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## STAT3 and HIF1 $\alpha$ cooperatively activate HIF1 target genes in MDA-MB-231 and RCC4 cells

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### Abstract

Solid tumors often exhibit simultaneously inflammatory and hypoxic microenvironments. The ‘signal transducer and activator of transcription-3’ (STAT3)-mediated inflammatory response and the hypoxia-inducible factor (HIF)-mediated hypoxia response have been independently shown to promote tumorigenesis through the activation of HIF or STAT3 target genes and to be indicative of a poor prognosis in a variety of tumors. We report here for the first time that STAT3 is involved in the HIF1, but not HIF2-mediated hypoxic transcriptional response. We show that inhibiting STAT3 activity in MDA-MB-231 and RCC4 cells by a STAT3 inhibitor or STAT3 small interfering RNA significantly reduces the levels of HIF1, but not HIF2 target genes in spite of normal levels of hypoxia-inducible transcription factor 1 $\alpha$  (HIF1 $\alpha$ ) and HIF2 $\alpha$  protein. Mechanistically, STAT3 activates HIF1 target genes by binding to HIF1 target gene promoters, interacting with HIF1 $\alpha$  protein and recruiting coactivators CREB binding protein (CBP) and p300, and RNA polymerase II (Pol II) to form enhanceosome complexes that contain HIF1 $\alpha$ , STAT3, CBP, p300 and RNA Pol II on HIF1 target gene promoters. Functionally, the effect of STAT3 knockdown on proliferation, motility and clonogenic survival of tumor cells *in vitro* is phenocopied by HIF1 $\alpha$  knockdown in hypoxic cells, whereas STAT3 knockdown in normoxic cells also reduces cell proliferation, motility and clonogenic survival. This indicates that STAT3 works with HIF1 to activate HIF1 target genes and to drive HIF1-dependent tumorigenesis under hypoxic conditions, but also has HIF-independent activity in normoxic and hypoxic cells. Identifying the role of STAT3 in the hypoxia response provides further data supporting the effectiveness of STAT3 inhibitors in solid tumor treatment owing to their usefulness in inhibiting both the STAT3 and HIF1 pro-tumorigenic signaling pathways in some cancer types.

### Keywords

cotranscriptional activation; HIF; hypoxia; STAT3; transcription

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## INTRODUCTION

A hypoxic microenvironment is common in solid tumors. The hypoxic microenvironment stabilizes hypoxia-inducible transcription factor 1 $\alpha$  (HIF1 $\alpha$ ) and 2 $\alpha$  (HIF2 $\alpha$ ) that are normally degraded under normoxia. The stabilized HIF1 $\alpha$  and HIF2 $\alpha$  proteins translocate to the nucleus, where they dimerize with aryl hydrocarbon receptor nuclear translocator (ARNT) to form HIF1 $\alpha$ /ARNT (HIF1) and HIF2 $\alpha$ /ARNT (HIF2) complexes. HIF1 and HIF2 bind to HIF-binding sites and activate genes involved in neovascularization, glycolysis, cellular proliferation and metastasis. Thus, the HIF-mediated hypoxia response is critical for tumor progression by allowing cancer cells to adapt to a low oxygen environment.<sup>1-4</sup>

In addition to HIF activity, many types of solid tumors such as breast, kidney, lung, prostate and head/neck tumors also exhibit constitutive 'signal transducer and activator of transcription-3' (STAT3) activity.<sup>5-9</sup> STAT3 is a latent cytoplasmic transcription factor, whose activity is induced by interleukin (IL)-6 and IL-10 family cytokines and growth factors such as epidermal growth factor and platelet-derived growth factor. The binding of cytokines to their receptors activates receptor-associated tyrosine kinases that are often members of the Janus kinase family or cytoplasmic SRC family kinases, whereas the binding of growth factors to their receptors activates intrinsic receptor tyrosine kinases. These activated tyrosine kinases phosphorylate the STAT3 protein at the tyrosine-705 (Y705) residue, which facilitates homodimerization and nuclear translocation of the STAT3 dimer. Nuclear STAT3 dimer then binds directly to STAT3-binding elements on target gene promoters to activate transcription. In normal cells, the activation of STAT3 is tightly controlled by the availability of cytokines or growth factors. In tumor cells, however, STAT3 activators such as IL-6, IL-10 and IL-11 are commonly overexpressed by cancer cells as well as by stromal cells in the solid tumor microenvironment. In addition, some cancers exhibit constitutive tyrosine kinase activity because of activating mutations, gene amplification or fusions of the GF receptors, GF or the cytoplasmic tyrosine kinases. A vast number of studies have established the role of STAT3 as an important oncogene in multiple cancer types by activating genes such as *VEGF*, *MMP2*, *MMP9*, *BCL-2*, *BCL-XL*, survivin (also called *BIRC5*), cyclin D1 and *MYC* that promote angiogenesis, cell proliferation, survival and metastasis.<sup>5-9</sup>

Recently hypoxia has been shown to activate STAT3 in several tumor cell lines and animal models.<sup>10-14</sup> In addition, STAT3 has been shown to work with HIF1 in activating vascular endothelial growth factor (VEGF) and haptoglobin gene transcription during hypoxia.<sup>13,15-19</sup> Given the fact that the hypoxic and inflammatory microenvironments often coexist in solid tumors and that hypoxia activates STAT3 activity, we hypothesized that STAT3 is a general cotranscription factor of the HIF-mediated hypoxia response. Here, the role of STAT3 in the general hypoxia response and the mechanism of STAT3 in contributing to the HIF-mediated hypoxia response were probed.

## RESULTS

### Hypoxia promotes STAT3 activity in a HIF-independent manner in cancer cell lines

A number of papers report activation of STAT3 by hypoxia in several cell types.<sup>10-14</sup> To confirm these results, we investigated STAT3 activity in RCC4 renal clear cell carcinoma cells, MDA-MB-231 breast cancer cells and Hep3B hepatoma cells. These cell lines express both HIF1 $\alpha$  and HIF2 $\alpha$  and have been extensively used in hypoxia studies.<sup>20-25</sup> Both Hep3B and MDA-MB-231 cells have functional pVHL, whereas RCC4 cells are deficient in functional pVHL and exhibit HIF activity even under normoxia.<sup>20,26,27</sup> Although all three cell lines expressed similar levels of total STAT3 protein under normoxia and hypoxia

(Figure 1a), levels of Y705 phosphorylated STAT3 (pY705STAT3), the transcriptionally active form of STAT3 protein, varied significantly among the cell lines (Figure 1a). RCC4 cells exhibited high levels of pY705STAT3 regardless of oxygen concentration; MDA-MB-231 cells displayed low levels of pY705STAT3 under normoxia while hypoxia increased STAT3 phosphorylation. However, no pY705STAT3 was detected even in hypoxic Hep3B cells (Figure 1a). Although STAT3 activation by hypoxia is well established, different pathways have been reported to activate STAT3 during hypoxia.<sup>11,12,28,29</sup> IL-6, IL-8, VEGF and pyruvate kinase, muscle, isoform 2 (PKM2) are reported STAT3 activators<sup>8,30-33</sup> and hypoxia-inducible genes.<sup>21,34</sup> The correlation between HIF activity and STAT3 activity in RCC4 and MDA-MB-231 cells promoted us to test whether HIF activity is responsible for STAT3 activation in these cell lines. VEGF and PKM2 are well-known HIF target genes; however, it is still not clear whether hypoxic induction of IL-6 and IL-8 is ARNT-dependent. Interestingly, like VEGF and PKM2, IL-6 and IL-8 were HIF target genes in MDA-MB-231 cells (Figure 1b). Furthermore, high levels of these genes in normoxic RCC4 cells were also HIF-/ARNT-dependent, as the levels of these STAT3 activators were significantly reduced in normoxic RCC4T cells or RCC4 cells targeted with ARNT small interfering RNA (siRNA; Figure 1c). In comparison with hypoxic MDA-MB-231 cells, hypoxic MDA-MB-231/ARNT siRNA cells exhibited similar pY705STAT3 levels according to densitometry scanning (Figure 1d). Furthermore, the levels of pY705STAT3 were found to be similar between normoxic RCC4 and normoxic RCC4T cells, and between normoxic RCC4T and hypoxic RCC4T cells (Figure 1e). These data suggested that the HIF-mediated hypoxia response did not have a significant role in hypoxia-mediated STAT3 activation in RCC4 and MDA-MB-231 cells.

