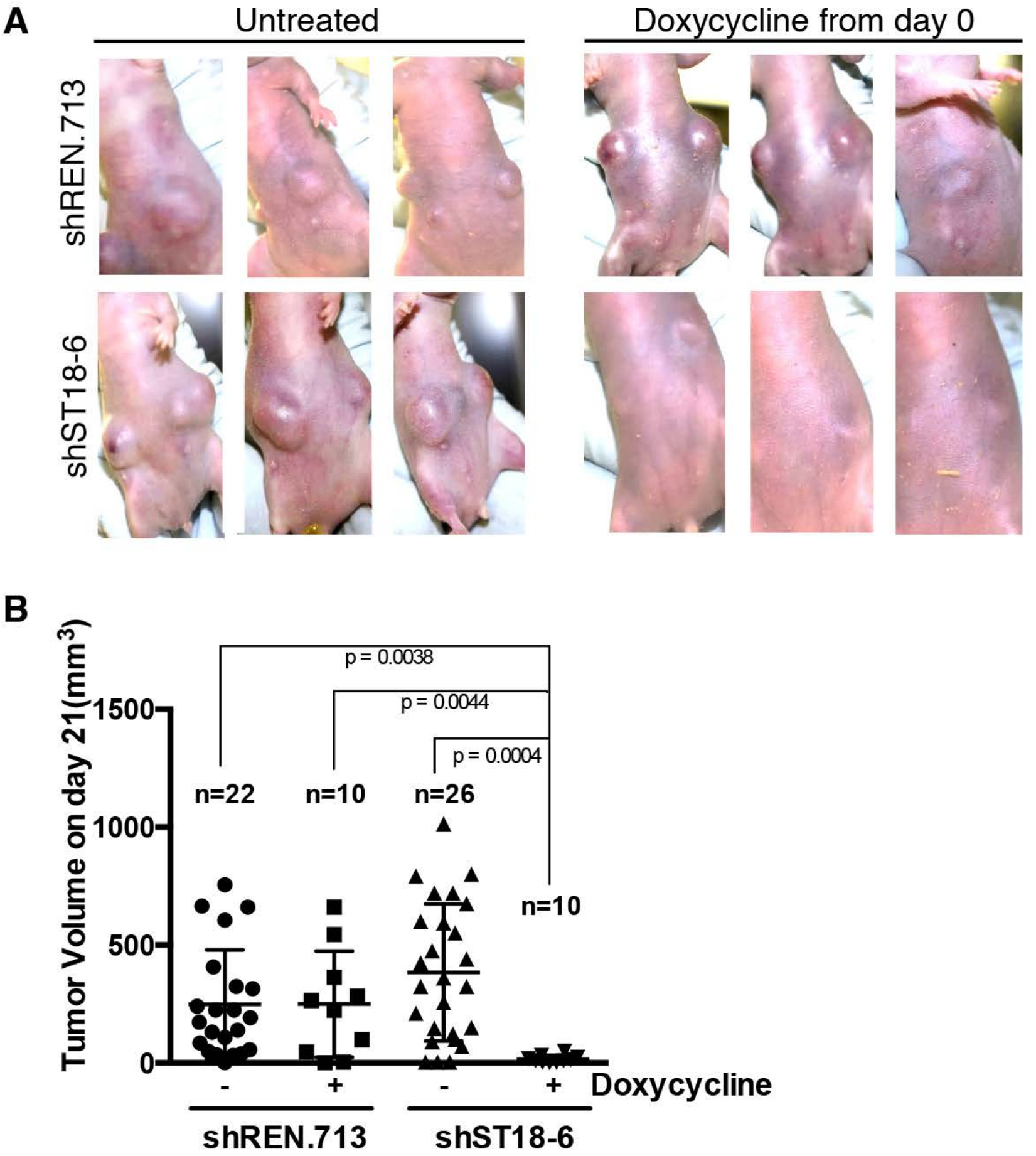
Supporting Fig. 4. Immunohistological analysis of subcutaneous tumors following *ST18* knockdown. A, Immunohistochemical staining reveals vascular ectasia (VE-cadherin), decrease in proliferation (Ki67) and apoptosis (cleaved Caspase 3) upon *ST18* silencing (with shST18-6 induced for 4h). **B,** None of the aforementioned changes occurred after induction

