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Overexpression of E2A proteins induces epithelial–mesenchymal transition in human renal proximal tubular epithelial cells suggesting a potential role in renal fibrosis

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Abstract **Epithelial–mesenchymal transition (EMT), a process whereby renal tubular epithelial cells lose phenotype and gain fibroblast-like characteristics, has been demonstrated to contribute significantly to the development of renal fibrosis. The immunosuppressant cyclosporine A (CsA) has been shown to induce renal fibrosis, a major complication of CsA therapy. The mechanisms that drive CsA-induced fibrosis remain undefined, however, CsA has been demonstrated to induce EMT in human renal proximal tubular epithelial cells (RPTEC). E2A transcription factors were identified as being upregulated by CsA treatment. To further examine the role of E2A proteins in EMT, E12 and E47 were overexpressed, alone and in combination, in human RPTEC. Both E12 and E47 elicited EMT effects on tubular epithelial cells with E47 more potent in inducing the fibroblast-like phenotype. These results indicate the important role of the E2A gene products in the progression of CsA-induced EMT and provide novel insights into CsA-induced renal fibrosis.**

Keywords: Epithelial–mesenchymal transition; Cyclosporine A; Renal fibrosis

1. Introduction

End stage renal disease (ESRD) is one of the most serious complications associated with hypertension, diabetes and the use of certain therapeutic drugs. The powerful immunosuppressant cyclosporine A (CsA) has revolutionised the field of whole organ transplantation leading to greatly improved survival and quality of life among graft recipients [1]. However, a major limiting factor in the use of CsA are toxic effects that occur in the kidney [2]. Long-term CsA treatment leads to irreversible ESRD, characterised by extensive tubulointerstitial fibrosis (TIF) [3]. TIF is characterised by interstitial thickening caused by the accumulation of extracellular matrix (ECM) components [4]. Previously, it was believed that the main source of the ECM that accumulates during TIF was infiltrating fibroblasts that became active in the interstitium [5]. Accumulated evidence now suggests that a major source of these active fibroblasts or myofibroblasts is the renal epithelium itself through a process termed epithelial–mesenchymal transition (EMT) [6]. EMT is a process where numerous phenotypic changes occur leading to the loss of epithelial markers and function and the acquisition of a fibroblastic phenotype, termed a myofibroblast [7]. We have previously reported that human proximal tubular epithelial cells (PTECs) exposed to CsA over 72 h underwent EMT [8,9]. Transforming growth factor β1 (TGF-β1) was identified as being a key mediator of this effect. Since EMT involves a gross change in cell phenotype, the process likely involves a remarkable alteration in the complement of genes being expressed. Large scale gene expression analysis of models of EMT has detected hundreds of genes with altered expression [10,11]. The main challenge lies is determining which changes are causative and which are consequential.

Yang and Liu [7] have proposed that EMT is an orchestrated stepwise process involving a number of key events that mark progress from a tubular epithelial cell to a myofibroblast. These include (i) loss of epithelial cell–cell adhesion and actin cytoskeletal rearrangement, (ii) de novo synthesis of myofibroblast specific marker proteins (e.g. α-SMA), (iii) disruption of the tubular basement membrane, and (iv) enhanced cell migration and invasiveness [7]. However, elucidation of the relationship between these events and the control mechanisms involved in the EMT process is still ongoing. Regulation of the morphological and phenotypic changes observed during EMT occurs, for the most part, at the level of transcription. Therefore, CsA-induced alterations in the expression of transcriptional regulators were investigated. The E2A gene was identified as being upregulated by CsA in this model [8]. The E2A gene encodes two distinct basic helix-loop-helix (bHLH) transcription factors, E12 and E47 [12]. The two proteins display differences only in their bHLH regions which still maintain over 72% amino acid sequence homology [13]. Both proteins contain a basic region necessary for DNA binding and a HLH structure that facilitates dimerisation with other bHLH proteins [12]. E12 and E47 modulate the expression of their target genes through the formation of homodimers (E12/E12 and E47/E47), heterodimers with each other (E12/E47) and heterodimers with other bHLH transcription factors (E12/MyoD, E47/snail) [14,15]. These bHLH proteins are thought to play critical roles in the regulation of cell commitment, growth and differentiation in a range of cell types including lymphocytes, muscle cells and neurons [15,16]. The

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Abbreviations: ESRD, end stage renal disease; TIF, tubulointerstitial fibrosis; CsA, Cyclosporine A; ECM, extracellular matrix; PTEC(s), proximal tubular epithelial cell(s); EMT, epithelial–mesenchymal transition; α-SMA, alpha-smooth muscle actin; HK-2, human kidney 2; RPTEC, renal proximal tubular epithelial cells; TGF-β1, transforming growth factor-beta 1

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potentially pivotal role of the E2A transcription factors in regulating cell phenotype suggested a role for E2A in this CsA-induced model of EMT and this was further investigated.

2. Materials and methods

2.1. Reagents

CsA was a generous gift from Novartis Pharmaceuticals Ltd. All tissue culture reagents were from Costar. CsA was prepared as a stock solution (4.2 mmol/L) by dissolving the powder in 100% ethanol. Primers for RT-PCR were obtained from Sigma Genosys Biotechnologies (Woodlands, TX). All other reagents were of the highest available purity from commercial sources. Vectors containing the full-length cDNAs encompassing the respective coding regions of human E12 and E47 were a generous gift from Dr. Carme Gallego, Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, 25008 Lleida, Catalunya, Spain, with the kind permission of Dr. Masataka Nakamura, Tokyo Medical and Dental University, Human Gene Sciences Center, Tokyo, Japan (previously described [17]) and Dr. Cornelis Murre, University of California, San Diego (previously described [18]).

