Sox2 suppresses the invasiveness of breast cancer cells via a mechanism that is dependent on Twist1 and the status of Sox2 transcription activity

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Abstract

Background: Sox2, an embryonic stem cell marker, is aberrantly expressed in a subset of breast cancer (BC). While the aberrant expression of Sox2 has been shown to significantly correlate with a number of clinicopathologic parameters in BC, its biological significance in BC is incompletely understood.

Methods: In-vitro invasion assay was used to evaluate whether the expression of Sox2 is linked to the invasiveness of MCF7 and ZR751 cells. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and/or Western blots were used to assess if Sox2 modulates the expression of factors known to regulate epithelial mesenchymal transition (EMT), such as Twist1. Chromatin immunoprecipitation (ChIP) was used to assess the binding of Sox2 to the promoter region of Twist1.

Results: We found that siRNA knockdown of Sox2 expression significantly increased the invasiveness of MCF7 and ZR751 cells. However, when MCF7 cells were separated into two distinct subsets based on their differential responsiveness to the Sox2 reporter, the Sox2-mediated effects on invasiveness was observed only in ‘reporter un-responsive’ cells (RU cells) but not ‘reporter responsive’ cells (RR cells). Correlating with these findings, siRNA knockdown of Sox2 in RU cells, but not RR cells, dramatically increased the expression of Twist1. Accordingly, using ChIP, we found evidence that Sox2 binds to the promoter region of Twist1 in RU cells only. Lastly, siRNA knockdown of Twist1 largely abrogated the regulatory effect of Sox2 on the invasiveness in RU cells, suggesting that the observed Sox2-mediated effects are Twist1-dependent.

Conclusion: Sox2 regulates the invasiveness of BC cells via a mechanism that is dependent on Twist1 and the transcriptional status of Sox2. Our results have further highlighted a new level of biological complexity and heterogeneity of BC cells that may carry significant clinical implications.

Keywords: Sox2, Transcription activity, Invasiveness, Twist1, Breast cancer

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Background

Tumor invasiveness is a complex process in which malignant cells dissociate and migrate from the primary site of growth, which may eventually lead to the formation of distant metastases [1]. In many types of solid tumor, it has been shown that epithelial-mesenchymal transition (EMT) is a crucial step for tumor invasiveness [2,3]. During EMT, malignant epithelial cells shed their differentiated characteristics (e.g. cell-cell adhesion, apical-basal polarity and immobility) and acquire mesenchymal features (e.g. increased motility and invasiveness) [4]. The induction of EMT can be triggered by cytokines, such as TGF-β and interleukin (IL)-8, as well as several transcriptional factors including Twist1, Snail, and ZEB [5-9]. Twist1 has been described to be one of the key promoters of EMT and invasiveness in a number of cancer types [10-12]. In several studies, Twist1 was found to be up-regulated by a number of proteins including STAT3 [13], BMP2 [14], SRC-1 [15], MSX2 [16], NF-κB [17], and ILK [18] and down-regulated by miR-580 and CPEB1/2 [19]. In breast cancer (BC), Twist1 has been found to promote EMT and invasiveness [5]. A number of immunohistochemical studies have described a significant positive correlation between Twist1 and the metastatic/invasive property of BC [5-8]. In an animal model, siRNA knockdown of Twist1 was found to inhibit BC cells to metastasize to the lungs [5]. Furthermore, the mechanisms by which Twist1 promotes tumor invasiveness in BC have been extensively examined; down-regulation of E-cadherin [9] and up-regulation of Twist1 expression by Sox2. When we assessed the roles of Sox2 in the two distinct cell subsets of MCF7 separated based on their differential responsiveness to the Sox2 reporter, as shown previously [39], we found that the Sox2-mediated effects on invasiveness in BC is restricted to ‘reporter un-responsive’ (RU) cells. We believe that our results have shed important insights into the biological significance of Sox2 in BC, the invasiveness property of BC, as well as a new level of biological complexity of this type of cancer.

Methods

Cell culture

MCF7 and ZR751 were purchased from American Type Culture Collection (ATCC, Rockville, MD). Both ZR751 and MCF7 cells were maintained in high glucose Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sigma, Oakville, ON, Canada) and were cultured under an atmosphere of 5% CO₂ at 37°C.

Generation of stable cell lines

Stable cells expressing the Sox2 GFP reporter were generated as previously described [39]. Cells stably expressing the Sox2 GFP reporter were cultured in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, 100 ng/ml streptomycin. 1 μg/ml of puromycin was added to the culture medium at all times. The generated stable cell clones were analyzed for GFP expression by flow cytometry every two weeks over a 4-month period. RR and RU cells were sorted out based on GFP expression and cultured separately. The two populations remained 98% pure over 4 months.

