Changes in N-acetyltransferase 8 in kidney tubular cell

Injury, recovery and mesenchymal stem/stromal cell treatment

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1. Abstract

Kidney disease is a worldwide health concern and a common consequence of acute insults (as hemorrhage, sepsis, drug toxicity) with unpredictable prognosis and lacking therapeutic options. Prolonged injury promotes progression to fibrosis and currently there are no drugs to reverse kidney disease progression. However, renal tubular epithelial cells (TECs) might dedifferentiate, proliferate and re-epithelize upon injury. N-acetyltransferase-8 (NAT8) is an enzyme highly expressed at the kidney and suggested to be renoprotective. This work assessed the expression of NAT8 throughout dedifferentiation and re-epithelization. As mesenchymal stem/stromal cells (MSCs)-based therapies have had positive outcomes, herein these were used as a tool to investigate NAT8 relevance for tubular repair. Hence, in an in vitro experimental setup the therapeutic potential of MSCs conditioned media (CM) and a possible link to NAT8 were investigated. TECs were exposed to transforming growth factor-β1 (TGF-β1, injury stimulus) promoting dedifferentiation and TGF-β1 withdrawal induced re-epithelization. Results showed that prolonged injury was associated with a decrease in NAT8. The re-epithelization was biphasic with a decrease of NAT8 followed by a late rescue. When MSC-CM was used in the absence of a harmful stimulus promoted re-epithelization, which was not related with increase in NAT8 transcription. Contrastingly, MSC-CM addition was associated with marked recovery of NAT8 transcription under continued TGF-β1 exposure. To the best of our knowledge, this is the first report of the paracrine effects of human MSCs on NAT8 in the kidney and the relevance of NAT8 to maintain regenerative potential when TECs undergo dedifferentiation.

Key words: Mesenchymal stem/stromal cell (MSC); N-acetyltransferase type 8 (NAT8); Renal injury; Renal tubular epithelial cell;

2. Background

The kidney has a major homeostatic role, regulating blood volume, pH, osmotic balance and hormone production. Also, it is a key player in organism detoxification [1], [2]. Kidney disease (KD) can have a widespread effect, disturbing the cardiovascular system, the immune system and leading to bone disorders [3], [4]. Acute kidney injury (AKI) and chronic KD (CKD), present particular pathophysiology but some hallmarks are common [5]–[7]. Including epithelial and endothelial injury, cytokine and growth factor release (for example, transforming growth fact β1, TGF-β1), and inflammatory response (including macrophages and leukocyte recruitment). Tissue and cell injury elicit a series of response mechanisms aimed at restoring homeostasis, including the unfolded protein response, autophagy, inflammation and apoptosis [5]–[7]. Upon injury, epithelial cells may undergo dedifferentiation concurrent with the loss of epithelial markers and increased expression of mesenchymal markers, a process called epithelial to mesenchymal transition (EMT). [8], [9]. EMT is a complex and possibly sequential multistep program comprising different stages. [8], [10], [11]. Initially, cells present an epithelial phenotype. Upon, initiation of EMT cells will acquire a partial EMT phenotype (pEMT) that progresses into a non-functional mesenchymal phenotype with advanced EMT. pEMT would be a reversible state expressing both epithelial (as e-cadherin, ZO-1, cytokeratin (CK)) and mesenchymal (vimentin (VM), α-smooth muscle actin, N-cadherin) markers [8], [10], [12]. EMT has been connected to progression towards fibrosis and loss of function [13]. Conversely, dedifferentiation is also associated with injury repair and tubular regeneration [14], [15]. The key to this discrepancy might lie in the different attributes of the different stages of EMT [10], [16]. So, although advanced EMT would be associated with renal damage, pEMT could mediate regeneration [10], [14], [15]. TGF-β1 is involved in the regulation of dedifferentiation, and this cytokine is up-regulated as part of the initial injury response [17]. And, under severe or persistent injury would, TGF-β1 would progressively increase possibly promoting fibrosis [15], [17]. Dysregulated TGF-β1 might induce the irreversible mesenchymal phenotype, representing the deleterious side of EMT (ultimately leading to fibrosis) [10], [15], [17]. So, dedifferentiation might mediate endogenous tubular repair mechanism [14], [15]. It was hypothesized by some authors that, after withstanding damage, functional terminally-