2.2. Cell culture and transfection

The human renal proximal tubular cell line, HK-2, was purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbeccos modified Eagle's medium containing 100 U/ml penicillin, 100 g/ml streptomycin, 5 μg/ml insulin, 10 ng/ml epidermal growth factor and 2 mM L-glutamine. Primary human renal proximal tubular epithelial cells (RPTEC) were purchased from Clonetics (Walkersville, MD). Primary tubular epithelial cells were maintained in Renal Epithelial Cell Growth medium (REGM) supplemented with REGM SingleQuots and Growth Factors (also Clonetics). Cell culture medium was changed every 48 h. Cells were maintained in 75 cm^2 Costar flasks at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. For transfections, HK-2 cells were seeded on 6 well plates at 5×10^3 cells/ml 24 h prior to treatment resulting in approximately 60% confluency at the time of transfection. Cells were transfected with FuGene 6 reagent (Roche), either with 1.75 μg of pcDNA mammalian expression vector containing a cDNA insert encoding E12 or 1.75 μg pcDNA mammalian expression vector containing a cDNA insert encoding E47. Cells were transfected for 24 h at which time further treatments were performed. The aim of this study was to examine if overexpression of E2A proteins, alone or in combination, could elicit similar effects to those observed in response to CsA treatment. Therefore, for the purposes of this study, transient transfectants were created.

2.3. Cell treatment

Cells were treated with a physiologically relevant dose of 4.2 μM CsA and the effects were examined at 12, 24, 48 and 72 h post-treatment. In all experiments with CsA, control cells were exposed to an appropriate concentration of ethanol vehicle.

2.4. Immunofluorescent microscopy

Cells were cultured on 8-well chamber slides. After transfection and treatment, cells were fixed in 3.7% (v/v) formaldehyde/PBS. F-actin was visualised by direct immunofluoresence using rhodamine phalloidin (Molecular Probes) was added as a 1:40 dilution. Cells being stained for E2A and α-SMA were incubated with a 1:100 dilution of mouse anti-E2A (Santa Cruz Technology), or mouse anti-α-SMA (Sigma). Subsequently the cells were incubated with secondary antibody (1:100 rabbit anti-mouse fluorescein isothiocyanate (DAKO)). Cells were incubated with secondary antibodies in the absence of primary antibody to demonstrate background staining. Slides were mounted and viewed using the Zeiss Axioplan 2 imaging fluorescent microscope.

2.5. Western blot analysis

Equal total protein amounts of cell lysates or nuclear fractions were electrophoresed using the procedure of Laemmli [19]. For detection of E2A proteins and E-cadherin, membranes were probed with antibodies specific for E2A proteins (1:500 dilution, Santa Cruz) and E-cadherin (1:1000 dilution, Transduction Labs) and results shown are representative of at least three experiments with similar results.

2.6. Detection of E2A/E47 DNA-binding activity by electrophoretic mobility shift assay (EMSA)

Nuclear fractions of adherent cells were obtained using the ProteoExtract subcellular proteome extraction kit (Calbiochem), as per manufacturer's instructions. Detection of E2A/E47 DNA-binding activity by EMSA was detected using the non-radioactive EMSA kit (Panomics), as per manufacturer's instructions.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) and quantitated by absorbance at 260 nm. RT-PCR primer pairs were designed using PRIMER 3 software (available at www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and using sequence data available at the NCBI database. Primers used for fibronectin were: GAPDH – forward: 5′ACCACAGTCCATGCCATCAC 3′, reverse: 5′ TCCACCACCCTGTTGCTGTA 3′; α-SMA – forward: 5′ GCGTGGCTATTCCTTCGTTAC 3′, reverse: 5′ CATAGTGGTGCCCCCTGATAG 3′.

A Gibco-BRL RT kit was used to generate cDNA. 2 μl of the cDNA was taken from each sample and inserted into 0.5 ml PCR tubes along with 1 μl of forward primer (100 ng/μl), 1 μl of reverse primer (100 ng/μl), 2 μl of 10 mM dNTPs, 5 μl of 10× buffer, 3 μl of 25 mM $MgCl_2$, 0.25 μl of *Taq* Polymerase (Promega) and 35.75 μl of sterile distilled water to bring the volume up to 50 μl. The number of PCR cycles used were determined to be within the linear range of the reactions. mRNA levels of the glyceraldehyde phosphate dehydrogenase gene (GAPDH) were used as a normalisation control.

3. Results

3.1. Effect of CsA on HK-2 tubular epithelial cell E2A expression levels

Exposure of HK-2 tubular epithelial cells to 4.2 μM CsA for periods up to 72 h resulted in significant upregulation of E2A proteins as demonstrated by Western blot analysis of nuclear fractions (Fig. 1A). This upregulation was also evident when E2A proteins were visualised using immunofluorescent microscopy demonstrating increased E2A expression at 72 h (Fig. 1B). Exposure of primary human proximal tubular epithelial cells to 4.2 μM CsA for periods up to 72 h resulted in significant upregulation of E2A proteins similar to that observed in HK-2 cells, as demonstrated by Western blot analysis of nuclear fractions (Fig. 1C).

3.2. Effect of CsA on E2A DNA-binding activity in HK-2 tubular epithelial cells

Transcription factors E12 and E47, encoded in the E2A gene bind to E-box DNA motifs in the promoters of target genes to modulate transcription. The effects of CsA on E12/E47 DNA-binding activity were assessed in HK-2 cells using the EMSA described in Section 2. Low, basal levels of E12/E47 DNA-binding activity were detected in control cells at 24, 48 and 72 h (Fig. 2). Markedly higher levels of E12/E47 DNA-binding activity were detected in cells treated with 4.2 μM CsA at 24, 48 and 72 h.