### **STAT3 activity corresponds with maximal hypoxia-inducible gene expression in MDA-MB-231 cells**

To uncover the role of STAT3 in the hypoxia response of MDA-MB-231 cells, we determined the kinetics of hypoxia-mediated STAT3 activation and hypoxia-mediated gene expression by conducting a time-course experiment. The levels of pY705STAT3 began to increase in cells exposed to hypoxia for 18 h, and this pY705STAT3 level was maintained during the 18–30-h hypoxia treatment (Figure 2a). Importantly, exposure of cells to hypoxia for 24 h dramatically increased the nuclear levels of pY705STAT3 protein (Figure 2b) in spite of similar levels of total nuclear STAT3 between normoxic cells and hypoxic cells (Figure 2b). STAT3 was reported to activate HIF1 $\alpha$  gene transcription in several other cell types;<sup>35-37</sup> we found that levels of HIF1 $\alpha$  mRNA were actually reduced about 50% in 6–30 h hypoxic MDA-MB-231 cells, whereas HIF2 $\alpha$ , ARNT and STAT3 mRNA levels were induced in cells treated with 18–30 h hypoxia (Figure 2c). However, HIF1 $\alpha$ , HIF2 $\alpha$ , ARNT and total STAT3 protein levels were not changed during 6–30 h hypoxia treatment (Figure 2a). Indeed, known STAT3 and HIF common target genes *PDK1* and *VEGF* exhibited upregulation in 6 and 12 h hypoxic MDA-MB-231 cells, and further increased induction in 18–30 h hypoxic cells in which STAT3 was also further activated (Figure 2d), whereas known STAT3 targets *MYC* and *BIRC5* were mainly induced in 18–30 h hypoxic cells in which STAT3 was activated by hypoxia (Figure 2d).<sup>16,38-43</sup> *CA9*, *NDRG1*, *PGK1* and *PAIL* genes are known HIF target genes,<sup>20,21,34</sup> but these genes have never been reported to be regulated by STAT3. Interestingly, these genes also exhibited higher levels of expression in MDA-MB-231 cells containing higher STAT3 activity (18–30 h hypoxia) than MDA-MB-231 cells having lower STAT3 activity (6–12 h hypoxia) (Figure 2e), demonstrating a possible role of STAT3 in enhancement of the hypoxia response in MDA-MB-231 cells.

## Optimal hypoxia activation of HIF1 target genes in MDA-MB-231 cells requires STAT3 activity

After having observed a correlation between STAT3 activity and optimal hypoxic induction of HIF target genes in MDA-MB-231 cells (Figure 2), we next attempted to determine the role of STAT3 in the HIF-mediated hypoxia response. Both 100 and 200  $\mu\text{M}$  STAT3 inhibitor S3I-201 significantly and similarly reduced the levels of pY705STAT3 in normoxic or 24 h hypoxic MDA-MB-231 cells (Figure 3a). In addition, 200  $\mu\text{M}$ , but not 100  $\mu\text{M}$  STAT3 inhibitor reduced the levels of HIF1 $\alpha$  and HIF2 $\alpha$ , but not ARNT protein (Figure 3a). Thus, 100  $\mu\text{M}$  STAT3 inhibitor was used to assess the role of STAT3 in the hypoxia response in MDA-MB-231 cells. To determine the role of STAT3 specifically in the HIF/ARNT-mediated hypoxia response, MDA-MB-231 cells were targeted with ARNT siRNA (Figure 3b). To assess the possible specific requirement of STAT3 for HIF1 and/or HIF2 target genes, we used siRNA to reduce HIF1 $\alpha$  or HIF2 $\alpha$  mRNA levels in MDA-MB-231 cells (Figure 3b).

Hypoxic induction of MYC and BCL-XL was reduced by STAT3 inhibitor, but not by ARNT siRNA, confirming MYC and BCL-XL as STAT3, but not HIF target genes (Figure 3c). Hypoxic induction of the known STAT3/HIF common target genes VEGF and PDK1 was significantly reduced by either ARNT siRNA or STAT3 inhibitor (Figure 3d), confirming both STAT3 and HIF/ARNT activate these genes under hypoxia. Interestingly, STAT3 inhibition reduced hypoxic induction of HIF1-specific genes *ANGPTL4* and *CA9* (Figure 3e), but not HIF2-specific target genes *GPR157* and *PLAC8* (Figure 3f), indicating a possible role of STAT3 in HIF1 target gene activation during hypoxia. As expected, combined inhibition of STAT3 and ARNT did not further reduce the levels of HIF2 target genes *GPR157* and *PLAC8* relative to their levels in MDA/ARNT siRNA cells (Figure 3f). Interestingly, combined inhibition of STAT3 and ARNT also exhibited no additional reduction of HIF1 target genes *ANGPTL4* and *CA9* (Figure 3e) and known STAT3/HIF common targets (Figure 3d), suggesting that STAT3 and ARNT activated these HIF target genes in the same, and not parallel pathway. STAT3 siRNA also significantly reduced hypoxic induction of HIF1-specific target genes *PDK1*, *ANGPTL4* and *CA9*, and HIF1/2 common target VEGF, but not HIF2-specific targets *GPR157* and *PLAC8* (data not shown). In summary, these data support a role of STAT3 in the HIF/ARNT-mediated hypoxia response, particularly in the induction of HIF1 target genes in MDA-MB-231 cells.

## STAT3 activity is required for hypoxia-induced HIF1 target gene expression in RCC4 cells

To extend the role of STAT3 in the hypoxia response to another cell type, and most importantly to assess the role of STAT3 in global HIF-mediated target gene activation, RCC4 cells were targeted with siRNA against HIF1 $\alpha$ , HIF2 $\alpha$ , HIF1 $\alpha$  + 2 $\alpha$  or STAT3 mRNA. Interestingly, knockdown of STAT3 exhibited 30% reduction of HIF1 $\alpha$  and HIF2 $\alpha$  mRNA (Figure 4a), but not HIF $\alpha$  protein (Figure 4b). HIF1-, HIF2-specific or HIF1/HIF2 common target genes were first identified using Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). Table 1 lists the top 10 most likely HIF1-specific, HIF2-specific and HIF1/HIF2 common target genes based on levels of reduction by HIF1 $\alpha$ , HIF2 $\alpha$  or HIF1 $\alpha$  + 2 $\alpha$  siRNAs, as determined by microarray (Table 1, microarray). Quantitative reverse transcription-PCR (qPCR) analysis of the levels of HIF target genes in RNA prepared from normoxic parental, HIF1 $\alpha$ , HIF2 $\alpha$ , ARNT or STAT3 siRNA-transfected RCC4 cells validated the microarray-identified top 10 HIF target genes. Importantly, STAT3 was found to be required for higher expression of all 20 HIF1 (including HIF1-specific and HIF1/2 common genes), but not the majority of top 10 HIF2-specific target genes (Table 1, qPCR). These results demonstrated for the first time that the STAT3 is specifically important for hypoxic activation of HIF1 target genes in RCC4 cells. Therefore, we focused specifically on STAT3's role in HIF1 target gene activation.

