FOXA2 attenuates the epithelial to mesenchymal transition by regulating the transcription of E-cadherin and ZEB2 in human breast cancer

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ARTICLE INFO
Article history:
Received 28 January 2015
Received in revised form 9 March 2015
Accepted 9 March 2015
Keywords:
FOXA2 transcription factor
EMT
E-cadherin transcription
ZEB2 transcription
Breast cancer

ABSTRACT
The Forkhead Box A2 (FOXA2) transcription factor is required for embryonic development and for normal functions of multiple adult tissues, in which the maintained expression of FOXA2 is usually related to preventing the progression of malignant transformation. In this study, we found that FOXA2 prevented the epithelial to mesenchymal transition (EMT) in human breast cancer. We observed a strong correlation between the expression levels of FOXA2 and the epithelial phenotype. Knockdown of FOXA2 promoted the mesenchymal phenotype, whereas stable overexpression of FOXA2 attenuated EMT in breast cancer cells. FOXA2 was found to endogenously bind to and stimulate the promoter of E-cadherin that is crucial for epithelial phenotype of the tumor cells. Meanwhile, FOXA2 prevented EMT of breast cancer cells by repressing the expression of EMT-related transcription factor ZEB2 through recruiting a transcriptional corepressor TLE3 to the ZEB2 promoter. The stable overexpression of FOXA2 abolished metastasis of breast cancer cells in vivo. This study confirmed that FOXA2 inhibited EMT in breast cancer cells by regulating the transcription of EMT-related genes such as E-cadherin and ZEB2.

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Introduction
Epithelial to mesenchymal transition (EMT) is a central, conservative process in which embryonic development is regulated based on specification and differentiation [1]. Typical features associated with EMT include dramatic changes in cellular morphology, loss and remodeling of cell–cell and cell–matrix adhesions, and gain in migratory and invasive capabilities. Additionally, EMT is involved in embryonic implantation, gastrulation and neural crest cell motility [2], wound healing and tissue regeneration [3], and inflammation and fibrosis [4,5]. EMT is also considered to be one of the important mechanisms regulating the progression of variable solid tumors, particularly for the invasive behavior of cancer cells [6]. In the prophase of cancer metastasis, EMT represents the conversion of differentiated epithelial cancer cells into migratory mesenchymal cancer cells, leading to cancer invasion, systemic cancer cell dissemination, and metastasis. EMT involves the loss of epithelial markers, such as the junction protein E-cadherin, as well as the concurrent acquisition of mesenchymal markers, such as vimentin [7]. Recent gene expression profiling experiments in various experimental cellular systems of EMT suggested that many genes significantly change in their expression during EMT [8].

Furthermore, EMT plays critical roles in the progression, especially metastasis, of breast cancer [9]. Breast cancer is the leading cause of mortality among women, and remains a public health issue on a global scale [10]. EMT also allows breast cancer cells to avoid apoptosis [11,12] and cellular senescence [13]. Moreover, EMT participates in the generation and maintenance of breast cancer stem cells [14–16], which is highly consistent with the notion that metastatic cells carry the ability to initiate new tumors [17,18]. EMT in breast cancer is controlled by a network of signaling pathways from various growth factors (i.e., epidermal growth factor (EGF) [19,20], transforming growth factor beta (TGFβ) [21–23], and FGF2 [24]). Growth factor signaling generally induces the expression of many transcription factors that are essential for EMT, such as ZEB1, ZEB2, Snail, Slug, β-catenin, and Twist [25–27], which repress the expression of E-cadherin and activate the mesenchymal transcriptionomes [8].

Transcription factor forkhead box protein A2 (FOXA2; also named HNF-3β) belongs to the forkhead/winged-helix family of transcription factors [28]. FOXA2 is involved in embryonic development, and broadly expresses and functions in multiple adult tissues from three
FOXA2 protein acts as a “pioneer” factor in transcriptional regulation because of its ability to bind to highly compacted chromatin [48], and it facilitates the binding of other proteins to the target genes [49,50]. In most cases, FOXA2 is revealed to activate the expression of its target genes. Nonetheless, a subset of FOXA2 bindings also results in the repression of its target gene transcription. For example, FOXA2 can bind to and repress the mesenchymal transcription factor Slug promoter in lung cancers [47]. FOXA2 protein was recently reported to interact with TLE3 [51], a transcriptional corepressor of the Gro/TLE/Grg family, whose complex with specific DNA-binding proteins causes gene repression [52], providing a mechanism of FOXA2-mediated transcriptional repression.

In the current study, we found that FOXA2 prevented EMT in human breast cancer. A strong correlation was confirmed between the expression levels of FOXA2 and the epithelial phenotype of breast cancer cells. Knockdown of FOXA2 promoted the mesenchymal phenotype, whereas stable overexpression of FOXA2 attenuated EMT in breast cancer cells. Our evidence suggested that FOXA2 stimulated the proliferation of epithelial phenotype-related E-cadherin, and repressed the expression of EMT-related transcription factor ZEB2 by recruiting a transcriptional corepressor TLE3 to the ZEB2 promoter. Furthermore, the stable overexpression of FOXA2 abolished the metastasis of breast cancer cells in vivo. This study confirmed that FOXA2 inhibited EMT in breast cancer cells by regulating the transcription of EMT-related genes, such as E-cadherin and ZEB2.

