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Article

Glucocorticoid receptor–IRS-1 axis controls EMT and the metastasis of breast cancers

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Glucocorticoid receptor (GR) is involved in the transcriptional regulation of genes that are important for various biological functions, including tumor growth and metastatic progression. However, the cellular and biological effects of GR remain poorly understood. Here, we investigated the role of GR and its underlying mechanism in mediating breast cancer cell survival and metastasis. We observed that the GR levels were increased in drug-resistant breast cancer cells and in metastatic breast cancer samples. GR promoted tumor cell invasion and lung metastasis *in vivo*. The GR expression levels were negatively correlated with the survival rates of breast cancer patients. Both ectopic expression and knockdown of GR revealed that GR is a strong inducer of epithelial-to-mesenchymal transition (EMT), which is consistent with its effects on cell survival and metastasis. GR suppressed the expression of insulin receptor substrate 1 (IRS-1) by acting as an IRS-1 transcriptional repressor. In addition, GR has an opposite effect on the expression levels of IRS-2, indicating that GR is able to differentially regulate the IRS-1 and IRS-2 expression. The cellular and biological effects elicited by GR were consistent with the reduced levels of IRS-1 observed in cancer cells, and GR-mediated IRS-1 suppression activated the ERK2 MAP kinase pathway, which is required for GR-mediated EMT. Taken together, our results indicate that GR–IRS-1 signaling axis plays an essential role in regulating the survival, invasion, and metastasis of breast cancer cells.

Keywords: GR, IRS-1, IRS-2, EMT, ERK2, breast cancer, tumor metastasis

Introduction

Breast cancer is one of the most common cancers and a leading cause of cancer mortality in women worldwide (Desantis et al., 2017). Breast cancers are generally classified as one of three subtypes based on their receptor expression profile (estrogen receptor, ER; progesterone receptor, PR; HER2): ER positive (ER^+), ER negative (ER^-), or triple negative (TN) (Tang et al., 2016). Although tumor metastasis is known to be the dominant cause of mortality in breast cancer patients (Buchheit et al., 2014), like many other solid tumors, the molecular mechanisms underlying the metastatic progression of breast cancers remain poorly understood. For decades, studies on the function of nuclear receptors in breast cancer biology have been largely limited to ER and PR (Hilton et al., 2018). In recent years, the GR has been suggested to be involved in cancers (Lin and Wang, 2016; McNamara et al., 2018). GR is a member of the steroid hormone receptor superfamily. GR-α, simply referred to as GR, is the major GR isoform and is ubiquitously expressed in various tissues. GR has been implicated in a broad range of physiological and pathological processes, such as glucose and lipid metabolism, immune suppression, cell survival, and differentiation (Zhou and Cidlowski, 2005). The role of GR in cancer progression is an important but complex issue. As relevant studies at the GR molecular level are lacking, the cellular and molecular effects of GR in mediating cancer progression are not well understood. Contradictory conclusions about the effect of GR on cancer progression and prognosis in breast cancers have been reported (Pan et al., 2011; Abduljabbar et al., 2015). Many conclusions were drawn from studies on the effects of Dexamethasone (Dex); however, drawbacks exist in using Dex to represent the biological functions of GR because the biological effects of different glucocorticoids (Gcs) can vary significantly. For example, a recent genomic study demonstrated a pronounced

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difference in the effects of Dex compared to those of compound A (another synthesized Gc) in regulating gene expression (Chen et al., 2015). In addition, GR also has ligand-independent functions in the cytoplasm (Yoon et al., 2014; Hapgood et al., 2016), and Gcs can also exert their effects via the mineralocorticoid receptor (Frey et al., 2004; Funder, 2005; Sacta et al., 2016). Thus, studies at the molecular level can be highly instrumental for improving our understanding of GR in cancer biology.

IRS-1, a cytoplasmic adaptor protein, transmits the insulin/insulin-like growth factor signals to elicit a cellular response. Nuclear location of IRS-1 can also be examined in certain cell samples (Reiss et al., 2012). The implication of IRS-1 in cancers has been suggested by several studies. For example, both tumor-suppressing and tumor-promoting effects of IRS-1 in mammary tumors have been reported (Dearth et al., 2006; Ma et al., 2006). However, its role in breast cancer progression remains unclear. In addition, the relationship between IRS-1 and GR in breast cancer has not been known. IRS-1 can act as a potent EMT suppressor by maintaining an epithelial phenotype in cells (Shi et al., 2009; Hu et al., 2013). Interestingly, the expression and activation of IRS-1 can be inhibited by Gc in skeletal muscle (Morgan et al., 2009). Dex has been shown to inhibit TGF-β-induced EMT and to induce a reverse process of EMT in Mv1-Lu cells (Zhang et al., 2010). Besides, Gc-related genes have been reported to be involved in EMT (Voutsadakis, 2016). Altogether, these data suggest a possibility that GR and IRS-1 may coordinately involved in the regulation of EMT, and GR may play a role in cancer by regulating EMT via a mechanism that involves IRS-1. Besides IRS-1, IRS-2 is also a major subtype of IRS family members. Although we focused on IRS-1 in this study, it is known that different roles of IRS-1 and IRS-2 have been reported in cancers (Gibson et al., 2007; Eckstein et al., 2017).

EMT is a fundamental event in embryonic development that is also employed in wound healing, tissue remolding, fibrotic diseases, and cancer metastasis (Chaffer et al., 2016; Nieto et al., 2016). EMT-associated fundamental changes at the molecular and cellular levels contribute to relevant alterations in cell properties and behaviors, such as increased cell mobility and acquired resistance to detachment-induced apoptosis and chemotherapy drugs (Tiwari et al., 2012; Huang et al., 2015). Until recently, the relationship between GR and EMT remained unidentified. In this study, we investigated the effects of GR on EMT and EMT-associated properties of breast cancer cells. The cellular effects of GR on the tumor cell survival and metastasis of breast cancer were determined, and the underlying molecular mechanisms were identified. We demonstrated that GR plays an essential role in cell survival, EMT and metastasis in breast cancers. Mechanistically, we identified that GR functions as a transcriptional repressor of IRS-1, leading to a pronounced decrease in IRS-1 expression. IRS-1 was found to be a potent inhibitor of extracellular regulated protein kinase 2 (ERK2) activation, which is essential for EMT induction. Our study demonstrated that GR levels and GR–IRS-1 signaling axis are essential in regulating EMT and EMT-associated cell survival and metastasis in breast cancer.

Results

GR is a critical survival factor for breast cancer cells

Although the effects of Dex on the survival and death of breast cancer cells have been reported (Wu et al., 2004), the role of GR has remained unclear. Analysis of Oncomine datasets showed that the GR levels were significantly higher in breast cancer cells compared to liver or lung cancer cells (Supplementary Figure S1), suggesting that GR may play an essential role in breast cancers. In addition, the GR expression levels were inversely correlated with the traditional drug sensitivity of breast cancer cells (Figure 1A). As non-traditional drugs, tumor necrosis factor α (TNF-α) and trichostatin A (TSA) have been widely used to induce apoptotic cell death. GR overexpression in MDA-MB-453 and MCF7 cells significantly reduced the TSA and TNF-α-induced apoptosis (Figure 1B–E). Similarly, GR protected cells from the traditional drugs paclitaxel- and 5-FU-induced apoptosis (Figure 1F and G). Besides, we found that GR overexpression upregulated apoptosis inhibitor Bcl-2 expression, which was not affected by Dex (Supplementary Figure S2A and B). Whereas, GR knockdown caused a reduced survival of MDA-MB-453 and MDA-MB-231 cells (Figure 1H–J). These results indicate that GR is a potent survival factor in breast cancer cells.

GR promotes breast cancer metastasis

Since the survival-promoting effect of GR suggests potentially a role of GR in the metastasis of breast cancer cells. Thus we examined this effect of GR on breast cancer metastasis. As shown in Figure 2A–C, GR overexpression strongly increased while GR knockdown markedly decreased lung metastasis. It is worth noting that both GR and vimentin (a mesenchymal marker) protein levels were higher in metastatic tumor nodules compared to adjacent normal lung sections (Figure 2A lower and Figure 2C middle and lower). Thus, these findings showed the ability of GR to promote metastasis of breast cancer cells.

