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## Mesenchymal-Epithelial Transition in epithelial response to injury: the role of Foxc2

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### Abstract

Over-expression of the forkhead family transcription factor Foxc2 has been shown to activate epithelial-mesenchymal transition (EMT) and correlate with tumor metastasis. In the present study we demonstrate that both mRNA and protein levels of Foxc2 increase one day after kidney ischemia/reperfusion in sublethally injured tubular cells, and that the protein is located in the cytoplasm rather than the nucleus of these cells. In vitro studies of cultured tubular cells confirm the cytoplasmic location of Foxc2 and demonstrate that increased cytoplasmic expression of Foxc2 correlates with epithelial differentiation rather than de-differentiation. Silencing of Foxc2 via RNAi in these cells led to EMT and increased cell migration. In contrast, Foxc2 is found in both the nucleus and cytoplasm of cultured fibroblasts, with RNAi leading to increased expression of epithelial markers and impaired cell migration. Consistent with a sub-cellular localization dependence of Foxc2 function, over-expression of Foxc2 in renal epithelial cells resulted in de novo nuclear expression of the protein and promotion of a mesenchymal/fibroblast phenotype. These results suggest that Foxc2 may have regulatory functions independent of its nuclear transcriptional activity, and that upregulation of endogenous Foxc2 in the cytoplasm of injured tubular cells activates epithelial cell re-differentiation rather than de-differentiation during organ repair.

### Keywords

Foxc2; kidney; E-cadherin; vimentin; migration

### Introduction

Epithelial cells in organs such as the kidney normally exist in a highly differentiated state that enables them to provide efficient vectorial transport while suppressing functions such as proliferation and migration. Following organ injury many of these cells can be lost, leading to permanent organ dysfunction unless they are replaced in some physiologic manner. Results from several laboratories demonstrate that repair after kidney injury occurs primarily via reconstitution of the injured tubule by surviving tubular epithelial cells (Duffield and

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Bonventre, 2005; Ishibe and Cantley, 2008; Lin *et al.*, 2005). Cells that survive the initial injury undergo a transient process of de-differentiation in which they lose their brush border, exhibit a flattened morphology, and express markers suggestive of a more mesenchymal phenotype (vimentin, fibroblast specific protein (FSP-1),  $\alpha$ -smooth muscle actin ( $\alpha$ SMA)) (Strutz *et al.*, 1995; Wallin *et al.*, 1992; Witzgall *et al.*, 1994). This de-differentiation is believed to promote cell survival and subsequent migration into the regions where cell necrosis, apoptosis, or detachment have led to denudation of the tubular basement membrane, where they proliferate in order to replace the lost cells (Bonventre, 2003). However, for tubule repair to be complete, the de-differentiated cells must ultimately re-differentiate back to an epithelial phenotype. If this re-differentiation fails to occur, the sustained de-differentiation (often referred to as epithelial-mesenchymal transition (EMT)) is believed to promote fibrosis, scarring and progressive kidney failure (Kalluri and Neilson, 2003; Liu, 2004). Therefore, identifying the factors that regulate the processes of de-differentiation and re-differentiation are critical to understanding how normal repair occurs.

Fox (Forkhead box) proteins are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity (Birkenkamp and Coffey, 2003; Calnan and Brunet, 2008; Obsil and Obsilova, 2008). The defining feature of Fox proteins is the forkhead box, a sequence of 80 to 100 amino acids forming a motif that binds to DNA. This forkhead motif is also known as the winged helix due to the butterfly-like appearance of the loops in the protein structure of the domain (Arden, 2006). The Fox family of transcription factors is expressed in various organs and tissues during development, and is involved in a variety of developmental and cellular differentiation processes (Coffey and Burgering, 2004). Foxc2, belonging to the “C” subfamily is required for cardiovascular development (Myatt and Lam, 2007), early organogenesis of the kidney (Xu and Massague, 2004), podocyte differentiation and glomerular basement membrane maturation (Greer and Brunet, 2005).

Mani *et al.* have recently proposed that Foxc2 can act as an activator of epithelial cell de-differentiation and metastasis in breast cancer (Mani *et al.*, 2007). By utilizing a mouse mammary tumor model, they showed that over-expression of Foxc2 increases the metastatic potential of otherwise poorly metastatic breast cancer cells. In addition, Foxc2 was found to be over-expressed in highly invasive and metastatic subtypes of breast cancer. In Madine-Darby canine kidney (MDCK) cells, Foxc2 over-expression resulted in phenotypic EMT with increased migratory and invasive behavior of these non-malignant tubular epithelial cells. These observations suggest that Foxc2 is an important transcriptional mediator of EMT, which can program cellular traits associated with de-differentiation, migration, and metastasis (Mani *et al.*, 2007).

It was therefore surprising that Bard *et al.* recently reported a possible role for Foxc2 in epithelial cell differentiation. They utilized a novel bioinformatics approach to identify common genes expressed by different tissues undergoing similar developmental programs. In an analysis of the genetic basis of mesenchyme-to-epithelium transition (MET), they identified Crabp1 and six transcriptional regulators including Cited1, Cited2, Meox1, Lhx1, Foxc1, and Foxc2 as widely involved in this process. Expression pattern analysis of these

transcriptional regulators showed that this unique gene set was expressed in no other tissues and that members of the set were thus candidates for regulating MET (Bard *et al.*, 2008).

