

SHORT COMMUNICATION

In vivo functional dissection of a context-dependent role for Hif1 α in pancreatic tumorigenesis

T Cheng^{1,9}, Z Jian^{1,9}, K Li², S Raulefs¹, I Regel³, S Shen⁴, X Zou⁴, J Ruland^{2,5,6}, GO Ceyhan¹, H Friess¹, CW Michalski⁷, J Kleeff^{8,9} and B Kong^{1,4,9}

Hypoxia-inducible factor 1 α (Hif1 α) is a key regulator of cellular adaptation and survival under hypoxic conditions. In pancreatic ductal adenocarcinoma (PDAC), it has been recently shown that genetic ablation of Hif1 α accelerates tumour development by promoting tumour-supportive inflammation in mice, questioning its role as the key downstream target of many oncogenic signals of PDAC. Likely, Hif1 α has a context-dependent role in pancreatic tumorigenesis. To further analyse this, murine PDAC cell lines with reduced Hif1 α expression were generated using shRNA transfection. Cells were transplanted into wild-type mice through orthotopic or portal vein injection in order to test the *in vivo* function of Hif1 α in two major tumour-associated biological scenarios: primary tumour growth and remote colonization/metastasis. Although Hif1 α protects PDAC cells from stress-induced cell deaths in both scenarios—in line with the general function Hif1 α —its depletion leads to different oncogenic consequences. Hif1 α depletion results in rapid tumour growth with marked hypoxia-induced cell death, which potentially leads to a persistent tumour-sustaining inflammatory response. However, it simultaneously reduces tumour colonization and hepatic metastases by increasing the susceptibility to anoikis induced by anchorage-independent conditions. Taken together, the role of Hif1 α in pancreatic tumorigenesis is context-dependent. Clinical trials of Hif1 α inhibitors need to take this into account, targeting the appropriate scenario, for example palliative vs adjuvant therapy.

Oncogenesis (2016) 5, e278; doi:10.1038/oncsis.2016.78; published online 12 December 2016

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a devastating cancer entity characterized by tissue hypoxia.^{1,2} Activated by hypoxia, hypoxia-inducible factor 1 α (Hif1 α) is a key downstream effector, which is involved in a variety of cellular processes mediating a number of adaptive changes. These adaptive changes are meant to alleviate cellular stress induced by hypoxic conditions and to promote cell survival under physiological circumstances.^{3,4} However, these responses are frequently used by PDAC cells to enhance the malignant potential such as chemoresistance or metastatic invasion.^{5,6} Indeed, high expression of Hif1 α is a negative predictor of PDAC patient's overall survival.⁷ Besides, under normoxic conditions, Hif1 α acts as the key downstream target of a number of putative oncogenic pathways of PDAC for example mTOR (mechanistic target of rapamycin). Hyperactivated mTOR signalling is able to stabilize Hif1 α in the absence of hypoxia and promotes tumour angiogenesis.⁸ Collectively, these data argue for an oncogenic role of Hif1 α in pancreatic tumorigenesis.

Recently, this notion has been challenged by a provocative study showing that genetic ablation of Hif1 α significantly accelerated PDAC development in mice by promoting B lymphocyte-mediated tumour-supportive inflammation.⁹ The crucial crosstalk with the immune system points to a complex

function of Hif1 α in pancreatic tumorigenesis. Thus, we hypothesised that Hif1 α might have a context-dependent role in PDAC. To test this, we generated murine PDAC cell lines with reduced Hif1 α expression from previously established cell lines.¹⁰ The *in vivo* function of Hif1 α was tested in two major tumour-associated biological scenarios: primary tumour growth and remote colonization/metastatic growth.

