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A novel miR-155/miR-143 cascade controls glycolysis by regulating *hexokinase 2* in breast cancer cells

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Cancer cells preferentially metabolize glucose through aerobic glycolysis. This phenomenon, known as the Warburg effect, is an anomalous characteristic of glucose metabolism in cancer cells. Chronic inflammation is a key promoting factor of tumourigenesis. It remains, however, largely unexplored whether and how pro-tumourigenic inflammation regulates glucose metabolism in cancer cells. Here, we show that pro-inflammatory cytokines promote glycolysis in breast cancer cells, and that the inflammation-induced miR-155 functions as an important mediator in this process. We further show that miR-155 acts to upregulate *hexokinase 2* (*hk2*), through two distinct mechanisms. First, miR-155 promotes *hk2* transcription by activation of signal transducer and activator of transcription 3 (STAT3), a transcriptional activator for *hk2*. Second, via targeting *C/EBP*β (a transcriptional activator for *mir-143*), miR-155 represses *mir-143*, a negative regulator of *hk2*, thus resulting in upregulation of *hk2* expression at the post-transcriptional level. The miR-155-mediated *hk2* upregulation also appears to operate in other types of cancer cells examined. We suggest that the miR-155/miR-143/HK2 axis may represent a common mechanism linking inflammation to the altered metabolism in cancer cells.

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Introduction

More than a century ago, chronic inflammation was proposed as a key promoting factor of tumourigenesis. Later on, the link between inflammation and cancer is continuously supported by clinical and epidemiological studies. Recent studies have significantly advanced our understanding of the molecular mechanisms underlying the inflammation-associated tumourigenesis (Aggarwal *et al*, 2009; Grivennikov and Karin, 2010; Morgan and Liu, 2011). Signal transducer and activator of transcription 3 (STAT3) signalling is a major pathway that connects inflammation to cancer (Yu *et al*, 2009; He and Karin, 2011). Mounting evidence supports that STAT3 induces and maintains a pro-tumourigenic inflammatory microenvironment during tumour initiation as well as malignant progression (Yu *et al*, 2009; He and Karin, 2011). Pro-inflammatory cytokines such as interleukin-6 (IL-6) and some growth factors relevant to tumourigenesis are potent STAT3 activators, while a number of genes associated with cell survival, proliferation, and angiogenesis are downstream targets of STAT3 (Aggarwal *et al*, 2009; Yu *et al*, 2009).

Recent studies show that microRNAs (miRNAs) function as novel pro-inflammatory regulators (Schetter *et al*, 2010; O'Neill *et al*, 2011). miRNAs are small, non-coding RNAs that negatively regulate protein-coding genes (Wu and Belasco, 2008). The causal roles of miRNAs in cancer have been well documented and miRNA-based anticancer therapies are in development (Garzon *et al*, 2010). Several miRNAs with evident roles in cancer are regulated by inflammatory signals. For example, *mir-155* is ubiquitously upregulated upon various inflammation stimuli; JNK, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) pathways are shown to be responsible for inflammation-induced *mir-155* expression (O'Connell *et al*, 2007; Bolisetty *et al*, 2009; Xiao *et al*, 2009). Of note, miR-155 is also an important target of Toll-like receptors (TLR) signalling in innate immune cells (O'Neill *et al*, 2011). On one hand, miR-155 negatively regulates innate immune signalling by targeting key signalling proteins; on the other hand, increased expression of miR-155 often results in the inappropriate activation of inflammatory pathways (O'Neill *et al*, 2011). Importantly, *mir-155* is upregulated in many types of tumours and acts as an oncomiR, as it promotes malignant transformation and cancer progression by negatively regulating tumour-suppressive genes *TP53INP1*, *RhoA*, *socs1*, etc. (Gironella *et al*, 2007; Kong *et al*, 2008; Jiang *et al*, 2010). Recently, we show that

mir-155 is induced by multiple inflammation mediators in breast cancer cells and boosts the pro-tumourigenic inflammatory STAT3 signalling by targeting *socs1* (Jiang *et al*, 2010), a potent repressor of JAK/STAT signalling (Davey *et al*, 2006). This study, along with a report by Tili *et al* (2009), indicates that miR-155 is a bridge linking inflammation and cancer. Similarly, recent reports show that the oncogenic miR-21 is induced by the IL-6-STAT3 inflammatory pathway, and mediates tumour initiation and malignant progression via targeting tumour suppressors PDCD4, TPM1, PTEN, and BTG2 (Meng *et al*, 2007; Zhu *et al*, 2007; Lu *et al*, 2008; Liu *et al*, 2009; Iliopoulos *et al*, 2010). Additionally, inflammatory response may also promote tumourigenesis through downregulation of tumour-suppressive miRNAs. For instance, let-7, a well-documented antitumourigenic miRNA, is repressed by inflammation stimulation, which in turn induces an epigenetic switch that controls cell transformation (Iliopoulos *et al*, 2009). These results clearly indicate that miRNAs are important mediators linking inflammation and cancer.

In cancer cells, glucose is preferentially metabolized by aerobic glycolysis, which differs from mitochondrial oxidative phosphorylation in normal, non-tumourigenic cells. This phenomenon, termed as the Warburg effect, is characterized by increased glycolysis and lactate production regardless of oxygen availability (Warburg, 1956). Based on the aerobic glycolysis accompanied by increased glucose uptake, a method named as [^{18}F]Fluorodeoxyglucose Positron Emission Tomography (^{18}FDG PET) imaging has been used worldwide as a diagnostic tool to detect malignant tumours (Di Chiro *et al*, 1982). ^{18}FDG PET combined with computer tomography (PET/CT) has a >90% sensitivity and specificity for detecting metastases of most epithelial cancers (Mankoff *et al*, 2007). Hexokinases catalyse the first and irreversible step of glucose metabolism (ATP-dependent phosphorylation of glucose to yield glucose-6-phosphate) (Robey and Hay, 2006). Hexokinase 2 (HK2) is the major isozyme that is overexpressed in tumours and contributes to aerobic glycolysis, and thus it is documented as a pivotal player in the Warburg effect and is proposed as a metabolic target for cancer therapeutic development (Mathupala *et al*, 2009; Vander Heiden, 2011). In addition to being a striking feature of cancer cell metabolism, the Warburg effect confers advantages to cancer cells, providing conditions favouring rapid proliferation and apoptosis resistance (Kroemer and Pouyssegur, 2008; Vander Heiden *et al*, 2009). Indeed, the Warburg effect, that is, the reprogramming of cellular energy metabolism, is recently added as an emerging hallmark of cancer (Hanahan and Weinberg, 2011).

In this study, we found that pro-inflammatory cytokines promote glucose consumption and lactate production in breast cancer cells and that this process is mediated by miR-155, an miRNA ubiquitously induced by inflammation. We further showed that miR-155 promoted glycolysis in breast cancer cells and increased ^{18}FDG uptake in breast tumours through upregulation of *hk2*, a key glycolytic enzyme in cancer cells. miR-155 appears to upregulate the expression of *hk2* through two distinct mechanisms. First, miR-155 facilitates the activation of STAT3, which promotes the transcription of *hk2*. Second, miR-155 represses *mir-143* by targeting *C/EBP*β, a transcriptional activator for *mir-143*, and subsequently facilitates *hk2* expression at the post-transcriptional level. Both the repression of *mir-143* and activation of STAT3 are required for miR-155 to enhance glycolysis in breast cancer cells and promote ^{18}FDG uptake in xenograft tumours. Additionally, this dual-miRNA-mediated regulation of *hk2* is also observed in liver and lung cancer cells, suggesting a common mechanism linking inflammation to the altered metabolism in cancer cells.

Results

IL-6 and miR-155 promote glycolysis and upregulate HK2 in breast cancer cells

Given that pro-tumourigenic inflammatory conditions promote tumour initiation and malignant progression (Grivennikov and Karin, 2010), and that the Warburg effect/reprogramming of energy metabolism is critical to the survival and proliferation of cancer cells (Kroemer and Pouyssegur, 2008; Vander Heiden *et al*, 2009), we asked whether inflammation potentiates cancer cell energy metabolism. To this end, we first examined the effect of pro-inflammatory cytokine IL-6 on glucose metabolism in breast cancer cells. The results showed that IL-6 dramatically increased the rates of glucose consumption and lactate production in MDA-MB-231 cells (Figure 1A).

Given that miR-155 is pro-inflammatory regulator that links inflammation and cancer (Tili *et al*, 2009; Jiang *et al*, 2010), we next asked whether miR-155 may mediate the effect of inflammatory signalling on glucose metabolism in breast cancer cells. We found that the rates of glucose consumption and lactate production were strongly increased by *mir-155* overexpression and significantly decreased by *mir-155* knockdown (Figure 1B). Interestingly, knockdown of *mir-155* significantly attenuated the effect of IL-6 on glucose consumption and lactate production (Figure 1A). Additionally, we found that other pro-inflammatory cytokines, including TNFα, IL-1β, and IFN-γ, also enhanced glycolysis in breast cancer cells, while knockdown of *mir-155* in all cases, significantly impaired the cytokine-mediated stimulation of glycolysis (Supplementary Figures S1A–C). We also examined two other breast cancer cell lines MCF-7 and SK-BR-3, and found that IL-6 treatment also stimulated glucose consumption and lactate production in these cells, while miR-155 knockdown attenuated the stimulatory effect of IL-6 (Supplementary Figures S1D and E). Collectively, these results indicate that inflammation enhances glycolysis in breast cancer cells and that miR-155 acts as an important mediator in this process.