Materials and methods

Cancer clinic tissue samples

A total of 39 breast cancer tissues were collected at Hunan Provincial Tumor Hospital (Changsha, China). The human tissues were obtained and studied in strict accordance with the protocol approved by Hunan Provincial Tumor Hospital Review Board.

Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the Chinese Academy of Sciences Cell Bank. The cells were maintained in DMEM containing 10% fetal bovine serum. MCF-10A was purchased from ATCC, USA and cultured with a MEGM BulletKit (Catalog No. CC-3150, Lonza/Clonetuc Corporations, Switzerland), in which GA-1000 (gentamycin–amphotericin B mix) was not used and 100 ng/ml cholina toxin (Sigma, USA) was added instead. For the EGF-induced EMT, MCF-10A cells were treated with 100 ng/ml EGF (Invitrogen, USA).

Western blot assays

To measure protein levels, lysates were resolved by denaturing gel electrophoresis and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with primary antibodies against proteins of interest. The signals from the primary antibodies were amplified by horseradish peroxidase-conjugated anti-mouse IgG (1:10,000; GELNA931VD, USA) or anti-rabbit IgG (1:10,000; GELNA934VAE, USA) and detected with Enhanced Chemiluminescence Plus (Beyotime, China). The following antibodies and dilutions were used: rabbit anti-FOXA2 (1:3000; Abcam ab23738, UK), mouse anti-E-cadherin (1:1000, Abcam ab1416, UK), rabbit anti-Vimentin (1:1000; Abcam ab27608, UK), rabbit anti-FOXAX1 (1:3000; Abcam ab237438, UK), rabbit anti-TLE3 (1:3000; Santa Cruz sc-9124, USA), rabbit anti-V5 (1:1000, Millipore ab3792, USA), rabbit anti-ZEB2 (1:3000; Abcam ab258373, UK), and mouse anti-β-actin (1:5000; Beyotime AA128, China).

Transwell assays

Cell migration assays were performed by using Transwell migration chambers (8 μm pore size; Corning, USA) according to the vendor’s instructions. Briefly, the cells were trypsinized and 1 × 105 cells were plated into the insert of the well. Twenty-four hours later, the cells in the insert of each well were removed and the cells under the bottom of the well were stained by 0.1% hexamethylenetetramine. All experiments were repeated three times. Representative photos were taken using a TE2000 microscope (Nikon Instruments Inc, Japan) (10×) or SMZ1500 stereomicroscope (Nikon Instruments Inc, Japan) (10×). The digital pixel densitometry from at least three different photos was measured with Image-J software (NIH, USA).

Total RNA isolation, blood RNA isolation, quantitative real-time PCR (qPCR) and RT-PCR

Total RNAs from cells and blood samples were prepared with Total RNA Kit I (Omega, USA) and blood RNAs from mice were isolated by the Blood RNA Kit (Omega, USA), according to the manufacturer’s protocols. The CDNAs were synthesized with M-MLV Reverse Transcriptase (Invitrogen, USA) from RNA samples (2 μg) to get 40 μl cDNAs. Quantitative real-time PCR (qPCR) was performed by using SYBR Green (Roche, Switzerland) in the realplex qPCR system (Eppendorf, Germany) with 0.8 μl cDNAs as templates in each reaction. The sequences for sense (S) and antisense (AS) primers are as follows: hFOXA2-5′-AAG ACC ACC AGG ACC GTG TA-3′ and hFOXA2-AS-5′-CCT TCA GGA AAC AGT CTT GC-3′, hE-cadherin-5′-CCG GAA TGC AGT TGA GGA TC-3′ and hE-cadherin-AS-5′-AGG AGT GTC TAAGC ATG GC-3′, hVimentin-5′-GAC GAC TTT GGC GAA GC-3′ and hVimentin-AS-5′-GCT TCC TGT TGG TCA TC-3′, hFOXA2-5′-GGA A TA ATC TTC TTA CCT GC-3′ and hFOXA1-AS-5′-TAC ACA CCT TGG TAC GAC-3′, hZEB2-5′-AGG CAG GGT GCA CAG GCA-3′ and hZEB2-AS-5′-AGG CAG GAT CCA CAA TTG-3′. All qPCR experiments were repeated at least three times.