Gene silencing

MCF7 and ZR751 cells were transfected with 1 nmol of SMARTpool siRNA designed against Sox2 (Thermo Scientific). Scramble non-targeting siRNA (Thermo Scientific) was used as the negative control. For all siRNA transfection, a BTX 830 electroporation instrument (Harvard Apparatus, Holliston, MA) was used. For double knockdown experiments, SMARTpool siRNA designed against Twist1 from Thermo Scientific was used.
Enforced expression of Sox2 in MCF7 cells was performed as previously described [39]. Briefly, phoenix packaging cells were transduced with either pMXs Sox2 retroviral vector (Addgene, MA, USA) or empty vector according to the manufacturer’s suggestion. MCF7 cells were infected with retroviral particles three times in 24 hour intervals. 48 hours after the final infection, cells were overnight starved and were then used to perform invasion assay.

Western blotting
Western blot analyses were performed as previously described [40,41]. The following antibodies were used: Sox2 (Cell Signaling Technologies), Twist1 (Santa Cruz), γ-Tubulin (Sigma).

Cell viability
Cell viability was determined using the 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI) according to the manufacturer’s protocol.

Cell invasion assay
As previously described, we assessed cell invasiveness using the Cytoselect™ 24-well cell invasion assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s protocol [42]. Briefly, cells were overnight starved prior to invasion assay. Approximately $1 \times 10^7$ cells in serum free medium were plated in the top chamber and medium supplemented with 10% FBS was used as a chemo-attractant in the lower chamber. The cells were then allowed to invade the reconstituted basement membrane matrix for 24 hours. The invasive cells passed the membrane were then dissociated from membrane, lysed and quantified using CyQuant GR fluorescent Dye.

Quantitative RT-PCR
Total RNA was extracted using TRIzol according to the manufacturer’s protocol. Quantitative RT-PCR was performed using Applied Biosystem Prism 7900HT instruments. The TaqMan gene expression assay (Applied Biosystems) used were: Hs01548727_m1 (MMP2), Hs00 234579_m1 (MMP9), Hs01675818_s1 (TGF-β). Primer sequences for Snail are: Forward 5′-acaaaggctg acagactcactg-3′, Reverse 5′-tgtctctctctctctctctc-3′. Primer sequences for Slug are: Forward 5′-gtctctctctctctctctctc-3′, Reverse 5′-tgctctctctctctctctctc-3′. Primer sequences for MMP3: Forward 5′-cactacagacc tgacctggtt-3′, Reverse 5′-aagcagacctggtgct-3′. Primer sequence for EAK are Forward 5′-gctctctctctctctctc-3′. Reverse 5′-ctgctctctctctctctc-3′. Human GAPDH was used as control. Expression of each gene was measured in triplicate.

Chromatin immunoprecipitation (ChIP) assay
ChIP assay was performed as our previously described [39]. The chromatin was extracted from MCF7-RR and -RU cells. A normal rabbit IgG antibody and anti-Sox2 antibody (Santa Cruz) was then incubated with the chro-matin. Isolated DNA was then amplified with Twist1 primers (−1478 to −1322 of transcriptional start site, 156 bp amplicons): Forward 5′-ggcgagtccgtactgagaag-3′ Reverse 5′-cggtgtcctcctaccc-3′.

Statistical analysis
All the statistical analyses were performed using the GraphPad Prism 5.1 program. Student T-test and One-way ANOVA were used to calculate p value. Results are presented as mean ± standard deviation.

Results
Sox2 suppresses the invasiveness of breast cancer cells
Using an in-vitro assay, we assessed if Sox2 regulates the invasiveness of two ER + breast cancer cell lines (i.e., MCF7 and ZR751), both of which have shown the highest expression level of Sox2 described in our previous study [39]. As shown in Figure 1A, siRNA knockdown of Sox2 resulted in a significant increase in the invasiveness of MCF7 and ZR751 cells. These changes were not due to a difference in cell growth between cells treated with Sox2 siRNA or scramble siRNA (Figure 1B). In contrast with the findings of another group [38], we found no significant difference in the invasiveness between MCF7 cells transfected with an empty vector or a Sox2 expression vector (Figure 1C-D).

