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ORIGINAL ARTICLE

Casein kinase 2 prevents mesenchymal transformation by maintaining Foxc2 in the cytoplasm

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Nuclear Foxc2 is a transcriptional regulator of mesenchymal transformation during developmental epithelial–mesenchymal transition (EMT) and has been associated with EMT in malignant epithelia. Our laboratory has shown that in normal epithelial cells Foxc2 is maintained in the cytoplasm where it promotes an epithelial phenotype. The Foxc2 amino terminus has a consensus casein kinase 2 (CK2) phosphorylation site at serine 124, and we now show that CK2 associates with Foxc2 and phosphorylates this site *in vitro*. Knockdown or inhibition of the CK2α/α′ kinase subunit in epithelial cells causes *de novo* accumulation of Foxc2 in the nucleus. Mutation of serine 124 to leucine promotes constitutive nuclear localization of Foxc2 and expression of mesenchymal genes, whereas an S124D phosphomimetic leads to constitutive cytoplasmic localization and epithelial maintenance. In malignant breast cancer cells, the CK2β regulatory subunit is downregulated and FOXC2 is found in the nucleus, correlating with an increase in α-smooth muscle actin (SMA) expression. Restoration of CK2β expression in these cells results in cytoplasmic localization of Foxc2, decreased α-SMA expression and reduced cell migration and invasion. In contrast, knockdown of CK2β in normal breast epithelial cells leads to FOXC2 nuclear localization, decreased E-cadherin expression, increased α-SMA and vimentin expression, and enhanced cell migration and invasion. Based on these findings, we propose that Foxc2 is functionally maintained in the cytoplasm of normal epithelial cells by CK2α/α′-mediated phosphorylation at serine 124 , which is dependent on proper targeting of the holoenzyme via the CK2β regulatory subunit.

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INTRODUCTION

Cells in a mature epithelium such as the kidney tubule or breast acini are maintained in a highly differentiated state. However, following organ injury these cells undergo transient dedifferentiation during which they lose their brush border, exhibit a flattened morphology and express mesenchymal markers such as vimentin and α-smooth muscle actin (α-SMA).[1–3] This dedifferentiation is believed to promote cell survival, migration and proliferation, thus enhancing regeneration of the mature epithelium.[4] However, sustained dedifferentiation (often referred to as epithelial–mesenchymal transition or EMT) can lead to fibrosis and scarring following organ injury[5,6] and can promote tumor metastasis in epithelial cancers.[7] Identifying the factors that regulate the processes of dedifferentiation and redifferentiation is therefore crucial to understanding normal epithelial biology and the forces that underlie aberrant tumor behavior.

Fox (forkhead box) proteins are a family of transcription factors that are important in regulating the expression of genes involved in cell growth, proliferation, differentiation and longevity.[8–10] Fox proteins contain the FHD, a sequence of ~100 amino acids forming a motif that binds to DNA.[11] Foxc2, belonging to the 'C' subfamily, is required for cardiovascular development,[12] early organogenesis of the kidney,[13] podocyte differentiation and glomerular basement membrane maturation.[14] Mani *et al.*[7] have demonstrated that Foxc2 can act as an activator of epithelial cell dedifferentiation during tumor metastasis, while Bard *et al.*[15] reported a possible function for Foxc2 in epithelial cell differentiation. Our laboratory previously investigated Foxc2 expression and localization following ischemia/reperfusion injury (I/R) of the kidney.[16] Foxc2 was transiently upregulated in tubular epithelial cells after ischemic injury but was detectable only in the cytoplasm of these cells both *in vitro* and *in vivo*. Suppression of cytoplasmic Foxc2 using RNA interference resulted in a partial loss of the epithelial phenotype. In contrast, *ex vivo* overexpression of Foxc2 in epithelial cells resulted in nuclear translocation and promotion of a mesenchymal phenotype. These results suggest that the mechanism by which epithelial cells control Foxc2 localization is critical for maintaining the epithelial phenotype.

The amino terminus of Foxc2 contains a region within the FHD that is known to be mutated in patients with autosomal-dominant inherited primary lymphedema associated with distichiasis.[17–19] One of those mutations, R120H, has been found to alter the subcellular localization of Foxc2 by an as yet undetermined mechanism. Our analysis of that region of Foxc2 reveals that it is adjacent to a potential casein kinase 2 (CK2) phosphorylation site at serine 124. In the current study, we show that CK2 can phosphorylate Foxc2 *in vitro* and that knockdown or inhibition of CK2 in cultured epithelial cells causes translocation of endogenous Foxc2 to the nucleus. Foxc2-S124D, containing a phosphomimetic of the putative CK2 phosphorylation site, was constitutively expressed in the cytoplasm, whereas Foxc2-S124L, a non-phosphorylatable mutant, was found predominantly in the nucleus and induced mesenchymal gene expression. In highly malignant breast cancer cells, FOXC2 was found in the nucleus, correlating with increased expression of mesenchymal

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markers. Examination of CK2 expression in these cells revealed downregulation of the CK2 regulatory subunit, CK2β. Overexpression of CK2β induced cytoplasmic localization of FOXC2, suppressed α-SMA expression and reduced cellular migration and invasion, whereas knockdown of CK2β in normal breast epithelial cells led to nuclear localization of Foxc2, downregulation of E-cadherin, upregulation of mesenchymal markers and increased cell migration and invasion. The ability of overexpressed CK2β to suppress the mesenchymal phenotype of malignant breast epithelial cells was prevented by the coexpression of Foxc2-S124L, confirming that Foxc2 is a key target of CK2β in epithelial maintenance.

RESULTS

Foxc2 localization

Cell fractionation of cultured MPT cells (immortalized epithelial cells derived from the mouse proximal tubule)[16] revealed that Foxc2 was only detectable in the cytoplasm, with no protein seen in the nuclear fraction (Figure 1a). In contrast, Foxc2 was localized in both the cytoplasm and nucleus of NIH3T3 fibroblasts. These results are consistent with our immunofluorescent analysis of renal tubular epithelial cells *in vivo*[16] and suggest that epithelial cells normally maintain Foxc2 in the cytoplasm. Transfection of MPT cells with a vector encoding FLAG-tagged Foxc2 resulted in the overexpression of Foxc2 with *de novo* detection of Foxc2 in the nucleus of MPT cells (Figure 1a). Examination of the sequence of Foxc2 revealed that the serine at position 124 (^{124}SLNE) is a potential phosphorylation site for CK2 (consensus sequence pS/TXXE/D). To determine whether phosphorylation at this site might regulate Foxc2 localization, constructs encoding full-length GFP-Foxc2-S124L (preventing phosphorylation of the site) and GFP-Foxc2-S124D (mimicking constitutive phosphorylation) were generated. Transient transfection of MPT cells with these constructs again demonstrated that overexpressed wild-type GFP-Foxc2 localized to both the nuclear and cytoplasmic compartments, whereas GFP-Foxc2-S124L localized predominantly to the nucleus in a speckled pattern and GFP-Foxc2-S124D localized to the cytoplasm (Figures 1b and c). Side-by-side comparison of whole-cell lysates confirmed that all three constructs were expressed at similar levels (Supplementary Figure 1). These results suggest that phosphorylation at serine 124 promotes cytoplasmic localization of Foxc2.