Transfection of siRNA

Human FOXA2 siRNA (sc-35569), human TLE3 siRNA (sc-36684), human ZEB2 siRNA (sc-38641) and control siRNA (sc-37007) were purchased from Santa Cruz, USA. Human FOXA2 siRNA was used as a smart pool of three different siRNAs: hFOXA1-S, 5′-GCA ATA CTC GCC TTA CGG CT-3′ and hFOXA1-AS, 5′-TAC ACA CCT TGG TAC GAC-3′; hZEB2-S, 5′-GCA ATA CTC GCC TTA CGG CT-3′ and hZEB2-AS, 5′-GCA ATA CTC GCC TTA CGG CT-3′; hVimentin-S, 5′-GCA ATA CTC GCC TTA CGG CT-3′ and hVimentin-AS, 5′-GCA ATA CTC GCC TTA CGG CT-3′. The annealing temperature (Ta) and number of PCR cycle (N) for RT-PCR; hTLE3: Ta 56 °C, N 35; hE-cadherin: Ta 58 °C, N 32; hvimentin: Ta 57 °C, N 33; hGAPDH: Ta 58 °C, N 26.

Lentivirus construction and infection

The FOXA2 cDNA or Firefly Luciferase cDNA was cloned in front of EGFP cDNA separated by an IRE sequence in the lentivirus plasmid vector described previously [53]. The constructed lentivirus plasmid vector (pLV-EF1αp-Luc-IRES-EGFP or pLV-EF1αp-FOXA2-ires-EGFP) was cotransfected into 293T cells with two packaging plasmids (pVSVG and Δ8.91) by calcium phosphate transfection to produce lentiviruses. Forty-eight hours post transfection, the medium of 293T was collected and the titration of the virus was measured by Flow cytometry. MDA-MB-231 cells were infected with the lentivirus (20 pfu/cell) containing either FOXA2 cDNA or Firefly Luciferase cDNA to establish cell lines stably expressing FOXA2 or Firefly Luciferase (Control).

Electrophoretic mobility shift assays (EMSA)

FAM-labeled double-strand DNA oligonucleotides were synthesized by Sangon (Shanghai), Co., Ltd, China, based on the sequence 5′-CAAC ACA AAC AAA AAA AAA ATT-3′ from Me-cadherin upstream region (−691 bp to −669 bp) and the sequence 5′-CGT TGT TGT TGT TGT TGT TGT GC-3′ from hZEB2 upstream region (−517 bp to −498 bp), which contained the FOXA2 consensus binding sites. In the binding reactions, 10 μg of nuclear proteins isolated from FOXA2-expressing MCF-7 cells was incubated with 1 pmol of the FAM-labeled probe and 2 μl of 5× binding buffer (Beyotime, China) in a total volume of 10 μl for 30 min at room temperature. The reactions were resolved in 4% native polyacrylamide gel electrophoresis in 0.5× TBE. The dose chosen for the competitive experiments was in the 100× molar excess of the unlabeled oligonucleotides. The oligonucleotides mutated in FOXA2 binding sites (Mut probes) 5′-CAAC ACA AAC AAA AAA AAA ATT-3′ for Me-cadherin or 5′-AGG CAG GCA AGA GAC-3′ for ZEB2-FOX2.
GGC G-3′ for hzE2B) were also used as controls in EMSA experiments. For the super-shift analysis, 1 μg of anti-FOX2 antibody (Abcam ab23738, UK) was added to the binding reaction.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described [54]. The following antibodies were used for immunoprecipitation: rabbit anti-FOX2 (Abcam ab23738, UK), rabbit anti-V5 (Millipore ab3792, USA), rabbit anti-IgG (Millipore PP64, USA). For immunoprecipitation, 2 μg of each interested antibody was used. The ChIP DNA sample or 1% total input (5 μl) was used in qPCR with the following primers: hE-cadherin upstream –704 bp forward: 5′-AAA AAT ACA ACA AAA AAC AAC A A-3′ and –586 bp backward: 5′-TCC TGG GAA GGC GTG-3′; hE-cadherin upstream –1892 bp forward: 5′-GCC CCA CTT TGG GAG GCC AC-3′ and –1862 bp backward: 5′-CCA CGA CGA CTG CCT AAT TT-3′; hZEB2 upstream –1746 bp forward: 5′-GCC TGG TCC CTC TCT TGA GTC CA-3′ and –1611 bp backward: 5′-AAA TCT GAA AAT AAT-3′.

Co-immunoprecipitation (Co-IP)

For Co-IP, MCF-7 cells were plated in 10-cm dishes and transfected with CMV-FOX2 expression plasmid (10 μg), CMV-V5-g3 expression plasmid (10 μg), or both. Two days later, the cells were collected, resuspended in 500 μl of lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl pH 7.4, protease inhibitor cocktail) and incubated for 20 min on ice. The lysates were centrifuged for 15 min at 14,000 g at 4 °C and the supernatant containing 500 μg of proteins was incubated with 2 μg anti-V5 antibody (Millipore ab3792, USA) overnight. Twenty μl of protein G crosslinked agarose beads was added to the sample and incubated for 1 h. The agarose beads were centrifuged and washed 4 times with the lysis buffer. The washed beads were subjected to SDS-PAGE followed by immunoblotting with anti-FOX2 (1:1000; Abcam ab23738, UK) and anti-V5 (1:1000; Millipore ab3792, USA).