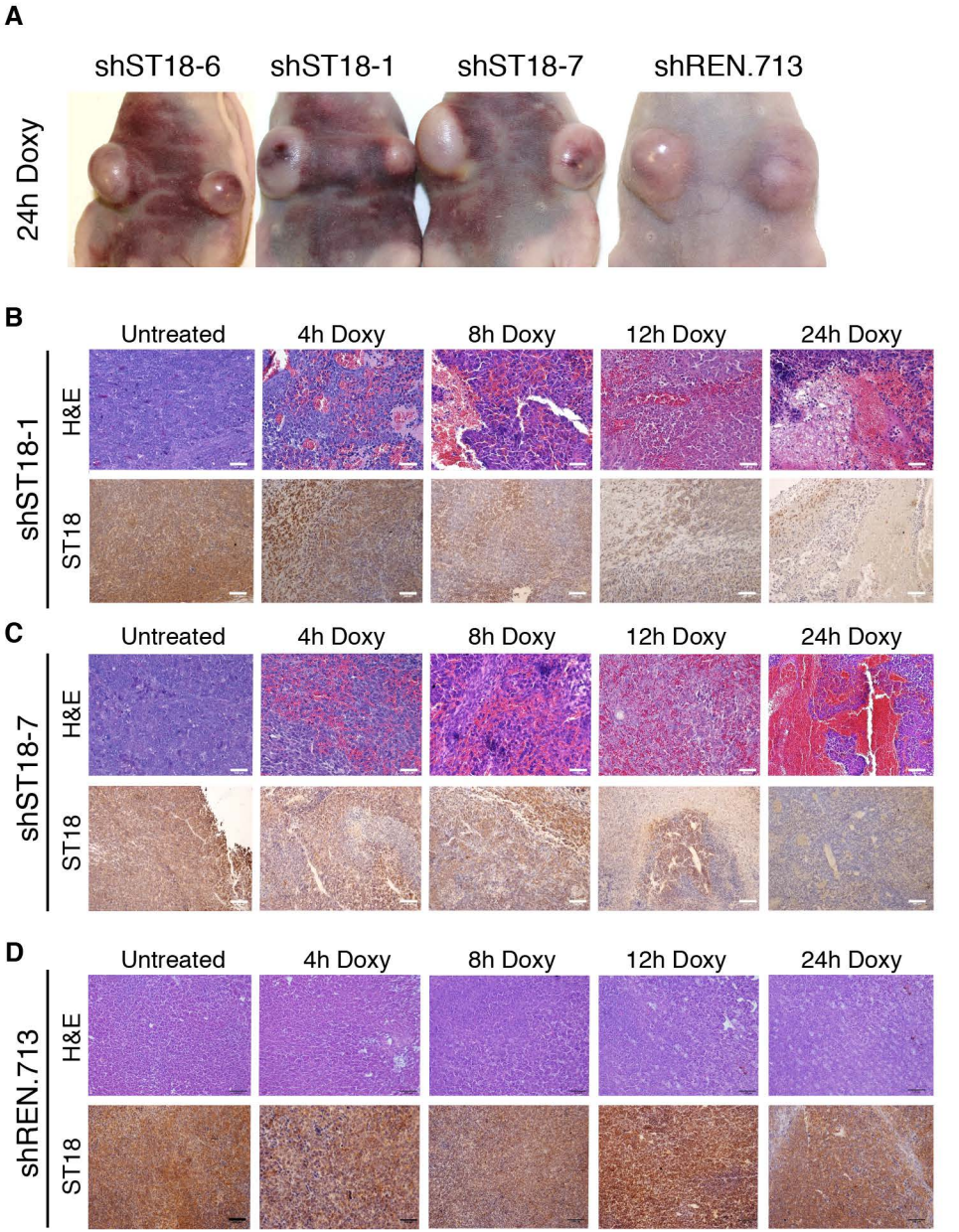
of the shREN.713 control. **C,** Staining for the macrophage marker IBA1 before (untreated) and after activation of shST18-6 or shREN.713, as indicated. *ST18* knockdown in the epithelial cells causes morphological changes in macrophages, in particular retraction of cytoplasmic processes and cellular rounding (insets). **D,** Reduction in macrophage numbers in shST18-6-expressing tumors after 4h (* p = 0.0003) and 8h (** p = 0.0001) of doxycycline treatment. Scale bars: 100μm.