RESULTS AND DISCUSSION

Previously, we characterized a number of murine PDAC cell lines from *p48^{Cre}; Kras^{G12D/+}; Tsc1^{fllox/+}* mice.^{1,10} Among these, 399 cells characterized by hyperactivated Mek/Erk/mTOR signalling reliably develop tumours in wild-type mice (C57BL/6J) upon orthotopic or portal vein injection, and were therefore chosen for this study. We stably transfected 399 cells with scramble control (shControl) or Hif1 α -specific shRNA-expressing plasmids (shHif1 α). Western blot analysis confirmed an approximate 90% reduction in Hif1 α expression in shHif1 α cells compared with shControl cells (Figure 1a). In comparison with the control cells, shHif1 α cells showed a significant reduction in glucose uptake (quantified by intracellular DG6P level: 50% reduction, $P=0.0005$) (Figure 1b), intercellular glutamate level (57% decrease, $P=0.0481$, Figure 1c) and lactate secretion (34% reduction, $P<0.0001$, Figure 1d),

¹Department of Surgery, Technical University of Munich (TUM), Munich, Germany; ²Institute of Clinical Chemistry and Pathobiochemistry, Munich, Germany; ³Institute of Pathology, Heinrich-Heine-University, Duesseldorf, Germany; ⁴Department of Gastroenterology, the Affiliated Drum Tower Hospital of Nanjing University, Medical School, Nanjing, Jiangsu, China; ⁵German Cancer Consortium (DKTK), Heidelberg, Germany; ⁶German Center for Infection Research (DZIF), Munich, Germany; ⁷Department of Surgery, University of Heidelberg, Heidelberg, Germany and ⁸NIHR Pancreas Biomedical Research Unit, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, UK. Correspondence: Dr B Kong, Department of Surgery, Technical University of Munich (TUM), Ismaninger Strasse 22, Munich 81675, Germany. E-mail: bo.kong@tum.de

⁹These authors contributed equally to this work.