The suppression of invasiveness by Sox2 is dependent on the status of the Sox2 transcription activity
As Sox2 is a transcription factor, we asked if Sox2 is transcriptionally active in BC cells, and whether the status of its activity has any impact on its effect on the invasiveness in BC.

To assess the Sox2 transcriptional activity, we have employed a previously characterized Sox2 reporter. The read-out of the reporter is provided by the inclusion of green fluorescence protein (GFP), driven by a mCMV promoter [39]. With the Sox2 reporter employed, we had identified that MCF7 and ZR751 cells are composed of two phenotypically distinct cell subsets that can be separated based on their differential responsiveness to the Sox2 reporter [39]. Specifically, cells showing Sox2 transcriptional activity are GFP-positive whereas those showing no evidence of Sox2 transcriptional activity are GFP-negative [39]. For the purpose of this study, the former cell population is labeled ‘reporter responsive’ or RR cells and the latter cell population is labeled ‘reporter
un-responsive or RU cells. To facilitate our studies, we generated stable cell clones expressing the Sox2 reporter construct. RR and RU cells were further isolated by flow cytometry and cultured separately. As shown in Additional file 1: Figure S1, the RR and RU cells were readily identified using flow cytometry. Cells stably transfected with the Sox2 reporter that have not been sorted into RR and RU cells are labeled ‘Sox2R’. We have previously excluded the possibility that the absence of GFP expression in RU cells is due to a lack of Sox2 protein as the vast majority of MCF7 and ZR751 cells expressed Sox2 detected by flow cytometry. Furthermore, by subcellular fractionation, we confirmed that Sox2 is present in the nuclei of these cells [39].

When the invasiveness of RR cells, RU cells and the unsorted Sox2R cells derived from MCF7 was compared, no significant difference was observed among these three cell populations (Figure 2A). However, as shown in Figure 2B, siRNA knockdown of Sox2 resulted in a significant increase in the invasiveness of MCF7-RU cells; in contrast, no significant change was seen in MCF7-RR cells. This difference between the two cell subsets was not due to a significant difference in their cell growth (Figure 2C). In keeping with our previous observation [39], siRNA knockdown of Sox2 also did not result in any significant change in the viability of MCF7-RR and -RU cell populations (Figure 2D). The similar experiments were performed using ZR751-RU cells. In keeping with the results of MCF7 cells, siRNA knockdown of Sox2 in ZR751-RU cells significantly increased in the invasiveness (Figure 2E).

Sox2 regulates Twist1 expression, but only in RU cells
To understand the mechanism by which Sox2 regulates the invasiveness of the RU cells, we examined if Sox2 modulates the expression of factors known to play key roles in regulating the invasiveness and/or EMT in various types of cancers, including Snail1, Slug, ZEB1, MMP2, MMP3, MMP9, Twist1, E-cadherin, N-cadherin, FAK and TGF-β [43-47]. Using quantitative RT-PCR, we found that siRNA knockdown of Sox2 in both MCF7-RR and -RU cells did not result in significant changes in the
mRNA levels of Snail1, Slug, ZEB1 (Figure 3B), as well as MMP2, MMP3, MMP9, FAK and TGF-β (not shown). As shown in Figure 3B and C, we found that siRNA knockdown of Sox2 led to a significant up-regulation of the Twist1 mRNA as well as an upregulation of the Twist1 protein, although these changes were confined to the RU cells. Correlating with these findings, the expression level of E-cadherin, one of the key Twist1 downstream targets, was down-regulated in RU cells but not RR cells (Figure 3B). N-cadherin, a cell-cell adhesion mediator, was significantly up-regulated in MCF7-RU cells but not RR cells. Using ChIP assay, we were able to demonstrate that Sox2 was bound to the promoter region of Twist1 in RU cells but not RR cells (Figure 3D).
Modulation of cell invasiveness by Sox2 is mediated via Twist1

We then asked if the Sox2-mediated modulation of invasiveness in RU cells is dependent on Twist1. As shown in Figure 4, siRNA knockdown of Sox2 in MCF7-RU cells led to a significant increase in invasiveness, whereas siRNA knockdown of Twist1 led to a significant decrease in invasiveness. Importantly, simultaneous silencing of Sox2 and Twist1 using siRNA largely abrogated the suppressive effect of Sox2 on invasiveness in MCF7-RU cells. These findings strongly suggest that Sox2 suppresses the invasiveness property of RU cells via down-regulating Twist1 in these cells. The same experiment was repeated using MCF7-RR cells and we found no significant change
in the invasiveness of these cells (Figure 5). Nevertheless, siRNA knockdown of Twist1 resulted in a significant decrease in the invasiveness of MCF7-RR cells, suggesting that Twist1, but not Sox2, is a key regulator of invasiveness in these cells. Again, the observed differences in invasiveness were not due to a significant difference in the cell growth among the negative controls and various treatment groups (Figure 5B).