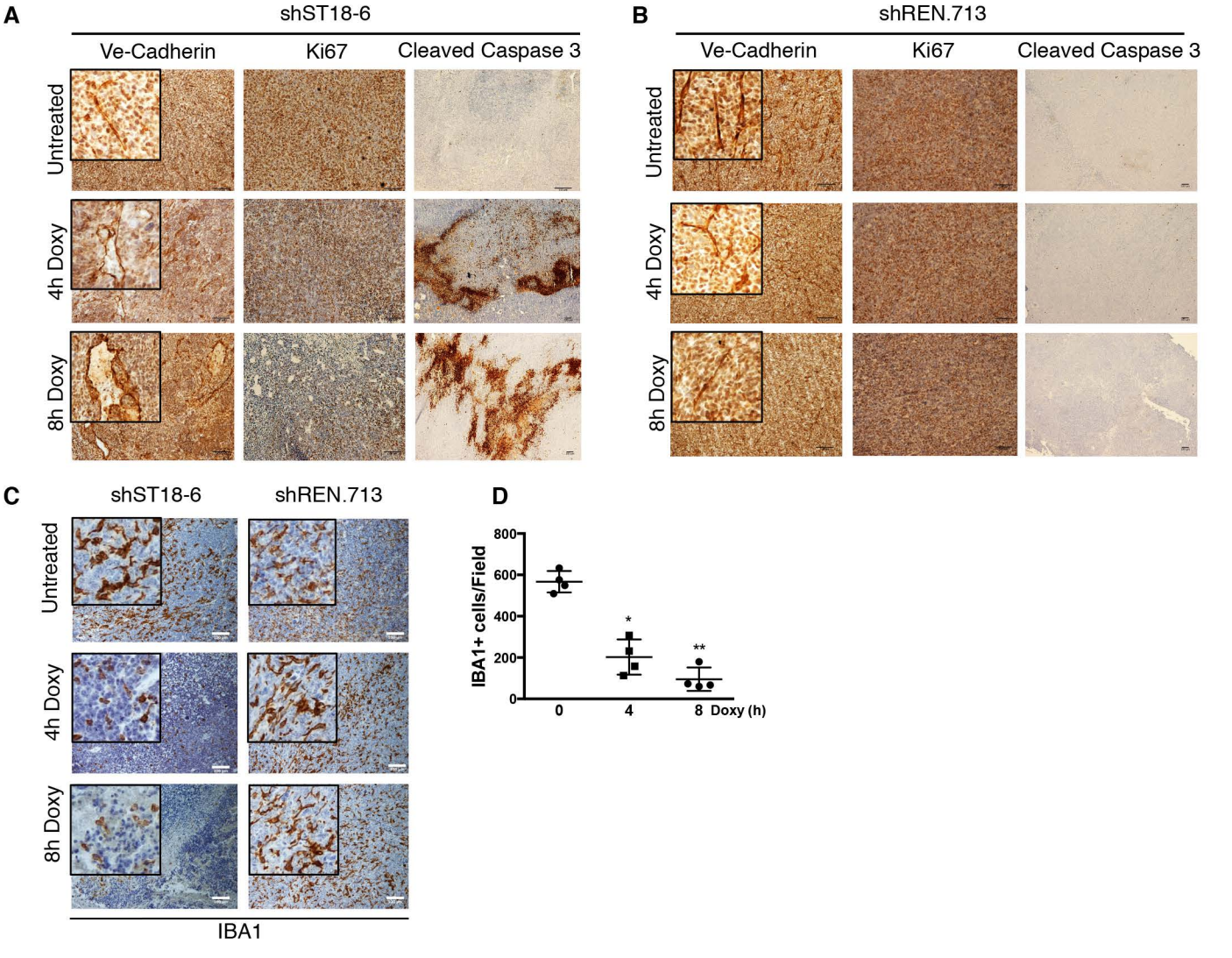
Supporting Fig. 5. Clodronate-mediated depletion of macrophages *in vivo* confirms their crucial role for *ST18* expression. A, Tumor-bearing animals were treated with liposome-encapsulated clodronate. Three days after treatment loss of IBA1+ macrophages is accompanied by hemorrhage, loss of ST18 expression and induction of cleaved Caspase 3. **B, C,** Clodronate treatment in pre-neoplastic (**B,** 7 months) and neoplastic (**C,** 17 months) *Mdr2*$^{-/-}$ mice causes reduced ST18 expression within 2 days. Scale bars: 100μm .