Received 4 October 2016; accepted 31 October 2016

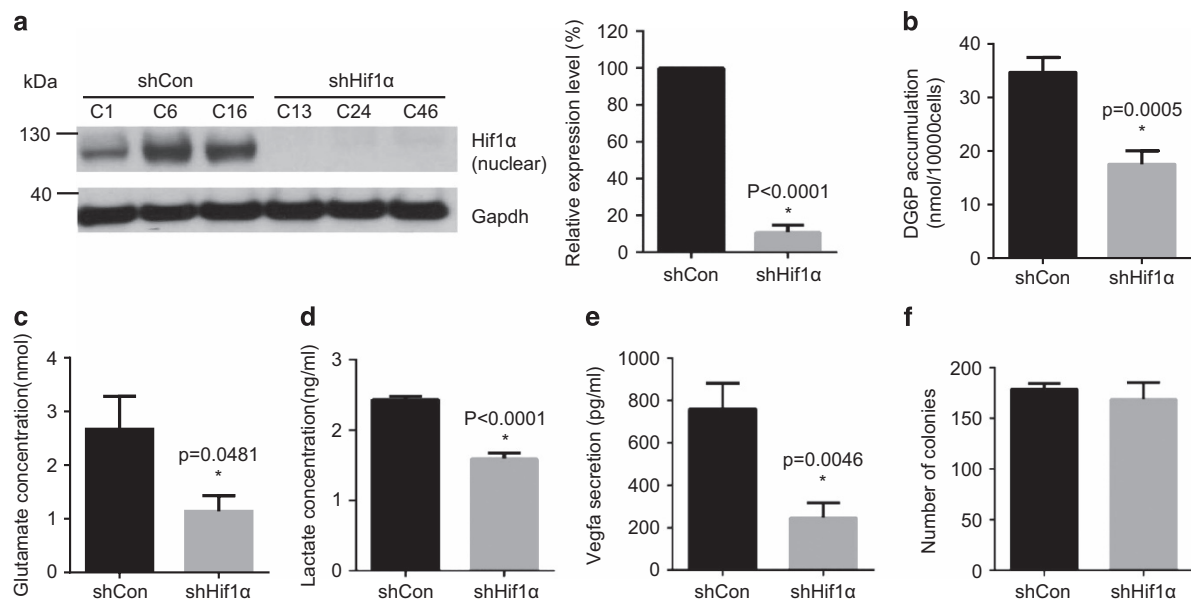


Figure 1. Hif1 α is downregulated in murine PDAC cells. (a) Western blot analyses show the downregulation of Hif1 α in shHif1 α cells but not in shControl cells: (left) western blot result; and (right) quantitative measurement; shHif1 α : transfection with Hif1 α -specific shRNA-expressing vector; shControl: transfection with control vectors. (b–d) The metabolic state of shControl cells or shHif1 α cells is determined by glucose uptake assay (b), glutamate assay (c) and lactate secretion assay (d). (e) Vegfa secretion between shControl and shHif1 α cells is measured by Vegfa ELISA assay. (f) The proliferation capacity of shControl or shHif1 α cells is determined by colony formation assay. All data are presented as mean \pm s.e.m. ($n=3$), unpaired t -test is used to examine statistical significance, $*P<0.05$. See Supplementary Materials and Methods.

which is in line with the influence of Hif1 α on tumour metabolism. Secretion of Vegfa (vascular endothelial growth factor A)—a downstream target of Hif1 α —was also decreased in shHif1 α cells (ELISA, 67% reduction, $P=0.0046$ Figure 1e). Downregulation of Hif1 α had no effect on cell proliferation *in vitro* under anchorage-dependent conditions in the colony formation assay (Figure 1f).

To test the influence of Hif1 α depletion on primary tumour growth, we performed orthotopic injections using control and shHif1 α cells. Interestingly, the shHif1 α cells gave rise to significantly larger tumours compared with control cells (Figure 2a, 11.8-fold increase, $P=0.0079$). Histological analysis revealed that the tumours derived from shHif1 α cells were more necrotic (Figure 2b). Indeed, the quantitative analysis confirmed that the necrotic region (boundary labelled by cleaved-caspase 3) was more pronounced in shHif1 α cells-derived tumours (Figure 2c, 33.8-fold increase, $P<0.0001$). In line, shHif1 α cells were more vulnerable to hypoxia-induced cell death *in vitro* (Figure 2d, 7.8-fold increase, $P=0.0297$). Since the shHif1 α cells-derived tumours were larger even after excluding necrotic areas (Figure 2c, shCon vs shHif1 α : 0.3 ± 0.16 vs 0.7 ± 0.08 cm², $P=0.0081$), we hypothesized that the outgrowth of shHif1 α tumours was due to elevated proliferation. Indeed, the quantitative analysis revealed that the proliferation index in viable tumour regions was significantly higher in shHif1 α tumours compared with control tumours (1.6-fold change, $P=0.0006$) (Figure 2e).

Since tumour-associated inflammation has been proven to affect tumour proliferation under certain conditions,¹¹ we hypothesized that the outgrowth of shHif1 α tumours resulted in necrosis that triggered an increased inflammatory response, which further supported rapid tumour growth. To test this, we measured the serum level of a number of inflammatory markers including serum amyloid A (SAA), Il6 (interleukin 6) and TNF α (tumour necrosis factor α). SAA levels in the serum of shHif1 α cells-transplanted animals were significantly higher than controls (262.0 ± 12.01 vs 199.8 ± 1.92 μ g/ml, $P=0.0083$) (Figure 3a). Similar results were obtained when serum levels of Il6 (Figure 3b, 19.0 ± 4.89 vs 5.1 ± 0.94 pg/ml, $P=0.0408$) and TNF α (Figure 3c, 19.0 ± 2.84 vs 8.7 ± 1.20 pg/ml, $P=0.0191$) were measured. Next,

we profiled the immune cell infiltration in shHif1 α and control cells-derived tumours by staining a number of immune cell markers including CD45 (immune cells), MPO (myeloperoxidase, neutrophils), CD3 (T cells), B220 (B cells) and F4/80 (macrophages). This analyses revealed that shHif1 α tumours were more densely infiltrated by immune cells (especially in necrotic areas) than control tumours (2.4-fold change, $P=0.001$, Figure 3d). Subsequent analysis revealed that these immune cells mainly were neutrophils (Figure 3e, 7.6-fold change, $P=0.0009$). T-cell infiltration, however, was reduced in shHif1 α tumours (Figure 3f). No difference in the number of infiltrating B cells (Figure 3g) and macrophages (Figure 3h) was observed. Taken together, the loss of Hif1 α promoted rapid primary tumour growth, resulting in necrosis and triggering tissue necrosis-associated inflammation, which potentially further facilitated tumour growth.