differentiated TECs have the ability to dedifferentiate and acquire a stem-like phenotype [18], expressing CD24, CD133 and VM. Then, these dedifferentiated cells would be able to proliferate and regenerate. [14], [15], [19]. Kramann and co-authors (2015) [14] specifically labelled terminally differentiated TECs and confirmed that, upon injury, these were the cells that mediated tubular repair. However, there is still controversy regarding the actual repair mechanism [20], [21]. Another contending theory suggests that an endogenous pool of stem cells resides within the tubule and following injury, these endogenous stem cells mediate repair [22]. Upon severe or prolonged injury the tubule might not be capable to fully repair which ultimately would lead to loss of function [7] and promote progression of KD. [4]. In fact, tubulointerstitial injury has been shown to correlate well with renal function impairment [7]. Nowadays, most available therapies only address comorbidities and slow down KD progression.[3], [6], [23], [24]. A possible novel target could be N-acetyltransferase 8 (NAT8) this enzyme is mostly expressed in kidney tubular cells [25], [26]. NAT8 has been implicated in detoxification [2], [27] associated (in genome-wide association studies) with systolic pressure, and glomerular filtration rate (GFR) and ultimately speculated to be renoprotective [28], [29]. In 2010 Veiga-da-Cunha and co-authors [27] identified NAT8 as the enzyme that N-acetylates cysteine residues and catalyses the last step in the mercapturic acid pathway (MAP). This pathway is important for renal detoxification of electrophilic compounds, which exert cellular damage. Other, more controversial hypothesised role for NAT8 is the capacity to acetylate lysine residues [30], [31] which is additionally connected with protein quality control mechanism [30] and acetylation of CD133 (mediating membrane trafficking) [31]. However, Veiga-da-Cunha and co-authors [27] investigated NAT8 ability to acetylate lysine residues and found that catalytic efficiency was 20-fold lower than for cysteine. Recently, Omata and co-authors (2016) found NAT8 to be altered in TEC injury and regeneration [32]. The authors confirmed that, in human proximal TECs, *NAT8* transcription was significantly reduced after 48 h of exposure to an injury stimulus that promoted dedifferentiation. Withdrawing injury stimulus lead to re-epithelization and progressive recovery of *NAT8* in the next 24 h. However, these authors also reported that *NAT8* knock-down did not prevent re-epithelization. Additionally, evidence supporting a role for NAT8 in mesenchymal stem/stromal cell (MSC) therapy was provided by Fu and co-authors (2014) [33] who observed an increased hepatic expression of NAT8 when umbilical cord derived MSCs were administered to treat ischemia-reperfusion injury. MSC treatment ameliorated hepatocellular damage and promoted regeneration and this was concurrent with rescued NAT8 expression which had been reduced upon liver injury. All these support the interest in the study of NAT8 and its potential for future therapies. Al, these also support the working hypothesis driving this work: that NAT8 expression could have a role in the therapeutic action of MSC.

MSCs are multipotent stem cells and, when therapeutically administered, they migrate preferentially towards the site of injury where MSCs are capable to engraft [34]. However, there is growing consensus that paracrine effects are more relevant to benefits associated with MSC [24], [35]. Paracrine effects may be mediated by biological active factors (including hepatocyte growth factor and insulin-like growth factor), microvesicles and exosomes (transporting molecules as mRNA, miRNA, surface receptors and lipids) produced and released by MSCs [24], [35]. MSCs have shown potential for the treatment of KD *in vitro* [36], *in vivo* [36], [37] and in clinical trial [38]. Their use has many advantages including that MSCs can be obtained from a multitude of tissues and their low immunogenicity. Notwithstanding, there is no standard isolation, characterization or expansion protocol [35], [39]. This work intended to investigate the expression of NAT8 in injury-induced dedifferentiation and re-epithelization processes. Also, it was aimed to clarify the paracrine effects of MSC on NAT8 in re-epithelization, while further validating the paracrine benefits of MSCs in repairing kidney damage.

3. Materials and methods

This was achieved by establishing an *in vitro* experimental design using an immortalized line of normal human proximal TECs (HK-2 [40]). These cells retain a series of phenotypical features characteristic of healthy adult TECs and have

been used in renal injury studies investigating the role of EMT in fibrosis/healing [13], [41], [42]. TGF-β1 was used as stimulus for cell dedifferentiation as it is up-regulated in renal injury, represents a main physiological driver of EMT and has been shown to induced dedifferentiation in human TECs and HK-2 [6], [13], [32]. Conversely, withdrawing TGF-β1 promoted re-epithelization [32]. Conditioned media (CM) from adipose tissue derived MSC, a validated source for therapy and previously used in HK-2 based assays [36], was administered to evaluate paracrine effects. Hallmarks of EMT were evaluated including increased VM concurrent with decreased CK and e-cadherin expression [12]. VM and CK were assessed by flow cytometry and e-cadherin by RT-qPCR which granted characterization of EMT at both protein and transcription level. NAT8 expression was assessed over time through RT-qPCR and these results were corroborated by immunofluorescence labelling.

Cell culture and experimental conditions

HK-2 (ATCC® CRL-2190™) cells were obtained from American Type Culture Collection [40], cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; 11320033, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, S 0615, Biochrom), 4 mM L-Glutamine (25030-081, Gibco) and 1% antibiotic-antimycotic (15240062, Anti-Anti, Invitrogen). Cells were kept at 37 ^{o}C, and 5.2% CO_2 and kept under 70-80% confluence. Cells were detached using trypsin - ethylenediaminetetraacetic acid (EDTA) (0.05%) (25300-054, Gibco). For all *in vitro* experiments cells were seeded in DMEM/F-12 for 24 h then, deprived of FBS for 16 h prior to establishing experimental conditions. TGF-β1 (100-21, Peprotech [43]) was reconstituted in citric acid (10 mM pH 3.0) and supplemented at 10 ng/mL. Media was refreshed every 24 h following a brief rinse with Phosphate Buffered Saline 1× (PBS).

Dedifferentiation assay: cells were seeded and exposed to control conditions (DMEM/F-12) or to TGF-β1 (DMEM/F-12 supplemented with 10 ng/mL TGF-β1 Experiments were performed in triplicates for each condition.

Dedifferentiation – re-epithelization assay: cells were seeded and exposed to four different conditions: **(1)** control (CTRL, DMEM/F-12); **(2)** TGF-β1 (TGF-β1, DMEM/F-12 supplemented with TGF-β1); **(3)** TGF-β1 exposure for 48 h followed by up to 96 h culture in TGF-β1-free media (TGF-β1 (48 h) + CTRL (96 h)); and **(4)** TGF-β1 exposure for 72 h followed by up to 72 h in TGF-β1-free media (TGF-β1 (72 h) + CTRL (72 h)). Upon TGF-β1 withdrawal, cells were cultured as control condition).