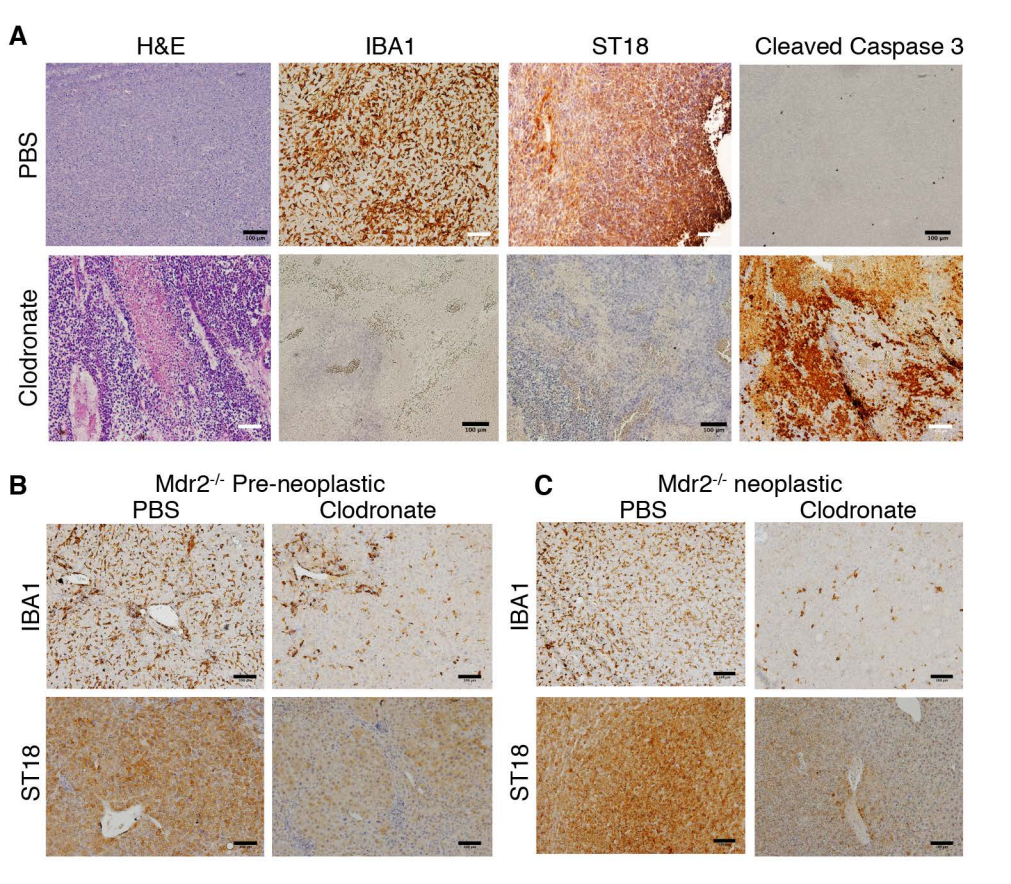
Supporting Fig. 6. *ST18* knockdown in HepG2 cells induces cell death *in vitro* and tumor regression *in vivo*. A, Quantitative RT-PCR analysis of mRNA levels in HepG2 cells shows expression of *ST18* at basal level and 24h after shST18 induction. Mouse hepatoblasts grown *in vitro* do not express ST18, and are shown for comparison. **B,** Activation of shST18 but not shREN.713 induces death of HepG2 cells, as measured by trypan blue staining. The shST18-6 hairpin, which targets both human and mouse *ST18*, was induced by Doxycyline treatment for the indicated periods of time. **C,** Hemorrhage and **D,** tumor regression following shST18 activation in subcutaneous HepG2 tumors. Following the seeding of $3x10^5$ HepG2 cells subcutaneously, tumors were left to develop in the recipient animals for 30 days, prior to doxycycline treatment (day 0). **E,** Tumors dissected following shST18 knockdown (shST18-6 ON, 24 hours after induction) show reduced sizes compared to either untreated mice (shST18 OFF) or to shREN.713 control tumors with or without doxycycline.