To test the function of Hif1 α in tumour colonization/metastasis, we inoculated shControl and shHif1 α cells into the portal vein. Here, shHif1 α cells developed significantly less hepatic metastasis than control cells (Figure 4a). The percentage of the area of metastasis in the liver (including median, left, right and caudate lobe) of shHif1 α cells-injected animals was 13.3-fold lower ($P<0.0001$) than that of control cells (Figure 4b). These data show that the ability of tumour colonization was dramatically decreased after Hif1 α knock-down. Since being able to survive under anchorage-independent conditions is an initial step of tumour colonization, we performed an anoikis assay to test this. In line, the viability of shHif1 α cells under anchorage-independent conditions was significantly reduced in comparison with control cells (Figure 4c, 43% reduction in viability, $P<0.0001$). In accordance with above described data, PDAC cells became more susceptible to anchorage-independent induced cell deaths after Hif1 α knock-down (Figure 4d, 14.0 fold change, $P=0.0093$).

In summary, we tested the *in vivo* function of Hif1 α in two major tumour-associated biological scenarios: primary tumour growth and remote colonization. Notably, although Hif1 α depletion generally renders PDAC cells more susceptible to stress-induced (that is, hypoxia or loss of cell/ECM contact) cell deaths, which is in line with the general function Hif1 α , it leads to different oncogenic

consequences. In particular, the results of our study partially support previous data showing that genetic ablation of Hif1 α significantly accelerated oncogenic Kras-driven pancreatic

tumorigenesis potentially by activating B cells-mediated inflammation. However, no difference was observed in B-cell infiltration in the current study. This disparity could be attributed to the