## STAT3, not HIF/ARNT, is primarily responsible for the hypoxia-mediated increased recruitment of CBP and p300 coactivators to endogenous HIF1 target promoters

Having showed that STAT3 is required for maximal hypoxic induction of HIF1 target genes, we wanted to determine the molecular mechanism(s) behind STAT3's contribution to HIF1 target gene activation during hypoxia. STAT3 activates gene transcription by recruiting histone acetylases CBP and p300, and basal components of the RNA polymerase II (Pol II) transcription complex.<sup>44–46</sup> Thus, we performed chromatin immunoprecipitation (ChIP) experiments to assess the binding of CBP, p300, the largest subunit of RNA Pol II, HIF1 $\alpha$  and STAT3 to HIF1 target gene promoters in 24 h normoxic or hypoxic parental MDA-MB-231 cells with or without 100  $\mu$ M S3I-201. We selected the VEGF promoter as a positive control, as VEGF is regulated by both HIF1 and STAT3.<sup>13,15–18</sup> CA9 and PGK1 genes were chosen as they are established HIF1 target genes, but they are newly identified STAT3 targets (Figure 2 and Table 1). As expected, hypoxia increased binding of HIF1 $\alpha$ , ARNT, STAT3, CBP, p300 and Pol II on CA9, PGK1 and VEGF promoter regions around the reported HREs at –6 for CA9, at –233 and –224 for PGK1 and at –975 for VEGF in MDA-MB-231 cells (Figures 5a–c). However, no significant binding of these factors to control regions of exon 8 for PGK1 and exon 3 for VEGF was detected (data not shown). S3I-201 significantly decreased STAT3 binding to all three promoters in hypoxic cells (Figures 5a–c). HIF1 $\alpha$  and ARNT binding to the VEGF (Figure 5c), but not CA9 and PGK1 promoters (Figures 5a and b) was reduced slightly in S3I-201-treated hypoxic MDA-MB-231 cells (Figure 5c). The most significant and consistent change in response to treatment with S3I-201 was the reduction or complete blockage of hypoxia-mediated increased binding of CBP, p300 and Pol II to all three promoters (Figures 5a–c), providing evidence for a critical role of STAT3 in hypoxic recruitment of CBP, p300 and Pol II to these genes. HIF/ARNT has been reported to activate hypoxia-inducible genes by recruiting CBP and p300 via N-terminal and C-terminal transactivation domains.<sup>47–49</sup> However, hypoxic MDA-MB-231/ARNT small hairpin RNA (shRNA) cells exhibited only a slight reduction in CBP, p300 and Pol II binding to CA9, PGK1 and VEGF promoters in spite of significant reduction of HIF1 $\alpha$  and ARNT binding (Figures 5a–c), demonstrating that STAT3, not HIF/ARNT, is the primary recruiter of CBP and p300 to HIF1 target genes CA9, PGK1 and VEGF promoters, whereas both STAT3 and HIF/ARNT contribute to Pol II recruitment. To confirm these results in a different cell type, RCC4 cells were targeted with ARNT shRNA or S3I-201 (Figures 5d–f). Again, STAT3 inhibitor was more effective in reducing CBP and p300 binding to CA9, PGK1 and VEGF promoters than ARNT shRNA, whereas both STAT3 and ARNT were equally important for Pol II recruitment (Figures 5d–f). In summary, these results demonstrate that STAT3 binds to the endogenous HIF1 target genes in an ARNT-independent fashion and participates in the HIF1-mediated hypoxia response by recruiting histone acetylases CBP and p300 and Pol II to HIF1 target gene promoters.

### STAT3 physically interacts with HIF1 $\alpha$ , but not with HIF2 $\alpha$

The above ChIP data demonstrated the binding of HIF1 $\alpha$ , STAT3, CBP, p300 and Pol II to HIF1 target gene promoters (Figure 5). In addition, HIF1 $\alpha$  (but not HIF2 $\alpha$ ) and STAT3 exhibited functional cooperation in activating HIF1 target genes (Figure 3 and Table 1). These data support a hypothesis that HIF1 $\alpha$  and STAT3 are components of the HIF1-specific target gene 'enhanceosome' complex. To test this hypothesis, we first assessed the physical interaction between STAT3 and HIF $\alpha$  proteins. STAT3 interaction with HIF1 $\alpha$  or HIF2 $\alpha$  was first tested using an overexpression system in which Flag-tagged HIF1 $\alpha$  but not Flag-tagged HIF2 $\alpha$  protein co-precipitated HA-tagged STAT3 protein in HEK293T cells transfected with STAT3C-HA (constitutively active STAT3 with HA-tag) alone for control, STAT3C-HA with human HIF1 $\alpha$ TM-Flag (triple mutation of HIF1 $\alpha$  with the Flag tag), or STAT3C-HA with human HIF2 $\alpha$ TM-Flag (Figure 6a). To confirm STAT3/HIF1 $\alpha$

interaction under physiological conditions, endogenous STAT3 protein in normoxic RCC4 cells was precipitated by STAT3 antibodies and protein-A/protein-G beads (Figure 6b, STAT3-IP lane); interestingly, STAT3 only co-precipitated HIF1 $\alpha$ , but not HIF2 $\alpha$  protein (Figure 6b, STAT3-IP lane).

### **STAT3, HIF1 $\alpha$ , p300 and Pol II form a transcriptional complex on endogenous HIF1 target promoters**

We next tested whether there were physical interactions among STAT3, HIF1 $\alpha$ , p300 and Pol II on endogenous HIF1 target gene promoters in 24 h hypoxia-treated MDA-MB-231 cells using ChIP/Re-chromatin Immunoprecipitation (Re-ChIP). Analysis of ChIP/ReChIP precipitated DNA by qPCR for CA9, PGK1 and VEGF promoters showed a high percentage of Pol II bound to the same physical promoter in proximity to STAT3, as was p300 with STAT3 and HIF1 $\alpha$  with STAT3 (Figure 7). These results were confirmed by the reciprocal ChIP/ReChIP of STAT3 with Pol II, STAT3 with p300 and STAT3 with HIF1 $\alpha$  (Figure 7). These results provided strong evidence that STAT3, HIF1 $\alpha$ , p300 and Pol II are components of a multifactorial transcription complex binding to HIF1 target gene promoters during hypoxia.

### **STAT3 knockdown phenocopies HIF1 $\alpha$ knockdown in tumorigenic properties of MDA-MB-231 and RCC4 cells in vitro**

Next we investigated whether STAT3 inhibition would phenocopy HIF1 inhibition in functional assays. To test this hypothesis, MDA-MB-231 cells were targeted with STAT3 or HIF1 $\alpha$  shRNAs to reduce STAT3 or HIF1 $\alpha$  protein levels (Figure 8a). To activate HIF and STAT3 activity for functional assays, parental, MDA-MB-231/HIF1 $\alpha$  shRNA or MDA-MB-231/STAT3 shRNA cell lines were cultured in media with 500  $\mu$ M dimethylxaloylglycine (DMOG). Importantly, like hypoxia, the hypoxia mimic DMOG induced STAT3 activation (Figure 8b). In comparison with parental MDA-MB-231 cells without DMOG, MDA-MB-231/HIF1 $\alpha$  shRNA cells exhibited slight reduction in proliferation, migration and clonogenic survival (Figures 8d–e), consistent with the fact that normoxic MDA-MB-231 cells did not express HIF1 $\alpha$  protein (Figure 3a). Interestingly, reduction of STAT3 also weakly reduced cell proliferation and migration, but strongly decreased clonogenic survival (Figure 8e). Compared with MDA-MB-231 cells without DMOG treatment, DMOG-treated parental MDA-MB-231 cells exhibited quicker proliferation during day 3–5 (Figure 8d), but had no significant changes in wound healing and clonogenic survival (Figures 8e and f). Furthermore, identical to MDA-MB-231/HIF1 $\alpha$  shRNA cells, DMOG-treated MDA-MB-231/STAT3 shRNA cells exhibited significantly decreased cell proliferation (Figure 8d), wound healing (Figure 8e) and clonogenic survival (Figure 8f), demonstrating that the HIF1/STAT3-mediated hypoxia response is important for maintaining cell proliferation, migration and survival of these cells under hypoxia.