3.3. Overexpression of E2A transcription factors in HK-2 tubular epithelial cells

The overexpression of E12 and E47 in whole cell lysates was assessed by Western blot analysis (Fig. 3). In mock transfected HK-2 cells, E12/E47 levels were barely detectable. Treatment of these cells with 4.2 μM CsA resulted in increased expression of E12/E47. In transfected cells, overexpression of E12 or E47

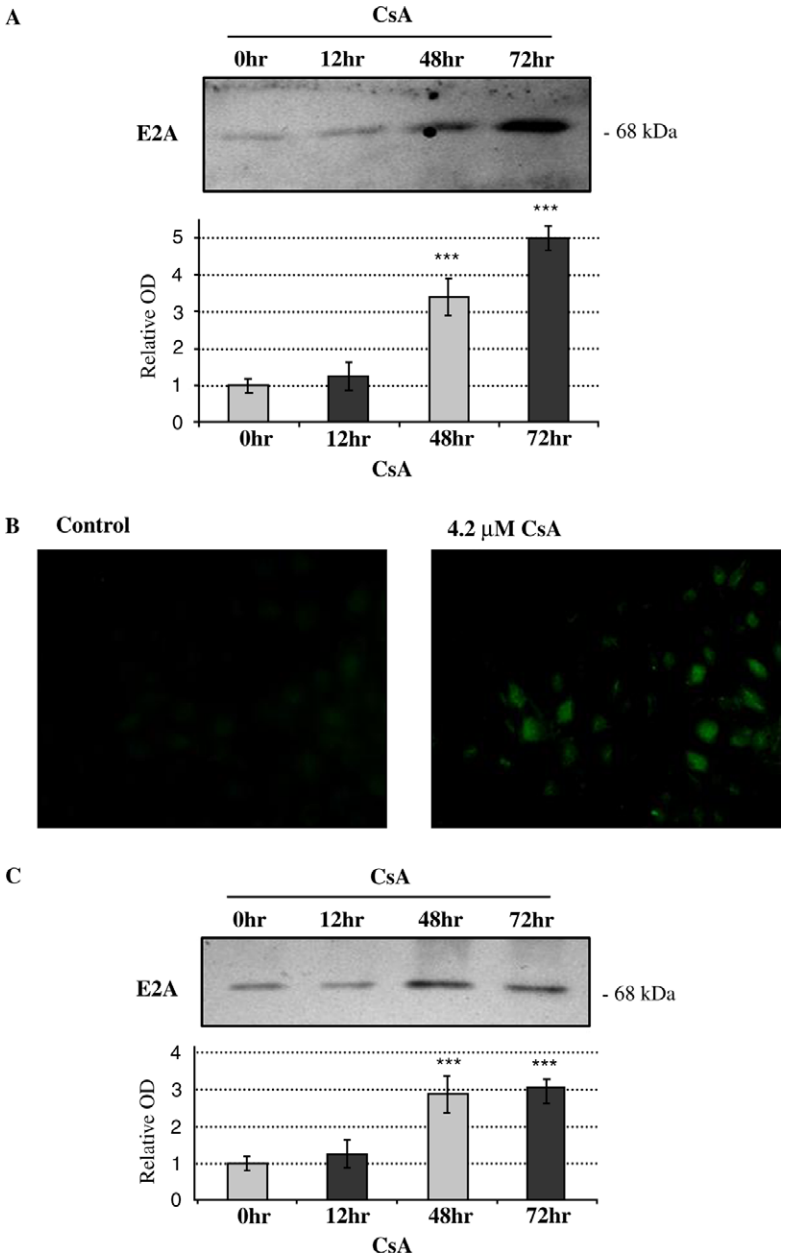

Fig. 1. Effect of 4.2 μM CsA on HK-2 tubular epithelial cell E2A expression. HK-2 cells and primary human tubular epithelial cells were grown on 6-well culture plates or tissue culture slides. Cells were treated with medium containing 4.2 μM CsA for 0, 12, 48 or 72 h. (A) Nuclear fractions obtained from HK-2 cells were subjected to SDS–PAGE and probed with an antibody directed against E2A. Bands were visualised by ECL. Images shown are representative of at least three independent experiments. Band intensity was quantified densitometrically. $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$ indicates statistically significant difference compared to control. (B) E2A proteins were visualised using indirect immunofluorescence staining (magnification 400×) Images are representative of at least three independent experiments. (C) Nuclear fractions obtained from primary human tubular epithelial cells were subjected to SDS–PAGE and probed with an antibody directed against E2A. Bands were visualised by ECL. Images shown are representative of at least three independent experiments. Band intensity was quantified densitometrically. $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$ indicates statistically significant difference compared to control.

was detected. β-Actin levels were determined to ensure equal protein loading.

3.4. Effects of E12 and E47 overexpression on the morphology of HK-2 tubular epithelial cells

The effects of E12 and E47 overexpression on HK-2 tubular epithelial cell morphology were assessed at 48 h by phase-contrast microscopy (Fig. 4). Cells were then cultured under the indicated conditions for a further 48 h. In all cases, mock-transfected HK-2 cells formed a confluent monolayer exhibiting normal epithelial characteristics. Individual cells displayed typical epithelial, polygonal shape with a high degree of attachment to neighboring cells (Fig. 4A). The morphology of E12-transfected HK-2 cells cultured with control medium was very similar to that of mock-transfected control cells, though some cell elongation was evident (Fig. 4C). E12-transfected cells exposed to 4.2 μM CsA exhibited very clear morphological changes similar to, but perhaps more pronounced