Mesenchymal stem/stromal cell conditioned media (MSC-CM) treatment assay: cells were seeded and exposed to five different conditions: **(1)** control (CTRL); **(2)** exposed to TGF-β1 (TGF-β1), **(3)** 72 h exposure to TGF-β1 followed by culture in TGF-β1 free DMEM/F-12 (TGF-β1 (72 h) + CTRL (72 h)), **(4)** 72 h long exposure to TGF-β1 followed by culture in TGF-β1 free DMEM/F-12 supplemented with 30% MSC-CM (TGF-β1 (72 h) + MSC-CM (72 h)) and **(5)** 72 h long exposure to TGF-β1 followed by culture in DMEM/F-12 supplemented with both 10 ng/mL TGF-β1 and 30% MSC-CM (TGF-β1 (MSC-CM)). MSC-CM was provided by the Stem Cell Bioengineering and Regenerative Medicine Laboratory, Técnico Lisboa, Campus TagusPark, Universidade de Lisboa and was obtained from the culture of adipose tissue derived MSCs from one donor. MSCs were cultivated in a 1000 mL New Brunswick® BioFlo (Eppendorf®) bioreactor with low glucose DMEM supplemented with 5% of human platelet lysate, kept at 37 ^{o}C, with 20% of dissolved oxygen provided by headspace aeration (N_2, O_2 and air) and pH 7.3. 20×10^6 cells were inoculated with 20 g/L of plastic microcarriers [44] in a final volume of 400 mL. After 7 days of culture supernatant was centrifuged (1500 rpm) and kept cryopreserved at –80 ºC. Prior to its use MSC-CM was thawed on ice aliquoted and stored at -80 ^{o}C until use.

Flow cytometry

Cells were seeded in 24 well plates (1×10^5 cells per well), for the *dedifferentiation* assay and in 12 well plates (2×10^5 cells per well) for *dedifferentiation – re-epithelization* and *MSC-CM treatment* assays. Each experimental condition was assayed in triplicates. Following a brief rinse with PBS 1× cells were detached with 2mM PBS-EDTA Fluorophore labelling was carried out by incubating cell for 30 min with 100 µL of allophycocyanin (APC) conjugated VM antibody (IC2105A, R&D Systems, Inc.) and phycoerythrin (PE) conjugated CK antibody (ab52469, Abcam) diluted 1:100 in PBS-Bovine Serum Albumin (BSA, A9647, Sigma) -TRITON X100 0.1% (T8787, Sigma). Then cells were centrifuged, washed, re-suspended in PBS-BSA 0.1% (200 µL) and examined by flow cytometry (FACScalibur – Becton Dickinson). 10,000 events per sample were acquired using *CellQuest* (Becton Dickinson). Collected data was analysed using FlowJo® software [45] and gates were set based on signal measured from a non-labelled sample.

Real time relative quantification reverse transcription polymerase chain reaction (Real time RT -PCR) analysis

2×10^5 cell cells were seeded in 12 well plates. Experimental conditions were as described in section *Cell culture and experimental conditions*. Then, cells were detached using PBS-EDTA (2 mM), and RNA extraction was carried out accordingly to manufacturer's instructions of the RNeasy® Mini Kit commercialized by QIAGEN™ [46] The obtained RNA was stored at -80ºC. RNA concentration was determined based on absorbance at 260 nm, using a Nanodrop 2000 (Thermo Scientific). cDNA was obtained from 1µg of RNA in bidistilled water (ddH_2O) by reverse transcriptase polymerase chain reaction (RT-PCR). RNA was incubated with random hexamers (11034731001, Roche) at 70 ^{o}C for 10 min. Then the temperature was lowered to 4 ^{o}C and 12.5 µL of a mixture of: Strand Buffer 5X (Y00146, Invitrogen),

0.1M dithiothreitol (Y00147, Invitrogen), 10mM deoxynucleotides mix (28-4065-22V, 28-4065-02V, 28-4065-12V and 28-4065-32V, GE Healthcare), RNAse OUT™ Recombinant RNase Inhibitor 40 U/µl (10777-019, Invitrogen), Superscript II® 200 U/µl (18064-022, Invitrogen) and ddH_2O was added to each sample. A Biometra® UNO II thermocycler was used with the following cycle specifications: 42 °C for 90 min, 75 °C for 15 min then cooled down to 4 °C. RNA was quantified relative to an endogenous reference *Hypoxanthine phosphoribosyltransferase* (HRPT) gene. Relative quantification real-time RT-PCR was performed using Power SYBR® Green PCR Master Mix (4367659, applied biosystems). The primers used for the genes of interest were acquired from SIGMA and the primer sequences were e-cadherin (forward 5' –CCACCAAAGTCACGCTGAATA and reverse 5' - GGAGTTGGGAAATGTGAGCAA), NAT8 (forward 5'–GGCTCCTTGTCACATCCGC and reverse 5'–GGTTCGAGGCAGCTTCAGC). HPRT (forward 5'–TGACACTGGCAAAACAATGCA and reverse 5'–GGTCCTTTTCACCAGCAGCT). To each sample a mix of reverse and forward primers, SYBR Green and ddH_2O was added. A total of 1 µL of sample (cDNA) was used. Samples were analysed in technical triplicates. A LightCycler®480 II (Roche) was used with the following program: 2 min at 50 °C, 10 min at 95 °C, then 45 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by 15 s at 95 °C, 15 s at 60 °C then continuous denaturation at 95 °C. Final cooling 40 °C for 10 s.