The conflicting nature of these findings raises the question of whether increased expression of Foxc2 activates EMT or MET. Although candidates for the regulation of renal epithelial cell de-differentiation are numerous and their mechanism of action widely investigated, the process of re-differentiation following injury is much less well understood and there has been little discussion about it in the literature, leading us to investigate the role of Foxc2 in this process using in vivo and in vitro approaches. Our results in models of kidney injury and in cultured cells lead us to propose that Foxc2 can have differential effects on cell de-differentiation and re-differentiation depending on the predominate sub-cellular localization of the protein, and that upregulation of endogenous Foxc2 is involved in regulating re-differentiation during the repair process after kidney injury.

## Material and Methods

### Surgery and experimental protocol

All animal protocols have been approved by the Yale animal care and use committee. Eight to ten week-old male C57BL6, weighing approximately 25-30 grams were anesthetized with a 0.03 ml intraperitoneal injection of 10:1 ketamine (100 mg/ml) and xylazine (10 mg/ml). The left flank region was shaved and the animals placed on a heating pad to keep a constant temperature. Flank incision was made, and the kidney exposed. For the Ischemia-Reperfusion (I/R) injury, a nontraumatic vascular clamp was applied across the renal artery for 30 min. The animals received 100 ml/kg of warm saline instilled into the peritoneal cavity during the procedure. After the clamps were released, the flanks were closed and animals allowed to recover with free access to food and water. At day 1, 3, 7 and 10 after surgery, the animals were sacrificed and kidneys harvested and snap-frozen. Sham kidneys were used for control.

### Histology and Immunohistochemistry

Mice underwent perfusion-fixation with 4% paraformaldehyde (PFA) through the left ventricle. Kidneys were embedded in paraffin and 5  $\mu$ m sections were cut. For histological evaluation of renal injury, sections were deparaffinized and stained with H&E. For immunohistochemistry experiments, sections were deparaffinized, boiled in Retrieval buffer (BD Pharmingen) and incubated overnight with anti-Foxc2 (Santa Cruz biotechnology: SC-31734 and Abcam: ab5060 (data not shown)) or anti-Megalin (generous gift from Dr Dan Biemesderfer).

### Real time polymerase chain reaction (qPCR)

Total RNA was extracted using the RNeasy Kit (Qiagen) and 1  $\mu$ g of RNA was reverse transcribed using random hexamer primers according to the manufacturer's instructions (SuperScript II, Invitrogen). qPCR was conducted using power SYBR green mix (Applied Biosystems) with a 7300 AB Real-time PCR machine (Applied Biosystems). Primer pairs were selected for their specificity and efficiency ( $> 0.9$ ) and target gene expression levels were determined by the comparative cycle threshold method (Ct) or ddCt (dCt of injured

kidney/dCt of uninjured contralateral kidney) method. PCR controls run in absence of template were constantly negative. Foxc2 (5'GCCACCTCCTGGTATCTGAAC, 5'GGACAGCCTCAGTATTTGGTG), GAPDH (5'GACCCCTTCATTGACCTCAAC, 5'CTTCTCCATGGTGGTGAAGA), Napi2a (5' TCTGATGCTGGCTTTCCCTTT, 5'ACCATGCTGACAATGATGGA), Aqp2 (5'ATCTTTGCCTCCACCGATGA, 5'GCCGGTGAAATAGATCCCAAG).

### Isolation of renal proximal tubules, cell transfection and cell culture

Isolation of primary cultures of mouse proximal tubule cells was done according to a modified protocol by Schafer et al. PTs were selected according to previously reported morphological criteria (Schafer *et al.*, 1997) and cultured in 24 well plates containing renal epithelial growth medium (REGM, Lonza). Immortalized mouse proximal tubule (MPT) cells were transfected with control plasmid or with plasmid encoding Foxc2 (Generous gift from Dr Robert A. Weinberg) using Lipofectamine 2000 (Invitrogen). Wild type and transfected cells were kept under identical conditions and maintained in 5% CO<sub>2</sub> at 37°C unless mentioned otherwise.

### Immunoblot analysis

Equal amounts of protein (50µg) were loaded and electrophoresis was performed in a 10% polyacrylamide separating gel/5% stacking gel. Proteins were transferred to PVDF membrane, and blocked with 5% milk in TBST for 1 hour. The membrane was incubated over night at 4°C with primary antibodies: anti-alpha Smooth Muscle Actin (α-SMA) (EPIT-MICS; 1184), anti-Vimentin (Santa Cruz Biotechnology; SC-7558), anti-E-Cadherin (BD Transduction Laboratories; 610181), anti-α-Catenin (BD Transduction Laboratories; 21620), or anti-Foxc2 (Santa Cruz Biotechnology; SC-31734). Blots were washed in 0.1% TBST and incubated with secondary antibody for 1 hour at room temperature. After extensive washing, the second antibody was visualized by chemiluminescence reagents. β-Actin expression as an equal loading control is also performed with an anti-Actin (Novus; NB600-503).

### Laser capture microdissection (LCM)

OCT frozen tissues were cut into 8 micron thick sections with a cryostat. Sections were placed onto PEN-membranes (Leica) that had been UV irradiated with maximum intensity for 30 min. Slides were immediately fixed with 95% ethanol for 10 minutes, washed with diethyl pyrocarbonate (DEPC)-treated water for five seconds and stained rapidly with Hematoxylin stain (Sigma) for 15 seconds. Sections were then washed with DEPC-treated water for 10 seconds, dehydrated with an ethanol gradient, washed again with 100% ethanol and xylenes, three times each respectively, and finally air dried for 3 minutes. LCM was performed on a Leica AS LMD microscope using a 7.0-µm laser spot diameter. Structures of interest were captured and isolated into RLT solution mixed with 0.2% linear acrylamid (Ambion). Total RNA was purified with the micro RNeasy kit (Qiagen) by following the manufacturer instructions.