Discussion

The aberrant expression of Sox2 in cancer cells has been found to correlate with the invasiveness of several types of solid tumors [30,34,35,37,48-50]. For instance, a high level of Sox2 expression detectable by immunohistochemistry was found to correlate with higher invasiveness and metastatic potential in gliomas and colorectal cancer [35,36]. Furthermore, siRNA knockdown of Sox2 can result in decreased invasiveness in cell lines derived from gliomas, melanomas and colorectal cancer [35-37]. However, it appears that Sox2 expression in cancer does not always correlate with increased invasiveness and metastasis. We found at least one previous study in which a relatively low level of Sox2 expression in gastric cancer correlates with increased invasiveness/metastatic potential [34]. In the current study, we also found evidence that Sox2 suppresses invasiveness in BC. Thus, the biological effects of Sox2 in cancer cells are likely to be tumor type-specific.

Our finding that Sox2 suppresses the invasiveness of BC is in contrast with that made by another group, who found that enforced expression of Sox2 in MCF7 cells can increases their invasiveness by approximately 60% [38]. In our study, we initially found that siRNA knockdown of Sox2 significantly increased the invasiveness of parental MCF7 cells and MCF7-RU cells. In view of the discrepancy between our conclusion and that described...
in the literature [38], we attempted to replicate the experiment that examined the effects of enforced Sox2 over-expression in MCF7 cells, as described previously [38], and we did not find any significant change in the invasiveness of these cells (Figure 1C). We would like to point out that the lack of response to enforced Sox2 expression in MCF7 is similar to the finding of one of previous studies, in which enforced expression of Sox2 in MCF7 cells was found to result in no significant change in mammosphere formation and cell growth [39]. While we do not have definitive explanations for the discrepancy between our results and the previously published results [38], we have considered the possibility that the MCF7 cell clones used in the two laboratories may be different. We also have considered the possibility that the \textit{in-vitro} invasiveness assays between the two laboratories have different characteristics. Lastly, since the exact Sox2 protein level has been shown to be functionally important in ESCs [51,52], it is possible that the total Sox2 protein levels after gene transfection are substantially different between the two laboratories, and thus, leading to substantially different biological responses.

The mechanisms by which Sox2 regulate tumor invasiveness have not been extensively studied. In the literature, we were able to identify only 3 studies that are directly relevant to this subject. In all of these three studies (using cell lines derived from colorectal cancer, melanomas and gliomas, respectively), siRNA knockdown of Sox2 was found to decrease invasiveness; in the same three studies, the decrease in invasiveness was found to correlate with a decreased expression level of one of the following molecules: MMP2, MMP3 or FAK [36,37,53]. To our knowledge, the mechanisms by which Sox2 regulates invasiveness in BC are not known. Thus, we screened a panel of factors known to play roles in regulating cell invasiveness/EMT in various types of cancer. In contrast with the previous reports, we did not find any appreciable changes in the expression levels of MMP3, MMP2 and FAK. Instead, we identified Twist1 as the only protein that is regulated by Sox2 in RU cells. Twist1 has been reported to be one of the master regulators of invasiveness and EMT, and dysregulation of Twist1 expression and function has been implicated to be associated with cancer progression [54-56]. In BC, a high level of Twist1 expression is more common in invasive lobular carcinomas [5]. While siRNA knockdown of Twist1 in BC cells led to a decrease in invasiveness [57], enforced expression of Twist1 in BC cells converts its normal epithelial cell morphology to a spindle-like/fibroblastic morphology [5,58]. In keeping with the concept that Twist1 plays a key role in regulating invasiveness in BC, siRNA knockdown of Twist1 decreased the invasive-

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**Figure 5** The role of Sox2 and Twist1 in RR cells. Similar experiments were performed in MCF7-RR cells as described in Figure 4. (A) siRNA knockdown of Sox2 in MCF7-RR cells did not lead to a significant increase in invasiveness. Nevertheless, siRNA knockdown of Twist1 significantly decreased the invasiveness. Cells treated with double scramble siRNA were used as a negative control. Triplicate experiments were performed. A representative experiment is shown (mean ± standard deviation) (n = 3). One-way ANOVA was used to calculated statistics. (B) By western blot analysis, Sox2 siRNA and Twist1 siRNA treatment dramatically decrease the expression level of Sox2 and Twist1, respectively. (C) Cell viability was measured by the MTS assay. No significant change was observed between the negative control and various treatments.
ness of both MCF7-RR and -RU cells by approximately 20-30% (Figures 4A and 5A).