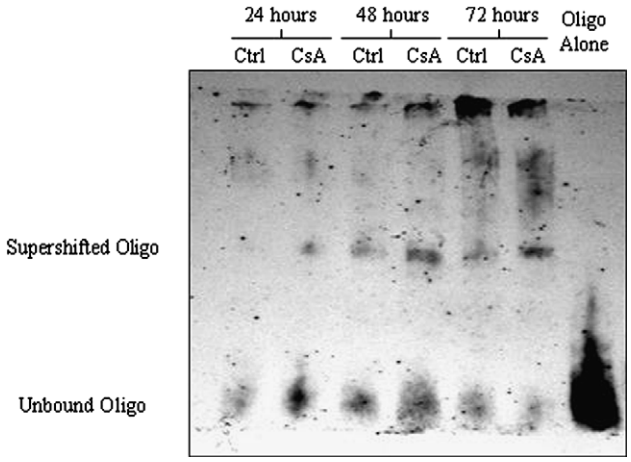

Fig. 2. Effect of 4.2 μM CsA on E47/E2A DNA-binding activity in HK-2 tubular epithelial cells. HK-2 cells grown on 6-well culture plates were treated with control medium or medium containing 4.2 mM CsA for 24, 48 or 72 h. Nuclear fractions obtained were analysed for E2A/E47 DNA-binding activity using the EMSA assay described in Section 2.6. Bands were visualised by ECL. Blot shown is representative of at least three independent experiments.

than those observed with CsA treatment of mock transfectants (Fig. 4B and D). E47-transfected cells exhibited striking morphological changes compared to mock transfected control cells (Fig. 4E). Cells displayed a more elongated shape with a large degree of cell detachment, similar to those observed previously in CsA-treated mock transfectants. CsA treatment of cells overexpressing E47 resulted in almost complete loss of epithelial morphology (Fig. 4F). Cells were notably elongated and spindle-like with widespread lack of cell–cell attachments, features associated with fibroblast-like cells. Overexpression of E12 and 47 in combination resulted in very significant morphological alterations similar to but more pronounced than those observed in E47-overexpressing cells (Fig. 4G). Treatment of these cells with CsA resulted in almost complete loss of epithelial morphological characteristics. In fact the morphology of cells overexpressing E12 and E47 was so altered that the addition of CsA did not significantly affect the morphology of these cells (Fig. 4H).

3.5. Effects of E12 and E47 overexpression on F-actin cytoskeletal arrangement in HK-2 cells

Immunofluorescent microscopy was used to examine the effects of E12 and E47 on F-actin cytoskeletal arrangement in human proximal tubular cells (Fig. 5). In all experiments, F-actin fibres in mock-transfected control cells were densely arranged just inside the cell periphery, where they associated to form a circular bundle (Fig. 5A). This organisation is characteristic of cells with apical-basolateral polarity, such as normal epithelial cells. In cells exposed to 4.2 μM CsA, reorganisation of the F-actin cytoskeleton was observed (Fig. 5B). The dense peripheral arrangement of F-actin was redistributed into stress fibres. In cells overexpressing E12, some alterations in F-actin cytoskeletal arrangement were observed but principle F-actin structures were preserved (Fig. 5C). Treatment of these cells with 4.2 μM CsA resulted in greater F-actin rearrangement compared to that observed in CsA-treated mock transfectants (Fig. 5D). Overexpression of E47 resulted in marked reorganisation of the F-actin cytoskeleton (Fig. 5E). Stress fibres were evident as was the disruption of cell–cell contacts. Treatment of these cells with CsA resulted in augmentation of these effects (Fig. 5F). Overexpression of E12 and E47 in combination produced some of the most striking effects observed (Fig. 5G). Widespread stress fibre formation and cell detachment was evident. Exposure of E12/E47 overexpressing cells to CsA did not result in any further stress fibre formation (Fig. 5H).

3.6. Effects of E2A transcription factor overexpression on E-cadherin protein expression in HK-2 tubular epithelial cells

E-cadherin expression in HK-2 tubular epithelial cells was assessed by Western blot analysis (Fig. 6). Treatment of mock-transfectants with 4.2 μM CsA resulted in reductions in E-cadherin levels. Overexpression of E12 resulted in a modest decrease in E-cadherin levels. Treatment of E12-overexpressing cells with CsA resulted in further downregulation of E-cadherin. Overexpression of E47 resulted in very significant decreases in E-cadherin levels. Treatment of E47-overexpressing cells with CsA resulted in almost complete loss of E-cadherin expression. In cells overexpressing E12 and E47 in combination, E-cadherin expression levels were comparable to levels in E47-overexpressing cells. Treatment of E12 and

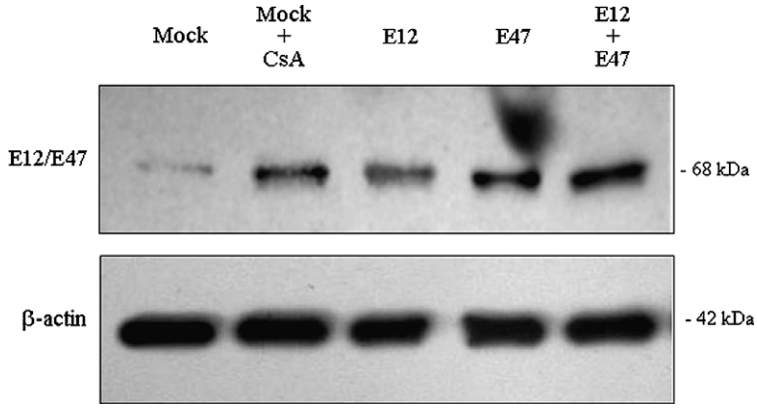

Fig. 3. E12/E47 overexpression in HK-2 tubular epithelial cells. HK-2 cells were grown on 6-well culture plates. Cells were transfected with equal amounts of a pcDNA3-E12 expression vector, a pcDNA3-E47 expression vector or both in combination. Cells were cultured for a further 48 h. (A) Whole cell lysates obtained were subjected to SDS–PAGE and probed with an antibody directed against E2A. (B) β-Actin was used as a loading control. Bands were visualised by ECL. Blots shown are representative of at least three independent experiments.