GR levels correlate with poor prognosis in breast cancer

To further understand the role of GR in breast cancer, we firstly analyzed the Oncomine datasets and found that GR expression was higher in aggressive subtypes such as TN (Figure 2D left) and invasive ductal carcinoma (Figure 2D right). Secondly, IHC staining of GR in a tissue microarray containing 150 human breast cancer samples showed that high levels of GR correlated with reduced survival rates, particularly in stage III breast cancers (Figure 2E and F). Consistently, the survival rates of GR-high patients in TN and invasive subtypes were significantly reduced (Figure 2G and H). GR overexpression in MCF7 cells and knockdown in MDA-MB-231 cells had no obvious effect on the expression levels of ER (Supplementary Figure S3A and B). By analysis of TCGA Breast

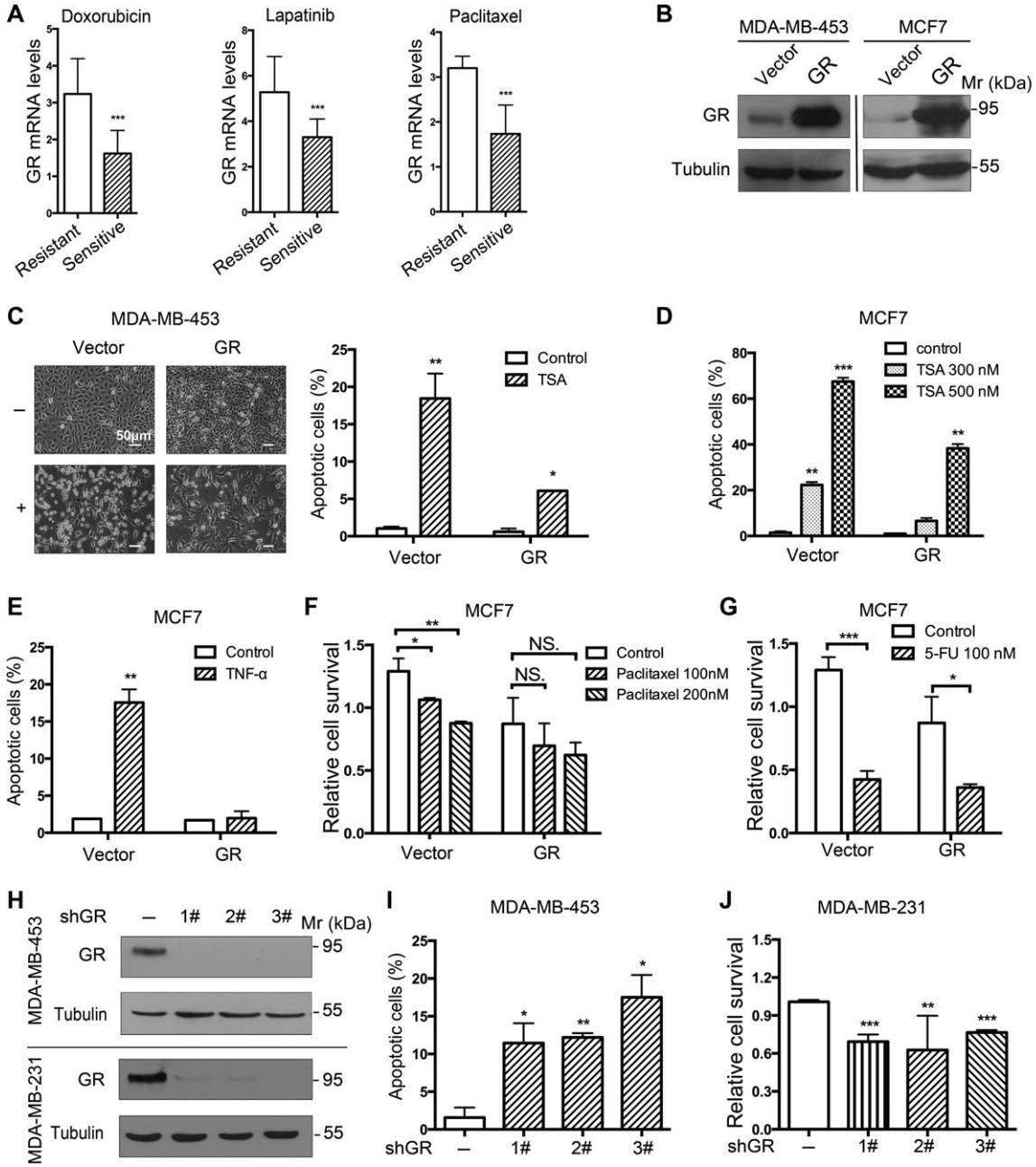

Figure 1 GR is a critical survival factor in breast cancer cells. (**A**) Increased GR expression in multiple drug-resistant breast cancer cell lines. Data were obtained from Oncomine datasets (left, GSE11812; middle and right, GSE36138). (**B**) Overexpression of GR in MDA-MB-453 (left) and MCF7 (right) cells, as examined by immunoblotting. (**C**) MDA-MB-453 cells were treated with TSA (300 nM) for 48 h, and cell apoptosis was examined by cell morphology (left) and FACS analysis (right). (**D** and **E**) MCF7 cells were treated with different concentrations of TSA for 48 h (**D**) and TNF-α (50 ng/ml) for 24 h (**E**), and cell apoptosis was measured by FACS analysis. (**F** and **G**) MCF7 cells were treated with different concentrations of Paclitaxel (**F**) and 5-FU (100 nM) for 48 h (**G**), and cell survival was measured by CCK-8 counting kit. (**H**) GR knockdown in MDA-MB-453 (upper) and MDA-MB-231 (lower) cells. (**I**) The apoptotic effect of GR knockdown in MDA-MB-453 was determined by FACS analysis. (**J**) Knockdown GR in MDA-MB-231 cells decreased cell survival, as determined with CCK-8 counting kit.

datasets from Oncomine database, we found GR mRNA level is not changed in ER^+ and ER^- invasive ductal breast cancer tissues (Supplementary Figure S3C), suggesting that there is no direct cross-talk between the GR and ER. However, Dex can downregulate ER expression in a dose- and time-dependent manner in MCF7 cells (Supplementary Figure S3D). Besides, we analyzed the effect of GR on the survival rate of patients in ER positive group (Supplementary Figure S3E and F) and obtained the similar results as that of TN and invasive subtypes. Collectively, these data indicate that, by acting as a survival factor, GR plays an essential role in breast cancer