Luciferase assays

The regions of human E-cadherin or ZEB2 promoter were PCR amplified from human genomic DNA with the following primers: hE-cadherin promoter –233 bp to +52 bp: Forward 5′-CCG CTC CAT GAG GGC CCC TTC TTT TTC CCG CT-3′ and Backward 5′-CCC AAT CCT CTT CCG CAA CAC AGG-3′; hE-cadherin promoter –733 bp to +60 bp: Forward 5′-CCG CTC CAT GAG GGC CCC TTC TTT TTC CCG CT-3′ and Backward 5′-CCC AAT CCT CTT CCG CAA CAC AGG-3′; hZEB2 promoter –885 bp to +66 bp: Forward 5′-CCG CTC CAT GAG GGC CCC TTC TTT TTC CCG CT-3′ and Backward 5′-CCC AAT CCT CTT CCG CAA CAC AGG-3′.

For luciferase assays, MCF-7 cells were transfected with 1.5 μg of different Luciferase reporter constructs and a certain amount of CMV-FOXA2, CMV-V5-TE3, or CMV-E-cadherin promoter plasmids. The luciferase activities were determined by using Promega Luciferase assay System (Promega, USA) and were used to measure luciferase enzyme activity following the manufacturer’s instruction.

Animal experiments

All animal experiments were conducted in accordance with institutional animal care and use guidelines, following approval by the Laboratory Animal Center of Hunan, China. For genotyping, the mice were bred and kept in a specific pathogen-free environment as described previously [55]. The wild-type and FOXA2-depleted mice were sacrificed by cervical dislocation, and the tissues were snap-frozen in liquid nitrogen and stored at –80 °C for further use. The experiments were conducted in accordance with institutional animal care and use guidelines, following approval by the Laboratory Animal Center of Hunan, China. All animal experiments were conducted in accordance with institutional animal care and use guidelines, following approval by the Laboratory Animal Center of Hunan, China.

Results

Expression levels of FOXA2 correlated with the epithelial phenotype of breast cancer cells

We first measured the protein levels of FOXA2, epithelial marker E-cadherin, and mesenchymal marker vimentin in the lyses of clinical human breast ductal carcinoma samples (total case number = 39). We found that the expression of FOXA2 was positively correlated with E-cadherin expression, but negatively correlated with vimentin expression (Figs. 1A and S1). We also found that E-cadherin expression and the epithelial phenotype of breast cancer cells was further confirmed by the EGF-induced EMT of the MCF-10A breast epithelial cell model (Fig. S2), in which the levels of FOXA2 mRNA and protein were downregulated during EMT progression, corresponding to the decreased E-cadherin expression and increased vimentin expression (Fig. 1B and C). We also used an epithelial-type breast cancer cell line MCF-7 and mesenchymal-type breast cancer cell line MDA-MB-231 to test whether the expression levels of FOXA2 vary between epithelial-type and mesenchymal-type breast cancer cells. MCF-7 cells exhibited low levels of the migration ability compared with MDA-MB-231 cells (Fig. S3). The expression of FOXA2, E-cadherin, and vimentin was measured in the two cell lines by qPCR and Western blot. Increased levels of FOXA2 mRNA and protein were found in epithelial-type MCF-7 cells (Fig. 1D and E).

FOX2 inhibited the migration of breast cancer cells

To test whether FOX2 is required for the epithelial phenotype of breast cancer cells, we used FOX2-specific siRNA to repress the expression of FOXA2 in MCF-7 cells. MCF-7 cells were transfected with control siRNA or FOX2 siRNA (200 nM). The knockdown of FOXA2 decreased the expression of FOXA2, E-cadherin, and vimentin in the lysates of cancer cell samples (Fig. S5). The expression of FOXA2, E-cadherin, and vimentin was measured in the two cell lines by qPCR and Western blot. Elevated levels of FOXA2 mRNA and protein were found in epithelial-type MCF-7 cells (Fig. 1D and E).

Animal experiments

All animal experiments were conducted in accordance with the institutional animal care and use guidelines, following approval by the Laboratory Animal Center of Hunan, China (protocol No. SYXX [Changsha] 2008-0001). 8 Balb/c nude mice (Female, 4-week old) were purchased from Slac Experimental Animal Company (Changsha, China). To generate mouse models of metastasis of breast cancer cells in vivo, MDA-MB-231 cells expressing Firefly Luciferase or MDA-MB-231 cells stably overexpressing FOXA2 were injected into the tail vein of each mouse (2 × 105 cells/mouse). At day 1, day 35, or day 60 post injection, the mice (n = 6, each group) were sacrificed and blood samples or tissue samples were collected. The total RNAs of blood samples or tissue samples were isolated and the relative concentration of human tumor cells in blood or in lung was determined by qPCR for the mRNA levels of human specific CYCLOPHILIN over the mRNA levels of mouse specific Cyclophilin. The lysates of tissue samples were prepared for Western blotting. The collected lung tissues were fixed overnight in 4% PFA and embedded in paraffin. Sections were stained with hematoxylin and eosin dyes.