To probe the potential mechanism by which inflammation and miR-155 regulate glycolysis in breast cancer cells, we examined the effects of IL-6 and miR-155 on the expression of a number of key genes involved in glycolysis, including *glucose transporter 1* (*Glut1*), *hk2*, *phosphofructokinase 2* (*PFK2*), *phosphoglycerate mutase 1* (*PGM1*), *pyruvate kinase isoform M2* (*PKM2*), *pyruvate dehydrogenase kinase 1* (*PDK1*), and *lactate dehydrogenase isoform A* (*LDHA*). Q-PCR analyses showed that all these genes were upregulated by IL-6 or miR-155 and downregulated by anti-miR-155; among them, *HK2* mRNA level was increased the most by IL-6 or miR-155 (Figure 1C). In line with our above results (Figures 1A–C), western blot assays showed that IL-6 dramatically enhanced HK2 protein expression, and HK2 protein level was dramatically increased by miR-155 and reduced by anti-miR-155 (Figure 1D). We have also examined the

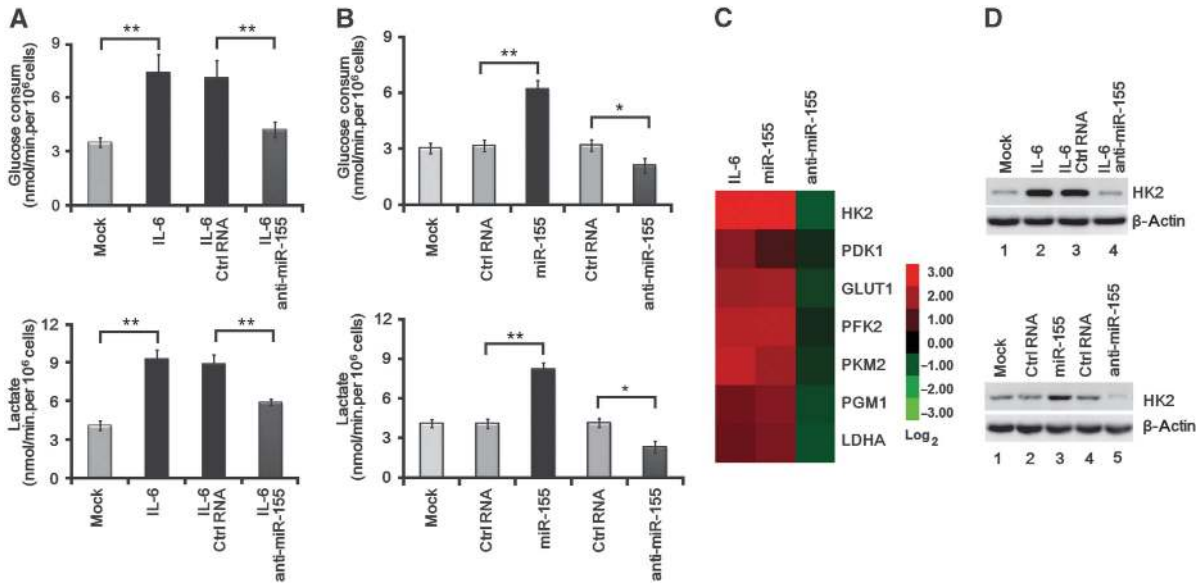


Figure 1 IL-6 and miR-155 enhance glycolysis in breast cancer MDA-MB-231 cells. (**A**, **B**) The effects of IL-6 (**A**) and miR-155 (**B**) on glycolysis. Top, the rates of glucose consumption; bottom, the rates of lactate production. (**A**) Cells were treated by IL-6, IL-6 + anti-miR-155, or IL-6 + control RNA (Ctrl RNA). (**B**) Cells were transfected with miR-155 mimics, anti-miR-155, or Ctrl RNA. (**C**) qRT–PCR analysis of the effect of IL-6 and miR-155 on the expression of genes related to glycolysis. Heatmap representation of differentially expressed mRNA levels 24 h after MDA-MB-231 cells were treated by IL-6, miR-155 mimics, or anti-miR-155. Upregulated mRNA expression is shown in red, whereas downregulated mRNA is shown in green. (**D**) Western blot analysis of HK2 protein expression by IL-6 treatment (top) or transfection with miR-155 (bottom). The average values ± s.d. of three separate experiments were plotted. $*P<0.05$, $**P<0.01$.

potential effect of miR-155 on several other glycolytic genes, and found that miR-155 expression enhanced the protein levels of additional glycolytic genes (PFK2 in particular; Supplementary Figure S1F), suggesting that miR-155 may control glycolysis at a broad level. Similarly, TNFα, IL-1β, and IFN-γ also upregulated HK2 protein levels, and their effects were virtually abrogated by knockdown of *mir-155* (Supplementary Figure S1). These results indicate that miR-155 plays a critical role in mediating inflammatory cytokine-stimulated upregulation of *hk2*. Given that HK2 is a critical enzyme catalysing the first and irreversible step of glycolysis (Robey and Hay, 2006), and that its expression is most dramatically regulated by inflammation or miR-155 (Figure 1C), we reasoned that *hk2* upregulation likely plays a major role in the enhancement of glucose consumption and lactate production under such conditions. Indeed, the stimulation of glycolysis by inflammatory cytokine treatment or miR-155 overexpression was dose dependently reduced by *hk2* knockdown (Supplementary Figures S1G and H). We thus focussed on the regulation of *hk2* for further mechanistic and functional studies.

STAT3 is a transcriptional activator of hk2

To dissect the molecular mechanism of the effects of inflammation and miR-155 on *hk2* expression, we used TransFac and Genomatix softwares (Wingender *et al*, 2001) to search for potential transcription factor binding sites in the human *hk2* promoter. Intriguingly, we found a putative binding site of STAT3 (Figure 2A), a transcription factor that is potently activated by IL-6 and miR-155 (Catlett-Falcone *et al*, 1999; Jiang *et al*, 2010). To explore whether STAT3 directly regulates *hk2*, we first performed chromatin immunoprecipitation (ChIP) assays in MDA-MB-231 cells, in which STAT3 is constitutively activated (Garcia *et al*, 2001). A genomic fragment containing the putative STAT3 binding was significantly enriched by antibodies against STAT3, compared with the IgG control (Figure 2B). Moreover, knockdown of *stat3* significantly reduced the *HK2* mRNA level in these cells (Figure 2C). Additionally, the mRNA levels of several other glycolytic genes were also affected by *stat3* siRNA treatment to different extent, but among these only PFK2 protein level appeared to be significantly reduced (Supplementary Figure S2). This is consistent with a recent report showing that IL-6-STAT3 signalling induces *PFK2* expression (Ando *et al*, 2010 named as *PFKFB3* in that paper). Next, we constructed luciferase reporters under control of either the wild-type human *hk2* promoter or a mutant version with a deletion of the 9-bp STAT3 binding site (termed as WT P_{hk2} reporter or the Mut P_{hk2} reporter, respectively) (Figure 2A, bottom). Either knockdown of *stat3* or addition of JSI-124, a pharmacological inhibitor of STAT3, in MDA-MB-231 cells significantly reduced the activity of the WT P_{hk2} (Figure 2D). The Mut P_{hk2} exhibited only ∼20% activity compared with the WT, but it was not affected by either *stat3* knockdown or addition of JSI-124. Collectively, these results support that STAT3 is an authentic and direct transcriptional activator for *hk2*.

As IL-6 is a potent activator of STAT3 (Catlett-Falcone *et al*, 1999), the finding that STAT3 directly activates *hk2* transcription (Figures 2A–D) is consistent with our earlier results that IL-6 treatment led to enhanced *hk2* expression in breast cancer cells (Figures 1C and D). Consistent with an important role of STAT3 in mediating the stimulation of *hk2* transcription by IL-6, we found that IL-6 induced an ∼8-fold increase of *HK2* mRNA level in MDA-MB-231 cells (Figure 2E) and led to enhanced STAT3 occupancy at the *hk2* promoter (Figure 2G), while either JSI-124 or *stat3* siRNA completely abolished the induction of *HK2* transcript levels (Figure 2E). JSI-124 and *stat3* siRNA also reduced the basal *HK2* mRNA

