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

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Abstract

Nuclear Foxc2 is a transcriptional regulator of mesenchymal transformation during developmental 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 phosphorylation site at serine 124, and we now show that CK2 associates with Foxc2 and phosphorylates this site in vitro. Knock-down 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-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 that is dependent on proper targeting of the holoenzyme via the CK2β regulatory subunit.

Keywords

Foxc2; E-cadherin; Epithelial Cell; Epithelial to Mesenchymal Transition; CK2 Signaling
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 de-differentiation during which they lose their brush border, exhibit a flattened morphology and express mesenchymal markers such as vimentin and α-smooth muscle actin.\(^1-3\) This de-differentiation is believed to promote cell survival, migration and proliferation, thus enhancing regeneration of the mature epithelium.\(^4\) However, sustained de-differentiation (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.\(^14\)

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.\(^7-9\) Fox proteins contain the FHD, a sequence of ~100 amino acids forming a motif that binds to DNA.\(^10\) Foxc2, belonging to the ‘C’ subfamily, is required for cardiovascular development\(^11\), early organogenesis of the kidney\(^12\), podocyte differentiation and glomerular basement membrane maturation.\(^13\) Mani et al.\(^14\) have demonstrated that Foxc2 can act as an activator of epithelial cell dedifferentiation during tumor metastasis, while Bard and colleagues\(^15\) 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 RNAi resulted in a partial loss of the epithelial phenotype. In contrast, \(ex vivo\) over-expression 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 phosphorylation site at serine 124. In the current study we show that CK2 can phosphorylate Foxc2 in vitro and that knock-down 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 markers. Examination of CK2 expression in these cells revealed down-regulation of the CK2 regulatory subunit, CK2β. Over-expression of CK2β induced cytoplasmic localization of FOXC2, suppressed α-SMA expression, 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 over-expressed CK2β to suppress the mesenchymal phenotype of malignant breast epithelial cells was prevented by the co-expression 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) 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 and suggest that epithelial cells normally maintain Foxc2 in the cytoplasm. Transfection of MPT cells with a vector encoding FLAG-tagged Foxc2 resulted in over-expression 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 (SLNE) is a potential phosphorylation site for casein kinase 2 (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 over-expressed 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 (Figure 1B, C). Side-by-side comparison of whole cell lysates confirmed that all three constructs were expressed at similar levels (Supplemental Figure 1). These results suggest that phosphorylation at serine 124 promotes cytoplasmic localization of Foxc2.

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 siRNA (Figure 2B, 65% knock-down of CK2α/α′), followed by analysis of the subcellular localization of endogenous Foxc2 24 hours later. Cell fractionation confirmed that endogenous Foxc2 is detectable only in the cytoplasm of control MPT cells, whereas CK2α/α′ knock-down 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 (a cell-permeable CK2 kinase inhibitor) 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 hour 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 over-expressed 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 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. In contrast, over-expression of Foxc2 by transfection or in epithelial tumors results in increased nuclear localization and a mesenchymal expression pattern (Figure 1A). 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 downregulation of E-cadherin and upregulation of both vimentin and α-SMA as compared to control cells transfected with GFP alone (Figure 4A, quantified in B). In contrast, cells over-expressing 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 (Supplemental Figure 2) revealed an epithelial morphology in MPT cells transfected with GFP only or GFP-Foxc2-S124D, while more of a 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, qRT-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 to 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.

**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.\textsuperscript{14} To determine whether FOXC2 is localized differently in normal breast epithelial cells as compared to 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.\textsuperscript{29} Whole cell lysates demonstrated an increase in total FOXC2 expression in highly metastatic MDA-MB-231 cells as compared to 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, while 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.

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

Over-expression of transfected Foxc2 in normal renal epithelia leads to nuclear localization,\textsuperscript{14, 16} and high levels of endogenous Foxc2 in MDA-MB-231 cells correlates 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.\textsuperscript{28} showed that unbalanced expression of CK2α and β subunits in a subset of breast tumor samples correlated with 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 to 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 B). Consistent with the findings of Deshiere et al.,\textsuperscript{28} these cells had the highest expression of CK2β with a CK2 α/α’ to β ratio of 2.5. In contrast, both MDA-MB-436 and 231 cells exhibited downregulation of CK2β and upregulation of α/α’, resulting in a reversal of the α/α’ to 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 to 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, CK2 α/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.