Statistical analysis

We used Microsoft Excel Program to calculate SD and statistically significant differences between samples. The asterisks in each graph indicate statistically significant changes with P values calculated by Student T Test: *P < 0.05, **P < 0.01, and ***P < 0.001. P values < 0.05 were considered statistically significant. The data of clinic cancer tissue samples (Fig. 1A) and the numbers of tumor metastasized in the lung (Fig. 1C) were analyzed with SPSS 15.0 (IBM, USA) and Windows 7 (Microsoft, USA).
promotes EMT [56]. In the present study, we found that the expression of E-cadherin relied on the FOXA2 levels in breast cancer cells (Fig. 2). Multiple putative FOXA binding sites were predicted in the 2 kb human E-cadherin promoter (in regions 691–669 bp and −341 bp to −317 bp), and ChIP assays confirmed that FOXA2 bound to endogenous E-cadherin promoter in MCF-7 cells (Fig. 3A). To further confirm the binding of FOXA2 at the E-cadherin promoter, we performed EMSAs with a FAM-labeled DNA probe synthesized from the E-cadherin promoter region from −691 bp to −669 bp and nuclear extracts containing FOXA2 proteins. We found that the probe could form a DNA/protein complex in EMSAs with FOXA2 protein, and the addition of either an unlabeled probe (100×) or FOXA2-specific antibody disturbed the formation of the FOXA2/DNA complex or resulted in a supershift complex, whereas the FAM-labeled mutated probe could not form the FOXA2/DNA complex (Fig. 3B). Consistent with this finding, the −733 bp to +60 bp E-cadherin promoter region could significantly mediate the transcriptional activity in FOXA2 expression vector-transfected MCF-7 cells compared with the control −233 bp to +52 bp E-cadherin promoter region containing non-putative FOXA binding sites (Fig. 3C). Thus, these results demonstrated that the E-cadherin gene was one of the direct transcriptional targets of FOXA2 in breast cancer cells.

**FOXA2 repressed the expression of ZEB2 in breast cancer cells**

As one of the representative inducers of EMT, ZEB2 is expressed in various types of human tumors, such as breast cancer [57], gastric cancer [58], and pancreatic cancer [59]. ZEB2 stimulates EMT by repressing the transcription of epithelial marker genes, such as E-cadherin [57], and upregulating the transcription of mesenchymal markers [60]. Interestingly, we found that the expression of ZEB2 was negatively correlated with that of FOXA2 in breast cancer cells. The overexpression of FOXA2 with Lv-FOXA2 decreased ZEB2 expression at mRNA and protein levels in MDA-MB-231 cells (Fig. 4A), whereas the knockdown of FOXA2 with FOXA2 siRNA increased ZEB2 in MCF-7 cells (Fig. 4B). These findings suggested that the expression of ZEB2 was negatively correlated with FOXA2 expression in breast cancer samples (Fig. S6). Multiple putative FOXA binding sites were predicted in the −2 kb human E-cadherin promoter.
human ZEB2 promoter (in regions −765 bp to −748 bp and −517 bp to −498 bp). ChIP assays confirmed that FOXA2 bound to the endogenous ZEB2 promoter in MCF-7 cells (Fig. 4C). EMSAs were performed to further confirm the binding of FOXA2 at the ZEB2 promoter with a FAM-labeled DNA probe synthesized from the ZEB2 promoter region from −517 bp to −498 bp and nuclear extracts containing FOXA2 proteins. We found that the probe could form a DNA/protein complex in EMSAs with FOXA2 protein. The addition of either an unlabeled probe (100×) or FOXA2-specific antibody disturbed the formation of a FOXA2/DNA complex or resulted in a supershift complex, whereas the FAM-labeled mutated probe could not form the FOXA2/DNA complex (Fig. 4D). Furthermore, a luciferase reporter plasmid containing the fragment of −885 bp to +66 bp of ZEB2 promoter was transfected with different amounts of CMV-FOXA2 expression vector into MCF-7 cells, and this ZEB2 promoter was repressed by FOXA2 in a dosage-dependent manner in the cells (Fig. 4E). Thus, these results implied that the transcription of ZEB2 was negatively regulated by FOXA2 in breast cancer cells. The idea that FOXA2 prevented EMT by repressing ZEB2 expression was further supported by the data of ZEB2 overexpression

![Image of graphs and diagrams related to the text]
FOXA2 bound to and stimulated the E-cadherin promoter. (A) FOXA2 bound to the endogenous E-cadherin promoter. Gene sequence analysis was performed to predict positions of putative FOXA2 binding sites in ~2 kb human E-cadherin promoter, and design the primers for ChIP assays. The chromatin of MCF-7 cells was cross-linked, sonicated, and immunoprecipitated (IP) with either FOXA2 antibody or rabbit IgG. The amount of promoter DNA associated with the IP chromatin was measured by qPCR with primers specific to E-cadherin promoter regions −704 bp to −586 bp and −1862 bp to −1828 bp (negative control). (B) FOXA2 bound to E-cadherin promoter region −691 bp to −669 bp. Nuclear extracts were prepared from MCF-7 cells and used for EMSAs with a FAM-labeled DNA probe synthesized from E-cadherin promoter sequence −691 bp to −669 bp. The unlabeled probe (100×) or 1 μg of FOXA2 antibody (α-FOXA2) was added to the reaction to show the specificity of FOXA2/DNA complex formation. EMSAs with a FAM-labeled mutated probe were also performed. (C) The E-cadherin promoter was activated by FOXA2 in MCF-7 cells. A luciferase reporter plasmid (1.5 μg) containing the fragment of −733 bp to −60 bp of E-cadherin promoter or a control luciferase reporter plasmid (1.5 μg) containing the fragment of −233 bp to +52 bp of E-cadherin promoter and loading control pRL-CMV luciferase reporter plasmid (20 ng) were transfected into MCF-7 cells with the CMV-FOXA2 expression vector (1000 ng) or a CMV empty expression vector (1000 ng). Protein lysates were prepared at 48 h following transfection and then used to measure dual luciferase enzyme activity. The asterisks indicate statistically significant changes: ***P ≤ 0.001.