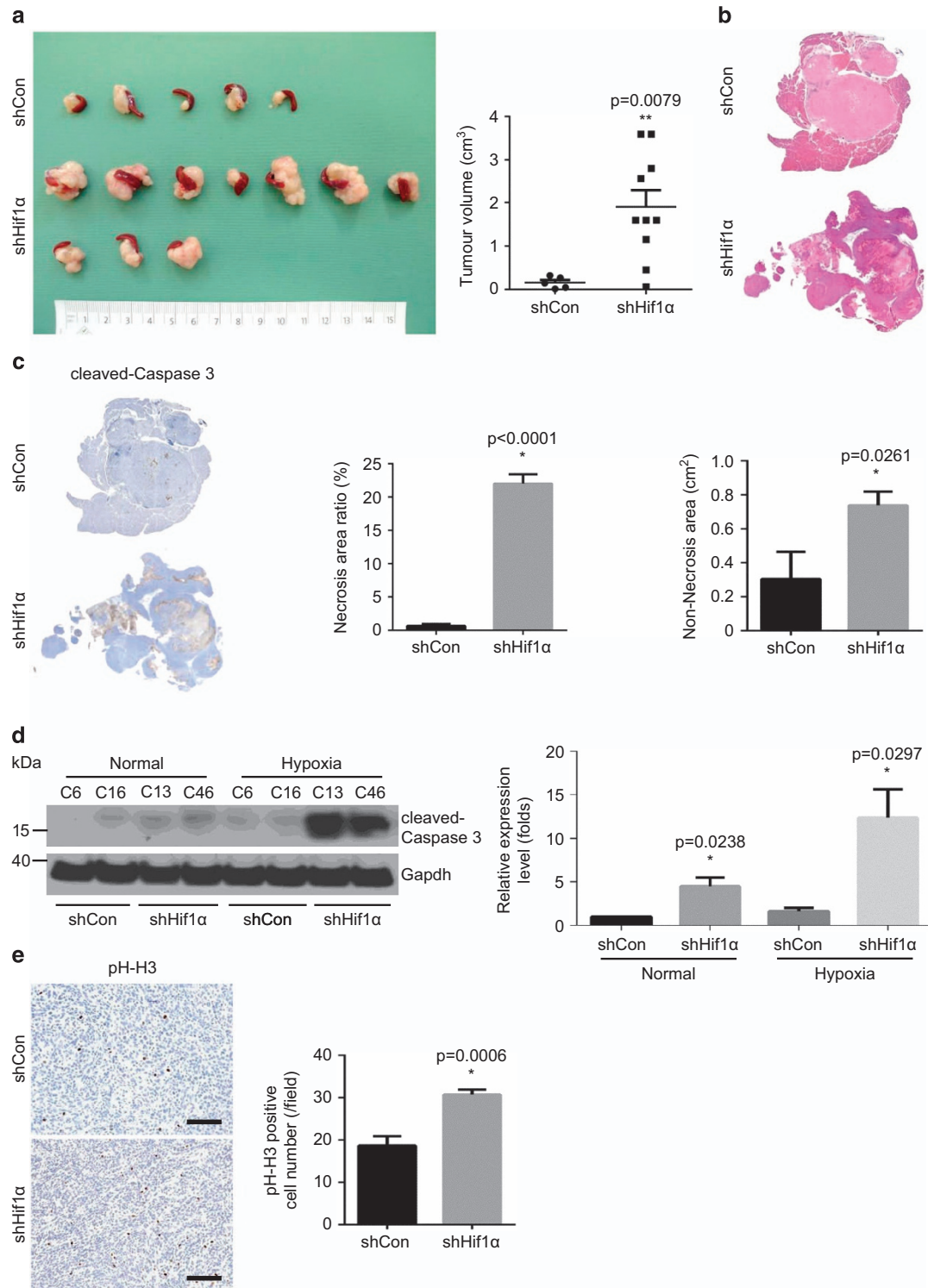


Figure 2. Hif1 α depletion promotes primary tumour growth with tissue necrosis *in vivo*. **(a)** The orthotopic transplantation experiment shows the volume of tumours derived from shControl and shHif1 α cells-injected animals: (left) gross pathology; and (right) quantitative measurement. **(b)** Representative H&E staining pictures show larger necrotic regions in shHif1 α tumours compared with shControl tumours. **(c)** Immunohistochemistry (IHC) stainings of cleaved-caspase 3 (left) demonstrate more apoptotic cells (middle) and larger non-necrosis area (right) in shHif1 α tumours in comparison with shControl tumours. **(d)** Western blot (left) and quantification results (right) show increased expression of cleaved-caspase 3 in shHif1 α cells under hypoxic conditions. **(e)** IHC staining of phosph-histone H3 (pH-H3) and quantitative analysis demonstrates increased proliferation in shHif1 α tumours; scale bar, 100 μ m. See Supplementary Materials and Methods.