Immunofluorescence microscopy (indirect)

Cells were seeded in an 8 well-chambered slide system (Thermo Fisher). For the *dedifferentiation – re-epithelization* assay, cells were seeded at a density of 1×10^3 cell/well. Experiments were performed in duplicate for the control and exposed to TGF-β1 conditions and triplicates for the TGF-β1 (48 h) + CTRL (96 h) condition. After exposure to experimental conditions media was discarded, cells were washed and then fixed with a solution of PBS-paraformaldehyde (PFA) (2%) – Glutaraldehyde (0.1%) for 10 min at RT. Cells were once again washed with PBS 1× and permeabilized with PBS-BSA (0.1%) – Triton X 100 (1%) for 10 min at RT. Afterward, cells were washed with PBS 1 × and incubated with primary antibody (anti-NAT8, ab197793, Abcam) diluted 1:100 in PBS-BSA (1%). Incubation was carried out overnight at 4°C in an orbital shaker set for slow agitation. Cells were washed and then incubated with the secondary antibody Invitrogen™ Alexa Fluor™ 488 (A-11008, Thermo Fisher) diluted 1:1000 in PBS-BSA (1%) for 2 hours, under slow agitation at RT then washed. Afterward, each sample was stained with VECTASHIELD mounting media with 4',6-diamidino-2-phenylindole (DAPI, H-1200, VECTOR laboratories). Finally, images were acquired using an inverted microscope (IX53, OLYMPUS), a fluorescence light source (U-HGLGPS, OLYMPUS) and the *CellSens* software.

Statistical Analysis

Statistical analysis were performed by using one–way ANOVA with *Dunnet*'s post-test or two-way ANOVA with *Bonferroni*'s post-test. Resorting to GraphPad Prism 5 (GraphPad Software). Statistical significance was defined as $p< 0.05$.

4. Results

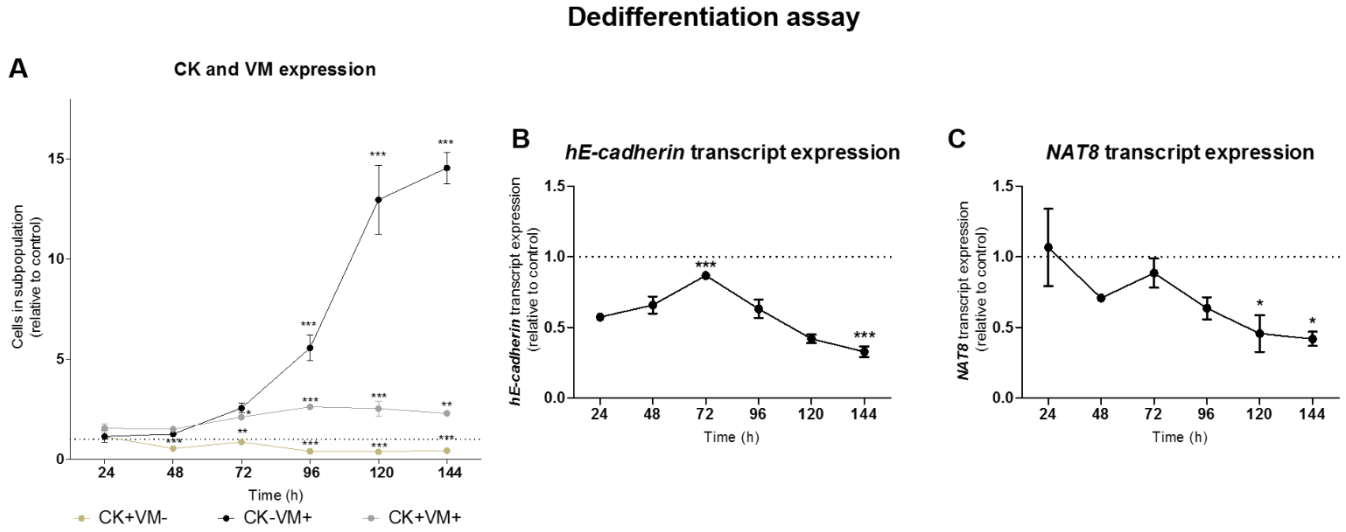
TGF-β1 administration mediated dedifferentiation and withdrawal promoted re-epithelization Exposing HK-2 cells to TGF-β1 (10 ng/mL) succeeded in initiating EMT as suggested by a raise in VM expression which, after 144 h , was nearly 15 times higher if exposed to TGF-β1 ($p<0.01$, **Panel a, A, black line**). This is also supported by the loss in CK (**Panel a, A, brown line**) and e-cadherin (**Panel a, B**) and acquisition of spindle-like morphology (

Panel b, A, middle row). The shift towards a more mesenchymal phenotype appears to be a gradual and time-dependent process. However, EMT gains evident emphasis beyond 96 h. The temporal curve of transcript levels of *NAT8* was assessed by RT-PCR. It was observed that TGF-β1-induced EMT is associated with a decrease in *NAT8* expression (**Panel a (C)**). In fact, after 48 h under TGF-β1, *NAT8* transcript levels remain consistently below those of the control group. An initial reduction in *NAT8* at 48 h was briefly followed by a transient rise at 72 h. And, from 96 h