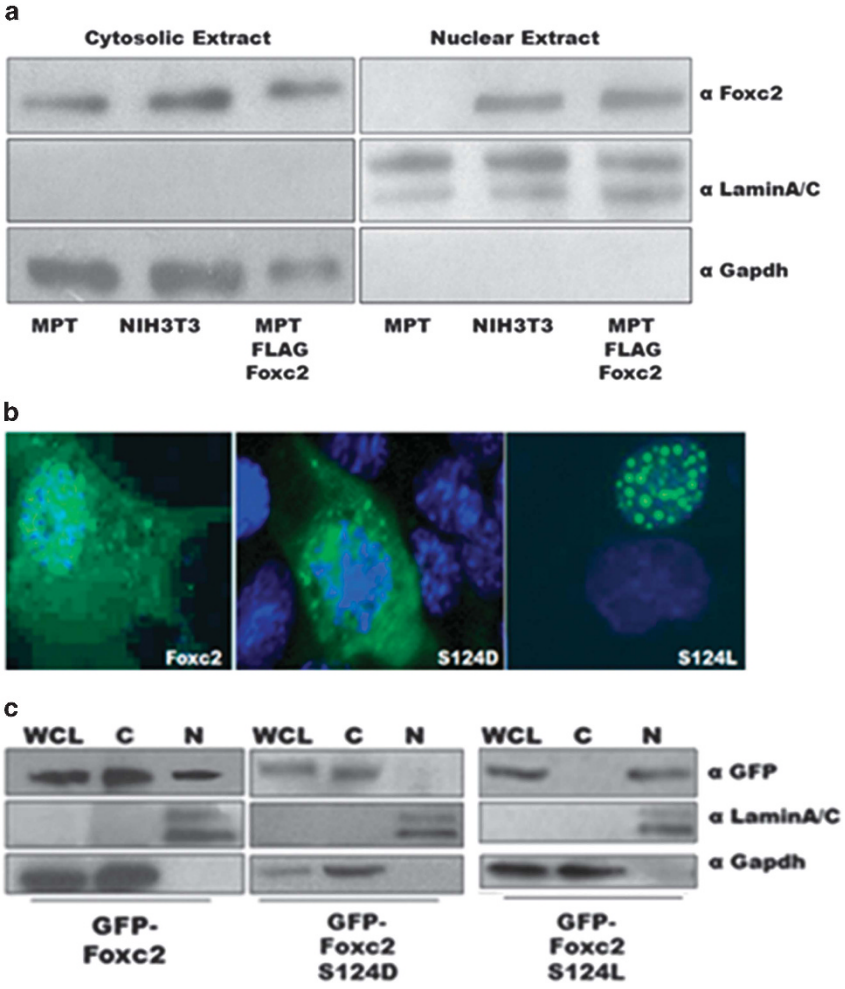

Figure 1. Foxc2 localization. (**a**) MPT cells, NIH3T3 cells and MPT cells transiently transfected with FLAG-Foxc2 were subjected to cell fractionation, followed by SDS–PAGE and immunoblotting for Foxc2, LaminA/C (nuclear marker) and GAPDH (cytoplasmic compartment marker). Representative images from an *n* of three replicates per condition. (**b**) MPT cells transiently transfected with either GFP-Foxc2, GFP-Foxc2-S124D or GFP-Foxc2-S124L were fixed and imaged for green fluorescent protein (GFP) (green) and DAPI (blue). Images were obtained at x40 magnification. (**c**) MPT cells transiently transfected as above were subjected to cell fractionation, followed by immunoblotting with anti-GFP, anti-LaminA/C and anti-GAPDH. Representative blots from an *n* of four to six replicates per condition.

CK2 is required to maintain cytoplasmic localization of endogenous Foxc2

To determine whether CK2 might be responsible for phosphorylation at S124, we first examined the potential association of CK2 with Foxc2. GFP-Foxc2 was transfected into MPT cells and the fusion protein immunoprecipitated with anti-GFP. These experiments revealed that GFP-Foxc2 co-immunoprecipitates with endogenous CK2 in renal epithelial cells (Figure 2a). MPT cells were then transfected with either siRNA directed against the CK2 kinase subunits (CK2α/α′) or scrambled small interfering RNA (siRNA) (Figure 2b, 65% knockdown of CK2α/α′), followed by analysis of the subcellular localization of endogenous Foxc2 24 h later. Cell fractionation confirmed that endogenous Foxc2 is detectable only in the cytoplasm of control MPT cells, whereas CK2α/α′ knockdown resulted in decreased cytoplasmic localization and *de novo* detection of Foxc2 in the nucleus (Figure 2c, quantified below). To determine whether the kinase activity of CK2 is required for cytosolic maintenance of Foxc2, MPT cells were treated ± TBCA (tetrabromocinnaic acid; a cell-permeable CK2 kinase inhibitor)[20] or vehicle, followed by examination of the localization of endogenous Foxc2. Cell fractionation revealed that Foxc2 was found in the cytoplasm of control MPT cells, whereas treatment with TBCA for 1 h resulted in decreased cytoplasmic localization and obvious detection of Foxc2 in the nucleus (Figure 2d). These results demonstrate that CK2 associates with Foxc2 and maintains its cytoplasmic localization in a kinase-dependent manner.

CK2-dependent cytoplasmic localization of Foxc2 requires serine 124

To determine whether CK2-mediated localization of Foxc2 is dependent on serine 124, MPT cells were transiently transfected with either full-length GFP-Foxc2 or GFP-Foxc2-S124D ± the CK2 kinase inhibitor TBCA. As described above, in the absence of TBCA overexpressed GFP-Foxc2 localized to both the cytoplasm and nucleus, whereas GFP-Foxc2-S124D localized to the cytoplasm (Figure 2e). Following CK2 inhibition with TBCA, GFP-Foxc2 was found almost entirely in the nucleus, whereas GFP-Foxc2-S124D remained localized in the cytoplasm (Figure 2f, quantified below). These results demonstrate that, in contrast to endogenous Foxc2, the S124D phosphomimetic does not require CK2 kinase activity for cytoplasmic localization, suggesting that CK2 may normally phosphorylate this site.

To determine if Foxc2 is indeed a CK2 substrate, an *in vitro* kinase assay was performed in which either immunoprecipitated GFP-Foxc2 or GFP-Foxc2-S124L was used as a substrate for