### Immunofluorescence microscopy

Cells were fixed with 4% PFA for 15 minutes and permeabilized with 0.075% saponin for 10 minutes. They were blocked with PBS containing 2% bovine serum albumin (BSA) and 0.2% gelatin. After labelling, the coverslips were mounted in Vectashield, and viewed with a Leica fluorescent microscope equipped with a 40X-Planapochromat objective and selective filters for fluorescein isothiocyanate, DAPI and Texas red.

### Knockdown of Foxc2 Expression

Three pairs of siRNA oligonucleotides were designed to target Foxc2 and were synthesized by the Yale Pathology Laboratory. siRNA were desalted using NAP10 columns (GE Healthcare) and eluted in 0.9% NaCl. The highest knock-down efficiency of Foxc2 was achieved with siRNA-3 targeting the sequence between nucleotides 1982 and 2000 of Foxc2 transcript: siRNA-1 (sense 5'-CUUACGACUGCACCAAAUAtt-3', antisense 5'UAUUUGGUGCAGUCGUAAGtt-3'), siRNA-2 (sense 5'-GCAACUCGCAGGUAACUUAtt-3', antisense 5'UAAGUUACCUGCGAGUUGCtt-3'), siRNA-3 (sense 5'-CCCAACUGUUACUGCCAAAtt-3', antisense 5'UUUGGCAGUAACAGUUGGGtt-3'). Transfections were performed using Lipofectamine 2000 (Invitrogen) together with 100 pmoles siRNA per well in 6 well plates. Cells were lysed 48 hrs after transfection for qPCR and immunoblot analysis of Foxc2 expression.

### Transwell migration assay

For Transwell migration assays, harvested cells ( $5 \times 10^4$  cells) were re-plated onto the upper chamber of a Transwell filter with 8  $\mu$ m pores (Costar) and serum loaded DMEM was used as chemotractant in the lower chamber. After 12 hours, cells were fixed with 4% PFA in PBS. Non-migrated cells on the upper side of the filter were removed with a cotton swab, and cells on the underside of the filter were stained with 0.4% crystal violet in 10% ethanol. Images were captured using a Nikon microscope system. For each experiment, the number of cells in nine random fields on the underside of the filter was counted, and three independent filters were analyzed.

### Statistics

All results are expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's *t* test. A *p* value less than 0.05 was considered to be statistically significant.

## Results

### Foxc2 is transiently upregulated following I/R

We performed unilateral ischemia/reperfusion (I/R) on eight week old male C57Bl6 mice to determine if Foxc2 is expressed in the adult kidney and whether its expression is altered following injury. The right kidney pedicle was subjected to 30 minute clamping followed by reperfusion and mRNA and proteins were harvested at 0, 1, 3, 7, and 10 days after I/R. Following ischemia and reperfusion, the expected morphological changes of injury were present in the proximal tubules. Histologic analysis showed widespread cell death along with cast formation and luminal obstruction at day 1, followed by almost complete recovery

on day 7 (Fig. 1A). Expression levels of Foxc2 mRNA were determined in whole kidney relative to Gapdh using real time PCR and normalized to expression in the control, uninjured kidney (normalized value expressed as the ddCt). Renal ischemia resulted in a 3-fold increase in the mRNA for Foxc2 at day 1 after injury with return to baseline by day 7 (Fig. 1B). Western blot analysis revealed that Foxc2 protein expression in whole kidney homogenates also peaked at day 1 after injury with a return to baseline by day 7 (Fig. 1C). Immunocytochemistry revealed that Foxc2 expression was low in uninjured kidneys, but increased in tubular cells of the cortex and outer medulla at one day after injury (Fig. 1D). Surprisingly, Foxc2 was present in the cytoplasm of these tubular cells and was not detected in the nuclei (Figure 1D, arrows).

### **Foxc2 is highly expressed in the proximal tubules after injury**

To more specifically determine the location of Foxc2 expression in the kidney; we utilized laser capture microdissection (LCM) (Fig. 2A). Cryosections from control and day 1 injured mouse kidneys were obtained and dissected by laser-capture to obtain mRNA from specific segments. To determine tubule specificity, 30 proximal tubule (PT) structures, 30 tubules morphologically identified as Henle's loop or DCT, 30 glomeruli and 3 intact papillas were laser microdissected from cryosections of 10 week old normal mouse kidney. Proximal tubules markers such as Napi2 and  $\gamma$ Gt1 were detected only in the PT cDNA pool while Aqp2 and Nkcc2 were only detected in Henle's loop/distal tubule (HL/DT) pool or papilla confirming the accuracy of the isolation/laser dissection (Fig. 2B). Following I/R, there was more than a 2-fold increase of Foxc2 mRNA expression in injured proximal tubules when compared to control proximal tubules from sham operated mice (Fig. 2C). In accordance with the data obtained from LCM experiments, paraffin sections from day 1 injured mouse kidney were co-immunostained with Foxc2 and the proximal tubule marker, megalin, and Foxc2 expression in the cytoplasm of injured proximal tubules was confirmed (Fig. 2D).