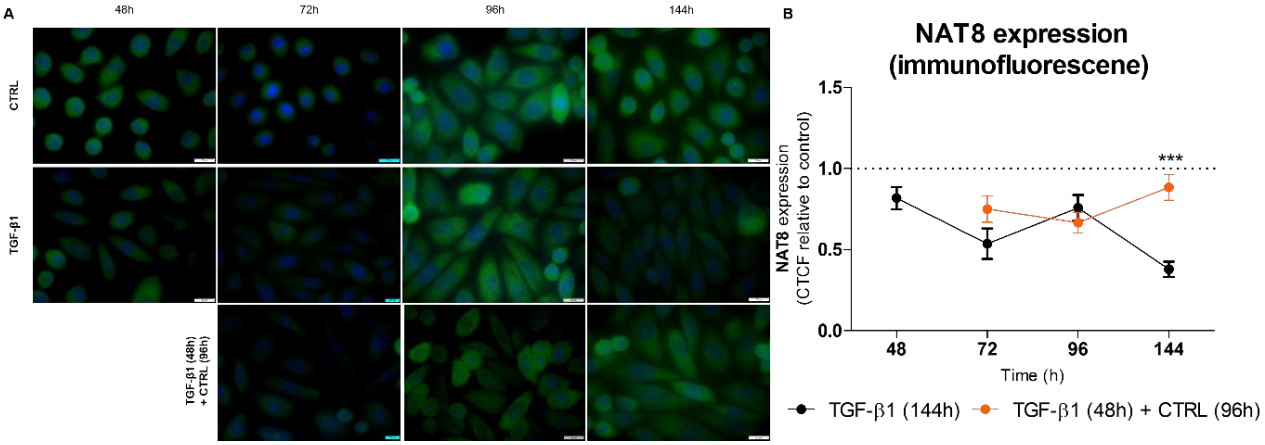
onward expression acquires a decreasing tendency that is sustained until the end of the assay ($p<0.05$). Data resulting from quantification of immunofluorescence imaging (

Panel b, B, black line) was consistent with *NAT8* transcript levels. Inclusively, the initial shift in *NAT8* transcription (decline followed by temporary recovery) seems to be patent in the protein expression, albeit delayed. Consistently, protein expression seems to be significantly reduced at the end of the assay.

These results confirm that dedifferentiation is associated with a decrease in NAT8. However, it would appear that just before dedifferentiation is firmly established a transient intensification in NAT8 expression occurs. Remarkably, at 96 h dedifferentiation becomes considerably more pronounced and the same is observed regarding the decrease in NAT8.



Panel a Dedifferentiation Assay: HK-2 cells were exposed to TGF-β1 (A) Expression of CK and VM was assessed by flow cytometry. CK+VM-, CK-VM+ and, CK+VM+ populations were quantified as percentage of cell count. **(B) *E-cadherin* transcript levels** were quantified by RT-PCR (relative to endogenous reference *HPRT*). **(C) *NAT8* transcript levels** were quantified by RT-PCR (relative to endogenous reference *HPRT*). Data are expressed as mean ± SEM and relative to control group (not exposed to TGF-β1). Statistical analysis of data was performed using one-way ANOVA with *Dunnett*'s post-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* 24 h time-point); TGF-β1 - transforming growth factor β1



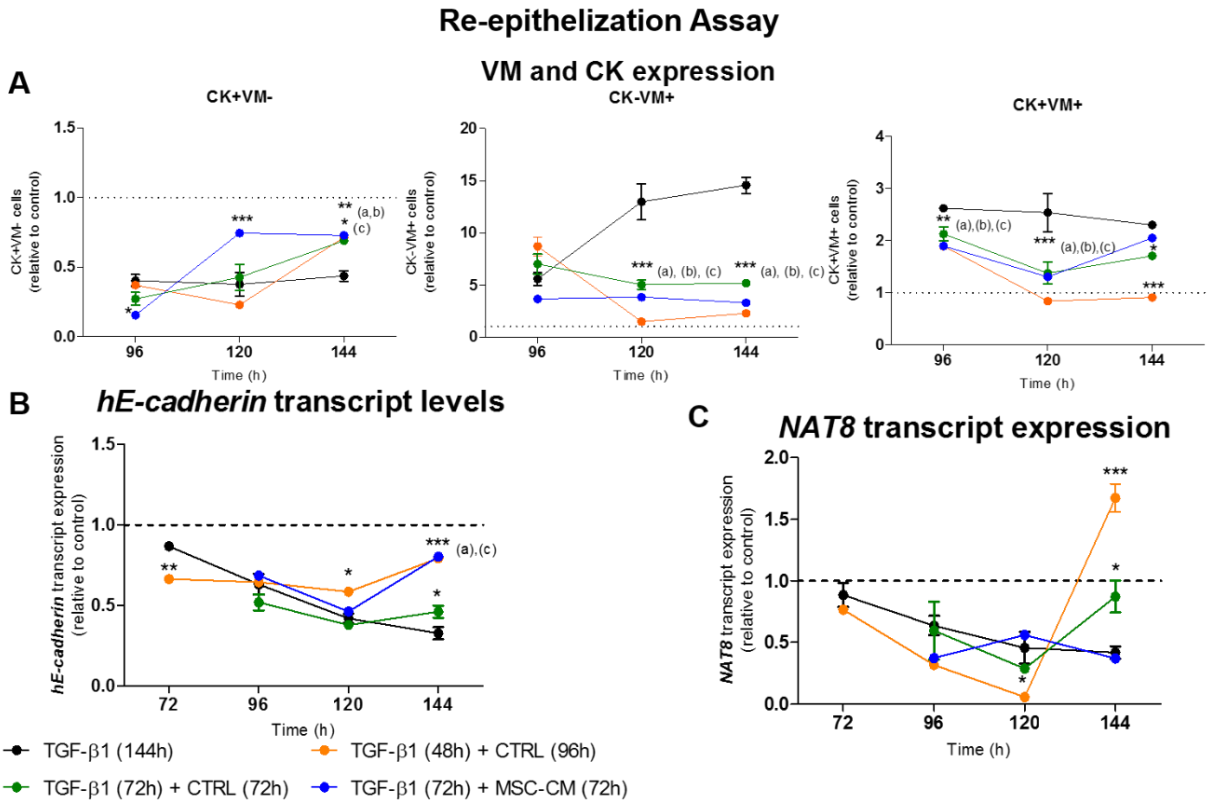
Panel b (A) NAT8 immunofluorescence (magnification x 600) and HK-2 cells were either culture under normal media (top row), exposed to TGF-β1 (middle row) or exposed to TGF-β1 for 48 h and then cultured for 96 h in normal conditions (bottom row) **(B) Quantification of NAT8 fluorescence** using image analysis software (*ImageJ*, SciJava). Data are expressed as mean ± SEM relative to control group mean. Statistical analysis of data was performed using one-way ANOVA test with *Dunnett*'s post-test (* $p<0.05$, *** $p<0.001$ *vs*. 48 h time-point); TGF-β1 - transforming growth factor β1