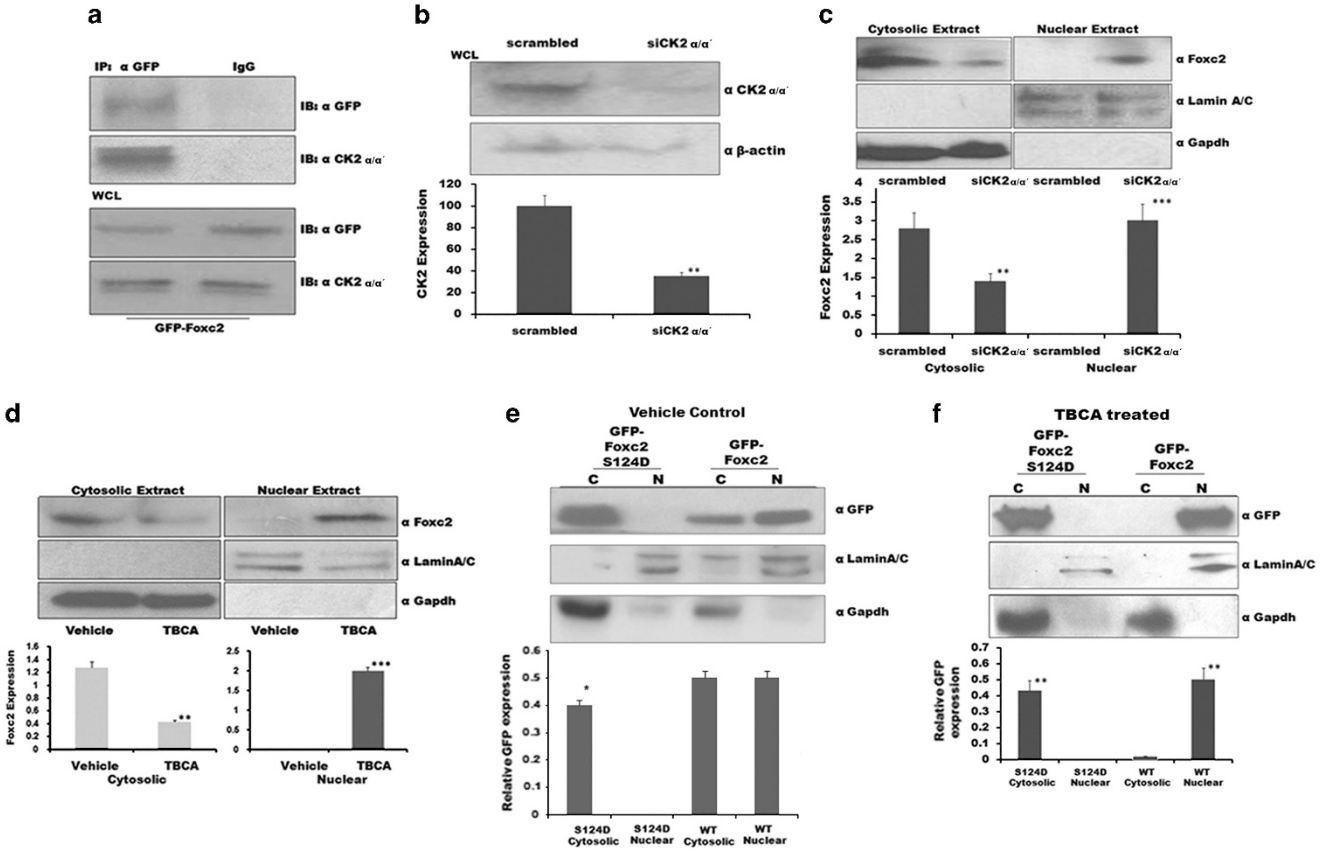

Figure 2. CK2 is required to maintain cytoplasmic localization of Foxc2. (**a**) MPT cells were transfected with GFP-Foxc2 and whole-cell lysates (WCLs) immunoprecipitated (IP) with anti-GFP or IgG isotype control and blotted for anti-GFP and anti-CK2. WCL is the input material for the IP. (**b**) MPT cells were transfected with scrambled siRNA or siRNA directed against CK2 followed 24 h later by lysis and immunoblotting to determine efficiency of CK2 protein reduction. Percentage knockdown was determined by normalizing to β-actin. Graph shows quantification for $n = 3$; $^{**}P < 0.005$ relative to siRNA scrambled control. (**c**) MPT cells were transfected as in (**b**) followed by cell fractionation and immunoblotting for Foxc2, LaminA/C and GAPDH. Graph shows quantification for $n = 3$; $^{***}P < 0.001$ and $^{**}P < 0.005$ relative to siRNA scrambled control. (**d**) MPT cells were treated with vehicle control or 100 μM TBCA for 1 h followed by cell fractionation and immunoblotting for endogenous Foxc2, LaminA/C and GAPDH. Relative Foxc2 expression in each compartment was determined as described in Materials and methods. $N = 4$; $^{***}P < 0.001$ and $^{**}P < 0.005$ relative to vehicle control. (**e** and **f**) MPT cells transiently transfected with the indicated constructs were treated with vehicle control (**e**) or 100 μM TBCA (**f**) for 1 h, and subjected to cell fractionation followed by immunoblotting with anti-GFP, anti-LaminA/C and anti-GAPDH. Relative expression was determined as described in Materials and methods. $N = 3$; $^{**}P < 0.005$.

purified CK2α/α′/β holoenzyme. GFP-Foxc2 was phosphorylated in the presence of added CK2, with less phosphorylation of GFP-Foxc2-S124L (Figure 3). These results show that CK2 can phosphorylate Foxc2 and suggest that serine 124 is the primary CK2 phosphorylation residue.

Cytoplasmic localization of Foxc2 maintains the epithelial phenotype

In normal renal epithelia, Foxc2 is upregulated after kidney injury, but remains localized to the cytoplasm.[16] In contrast, overexpression of Foxc2 by transfection or in epithelial tumors results in increased nuclear localization and a mesenchymal expression pattern (Figure 1a).[7,16] To determine whether nuclear translocation of Foxc2 is required for mesenchymal expression, MPT cells were transfected with either GFP alone, GFP-Foxc2-S124D or GFP-Foxc2-S124L, followed by examination of cell morphology and expression of epithelial and mesenchymal markers. Transfection with GFP-Foxc2-S124L (found predominantly in the nucleus) resulted in the downregulation of E-cadherin and upregulation of both vimentin and α-SMA as compared with control cells transfected with GFP alone (Figure 4a, quantified in Figure 4b). In contrast, cells overexpressing GFP-Foxc2-S124D (found predominantly in the cytoplasm) exhibited a modest increase in E-cadherin expression and failed to upregulate either vimentin or α-SMA, even though the total amount of GFP-Foxc2 was indistinguishable in the two groups. F-actin staining (Supplementary Figure 2) revealed an epithelial morphology in MPT cells transfected with GFP only or GFP-Foxc2-S124D, whereas more of a spindled shape was observed in the GFP-Foxc2- and GFP-Foxc2-S124L-expressing cells.

To determine if the difference in protein expression observed between Foxc2-S124D- and Foxc2-S124L-transfected cells was due to transcriptional regulation, quantitative reverse transcription–PCR was performed to quantify mRNA. These studies demonstrated that cells expressing Foxc2-S124L exhibited a significant reduction in the mRNA for E-cadherin and increased mRNA for both vimentin and α-SMA as compared with either control cells or those expressing GFP-Foxc2-S124D (Figure 4c). Cumulatively, these data suggest that activation of a mesenchymal expression phenotype by Foxc2 requires trafficking to the nucleus and subsequent regulation of gene transcription.

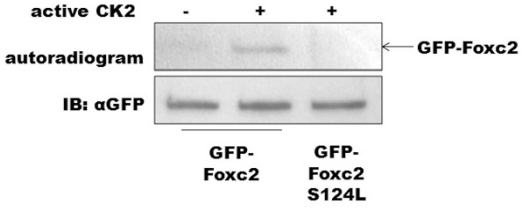

Figure 3. Serine 124 is phosphorylated *in vitro* by CK2. MPT cells were transfected with GFP-Foxc2 or GFP-Foxc2-S124L, followed by immunoprecipitation with anti-GFP antibody and *in vitro* phosphorylation ± CK2. Paired samples were subjected to SDS–PAGE and immunoblotting with anti-GFP antibody to confirm immunoprecipitation of GFP-Foxc2 and GFP-Foxc2-S124L.

FOXC2 nuclear localization is increased in metastatic breast cancer cells

Increased FOXC2 expression in breast cancer biopsies has been shown to correlate with increased metastasis.[7] To determine whether FOXC2 is localized differently in normal breast epithelial cells as compared with cells derived from metastatic tumors, we compared MCF10A cells derived from normal human breast tissue with MDA-MB-436 (derived from a weakly metastatic tumor) and MDA-MB-231 (derived from a highly metastatic tumor) breast cancer cell lines.[21] Whole-cell lysates demonstrated an increase in total Foxc2 expression in highly metastatic MDA-MB-231 cells as compared with the normal breast MCF10A cells and weakly metastatic MDA-MB-436 cells (Figure 5a, quantified below). Subcellular fractionation reveals that FOXC2 is detected only in the cytoplasm of MCF10A cells, similar to the pattern seen in normal renal tubular epithelial cells (Figure 5b, quantified below). In contrast, MDA-MB-436 cells had mixed cytosolic and nuclear expression, whereas MDA-MB-231 cells expressed Foxc2 predominantly in the nuclear compartment. Thus, FOXC2 is easily detected in the nucleus in cells derived from metastatic tumors, but is maintained in the cytoplasm of normal breast epithelial cells.

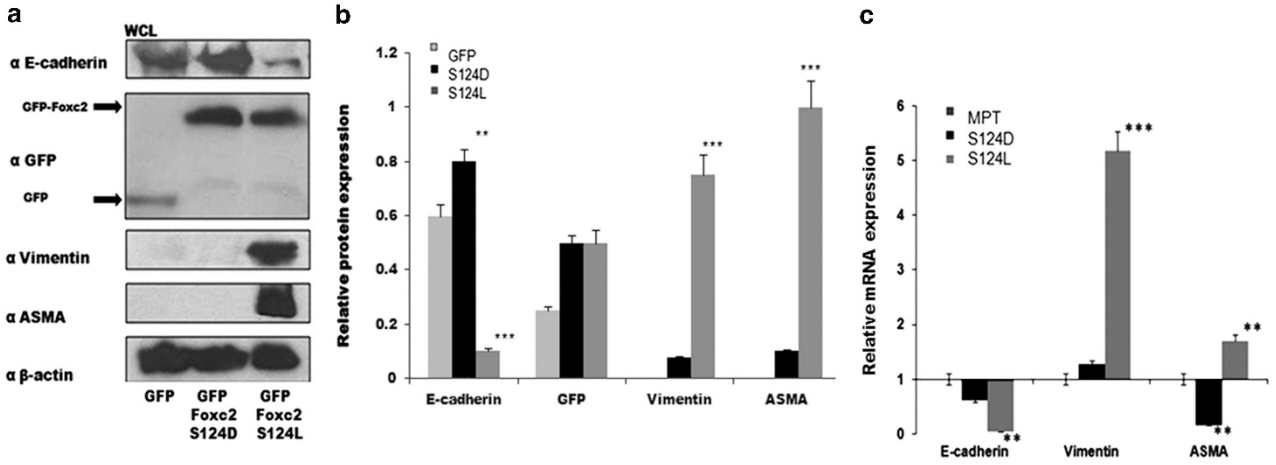

Figure 4. Cytoplasmic localization maintains an epithelial phenotype. (**a**) MPT cells were transfected with GFP, GFP-Foxc2-S124D or GFP-Foxc2-S124L constructs three times to achieve ~70% transfection efficiency and grown to 90% confluency. Whole-cell lysates (WCLs) were then blotted with the indicated antibodies. (**b**) Quantification of three experiments performed as in (**a**) with protein expression normalized to β-actin. $N = 3$; $^{***}P < 0.001$, $^{**}P < 0.005$ (compared with GFP control cells). (**c**) Real-time PCR was performed using RNA from cells transfected as in (**a**) with RNA expression normalized to Hprt1 and MPT control. $N = 3$; $^{***}P < 0.001$ and $^{**}P < 0.005$ (compared with control cells).