### **Foxc2 upregulation correlates with epithelialization in vivo and in vitro**

The upregulation of Foxc2 after renal I/R, coupled with prior demonstrations that over-expression of Foxc2 leads to EMT, led us to examine whether Foxc2 acts in normal epithelia to promote de-differentiation. This was first investigated by determining the expression of Foxc2 during kidney development, where mesenchymal-epithelial transformation (MET) and subsequent epithelial differentiation is required for normal tubule formation. Immunostaining of embryonic day 16 (E16) kidneys reveals that Foxc2 is nearly undetectable in the poorly differentiated cells of the cap mesenchyme (MM), early renal vesicles (RV), and ureteric bud (UB) tips present in the nephrogenic zone, but that it is highly expressed in the more differentiated tubules located in the medulla and papilla (Fig. 3A-C). High magnification images reveal that Foxc2 is localized to the cytoplasm in these cells (Fig. 2B, arrowheads), similar to the location in the injured tubular cells of the adult kidney. Immunostaining with the proximal tubule marker megalin and the UB/collecting duct marker dolichos biflorus (DBA) demonstrates that the Foxc2 positive cells are primarily located in the proximal tubule and collecting duct (Fig. 3D-J). There was also detectable Foxc2 expression in glomerular cells that were localized in a podocyte-specific pattern (data not shown). Thus, Foxc2 upregulation correlates with epithelial differentiation rather than de-differentiation in the developing kidney.

To further determine the normal expression pattern of Foxc2 during epithelial cell differentiation, we utilized in vitro models of cell differentiation and de-differentiation. Culture of mouse proximal tubule (MPT) cells derived from the ImmortoMouse (Karihaloo *et al.*, 2005; Sinha *et al.*, 2003) under the permissive conditions for large T expression (33°C +  $\gamma$ -interferon) results in de-differentiation as judged by the downregulation of E-cadherin and upregulation of vimentin expression, whereas culture at 37°C in the absence of  $\gamma$ -Ifn results in re-expression of E-cadherin and suppression of vimentin (Fig. 3B). In order to determine how Foxc2 expression correlates with the level of cell differentiation, we maintained MPT cells for 10 days at 33°C +  $\gamma$ -Ifn or at 37°C minus  $\gamma$ -Ifn and found that Foxc2 expression levels were low in de-differentiated cells contrasting with high levels of expression in the more differentiated cells (Fig. 3C). The increase in Foxc2 expression under the non permissive condition occurred in parallel with an increase in the expression of epithelial markers E-cadherin and  $\alpha$ -catenin and a decrease in the expression of the mesenchymal markers  $\alpha$ -Sma and vimentin (Fig. 3C).

We have found that even when cultured at 37°C minus  $\gamma$ -Ifn, MPT cells can be induced to de-differentiate or re-differentiate simply by altering the degree of confluency. To achieve this, maximally differentiated highly confluent MPT cells (more than 7 days in culture at 37°C minus  $\gamma$ -interferon) are passaged to sparse, sub-confluent, or highly confluent cell densities for 24 hours, then proteins extracted and total cell lysates blotted for Foxc2, epithelial markers (E-cadherin and  $\alpha$ -catenin) and mesenchymal markers (vimentin and  $\alpha$ -Sma). Foxc2 expression levels were maintained in those cells passaged from high confluency to high confluency in correlation with the epithelial markers, whereas passage to low confluency resulted in marked downregulation of Foxc2, E-cadherin and  $\alpha$ -catenin. In contrast, vimentin and  $\alpha$ -Sma proteins levels were induced at low density, consistent with these cells de-differentiating towards a “mesenchymal” phenotype (Fig. 3D). Thus, in vivo and in vitro models of tubular cell differentiation demonstrate an upregulation of Foxc2 expression as cells develop a more epithelial phenotype.

### Localization of Foxc2 is cell type-specific

To more accurately examine the sub-cellular localization of Foxc2, we performed immunofluorescence staining of cultured proximal tubule cells. In both proximal tubule epithelial cells (PTEC) freshly isolated from 10 week old wild-type mice (Fig. 4A) as well as immortalized MPT cells (Fig. 4B), Foxc2 was localized to the cytoplasm. To determine the specificity of this signal, we performed RNAi against Foxc2 which demonstrated a significant reduction of the Foxc2 immunoreactivity in the cytoplasm of MPT cells (Fig. 4B, lower panels). Furthermore we separated MPT nuclear and cytoplasmic proteins (confirmed by blotting for nuclear and cytoplasmic markers Lamin and Gapdh, respectively). Western blotting of Foxc2 revealed localization in the cytoplasm of MPT cells contrasting with no detectable signal in the nuclear fraction of these cells (Fig. 4C). Thus, both in vitro and in vivo data support a cytoplasmic localization of Foxc2 in renal tubular cells.

In contrast, Foxc2 was localized in both the cytoplasm and nuclei of NIH 3T3 cells with a diminution of signal from both compartments after silencing with the Foxc2 siRNA (Fig 4D, upper and lower panels, respectively). Cell fractionation of 3T3 cells followed by protein

blotting against Foxc2 confirmed the nuclear + cytoplasmic localization in these cells (Fig 4E).

### **Differentiation effects of Foxc2 correlate with subcellular localization**

We took advantage of RNAi to investigate the regulatory role of Foxc2 in epithelial cell differentiation. Transfection with siRNA directed against Foxc2 in confluent, maximally differentiated MPT cells resulted in a significant decrease of E-cadherin and  $\alpha$ -catenin protein levels after 48 hours, but had little effect on the mesenchymal markers vimentin and  $\alpha$ -SMA (Fig. 5A).

In contrast to the loss of epithelial differentiation seen following Foxc2 knock-down in MPT cells, silencing of Foxc2 in NIH 3T3 cells under the same conditions revealed a significant increase in the expression of epithelial markers (E-cadherin and  $\alpha$ -catenin) with a concomitant decrease of the mesenchymal markers vimentin and  $\alpha$ -Sma (Fig. 5B). Thus, the subcellular location of Foxc2, and its role in regulating the expression of epithelial and mesenchymal markers, appears to be cell-type specific.