These functional assays were also conducted in normoxic RCC4 cells that exhibited constitutive HIF1 $\alpha$  and STAT3 activity. Reduction of STAT3 and HIF1 $\alpha$  activity was achieved by shRNA targeting HIF1 $\alpha$  or STAT3 mRNA (Figure 8c). Again, HIF1 $\alpha$  or STAT3 shRNAs greatly and similarly reduced RCC4 cell proliferation (Figure 8g) and motility (Figure 8h). However, clonogenic survival of RCC4 cells was similarly enhanced by HIF1 $\alpha$  or STAT3 shRNA (Figure 8i), demonstrating HIF1 $\alpha$  or STAT3 activity in RCC4 cells actually decreases clonogenic survival, consistent with HIF1 $\alpha$ 's tumor suppressor role in the VHL-deficient RCC4 cells.<sup>50</sup>

## DISCUSSION

Cytokines and growth factors are well-known activators of the inflammatory response mediator STAT3.<sup>5-9,38</sup> Recent studies show that hypoxia also activates STAT3 via different mechanisms, including activation of SRC kinase in pancreatic carcinoma BxPC3 and PANC1 cells,<sup>28</sup> activation of Janus kinase 2 in lung carcinoma IGR-Heu cells,<sup>11</sup> activation of nuclear factor- $\kappa\beta$  and activator protein-1 (AP1) and IL-6 expression in several head and neck carcinoma cells,<sup>29</sup> or increased levels of reactive oxygen species in the human ovarian A2780 cancer cells.<sup>12</sup> However, it is not clear whether all or some of these pathways are activated in specific cancer cell types. We found that hypoxia activates STAT3 in MDA-MB-231 cells, but not in Hep3B cells. In addition, we show that hypoxic activation of STAT3 in MDA-MB-231 cells and constitutive STAT3 activity in RCC4 cells is ARNT independent (Figure 1). However, it is still possible that STAT3 activators induced by hypoxia could activate STAT3 in the absence of SRC or other STAT3 activators.

Several previous reports indicate that STAT3 functions as an activator of HIF1 $\alpha$  gene transcription and/or a promoter of HIF1 $\alpha$  protein stability.<sup>35-37,51</sup> However, other studies reported that inhibition of STAT3 activity has no effect on the levels of HIF1 $\alpha$  mRNA and/or protein. For example, the STAT3 inhibitor STATTIC inhibits STAT3 phosphorylation, but not the levels of HIF1 $\alpha$  protein in hypoxic head and neck cancer UM-SCC-17B and SC-19 cells;<sup>52</sup> hypoxia activates Janus kinase 2 and STAT3 in pulmonary arterial smooth muscle cells, but STAT3 siRNA does not change the levels of HIF1 $\alpha$  protein in the hypoxic PASM cells.<sup>53</sup> Furthermore, c-Src inhibitor saracatinib significantly reduces p-STAT3, but has no effects on the HIF1 $\alpha$  protein levels in hypoxic kidney cancer Caki-1 cells.<sup>54</sup> We also show that Hep3B cells express high levels of HIF1 $\alpha$  mRNA<sup>20</sup> although STAT3 is not active in Hep3B cells (Figure 1a). In addition, we found that STAT3 shRNA (Figure 8a) or 100  $\mu$ M STAT3 inhibitor S3I-201 (Figure 3a) does not reduce the levels of HIF1 $\alpha$  mRNA and/or protein in MDA-MB-231 cells. In addition, STAT3 target genes *STAT3*, *MYC* and *BIRC5*, but not *HIF1 $\alpha$*  (Figures 2c and d) were induced in 18–30 h hypoxia-treated MDA-MB-231 cells. In contrast, we found that STAT3 is a weak activator of the HIF1 $\alpha$  gene in RCC4 cells. Furthermore, we found that STAT3 activates the HIF1 $\alpha$  and HIF2 $\alpha$  promoter reporters in HEK293T cells (Supplementary Figure S1). Thus, these different findings are likely due to cell-type differences whereby reduction of STAT3 activity is not enough to reduce HIF1 $\alpha$  gene expression in some cell types in which *HIF1 $\alpha$*  gene expression could be maintained by other *HIF1 $\alpha$*  gene activators such as nuclear factor- $\kappa\beta$ , PI3K/AKT and activator protein-1.<sup>55</sup>

VEGF and haptoglobin are known STAT3 target genes<sup>16,56,57</sup> and hypoxia inducible genes.<sup>58-60</sup> It makes sense that STAT3 and HIF1 $\alpha$  cooperatively activate VEGF and haptoglobin genes during hypoxia.<sup>13,15,17-19</sup> We confirmed the activation of VEGF by HIF1 $\alpha$  and STAT3 in MDA-MB-231 and RCC4 cells (Figure 2 and Table 1). More importantly, for the first time, we establish the role of STAT3 as a general cotranscriptional activator to a larger set of HIF target genes that have never been reported as STAT3 targets (Table 1).

Although the role of STAT3 in the hypoxia response was also probed previously,<sup>11,14</sup> these studies used IGR-Heu lung and U87 glioblastoma cells in which knockdown of STAT3 also significantly reduced HIF1 $\alpha$  mRNA and protein.<sup>11,14</sup> Thus, these studies did not separate STAT3's role in regulating *HIF1 $\alpha$*  gene expression from its role as a HIF coactivator during hypoxia. In contrast, we studied the role of STAT3 in the hypoxia response by using cell lines that have reduced STAT3 activity, but normal levels of HIF1 $\alpha$ , HIF2 $\alpha$  and ARNT. In addition, by using siRNAs targeting HIF1 $\alpha$ , HIF2 $\alpha$  or both HIF $\alpha$  mRNAs, we specifically studied the role of STAT3 in the HIF-mediated hypoxia response. Most importantly, we

found for the first time that STAT3 activity is required for HIF1, but not HIF2 target gene activation in RCC4 and MDA-MB-231 cells.

Although increased binding of STAT3, HIF1 $\alpha$ , p300 and RNA Pol II to the VEGF promoter during hypoxia has been reported,<sup>13,15</sup> we for the first time show that STAT3 is primarily responsible for p300 and CBP recruitment, whereas both STAT3 and HIF1 $\alpha$ /ARNT recruit RNA Pol II to the VEGF promoter. In addition, we demonstrate the role of STAT3 in recruiting CBP, p300 and RNA Pol II to two novel STAT3 target genes *CA9* and *PGK1* (Figures 6 and 7).

Although STAT3 was found to be involved in the hypoxia response, STAT3 clearly has HIF-independent activity. For example, STAT3 specifically activates MYC and BCL-XL (Figures 2c and 3c), HIF2 $\alpha$ , ARNT and STAT3 in MDA-MB-231 cells (Figure 2b). This is consistent with functional assays demonstrating that inhibition of STAT3 in normoxic MDA-MB-231 cells reduces cell survival (Figure 8).

Our target gene and functional studies demonstrate integration between the inflammatory STAT3 pathway and the hypoxic HIF1 pathway under hypoxia. Our findings suggest that pharmacological inhibition of STAT3 activity could prove anti-tumorigenic and especially effective in some solid tumors in which the HIF1-mediated hypoxia response has a critical role in cancer progression, whereas anti-STAT3 treatment could be less effective in cells such as RCC4 in which HIF1 could function as a tumor suppressor.

## MATERIALS AND METHODS

### Cell culture

HEK293T, RCC4, RCC4T, Hep3B and MDA-MB-231 cells were grown in high-glucose Dulbecco's modified Eagle medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum. For hypoxia, 25 mM hydroxyethyl piperazineethanesulfonic acid was added to growth media and cells were incubated at 21 or 1.2% O<sub>2</sub> for 24 h (or otherwise noted). Phosphorylation of Y705-STAT3 was inhibited by adding 100  $\mu$ M S3I-201 (sc-204304; Santa Cruz, Dallas, TX, USA) to media 1 h before hypoxia treatment.

### Knockdown of HIF $\alpha$ , ARNT and STAT3 mRNAs using siRNAs or shRNAs

Control or siRNAs specific for human HIF1 $\alpha$ , HIF2 $\alpha$  and ARNT mRNAs were described previously.<sup>61</sup> STAT3 siRNA (siSGENOME M-003544-02) was purchased from Dharmacon (Waltham, MA, USA). Transient knockdown of mRNA in RCC4 or MDA-MB-231 cells were performed as described.<sup>61</sup> Stable knockdown of HIF1 $\alpha$ , ARNT or STAT3 mRNA in RCC4 and MDA-MB-231 cells were achieved by pLKO.1 lentiviruses expressing shRNAs targeting mRNAs of HIF1 $\alpha$  (TRCN000003810), ARNT (TRCN000003816) or STAT3 (TRCN0000020840).