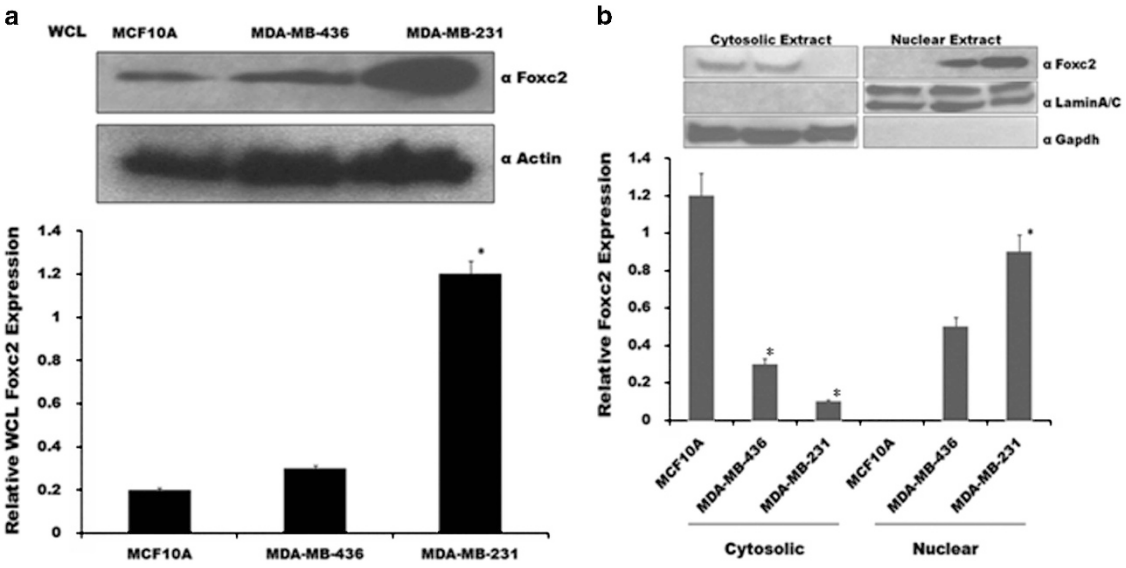

Figure 5. Foxc2 nuclear expression correlates with metastatic phenotype in breast cancer cell lines. (**a**) MCF10A, MDA-MB-436 and MDA-MB-231 whole-cell lysates (WCLs) were subjected to immunoblotting with anti-Foxc2 or anti-β-actin and quantified relative to β-actin. $N = 3$; $^*P < 0.05$ relative to MCF10A cells. (**b**) MCF10A, MDA-MB-436 and MDA-MB-231 cells were subjected to cell fractionation, followed by SDS–PAGE and immunoblotting for Foxc2, Lamin A/C and GAPDH, and quantified as below. Relative Foxc2 expression was determined as described in Materials and methods. $N = 3$; $^*P < 0.05$ relative to MCF10A cells. C, cytosolic; N, nuclear.

Metastatic breast cancer cell lines have decreased CK2β and upregulate mesenchymal proteins

Overexpression of transfected Foxc2 in normal renal epithelia leads to nuclear localization,[7,16] and high levels of endogenous Foxc2 in MDA-MB-231 cells correlate with nuclear localization (Figure 5). Surprisingly, MDA-MB-436 cells also exhibited nuclear localization of Foxc2 despite having normal levels of the protein (Figure 5a). Recently, Deshiere *et al.*[22] showed that unbalanced expression of CK2α and β-subunits in a subset of breast tumor samples correlated with the expression of EMT-related markers. We therefore examined CK2 subunit expression and mesenchymal markers in the metastatic MDA-MB-436 and MDA-MB-231 cells as compared with non-malignant MCF10A breast epithelial cells and MPT kidney epithelial cells. Similar to MPT cells, MCF10A cells expressed high levels of E-cadherin and low levels of the mesenchymal proteins vimentin and α-SMA (Figure 6a, quantified in Figure 6b). Consistent with the findings of Deshiere *et al.*,[22] these cells had the highest expression of CK2β with a CK2α/α′:β ratio of 2:5. In contrast, both MDA-MB-436 and -231 cells exhibited the downregulation of CK2β and upregulation of α/α′, resulting in a reversal of the α/α′:β ratio to 3:1 and 10:1, respectively. This correlated with suppression of E-cadherin expression and upregulation of vimentin and α-SMA in the metastatic cell lines. This same reversal of the α/α′:β ratio was detected when MDA-MB-231 cells were compared with non-malignant kidney MPT cells (Figure 6c).

NIH3T3 fibroblasts, which express Foxc2 in the nucleus and exhibit a mesenchymal phenotype, were also analyzed for their Foxc2, CK2α/α′ and CK2β expression. Similar to MDA-MB-436 cells, NIH3T3 cells had total Foxc2 expression levels comparable to those found in non-malignant MPT and MCF10A epithelial cells, but exhibited increased CK2α/α′ expression with low levels of CK2β expression (Figure 6c). These data suggest that downregulation of CK2β may be sufficient to prevent CK2-mediated maintenance of Foxc2 in the cytoplasm, even in the setting of normal levels of total Foxc2 expression.

Overexpressing CK2β in malignant breast cancer cells rescues FOXC2 cytoplasmic localization

In light of the correlation between downregulation of CK2β and nuclear localization of FOXC2, we pursued the possibility that normalization of CK2β expression in MDA-MB-231 cells could promote FOXC2 localization to the cytoplasm. MDA-MB-231 cells transfected with a vector encoding CK2β demonstrated an increase in CK2β expression as well as a decrease in CK2α/α′ (Figure 7a). This partial normalization of the CK2α/α′:CK2β ratio led to a decrease in α-SMA expression by the MDA-MB-231 cells, but failed to reduce their vimentin expression or rescue E-cadherin expression. Subcellular fractionation revealed that re-expression of CK2β in MDA-MB-231 cells induced a shift of most of the FOXC2 from the nucleus to the cytoplasm, although FOXC2 was still detected in the nucleus at low levels (Figure 7b). This resulted in a decrease in both serum- and serum-independent cell migration (Figure 7c) and cell invasion through a basement membrane extract (Figure 7d). These data suggest that reduced levels of CK2β may impair CK2α/α′-mediated FOXC2 phosphorylation, thus promoting FOXC2 nuclear localization and an increase in cellular migration and invasion.

Reducing CK2β expression in normal epithelial cells promotes FOXC2 nuclear localization and a mesenchymal phenotype

To better clarify the role of CK2β in regulating CK2-dependent FOXC2 localization, MCF10A cells were transfected with siRNA against CK2β. This resulted in decreased CK2β expression and an increase in CK2α/α′ (Figure 8a), similar to the pattern seen in MDA-MB-436 and MDA-MB-231 cells (Figure 7a). This reversal of the CK2α/α′:CK2β ratio resulted in the translocation of FOXC2 from the cytoplasm to the nucleus (Figure 8b), along with downregulation of E-cadherin and upregulation of α-SMA (Figure 8a). Functionally, these CK2β knockdown cells exhibited increased cell migration and increased cell invasion (Figures 8c and d). These data demonstrate that maintenance of the proper ratio of CK2β:CK2α/α′ in epithelial cells is required for the maintenance of FOXC2 in the cytoplasm and prevention of EMT.

