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Twist induces epithelial-mesenchymal transition and cell motility in breast cancer via ITGB1-FAK/ILK signaling axis and its associated downstream network

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ABSTRACT

Twist, a highly conserved basic Helix-Loop-Helix transcription factor, functions as a major regulator of epithelial-mesenchymal transition (EMT) and tumor metastasis. In different cell models, signaling pathways such as TGF-β, MAPK/ERK, WNT, AKT, JAK/STAT, Notch, and P53 have also been shown to play key roles in the EMT process, yet little is known about the signaling pathways regulated by Twist in tumor cells. Using iTRAQ-labeling combined with 2D LC–MS/MS analysis, we identified 194 proteins with significant changes of expression in MCF10A-Twist cells. These proteins reportedly play roles in EMT, cell junction organization, cell adhesion, and cell migration and invasion. ECM–receptor interaction, MAPK, PI3K/AKT, P53 and WNT signaling were found to be aberrantly activated in MCF10A-Twist cells. Ingenuity Pathways Analysis showed that integrin β1 (ITGB1) acts as a core regulator in linking integrin-linked kinase (ILK), Focal-adhesion kinase (FAK), MAPK/ERK, PI3K/AKT, and WNT signaling. Increased Twist and ITGB1 are associated with breast tumor progression. Twist transcriptionally regulates ITGB1 expression. Over-expression of ITGB1 or Twist in MCF10A led to EMT, activation of FAK/ILK, MAPK/ERK, PI3K/AKT, and WNT signaling. Knockdown of Twist or ITGB1 in BT549 and Hs578T cells decreased activity of FAK, ILK, and their downstream signaling, thus specifically impeding EMT and cell invasion. Knocking down ILK or inhibiting FAK, MAPK/ERK, or PI3K/AKT signaling also suppressed Twist-driven EMT and cell invasion. Thus, the Twist-ITGB1-FAK/ILK pathway and their downstream signaling network dictate the Twist-induced EMT process in human mammary epithelial cells and breast cancer cells.

1. Introduction

Invasiveness is a feature of malignant tumor cells, which reflects their dissemination ability from the primary site to distant organs to form metastases. The epithelial-mesenchymal transition (EMT) process is required for tumor cell invasion and metastasis in many solid tumor types (Yang et al., 2004; Kouso et al., 2013; Xu et al., 2013; Liu et al., 2014). During the EMT process, polarized epithelial cells lose the adherent and tight cell junctions, and become more migratory and invasive.

EMT can be triggered or mediated by several transcription factors including Snail, Twist, Slug, and ZEB1 (Martin et al., 2005). It can also be induced by some of the cellular secreting factors, such as transforming growth factor beta1 (TGF-β1), bone morphogenetic protein 4 (BMP4), vascular endothelial cell growth factor (VEGF) (Boyer et al., 1999; Xu et al., 2011; Desai et al., 2013), inflammatory factors (e.g. IL-8) (Bates et al., 2004), hypoxia associated factors (e.g. HIF-1) (Yang et al., 2008), and cell junction proteins (e.g. Claudin-1) (Stebbing et al., 2013).

Twist (also called Twist1) is a critical transcriptional factor essential for neural tube formation, cell migration, and differentiation in embryonic development (Perez-Pomares and Munoz-Chapuli, 2002). Twist has been found to induce EMT in different cells and tumor tissues, including murine isogenic breast

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cancer (Yang et al., 2004), prostate cancer (Yuen et al., 2007), hepatocellular carcinoma (HCC) (Yang et al., 2009), gastric cancer (Feng et al., 2009), oesophageal squamous cell carcinoma (Sasaki et al., 2009), and bladder cancer (Wallerand et al., 2010). Oncogenes such as RAS, ErbB2, and c-Myc can elicit EMT in mammary tumors by up-regulating Twist (Liu et al., 2009). STAT3 and HIF-1alpha up-regulate Twist expression to induce EMT in prostate cancer by direct binding to the TWIST1 promoter (Cho et al., 2013). Epidermal growth factor (EGF) or TGF-β1 treatment also increases Twist1 to modulate EMT in HCC cells (Wang et al., 2013a,b). Twist regulates EMT through miRNA10b, AKT2, IL-8 (Ma et al., 2007), MMPs, TIMP, E-cadherin, and Vimentin (Okamura et al., 2009; Zhao et al., 2011; Smith et al., 2013). In addition, experiments have shown that Twist plays an essential role in cancer metastasis through different signaling pathways, such as AKT signaling in breast cancer (Zhang et al., 2015), WNT in cervical cancer (Li and Zhou, 2011), VEGF in HCC (Niu et al., 2007), and P53 in gastric cancer cells (Feng et al., 2009). Thus, induction and regulation of EMT by Twist may involve multiple molecular mechanisms in different cells and tumor tissues. Here we intended to address which signaling pathways are critically involved in Twist-induced EMT in Twist-positive breast cancer and how Twist can regulate these signaling in the EMT process.

In this study, we discovered that a signaling network including FAK/ILK signaling, MAPK/ERK, PI3K/AKT, WNT, and P53 is critical for Twist-induced EMT in human mammary epithelial cells using iTRAQ-based proteomic analysis. Notably, integrin β1 (ITGB1) was found to act as a core regulator in this network.