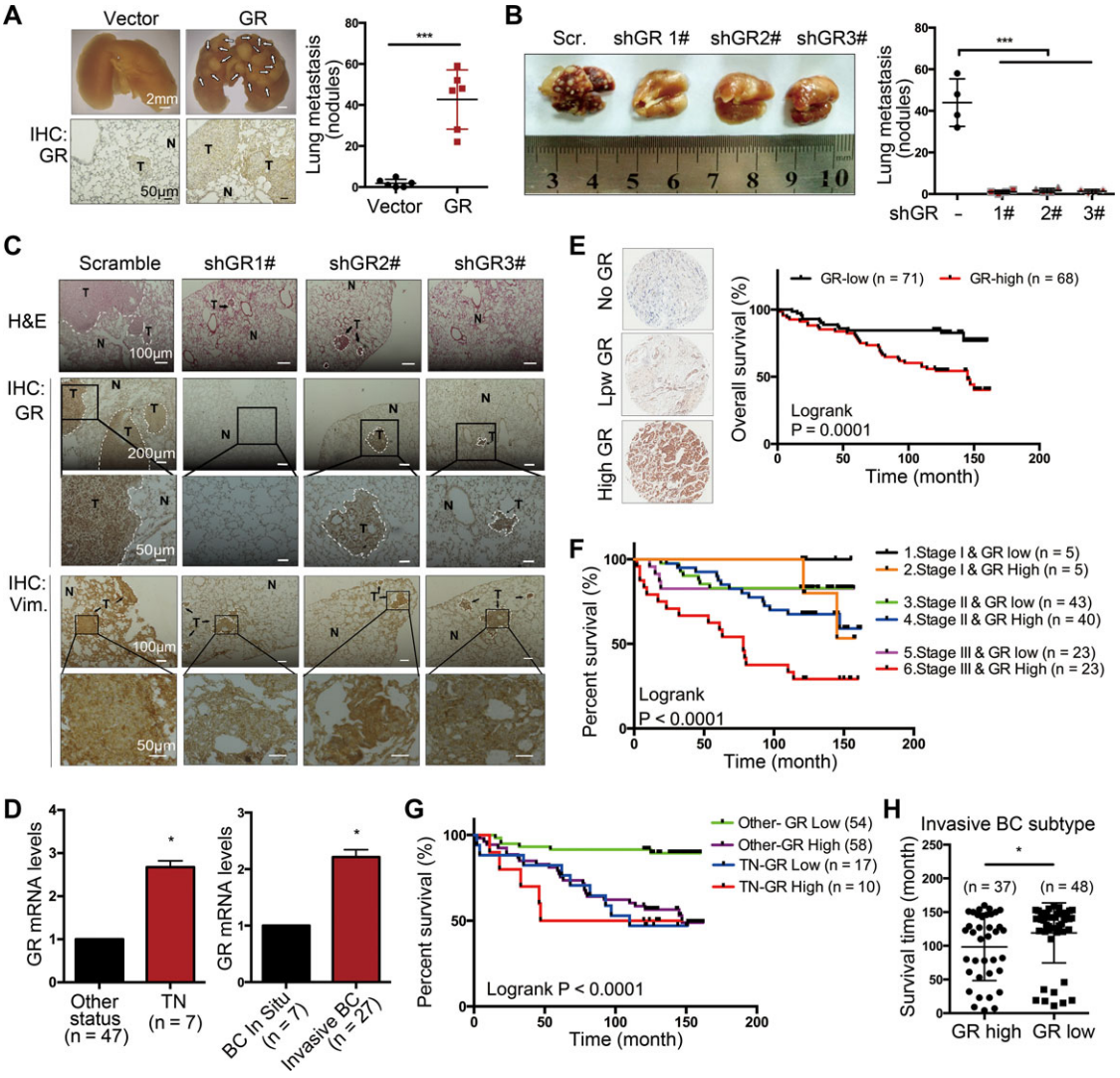

Figure 2 GR is a risk factor in breast cancers. (**A**) GR overexpression promotes lung metastasis. Upper, representative images of gross lung views at 8 weeks after tail vein injection of MCF7 cells were shown under a stereoscope and the metastatic nodules are indicated by white arrows; lower, IHC staining of GR in lungs dissected from individual groups. N, normal; T, tumor; $n = 6$ per group. Right, statistics of nodules per lung. (**B** and **C**) GR knockdown inhibits lung metastasis of breast cancer cells. Experimental mice were injected with MDA-MB-231 cells, and metastatic lung colonization was examined 4 weeks later. $n = 4$ per group. (**B**) Representative images of lungs with metastatic nodules from different groups (left) and counted lung metastasis nodules (right). (**C**) GR and EMT marker vimentin (Vim.) levels in lung colonization of tumor cells, as examined by H&E staining (upper) and IHC staining of GR (middle) and Vim. (lower) in lung tissue. (**D**) GR mRNA levels in TN (left) and invasive (right) breast cancers (BC) are higher. Data were obtained from the Oncomine datasets (left, GSE3971; right, GSE7390). TN, triple negative. (**E**–**H**) Breast cancer tissue microarray (150 cases) was conducted by IHC staining of GR, and GR is negatively correlated with the survival rates of breast cancer patients. Kaplan-Meier survival plots for the overall survival rates (**E**) and survival percentages for patients in three clinical stages (TNM system) (**F**) or in TN subtype (**G**) according to IHC staining of GR. GR high, H-score ≥ 7; GR low, H-score < 7. (**H**) Statistics of the survival time of patients in invasive BC subtype according to IHC staining of GR. GR high, H-score ≥ 7; GR low, H-score < 7.

progression and that the GR levels can be instrumental in predicting the prognosis of breast cancer patients.

GR overexpression induces EMT and EMT-associated cell behavior changes in breast cancer cells

EMT is an important event that confers cells increased mobility, which is implicated in cancer cell survival and metastasis. However, the role of GR in EMT is unknown. MCF7, MDA-MB-453, and MDA-MB-231 are three breast cancer cell lines with different degrees of epithelial characteristics (decreasing in order), which are related to their corresponding aggressiveness (Figure 3A upper). We observed that the both GR mRNA and protein levels positively correlated with vimentin levels and negatively correlated with E-cadherin levels (Figure 3A lower and B). Linear regression analysis also showed correlations between the GR and EMT marker levels. A negative correlation was observed