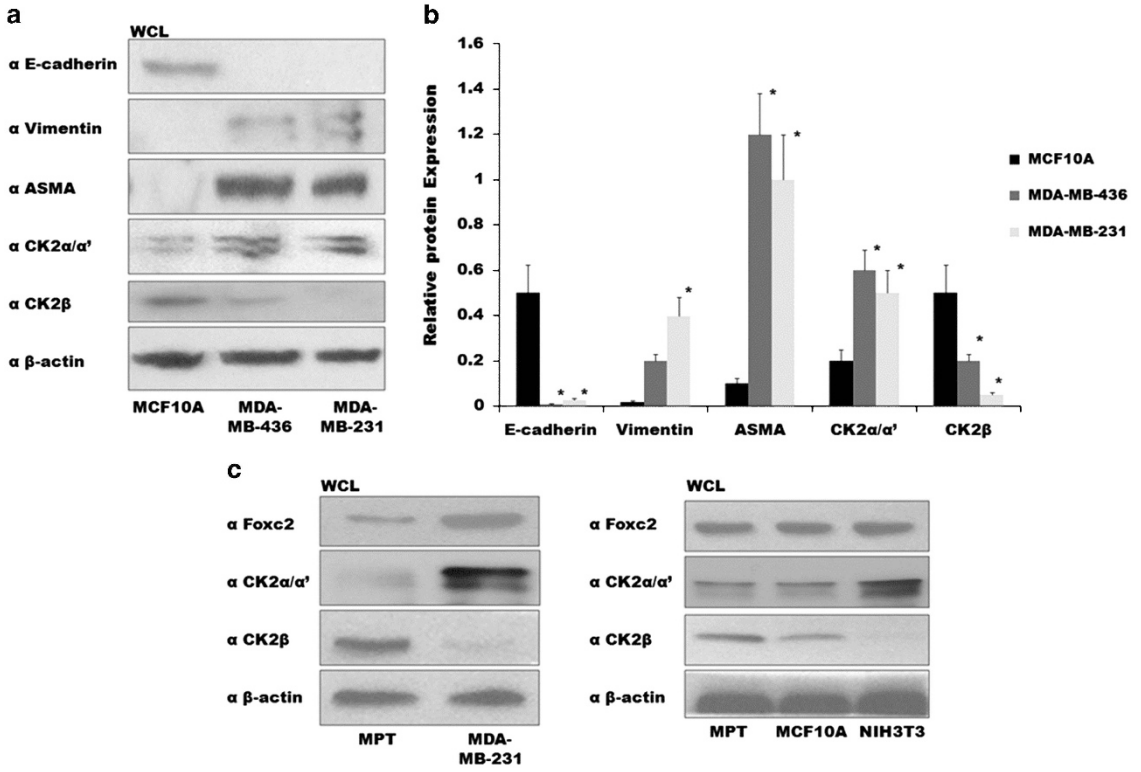

Figure 6. Mesenchymal cell lines expressing nuclear Foxc2 have higher expression levels of EMT markers and altered CK2α/α′/CK2β expression. (**a**) MCF10A, MDA-MB-436 and MDA-MB-231 whole-cell lysates (WCLs) were subjected to immunoblotting with the indicated antibodies. (**b**) Quantification of three experiments performed as in (**a**) with protein expression normalized to β-actin loading control. $N = 3$; $^*P < 0.05$ relative to MCF10A cells. (**c**) MPT and MDA-MB-231 WCLs (left panel) along with MPT, MCF10A and NIH3T3 cell lysates (right panel) were subjected to immunoblotting with the indicated antibodies.

FOXC2 is a critical CK2 target for epithelial maintenance

To determine the functional significance of FOXC2 phosphorylation in the overall spectrum of CK2 signaling, MCF10A cells were transfected with either WT Foxc2 or Foxc2-S124L ± CK2β. As predicted from the results in kidney epithelial cells, overexpression of either Foxc2 or Foxc2-S124L alone led to a marked increase in MCF10A cell migration and invasion (Figures 9a and c). This increase in mesenchymal behavior was suppressed in cells coexpressing CK2β along with wild-type Foxc2, whereas CK2β failed to suppress the increased migration and matrix invasion of Foxc2-S124L-expressing MCF10A cells.

In the metastatic MDA-MB-231 breast cancer cell line (in which Foxc2 is already localized to the nucleus), migratory and matrix invasion rates were high at baseline and did not increase further with the expression of Foxc2 or Foxc2-S124L alone (Figures 9b and d). As before, overexpression of CK2β suppressed the migratory and invasive phenotype of these cells, and this suppressive effect was maintained in cells in which CK2β was coexpressed with wild-type Foxc2. In contrast, overexpression of CK2β in cells coexpressing Foxc2-S124L failed to suppress migration and invasion (Figures 9b and d). These data demonstrate that cytoplasmic maintenance of Foxc2 has a key effector role in CK2-dependent epithelial maintenance.

DISCUSSION

Our previous results revealed that normal epithelial cells maintain Foxc2 in the cytoplasm even when Foxc2 is physiologically upregulated following epithelial injury.[16] However, when Foxc2 is overexpressed, such as following transfection of cultured cells or in epithelial-derived tumors, it can be readily detected in both the cytoplasm and the nucleus.[7,16] These results suggest that Foxc2 is maintained in the cytoplasm of normal epithelial cells by a regulated signaling pathway that can either be overwhelmed in the setting of very high levels of Foxc2 and/or dysregulated following transition to a malignant phenotype. In the current study, we demonstrate that a core feature of this signaling pathway is the CK2α/α′-dependent phosphorylation of Foxc2 at serine 124 in the amino terminus of the protein, and that this process is regulated in a cell-type-specific manner by the relative expression of the CK2α/α′ and CK2β subunits.

Serine 124 sits in a highly conserved region of Foxc2 that is also present in other Fox family members (Table 1). Analysis of the flanking sequence around serine 124 reveals that this is a potential CK2 phosphorylation site. Our results show that CK2 associates with Foxc2, and that in the absence of CK2 or following inhibition of CK2 kinase activity, Foxc2 predominantly traffics into the nucleus even in the absence of overexpression. These observations provide strong evidence that Foxc2 is maintained in the cytoplasm in a CK2-dependent manner, but do not define the CK2 regulatory site. To address this, we generated a non-phosphorylatable mutant of Foxc2 that mimics one of the mutations seen in patients with LD (S124L) as well as a phosphomimetic mutation at the site (S124D) and compared the localization of these constructs with that of wild-type Foxc2. Consistent with the model that phosphorylation of Foxc2 at serine 124 specifically maintains Foxc2 in the cytoplasm, S124D mutants were almost exclusively localized to the cytoplasm, whereas S124L mutants were predominantly found in the nucleus. The additional observation that treatment of cells expressing the S124D mutant with the CK2 inhibitor TBCA did not cause Foxc2-S124D to traffic into the