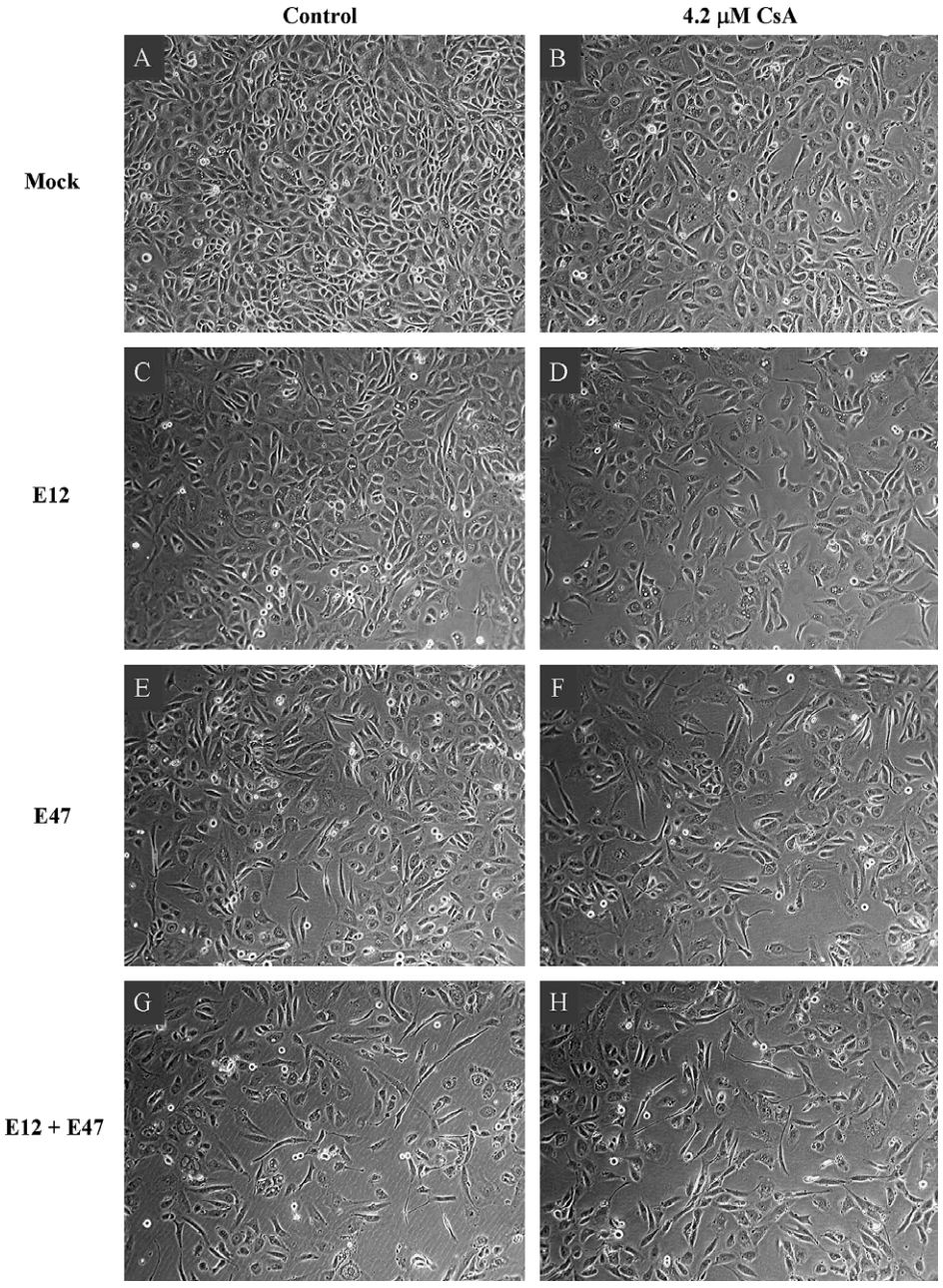

Fig. 4. Effect of E12 and E47 overexpression on tubular epithelial cell morphology. Cellular morphology was assessed using phase contrast microscopy. HK-2 cells grown on 6-well culture plates were transfected with equal amounts of pcDNA3-E12 expression vector (C,D), pcDNA3-E47 expression vector (E,F) or both in combination (G,H). Cells were treated with control medium (A,C,E,G) or medium containing 4.2 μM CsA (B,D,F,H) for 48 h (magnification 100×). Images are representative of at least three independent experiments.

E47 overexpressing cells with CsA resulted in almost complete loss of E-cadherin expression.

3.7. Effects of E2A transcription factor overexpression on α-SMA expression in HK-2 tubular epithelial cells

α-SMA mRNA levels in HK-2 tubular epithelial cells were assessed by RT-PCR and α-SMA protein was visualised using immunofluorescent microscopy (Fig. 7). Treatment of mock-transfectants with 4.2 μM CsA resulted in increased α-SMA mRNA levels (Fig. 7A). Overexpression of E12 and E47 individually also increased α-SMA mRNA levels but not to the levels observed with CsA treatment. Overexpression of E12 and E47 in combination resulted in similar α-SMA mRNA levels as those observed in CsA-treated mock-transfectants. α-SMA protein levels, as assessed by immunofluorescent microscopy correlated with observed mRNA levels (Fig. 7B) with overexpression of E12 and E47 in combination resulting in the highest levels of α-SMA protein expression.