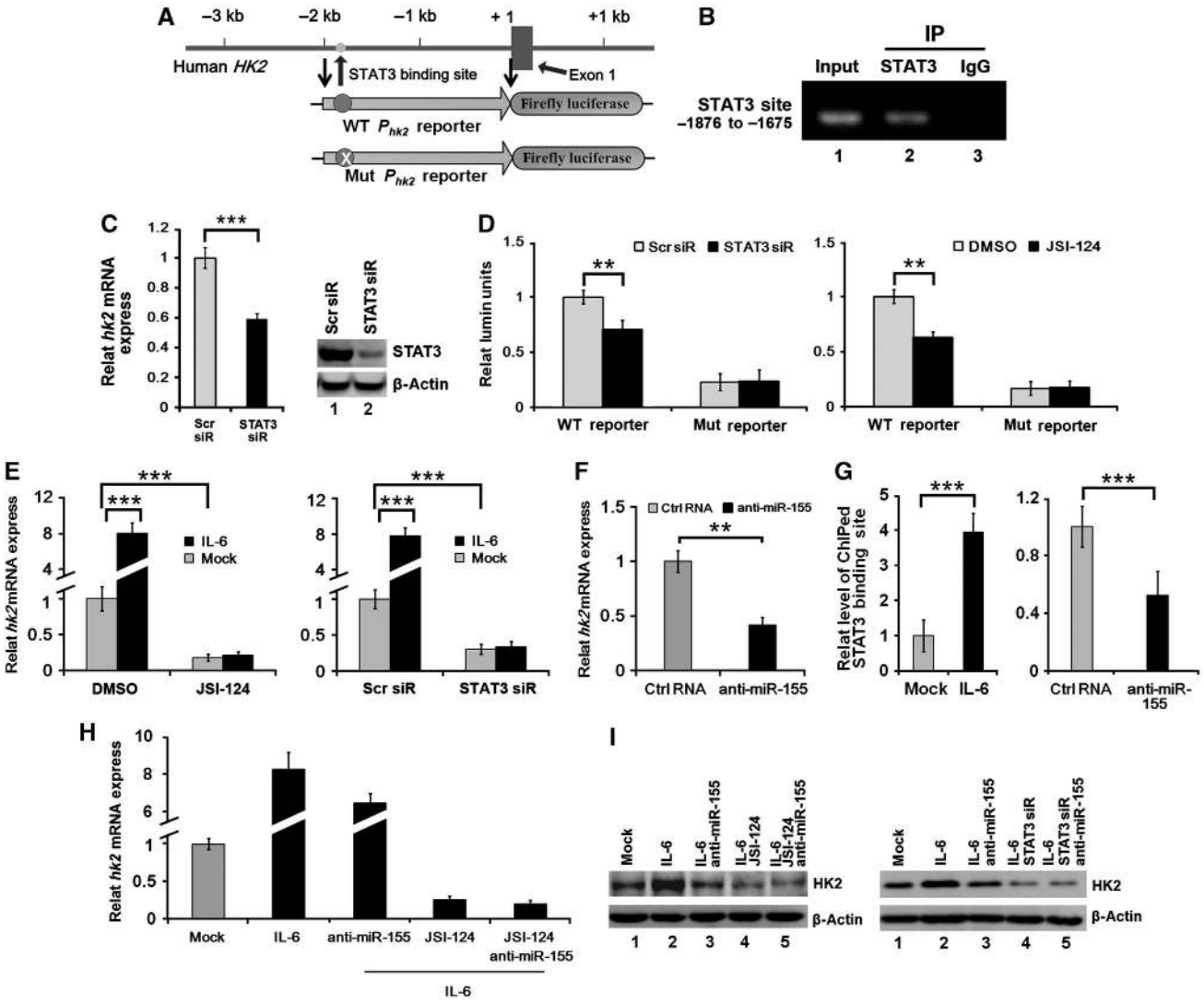


Figure 2 STAT3 is a transcription activator for *hk2* in MDA-MB-231 cells. (**A**) Schematic representation of predicted STAT3 binding site in human *hk2* promoter. The constructions of WT P_{hk2} reporter and Mut P_{hk2} reporter were shown at bottom. (**B**) ChIP analysis of the *hk2* promoter using antibodies against STAT3. (**C**) The effect of *stat3* knockdown on *hk2* expression. Left, qRT–PCR analysis of *HK2* mRNA; right, western blot of STAT3 protein. (**D**) The effect of *stat3* knockdown on the activity of the P_{hk2} reporter. (**E**) qRT–PCR analysis of *hk2* expression with IL-6 treatment. (**F**) qRT–PCR analysis of *hk2* expression with anti-miR-155 transfection. (**G**) ChIP assays of STAT3 occupancy on the *hk2* promoter in IL-6-treated (left) or anti-miR-155-transfected cells (right). The level of ChIPed STAT3 binding site in mock and Ctrl RNA-treated cells was set to 1.0, and that in others was normalized relative to the respective control. (**H**) Modulation of *HK2* mRNA levels by IL-6, JSI-124, and miR-155 inhibition. (**I**) Modulation of HK2 protein levels by IL-6, JSI-124, *stat3* siRNA, and miR-155 inhibition. The average values ± s.d. of three separate experiments were plotted. $^{**}P<0.01$, $^{***}P<0.001$.

levels (Figure 2E), which is in agreement with that STAT3 is constitutively activated in these cells (Garcia *et al*, 2001). As we have previously shown that miR-155 contributes to STAT3 activation in breast cancer cells via suppression of *socs1* (Jiang *et al*, 2010), we tested whether this regulation also extends to the *hk2* gene. Indeed, we found that knockdown of *mir-155* decreased *HK2* mRNA levels in MDA-MB-231 cells (Figure 2F). Moreover, ChIP assays showed that STAT3 occupancy at the *hk2* promoter was significantly reduced by anti-miR-155 (Figure 2G). Collectively, these results reveal that IL-6 and miR-155 promote *hk2* transcription through activation of STAT3.

Although miR-155 plays a role in augmenting STAT3 activation via suppression of *socs1*, it nevertheless is not absolutely required for STAT3 activation by IL-6 (Jiang *et al*, 2010). Indeed, in the presence of anti-miR-155, IL-6 still induced an ~6-fold increase of *HK2* mRNA levels (Figure 2H). Intriguingly, western blot analyses showed that anti-miR-155 treatment strongly reduced HK2 protein levels in IL-6-treated cells (Figure 2I), to a level comparable to that in mock-treated cells, despite that *HK2* mRNA level was significantly induced by IL-6 (~6-fold; Figure 2H). This result strongly suggests that miR-155 employs an additional mechanism to positively regulate HK2 protein expression, likely at the post-transcriptional level.

hk2 is a target of miR-143

As miR-155 appears to positively regulate HK2 protein expression at the post-transcriptional level, it is unlikely that *hk2* constitutes a direct target of miR-155 given that miRNAs are

generally known to repress the expression of their direct targets. Nevertheless, we considered the possibility that the post-transcriptional regulation of HK2 protein expression might be mediated by other miRNA(s). To explore this, we searched the human *hk2* 3′UTR using miRNA target prediction tools (Krek *et al*, 2005; Lewis *et al*, 2005), and found a putative binding site for miR-143, an miRNA with a well-documented role in tumour suppression (Chen *et al*, 2009; Kent *et al*, 2010; Osaki *et al*, 2011; Figure 3A). To test whether miR-143 indeed regulates *hk2*, we first used luciferase reporter assays. The wild-type *hk2* 3′UTR or a mutant version with deletion of the 7-bp sequence complementary to the 5′ part of miR-143 (*hk2* 3′UTR Mut) was cloned downstream of the *Renilla* luciferase gene (Figure 3A, right), and the reporter construct was transfected into 293T cells along with miR-143 mimics. As expected, co-transfection of miR-143 dramatically reduced the wild-type reporter activity, whereas the mutant reporter was not affected (Figure 3B). Interestingly, systematic screening of a number of breast cancer cell lines revealed that *mir-143* expression was inversely correlated with HK2 protein expression (Figure 3C): the *hk2* expression was the highest and *mir-143* lowest in MDA-MB-231 cells, while the opposite was observed in ZR-75-30 cells. In addition, *mir-143* overexpression significantly reduced both the protein and mRNA levels of *hk2* in MDA-MB-231 cells (Figure 3D), whereas *mir-143* knockdown in ZR-75-30 cells led to enhanced *hk2* expression (Figure 3E). Collectively, these results indicate that *hk2* is a direct target of miR-143 in breast cancer cells.

miR-155 represses mir-143 by targeting C/EBPβ and upregulates hk2 at the post-transcriptional level

Our finding that miR-143 directly suppresses *hk2* expression raised an intriguing possibility that miR-155 might mediate its regulatory effect on *hk2* via miR-143. In support of this notion, we found that *mir-143* expression was inversely correlated with *mir-155* expression in breast cancer cell lines (Supplementary Figure S3A). To directly test whether miR-155 regulates miR-143 expression and does so at the transcriptional level, we overexpressed miR-155 in ZR-75-30 cells, which harbour low endogenous levels of miR-155 (Supplementary Figure S3A), and found that introduction of exogenous miR-155 reduced *pri-mir-143* expression by ~60% (Figure 4A, left). We also performed knockdown of *mir-155* in MDA-MB-231 cells, which have high endogenous *mir-155* expression (Supplementary Figure S3A), and found that *mir-155* knockdown significantly elevated miR-143 expression in these cells (by ~4-fold) (Supplementary Figure S3B), further supporting that miR-155 represses *mir-143* expression. We next constructed a luciferase reporter controlled by the ~2.6-kb human *mir-143* promoter $P_{mir\text{-}143}$ (Figure 4B, bottom). Reporter assays showed that the $P_{mir\text{-}143}$ activity was strongly inhibited by co-transfection of miR-155 (Figure 4A, right), indicating that the promoter activity of *mir-143* is indeed suppressed by miR-155.

We next asked how miR-155 regulates the promoter activity of *mir-143*. Using both the TransFac and Genomatix programs (Wingender *et al*, 2001), we searched for potential transcription factor binding sites in the $P_{mir\text{-}143}$ promoter. Interestingly, two known miR-155 targets, C/EBPβ (Costinean *et al*, 2009; He *et al*, 2009) and Ets-1 (Romania *et al*, 2008), stood out as the candidate transcription factors (Figure 4B). We thus performed ChIP assays using anti-C/EBPβ, anti-Ets-1, or rabbit IgG antibodies in ZR-75-30 cells, which exhibit high endogenous levels of C/EBPβ and miR-143 (Figure 3C and data not shown), and found that the promoter fragment containing the C/EBPβ and Ets-1 sites was enriched