Although, NAT8 is ultimately recovered through re-epithelization ($p<0.001$) initial re-epithelization did not correlate with NAT8 up-regulation. MSC-CM promotes re-epithelization. Withdrawal of TGF-β1 after 48 or 72 h long exposures was followed by a recovery of the epithelial phenotype, as confirmed by decrease in VM expression, increased CK (**Panel c, A**), up-regulation of *e-cadherin* transcription (**Panel c, C**) and suggested by morphology changes

(data not presented). Also, the more prolonged the exposure to TGF-β1, the less effective seems to be the recovery of the epithelial phenotype.

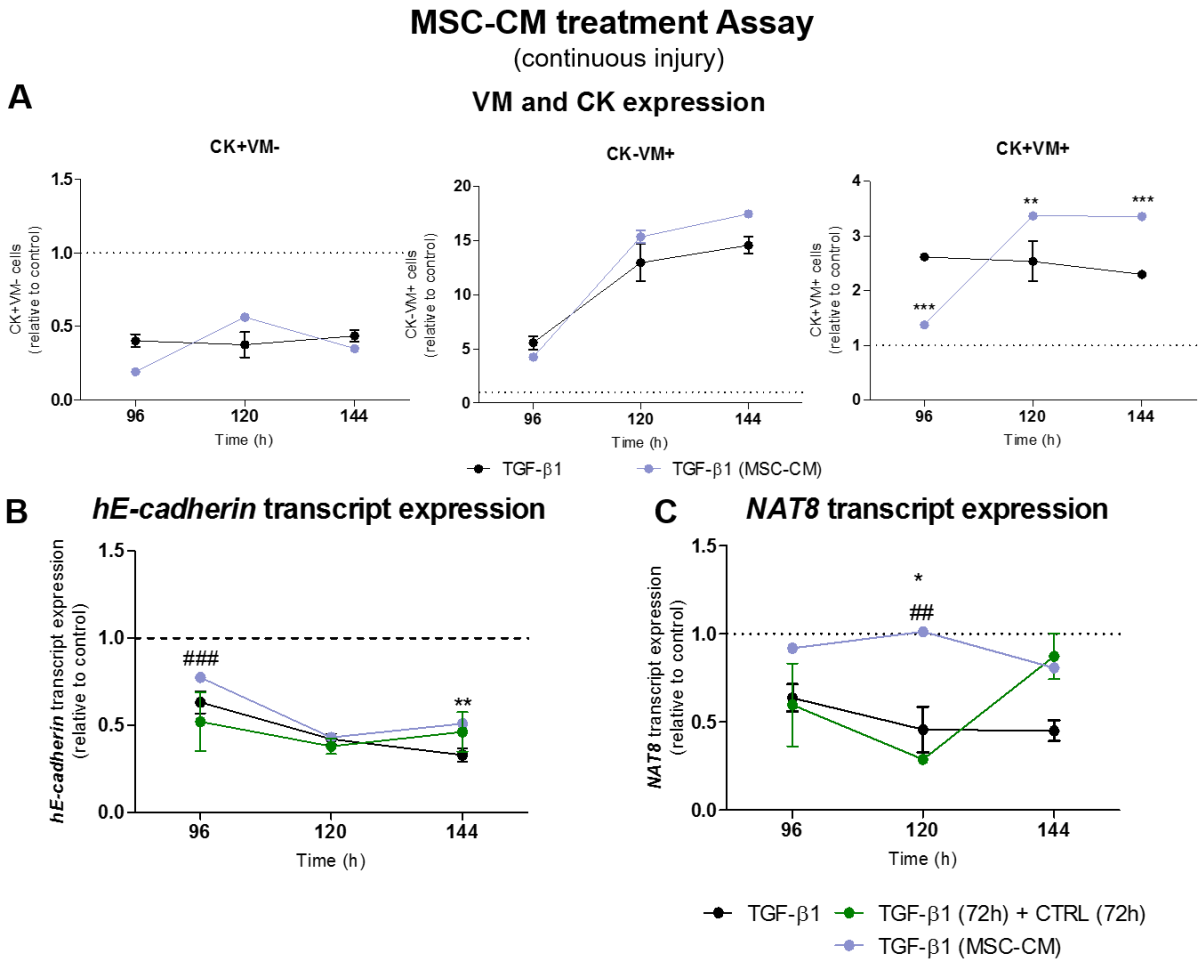
Following TGF-β1 withdrawal MSC-CM was added to the media resulting in more effective re-epithelization than under normal culture conditions. Since, changes in CK and VM expression (**Panel c, A, dark blue line**), *e-cadherin* transcript expression (**Panel c, B, dark blue line**) and morphology (data not presented) showed more pronounced and rapid recovery of epithelial phenotype. Nonetheless, recovery under MSC-CM stimulation, which seems more effective, was not accompanied by an increase in *NAT8* expression **Panel c (C, blue line)**.