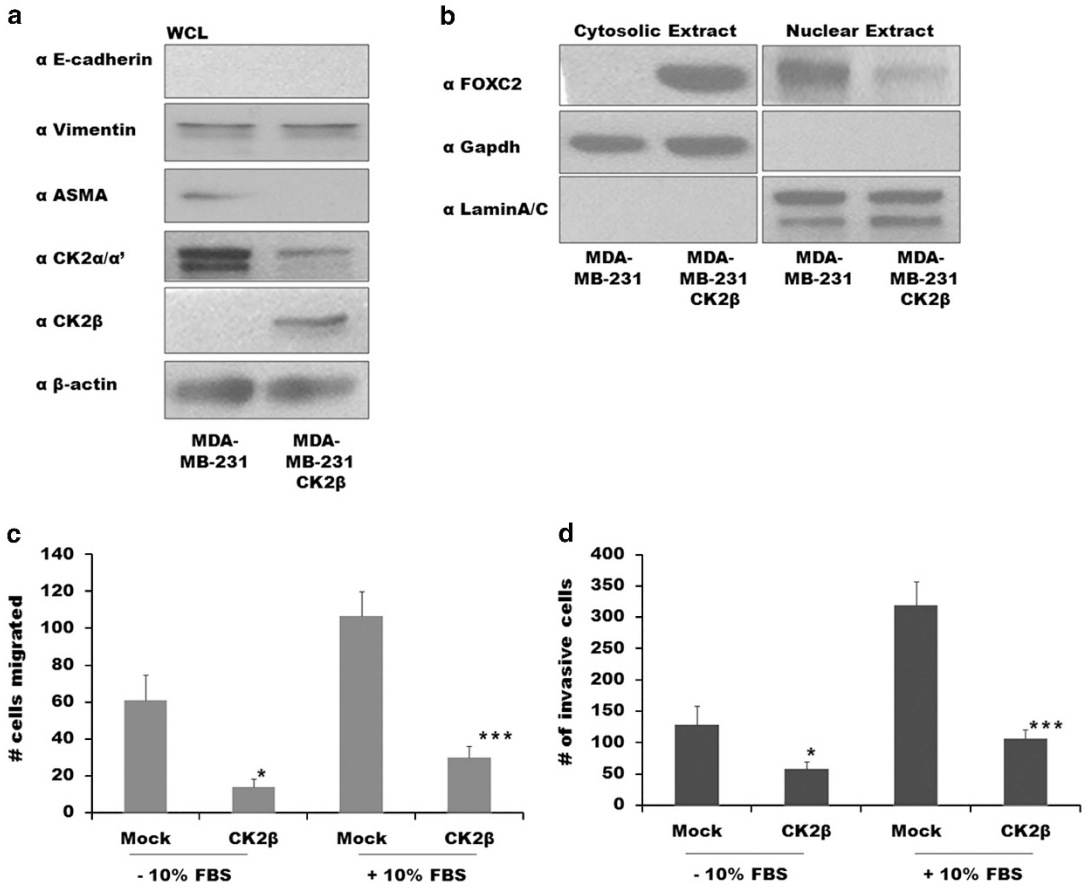

Figure 7. Restoration of CK2β expression in metastatic breast cancer cells promotes Foxc2 cytoplasmic localization and reduced migration. (**a**) MDA-MB-231 cells were transfected with either an empty vector or a CK2β expression construct two times and grown to 90% confluency. Whole cell lysates (WCLs) were then blotted with the indicated antibodies. (**b**) MDA-MB-231 cells transfected as in (**a**) were subjected to cell fractionation and fractions immunoblotted for FOXC2, LaminA/C and GAPDH. (**c**) MDA-MB-231 cells transfected as in (**a**) were subjected to cellular migration assays ± 10% FBS. $N = 3$; ***$P < 0.001$ and *$P < 0.05$ (compared with control cells). (**d**) MDA-MB-231 cells transfected as in (**a**) were subjected to a basement membrane extract cellular invasion assay ± 10% FBS. $N = 3$; ***$P < 0.001$ and *$P < 0.05$ (compared with control cells). C, cytosolic; N, nuclear.

nucleus suggests that serine 124 is the CK2-dependent phosphorylation site that specifically mediates cytoplasmic retention of Foxc2. This model is supported by our *in vitro* kinase assay demonstrating that CK2 directly phosphorylates Foxc2, with reduced phosphorylation of the S124L mutant.

CK2 is a constitutively active kinase that participates in many cellular processes including replication, transcription, translation and signal transduction.[23] CK2 consists of a heterodimer of the CK2α serine/threonine kinase and the CK2β regulatory subunit that is required for substrate binding.[23] CK2 has been described as a nuclear protein kinase, but has been found to be present in both the cytosol and the nucleus.[24] Consistent with a functional role of CK2 in both locations, Cdc25 has been shown to be exported from the nucleus by CK2-dependent phosphorylation,[25] whereas CK2-mediated phosphorylation of survivin and PPARγ has been implicated in the maintenance of their cytoplasmic localization.[26–28]

The mechanism by which CK2-dependent phosphorylation of Foxc2 is regulated appears to be similar to that described for CK2-dependent phosphorylation of Snail. MacPherson *et al.*[29] demonstrated that CK2 phosphorylates Snail1 to regulate its transcriptional activity and Deshiere *et al.*[22] found that the level of expression of the CK2β regulatory subunit was critical in determining the extent of Snail1 phosphorylation. CK2β regulates binding of CK2α/α′ to target proteins, and Deshiere *et al.*[22] found that low levels of CK2β resulted in the failure of CK2α/α′-dependent phosphorylation of Snail and subsequent Snail stabilization and induction of EMT. Our results are consistent with these findings in that downregulation of CK2β was detected in both of the breast metastatic tumor cell lines that we examined, resulting in a progressive reversal of the CK2α/α′:β-subunit ratio (as determined using our antibodies) in moving from normal breast epithelia (2:5) to weakly metastatic (3:1) and highly metastatic (10:1) breast tumor cells. This correlated with increased nuclear localization of FOXC2 even in the absence of FOXC2 overexpression. Mesenchymal fibroblasts, which express Foxc2 in both compartments, also demonstrated a decrease in β-subunit expression relative to α.

More interestingly, re-expression of the CK2β subunit in MDA-MB-231 cells partially normalized the α/α′:β-subunit ratio, induced cytoplasmic localization of the majority of FOXC2 and reduced migration and invasion of these highly metastatic breast cancer cells. However, coexpressing the non-phosphorylatable Foxc2-S124L mutant along with CK2β significantly suppressed the ability of CK2 to restore a more epithelial phenotype in MDA-MB-231 cells and promoted a mensenchymal phenotype in the non-malignant MCF10A breast cell line. Furthermore, CK2β knockdown in the MCF10A normal breast epithelial cell line resulted in a shift of FOXC2 from the cytoplasm to nucleus even though CK2α/α′ levels were increased. The increase in nuclear FOXC2 in these cells correlated with a mesenchymal expression pattern and increased migration/invasion, recapitulating the phenotype of the

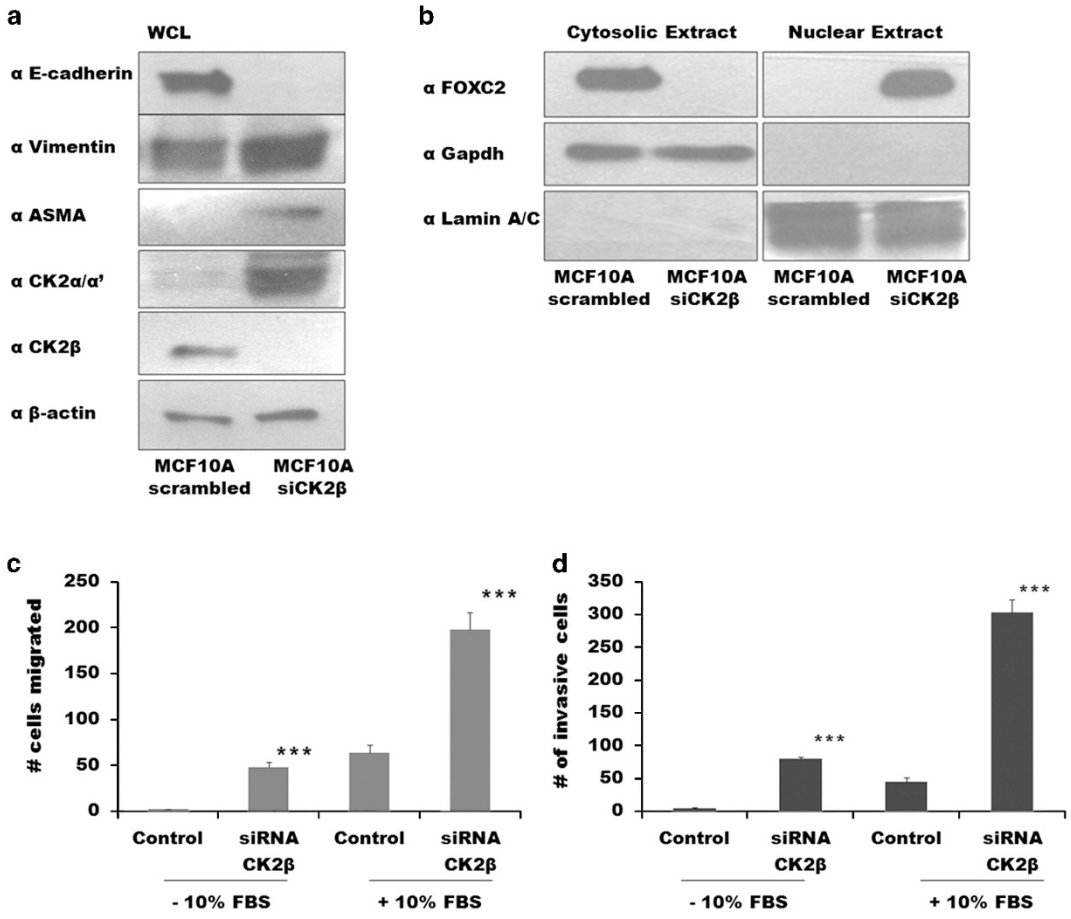

Figure 8. CK2β knockdown in normal breast epithelial cells promotes Foxc2 nuclear localization and increased migration/invasion. (**a**) MCF10A cells were transfected with either a scrambled siRNA or siRNA directed against CK2β and grown to 90% confluency. Whole-cell lysates (WCLs) were then blotted with the indicated antibodies. (**b**) MCF10A cells transfected as in (**a**) were subjected to cell fractionation and fractions immunoblotted for Foxc2, LaminA/C and GAPDH. (**c**) MCF10A cells transfected as in (**a**) were subjected to cellular migration assays ± 10% FBS. $N = 3$; ***$P < 0.001$ (compared with control scrambled siRNA cells). (**d**) MCF10A cells transfected as in (**a**) were subjected to a basement membrane extract cellular invasion assay ± 10% FBS. $N = 3$; ***$P < 0.001$ (compared with control cells). C, cytosolic; N, nuclear.

metastatic breast cancer cell lines. Cumulatively, these studies support the model that the ratio of CK2α/α′:CK2β determines the phosphorylation targets of the holoenzyme and show that FOXC2 serves as an important CK2 target for epithelial maintenance.