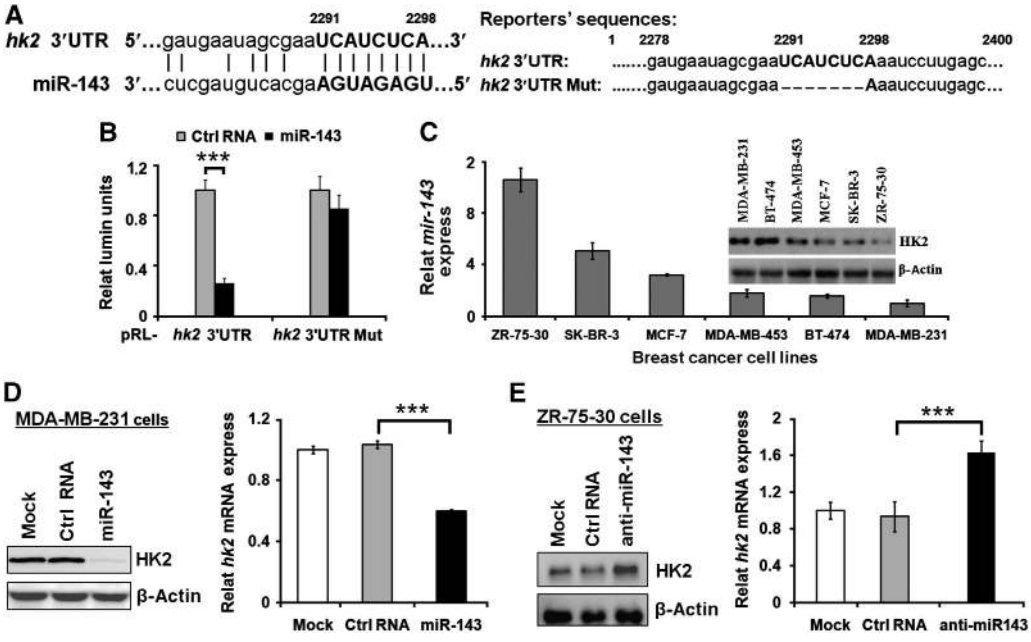


Figure 3 *hk2* is a target of miR-143. (**A**) *hk2* is predicted to be a target of miR-143. The seed sequence of miR-143 is shown in upper case. The sequences of wild-type (pRL-*hk2* 3′UTR) and mutated *hk2* 3′UTR-*Renilla* luciferase reporters (pRL-*hk2* 3′UTR Mut) are shown on right. (**B**) *hk2* 3′UTR luciferase reporter assays in 293T cells. (**C**) Expression of *mir-143* and HK2 protein in breast cancer cell lines. Upper right, western blot of HK2 protein; bottom, qRT–PCR analysis of miR-143. (**D**) *hk2* expression was downregulated by miR-143 in MDA-MB-231 cells. (**E**) *hk2* expression was upregulated by anti-miR-143 in ZR-75-30 cells. The respective treatment was indicated in the figures. The average values ± s.d. of three separate experiments were plotted. $^{***}P<0.001$.

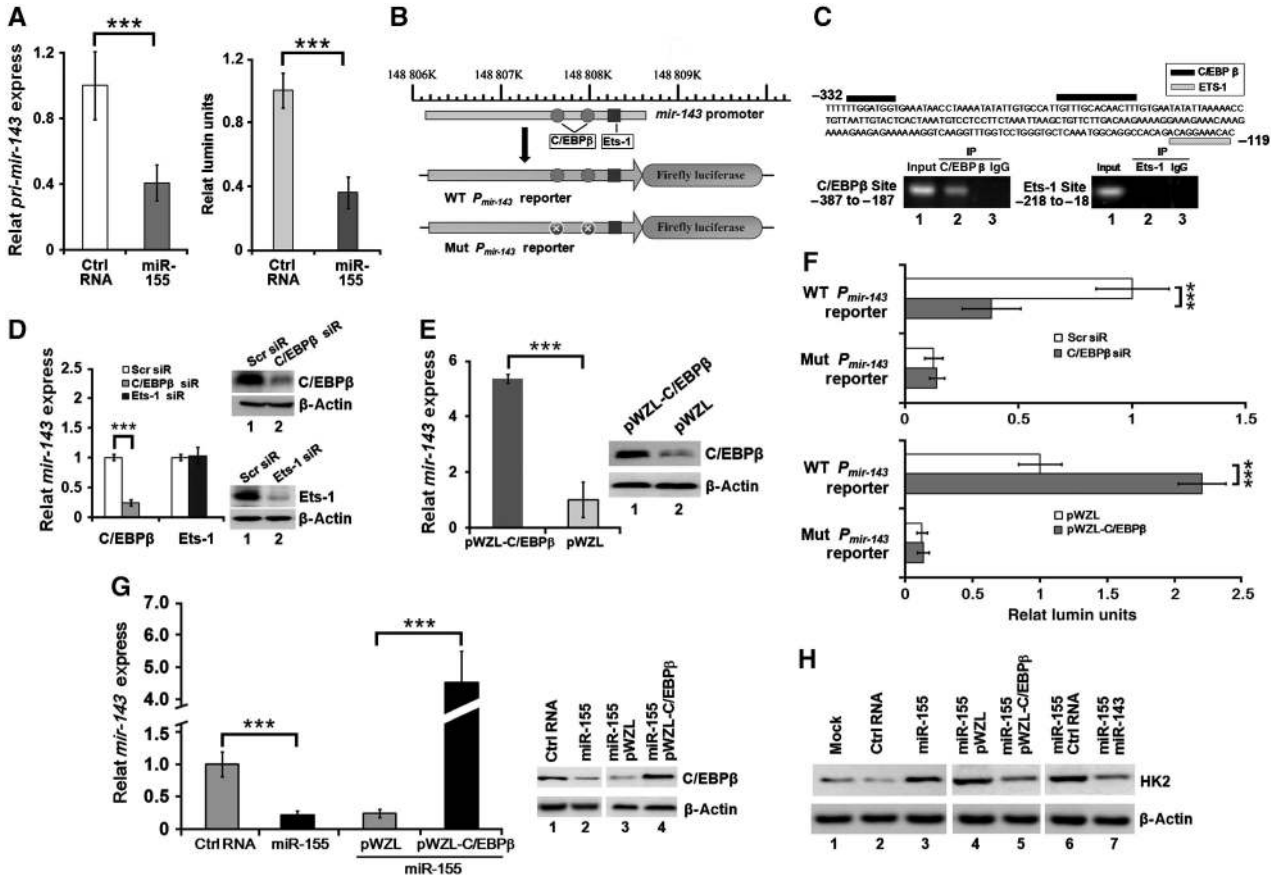


Figure 4 miR-155 downregulates *mir-143* via targeting *C/EBP*β in ZR-75-30 cells. (**A**) The effect of miR-155 on the expression of the primary *mir-143* transcript (left) and the activity of the $P_{mir\text{-}143}$ reporter (right). (**B**) Schematic representation of predicted C/EBPβ and Ets-1 binding sites in the human *mir-143* promoter. The constructions of WT $P_{mir\text{-}143}$ reporter and Mut $P_{mir\text{-}143}$ reporter were shown at bottom. (**C**) ChIP analysis of the *mir-143* promoter using antibodies against C/EBPβ or Ets-1. (**D**) Regulation of miR-143 by knockdown of *C/EBP*β or *Ets-1*. (**E**) Regulation of miR-143 by ectopic overexpression of C/EBPβ. (**F**) Modulation of the $P_{mir\text{-}143}$ promoter activity by *C/EBP*β knockdown or overexpression. (**G**) Ectopic overexpression of *C/EBP*β reversed the inhibition of *mir-143* expression by miR-155. (**H**) Overexpression of *C/EBP*β or *mir-143* attenuated the induction of HK2 expression by miR-155. The average values ± s.d. of three separate experiments were plotted. $^{***}P<0.001$.

by anti-C/EBPβ, but not by anti-Ets-1 (Figure 4C). Consistent with this, knockdown of *C/EBP*β strongly reduced *mir-143* expression in these cells, whereas knockdown of *Ets-1* only had a marginal effect (Figure 4D). Furthermore, overexpression of *C/EBP*β led to an ~5-fold increase of miR-143 level (Figure 4E). Finally, we generated a Mut $P_{mir\text{-}143}$ reporter by mutating the two putative C/EBPβ binding sites (Figure 4B, bottom). This mutant promoter retained ~10% of the activity compared with the wild type (Figure 4F). As expected, the activity of wild-type $P_{mir\text{-}143}$ was reduced by *C/EBP*β knockdown and increased by *C/EBP*β overexpression, while that of the Mut $P_{mir\text{-}143}$ was only marginally affected (Figure 4F). Collectively, these data strongly suggest that C/EBPβ is a direct transcriptional activator for *mir-143*.

We next examined whether miR-155 regulates miR-143 expression through targeting *C/EBP*β. Transfection of miR-155 into ZR-75-30 cells (which show low endogenous levels of miR-155 expression; Supplementary Figure S3A) indeed significantly decreased miR-143 expression (Figure 4G, left), while at the same time also reduced C/EBPβ protein levels (Figure 4G, right), in agreement with previous reports that *C/EBP*β is a direct target of miR-155 (Costinean *et al*, 2009; He *et al*, 2009). Interestingly, the inhibition of miR-143 expression by miR-155 was completely rescued when an miR-155-resistant form of C/EBPβ was co-expressed in these cells (Figure 4G). Moreover, the activity of the Mut $P_{mir\text{-}143}$ with no C/EBPβ binding sites was not regulated by miR-155 (Supplementary Figure S3C). These results together strongly support that miR-155 represses *mir-143* expression via targeting *C/EBP*β.

To directly test the regulatory axis comprising all four players (miR-155, *C/EBP*β, miR-143, and *hk2*), we examined the regulation of *hk2* in ZR-75-30 cells, which harbour low endogenous levels of miR-155 (Supplementary Figure S3A). Introduction of exogenous miR-155 significantly upregulated HK2 protein expression in these cells (Figure 4H, lane 3), while overexpression of either *C/EBP*β (lane 5) or *mir-143* (lane 7) dramatically suppressed the miR-155-mediated upregulation of HK2 protein expression. It should be noted that we have also shown in earlier parts that knockdown of *mir-155* in MDA-MB-231 cells, which harbour high endogenous levels of miR-155 (Supplementary Figure S3A), led to both elevation of miR-143 expression (Supplementary Figure S3B) and suppression of HK2 protein levels (Figure 1D). Taken together, these results indicate that miR-155, through targeting *C/EBP*β, downregulates *mir-143* expression, and the

downregulation of miR-143, in turn, promotes *hk2* expression at the post-transcriptional level.