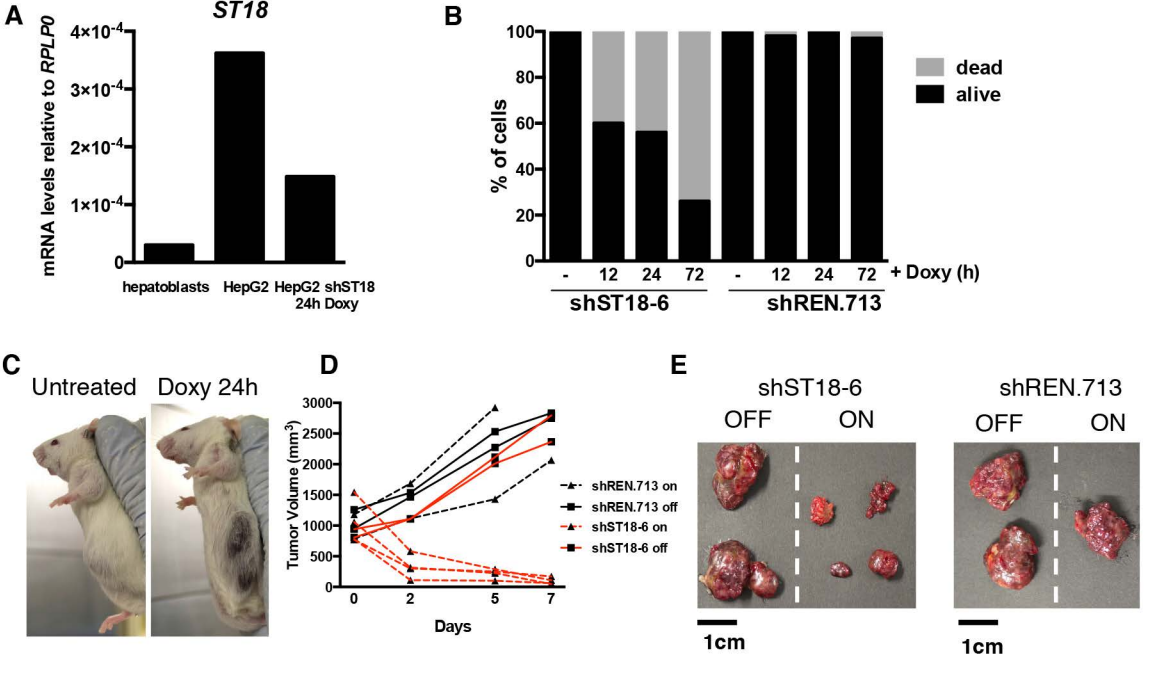

A
Solid
Pseudoglandular
Trabecular
Tumor
100 µm
B
Albumin
α-fetoprotein
Cytokeratin 19
Tumor
100 µm
C
IBA1
Tumor
D
ST18 mRNA
6×10-4
4×10-4
2×10-4
0
*
**
- + + BM MØ
- - + LPS (1h)
ST18 mRNA
1.5×10-3
1.0×10-3
5.0×10-4
0.0
***
****
- + + RAW.264
- - + LPS (1h)
E
ST18 mRNA
1×10-4
8×10-5
6×10-5
4×10-5
2×10-5
0
n=5
*
n=6
- +
RAW.264
Supernatant


A
Untreated
Doxycycline from day 0
shREN.713
shST18-6
B
Tumor Volume on day 21(mm3)
1500
1000
500
0
p = 0.0038
p = 0.0044
p = 0.0004
n=22
n=10
n=26
n=10
-
+
-
+
Doxycycline
shREN.713
shST18-6


A
shST18-6
shST18-1
shST18-7
shREN.713
24h Doxy
B
Untreated
4h Doxy
8h Doxy
12h Doxy
24h Doxy
shST18-1
H&E
ST18
C
Untreated
4h Doxy
8h Doxy
12h Doxy
24h Doxy
shST18-7
H&E
ST18
D
Untreated
4h Doxy
8h Doxy
12h Doxy
24h Doxy
shREN.713
H&E
ST18


A
shST18-6
Ve-Cadherin
Ki67
Cleaved Caspase 3
Untreated
4h Doxy
8h Doxy
B
shREN.713
Ve-Cadherin
Ki67
Cleaved Caspase 3
Untreated
4h Doxy
8h Doxy
C
shST18-6
shREN.713
Untreated
4h Doxy
8h Doxy
IBA1
D
IBA1+ cells/Field
800
600
400
200
0
*
**
0
4
8
Doxy (h)


A
H&E
IBA1
ST18
Cleaved Caspase 3
PBS
Clodronate
B
Mdr2-/- Pre-neoplastic
PBS
Clodronate
IBA1
ST18
C
Mdr2-/- neoplastic
PBS
Clodronate
IBA1
ST18


A
ST18
mRNA levels relative to RPLP0
4x10-4
3x10-4
2x10-4
1x10-4
0
hepatoblasts
HepG2
HepG2 shST18 24h Doxy
B
% of cells
100
80
60
40
20
0
dead
alive
-
12
24
72
-
12
24
72
+ Doxy (h)
shST18-6
shREN.713
C
Untreated
Doxy 24h
D
Tumor Volume (mm3)
3000
2500
2000
1500
1000
500
0
0
2
5
7
Days
shREN.713 on
shREN.713 off
shST18-6 on
shST18-6 off
E
shST18-6
OFF
ON
shREN.713
OFF
ON
1cm
1cm