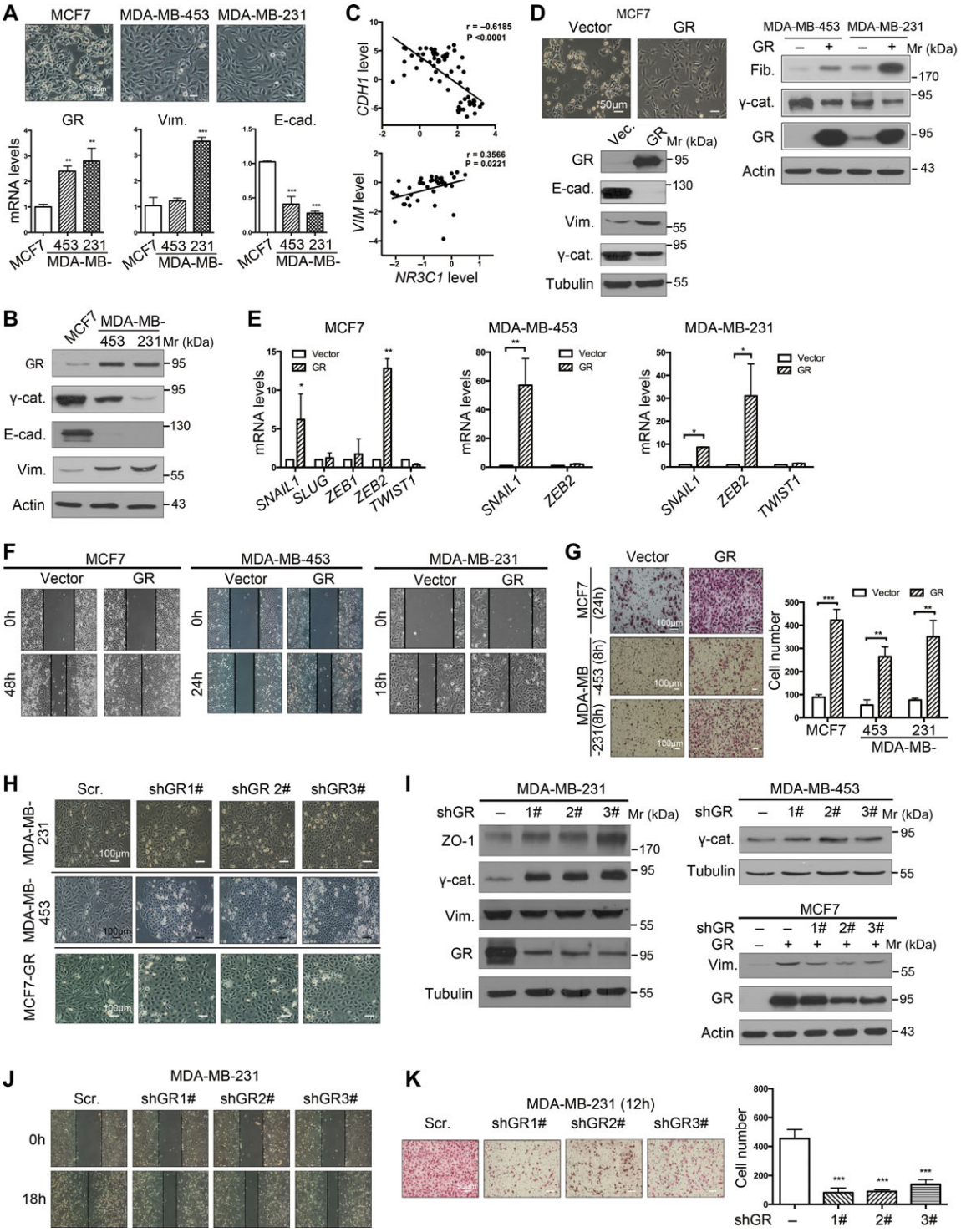

Figure 3 GR induces EMT and cell migration. (**A**) Morphological differences of three human breast cancer cell lines (upper) and mRNA levels of GR, vimentin, and E-cadherin determined by qPCR (lower). (**B**) GR levels and EMT marker protein levels in three breast cancer cell lines. (**C**) The correlation of GR mRNA level with *CDH1* (upper) and *VIM* (lower) levels in Oncomine clinical breast cancer datasets (upper, GSE2603; lower, GSE3281). (**D**) GR overexpression induces EMT in MCF7 (left), MDA-MB-453, and MDA-MB-231 (right) cells, as determined by changes in cell morphology and EMT-related genes that were observed by immunoblotting. (**E**) GR overexpression induces expression of EMT-associated transcription factors in three breast cancer cell lines, as examined by qPCR. (**F**) GR overexpression increases cells migration in MCF7 (left), MDA-MB-453 (middle), and MDA-MB-231 (right) cells, as examined by wound healing assay. (**G**) GR overexpression increases transwell cell migration. Right, the statistics of cell transwell. (**H** and **I**) Knocking down GR induces MET-like changes in three breast cancer cell lines, as determined by cell morphology (**H**) and EMT marker protein levels (**I**). (**J** and **K**) The effect of GR knockdown on cell migration of MDA-MB-231 cells, as examined by wound healing assay (**J**) and transwell migration assay (**K**).

between *NR3C1* (gene encoding GR) and *CDH1* (encoding E-cadherin), while a positive correlation was observed between *NR3C1* and *VIM* (encoding vimentin) (Figure 3C). These observations suggest that GR is important in EMT induction. GR overexpression in MCF7 cells caused a spindle-like cell morphological change (Figure 3D left upper) and reduced the E-cadherin and γ-catenin levels, but increased the vimentin levels (Figure 3D left lower). Similar results were observed in MDA-MB-453 and MDA-MB-231 cells (Figure 3D right). Besides, GR also increased the EMT-associated transcriptional factors such as Snail1 and/or Zeb2 in breast cancer cell lines (Figure 3E), suggesting their involvement in GR-induced EMT. To investigate the cellular and biological impact of GR-mediated EMT, we examined the effect of GR on EMT-associated cell behaviors. As shown by wound healing (Figure 3F) and transwell (Figure 3G) assays, GR overexpression markedly increased cell migration in three breast cancer cell lines. These results confirmed the EMT-promoting function of GR. Interestingly, phosphorylated GR was found in the cytoplasm and nucleus in both the presence and absence of Dex in MCF7 cells (Supplementary Figure S4A). In addition, Dex treatment did not affect GR-induced or TGF-β1-induced EMT in MCF7 and A549 cells (Supplementary Figure S4B and C). Inhibition of GR phosphorylation by GR antagonist RU486 had no obvious effect on GR-induced EMT (Supplementary Figure S4D), suggesting that the GR level, but not its ligand of phosphorylation status, is essential for EMT induction.

GR knockdown inhibits EMT and EMT-associated cell behavior changes

MDA-MB-231 is a breast cancer cell line with a markedly increased GR level and correspondingly high cell mobility. To further examine the effect of GR on EMT, we knocked down GR in these cells and found that GR knockdown induced MET-like changes, as shown by cell morphology (Figure 3H upper) and EMT marker protein levels (Figure 3I left). Similar results were found in MDA-MB-453 cells and MCF7-GR cells (Figure 3H middle and lower and I right). Besides, GR knockdown significantly reduced MDA-MB-231 cell migration as examined by wound healing assay (Figure 3J) and transwell assay (Figure 3K). Altogether, these results support the role of GR as a critical inducer of EMT and EMT-associated responses, which accounted at least in part to its roles in breast cancer cell survival and metastasis.

GR functions as a transcriptional repressor of IRS-1

To explore the mechanism of GR-mediated EMT in breast cancer cells, we examined several genes related with EMT or/and cell survival. GR overexpression can induce pronounced changes of IRS-1 in breast cancer cell lines (Supplementary Figure S5A). As IRS-1 is an essential EMT regulator, these observations suggest that the effect of GR may be linked with it. Further examination showed that both mRNA (Figure 4A) and protein (Figure 4B) levels of IRS-1 were significantly higher in cell lines with strong epithelial properties while lower in cell lines with relatively weak epithelial properties, suggesting a link between GR and IRS-1 signaling in EMT regulation and breast cancer metastasis. GR overexpression decreased IRS-1 protein levels in MCF7, MDA-MB-453 and MDA-MB-231 cells (Figure 4C and Supplementary Figure S5A), while its knockdown in MDA-MB-231 cells increased the IRS-1 levels (Figure 4D and Supplementary Figure S5B), conforming an inhibitory effect of GR on IRS-1 expression. To explore how GR inhibits IRS-1 expression, we examined the effect of MG132, a proteasome inhibitor, on the IRS-1 protein levels. As MG132 treatment could not restore the IRS-1 levels that were decreased due to GR overexpression (Supplementary Figure S5C), it suggests that the decreased IRS-1 levels by GR were not due to the changes of IRS-1 protein turnover. Using qPCR, we determined that GR significantly inhibits IRS-1 gene transcription (Figure 4E). A luciferase reporter assay also showed that IRS-1 promoter activity was significantly decreased in GR-overexpressing MCF7 cells (Figure 4F) and increased in GR-knockdown MDA-MB-231 cells (Figure 4G). To further investigate the relationship between GR and IRS-1, we designed five pairs of primers according to different regions of the promoter sequence of IRS-1 (Supplementary Table S6) to determine which region is required for regulation of GR. Three of the five regions were found to bind the IRS-1 promoter, among which the region of −1504 to −1336 exhibited the strongest binding ability (Figure 4H and I). Supplementary Figure S5A suggests that other factors (NF-κB, p65, p53, E2F1) may be involved in the GR mediated transcriptional repression of IRS-1. Together, these results confirmed the role of GR in suppressing IRS-1 gene transcription. Although GR overexpression decreased IRS-1 levels, it surprisingly increased IRS-2 protein and mRNA levels (Figure 4C; Supplementary Figure S5A, B, and D). While GR knockdown in MDA-MB-231 cells increased the IRS-1 levels but decreased IRS-2 levels (Figure 4D; Supplementary Figure S5B and D), It is interesting to note that this inhibitory effect of GR on IRS-1 is also independent of Dex even though Dex-mediated IRS-2 upregulation is dependent on GR (Supplementary Figure S5E and F).

Implication of IRS-1 in GR-mediated EMT

As IRS-1 is a direct downstream target of GR, we assessed whether IRS-1 is critical in GR-mediated EMT. Strikingly, knockdown of IRS-1 induced EMT (Figure 5A and B) while IRS-1 overexpression alone induced obvious MET-like changes in MCF7 cells (Figure 5C). IRS-1 overexpression completely suppressed GR-induced EMT in MCF7 cells (Figure 5D and E). In addition, the inhibitory effect of IRS-1 overexpression on GR-mediated actin rearrangement and downregulation of E-cadherin was also determined (Figure 5F). Thus, it is clear that there is an opposing effect on EMT and a regulatory relationship between GR and IRS-1 molecules.