Functional role of the miR-143:hk2 axis in regulating glucose metabolism and controlling tumourigenesis in breast cancer cells

Given the importance of HK2 in aerobic glycolysis in cancer cells, it seems likely that the newly discovered miR-143:*hk2* axis would serve an important function in regulating glucose metabolism in breast cancer cells. To explore this, we overexpressed miR-143 in MDA-MB-231 cells, in which its endogenous expression level is low (Figure 3C), and found that miR-143 overexpression significantly reduced the rates of glucose consumption and lactate production (Figure 5A). Correspondingly, a MicroPET assay showed that miR-143-overexpressing xenograft tumours exhibited a significantly lower level of ^{18}FDG uptake (as normalized to tumour mass) than the control tumours (Figure 5B). We also performed the reciprocal experiment by knocking down *mir-143* in ZR-75-30 cells, in which its endogenous expression level is high

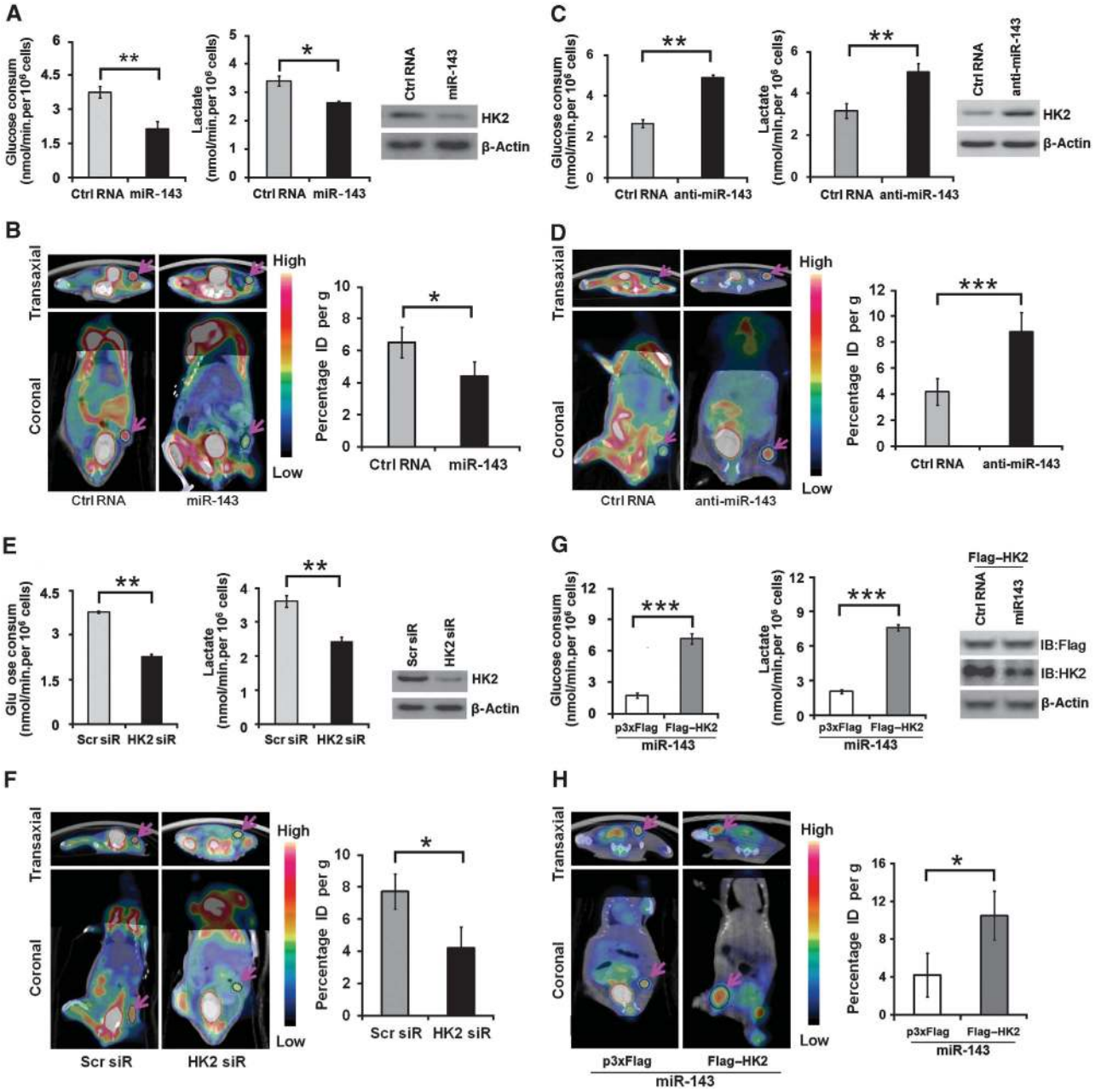


Figure 5 The miR-143-*hk2* axis regulates glycolysis in breast cancer cells. (**A**, **C**, **E**, and **G**) Glucose metabolism in cells with *mir-143* overexpression (**A**), *mir-143* knockdown (**C**), *hk2* knockdown (**E**), and ectopic overexpression of Flag–HK2 (**G**). Left, the rates of glucose consumption; middle, the rates of lactate production; right, western blot of HK2 protein. (**A**) MDA-MB-231 cells were transfected with Ctrl RNA or miR-143 mimics. (**C**) ZR-75-30 cells were transfected with Ctrl RNA or anti-miR-143 mimics. (**E**) MDA-MB-231 cells were transfected with Scr siR or *HK2* siR. (**G**) MDA-MB-231 cells were co-transfected with miR-143 mimics and Flag–HK2 or control vectors. (**B**, **D**, **F**, and **H**) ^{18}FDG uptake in xenograft tumours with *mir-143* overexpression (**B**), *mir-143* knockdown (**D**), *hk2* knockdown (**F**), and ectopic overexpression of Flag–HK2 (**H**). Left, a representative microPET/CT image; right, quantification of ^{18}FDG uptake in tumours (in percentage ID/g tumour) (mean ± s.d., $n = 4$–5 per group). The average values ± s.d. of three separate experiments were plotted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

(Figure 3C). *mir-143* knockdown dramatically promoted glycolysis in cultured ZR-75-30 cells and enhanced ^{18}FDG uptake in xenograft tumours (Figures 5C and D). Western blot analyses showed that HK2 expression was altered by modulation of *mir-143* (Figures 5A and C, right), which was further confirmed by immunohistochemical examination of xenograft tumour sections (Supplementary Figures S4A and B). These results support that miR-143 targets *hk2* and negatively regulates glycolysis in breast cancer cells.

To further corroborate that miR-143 exerts its effects on glycolysis by targeting *hk2*, we found that knockdown of *hk2* dramatically reduced glucose consumption and lactate production in MDA-MB-231 cells (which have low endogenous miR-143 and high endogenous HK2; Figure 3C) and significantly decreased ^{18}FDG uptake in xenograft tumours (Figures 5E and F), indicating that RNAi-mediated silencing of *hk2* phenocopies the effect of miR-143 on glycolysis (compare with Figures 5A and B). Additionally, we constructed an *hk2* expression vector (p3 × Flag–HK2), which lacks the *hk2* 3′UTR, for ectopic expression of Flag–HK2. Restoration of HK2 protein expression in MDA-MB-231 cells dramatically rescued the effect of miR-143 on glucose consumption and lactate production in cultured cells as well as ^{18}FDG uptake in xenograft tumours (Figures 5G and H), indicating that reduction of *hk2* expression is critical to the inhibitory effect of miR-143 on glycolysis. Western blot analyses confirmed that *hk2* RNAi significantly reduced HK2 protein expression while p3 × Flag–HK2 successfully rescued HK2 protein levels in these cells (Figures 5E and G, right). Immunohistochemical assays verified that HK2 protein levels were reduced in *hk2* siRNA tumours and restored in p3 × Flag–HK2 tumours (Supplementary Figures S4C and D).

We noted that the regulation of *hk2* and *mir-143* expression in xenograft breast tumours also affected tumour volume in addition to ^{18}FDG uptake in tumours (Figures 5B, D, F, and H). We then further explored the miR-143:*hk2* axis in controlling tumourigenesis in breast cancer cells. We first examined the effect of miR-143 on breast cancer cell proliferation and survival. We found that *mir-143* introduction in MDA-MB-231 cells, in which endogenous miR-143 level is low (Figure 3C), severely reduced cell proliferation, anchorage-independent growth, cell survival, as well as the rate of xenograft tumour growth in nude mice (Figure 6A; Supplementary Figure S5A). These results validate that miR-143 has anti-proliferative and pro-apoptotic effects in breast cancer cells. In addition, we examined the effects of miR-143 on breast cancer cell migration and metastasis *in vitro* and *in vivo*. Transfection of miR-143 mimics in MDA-MB-231 cells significantly reduced cell migration in a wound healing assay and transwell migration assay (Figure 6B, left; Supplementary Figure S5A). To determine the function of miR-143 on metastasis *in vivo*, we transfected miR-143 mimics into firefly luciferase-labelled MDA-MB-231 cells and performed tail vein xenografts. We observed strong luciferase foci in the lung fields of mice injected with control RNA transfected cells but a dramatic reduction in the luciferase signal in cells transfected with miR-143 mimics (Figure 6B, right), suggesting that miR-143 inhibits the lung metastasis of breast cancer cells. In a reciprocal experiment, downregulation of *mir-143* in ZR-75-30 cells, in which endogenous miR-143 level is high (Figure 3C), led to a significant increase of cell proliferation, anchorage-independent growth, cell survival, xenograft tumour growth, as well as cell migration in wound healing and transwell migration (Supplementary Figure S6). Western blot analyses showed that HK2 protein and the cell proliferation marker proliferating cell nuclear antigen (PCNA) in breast cancer cells were downregulated by miR-143 mimics and upregulated by anti-miR-143 (Figure 6A; Supplementary Figure S5A). These results together indicate that miR-143 inhibits breast cancer cell proliferation and migration.