De-differentiation of epithelial cells results in simplification of adherens junctions as well as upregulation of structural elements such as vimentin and  $\alpha$ -Sma that jointly promote cell migration. Therefore to investigate the phenotypic outcome of Foxc2-dependent alterations in cell epithelialization, we performed Transwell migration assays to assess single cell migration. MPT or NIH 3T3 cells transfected with either control or Foxc2 siRNA were seeded on the upper side of an 8 $\mu$ m pore size polycarbonate membrane, and then culture medium containing 10% FBS was added to the lower chamber as the chemoattractant. After 12 h, the number of cells that had migrated through the pores was quantified. These experiments demonstrated that loss of epithelialization in MPT cells subjected to RNAi of Foxc2 correlated with an increase in single cell migration (Fig. 5C), whereas silencing Foxc2 in NIH 3T3 cells, in which epithelial markers were upregulated and mesenchymal markers downregulated, led to a decrease in cell migration (Fig. 5D).

### **Overexpression of Foxc2 in epithelial cells results in nuclear localization and epithelial-mesenchymal transition**

Our results demonstrate that endogenous Foxc2 is only detected in the cytoplasm of renal tubular cells and that loss of this expression results in epithelial de-differentiation. However, it has been demonstrated that over-expression of Foxc2 in a different renal cell line, MDCK cells, also results in cell de-differentiation. To determine whether these contrasting results are due to differences within individual tubule cell lines or could relate to sub-cellular localization of the over-expressed Foxc2, we transfected MPT cells with an empty vector or a vector encoding human Foxc2. Over-expression of Foxc2 in these cells was confirmed at the mRNA (data not shown) and the protein levels (Fig. 6A). Examination of markers of epithelial differentiation revealed that over-expression of Foxc2 resulted in a decrease in the epithelial markers E-cadherin and  $\alpha$ -catenin with an upregulation of the mesenchymal markers  $\alpha$ -Sma and vimentin (Fig. 6A), consistent with the results of Mani *et al.* (Mani *et al.*, 2007). Subcellular fractionation of these cells revealed that over-expression of Foxc2 resulted in the de novo detection of Foxc2 in the nucleus of MPT cells (Figure 6B), in a



pattern reminiscent of the expression in NIH 3T3 cells. This over-expression led to phenotypic de-differentiation of the MPT cells to a morphology more typical of mesenchymal cells (Fig. 6C), with detection of Foxc2 in the nucleus of many of the transfected cells (Fig. 6D). Thus over-expression of Foxc2 in MPT cells results in nuclear expression and activation of a de-differentiated phenotype similar to that seen in cultured fibroblasts.

## Discussion

We used the model of renal ischemia/reperfusion (Kale *et al.*, 2003) to determine if Foxc2 is expressed in tubular cells of the adult kidney and whether its expression is induced after tubular injury. Based on the modest literature in regards to this factor, we originally predicted that Foxc2 would be upregulated in the nucleus of sub-lethally injured tubular cells where it might be involved in activating cell de-differentiation and migration by mediating the expression of mesenchymal markers such as  $\alpha$ -Sma and vimentin while downregulating the expression of epithelial markers. Our results indeed demonstrate that Foxc2 expression is increased after IRI in both cortex and outer medulla between days 1-3, but surprisingly this increase is seen in the cytoplasm of the tubular cells rather than in the nucleus. Furthermore, Foxc2 is upregulated rather than downregulated in nascent tubular cells during the process of MET in the developing kidney.

In order to more accurately determine the site of Foxc2 localization and to better address the role of endogenous Foxc2 expression in non-malignant epithelial cells, we utilized several in vitro models of reversible cell de-differentiation. One model involves induction of de-differentiation by expression of the large T antigen under control of the  $\gamma$ -Ifn inducible, temperature sensitive promoter (Karihaloo *et al.*, 2005; Sinha *et al.*, 2003), whereas the other model utilizes degree of cell confluency to regulate the level of expression of epithelial markers (Ishibe *et al.*, 2006). In both cases, epithelial cell differentiation correlated with increased levels of cytoplasmic Foxc2 whereas de-differentiation correlated with decreased levels of Foxc2.

These data are consistent with the surprising possibility that Foxc2 upregulation might be acting as a check-point to inhibit epithelial cell de-differentiation and/or to activate epithelial cell re-differentiation during kidney repair. This was unexpected since Foxc2 upregulation has been shown to correlate with cancer metastases and its over-expression can lead to epithelial-mesenchymal transition (EMT) (Mani *et al.*, 2007). However, as noted earlier, a bioinformatics approach has also identified Foxc2 along with several other transcription factors as candidate regulators of mesenchyme-to-epithelium transition (MET) in mouse embryos (Bard *et al.*, 2008).

We believe that the resolution of these contrasting results may reside in the subcellular localization of Foxc2. Foxc2 is considered to be a nuclear protein that undergoes sequence-specific DNA binding and promotion of gene transcription (Hayashi and Kume, 2008; Hayashi *et al.*, 2008). However, we find that Foxc2 cannot be detected in the nucleus in tubular epithelial cells either in vitro and in vivo. In contrast, Foxc2 is localized to both the cytoplasm and nucleus in 3T3 fibroblasts as well as following over-expression in cultured

epithelial cells. In both cases where we detected Foxc2 in the nucleus, the Foxc2 expression correlated with promotion of a mesenchymal/fibroblast phenotype. For example, overexpression of Foxc2 in MPT cells led to downregulation of the normal expression of epithelial markers, while knock-down of Foxc2 in 3T3 cells resulted in an increase in expression of epithelial markers. In contrast, Foxc2 was found only in the cytoplasm in non-malignant tubular epithelial cells. In this location, Foxc2 appears to promote an epithelial phenotype since knock-down results in loss of epithelialization.