Regarding the expression of NAT8, following both 48 and 72 h long exposures there was a drop in expression levels of NAT8 further below that of HK-2 cells continuously exposed to TGF-β1 **Panel c (C, black line)**. Later, in the last endpoint of the assay, concurrent with clear signs of CK and *e-cadherin* rescue NAT8 expression reverts to values near or above control level. These results were supported by immunofluorescence data (**Panel b, A bottom row, B orange line**) and suggest that a latency period occurred before the rise in NAT8 is effectively established.



Panel c Re-epithelization assay. HK-2 cells were either exposed to TGF-β1 for 48 h and then either cultured for 96 h in TGF-β1 free-media **(orange line)** or exposed to TGF-β1 for 72 h and then cultured for 72 h in either TGF-β1 free-media **(green line)** or exposed to TGF-β1 for 72 h and then cultured for 72 h in media supplemented with MSC-CM (30%) **(dark blue line)** or continuously exposed to TGF-β1 **(black line) (A) CK and VM expression** was assessed (flow cytometry **(B) *hE-cadherin* transcript levels** were quantified by RT-PCR (relative to endogenous reference *HPRT*) **(C) *NAT8* transcript levels** were quantified by RT-PCR (relative to endogenous reference *HPRT*)**.** Statistical analysis of data was performed using two-way ANOVA test with *Bonferroni*'s post-test (*p<0.05, ** p<0.01, *** p<0.001, vs. TGF-β1 (a) (b) and (c) refer to orange, green or dark blue respectively); TGF-β1 – Transforming growth factor – β1

MSC-CM treatment under continuous injury promotes improvement and is correlated with clear recovery of NAT8 transcription levels. Although, administration of MSC-CM in the continuous presence of TGF-β1 did not suffice reverse changes in VM and CK expression (**Panel d, A, light blue line**) it did lead to an increase in *e-cadherin* (**Panel d, B, light blue line**) comparing to continuous exposure to TGF-β1 alone (**Panel d, B, black line**). Moreover, at the end of the assay (144 h), regarding recovery in *e-cadherin* expression the effect of MSC-CM supplementation was comparable to TGF-β1 withdrawal (**Panel d, B, green line**). In this scenario, the observed effects were associated with a clear recovery of *NAT8* expression (**Panel d, C, light blue line**).



Panel d MSC-CM treatment under continuous injury HK-2 cells were first exposed to TGF-β1 for 72 h and then cultured for another 72 h in either: media supplemented with both TGF-β1 and MSC-CM (30%) **(light blue line)**, normal media **(green line)** or continuously exposed to TGF-β1 **(black line). (A) Expression of CK and VM** was assessed by flow cytometry. CK+VM-, CK-VM+ and, CK+VM+ populations were quantified as percentage of cell count. **(B) *hE-cadherin* transcript level** was quantified by RT-PCR (relative to endogenous reference *HPRT*). **(C) *NAT8* transcript level** was quantified by RT-PCR (relative to endogenous reference *HPRT*). Data are expressed as mean ± SEM and relative to control group mean. Statistical analysis of data was performed using two-way ANOVA test with *Bonferroni*'s post-test (** p<0.01, *** p<0.001, vs. TGF-β1 and # p<0.05, ### p<0.001, vs, TGF-β1 (72 h) + CTRL (72 h); TGF-β1 – Transforming growth factor – β1