ERK2 plays a role in GR–IRS-1-mediated EMT

Mitogen-activated protein kinase (MAPK) cascades are well-known signaling pathways that regulate diverse cellular processes (Meloche and Pouysségur, 2007; Deschênes-Simard et al., 2014). ERK1/2, important survival factors and components of the MAP kinase pathway, are required for TGF-β1-induced EMT (Xie

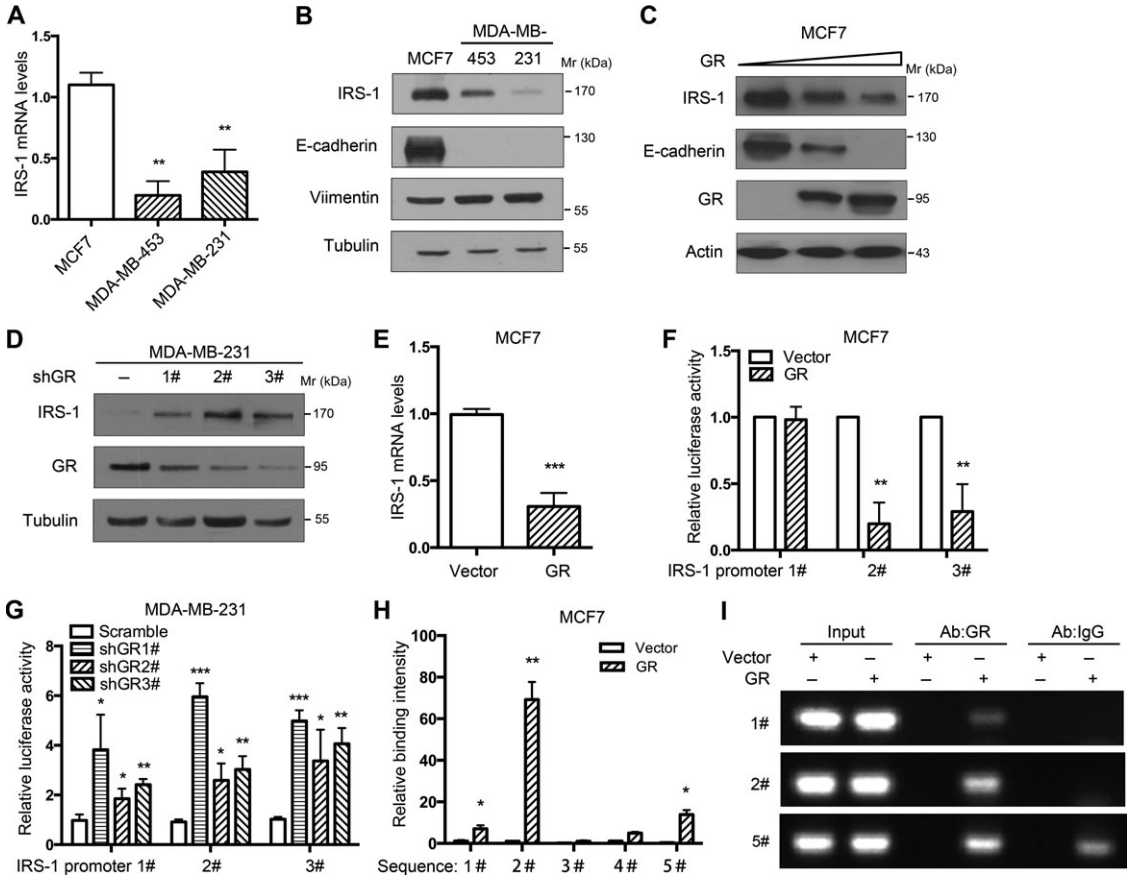

Figure 4 IRS-1 is transcriptionally repressed by GR. (**A** and **B**) IRS-1 level is positively correlated with an epithelial phenotype and negatively correlated with a mesenchymal phenotype in breast cancer cell lines, as measured by qPCR (**A**) and immunoblotting (**B**). (**C**) GR overexpression markedly decreased the IRS-1 protein levels in MCF7 cells, as examined by immunoblotting. (**D**) GR knockdown in MDA-MB-231 cells markedly increased the IRS-1 protein levels. (**E**) The effect of GR overexpression on the IRS-1 mRNA levels in MCF7 cells, as determined by qPCR. (**F** and **G**) The effect of GR on IRS-1 promoter luciferase activity in MCF7 cells (**F**) and MDA-MB-231 cells (**G**). 1#: −1123 to +77; 2#: −1123 to +241; 3#: −2091 to +241. (**H** and **I**) GR binds to IRS-1 promoter regions. ChIP–qPCR statistics using five pairs of primers corresponding to the promoter region of IRS-1 (**H**) and representative images of DNA-PAGE (**I**).

et al., 2004; Shin et al., 2010). We found significantly increased activation of ERK1/2 (pERK1/2) in cells with high basal GR levels, suggesting a positive relevance between ERK1/2 activation and GR levels (Figure 6A). GR overexpression not only downregulated the IRS-1 levels but also significantly increased ERK2 phosphorylation in three breast cancer cell lines (Figure 6B and Supplementary Figure S5A). Conversely, GR knockdown increased IRS-1 expression and decreased pERK2 (Figure 6C). These results demonstrate a relationship between GR–IRS-1 signaling and ERK1/2 activation. Further investigation showed that IRS-1 knockdown increased ERK2 activation (Figure 6D), whereas IRS-1 overexpression inhibited GR-mediated ERK2 activation (Figure 6E), suggesting that IRS-1 functions as a suppressor of ERK2. In addition, treatment of cells with U0126, a selective inhibitor of ERK1/2, increased the epithelial marker protein levels (Supplementary Figure S5G and H). Moreover, ERK2 knockdown not only induced significant MET-like changes in MDA-MB-231 cells (Figure 6F) but also blocked GR-induced EMT in MCF7 cells (Figure 6G). The above results demonstrate that ERK2 is a downstream signaling effector of GR-IRS-1 axis, which plays a role in GR–IRS-1-mediated EMT and relevant cellular behaviors.

GR–IRS-1 axis promotes cell migration and metastasis of breast cancer

The above findings indicate that GR–IRS-1 signaling is an essential pathway in the control of EMT. By wound healing assay, we observed that IRS-1 knockdown enhanced cell migration while its overexpression significantly inhibited cell migration mediated by GR in MCF7 cells (Figure 7A and B), which was confirmed by transwell-migration and transwell-invasion assays (Figure 7C and D). These results demonstrate an inverse effect of GR and IRS-1 on EMT-associated cellular properties. By using a tail vein injection assay, we further examined the relationship between GR and IRS-1 in breast cancer lung metastasis. GR