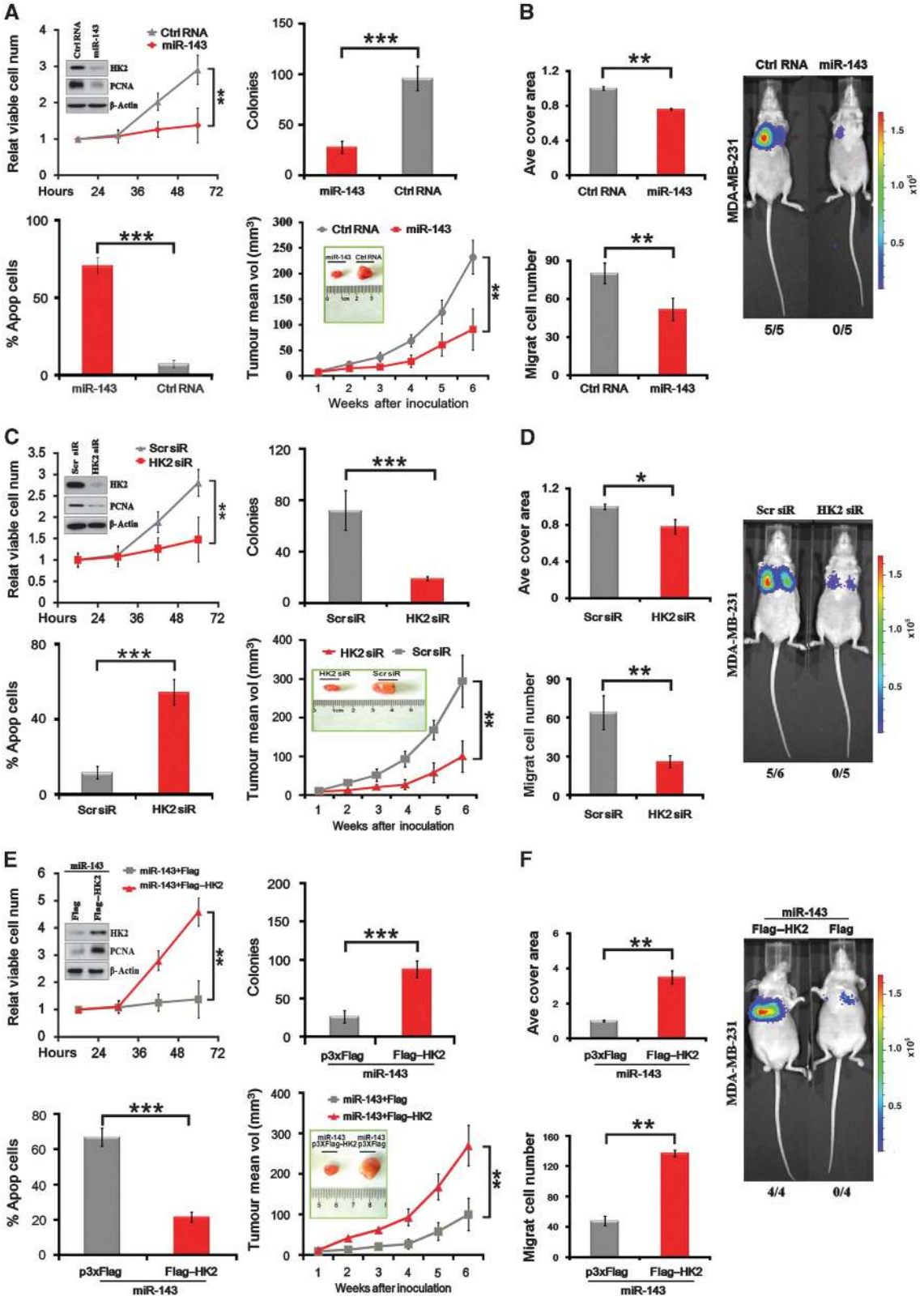
Next, we examined whether miR-143 exerts its antitumour effects by targeting *hk2*. We found that knockdown of *hk2* in MDA-MB-231 cells significantly reduced cell proliferation, anchorage-independent growth, cell survival, and xenograft tumour growth (Figure 6C; Supplementary Figure S5B). *hk2* knockdown also inhibited MDA-MB-231 cell migration in wound healing, transwell migration, and tail vein xenograft assays (Figure 6D; Supplementary Figure S5B). These results indicate that *hk2* is oncogenic in breast cancer cells and that RNAi-mediated silencing of *hk2* phenocopies the effect of miR-143 on cell proliferation and migration. Importantly, ectopic expression of HK2 protein in miR-143-overexpressing MDA-MB-231 cells overrode the antitumour effects of miR-143 (Figures 6E and F; Supplementary Figure S5C), suggesting that targeting *hk2* represents an important mechanism of the antitumour activity of miR-143. Collectively, these results indicate that the miR-143:*hk2* axis also plays an important role in regulating cell growth and migration of breast cancer cells.

Functional importance of the miR-155/miR-143 cascade in regulating glycolysis in breast cancer cells

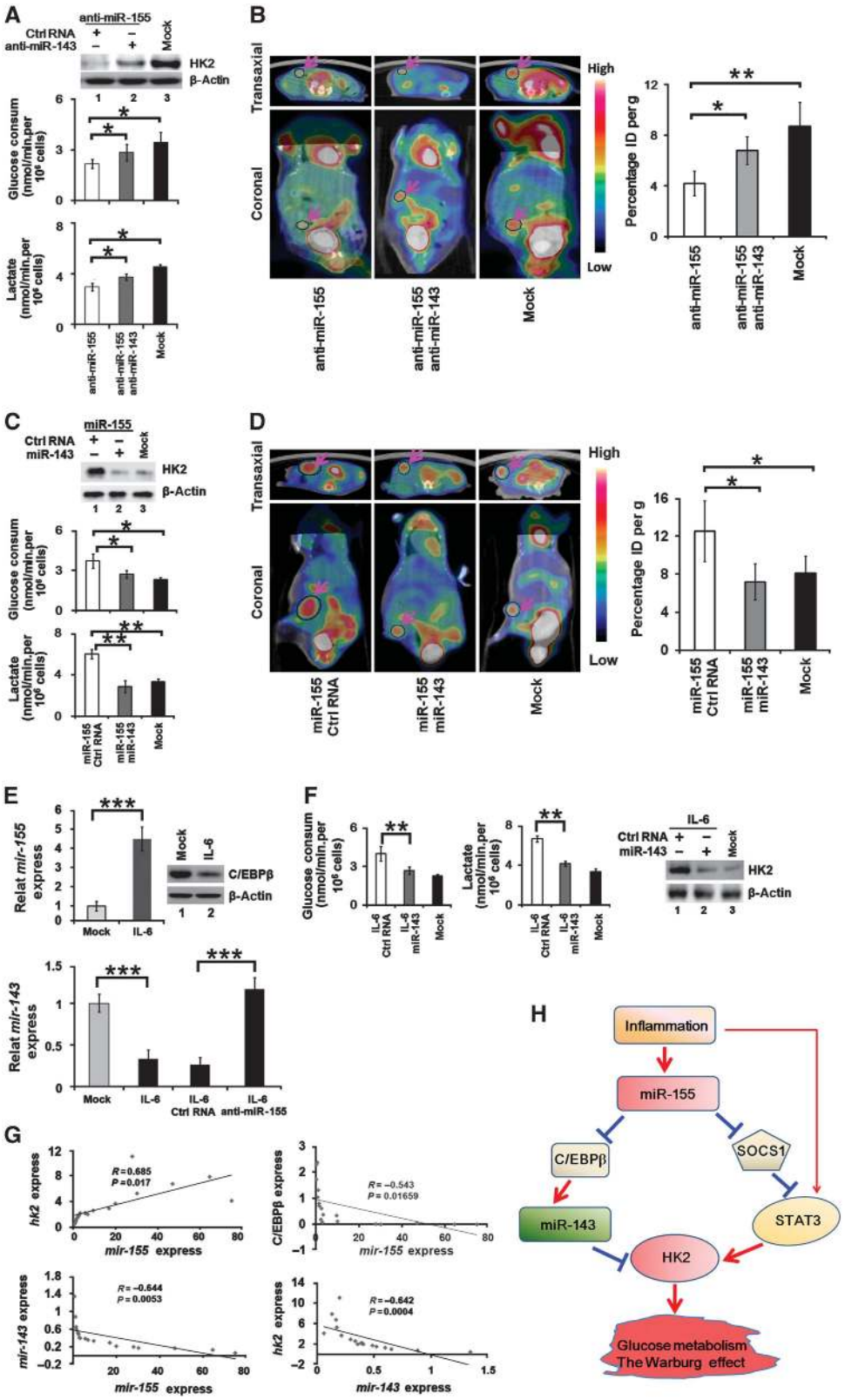
Our above results identify two pathways by which miR-155 acts to upregulate *hk2* and subsequently enhance glycolysis: one through the *C/EBP*β-miR-143 axis and the other through SOCS1-STAT3. To probe the functional importance of the first route, we found that transfection of anti-miR-155 into MDA-MB-231 cells, in which endogenous *mir-155* is highly