**Over-expressing 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 dependent 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 6A). This reversal of the CK2α/α’:CK2β ratio resulted in 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 knock-down cells exhibited increased cell migration and increased cell invasion (Figure 8C, D). These data demonstrate that maintenance of the proper ratio of CK2β to CK2α/α’ in epithelial cells is required for maintenance of FOXC2 in the cytoplasm and prevention of EMT.

**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, over-expression of either Foxc2 or Foxc2-S124L alone led to a marked increase in MCF10A cell migration and invasion (Figure 9A, C). This increase in mesenchymal behavior was suppressed in cells co-expressing CK2β along with wild-type Foxc2, whereas CK2β failed to suppress the increased migration and matrix invasion of Foxc2-S124L expressing MCF10A cells.

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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 expression of Foxc2 or Foxc2-S124L alone (Figure 9B, D). As before, over-expression of CK2β suppressed the migratory and invasive phenotype of these cells, and this suppressive effect was maintained in cells in which CK2β was co-expressed with wild-type Foxc2. In contrast, over-expression of CK2β in cells co-expressing Foxc2-S124L failed to suppress migration and invasion (Figure 9B, D). These data demonstrate that cytoplasmic maintenance of Foxc2 plays 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. However, when Foxc2 is over-expressed, such as following transfection of cultured cells or in epithelial-derived tumors, it can be readily detected in both the cytoplasm and the nucleus. 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 over-expression. 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 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. CK2 consists of a heterodimer of the CK2α serine/threonine kinase and the CK2β regulatory subunit that is...
required for substrate binding.\textsuperscript{21} CK2 has been described as a nuclear protein kinase but has been found to be present in both the cytosol and the nucleus.\textsuperscript{22} Consistent with a functional role of CK2 in both locations, Cdc25 has been shown to be exported from the nucleus by CK2 dependent phosphorylation,\textsuperscript{23} while CK2-mediated phosphorylation of survivin and Ppar\(\gamma\) has been implicated in maintenance of their cytoplasmic localization.\textsuperscript{24–26}

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.\textsuperscript{27} demonstrated that CK2 phosphorylates Snail1 to regulate its transcriptional activity and Deshiere and colleagues\textsuperscript{28} found that the level of expression of the CK2\(\beta\) regulatory subunit was critical in determining the extent of Snail1 phosphorylation. CK2\(\beta\) regulates binding of CK2\(\alpha/\alpha'\) to target proteins, and Deshiere et al.\textsuperscript{28} found that low levels of CK2\(\beta\) resulted in failure of CK2\(\alpha/\alpha'\) dependent phosphorylation of Snail and subsequent Snail stabilization and induction of EMT. Our results are consistent with these findings in that downregulation of CK2\(\beta\) was detected in both of the breast metastatic tumor cell lines that we examined, resulting in a progressive reversal of the CK2\(\alpha/\alpha'\) to \(\beta\) 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 over-expression. Mesenchymal fibroblasts, which express Foxc2 in both compartments, also demonstrated a decrease in \(\beta\) subunit expression relative to \(\alpha\).

More interestingly, re-expression of the CK2\(\beta\) subunit in MDA-MB-231 cells partially normalized the \(\alpha/\alpha':\beta\) subunit ratio, induced cytoplasmic localization of the majority of FOXC2, and reduced migration and invasion of these highly metastatic breast cancer cells. However, co-expressing the non-phosphorylatable Foxc2-S124L mutant along with CK2\(\beta\) significantly suppressed the ability of CK2 to restore a more epithelial phenotype in MDA-MB-231 cells and promoted a mesenchymal phenotype in the non-malignant MCF10A breast cell line. Furthermore, CK2\(\beta\) knockdown in the MCF10A normal breast epithelial cell line resulted in a shift of FOXC2 from the cytoplasm to nucleus even though CK2\(\alpha/\alpha'\) 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 metastatic breast cancer cell lines. Cumulatively, these studies support the model that the ratio of CK2\(\alpha/\alpha':\beta\) determines the phosphorylation targets of the holoenzyme and show that FOXC2 serves as an important CK2 target for epithelial maintenance.

Studies utilizing transfection of cultured epithelial cells with wild type Foxc2 have proven that over-expression of Foxc2 results in reduced levels of E-cadherin and increased expression of mesenchymal markers such as vimentin and smooth muscle actin.\textsuperscript{14} However, the specific mechanism of this effect has been unclear since Foxc2 is increased in both the cytoplasmic and nuclear compartments in that setting.\textsuperscript{16} The current studies demonstrate that nuclear localization of Foxc2 is required for expression of mesenchymal proteins and suppression of E-cadherin since over-expression of cytoplasmically localized Foxc2-S124D fails to increase vimentin or \(\alpha\)-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 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

Mouse proximal tubule (MPT) cells were cultured and maintained in our laboratory as previously reported.16 MCF10A cells, MDA-MB-436 and MDA-MB-231 cells were maintained by American Type Culture Collection standards. 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. The pAB07-CK2β expression vector was obtained from Addgene.