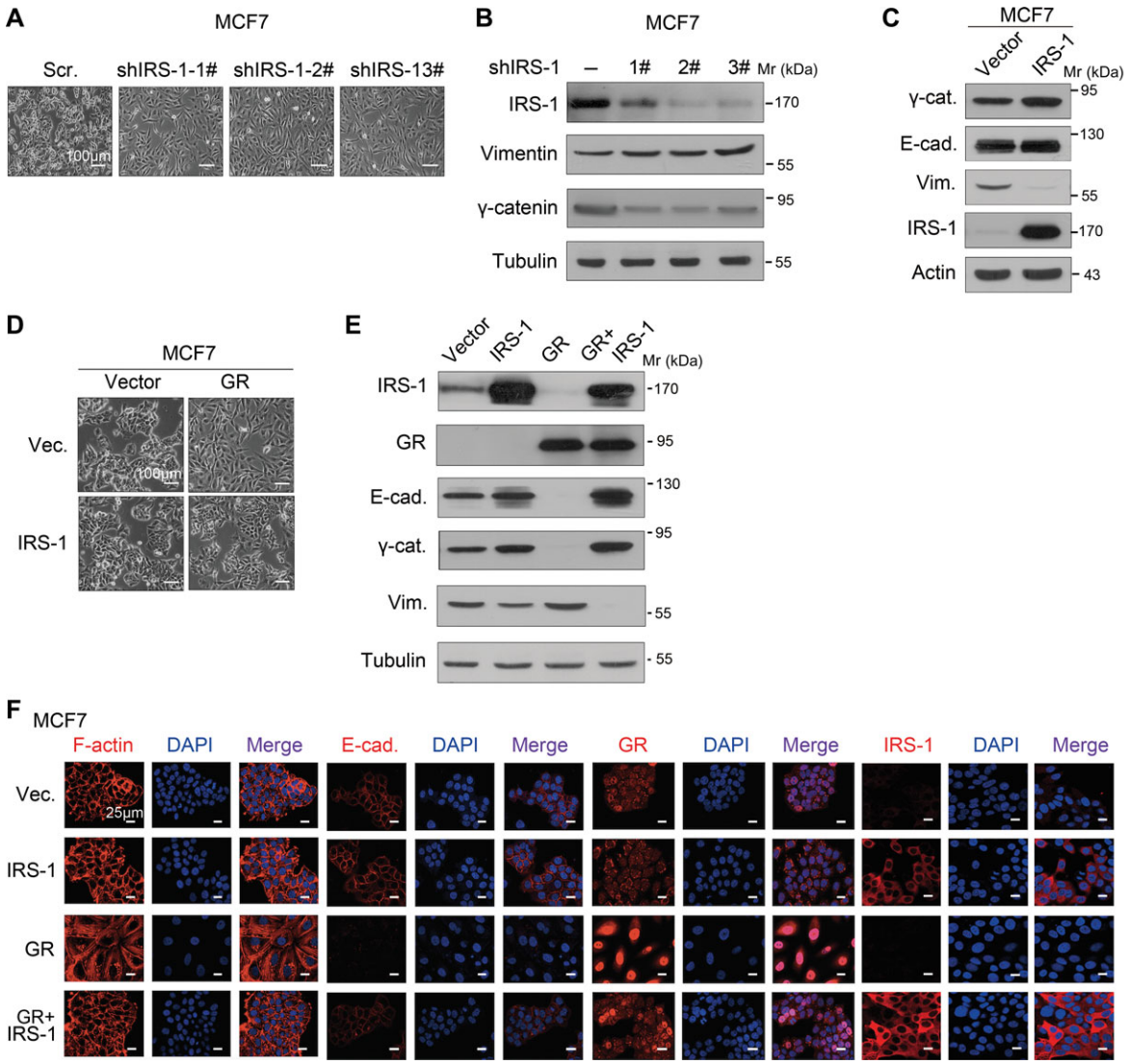

Figure 5 IRS-1 is involved in GR-mediated EMT. (**A** and **B**) IRS-1 knockdown induces spontaneous EMT in MCF7 cells, as determined by cell morphology (**A**) and immunoblotting of γ-catenin and vimentin (**B**). (**C**) IRS-1 overexpression induces MET-like changes in MCF7 cells, as examined by immunoblotting. (**D** and **E**) IRS-1 overexpression inhibits GR-induced EMT in MCF7 cells, as determined by cell morphology (**D**) and immunoblotting of E-cadherin, γ-catenin, and vimentin (**E**). (**F**) The effect of IRS-1 on GR-mediated F-actin formation with decreased E-cadherin levels in MCF7 cells, as examined by immunofluorescent staining with xyz scanning.

overexpression increased lung colonization, whereas IRS-1 overexpression abolished this effect (Figure 7E and F). A slight to moderate increase in lung weight was observed in the GR-overexpression group, which was also inhibited by IRS-1 overexpression (Figure 7G). Besides, increased IRS-1 expression also abolished GR-mediated increases in blood circulating tumor cells (CTCs) (Figure 7H). Analyses of Oncoming datasets showed that the IRS-1 mRNA levels were significantly decreased in aggressive breast cancers (Figure 7I), and consistently its expression was negatively correlated with GR expression in breast cancers (Figure 7J). Together, these results indicated a suppressor and downstream effector relationship between GR and IRS-1, which is essential in controlling EMT and metastasis of breast cancer cells.

Discussion

GR plays important roles in various biological processes that are essential for health and diseases (Kadmiel and Cidlowski, 2013). Although GR has been implicated in the development and progression of cancers, understanding of its biological function has largely been limited to studies on the pharmacological effects of Dex. More extensive studies are therefore needed to gain a better understanding of GR biology.

TGF-β signaling has been closely implicated in the development and progression of cancers. In the early stages of this study, we observed that in response to TGF-β, there was a dose- and time-dependent decrease in the GR levels in AML12 hepatocytes (Supplementary Figure S6A and B) and a strong increase in the GR levels in A549 lung cancer cells (Supplementary Figure S6C

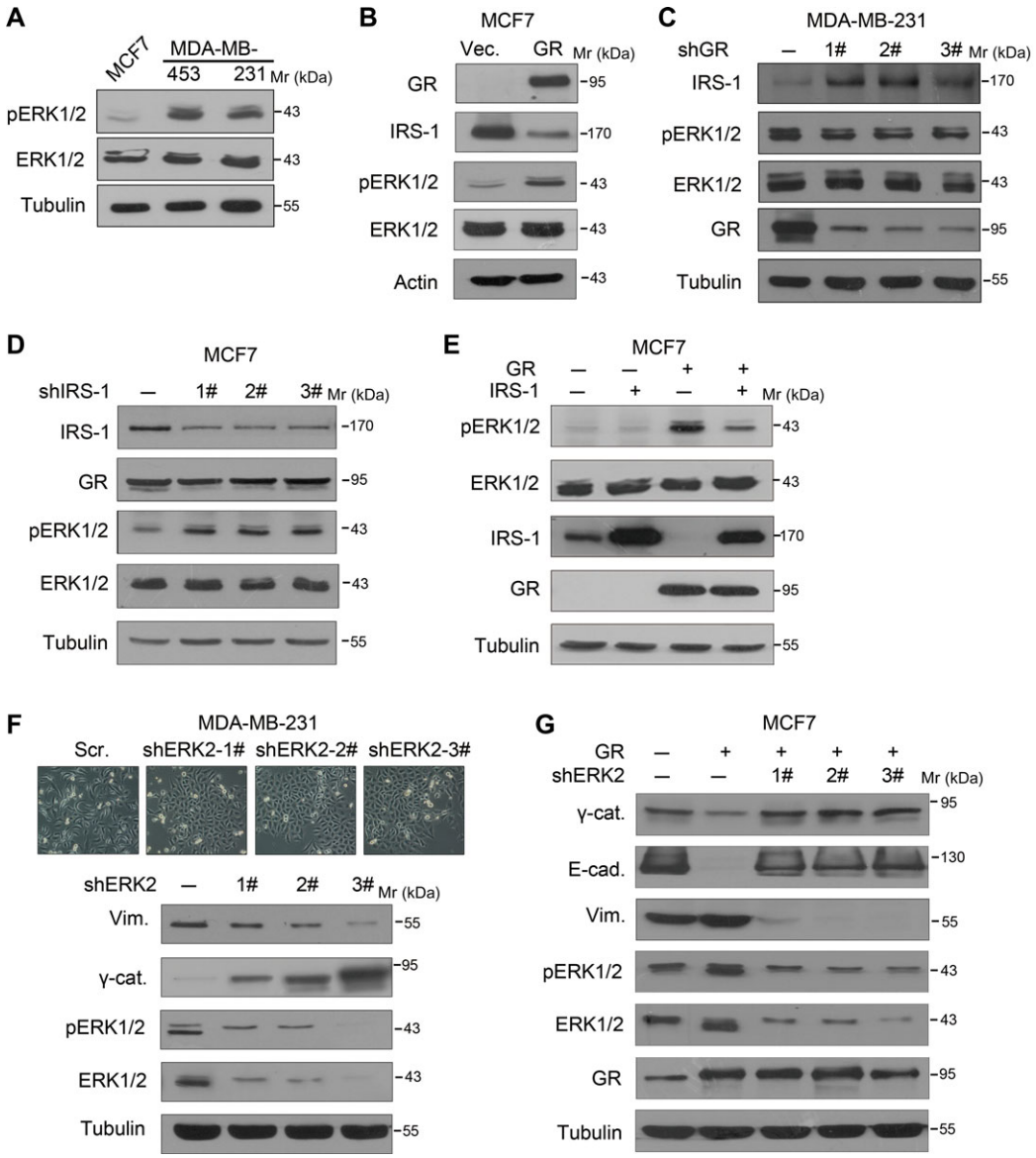

Figure 6 ERK2 plays a role in the regulation of EMT by GR–IRS-1 axis. (**A**) ERK1/2 and pERK1/2 levels are higher in breast cancer cell lines with a stronger mesenchymal phenotype, as determined by immunoblotting. (**B**) GR overexpression increases the activation of ERK2 in MCF7 cells, as examined by immunoblotting. (**C**) GR knockdown decreases pERK2 in MDA-MB-231 cells. (**D**) IRS-1 knockdown increases the activation of ERK2 in MCF7 cells. (**E**) IRS-1 overexpression inhibits GR-mediated activation of ERK2 in MCF7 cells. (**F**) ERK2 knockdown induces MET-like changes in MDA-MB-231 cells, as determined by cell morphology (upper) and immunoblotting of γ-catenin and vimentin (lower). (**G**) ERK2 knockdown inhibits GR-induced EMT in MCF7 cells.

and D). This striking inverse alterations in GR levels suggests that GR is not only important for cell survival but may also be involved in regulating EMT because TGF-β can induce strong concomitant apoptosis and EMT in normal hepatocytes but only induce EMT in A549 cells. Additional experiments showed that GR levels are significantly higher in breast cancer cells (Supplementary Figure S1), particularly in chemotherapy-resistant and aggressive cell lines (Figures 1A and 2D), suggesting a role for GR in EMT and breast cancer progression. The identification of GR as a survival factor that is essential in breast cancer cell lines provides a mechanistic explanation for drug-resistance in these cells (Figure 1). This survival effect of GR also correlates with its effect on EMT and metastasis (Figures 2 and 3). Analysis of bioinformatic data showed that GR levels correlate with an increased mesenchymal phenotype in breast cancer cells, supporting the conclusion that GR promotes EMT in breast cancer cells.