Figure 6 The miR-143-*hk2* axis regulates proliferation and migration of MDA-MB-231 cells. (**A**) miR-143 inhibited the proliferation and survival of MDA-MB-231 cells. Cells were transfected with Ctrl RNA or miR-143 mimics, and the assays were performed 24 h post transfection except where indicated otherwise. Upper left: MTT assays; upper right, soft agar colony formation assays; lower left, cell apoptosis assays; lower right, the time course of orthotopic xenograft tumour growth with representative tumours 6 weeks after inoculation (inset photo) (mean ± s.d., $n = 5$ per group). (**B**) miR-143 inhibited the migration and lung colonization of MDA-MB-231 cells. Cell transfections were similar to (**A**). Upper left: wound healing assays; lower left, transwell migration assays; right, representative images of tail vein xenograft assay ($n = 5$). (**C**) *hk2* knockdown inhibited the proliferation and survival of MDA-MB-231 cells. Procedures are similar to (**A**) but with knockdown of *hk2*. (**D**) *hk2* knockdown inhibited the migration and lung colonization of MDA-MB-231 cells. Procedures are similar to (**B**) but with knockdown of *hk2*. (**E**) Ectopic expression of HK2 protein overrode the antitumourigenesis effects of miR-143 in MDA-MB-231 cells. Procedures are similar to (**A**) but with ectopic overexpression of Flag–HK2 in the absence or presence of miR-143 overexpression. (**F**) Ectopic expression of HK2 protein overrode the antimigration effects of miR-143 in MDA-MB-231 cells. Procedures are similar to (**B**) but with ectopic overexpression of Flag–HK2 in the absence or presence of miR-143 overexpression. Values represent the means ± s.d. of three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expressed (Supplementary Figure S3A), dramatically reduced HK2 protein expression (Figure 7A); and such downregulation of HK2 was partially reversed when anti-miR-143 was introduced (Figure 7A). Moreover, miR-155 inhibition in these cells also reduced the rates of glucose consumption and lactate production in cultured cells and ^{18}FDG uptake in xenograft tumours, while anti-miR-143 significantly reversed these effects as well (Figures 7A and B). Consistently,



introduction of miR-155 mimics into ZR-75-30 cells, in which endogenous *mir-155* expression is low (Supplementary Figure S3A), dramatically increased HK2 protein expression, the rates of glucose consumption and lactate production in cultured cells, and ^{18}FDG uptake in xenograft tumours, while all these effects were attenuated with the introduction of miR-143 (Supplementary Figures 7C and D). Collectively, these results support that the miR-155/miR-143 regulatory cascade indeed plays an important role in regulating *hk2* expression and glycolysis in breast cancer cells.



To probe the functional role of the second route, we used JSI-124 to inhibit STAT3 activity in miR-155-overexpressing ZR-75-30 cells and tumours. This STAT3 inhibitor completely suppressed the abilities of miR-155 to upregulate *hk2* expression, enhance glycolysis in cultured cells, and promote ^{18}FDG uptake in xenograft tumours (Supplementary Figures S7A and B). These results indicate that STAT3 activity is also essential for miR-155 to enhance glycolysis in breast cancer cells. This perhaps comes as no surprise given the essential role of STAT3 in *hk2* transcription (Figure 2).

As miR-155 plays an important role in linking IL-6 signalling to promotion of glycolysis (Figure 1A), we further asked whether the newly elucidated miR-155/miR-143 regulatory cascade indeed mediates the effect of IL-6 on cancer cell metabolism. To this end, we used ZR-75-30 cells, which have high levels of endogenous miR-143 and low levels of endogenous miR-155 (Supplementary Figure S3A). IL-6 treatment strongly induced *mir-155* expression (by about five-fold) (Figure 7E, top left). Intriguingly, the NF-κB inhibitor BAY-117082 completely blocked IL-6-induced *mir-155* expression (Supplementary Figure S7C), consistent with the previous finding that the NF-κB pathway is involved in inflammation-induced *mir-155* expression (Xiao *et al*, 2009; O'Neill *et al*, 2011). At the same time, IL-6 also reduced C/EBPβ protein level (Figure 7E, top right) and *mir-143* expression (by ∼70%) in these cells (Figure 7E, bottom), accompanied by a significant elevation of glucose consumption, lactate production, and HK2 protein expression (Figure 7F). By contrast, treatment with anti-miR-155 completely abolished the effects of IL-6 on *mir-143* expression (Figure 7E). Importantly, we also found that introduction of exogenous *mir-143* in ZR-75-30 cells severely suppressed the stimulatory effect of IL-6 on HK2 protein expression, and glucose consumption and lactate production (Figure 7F). Together, these results suggest that the inflammatory cytokine IL-6 regulates glucose metabolism through the miR-155/miR-143 microRNA cascade.

Correlation of mir-155, mir-143, and hk2 expression in breast cancer patients

To test the clinical relevance of the above findings, we examined the concentration of IL-6 in the sera of breast cancer patients and assessed STAT3 phosphorylation status in primary breast tumours by ELISA. We found that IL-6 levels from patient sera were dramatically elevated compared with healthy controls (Supplementary Figure S7D), consistent with a previous report (Salgado *et al*, 2003). Phospho-STAT3 levels were significantly increased in breast tumours relative to normal tissues ($n=18$) (Supplementary Figure S7E), in agreement with previous findings showing that STAT3 is constitutively activated in human breast cancer cell lines and breast tumours (Clevenger, 2004; Sato *et al*, 2011). Also, in the same set of breast tumours, we examined the levels of miR-155, miR-143, and *HK2* and *C/EBP*β mRNAs by qRT–PCR. Interestingly, we observed a positive correlation between *HK2* mRNA and miR-155 levels (Figure 7G, upper left; $R=0.685$, $P=0.017$), a negative correlation between *C/EBP*β mRNA and miR-155 levels (Figure 7G, upper right; $R=-0.543$, $P=0.017$), a significant inverse correlation between miR-155 and miR-143 levels (lower left; $R=-0.644$, $P=0.005$), and a significant inverse correlation between *HK2* mRNA and miR-143 levels (lower right; $R=-0.642$, $P=0.0004$). These results support that the regulatory axis of miR-155/miR-143/HK2 uncovered in our study is of clinical relevance in human breast cancer.

We also asked whether the regulation of *hk2* by the miR-155/miR-143 cascade operates in other cancer types. We transfected miR-155 into human liver and lung cancer cell lines, Huh-7 and NCI-H460, respectively, and examined the HK2 protein expression in these cells. miR-155 increased HK2 protein expression in both cell lines, which was reversed by the addition of miR-143 (Supplementary Figure S7F). This result suggests that regulation of *hk2* by the miR-155/miR-143 cascade may represent a common mechanism linking inflammation to glucose metabolism in cancer cells.

Discussion

The importance of inflammation in tumour initiation and malignant progression is well documented (Aggarwal *et al*, 2009; Grivennikov and Karin, 2010; Schetter *et al*, 2010), so is the Warburg effect on survival and proliferation of cancer cells in the tumour microenvironment (Kroemer and Pouyssegur, 2008; Vander Heiden *et al*, 2009). However, whether and how inflammation is attributed to the cancer cell metabolism remains largely unexplored. In this study, we found that pro-inflammatory cytokines, including IL-6, TNFα, IL-1β, and IFN-γ, significantly accelerates glycolysis in breast cancer cells (Figure 1; Supplementary Figure S1), providing direct evidence that inflammation potentiates glucose metabolism in cancer. We demonstrated that knockdown of *mir-155* fully attenuated the effect of inflammatory cytokines on glycolysis in breast cancer cells. As *mir-155* is ubiquitously induced by inflammation (O'Connell *et al*, 2007; Bolisetty *et al*, 2009; Tili *et al*, 2009; Xiao *et al*, 2009; Jiang *et al*, 2010; O'Neill *et al*, 2011), these data point miR-155 as a vital mediator to link inflammation and glucose metabolism in cancer cells.

Figure 7 The miR-155/miR-143 cascade regulates glycolysis in breast cancer cells *in vitro* and *in vivo*. (**A**, **B**) Anti-miR-143 attenuated the effect of anti-miR-155 on glycolysis in MDA-MB-231 cells (**A**) and ^{18}FDG uptake in MDA-MB-231 tumours (**B**). (**A**) Cells were transfected with anti-miR-155 (lane 1) or co-transfected with anti-miR-155 and anti-miR-143 (lane 2). Top, western blot of HK2 protein; middle, the rate of glucose consumption; bottom, the rate of lactate production. (**B**) Cell transfections were same as that in (**A**). Left, a representative microPET/CT image; right, quantification of ^{18}FDG uptake in tumours (in percentage ID/g tumour) (mean ± s.d., $n=4$–5 per group). (**C**, **D**) *mir-143* expression abolished the effect of miR-155 on glycolysis in ZR-75-30 cells (**C**) and ^{18}FDG uptake in ZR-75-30 tumours (**D**). Cells were transfected with miR-155 or co-transfected with miR-155 and miR-143. Procedures are similar to that in (**A**, **B**). (**E**) Knockdown of *mir-155* abolished the effect of IL-6 on *mir-143* expression in ZR-75-30 cells. Top left, qRT–PCR analysis of *mir-155* expression; top right, western blot of C/EBPβ protein; bottom, qRT–PCR analysis of *mir-143* expression. (**F**) *mir-143* expression abolished the effect of IL-6 on glucose consumption (left), lactate production (middle), and HK2 protein expression (right) in ZR-75-30 cells. (**G**) miR-155, miR-143, and *HK2* mRNA levels in breast cancer patients. Correlation coefficients (*R*) and *P*-values were indicated. (**H**) Model of the dual-switch mechanism through which miR-155 conveys the inflammatory signals to the Warburg effect. Values represent the means ± s.d. of three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Mechanistically, miR-155 exerts its role in glycolysis mainly by upregulation of *hk2*, a key glycolytic enzyme and pivotal player in the Warburg effect (Mathupala *et al*, 2009). We found two switches controlling the regulation of *hk2* by miR-155 (Figure 7H). The first one is STAT3 that binds to the *hk2* promoter to promote its transcription. This is conserved in mouse as shRNA knockdown of *stat3* in mouse embryonic fibroblast cells represses *HK2* mRNA expression (Ando *et al*, 2010). miR-155 can turn on the STAT3 switch through downregulation of one of the STAT3 inhibitors, SOCS1 (Jiang *et al* 2010). The other one is miR-143 that binds to the 3′UTR of *HK2* mRNA to repress HK2 protein production (Figure 3). To restore *HK2* translation, miR-155 needs to flip this miR-143 switch off by suppressing the expression of *C/EBP*β, which is revealed as an *mir-143* transcriptional activator (Figure 4; Supplementary Figure S3). In one hand, without the STAT3 turned on, there is no sufficient *HK2* mRNA; in other hand, without turning off the miR-143 switch, increased mRNA levels of *HK2* by IL6/miR-155/STAT3 do not result in more HK2 proteins. The importance of this dual-control system is reflected by the results that both repression of *mir-143* and activation of STAT3 are required for miR-155 to accelerate glycolysis in breast cancer cells and increase ^{18}FDG uptake in breast tumours (Figure 7; Supplementary Figure S7). Thus, our findings reveal a novel miR-155/miR-143/HK2 cascade that links inflammation and glucose metabolism in breast cancer cells.