As mentioned in the introduction, the expression of Twist1 has been shown to be regulated by a number of proteins such as STAT3, BMP2 and SRC-1. The expression of Sox2 has been shown to correlate with that of Twist1 in human glioblastoma cells [59], although direct proof that Sox2 regulates the expression of Twist1 is lacking. For the first time, we have provided direct evidence that the expression of Twist1 in BC is regulated by Sox2, and this regulation only occurs in the RU cells. Results from our ChIP studies further support the fact that Twist1 is regulated by Sox2 only in RU cells. Although Sox2 does not respond to the reporter in RU cells, possibly due to the fact that Sox2 in RU cells cannot bind to the Sox2 binding motif present in the Sox2 reporter [39], Sox2 in RU cells can bind to the alternative Sox2 binding motif present in the Twist1 gene promoter and thus suppress its expression as well as invasiveness. These findings are in parallel to the findings that Sox2 is known to negatively regulate a set of genes in ESCs. In contrast, in RR cells, Sox2 does not bind to the promoter region of Twist1 and the expression of Twist1 is regulated by other factors. The mechanism underlying the decision as to whether Sox2 binds to the Twist1 gene promoter is under active investigation in our laboratory. The transcription activity of Sox2 in normal ESCs has been shown to be modulated by its binding partners, we speculated that a similar scenario may occur in BC cells. Taken together, our findings suggest that the Sox2 transcriptional activity and Twist1 can serve as markers to predict invasiveness in breast cancer cells.

An important concept emerged from the results of this study is related to the significance of the dichotomy of BC cells separated based on the differential responsiveness to the Sox2 reporter. Specifically, based on our double siRNA knockdown experiments (Figure 4), the Sox2-Twist1 axis plays a key role in regulating the invasiveness in RU cells. In contrast, Twist1, but not Sox2, plays a key role in regulating the invasiveness of RR cells. While the true biological significance of these observations requires further studies, we believe that our results have highlighted a new level of biological complexity of BC. In view of this new knowledge, one may wonder if our current treatments of BC, which are designed based on the assumption that BC cells within a tumor are composed of a biologically uniform population of cancer cells, are fundamentally inadequate. This newly discovered biological complexity of BC cells may prompt us to consider treatment strategies that are based on the recognition of phenotypically distinct cell subsets in BC that are driven by different biochemical pathways.

**Conclusion**

In summary, we reported for the first time that Sox2 suppresses invasiveness in BC cells, but only in RU subset. Moreover, Sox2 was found to be a major regulator of Twist1 by controlling the expression level of Twist1. Results from our studies have further supported that the dichotomy of BC based in their differential responsiveness to the Sox2 reporter carries biological importance, highlighting a new level of biological complexity of BC.

**Additional file**

**Additional file 1: Figure S1. Identification of the dichotomy of BC cells based on the differential responsiveness to the Sox2 reporter. (A) MCF7 was stably transfected with either the Sox2 GFP reporter or mCMV lentiviral vector. Cells stably transfected with the Sox2 GFP reporter were labeled as ‘MCF7 Sox2R’. Cells stably transfected with mCMV control were labeled as ‘MCF7 mCMV’. GFP expression was measured by flow cytometry. Cells showing Sox2 transcriptional activity are GFP-positive whereas those showing no evidence of Sox2 transcriptional activity are GFP-negative. For the purpose of this study, the former cell population is labeled ‘reporter responsive’ or RR cells and the latter cell population is labeled ‘reporter un-responsive’ or RU cells. (B) To further examine the biology of these two cell subsets, we isolated and cultured the GFP-positive (labeled as ‘RR’) and GFP-negative cells (labeled as ‘RU’) separately from MCF7 cells.

**Abbreviations**

Sox2: Sex-determining region Y-box 2; GFP: Green fluorescent protein; ChIP: Chromatin immunoprecipitation; ESC: Embryonic stem cell; BC: Breast cancer; EMT: Epithelial-mesenchymal transition.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

FW and XY performed experiments and analyzed data; PW, KJ, OW, DD, NK, YM and GB assisted with experiments; FW and RL designed the research plan; FW and RL wrote the manuscript. All authors’ read and approved the final manuscript.

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