As EMT is essential for increased migration and invasion abilities of cells, and as chemotherapy-resistant cells arise from mesenchymal stem cells (Cao et al., 2016; El-Badawy et al., 2017), the regulatory effect of GR on EMT may be contributed to its metastatic function. *In vivo* studies showed that GR overexpression increased the number of CTCs in mouse blood as well

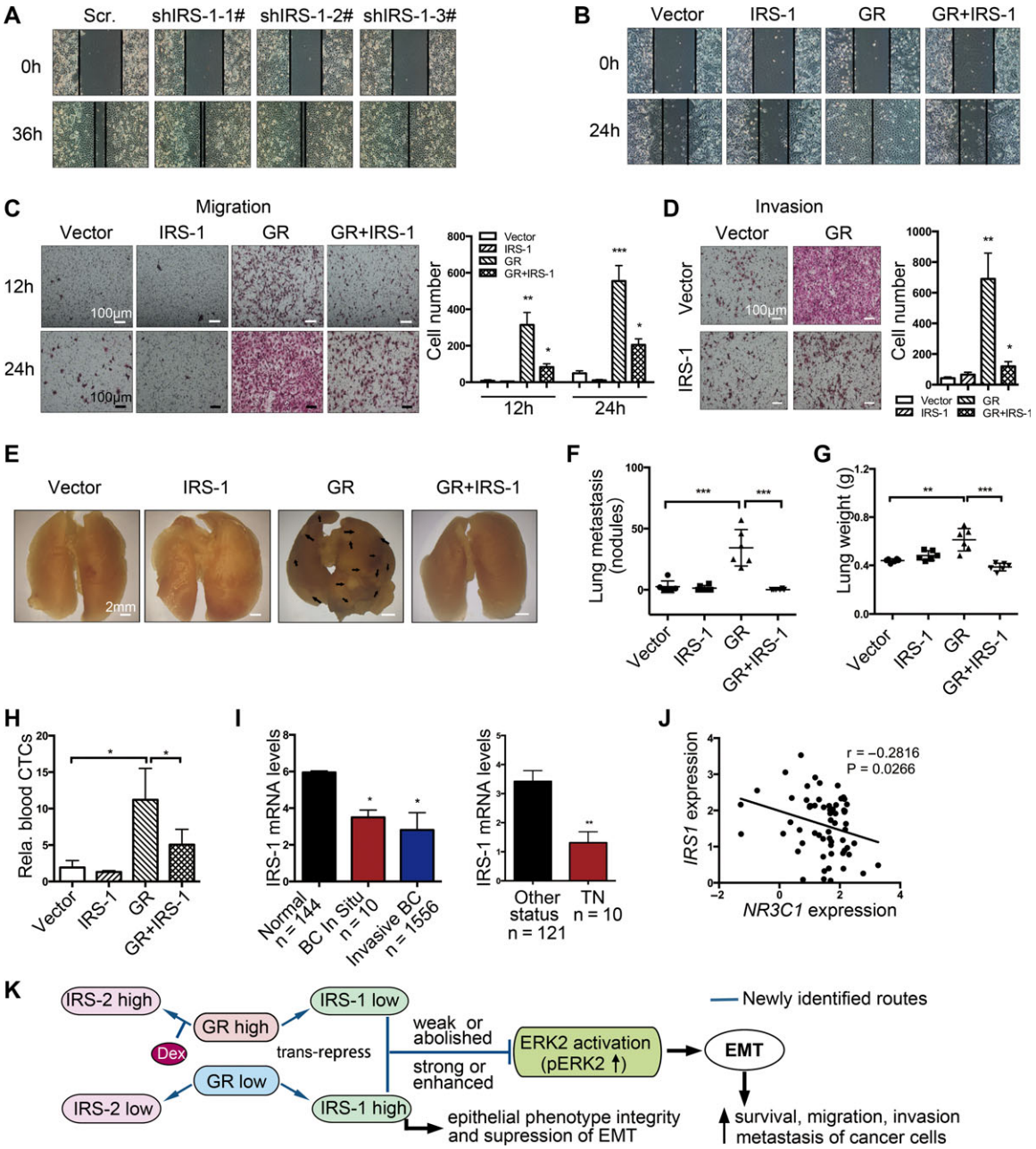

Figure 7 IRS-1 is involved in GR-mediated cell migration and tumor metastasis. (**A**) IRS-1 knockdown increases cell migration in MCF7 cells. (**B**) IRS-1 overexpression inhibits GR-induced cell migration, as determined by the wound healing assay. (**C** and **D**) IRS-1 overexpression blocks GR-mediated increase in cell migration (**C**) and cell invasion (**D**) in MCF7 cells. Right are the statistics. (**E**–**H**) The effect of IRS-1 on GR-mediated lung metastasis of breast cancer cells. Mice were sacrificed 8 weeks after tail vein injection. Lungs are shown under a stereoscope (**E**), and the metastatic nodules (**F**) and lung weight (**G**) were counted. $n = 6$ per group. (**H**) Blood CTCs were measured by qPCR. (**I**) Lower expression of IRS-1 mRNA in both invasive (left) and TN (right) breast cancer subtypes compared to the respective controls. The data were obtained from the Oncomine clinical datasets (left, EGAS0083; right, GSE3143). (**J**) The negative correlation of IRS-1 and GR mRNA expression in clinical breast cancers from Oncomine dataset (GSE3726). (**K**) A schematic illustration of the main findings of this work. GR differentially regulates the IRS-1 and IRS-2 expression levels. The GR–IRS-1 signaling pathway was identified to be implicated in the regulation of EMT and metastasis of breast cancer cells.

as increased lung colonization of breast cancer cells (Figures 2A–C and 7E–H). The observations that GR reduced xenograft growth (Supplementary Figure S7A–F) but increased distal lung colonization of breast cancer cells (Figures 2A–C and 7E, F) suggest that the ability of GR to promote metastasis is not due to increased cell proliferation but due to the induction of EMT and

EMT-associated property changes in cells. Besides, we examined the cell proliferation by BrdU incorporation assay and observed that IRS-1 overexpression impaired the effect of GR on cell proliferation (Supplementary Figure S7G). Soft agar colony formation experiments showed that colony formation ability of MDA-MB-231 (stronger mesenchymal phenotype) was weaker than MCF7 cells (stronger epithelial phenotype) (Supplementary Figure S7H), suggesting a negative relation between colony formation and mesenchymal properties. In addition, this was confirmed by the evidence that GR overexpression and knockdown respectively decreased and increased the colony formation (Supplementary Figure S7I and J). IRS-1 can rescue the colony formation ability lost by GR (Supplementary Figure S7I). These opposing effects of GR and IRS-1 correlate with their regulatory effects on EMT, as growth inhibition is generally a precondition of EMT induction. GR levels positively correlate with breast cancer CTCs and also correlate with poor prognosis of breast cancer patients (Figures 2E–H and 7H). These findings demonstrate a strong promoting effect of GR in the progression of breast cancers.

The potent suppressor effect of IRS-1 in TGF-β-induced EMT in lung cancer cells (Shi et al., 2009; Hu et al., 2013) suggests a suppressive effect on tumor metastasis. In contrast to IRS-1, the GR levels are markedly increased during TGF-β-induced EMT (Supplementary Figure S6C and D). These inverse changes in response to TGF-β also suggest an opposing effect of GR and IRS-1 in EMT and imply an important relationship of them. We also identified that IRS-1 is a downstream target of GR (Figure 4 and Supplementary Figure S5A–F). GR-mediated down regulation of IRS-1 is essential for GR-mediated cellular effects, which revealed a mechanism underlying the GR-mediated EMT. As the effect of GR or IRS-1 can be modulated by alterations in their levels, the relationship between them constitutes a signaling axis that can impact EMT and EMT-associated events. In this study, we found that GR and IRS-1 have opposing effects on ERK2 MAP kinase activation, which plays an important role in the regulation of GR–IRS-1-mediated EMT (Figure 6). Decreased ERK2 activation increases the epithelial phenotype and blocks GR-mediated EMT. ERK MAP kinase is a well-known survival factor and increased cell survival can contribute on the extent of EMT (Liu et al., 2017). Thus, we postulate that it is likely that the involvement of ERK2 in GR–IRS-1-mediated EMT was due to its survival promoting effect in this case of study. These data confirm that the GR and IRS-1 signaling axis is important in regulating EMT and EMT-associated cell behaviors.