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% FBS in 5% CO2 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 PBS and rinsed in PBS, prior to applying mounting solution with DAPI (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, Burlingame, CA, USA). Images were acquired using an inverted fluorescent microscope with a 40x objective (Olympus) and OpenLab software (Improvision).

**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 re-fed with DMEM:F12 plus 10%FBS, 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, Waltham, MA, USA) protease/phosphatase inhibitors. Whole cell lysates were cleared by 10 min centrifugation at 14000 rpm at 4°C. Protein concentration was determined by the Bradford assay (Bio-Rad). 50 μg of protein lysate was loaded on to gels and transferred to PVDF membranes. Primary antibodies used overnight at 4°C: anti-α-SMA (EPIT-MICS, 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, Santa Cruz, CA, USA; SC-9996), anti-CK2α/α′ (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-136281), anti-CK2β (Pierce Biotechnology, Rockford, USA, PA5-27416) or anti-Foxc2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 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, Waltham, MA, USA). Cells were washed with cold PBS. Plates were tipped on side to drain excess PBS which was aspirated off. CERI mixture was then added to the well (200μl CERI, 2μl Halt protease/phosphatase inhibitors per well of a 6-well plate) and vortexed vigorously for 15 seconds. Tubes were incubated on ice for 10 min. Then 11μl of cold CERII was added to each tube and vortexed vigorously for 10 sec. Tubes were incubated on ice 1 min, vortexed 10 sec, and centrifuged at 13200 rpm for 5 min at 4°C. Immediately supernatant (Cytosolic Fraction) was transferred to a clean pre-chilled 1.5ml. A quick spin was performed to remove excess cytosolic fraction with a syringe. The pellet was washed 2 times with PBS to remove any remaining cytosolic fraction. After the nuclear pellet was collected, the NER cocktail was added (100μl NER, 1μl Halt protease/phosphatase inhibitors) to pellet, incubated on ice for 10 min intervals, vortexing for 15 sec at the start of intervals for a total of 40 min, finally centrifuging at 13200 rpm for 10 min at 4°C. Supernatant (nuclear fraction) was transferred to a clean pre-chilled 1.5ml 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 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, Santa Cruz, CA, USA) at 4°C overnight with of 20 μl of 50% (v/v) Protein A/G plus–agarose bead slurry (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Beads were pelleted by centrifugation for 2 min at 1000 g, and then washed three times with lysis buffer, and twice 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 SDS/PAGE and transferred 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, Santa Cruz, CA, USA), then horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

CK2 inhibition

MPT cells were treated with vehicle control or 100μM of TBCA (Calbiochem, Billerica, CA, USA) for 1h and subjected to cell fractionation. For RNAi, MPT cells were transfected with scrambled siRNA (Ambion, Foster City, CA, USA), siRNA directed to knock-down CK2α/α′ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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 RNaseasy Kit (Qiagen, Valencia, CA, USA) and 1 mg of RNA was reverse transcribed using random hexamer primers according to the manufacturer’s instructions (iScript; Biorad, Hercules, CA, USA). qPCR was conducted using power SYBR green mix (SsoFast; Biorad, Hercules, CA, USA) with a iCycler real-time PCR machine (Biorad, Hercules, CA, USA). 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 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 500U of purified protein kinase CK2 (New England Biolabs, Ipswich, MA, USA) in the presence of kinase buffer (New England Biolabs, Ipswich, MA, USA), 20 μM cold ATP (Sigma, St. Louis, MO, USA) and 2 μCi of [γ-32P]ATP (Perkin Elmer, Waltham, MA, USA). In vitro kinase reactions were terminated by addition of SDS-PAGE sample buffer and boiling for 5 min. Samples were subjected to SDS-PAGE, and the amount of 32P 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 8μm. The underside of filters was coated with 1mg/mL of collagen-I (Trevigen, Gaithersburg, MD, USA) in PBS for 1 h at room temperature, rinsed twice 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/ml in DMEM supplemented with 1 mg of BSA/ml (DMEM/BSA) to prevent cell clumping. The bottom wells of the ChemoTx microplate were filled with DMEM/BSA ± 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% CO₂. 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 done using Cultrex® 96-well Basement Membrane Extract (BME) Cell Invasion Assay (Trevigen, Gaithersburg, MD). Briefly, triplicate transwell chambers with 8-μm pore polycarbonate filters were coated with 50μl of ice-cold 0.5× BME in 1x Coating Buffer and incubated for one hour at 37°C. To monitor cell invasion, 5000 cells in 50 μL of serum-free media were seeded on the BME-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 violet 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 less than 0.05 was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 3 replicates per condition. (B) MPT cells transiently transfected with either GFP-Foxc2, GFP-Foxc2-S124D or GFP-Foxc2-S124L were fixed and imaged for GFP (green) and DAPI (blue). Images obtained at 40x 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 4–6 replicates per condition.
Figure 2. CK2 is required to maintain cytoplasmic localization of Foxc2