4. Discussion

In this current study, we have demonstrated that E2A overexpression can induce EMT in human RPTEC. EMT is now

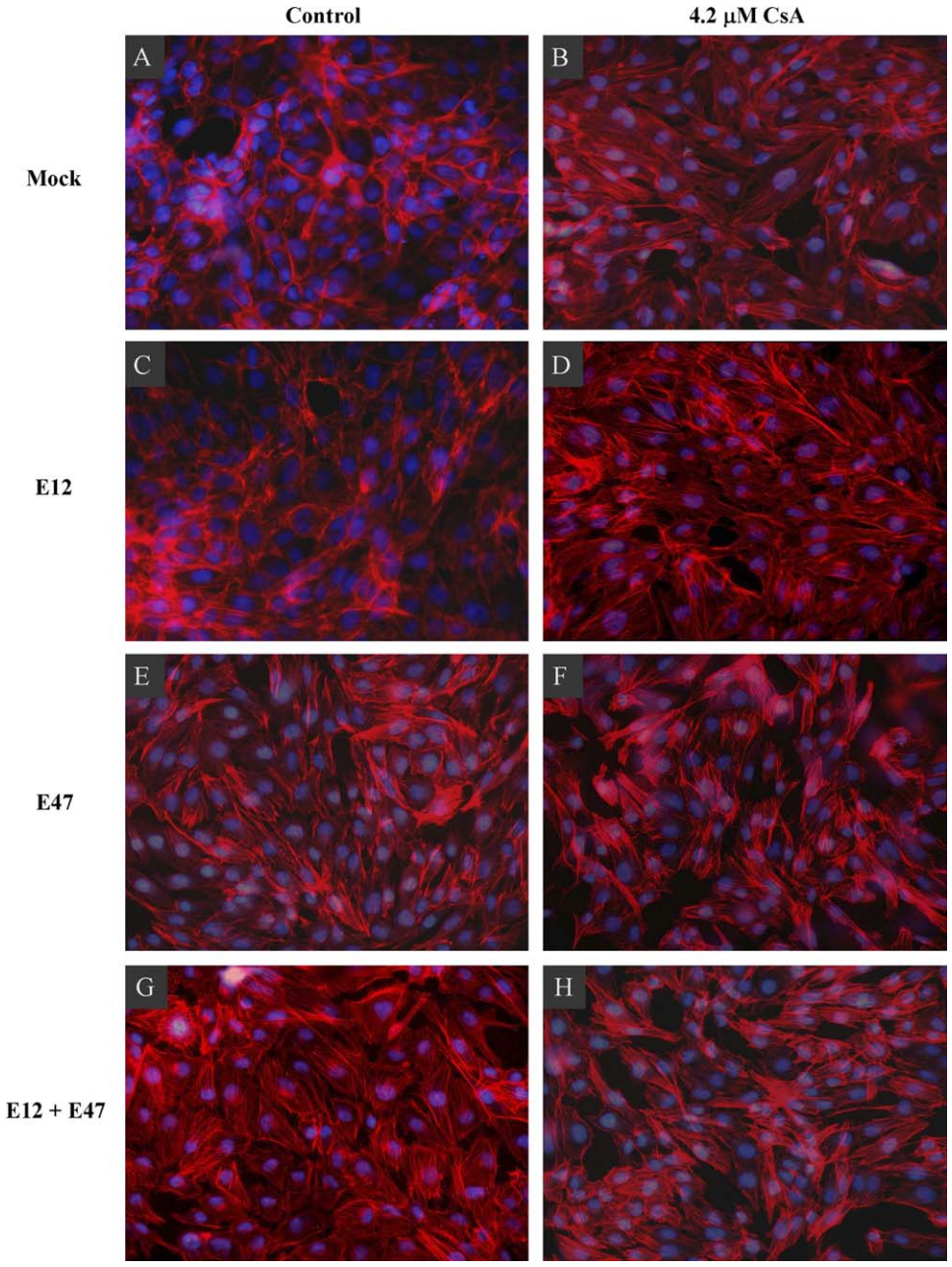

Fig. 5. Effect of E12 and E47 overexpression on tubular epithelial cell F-actin cytoskeletal arrangement. HK-2 cells grown on tissue culture slides were transfected with equal amounts of pcDNA3-E12 expression vector (C,D), pcDNA3-E47 expression vector (E,F) or both in combination (G,H). Cells were treated with control medium (A,C,E,G) or medium containing 4.2 μM CsA (B,D,F,H) for 48 h. The F-actin cytoskeleton was visualised using direct immunofluorescence staining with rhodamine phalloidin. Cell nuclei were visualised using direct immunofluorescence staining with DAPI (magnification 400×). Images are representative of at least three independent experiments.

acknowledged to play a significant role in a number of disease states including renal fibrosis and tumor metastasis [6,20,21]. The development of renal fibrosis and the enhancement of tumour formation with long term CsA use is now well established [22,23]. We have previously demonstrated that CsA-induced EMT in RPTEC [8,9].

PTECs may no longer be regarded as bystanders in renal disease development, but as active contributors to renal fibrosis [24]. After EMT, tubular epithelial cells can migrate to the interstitium and contribute to disease progression through production of ECM, cytokines promoting fibrosis and factors inducing other epithelial cells to undergo EMT, forming part of a vicious cycle of fibrosis [4]. EMT can be induced in vitro by a range of stimuli including fibroblast growth factor, advanced glycation end products, factors released by activated immune cells and TGF-β1 [25–28]. The variety of EMT inducers seems to suggest a multitude of potential mechanisms. However, Yang and Liu [7] have succinctly described key events critical to EMT progression, suggesting common downstream mechanisms must be involved regardless of the initiating factors. Since EMT is a gross change in cell phenotype, it follows that the key events governing the changes occur at the