Although it has been generally assumed that GR is located and sequestered in the cytoplasm by heat-shock proteins and that, upon ligand binding, it dissociates from HSP, becomes phosphorylated and translocated to the nucleus, where it can regulate gene expression by binding to DNA elements or transcriptional co-factors (Wang and Harris, 2015), we observed that phosphorylated GR was present both in the cytoplasm and nucleus. Exogenous Dex stimulated GR phosphorylation and its nuclear translocation, but had no obvious effect on the level of nuclear un-phosphorylated GR and EMT induction (Supplementary Figure S4A–C). These data suggest that phosphorylation is not strictly required for GR nuclear location or translocation. Dex treatment enhanced GR-mediated increase in IRS-2 but had no effect on GR-mediated decrease in the expression of IRS-1 (Supplementary Figure S5E and F). Besides, inhibition of GR phosphorylation by RU486 had no effect on GR-mediated EMT, implying that GR-mediated transcriptional suppression of IRS-1 is independent of its phosphorylation status. These findings may suggest that phosphorylated and un-phosphorylated GR may have different biological effects.

As IRS-1 is an essential component in insulin signaling, the finding that GR functions as a suppressor of IRS-1 suggests that GR or the GR–IRS-1 axis can function as potent modulators of insulin-resistance and insulin-sensitivity. This observation could be important considering human health and relevant diseases, such as type II diabetes, Gc actions, and glucose homeostasis. For example, the opposing effect between GR and IRS-1 provides a possible explanation for the occurrence of insulin resistance in patients undergoing systemic Gc therapy (Ferris and Kahn, 2012). A schematic illustration is provided for helping to summarize the conclusions (Figure 7K). Briefly, our study identified GR as a potent inducer of EMT. GR promotes breast cancer progression as the GR levels correlate with increased metastasis, reduced survival rates, and poor prognosis in breast cancer patients. IRS-1 is a downstream molecular target of GR and its suppression by GR activates ERK2 and induces EMT. The GR–IRS-1 axis plays essential role in the regulation of EMT and metastatic lung colonization of breast cancer cells. GR–IRS-1 signaling can be modulated by alterations in the level of either GR or IRS-1. Thus, modulation of GR–IRS-1 signaling is important in understanding the biological functions and potential therapeutic significance of GR. Although IRS-2 has also been identified as a different downstream target of GR, its molecular function remains to be identified.

Materials and methods

Reagents and antibodies

TGF-β1, TSA, TNF-α, paclitaxel, 5-FU, and insulin were purchased from Sigma-Aldrich. Lentivirus system plasmids pCDH-CMV-MCSEF1-Puro, psPAX2, pMD2.G and pLKO.1-TRC were purchased from Addgene. Antibody information is listed in Supplementary Table S1.

Cell culture

All the cells used in this study were purchased originally from ATCC. MCF7 cells were maintained as described in ATCC. Briefly, cells were cultured in Modified Eagle Medium (MEM) supplemented with 10 μg/ml insulin, 10% FBS, and 1% penicillin-streptomycin. MDA-MB-453 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and 1% penicillin–streptomycin. All cells were cultured in a humidified atmosphere with 5% CO_2 incubator at 37°C.

Plasmid construction

The primers used to amplify GR and IRS-1 are listed in Supplementary Table S2. GR and IRS-1 were generated by ligating the full-length open-reading frame into the pCDH-IRES-GFP and PCDH-CMV vectors separately. shRNAs (shGR, shIRS-1, and shERK2) construct were generated by pLKO.1-TRC. A scrambled sequence served as the control shRNA. The optimal targeting sequences identified for each gene are shown in Supplementary Table S3. For IRS-1 promoter reporter plasmids, pGL 3.0-luciferase was used as the vector, and the promoter regions were amplified using the primers shown in Supplementary Table S4.

Stable transfection

Package of high-titer lentivirus in 293T and cell transfection were conducted as previously described (Wang et al., 2017).

Quantitative real time PCR (qPCR)

qPCR assays were conducted as previously described (Yuan et al., 2013). All values were normalized against the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primer sequences are shown in Supplementary Table S5.

Immunoblotting

Cell lysates were prepared and western blots were performed as previously described (Wang et al., 2010). β-actin or tubular was used as a loading control.

Analysis of cell death and survival

Cell death was performed with an Annexin V-FITC/PI apoptosis detection kit (Vazyme) (Liu et al., 2017), and cell survival was examined using CCK-8 counting kit (Yeasen), according to the manufacturer's instructions.

Wound healing migration assay

The assay was performed as previously described (Yuan et al., 2013).

Trans-well migration and invasion assays

These assays were performed as previously described (Tang et al., 2011, 2015) using a modified Boyden chamber (Costar). The trans-well cells were fixed with methanol, stained with hematoxylin and eosin (H&E), photographed under an inverted microscope, and quantified by counting the number of stained cells from five random fields (100×).

Immunofluorescent staining

The assay was performed as previously described (Tang et al., 2015). F-actins and the nuclei were stained with phalloidin (Invitrogen, A22281) and 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen, P-36931), respectively, according to the vendor's instructions. The fluorescence was visualized under a confocal microscopy (Leica TCS SP5 MP).

Chromatin immunoprecipitation (ChIP)

The assay was performed with an EZ-Zyme Chromatin Prep Kit (Millipore) according to the manufacturer's protocol (Tang et al., 2015). Five sets of primers that cover IRS-1 promoters are shown in Supplementary Table S6.

Gene reporter luciferase assay

MCF7 and MDA-MB-231 cells were pre-transfected with plasmids to overexpress or knockdown GR, then pGL-IRS-1-promoter-luciferase plasmids (range from −2091 bp to +241 bp) were co-transfected with a Renilla luciferase plasmid (pRL-CMV from Promega). Cells were collected 36 h after the transfection and the luciferase activities were measured by using the Dual-luciferase Reporter Assay System (Promega).

Tail vein injection and in vivo metastasis analysis

Mouse care and treatment were conducted as previously described with minor modifications (Tang et al., 2015). Tumor cells (1×10^6) were injected intravenously into six-week-old BALB/c (SLAC) nude mice. The lungs were resected and photographed. The number of metastatic nodules on the lung surface was counted.

Examination of blood CTCs

Freshly isolated blood was lysed to remove red blood cells, total RNA was extracted and reverse transcribed using ReverTra Ace-α (Toyobo). The relative number of CTCs was determined by comparing the amount of human *GAPDH* expression to mouse *GAPDH* expression using qPCR. The primers used in this experiment are shown in Supplementary Table S5.

Histology and immunohistochemistry (IHC) staining

IHC of human breast cancer tissues was examined using SPlink Detection Kits (SP-9001, ZSGB-BIO) according to the manufacturer's instructions. The bound antibody was revealed by DAB kit (ZLI-9018, ZSGB-BIO) and the nucleus was stained with haematoxylin.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Care and use Committee at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Human breast cancer tissues were purchased from Outdo Biotechnology and the product ID is HBre-Duc150Sur-01. The archived samples from Shanghai National Engineering Research Center for the biological chip assay were anonymous, and informed consent was not required.

H-score quantification

The H-score method assigned a score of 0–12 to each sample based on the percentage of cells stained at different intensities as viewed under a microscope. The discriminatory threshold was set at 7, and H-score ≥ 7 were considered as GR high, while H-score < 7 were considered as GR low.

Statistical analysis

Statistics are expressed as the mean ± SD with Student's *t*-test and ANOVA. *P*-values < 0.05 were considered statistically significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s. non-significance. Gene expression correlations were determined using the Pearson coefficient. The Kaplan–Meier and Log-rank tests were used for analysis of survival rates.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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