2. Materials and methods

2.1. Cell culture, reagents, plasmids, retroviral infections and reporter assays

MCF10A cells and the breast cancer cells (including T47D, MCF7, BT474, MDA-MB-453, MDA-MB-468, MDA-MB-231, BT549 and Hs578T) used in this study were obtained from ATCC. MCF10A cells were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 5% horse serum (Invitrogen) as previously described (Liu et al., 2009). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Gibco). Breast cancer cells were cultured in RPMI-1640 medium (Invitrogen) with 10% FBS (Gibco). The signaling-specific inhibitors PD98059 (Cell signaling Technology) (5 μM) for MAPK, LY294002 (Cell signaling Technology) (10 μM) for PI3K/AKT, and PF-562271 (Selleck Chemicals) (5 μM) for FAK were used in this study as described in the Figure legends.

The retroviral expression vectors encoding c-Myc-tagged Twist (Liu et al., 2009) and the human ITGB1 (NM_002211) purchased from Origene (http://www.origene.com) were subcloned into pBABE-Puro-based vector. The viral supernatant was prepared as previously described (Liu et al., 2009) to establish Twist- or ITGB1-overexpressing cells. Infectious lentivirus encoding ITGB1 shRNAs were produced and used in MCF10A-Twist, BT549 and Hs578T cells. The small interfering RNAs (siRNAs) against Twist and ILK, and non-targeting siRNA acquired from Dharmacon were listed in Supplemental Table 1.

The ITGB1 promoter was inserted into pGL3-based reporter vector to get ITGB1-Luc constructor. For luciferase reporter assays, pGL3-Luc or ITGB1-Luc reporter and the expression vector encoding ITGB1 or its control vector were co-transfected into HEK-293; MCF10A-Twist, Hs578T, and BT549 cells or their corresponding Twist-silenced cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The medium was changed after 5 h, and luciferase activity was determined after 24 h. The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the Renilla luciferase activities.

2.2. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells or tissue samples with Trizol (Invitrogen) according to the manufacturer's protocol. The primers used for each gene were listed at Table 1. qRT-PCR was carried out using SYBR Premix Ex Taq™ II (Takara, Dalian, China) and a Bio-Rad CFX Manager instrument (Applied Biosystems). Actin was used as internal control for normalizing different samples. All experiments were performed at least three times.

2.3. iTRAQ-based quantitative proteomic analysis

Cells were cultured to 80% confluence and protein extraction was performed using standard RIPA buffer with 1 mM of phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (Sigma). Protein samples were digested with sequencing grade modified trypsin (Promega Corp) (Maurer et al., 2014). The tryptic peptides were labeled with an iTRAQ-8 plex kit (Applied Biosystems, USA) according to the manufacturer's protocol. Specifically, proteins from MCF10A-Twist cells were labeled with 113 and 115 tags, while those from MCF10A-Vector cells were labeled with 114 and 116 tags. The labeled peptide mixture was fractionated with a strong cation exchange column (SCX) (Thermo Scientific) into 12 fractions.

The lyophilized SCX fractions were re-dissolved in 2% acetonitrile, 0.1% formic acid, and loaded on ChromXP C18 (3 μm, 120 Å) nanoLC trap column. The online trapping and desalting procedures were carried out at 2 μL/min for 10 min with 100% solvent A and then with solvent B. Solvents were composed of water/acetonitrile/formic acid (A, 98/2/0.1%; B, 2/98/0.1%). Then, an elution gradient of 5–35% acetonitrile (0.1% formic acid) in 90 min gradients was used on an analytical column (75 μm × 15 cm C18-3 μm 120 Å, ChromXPEk sigent). LC–MS/MS analysis was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON). Data was acquired using an ion spray voltage of 2.4 kV, curtain gas of 30 PSI, nebulizer gas of 5 PSI, and an interface heater temperature of 150 °C. The MS was operated with TOF-MS scans. For IDA, survey scans were acquired in 250 ms, and as many as 30 product ion scans (80 ms) were collected if exceeding a threshold of 150 counts per second (counts/s) and with a +2 to +5 charge-state. A Rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (~12 s).

The raw MS data in wiff format was submitted to ProteinPilot software v4.5 (AB Sciex, USA) for protein identification and quantification analysis. The global false discovery rate was estimated, with the integrated PSPEP tool in the ProteinPilot Software, to be 1.0% at both protein and peptide level after compared with human proteome set database (release 10/2013, 44,166 entries) and an automatic "Decoy Database" downloaded from http://www.uniprot.org/uniprot/. Search parameters were set as follow: sample type of iTRAQ-8 plex (peptide labeled), cysteine modified with iodoacetamide, trypsin digestion, thorough searching mode, and minimum protein threshold of 95% confidence (unused protein score >1.3). For this study, proteins with <2 distinct peptides (95% confidence) or error factor (EF) >2.0 were eliminated to improve the confidence of protein quantitation. Peptide summaries were exported from ProteinPilot, and isotope correction and relative quantification were calculated. Based on the biological variation, the observed proteins with iTRAQ ratios below 0.83 were considered to be reduced in expression, and the proteins with iTRAQ