(A) MPT cells were transfected with GFP-Foxc2 and whole cell lysates immunoprecipitated 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 hours later by lysis and immunoblotting to determine efficiency of CK2 protein reduction. Percentage knock-down determined by normalizing to β-actin. Graph shows quantification for n=3; **p<0.005 relative to siRNA scrambled control. (C) MPT cells 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, **p<0.005 relative to siRNA scrambled control. (D) MPT cells were treated with vehicle control or 100μM TBCA for 1h, followed by cell fractionation and immunoblotting for endogenous Foxc2, LaminA/C and GAPDH. Relative Foxc2 expression in each compartment was determined as described in Methods. n=4; ***p<0.001, **p<0.005 relative to vehicle control. (E–F) MPT cells transiently transfected with the indicated constructs were treated with vehicle control (E) or 100μM TBCA (F) for 1h, and subjected to cell fractionation followed by immunoblotting with anti-GFP, anti-LaminA/C and anti-GAPDH. Relative expression was determined as described in Methods. n=3; **p<0.005.
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.
Figure 4. Cytoplasmic localization maintains an epithelial phenotype
(A) MPT cells were transfected with GFP, GFP-Foxc2-S124D or GFP-Foxc2-S124L constructs x3 to achieve ~70% transfection efficiency and grown to 90% confluency. Whole cell lysates were then blotted with the indicated antibodies. (B) Quantification of 3 experiments performed as in (A) with protein expression normalized to β-actin. n=3; ***p<0.001, **p<0.005 (compared to 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. WCL=whole cell lysate. n=3; ***p<0.001, **p<0.005 (compared to 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 were subjected to immunoblotting with anti-Foxc2 or anti-β-actin and quantified relative to β-actin. *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 below. Relative FOXC2 expression was determined as described in Methods. WCL=whole cell lysate, C=Cytosolic, N=Nuclear. *p<0.05 relative to MCF10A cells.
Figure 6. Mesenchymal cell lines expressing nuclear FOXC2 have higher expression levels of EMT markers and altered CK2α/CK2β expression

(A) MCF10A, MDA-MB-436 and MDA-MB-231 whole cell lysates were subjected to immunoblotting with the indicated antibodies. (B) Quantification of 3 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 whole cell lysates (left panel) along with MPT, MCF10A and NIH3T3 cell lysates (right panel) were subjected to immunoblotting with the indicated antibodies. WCL=whole cell lysate.
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 x2 and grown to 90% confluency. Whole cell lysates 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, *p<0.05 (compared to 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, *p<0.05 (compared to control cells). WCL=whole cell lysate, C=Cytosolic, N=Nuclear, FBS=Fetal Bovine Serum.
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 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, *p<0.05 (compared to 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 to control cells). WCL=whole cell lysate, C=Cytosolic, N=Nuclear, FBS=Fetal Bovine Serum.
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 fetal bovine serum (FBS). n=3; ***p<0.001 (compared to 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 to 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, *p<0.05 (compared to 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 to control cells).
Table 1

Potential CK2 site is conserved amongst Fox family members.

| (FOXC2)  | Homo sapiens | RHNLSDLNCF |
|----------|--------------|-------------|
| (Foxc2)  | Mus musculus | RHNLSDLNCF |
| (Foxc2)  | Gallus gallus | RHNLSDLNCF |
| (Foxc2)  | Xenopus tropicalis | RHNLSDLNCF |
| Foxc1    |              | RHNLSDLNCF |
| Foxf1a   |              | RHNLSDLNCF |
| Foxf2    |              | RHNLSDLNCF |
| Foxl1    |              | RHNLSDLNCF |
| Foxl2    |              | RHNLSDLNCF |
| Foxs1    |              | RHNLSDLNCF |

**CK2 consensus sequence**

*SxxE*