### DNA constructs

pcDNA3.1hHIF1 $\alpha$ TM-Flag (triple mutations of P402A/P577A/N813A of human HIF1 $\alpha$  protein with Flag-tag) and pcDNA3.1hHIF2 $\alpha$ TM-Flag have been described.<sup>61</sup> The pRC/CMV/STAT3C-Flag construct was purchased from Addgene (Cambridge, MA, USA; plasmid 8722, deposited by Jim Darnell) and used as a template to make pcDNA3.1 STAT3C-HA (HA-tagged at STAT3 C-terminus).

### RNA preparation, microarray and qPCR

RNA was isolated from cells using the RNeasy Plus mini kit (Qiagen, Germantown, MD, USA). RNA was reverse-transcribed using the iSCRIPT Advanced complementary DNA



synthesis kit (Bio-Rad, Hercules, CA, USA). Levels of mRNA were quantified by Sybr Green qPCR using the CFX384 Real-Time System (Bio-Rad). All primer sets designed to measure target gene mRNA levels or used in ChIP were validated for their specificity and amplification efficiency (85–110%) using melt curve analysis, qPCR product sequencing and standard dilution analysis. qPCR results were normalized using 18S rRNA. Results were the average of a minimum of three independent experiments performed in triplicate. Microarray analysis of HIF target gene specificity in RCC4 cells was basically identical to HIF target gene analysis in Hep3B cells, as described.<sup>61</sup>

### Protein analysis

Typically total cell lysates were used for western blot analysis. To detect nuclear and cytoplasmic protein, the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo, Waltham, MA, USA, 78833) was used. Protein concentration was determined by BCA protein assay kit (Pierce, Waltham, MA, USA, 23223 and 23224) and the same amount of protein was loaded in western blot procedures, as described below. Western blot analysis was performed using standard protocols with the following primary antibodies: anti-HIF1 $\alpha$  monoclonal antibodies (NB 100–105; Novus Biologicals, Littleton, CO, USA), anti-HIF2 $\alpha$  polyclonal antibodies (NB100–122; Novus Biological), anti-ARNT mAb (NB 100–124; Novus Biological), anti-STAT3 (total) pAb (K-15, sc-483; Santa Cruz), anti-Flag M2 mAb (F-3165; Sigma-Aldrich, St Louis, MO, USA), anti-HA (MMS-101P, Covance, Alice, TX, USA) and anti-beta Actin (SC-1616; Santa Cruz). Anti-STAT3 western blot was blocked in 5% bovine serum albumin in Tris buffered saline with Tween 20 (TBST) and probed using anti-pSTAT3 (pY705) mAb (B-7, sc-8059; Santa Cruz).

### STAT3/HIF $\alpha$ protein interaction

For *in vitro* STAT3 and HIF1 $\alpha$  (or HIF2 $\alpha$ ) protein interaction, HEK293T cells were transfected with HA-tagged constitutively active STAT3 alone, or STAT3C-HA with Flagged constitutively active HIF1 $\alpha$  or HIF2 $\alpha$ . Lysates from transfected 293T cells were immunoprecipitated with anti-Flag M2 beads (Sigma-Aldrich) to pull down Flag-tagged HIF1 $\alpha$  or HIF2 $\alpha$ , in which HA-tagged STAT3 was detected. For endogenous HIF/STAT3 protein interaction, nuclear extracts from RCC4 cells were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit in which STAT3 antibody and protein-A/protein-G beads were added.

### ChIP and ReChIP

ChIP assays were performed as described.<sup>62</sup> Anti-HIF1 $\alpha$  (NB 100-134B3; Novus Biologicals), anti-ARNT (NB 100–110; Novus Biologicals), anti-CBP (A-22, sc-369; Santa Cruz), anti-p300 (N-15, sc-584; Santa Cruz), anti-STAT3 (K-15, sc-483; Santa Cruz) and anti-Pol II (H-224, SC-9001, Santa Cruz) antibodies were used for protein–DNA complex precipitation, whereas rabbit preimmune serum served as a control. DNA from input or immunoprecipitated samples was assayed using Sybr Green-based quantitative PCR with specific primers designed to amplify the CA9, PGK1 or VEGF promoter around the reported HREs or in exons for negative controls. ChIP/ReChIP: ChIP was performed as above, binding complexes from the first immunoprecipitation were eluted from the sepharose beads using Re-ChIP buffer as described.<sup>61</sup> The eluted protein–DNA complexes were diluted in radioimmunoprecipitation buffer and resubjected to ChIP using a different antibody.

### *In vitro* tumorigenic assays

Proliferation assays, scratch assays and clonogenic survival assays were performed for MDA-MB-231 cells or MDA/HIF1 $\alpha$  shRNA or MDA/STAT3 shRNA cells with or without

500  $\mu$ M DMOG under normoxia. The same experiments were carried out in RCC4 cells or RCC4/HIF1 $\alpha$  shRNA or RCC4/STAT3 shRNA cells under normoxia, as described.<sup>61</sup>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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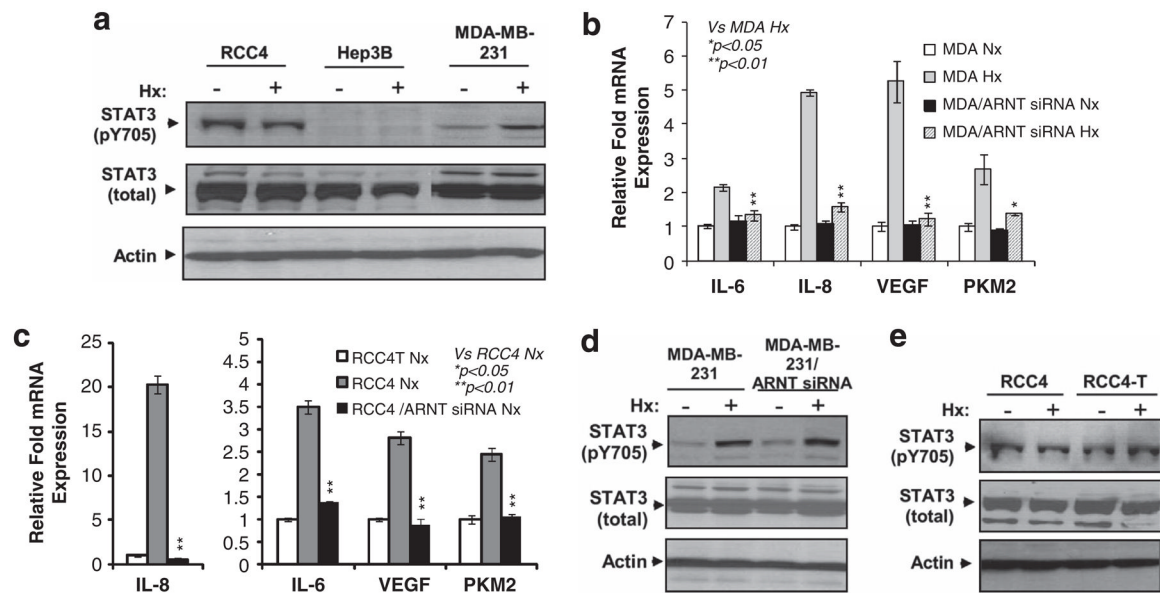
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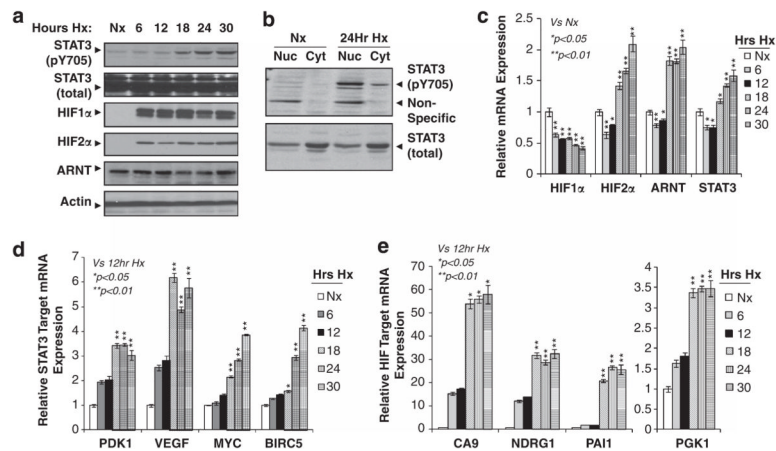
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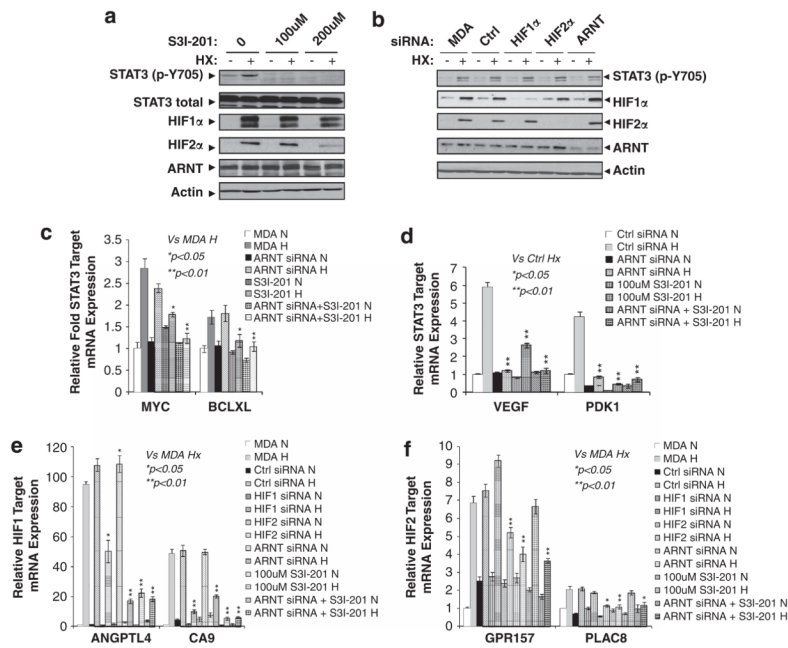
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**Figure 1.**