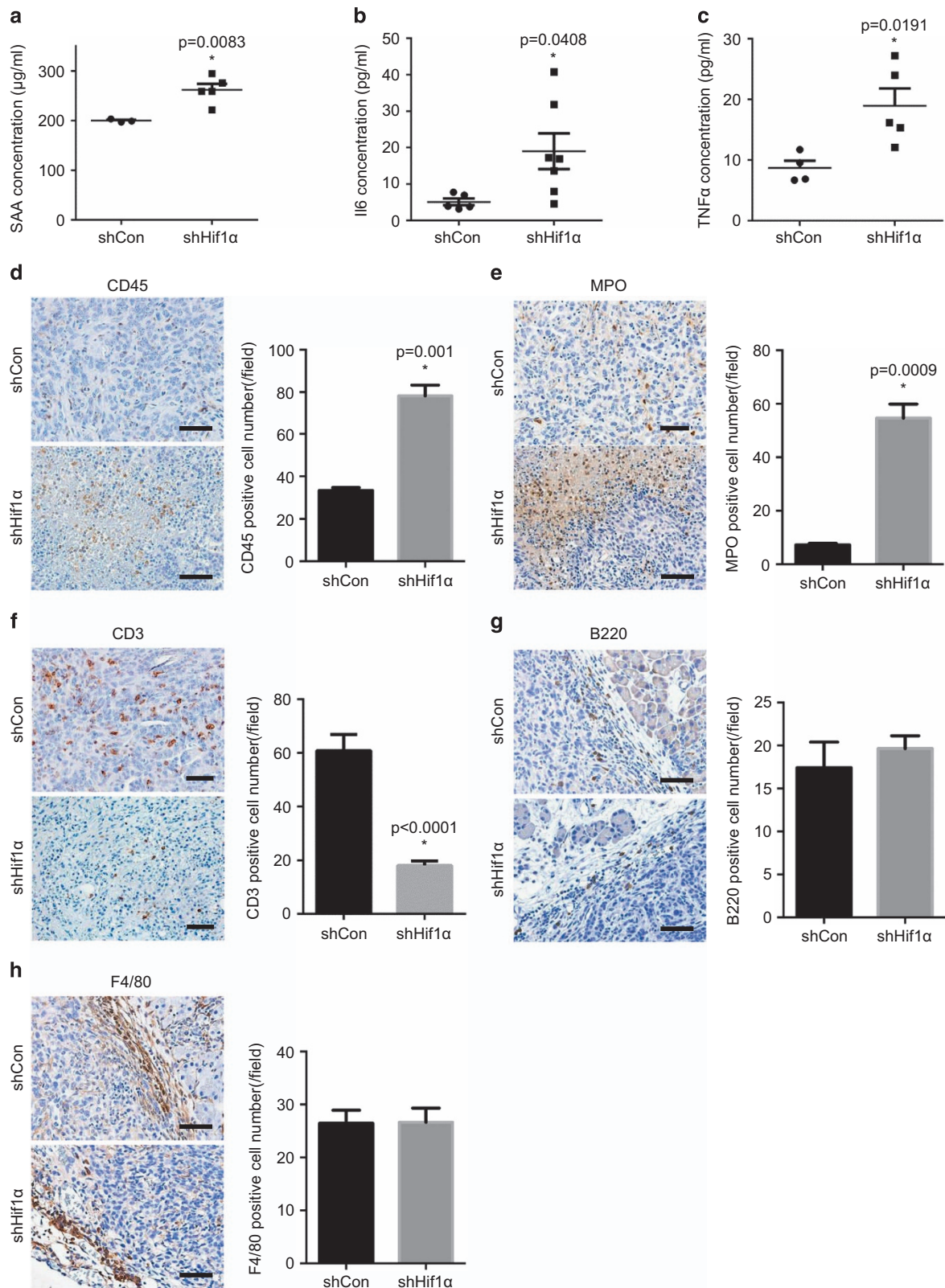


Figure 3. Increased inflammatory response triggered by Hif1 α depletion. (**a–c**) Elevated levels of SAA (**a**), IL6 (**b**) and TNF α (**c**) are detected in the serums of shHif1 α cells-transplanted animals compared with controls. (**d**) Representative IHC pictures of CD45 and quantitative analysis demonstrate increased immune cells infiltration (especially in necrotic areas) in the shHif1 α tumours. (**e**) Representative IHC pictures of MPO and quantitative analysis reveal that neutrophils are the most infiltrated immune cells in the shHif1 α tumours, which is confirmed by the quantitative analysis. Scale bar, 200 μ m. (**f–h**) Representative IHC pictures and quantitative analysis of B220 (**g**) and F4/80 (**h**) show no difference in B cells and macrophages infiltration between shControl and shHif1 α tumours. Scale bar, 200 μ m. All data are presented as mean \pm s.e.m., and the statistical difference is determined by unpaired *t*-test. **P* < 0.05. See Supplementary Materials and Methods.