FOXA2 repressed the expression of ZEB2 in breast cancer cells. The samples of FOXA2 overexpression with Lv-FOXA2 in MDA-MB-231 cells and FOXA2 knockdown with FOXA2 siRNA in MCF-7 cells were analyzed, as shown in Fig. 2. (A) The overexpression of FOXA2 decreased ZEB2 expression at mRNA and protein levels in MDA-MB-231 cells. (B) Knockdown of FOXA2 increased ZEB2 expression at mRNA and protein levels in MCF-7 cells. (C) FOXA2 bound to the endogenous ZEB2 promoter. Gene sequence analysis was performed to predict positions of putative FOXA2 binding sites in ~2 kb human ZEB2 promoter, and design the primers for ChIP assays. The chromatin of MCF-7 cells was cross-linked, sonicated, and immunoprecipitated (IP) with either FOXA2 antibody or rabbit IgG. The amount of promoter DNA associated with the IP chromatin was measured by qPCR with primers specific to ZEB2 promoter regions −579 bp to −463 bp and −1756 bp to −1611 bp (negative control). (D) FOXA2 bound to ZEB2 promoter region −517 bp to −498 bp. Nuclear extracts were prepared from MCF-7 cells and used for EMSAs with a FAM-labeled DNA probe synthesized from ZEB2 promoter sequence −517 bp to −498 bp. The unlabeled probe (100×) or 1 μg of FOXA2 antibody (α-FOXA2) was added to the reaction to show specificity of FOXA2/DNA complex formation. EMSAs with a FAM-labeled mutated probe were also performed. (E) The ZEB2 promoter was repressed by FOXA2 in MCF-7 cells. A luciferase reporter plasmid (1.5 μg) containing the fragment of −885 bp to −66 bp of ZEB2 promoter and loading control pRL-CMV luciferase reporter plasmid (20 ng) were transfected into MCF-7 cells with different amounts of CMV-FOXA2 expression vector (0, 1, 2, and 4 μg, balanced with the different amounts of CMV empty expression vector). Protein lysates were prepared at 48 h following transfection and then used to measure dual luciferase enzyme activity.
experiments in FOXA2-overexpressed MDA-MB-231 cells and ZEB2 knockdown experiments in FOXA2-downregulated MCF-7 cells. The results of these experiments showed that the different levels of manipulated ZEB2 expression could recover changes in the epithelial or mesenchymal phenotype caused by the high or low levels of FOXA2 expression (Fig. 5I).

**TLE3 was involved in the repression of ZEB2 expression by FOXA2 in MCF-7 cells**

In this study, we found that FOXA2 negatively regulated the transcription of ZEB2 in breast cancer cells. FOXA2 was able to interact with the transcriptional corepressor TLE3 [51], thereby providing a plausible mechanism of FOXA2 to repress the transcription of its target genes. To test this hypothesis, we first confirmed the differential expression levels of TLE3 in different types of breast cancer cells, in which the TLE3 levels were much higher in epithelial MCF-7 cells than that in mesenchymal MDA-MB-231 cells (Fig. 5A). The involvement of TLE3 in EMT of breast cancer cells was further confirmed by the TLE3 knockdown experiments in MCF-7 cells. The transfection of TLE3 siRNA dramatically increased the cell migration of MCF-7 cells (Fig. 5B). The knockdown of TLE3 decreased the expression of epithelial-related E-cadherin, and resulted in the increased expression of mesenchymal-related vimentin in MCF-7 cells (Fig. 5C and D). To confirm that FOXA2 interacts with TLE3, we performed co-immunoprecipitation (Co-IP) with the lysates from MCF-7 cells transfected with FOXA2 expression vector, TLE3-V5 expression vector, or both. With the immunoprecipitation of a V5-tag specific antibody, a strong interaction between TLE3 and FOXA2 was observed (Fig. 5E). To test whether FOXA2 recruits TLE3 to the ZEB2 promoter, we performed EMAs with the FAM-labeled ZEB2 promoter (−517 bp to −498 bp) DNA probe and nuclear extracts from CMV-FOXA2-transfected, CMV-V5TLE3-transfected, or both vector-transfected MCF7 cells. Consistent with the published data that TLE3 does not bind to DNA directly [52], we found that TLE3 itself was not able to form the protein/DNA complex with the ZEB2 probe, but it could result in a supershift of the FOXA2/DNA complex on this ZEB2 DNA fragment (Fig. 5F). The endogenous binding of TLE3 on the ZEB2 promoter was observed by ChIP assays, and the knockdown of FOXA2 resulted in the significantly decreased binding levels of TLE3 on this promoter (Fig. 5G). This finding suggested that FOXA2 mediated the ZEB2 promoter binding of this transcriptional corepressor. To test whether TLE3 participates in the repression of the ZEB2 promoter bound by FOXA2, the luciferase reporter plasmid containing the −885 bp to +66 bp fragment of ZEB2 promoter was transfected into MCF-7 cells with the FOXA2 expression vector, with an increased amount of TLE3 expression vector. We found that the increased levels of TLE3 resulted in a continued decrease in the ZEB2 promoter activities when FOXA2 was present at the same levels (Fig. 5H). All these results suggested that FOXA2 recruited TLE3 to the ZEB2 promoter and repressed ZEB2 expression in breast cancer cells.