There are two potential explanations for our findings. First it is possible that Foxc2 functions solely as a transcription factor and that the amount present in the nucleus of normal epithelial cells is simply below the limits of detection of our assays. In this model, small amounts of Foxc2 in the nucleus would be predicted to activate pro-epithelial genes whereas larger amounts of Foxc2 might inhibit the expression of those same genes as well as activating the expression of mesenchymal/fibroblast genes such as vimentin. In that case, during differentiation epithelial cells would activate nuclear export pathways and/or inhibit nuclear import in order to limit the amount of Foxc2 present in the nucleus. Alternatively, Foxc2 might function to promote epithelial differentiation by shuttling Id proteins or other inhibitors of epithelial differentiation out of the nucleus, or by other cytoplasmic functions independent of its transcriptional activity.

The mechanisms of non-nuclear signaling by Foxc2 and of Foxc2 shuttling between the nucleus and cytoplasm remain to be determined. Foxc2 is believed to be localized exclusively to the nucleus of COS-7 cells (Fujita *et al.*, 2006), another fibroblast cell line, and mutation in the DNA binding domain (also called the forkhead domain (FHD)) will affect its nuclear localization (Berry *et al.*, 2005). It has been shown that Foxc2 protein expression can be activated downstream of insulin and TNF- $\alpha$  via a PI3K-Akt and ERK 1/2-dependent pathway (Gronning *et al.*, 2002). Interestingly, Akt-dependent phosphorylation of several of the forkhead proteins belonging to the "O" subfamily, including FOXO1a, 3a and 4, inhibits their transcriptional activity by causing their translocation from the nucleus into the cytoplasm (Bois and Grosveld, 2003; Schwab *et al.*, 2005). These observations suggest that multiple external signals might activate signaling responses that could determine the balance between nuclear and cytoplasmic Foxc2, which in turn determines whether this protein acts to promote EMT or MET.

Cumulatively, the present study along with results from several other groups suggest that Foxc2 can activate the expression of either epithelial or mesenchymal genes depending on the cell type and the subcellular localization of the protein. Based on our *in vitro* and *in vivo* findings, we believe that the upregulation of endogenous Foxc2 in the cytoplasm of injured tubular cells serves to moderate the de-differentiation response to acute injury and to promote epithelial re-differentiation during the repair process.

## Acknowledgements

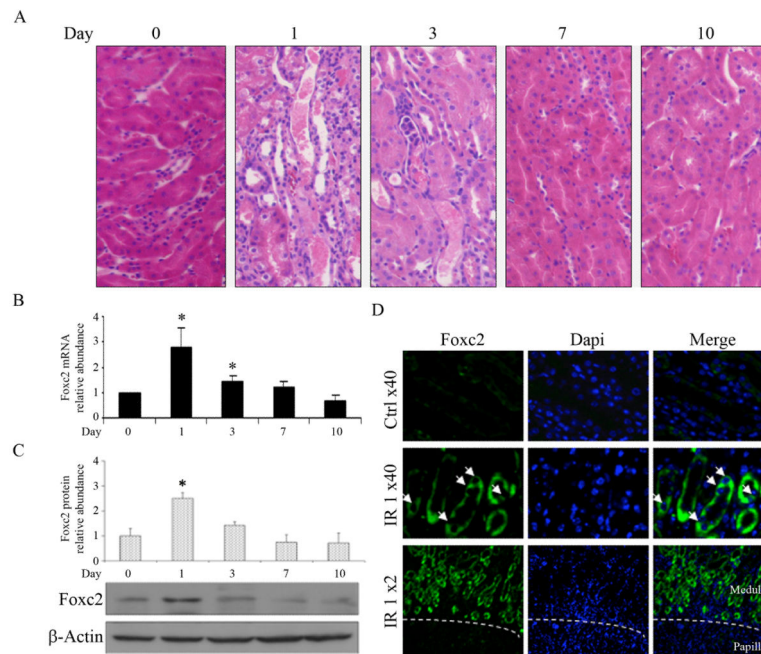
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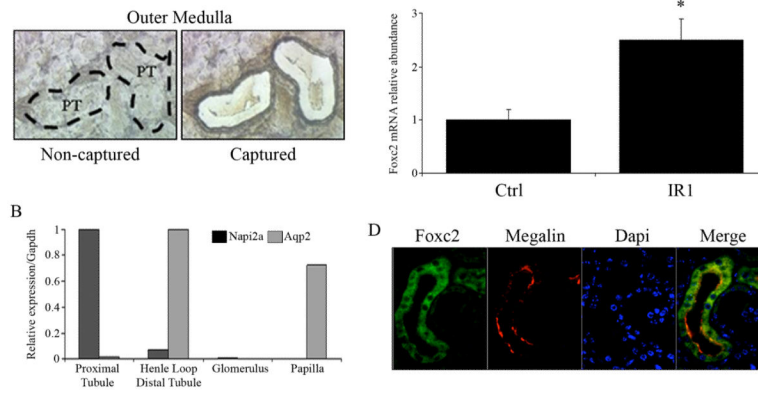
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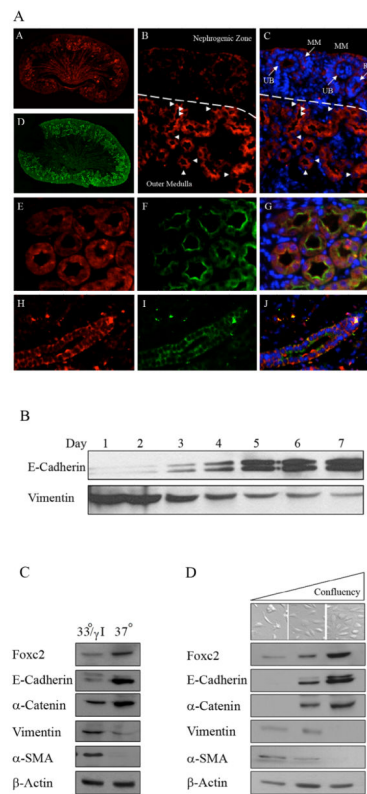
### Figure 1. Foxc2 is transiently expressed following I/R