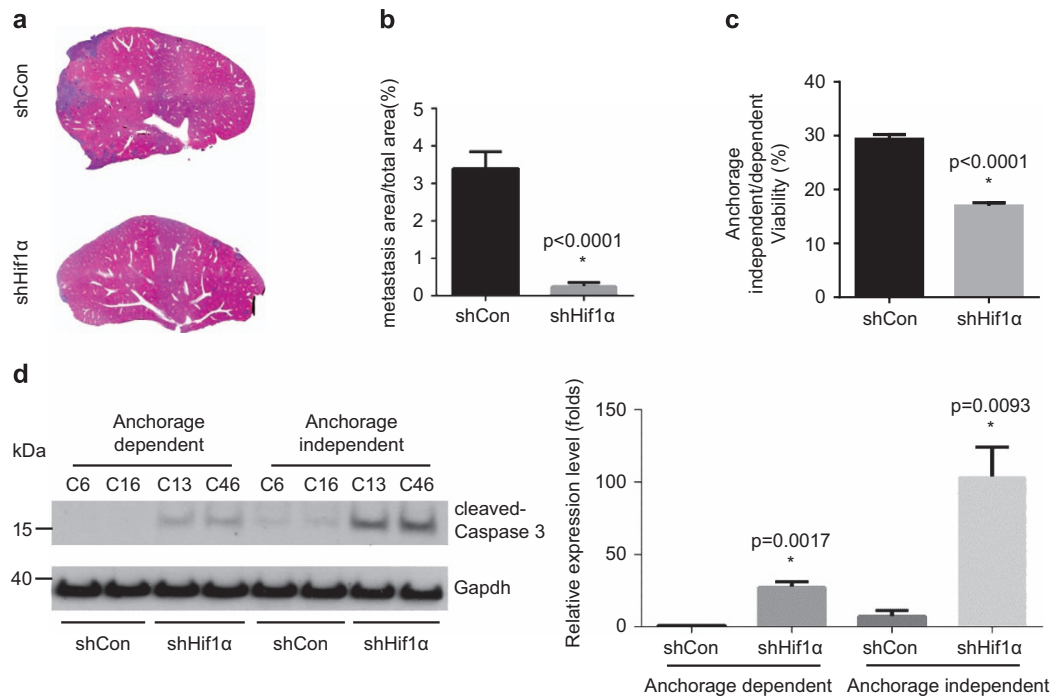


Figure 4. Loss of Hif1 α impairs hepatic metastasis and resistance toward anoikis state. **(a–b)** Representative H&E staining pictures **(a)** and metastasis area calculation **(b)** reveal decreased metastatic foci after portal vein injection of shHif1 α cells as compared with shControl cells. **(c)** Anoikis assay shows diminished cell survival in the shHif1 α cells under anchorage-independent conditions. **(d)** Western blot and quantified measurements show expression of cleaved-caspase 3 in shControl or shHif1 α cells under anchorage-dependent and -independent conditions. The data were a result of three independent experiment. An unpaired *t*-test was used for determining statistical significance. **P* < 0.05. See Supplementary Materials and Methods.

different mouse models (xenograft vs genetic models). The used xenograft model lacks the co-evolution of PDAC cells and the immune system. In the genetic model, Hif1 α is concomitantly inactivated in the endocrine cells (for example, β cells), which is known to cause insulin resistance,¹² which may influence the subsequent immune response. Despite these limitations, two studies led to the similar conclusion that Hif1 α is crucial for tumour growth and in modulating the immunogenic reactions towards PDAC.

Considering this context-dependent role Hif1 α in pancreatic tumorigenesis, clinical trials of Hif1 α inhibitors (for example, PX-478) in PDAC need to be carried out with caution.^{13,14} Based on the evident crosstalk between Hif1 α and the immune system, Hif1 α inhibitors could be tested in combination with various immune therapies in PDAC (for example, PD-1 inhibitors¹⁵). On the other hand, since Hif1 α dramatically affects the capacity of PDAC in tumour colonization/metastasis, Hif1 α inhibitors might be effective in targeting circulating cancer cells. In this regard, both genetic and xenograft models are useful in testing the effectiveness of such therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This project was supported in part by a grant from the TU Munich commission for clinical research (KKF C21-11, to BK), the Deutsche Forschungsgemeinschaft (MI 1173/5-1, to CWM, BK and JK). This work was supported by research grants from the DFG (SFB 1054 and RU 695/6-1) and an ERC Advanced Grant t (FP7, grant agreement No 322865) to JR. We thank Manja Thorwirth and Nadja Maeritz for their excellent technical support.