**Stable overexpression of FOXA2 abolished the migration ability of breast cancer cells in vivo**

To determine the effect of FOXA2 on the metastasis of breast cancer cells in vivo, we generated metastasis models in nude mice via tail vein injection of lentivirus-infected MDA-MB-231 cells (control) or FOXA2-expressing MDA-MB-231 cells. The mice were sacrificed at days 1, 35, and 60 post-injection (n = 6 for each group). The stable overexpression of FOXA2 in MDA-MB-231 cells decreased the number of circulating tumor cells in the blood of tested mice of the day 35 group (Fig. 5A). The overexpression of FOXA2 also decreased the amount of metastasized tumor cells in lungs (Fig. 6B), as determined by the mRNA levels of human-specific CYCLOPHILIN compared with those of mouse-specific Cyclophilin at the day 60 group. Abolished metastasis and tumor formation were found in the lungs of FOXA2-expressing MDA-MB-231 cell-injected mice at day 60 (Fig. 6C), and the expression of exogenous human FOXA2 in the tissue samples was detected by qPCR and Western blot (Fig. 5E). Compared with the control cells, FOXA2-expressing MDA-MB-231 cells also produced less malignant tumors in the lungs of tested mice with H&E staining (Fig. 6D). These results indicated that FOXA2 prevented the metastasis of breast cancer cells in vivo.

**Discussion**

EMT plays critical roles in the progression of breast cancer metastasis [61]. In the present study, we found a strong correlation between the expression levels of FOXA2 and the epithelial phenotype of clinical human breast ductal carcinoma samples. With two in vitro cell models of epithelial-type or mesenchymal-type breast cancer cells, we demonstrated that FOXA2 prevented EMT of breast cancer cells by stimulating the transcription of epithelial-related E-cadherin and repressing the expression of EMT-related transcription factor ZEB2 (Fig. 6E). We propose that FOXA2 attenuates the transcription of ZEB2 by recruiting a transcriptional corepressor TLE3 to the ZEB2 promoter. This study provides evidence to suggest that FOXA2 prevented the metastasis of breast cancer cells in vivo.

Breast cancer metastasis involves multiple steps, such as local tissue invasion (undergoing EMT in the primary cancers), intravasation, survival in the circulation, extravasation, seeding, and colonization at distant tissues where MET (mesenchymal to epithelial transition) is needed for secondary cancer formation [62]. The tendency of invading cancer cells to undergo MET is generally considered to be influenced by the local microenvironment they encounter after extravasation into the parenchyma of the distant organs [63]. Accumulated evidence from previous studies suggested that successful metastasis is facilitated by both EMT-enhanced mesenchymal traits of cancer cells at the early steps and MET-mediated epithelial traits of cancer cells at the final step [64,65]. The idea that the colonization at distant tissues is promoted by epithelial traits of invading cancer cells is supported by recent studies, which showed that a well-known epithelial-related microRNA, miR-200, enhances the lung metastasis of breast cancer; moreover, miR-200-containing extracellular vesicles can transfer the metastatic capability from metastatic cancer cells to nonmetastatic cancer cells [66]. MiR-200 maintains epithelial traits of cancer cells by suppressing EMT and enhancing MET [67]. In the current study, FOXA2 was found to prevent EMT of breast cancer cells, and the overexpression of FOXA2 caused MET of mesenchymal MDA-MB-231 cells. In contrast to the results of the miR-200 studies mentioned above, our in vivo studies found that the overexpression of FOXA2 abolished the metastatic capability of FOXA2-expressing MDA-MB-231 cells, which possessed epithelial traits but exhibited decreased colonization in the lungs. This observation could not be explained only by the effect of FOXA2 on the maintenance of epithelial traits of the cancer cells. As an important transcription factor, FOXA2 has great potential in regulating different properties of cancer cells during metastasis. FOXA2 may be involved in downregulating the potential of cancer stem cells, abolishing the survival ability of circulating cancer cells (see Fig. 6A), and preventing the proliferation of cancer cells by inducing stem cell differentiation [68,69], causing apoptosis [70], and inhibiting cell proliferation [71]. Therefore, besides its EMT-related role confirmed in this study, FOXA2′s multiple functions in the different steps of metastasis need to be further investigated to describe the overall picture of FOXA2-regulated metastasis of breast cancer cells.