Hypoxia activates STAT3 activity in breast cancer cell line MDA-MB-231. (a) Western blot analysis of total and phosphorylated (Y705) STAT3 protein levels in normoxic and hypoxic (Hx) RCC4 (pVHL-null), Hep3B and MDA-MB-231 cancer cell lines. Beta-actin serves as loading control for this and other western blot analysis in the paper. (b) qPCR analysis of mRNA levels of reported STAT3 activators in normoxic and hypoxic MDA-MB-231 cells or cells targeted with ARNT siRNA. qPCR results were normalized to 18S rRNA expression and calibrated to normoxia control cells; error bars are  $\pm 1$  s.d. from at least three independent experiments in this and other figures. Two-tail *t*-tests were performed for this and other studies in the paper with ‘\*’ indicating  $P < 0.05$  and ‘\*\*’ indicating  $P < 0.01$ . Controls for the *t*-tests are indicated in the figures. (c) qPCR analysis of mRNA levels of reported STAT3 activators in normoxic RCC4 cells, RCC4 cells targeted with ARNT siRNA or normoxic pVHL-transfected RCC4-T cells. (d) Western blot analysis of total and pY705STAT3 protein in normoxic and hypoxic MDA-MB-231 cells or MDA-MB-231 cells targeted with ARNT siRNA. (e) Western blot analysis of total and pY705STAT3 protein in normoxic or hypoxic RCC4 and RCC4 cells reconstituted with pVHL (RCC4-T cells).

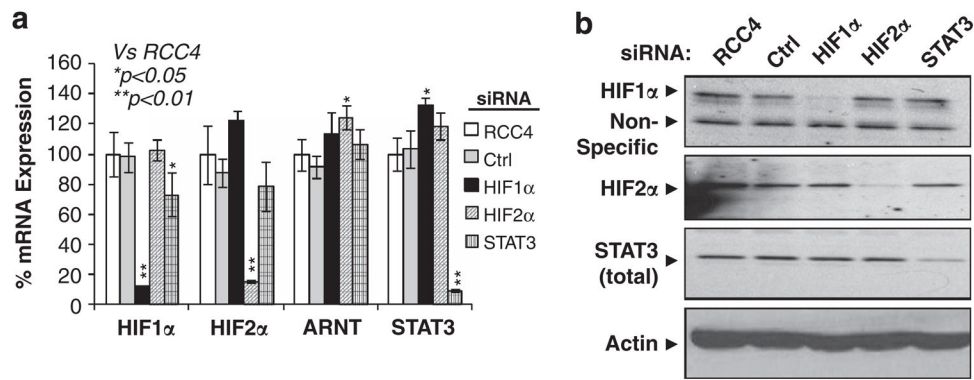
**Figure 2.**

Hypoxia-mediated STAT3 activation corresponds with maximal HIF target gene expression in MDA-MB-231 cells. **(a)** Western blot analysis of pY705STAT3, total STAT3, HIF1 $\alpha$ , HIF2 $\alpha$  and ARNT protein in MDA-MB-231 cells cultured under normoxia (Nx) or 6–30 h hypoxia (Hx). **(b)** Western blot analysis of pY705STAT3 and total STAT3 in nuclear (Nuc) and cytosolic (Cyt) fractions of the MDA-MB-231 cells cultured under Nx or 24 h Hx. **(c)** qPCR analysis of mRNA levels of HIF1 $\alpha$ , HIF2 $\alpha$ , ARNT and STAT3 in MDA-MB-231 cells cultured under Nx or 6–30 h of Hx. **(d)** qPCR analysis of mRNA levels of known STAT3/HIF common targets (PDK1 and VEGF) or STAT3 targets (MYC and BIRC5) in MDA-MB-231 cells under Nx and 6–30 h Hx. **(e)** qPCR analysis of mRNA levels of known HIF target genes in MDA-MB-231 cells under Nx and 6–30 h Hx.

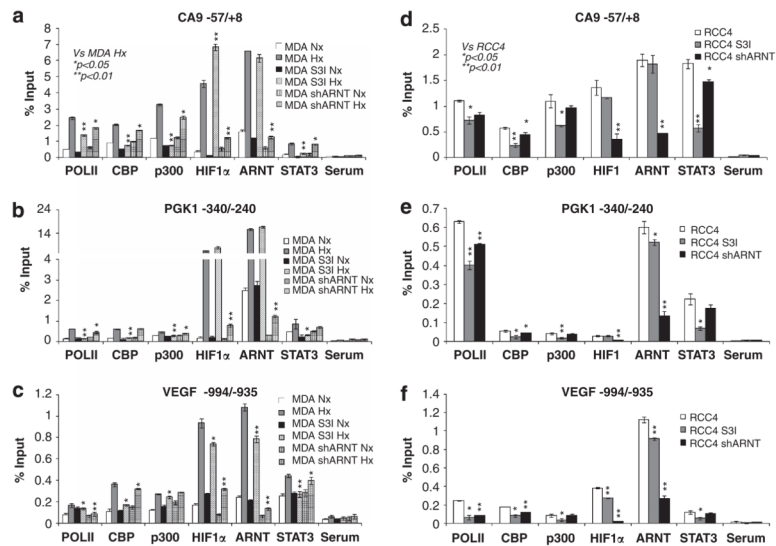
**Figure 3.**

Optimal hypoxic induction of HIF1 target genes in MDA-MB-231 cells requires STAT3 activity. **(a)** Western blot analysis of levels of pY705STAT3, total STAT3, HIF1 $\alpha$ , HIF2 $\alpha$  and ARNT protein in MDA-MB-231 cells cultured under normoxia (Nx) or 24 h hypoxia (Hx) in the absence or presence of 100 or 200  $\mu$ M of the STAT3 inhibitor S3I-201. **(b)** Western blot analysis of indicated protein in Nx or 24 h Hx MDA-MB-231 cells (MDA), or MDA-MB-231 cells targeted with control siRNA or siRNA against HIF1 $\alpha$ , HIF2 $\alpha$ , or ARNT mRNA. **(c)** qPCR analysis of mRNA levels of known STAT3 target genes *MYC* and *BCL-XL* in Nx or 24 h Hx MDA cells, or cells targeted with ARNT siRNA, or with 100  $\mu$ M STAT3 inhibitor S3I-201 or both ARNT siRNA and STAT3 inhibitor. **(d)** qPCR analysis of mRNA levels of known STAT3/HIF common target genes *VEGF* and *PDK1* in Nx or 24 h Hx MDA cells as for Figures 3c, e and f) qPCR analysis of mRNA levels of **(e)** HIF1- and **(f)** HIF2-specific target genes in Nx or 24 h Hx MDA cells, siRNA-targeted MDA cells, 100  $\mu$ M S3I-201-treated MDA cells or ARNT siRNA with STAT3 inhibitor.