AUTHOR CONTRIBUTIONS

BK and JK designed the study. TC, ZJ, KL, SR and IR performed experiments and acquired the data. TC, ZJ, BK and JK drafted the manuscript. JR, SS, XZ, GC, HF and CWM revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

REFERENCES

- Kong B, Cheng T, Wu W, Regel I, Raulefs S, Friess H *et al*. Hypoxia-induced endoplasmic reticulum stress characterizes a necrotic phenotype of pancreatic cancer. *Oncotarget* 2015; **31**: 32154–32160.
- Koong AC, Mehta VK, Le QT, Fisher GA, Terris DJ, Brown JM *et al*. Pancreatic tumors show high levels of hypoxia. *Int J Radiat Oncol Biol Phys* 2000; **48**: 919–922.
- Semenza GL. Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 1999; **15**: 551–578.
- Kumar H, Choi DK. Hypoxia inducible factor pathway and physiological adaptation: a cell survival pathway? *Mediators Inflamm* 2015; **2015**: 584758.
- Colbert LE, Fisher SB, Balci S, Saka B, Chen Z, Kim S *et al*. High nuclear hypoxia-inducible factor 1 alpha expression is a predictor of distant recurrence in patients with resected pancreatic adenocarcinoma. *Int J Radiat Oncol Biol Phys* 2015; **91**: 631–639.
- Ye LY, Zhang Q, Bai XL, Pankaj P, Hu QD, Liang TB. Hypoxia-inducible factor 1alpha expression and its clinical significance in pancreatic cancer: a meta-analysis. *Pancreatol* 2014; **14**: 391–397.
- Shibaji T, Nagao M, Ikeda N, Kanehiro H, Hisanaga M, Ko S *et al*. Prognostic significance of HIF-1 alpha overexpression in human pancreatic cancer. *Anticancer Res* 2003; **23**: 4721–4727.
- Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM *et al*. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* 2000; **60**: 1541–1545.
- Lee KE, Spata M, Bayne LJ, Buza EL, Durham AC, Allman D *et al*. Hif1a deletion reveals pro-neoplastic function of B cells in pancreatic neoplasia. *Cancer Discov* 2016; **6**: 256–269.

- 10 Kong B, Wu W, Cheng T, Schlitter AM, Qian C, Bruns P *et al*. A subset of metastatic pancreatic ductal adenocarcinomas depends quantitatively on oncogenic Kras/Mek/Erk-induced hyperactive mTOR signalling. *Gut* 2016; **65**: 647–657.
- 11 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436–444.
- 12 Cheng K, Ho K, Stokes R, Scott C, Lau SM, Hawthorne WJ *et al*. Hypoxia-inducible factor-1 α regulates beta cell function in mouse and human islets. *J Clin Invest* 2010; **120**: 2171–2183.
- 13 Koh MY, Spivak-Kroizman T, Venturini S, Welsh S, Williams RR, Kirkpatrick DL *et al*. Molecular mechanisms for the activity of PX-478, an antitumor inhibitor of the hypoxia-inducible factor-1 α . *Mol Cancer Ther* 2008; **7**: 90–100.
- 14 Zhao T, Ren H, Jia L, Chen J, Xin W, Yan F *et al*. Inhibition of HIF-1 α by PX-478 enhances the anti-tumor effect of gemcitabine by inducing immunogenic cell death in pancreatic ductal adenocarcinoma. *Oncotarget* 2015; **6**: 2250–2262.
- 15 Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P *et al*. PD-L1 is a novel direct target of HIF-1 α , and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J Exp Med* 2014; **211**: 781–790.



Oncogenesis is an open-access journal published by Nature Publishing Group. This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016

Supplementary Information accompanies this paper on the *Oncogenesis* website (<http://www.nature.com/oncsis>)