5. Discussion

The results herein obtained suggest a role for NAT8 in the maintaining the regenerative capacity of TECs undergoing injury induced dedifferentiation. The first indication in this sense was that NAT8 expression was only firmly decreased when dedifferentiation was intensified. Initial dedifferentiation, which could correspond to a reversible phenotype with regenerative properties was associated with a transient increase in NAT8. The period of established but not drastic dedifferentiation (from 48 to around 96 h of exposure) is characterized by a significant increase in the double positive CK+VM+ population and expression of both epithelial and mesenchymal markers is consistent with partial EMT stage [8], [10]. Remarkably, apparent transient rises in NAT8 expression both considering transcript level (**Panel a** C**,** observed around 72 h) and estimated protein level (around 96 h, **Panel b** B) occurred during this period. Secondly, an escalation in dedifferentiation correlates with an intensification in NAT8 loss. Prolonging the exposure for over 96 h resulted in an emphasized expression of VM (**Panel a** A, black line) and sustained decrease of epithelial markers (**Panel a** A brown line and B). From that point on, NAT8 transcription follows a significant decreasing tendency (see **Panel a C**). So, initial differentiation tied to regenerative potential appears to be associated with a transient rise in expression of NAT8. Further, temporal EMT escalation, associated with progression to an irreversible and possibly pro-fibrotic phenotype, was accompanied by NAT8 loss. Another observation was that initial re-epithelization, in the absence of an injury stimulus, does not appear to be dependent of NAT8. In both injury scenarios (48 and 72 h pulses) TGF-β1 withdrawal was

immediately followed by a decrease in NAT8 transcript expression (**Panel c** C). Nonetheless, at the end of the assay (144 h, 48 h +96 h or 72 h+72 h) significant recovery of epithelial markers was observed and this was associated with an increase in NAT8 transcript levels. Similarly, at the protein level, following removal of TGF-β1 there seems to be a latency period before recovery in NAT8 is effectively established (**Panel b** B). Furthermore, HK-2 cells were exposed to TGF-β1 for 72 h and then cultured under TGF-β1 free media supplemented with MSC-CM to study the reparative potential of MSCs *via* paracrine effects. This led to a more rapid and more complete re-epithelization process (**Panel c** A and B, dark blue line) and this was also not associated with recovery of NAT8 transcription (**Panel c** C, dark blue line).

Given the postulated renoprotective role of NAT8 [28], [32], it would be expected that recovery of tubular epithelial phenotype would be complemented by an increase in NAT8. Nonetheless, either with or without the addition of MSC-CM to media, re-epithelization seems to initiate independently of NAT8 expression. Supporting the speculation that NAT8 mediates its protective effect through another pathway rather than re-epithelization. However, the decrease in NAT8 transcription in the sequence of TGF-β1 withdrawal was in disagreement with the literature. Omata and co-authors (2016) [32] reported NAT8 as progressively increasing through re-epithelization after TGF-β1 removal (in the sequence of 48 h exposure to TGF-β1 3 ng/mL). This contradiction might be due to the difference in concentration of TGF-β1 supplemented (3 ng/mL in [32] *vs.* 10 ng/mL in the present work), which may have resulted in a more pronounced and lasting response in the experiment being described here. Additionally, intrinsic differences in the cellular model, might account for the disparity since Omata and his group used primary human TECs.

Another aspect that supports the proposed role for NAT8 in in the maintaining the regenerative capacity of dedifferentiated TECs is that MSC-CM treatment under continuous injury induced signs of improvement in association with a clear rescue of NAT8. HK-2 cells were exposed to TGF-β1 for 72 h afterwards MSC-CM was added to the media (without withdrawing TGF-β1). This led to an improvement in *e-cadherin* expression (**Panel d** B, light blue line) and in morphology (data not presented) resulting in nearly the same effect as withdrawing TGF-β1 altogether. Considering this, and the extensive literature backing the benefits of MSC-CM, it is acceptable to suggest that MSC-CM show the potential to attenuate the action of TGF-β1. Leading to the hypothesis that when under continuous injury MSC mediate their effect by promoting the survival and maintenance of a dedifferentiated population that is able to replenish the tissue. This effect was coupled with a significant and consistent increase in NAT8 expression, suggesting NAT8 is part of the mechanisms underlying this renoprotective effect. No protein expression or activity was evaluated limiting the strength of this conclusion. However, evidence of augmented protein expression NAT8 in the context of MSC treatment has already been reported albeit in liver injury[33]. NAT8 is implicated in the MAP [27], a detoxifying mechanism, this might suffice to significantly improve the survival capacity of dedifferentiated cells. Otherwise, NAT8 might play a more regulatory role, in fact, N-acetylation has been shown to have a profound impact on protein metabolism [47]. NAT8 has been shown to acetylate CD133, and this modification is required for successful trafficking of CD133 to the cell membrane [31], which has been identified as a characteristic marker of dedifferentiation. The work developed here was very exploratory and some aspects require further validation. First, classification of the different phenotypes (epithelial, pEMT/dedifferentiated cells and mesenchymal/pro-fibrotic) should be improved. Preferentially by defining quantitative criteria to assess EMT marker expression. Also, other markers associated with renal dedifferentiated and regenerative capable cells, such as CD133 and CD24 [14], [19], [48] should be included as well as, markers more closely associated with extra cellular matrix production and fibrosis, such as α-smooth muscle actin or fibronectin [8], [13]. Importantly, sorting and re-culture of cells specific to each population should be performed to evaluate their ability to proliferate and revert back to the healthy epithelial phenotype. Such an approach has been implemented [10] successfully in the MCF10A cell line using TGF-β1 to induce EMT. Also, all the supporting evidence is associative therefore only correlation can be claimed. Functional evidence will be necessary so, in the future enzyme activity should be measured and NAT8

knock-down and overexpression experiments must be performed. To investigate how these would impact injury, repair and response to MSC treatment.

6. Conclusion

In general, the results obtained support suggest that NAT8 has a role in maintaining the regenerative capacity of tubular epithelial cells undergoing injury induced dedifferentiation. Also, they underscore the initial hypothesis that the paracrine action of MSCs might be linked to changes in NAT8 expression, particularly in the context of kidney injury.

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