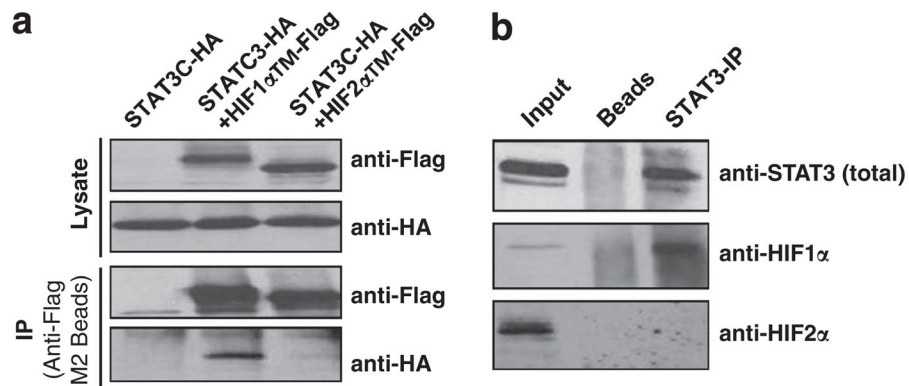


**Figure 4.** HIF1 $\alpha$ , HIF2 $\alpha$  or STAT3 siRNA efficiently reduced mRNA and protein expression of HIF1 $\alpha$ , HIF2 $\alpha$  and STAT3 in RCC4 cells. **(a)** qPCR analysis of mRNA levels of HIF1 $\alpha$ , HIF2 $\alpha$ , ARNT and STAT3 in normoxic RCC4 cells and RCC4 cells targeted with HIF1 $\alpha$ , HIF2 $\alpha$  or STAT3 siRNA. **(b)** Western blot analysis of HIF1 $\alpha$ , HIF2 $\alpha$  or STAT3 protein levels in parental or siRNA-transfected RCC4 cells.

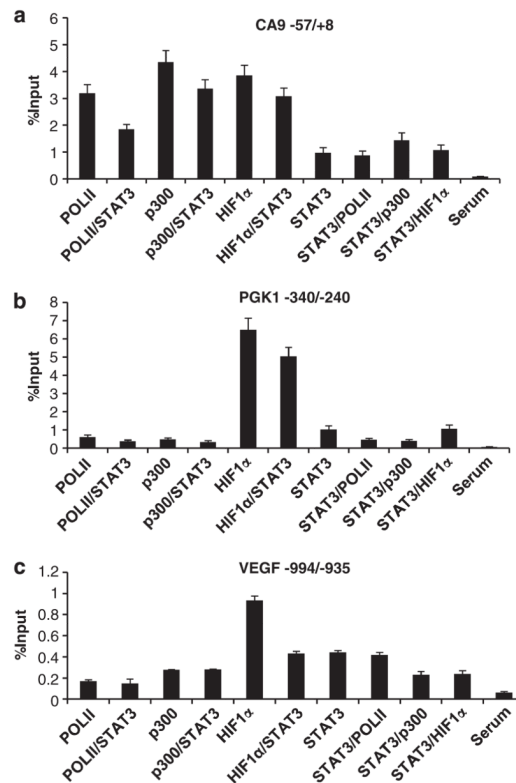


**Figure 5.**

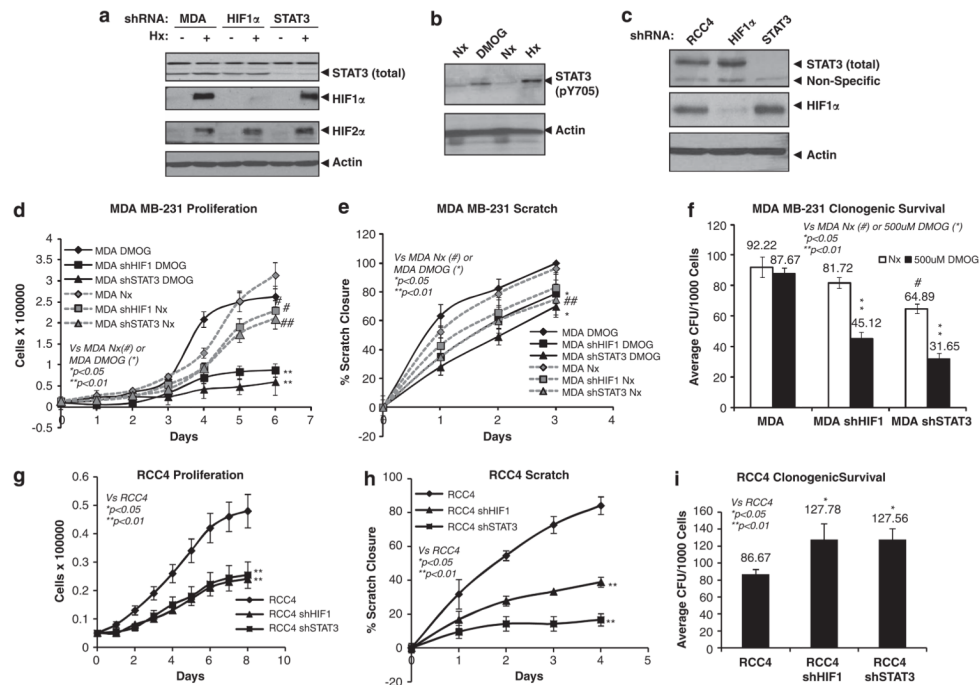
STAT3, but not HIF1 $\alpha$ /ARNT is primarily responsible for recruiting CBP and p300 coactivators to the endogenous HIF1 target promoters. ChIP analysis of Pol II, CBP, p300, HIF1 $\alpha$ , ARNT and STAT3 binding to the HIF1 target promoters around the reported HREs of the *CA9* (a), *PGK1* (b) and *VEGF* genes (c) in MDA-MB-231 cells under normoxic (Nx) or 24 h hypoxic (Hx) conditions or MDA cells treated with STAT3 inhibitor, or with ARNT stably knocked down. Co-precipitated DNA is presented as percent DNA compared with input controls and background level is quantified by DNA pulled down using non-reactive serum (Serum). Similar ChIP was performed in normoxic RCC4 cells, or RCC4 cells targeted with STAT3 inhibitor or ARNT stably knocked down for CA9 promoter (d), PGK1 promoter (e) and VEGF promoter (f).



**Figure 6.** STAT3 protein specifically interacts HIF1 $\alpha$ , but not HIF2 $\alpha$  protein. **(a)** Western blot analysis of Flag-tagged HIF1 $\alpha$  and HIF2 $\alpha$  proteins (anti-Flag) or HA-tagged STAT3 (anti-HA) in the lysates (Lysate) or anti-Flag M2 bead immunoprecipitated materials (IP) from 293T cells transfected with STAT3C-HA (HA-tagged constitutively active STAT3), STAT3C-HA +HIF1 $\alpha$ TM-Flag (Flag tagged HIF1 $\alpha$  with triple mutation to make HIF1 $\alpha$  stable and active under normoxia), or STAT3C-HA +HIF2 $\alpha$ TM-Flag. **(b)** Western blot analysis of STAT3, HIF1 $\alpha$  or HIF2 $\alpha$  protein in the RCC4 lysates (input) or IP materials from the protein-A/protein-G beads (beads) or IP materials using anti-STAT3 pY705 antibody and protein-A/protein-G beads.