Studies using transfection of cultured epithelial cells with wild-type Foxc2 have proven that overexpression of Foxc2 results in reduced levels of E-cadherin and increased expression of mesenchymal markers such as vimentin and SMA.[7] However, the specific mechanism of this effect has been unclear as Foxc2 is increased in both the cytoplasmic and nuclear compartments in that setting.[16] The current studies demonstrate that nuclear localization of Foxc2 is required for the expression of mesenchymal proteins and suppression of E-cadherin as overexpression of cytoplasmically localized Foxc2-S124D fails to increase vimentin or α-SMA protein expression or suppress E-cadherin expression. The observation that transfection with nuclear localized Foxc2-S125L reduced the mRNA levels for E-cadherin while increasing the message for both vimentin and α-SMA is consistent with the model that Foxc2-mediated EMT is dependent on transcriptional regulation. Interestingly, the shift of FOXC2 from the nucleus to cytoplasm seen following re-expression of CK2β in MDA-MB-231 cells was sufficient to reduce α-SMA expression, but was not sufficient to reduce vimentin expression or relieve the tonic inhibition of E-cadherin expression seen in these cells. Whether this differential effect is due to the transcriptional activity of the small amount of FOXC2 that remains in the nucleus of CK2β-re-expressing MDA-MB-231 cells, or is due to non-FOXC2-dependent regulation of vimentin and E-cadherin in these malignant cells remains to be determined.

In conclusion, we show that CK2-mediated phosphorylation of Foxc2 promotes cytoplasmic localization of Foxc2 in normal epithelial cells, and that interruption of CK2 activity by either kinase inhibition or knock-down of the enzyme results in Foxc2 nuclear localization, increased migration, invasion, and partial loss of the epithelial phenotype. These data suggest that the CK2 holoenzyme normally acts to maintain cells in the epithelial state, but that this regulatory mechanism can be overcome under pathologic conditions. Thus stimulation of CK2 dependent phosphorylation of Foxc2, or suppression of Foxc2 dephosphorylation, might serve as therapeutic targets to promote maintenance of the epithelial phenotype and reduce the malignant phenotype in breast cancer cells.

MATERIALS AND METHODS

Cell lines and cell culture

MPT cells were cultured and maintained in our laboratory as reported previously.[16] MCF10A cells, MDA-MB-436 cells and MDA-MB-231 cells were maintained by American Type Culture Collection standards (Manassas, VA,

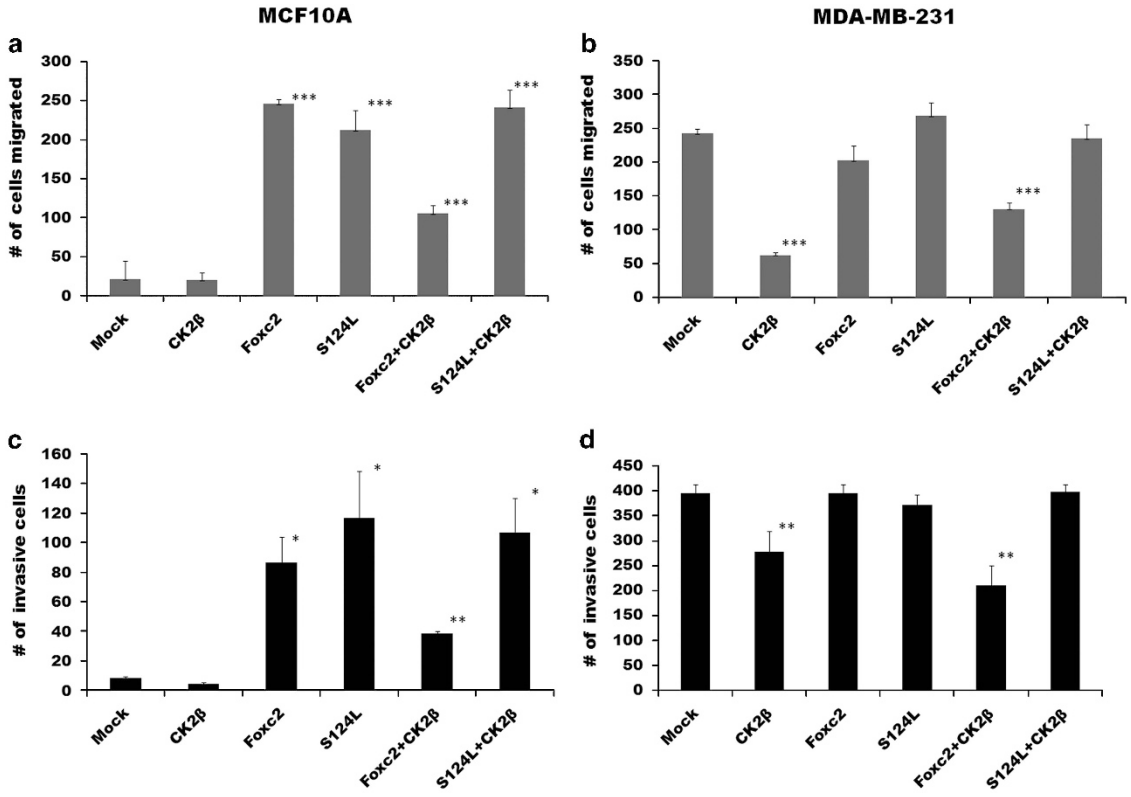

Figure 9. Foxc2-S124L can overcome the CK2β-mediated normalization of migration. (**a**) MCF10A cells were transfected with either empty vector, CK2β, GFP-Foxc2 or GFP-Foxc2-S124L in the indicated combinations, grown to 90% confluency and subjected to cell migration assays without FBS. $N = 3$; $^{***}P < 0.001$ (compared with control cells). (**b**) MDA-MB-231 cells transfected as in (**a**) were subjected to cell migration assays without FBS. $N = 3$; $^{***}P < 0.001$ (compared with control cells). (**c**) MCF10A cells transfected as in (**a**) were subjected to a basement membrane extract cellular invasion assay without FBS. $N = 3$; $^{**}P < 0.01$ and $^{*}P < 0.05$ (compared with control cells). (**d**) MDA-MB-231 cells transfected as in (**a**) were subjected to a basement membrane extract cellular invasion assay without FBS. $N = 3$; $^{**}P < 0.01$ (compared with control cells).

Table 1. Potential CK2 site is conserved among Fox family members

(FOXC2) *Homo sapiens*	RHNLSLNECF
(Foxc2) *Mus musculus*	RHNLSLNECF
(Foxc2) *Gallus gallus*	RHNLSLNECF
(Foxc2) *Xenopus tropicalis*	RHNLSLNECF
Foxc1	RHNLSLNECF
Foxf1a	RHNLSLNECF
Foxf2	RHNLSLNECF
Foxl1	RHNLSLNECF
Foxl2	RHNLSLNECF
Foxs1	RHNLSLNECF
CK2 consensus sequence	SxxE

Abbreviation: CK2, casein kinase 2.

USA). MCF10A and MDA-MB-231 cells were a generous gift from Dr David Rimm. MDA-MB-436 cells were a generous gift from Dr Marc Hansen.

Expression vectors and cell transfection studies

Full-length murine Foxc2 (a generous gift from Dr Tsutomu Kume) was subcloned in-frame with the N-terminal GFP-tag in the pEGFP-C1 vector (Clontech, Mountain View, CA, USA) or the N-terminal FLAG-tag in pFLAG-CMV (Sigma, St Louis, MO, USA). Residues in the murine Foxc2 cDNA were mutated and restriction sites for subcloning created by site-directed mutagenesis in the pEGFP-C1 vector according to the manufacturer's directions. Mutations were confirmed by sequencing at the Yale Keck Sequencing Facility (New Haven, CT, USA). The pAB07-CK2β expression vector was obtained from Addgene (Cambridge, MA, USA).