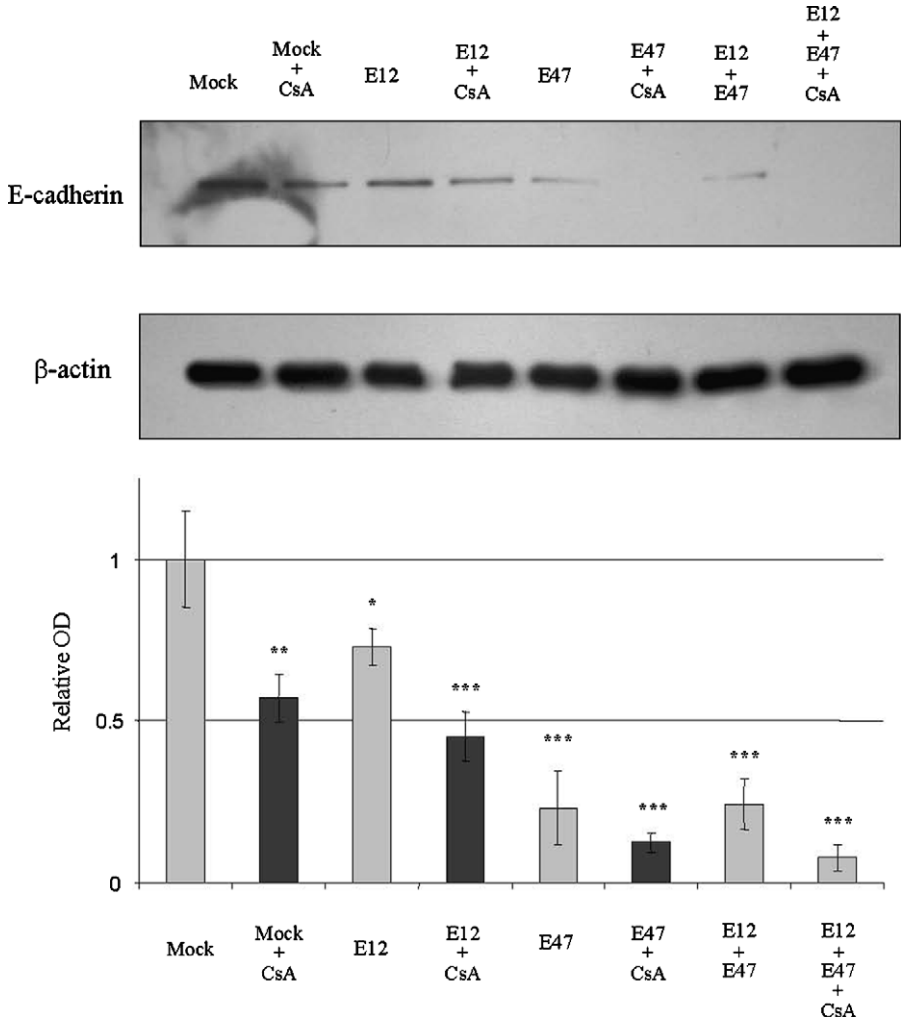

Fig. 6. Effect of E12 and E47 overexpression on E-cadherin expression in tubular epithelial cells. HK-2 cells were grown on 6-well culture plates. Cells were transfected with equal amounts of a pcDNA3-E12 expression vector, a pcDNA3-E47 expression vector or both in combination. Cells were cultured in control medium or medium containing 4.2 μM CsA for 48 h. Whole cell lysates obtained were subjected to SDS–PAGE and probed with an antibody directed against E-cadherin. β-actin was used as a loading control. Blots shown are representative of at least three independent experiments. Band intensity was quantified densitometrically. $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$ indicates statistically significant difference compared to control.

level of gene expression. E2A gene mRNA was previously identified as being upregulated in tubular epithelial cells in response to CsA exposure [8]. This was confirmed in the current study at protein level in nuclear lysates and by immunofluorescent microscopy. The E2A gene encodes two basic helix-loop-helix (bHLH) transcription factors, E12 and E47 [29], which in combination with other bHLH factors, interact with E-boxes in the promoters of target genes to modulate their transcription [15]. E2A products have been identified as being involved in the regulation of a number of genes with relevance to the EMT process [15]. While mechanisms involved in promoting E12/E47 transcriptional activity are largely undefined, it has been proposed that a major factor is the balance between cellular E2A levels and the levels of their inhibitors, Id proteins (Id1 and Id4 principally) [30]. Thus, the expression levels of the transcription factors may be a critical determinant of their activity. In a recent study, Kondo et al. demonstrated that TGF-β1 treatment resulted in upregulation of E2A transcription factors and a concomitant downregulation of Id proteins [31]. Perez-Moreno et al. demonstrated that transfection of MDCK renal epithelial cells with a construct encoding the E47 transcript caused EMT, resulting from repression of E-cadherin expression in the absence of any other stimulus [32]. In addition, Kumar et al. have demonstrated the involvement of E2A transcription factors in α-SMA induction in smooth muscle cells in vivo [33].

In the current model, CsA treatment was seen to result in marked increases in E12/E47 transcriptional activity tubular epithelial cells, detected by EMSA, which would result in modulation of target genes. To further examine the involvement of E2A transcription factors in this model, E12 and E47 were overexpressed alone and in combination in tubular epithelial cells. The effects of E2A protein overexpression were observed in the absence and presence of CsA to identify what synergistic effects, if any, might exist. Overexpression of E12 alone had some effects on cell morphology and F-actin cytoskeletal arrangement. E12 overexpression also resulted in modest reductions in E-cadherin expression levels and a concomitant increase in α-SMA levels. These results suggest that while E12 is capable of eliciting transcriptional events associated