**Figure 7.** STAT3, HIF1 $\alpha$ , p300 and Pol II form a transcriptional complex on the endogenous HIF1 target promoters. ChIP/ReChIP analysis of STAT3, HIF1 $\alpha$ , Pol II and P300 binding to the endogenous HIF1 target gene promoters CA9 (a), PGK1 (b) and VEGF (c) in 24 h hypoxic MDA-MB-231 cells. Antibodies used for the primary and secondary immunoprecipitation are listed as first/second. The co-precipitated DNA is presented in comparison with pull-down of each individual factor and non-reactive serum controls (Serum).



**Figure 8.** STAT3 knockdown phenocopies HIF1 $\alpha$  knockdown in tumorigenic properties of MDA-MB-231 and RCC4 cells *in vitro*. **(a)** Western blot analysis of total STAT3, HIF1 $\alpha$  and HIF2 $\alpha$  in normoxic (Nx) or hypoxic (Hx) MDA-MB-231 cells or MDA-MB-231 cells with stably knocked down HIF1 $\alpha$  or STAT3 protein. **(b)** Western blot analysis of pY705STAT3 in Nx or 24 h Hx MDA-MB-231 cells or Nx cells treated with or without 500  $\mu$ M DMOG. **(c)** Western blot analysis of HIF1 $\alpha$  or total STAT3 protein in RCC4 cells or RCC4 cells with HIF1 $\alpha$  or STAT3 shRNA. **(d)** Proliferation of MDA-MB-231 cells, MDA/shHIF1 $\alpha$  and MDA/shSTAT3 cells treated with or without 500  $\mu$ M DMOG. **(e)** Motility of MDA-MB-231, MB-231/shHIF1 and MDA-MB-231/shSTAT3 cells grown with or without 500  $\mu$ M DMOG was measured and presented as percent closure of a scratch assay. **(f)** Percent survival of colony-forming units of MDA-MB-231, MDA-MB-231/shHIF1 and MDA-MB-231/shSTAT3 cells grown in the presence or absence of 500  $\mu$ M DMOG in a clonogenic survival assay. **(g)** Proliferation of RCC4, RCC4/shHIF1 and RCC4/shSTAT3 cells under Nx. **(h)** Motility of Nx RCC4, RCC4/shHIF1 and RCC4/shSTAT3 cells was measured and presented as percent closure in a scratch assay. **(i)** Percent survival of colony-forming units of RCC4, RCC4/shHIF1 and RCC4/shSTAT3 cells in a clonogenic survival assay.

**Table 1**  
Efficient expression of HIF1 target genes in RCC4 cells requires STAT3 activity

Probe ID	Accession	Gene symbol	Microarray				qPCR			
			HIF1 siRNA	HIF2 siRNA	HIF1 + HIF2 siRNA	HIF1 siRNA	HIF2 siRNA	ARNT siRNA	STAT3 siRNA	
<i>HIF1-specific targets</i>										
213943_at	NM_000474	TWIST1	0.06	0.82	0.06	0.43**	1.39	0.21**	0.29**	
1553036_at	NM_153839	GPR111	0.08	0.89	0.11	0.23**	0.92	0.28**	0.55*	
218995_s_at	NM_001168319	EDN1	0.16	0.83	0.06	0.61**	0.94	0.35**	0.6**	
205199_at	NM_001216	CA9	0.25	0.94	0.31	0.25**	0.86	0.22**	0.38**	
203685_at	NM_000633	BCL2	0.31	0.62	0.47	0.38*	0.67	0.14**	0.42*	
206367_at	NM_001002914	KCTD11	0.39	0.93	0.54	0.31**	1.28	0.3**	0.37**	
200650_s_at	NM_001135239	LDHA	0.44	1.23	0.31	0.36**	0.7	0.17**	0.24**	
202095_s_at	NM_001012270	BIRC5	0.46	2.14	0.47	0.51**	0.92	0.09**	0.64*	
202364_at	NM_001008541	MXI1	0.47	0.87	0.44	0.43**	0.89	0.14**	0.52**	
200737_at	NM_000291	PGK1	0.5	1.41	0.66	0.49**	1.02	0.27**	0.51**	
<i>HIF2-Specific Targets</i>										
217287_s_at	NM_004621	TRPC6	0.88	0.06	0.04	0.84	0.2**	0.2**	0.85	
202627_s_at	NM_000602	PAI1	2.46	0.37	0.28	0.78	0.28*	0.16**	0.85	
1552846_s_at	NM_152304	RAB42	0.93	0.38	0.27	1.65*	0.61*	0.44**	1.08	
205670_at	NM_004861	CST	1.15	0.41	0.41	0.77*	0.34**	0.33**	0.63**	
209652_s_at	NM_001207012	PGF	2	0.41	0.35	0.62*	0.09**	0.08**	0.54**	
230250_at	NM_001109754	PTPRB	0.93	0.47	0.35	1.37	0.47**	0.28**	1.43*	
219888_at	NM_003116	SPAG4	1.05	0.5	0.13	1.37*	0.64**	0.05**	1.04	
227970_at	NM_024980	GPR157	0.81	0.52	0.41	0.88	0.29**	0.28**	1.22	
208286_x_at	NM_001173531	POU5F1	0.95	0.63	0.62	0.52**	0.2**	0.26**	0.55*	
205015_s_at	NM_001099691	TGFA	2.46	0.71	0.71	1	0.38*	0.25**	1.12	
<i>HIF1/2 common targets</i>										
202912_at	NM_001124	ADM	0.5	0.5	0.16	0.65*	0.63*	0.28**	0.75*	

Probe ID	Accession	Gene symbol	Microarray				qPCR			
			HIF1 siRNA	HIF2 siRNA	HIF1 + HIF2 siRNA	HIF1 siRNA	HIF2 siRNA	ARNT siRNA	STAT3 siRNA	
204298_s_at	NM_001178102	LOX	0.35	0.76	0.2	0.41**	0.3**	0.34**	0.42**	
200632_s_at	NM_001135242	NDRG1	0.7	0.62	0.29	0.54**	0.31**	0.16**	0.34**	
221478_at	NM_004331	BNIP3L	0.57	0.5	0.33	0.26**	0.59*	0.28**	0.29**	
227353_at	NM_152468	TMC8	0.44	0.66	0.35	0.54*	0.47**	0.42**	0.36**	
206686_at	NM_002610	PDK1	0.47	0.47	0.36	0.31**	0.53**	0.16**	0.43**	
201250_s_at	NM_006516	GLUT1	0.71	0.62	0.38	0.68*	0.37**	0.26**	0.79*	
221748_s_at	NM_022648	TNSI	0.35	0.66	0.41	0.48**	0.39**	0.42**	0.44**	
208329_at	NM_021635	PBOV1	0.71	0.66	0.5	0.25**	0.4**	0.19**	0.3**	
210512_s_at	NM_001025366	VEGFA	0.63	0.57	0.71	0.69*	0.52*	0.27**	0.7*	

Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; HIF1, hypoxia-inducible factor-1; STAT3, signal transducer and activator of transcription-3. Vs RCC4.

\*  $P < 0.05$ .

\*\*  $P < 0.01$ . DNA microarray was performed to determine global HIF1-, HIF2-specific and HIF1/2 common target genes in RCC4 cells or RCC4 cells targeted with HIF1 $\alpha$ , HIF2 $\alpha$  or HIF1 $\alpha$  + HIF2 $\alpha$  siRNA. Listed are the top most likely HIF1-, HIF2-specific and HIF1/HIF2 common target genes (Microarray). HIF target gene specificity was confirmed by qPCR using RNAs from RCC4 cells targeted with HIF1 $\alpha$ , HIF2 $\alpha$  or ARNT siRNA. In addition, the role of STAT3 in HIF target gene regulation was determined by qPCR using complementary DNA generated from RCC4 cells targeted with STAT3 siRNA (qPCR).