When EMT of cancer cells is triggered by multiple stimuli, a number of transcription factors and feedback loops among them function in this complex process [72]. Extensive cross-talk among
these transcription factors is necessary to mediate EMT [73]. Most of the identified transcription factors involved in EMT, such as ZEB2, are upregulated by EMT-inducing signals and stimulate the EMT progression of cancer cells. By contrast, as a suppressor of tumor metastasis, FOXA2 was downregulated during the EGF-induced EMT progression of breast cancer cells (see Fig. 1). We observed that the overexpression of ZEB2 repressed FOXA2 expression in MCF-7 cells, and the knockdown of ZEB2 stimulated FOXA2 expression in MDA-MD-231 cells (Fig. S9), providing a plausible mechanism of FOXA2 downregulation by ZEB2 during EMT of breast cancer cells. ZEB2 has been found to repress the transcription of E-cadherin [57], whose promoter is hypermethylated during EMT [74]. Interestingly, the increased methylation in the FOXA2 gene has also been detected in clinical breast cancer samples [43]. The sustained induction of EMT activates DNA methylation of multiple genes in breast cancers [75]. Given that ZEB2 interacts with DNA methyltransferase
1 and increases the methylation of E-cadherin promoter in breast cancer cells [76], the reduction in FOXA2 expression during EMT may involve a ZEB2-mediated epigenetic modification, such as DNA methylation in the FOXA2 gene. This hypothesis will be elucidated by future studies.

FOXA2 can scan chromatin and bind to its recognition motif in condensed DNA structures [48,77]. The epigenetic marker H3K4me2 that correlates with active enhancers in promoters [78] is thought to facilitate FOXA2 binding [79]. In most studies, FOXA2 has been revealed to activate the expression of its target genes, such as E-cadherin. However, in a recent study, a significant fraction of FOXA-bound sites was identified to have a relatively closed chromatin conformation, which is linked to the epigenetic signature toward repressive histone markers [80]. Gro/TLE/Grg proteins, which inhibit the transcriptional machinery by recruiting HDACs or PRC2 proteins to the locus of certain genes [81–83], interact with FOXA and switch FOXA from an activator to a repressor of transcription [51]. We have confirmed that Foxa1, which is highly homologous to Foxa2, can facilitate the differentiation of mouse pluripotent stem cells by repressing the transcription of the Nanog gene via recruiting Grg3 (the mouse homolog of TLE3) to the Nanog promoter and then switching this promoter to an inactive chromatin status with typical modifications in histone H3 [84]. In the present study, we confirmed that FOXA2 interacted with TLE3 and recruited TLE3 to the ZEB2 promoter. Given that TLE3 can interact with HDACs and PRC2 proteins, it may recruit these histone modification enzymes to the ZEB2 gene region and then elicit histone modification changes at the ZEB2 locus, resulting in the following repression of ZEB2 in epithelial breast cancer cells. This possibility needs to be further investigated. Furthermore, future analysis of genome-wide FOXA2 binding and changes in FOXA2-mediated gene expression patterns is needed to provide an entire picture of transcription regulation by FOXA2 during EMT of breast cancer.

To date, several members of the FOX transcription factor family have been found to participate in the regulation of EMT of breast cancer cells. Among them, FOXC2 plays a key role in metastasis and is associated with aggressive basal-like breast cancers, suggesting that it may be a specific marker for human basal-like breast cancers [85]. FOXC2 expression is significantly correlated with the aggressive basal-like subtype of human breast cancers, and it is induced during EMT triggered by TGF-β1 and several EMT-inducing transcription factors [85]. FOXC2 may also play a role in EMT maintenance of breast cancer cells [86]. Our own studies have confirmed that FOXM1 promotes EMT by stimulating the transcription of EMT-related transcription factor Slug, and the knockdown of FOXM1 inhibits the mesenchymal phenotype in breast cancer cells [87]. We found that FOXM1 is a potential therapeutic target for breast cancer, and developed a gene therapy method with an adenovirus-mediated RNA interference of FOXM1 for breast cancer treatment [88]. The present paper presents evidence that strongly supports the role of FOXA2 as a transcriptional repressor of epithelial EMT in breast cancer.
FOXA2 in preventing EMT and metastasis of breast cancer. Based on these results, the upregulation of FOXA2 could potentially be an effective therapeutic approach for inhibiting the migration, invasion, and metastasis of breast cancer.

Acknowledgements
This work was supported by National Natural Science Foundation of China [grant number 81117949, 31161160558, 81472718 to Y.T.]; and the Institute of Science and Technology of Changsha, China [grant number K1403008-31].

Conflict of interest
The authors declare no conflict of interest.

Appendix: Supplementary material
Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.03.008.

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