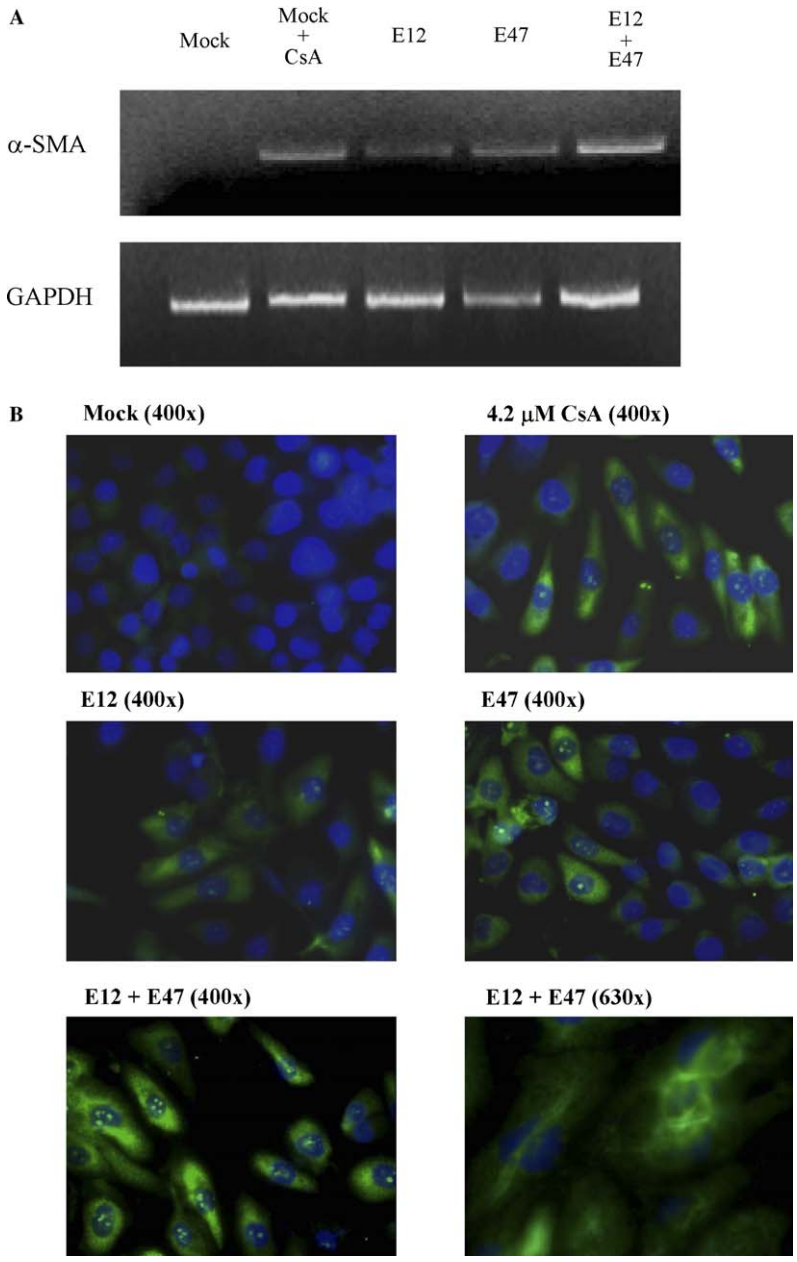

Fig. 7. Effect of E12 and E47 overexpression on α-SMA expression in tubular epithelial cells. HK-2 cells were grown on 6-well culture plates or tissue culture slides. Cells were transfected with equal amounts of a pcDNA3-E12 expression vector, a pcDNA3-E47 expression vector or both in combination. Cells were cultured for a further 48 h. (A) RT-PCR analysis was carried out on RNA obtained using primers specific to α-SMA. The GAPDH gene was used as a loading control. Images shown are representative of at least three independent experiments. (B) α-SMA protein was visualised using indirect immunofluorescence staining. Images are representative of at least three independent experiments.

with EMT to some degree. The effects were not significant enough to effect a gross change in cell morphology. However, the overexpression of E12 did appear to augment some CsA-induced events, albeit subtly. Overexpression of E47 alone had major effects on cell morphology and F-actin cytoskeletal arrangement. The morphology of E47 overexpressing cells was fibroblast-like with significant F-actin rearrangement. E47 overexpression resulted in significant reductions in E-cadherin expression levels and α-SMA expression was also induced. The effects of CsA were significantly enhanced by the overexpression of E47. These findings are in agreement with observations in other studies that identified E47 homodimers as being more potent repressors of E-cadherin than E12 homodimers [34,35]. In the present study, the most significant effects on tubular epithelial cell morphology and phenotype were observed with overexpression of E12 and E47 in combination. E-cadherin expression was severely diminished, α-SMA was strongly induced and the effects on morphology and F-actin arrangement were striking with overexpression of both E12 and E47. These effects were augmented slightly by CsA treatment. These results suggest that while overexpression of E2A transcription factors, individually or in combination, is sufficient to induce EMT to varying degrees, other signals induced by CsA, such as TGF-β1 or PKC-β upregulation [8], also

promote the progression of EMT in the current model, to provide the maximal response.

This potential role of E2A proteins in CsA-induced EMT is further supported by investigations into the expression pattern of E2A in human tissues. Rutherford et al. reported that E2A proteins were not detectable in adult human kidney samples [36]. However, E2A expression was detected in the first trimester kidney samples, most abundantly in the primitive, developing renal tubules, pre-epithelioid cells and renal interstitial cells, suggesting an association between E2A expression and mesenchymal cells. This is also in keeping with the proposed reactivation of developmental pathways in an effort to facilitate repair in the damaged kidney [37]. E2A proteins also play a key role in B and T cell development [38]. E2A protein expression has also been shown to regulate IgG isotype and class switching in B-cells [14,39]. Considering this may also suggest a novel mechanism of CsA's immunosuppressive effects.

In conclusion, we provide evidence that upregulation of the E2A gene may play a key role in CsA-induced EMT in PTECs. Overexpression of the E2A gene products induced alterations in cell morphology, cytoskeletal arrangement, and E-cadherin and α-SMA expression, all hallmarks of the EMT process. Exploration of the mechanisms involved in this model of CsA-induced EMT may lead to the development of novel therapeutic targets for the treatment of renal fibrosis, and provide further insight into the roles and relevance of the E2A transcription factors in disease processes.

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