miR-155 is well documented as an oncomiR in various cancers. Through negatively regulating tumour suppressive genes *TP53INP1*, *RhoA*, and *socs1* (Gironella *et al*, 2007; Kong *et al*, 2008; Jiang *et al*, 2010), miR-155 promotes malignant transformation and cancer progression of pancreatic cancer, breast cancer, etc. Our data here show that miR-155 upregulates *hk2* and contributes to glycolysis in breast cancer cells (Figures 1 and 7). This finding, along with a recent report that hypoxic conditions induce miR-155 expression in lung cancer cells (Babar *et al*, 2011), suggest that a novel function of miR-155 in regulating cancer cell glucose metabolism.

In contrast, miR-143 is found as a tumour-suppressive miRNA (Chen *et al*, 2009; Kent *et al*, 2010; Osaki *et al*, 2011). In this study, we showed that miR-143 reduced glycolysis in breast cancer cells and ^{18}FDG uptake in breast tumours (Figure 5), revealing that miR-143 represses glucose metabolism in cancer cells. To our knowledge, this is the first report that this tumour-suppressive miRNA also plays a critical role in regulating cancer cell energy metabolism. We identified *hk2*, which catalyses the irreversible first step of glucose metabolism and is a key glycolytic enzyme for aerobic glycolysis, as a novel target of miR-143 and further demonstrated that miR-143 represses glycolysis by targeting *hk2* in breast cancer cells.

Moreover, we showed that *hk2* promotes the growth and migration of breast cancer cells (Figure 6), suggesting that *hk2* is oncogenic in breast cancer. This is in agreement with a recent report showing that *hk2* promotes tumour growth in glioblastoma multiforme (Wolf *et al*, 2011). Previous studies suggest that miR-143 exerts its tumour-suppressive function through targeting oncogenes such as *KRAS* and *MMP-13* (Chen *et al*, 2009; Kent *et al*, 2010; Osaki *et al*, 2011). Our findings here showed that targeting *hk2* also contributes to the antitumour activity of miR-143. These results indicate that miR-143:*hk2* axis, in addition to regulating glucose metabolism, plays an important role in controlling tumourigenesis in breast cancer cells. These findings not only further support the notion that cancer cells use aerobic glycolysis to generate biosynthetic precursors for sustaining cancer cell proliferation and shape a tumour favourable microenvironment for facilitating invasion and metastasis (Kroemer and Pouyssegur, 2008; Vander Heiden *et al*, 2009), but also add a novel molecular link between tumour biology and tumour metabolism.

In addition, regulation of *hk2* in tumours has been extensively investigated due to its role in aerobic glycolysis in cancer cells. Epigenetic events (e.g., demethylation) and/or gene amplification have been implicated to as upregulation of *hk2* during tumourigenesis (Mathupala *et al*, 2009). Additionally, oxidative stress, glucose, insulin, and cAMP also modulate *hk2* expression and activity (Mathupala *et al*, 1995, 2001). Our results showed that *hk2* is transactivated by the pro-tumourigenic inflammatory signalling STAT3, revealing a new mechanism for *hk2* regulation. Moreover, our data indicate that *hk2* is downregulated by miR-143 and upregulated by miR-155, providing the first experimentally demonstration that *hk2* is regulated by miRNAs.

In summary, our study here provides direct evidence that inflammation promotes cancer cell glucose metabolism through the cascades of miR-155-SOCS1-STAT3-HK2 and miR-155-C/EBPβ-miR-143-HK2. As miR-155, miR-143, and HK2 are potential cancer therapeutic targets (Ko *et al*, 2004; Kim *et al*, 2007; Garzon *et al*, 2010; Kitade and Akao, 2010; Tazawa *et al*, 2011), these findings provide direct support for such antineoplasia strategies.

Materials and methods

Cell lines and patient specimens

All cell lines were cultured at 37°C in an atmosphere containing 5% CO_2. HEK293T, ZR-75-30, MDA-MB-231, MCF-7, MDA-MB-453, BT-474, and SK-BR-3 cells were obtained from ATCC (Manassas, VA) and cultured according to ATCC guidelines. For inflammatory stimulation, 10 ng/ml IL-6, 50 ng/ml IL-1β, 100 ng/ml TNFα, or 50 ng/ml IFN-γ (Ebioscience, San Diego, CA) was used to treat cells. Breast tumour specimens and their matching normal adjacent tissues were collected during surgery from patients in Zhongshan Hospital affiliated to Fudan University. Samples were immediately snap frozen and stored at −80°C. Specimen collection was approved by the hospital authorities.

Plasmid constructs

Human *hk2* coding sequences were cloned into the p3 × Flag–CMV^{TM}-14 expression vector (Sigma, St. Louis, MO) to construct p3 × Flag–HK2. The *hk2* promoter and *mir-143* promoter were cloned into the pGL3-Basic firefly luciferase plasmid (Promega, Madison, WI, USA) to construct WT P_{hk2} reporter (Figure 2A) and WT $P_{mir\text{-}143}$ reporter (Figure 4B), respectively. A 9-bp sequence (TTCCCTGAA) as the putative STAT3 binding site was deleted in the Mut P_{hk2} reporter (Figure 2A). Two predicted C/EBPβ binding sites (TTGGATGG and TTGCACAA) were deleted in the Mut $P_{mir\text{-}143}$ reporter (Figure 4B). For reporter pRL-*hk2* 3′UTR, the ∼2.4-kb human *hk2* 3′UTR was cloned downstream of the *Renilla* luciferase gene in pRL-TK (Promega). Seven nucleotides in *hk2* 3′UTR corresponding to 5′ part of miR-143 were deleted in the pRL-*hk2* 3′UTR Mut construct (Figure 3A). All constructs were confirmed by DNA sequencing.

Luciferase reporter assay

For the promoter-firefly luciferase reporter assay, each reporter construct was co-transfected into 293T cells in 24-well plates with *Renilla* luciferase plasmid pRL-TK and RNA oligonucleotides or vectors as indicated in the Figures 2 and 4. For *hk2* 3′UTR-*Renilla*

luciferase reporter assay, each reporter construct was co-transfected into 293T cells together with firefly luciferase plasmid pGL3 and miR-143 mimics or Ctrl RNA (Ambion). Cells were harvested 48 h after transfection. Luciferase activity was measured using the Dual-luciferase Reporter Assay System (Promega) and normalized to respective controls as described previously (Jiang *et al*, 2010).

ChIP assay

The ChIP assay was performed as previously described (Boyd *et al*, 1998). Mouse monoclonal anti-C/EBPβ antibody (1:500; Santa Cruz), rabbit polyclonal anti-Ets-1 antibody (1:500; Santa Cruz), and anti-STAT3 antibody (1:500; Santa Cruz) were used in ChIP assays with a mouse monoclonal IgG (1:500; Sigma) as a negative control. The presence of predicted transcription factor binding regions pulled by these antibodies was assessed by PCR. A small amount of pre-cleared DNA (before addition of antibodies) was set aside as an input control. The PCR primer sequences for DNA fragments as parts of the targeted promoters are provided in Supplementary Table S1.

Measurement of glucose consumption and lactate production

Glucose consumption and lactate production were analysed as described previously (Kawauchi *et al*, 2008). Glucose levels were determined using a glucose assay kit (Sigma). Lactate levels were determined using the Lactate Assay kit (Biovision, Mountain View, CA).

MicroPET/CT imaging of mice

Approximately 4×10^6 cells were orthotopically xenografted in 6–8-week-old nude mice. Five mice were included in one experimental group. In all, 2–3 weeks after inoculation, tumour-bearing mouse was anaesthetized using 2% isoflurane prior to injection of 100–200 uCi ^{18}FDG via the tail vein. Sixty minutes after ^{18}FDG injection, mouse was scanned on a dedicated small animal microPET/CT scanner (Siemens Inveon MM STD MicroPET/CT 3074; Siemens). Animals were imaged with 20-min microCT scan and immediately followed by a 20-min microPET scan. Images were analysed with the Inveon software (Siemens). Regions of interest (ROIs) were manually drawn by qualitative assessment to cover the entire tumour. The ROI counts were then converted to the %ID/g tumour using filtered back projection as previously described (Gambhir *et al*, 2000). Tumour volume was generated by summation of voxels within the tomographic planes.

Statistical analysis

All results were presented as the mean ± standard error of the mean (s.e.m.). A Student's *t*-test was performed to compare the differences between treated groups relative to their paired controls. *P*-values are indicated in the text and figures above the two groups compared with a value <0.05 (denoted by *) considered as significant (***$P<0.001$, **$P<0.01$).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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Author contributions: SJ, LFZ, HWZ, YL, EDW, and MFL planned the project; SJ, LFZ, DL, and MFL designed the experiments; SJ, LFZ, HWZ, SH, MHL, SL, and BL performed the experiments; SJ, LFZ, SL, BL, and MFL analysed the data; YL, DL, and MFL wrote the paper; all authors discussed the results and commented on the manuscript; MFL supervised the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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