Mice underwent I/R and kidneys were harvested at day 0, 1, 3, 7 and 10. (A) Histologic evaluation of kidney sections stained with hematoxylin/eosin. (B) Real-time PCR analysis of Foxc2 mRNA expression from injured kidneys at the indicated days versus control kidneys at day 0; normalized to Gapdh.  $n=3$  animals per group,  $*P < 0.05$  vs. day 0. (C) Western blotting and densitometric quantification of Foxc2 expression from injured kidneys versus day 0,  $n=3$  animals per group,  $*P < 0.05$ . (D) Shown are representative immunofluorescence images of control mouse kidney (Ctrl) and ischemically injured kidney on day 1 (IRI) stained with the anti-Foxc2 antibody (40x magnification). Arrows demonstrate the absence of Foxc2 in cell nuclei. The bottom panels show a low power view (2x) demonstrating that Foxc2 upregulation after IRI was confined to the outer medulla and not seen in the papilla.

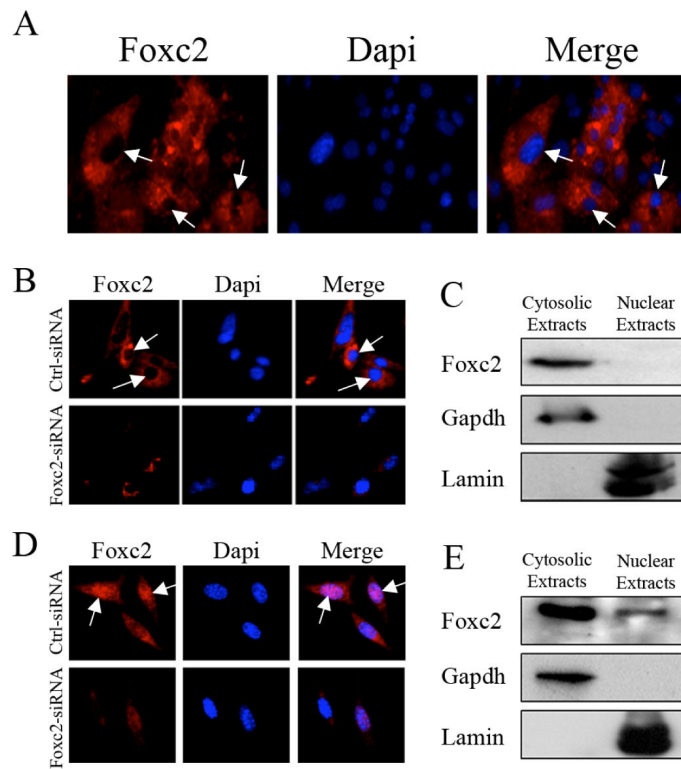


**Figure 2. Foxc2 is highly expressed in the proximal tubules after injury**

Representative image before and after proximal tubule laser capture (A) and real time PCR of tubule segment specific gene markers from the listed kidney regions (B). (C) RT-PCR analysis of Foxc2 mRNA expression from laser captured proximal tubules of control mouse kidney and ischemically injured kidney on day 1. Gapdh was used as an internal control for normalization.  $n=3$  animals per group,  $*P<0.05$ . (D) Double immunofluorescence staining of Foxc2 with the proximal tubule marker megalin, 24 hours post ischemia/reperfusion injury. Note that post I/R injury the brush border is lost and megalin expression is confined to linear staining at the apical surface of the PT cells.