Immunofluorescence

For localization studies, MPT cells were plated on glass coverslips and fed with Opti-MEM medium (Invitrogen, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. Cells were transfected with 0.8 μg of plasmid DNA with Lipofectamine 2000 for 4 h. Transfected cells were stabilized for 48 h before fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) and rinsed in PBS, before applying mounting solution with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA). For F-actin staining, cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100, stained with Rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) and mounted with DAPI (Vector Laboratories). Images were acquired using an inverted fluorescent microscope with a x40 objective (Olympus, Leeds, MA, USA) and OpenLab software (Improvision, Waltham, MA, USA).

Immunodetection

Immortalized MPT cells were plated in 100 mm dishes and allowed to grow in Opti-MEM with 5% FBS for 48 h. Cells were refed with Dulbecco's modified Eagle's medium:F12 plus 10% FBS, and then transfected with 16 μg of DNA. Cells were lysed with RIPA buffer (Teknova, Hollister, CA, USA) or the NE-PER Kit (Thermo Scientific, Waltham, MA, USA) containing Halt (Thermo Scientific) protease/phosphatase inhibitors. Whole-cell lysates were cleared by 10 min centrifugation at 14 000 r.p.m. at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Fifty micrograms of protein lysate was loaded on to gels and transferred to PVDF membranes. Primary antibodies used overnight at 4 °C were as follows: anti-α-SMA (Epitomics, Burlingame, CA,

USA; 1184), anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-7558), anti-E-cadherin (BD Transduction Laboratories, San Jose, CA, USA; 610181), anti-β-actin (Novus, Littleton, CO, USA; NB600-503), anti-GFP (Santa Cruz Biotechnology; SC-9996), anti-CK2α/α′ (Santa Cruz Biotechnology; SC-136281), anti-CK2β (Pierce Biotechnology, Rockford, IL, USA; PA5-27416) or anti-Foxc2 (Santa Cruz Biotechnology; SC-28704 or SC-21397). Antibody–antigen complexes were identified by chemiluminescence (ECL +System; Amersham Biosciences, Piscataway, NJ, USA).

NE-PER fractionation

MPT cells grown in culture plates were placed on ice and fractionated using the NE-PER Kit (Thermo Scientific). Cells were washed with cold PBS. Plates were tipped on side to drain excess PBS, which was aspirated off. The proprietary CERI mixture was then added to the well (200 µl CERI, 2 µl Halt protease/phosphatase inhibitors per well of a 6-well plate) and vortexed vigorously for 15 s. Tubes were incubated on ice for 10 min. Then, 11 µl of cold CERII was added to each tube and vortexed vigorously for 10 s. Tubes were incubated on ice 1 min, vortexed 10 s and centrifuged at 13 200 r.p.m. for 5 min at 4 °C. Immediately, supernatant (cytosolic fraction) was transferred to a clean prechilled 1.5 ml. A quick spin was performed to remove excess cytosolic fraction with a syringe. The pellet was washed two times with PBS to remove any remaining cytosolic fraction. After the nuclear pellet was collected, the NER cocktail was added (100 µl NER, 1 µl Halt protease/phosphatase inhibitors) to the pellet, incubated on ice for 10 min intervals, vortexing for 15 s at the start of intervals for a total of 40 min, and finally centrifuging at 13 200 r.p.m. for 10 min at 4 °C. The supernatant (nuclear fraction) was transferred to a clean prechilled 1.5 ml tube. All fractions were stored at − 80 °C unless immunoprecipitation was performed. Quantification of relative levels of Foxc2 in each compartment was performed using normalization to the loading controls (LaminA/C and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) and to the proportion of total cell protein isolated from each compartment (4.65:1, cytosolic: nuclear).

Immunoprecipitation

Cell lysates (1000 µg of protein) from cells transfected with GFP-Foxc2 were incubated with 10 µl of monoclonal antibody against GFP (Santa Cruz Biotechnology) at 4 °C overnight with 20 µl of 50% (v/v) Protein A/G plus–agarose bead slurry (Santa Cruz Biotechnology). Beads were pelleted by centrifugation for 2 min at 1000 *g*, and then washed three times with the lysis buffer and two times with PBS. Proteins were eluted and prepared for analysis by heating in reduced Laemmli sample buffer at 100 °C for 5 min. Proteins were resolved by 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred on to PVDF membranes for western blotting. Membranes were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline, then probed either with antibody–horseradish peroxidase conjugates containing antibody against GFP or CK2 proteins (Santa Cruz Biotechnology) and finally with horseradish peroxidase conjugates (Santa Cruz Biotechnology).

CK2 inhibition

MPT cells were treated with vehicle control or 100 µM of TBCA (Calbiochem, Billercia, CA, USA) for 1 h and subjected to cell fractionation. For RNA interference, MPT cells were transfected with scrambled siRNA (Ambion, Foster City, CA, USA), siRNA directed to knock down CK2α/α′ (Santa Cruz Biotechnology) or siRNA directed to knock down CK2β (Life Technologies, Grand Island, NY, USA) and subjected to cell fractionation.

Quantitative real-time PCR

Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA) and 1 mg of RNA was reverse transcribed using random hexamer primers according to the manufacturer's instructions (iScript; Bio-Rad). Quantitative real-time PCR was conducted using power SYBR green mix (SsoFast; Bio-Rad) with a iCycler real-time PCR machine (Bio-Rad). Primer pairs were selected for their specificity and efficiency, and target gene expression levels were determined by the comparative cycle threshold method (Ct) or ddCt (dCt of target/dCt of control) method. PCR controls run in the absence of template were consistently negative.

In vitro kinase assay

Immunoprecipitated GFP-Foxc2 or GFP-Foxc2-S124L constructs were incubated for 60 min at 30 °C with and without 500 U of purified protein kinase CK2 (New England Biolabs, Ipswich, MA, USA) in the presence of kinase buffer (New England Biolabs), 20 µM cold ATP (Sigma) and 2 µCi of [γ-^{32}P]ATP (Perkin-Elmer, Waltham, MA, USA). *In vitro* kinase reactions were terminated by the addition of SDS–PAGE sample buffer and boiling for 5 min. Samples were subjected to SDS–PAGE, and the amount of ^{32}P incorporated into GFP-Foxc2 or GFP-Foxc2-S124L was analyzed by autoradiography, followed by immunoblot analysis.

Migration assay

Cell migration was determined by using a 96-well ChemoTx (Neuroprobe, Gaithersburg, MD, USA) cell migration microplate with a pore size of 8 µm. The underside of filters was coated with 1 mg/ml of collagen-I (Trevigen, Gaithersburg, MD, USA) in PBS for 1 h at room temperature, rinsed two times in distilled water and air dried. Serum-starved cells were trypsinized to yield single cells. The cells were pelleted and diluted to a final concentration of 3.3×10^4 cells per ml in Dulbecco's modified Eagle's medium supplemented with 1 mg of bovine serum albumin per ml (Dulbecco's modified Eagle's medium/bovine serum albumin) to prevent cell clumping. The bottom wells of the ChemoTx microplate were filled with Dulbecco's modified Eagle's medium/bovine serum albumin ± 10% FBS, covered with the filter and 1000 cells added to each top well. The chamber was incubated for 12 h at 37 °C in 5% CO_2. Cells adhering to the top of the filter were removed, and cells adhering to the bottom of the filter were fixed in 100% methanol, washed and adherent cells stained with 1% (wt/vol) crystal violet in 20% (vol/vol) methanol for 10 min and counted visually. All experiments were performed in quadruplicate.

Invasion assay

Invasion assays were carried out using Cultrex 96-well Basement Membrane Extract Cell Invasion Assay (Trevigen). Briefly, triplicate transwell chambers with 8-µm pore polycarbonate filters were coated with 50 µl of ice-cold 0.5× basement membrane extract in 1x coating buffer and incubated for 1 h at 37 °C. To monitor cell invasion, 5000 cells in 50 µl of serum-free media were seeded on the basement membrane extract-coated filters and the lower chamber was filled with 150 µl of medium ± 10% FBS as a chemoattractant. After incubation for 24 h, the cells on the underside of the filter were stained with crystal violent and quantified visually.

Statistics

All results are expressed as mean ± s.e.m. Statistical significance was assessed by Student's *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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