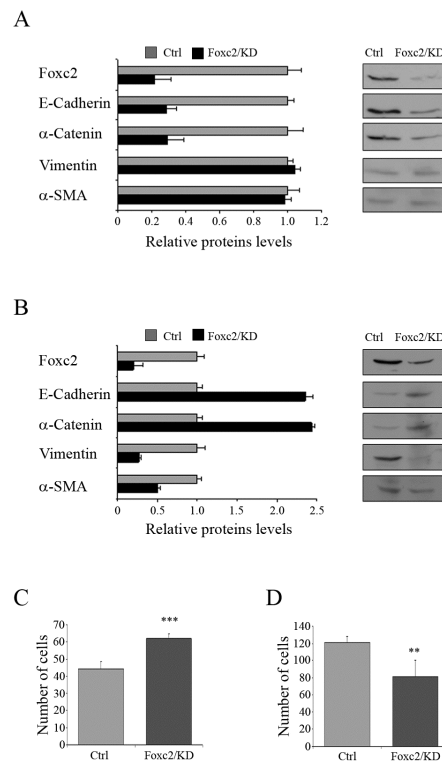
**Figure 3. Foxc2 upregulation correlates with epithelialization in vivo and in vitro**  
 (A-C) Immunostaining of E16 mouse kidney for Foxc2 (A- 4x; B,C - 40x) reveals strong cytoplasmic expression in developing tubules inferior to the nephrogenic zone with minimal expression in the structures of the nephrogenic zone itself (MM-metanephric mesenchyme; UB-ureteric bud; RV-renal vesicle; arrowheads-exclusion of Foxc2 from the nucleus). (D-G) Double immunostaining for megalin (green, D-4x; F,G-40x) and Foxc2 (red, E,G-40x) reveals that Foxc2 is upregulated in megalin-positive proximal tubules. (H-I) Double immunostaining with Foxc2 (red, H,I-40x) and dolichos biflorus (green, I,J-40x) reveals expression of Foxc2 in the maturing collecting duct of the renal papilla. (B) Western analysis of MPT cells changed from 33°C+γ-IFN to 37°C-γIFN at day 0 and cultured for 7 days. E-cadherin is progressively upregulated coincident with downregulation of vimentin. (C) Undifferentiated MPT cells maintained under permissive conditions (33°C with interferon (IFN)) at high confluency for 10 days were compared to maximally differentiated MPT cells grown under non-permissive conditions (37°C without IFN) at high confluency for the same time. Lysates were immunoblotted for the indicated proteins with β-actin serving as a loading control. (D) Maximally differentiated, highly-confluent MPT cells grown under non-permissive condition for 7 days were trypsinized and passaged to three different confluency levels (sparse, sub-confluent, highly confluent; representative Hoffman contrast images included). Cell lysates were blotted for Foxc2, epithelial markers (E-cadherin and a-catenin) and mesenchymal markers (Vimentin and α-Sma).



**Figure 4. Foxc2 has a cell type-specific localization pattern**

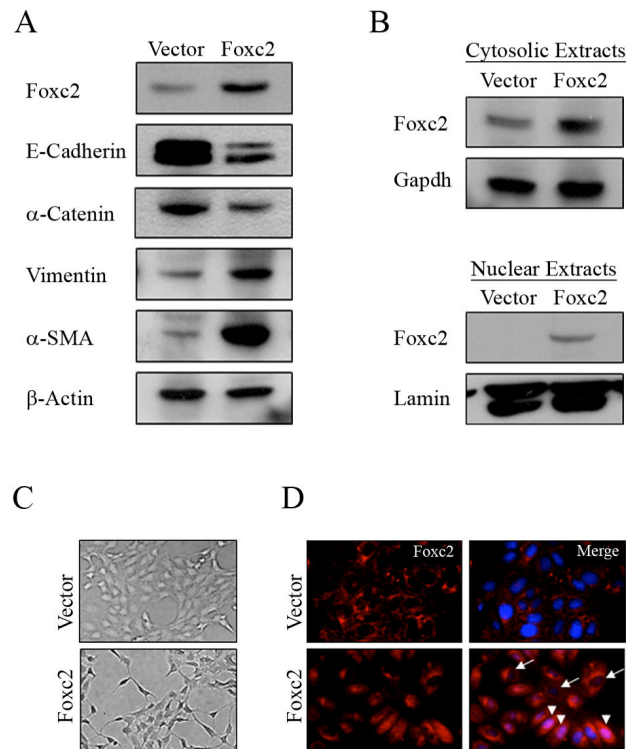
Immunofluorescence images of Foxc2 expression in proximal tubule epithelial cells (PTEC, A), MPT cells (B) or NIH 3t3 cells (D) showing cytoplasmic location in the two epithelial cell types but joint cytoplasmic and nuclear localization in the NIH 3t3 cells (arrows denote nuclei). Transfection of MPT or NIH 3t3 cells with siRNA directed against Foxc2 (B,D, lower panels) results in marked loss of the Foxc2 signal. (C,F) Nuclear and cytoplasmic proteins were extracted from MPT (C) and NIH3t3 (E) cells and analyzed for expression of Foxc2, the nuclear protein lamin and cytoplasmic protein Gapdh.





**Figure 5. Differentiation effects of Foxc2 correlate with subcellular localization**

(A,B) Protein expression was examined in MPT cells (A) and NIH 3T3 cells (B) following transfection with control siRNA (Ctrl) or anti-Foxc2 siRNA (Foxc2/KD). Densitometric quantification of three different experiments is shown on the left (normalized to  $\beta$ -actin). Representative immunoblotting of Foxc2, mesenchymal markers vimentin and  $\alpha$ -Sma and epithelial markers E-cadherin and  $\alpha$ -catenin are shown on the right. (C,D) Relative migration of MPT cells (C) and NIH3T3 cells (D) transfected with Foxc2 siRNA or control siRNA was examined using a Transwell assay ( $n = 3$ ; \*\*\* $P < 0.001$ , \*\* $P < 0.005$ ).



**Figure 6. Over-expression of Foxc2 in epithelial cells results in nuclear localization and EMT**  
MPT cells were transiently transfected with empty vector or vector encoding Foxc2. (A) Western blotting of Foxc2, epithelial markers (E-cadherin and  $\alpha$ -catenin) and mesenchymal markers ( $\alpha$ -Sma and Vimentin) from whole cell lysates. (B) Cytosolic and nuclear proteins were separated from vector control and Foxc2-expressing cells and blotted for Foxc2, cytoplasmic marker (Gapdh) and nuclear marker (Lamin). (C,D) MPT cells expressing either the control vector or Foxc2 are shown using Hoffman modulation (C, 10x) and immunofluorescence microscopy (D, 40x). Arrows in (D) demonstrate cells in which Foxc2 remains excluded from the nucleus, arrowheads demonstrate cells in which Foxc2 is seen in the nucleus and cytoplasm.