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ORIGINAL ARTICLE

Polyamines modulate epithelial-to-mesenchymal transition

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Abstract Epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition are biologic processes responsible for conversion of epithelial cells into a mesenchymal phenotype or viceversa, respectively. They occur during embryo- and foetal-development and, in adult organisms, are involved in wound healing, in the genesis and progression of organ fibrosis as well as in the invasiveness of epithelial cancer cells. The key event of epithelial-to-mesenchymal transition is the loss of E-cadherin expression due to repressor activity of the transcriptional factor Snai1. Intracellular Snai1 levels are controlled through translational and post-translational events such as phosphorylation and de-phosphorylation, potentially modulated by polyamine content. Epithelial MDCK cells exposed to TGF-β_1 acquired a fibroblastoid phenotype and expressed mesenchymal markers. These changes were emphasized in cells that were also exposed to DFMO in order to decrease the intracellular levels of polyamines. Addition of exogenous polyamines almost completely abolished the combined action of DFMO and TGF-β_1 and rapidly reverted to epithelial phenotype MDCK cells previously undergone to mesenchymal phenotype. Nuclear extracts of cells treated with DFMO + TGF-β_1 revealed the presence of Snai1 immunopositive bands in a range of molecular weight between 55 and 72 kDa, with additional positive bands detected at MW greater than 170 kDa. Same bands resulted positive to anti-Sumo 2/3 antibody, suggesting that an intracellular low level of polyamines favours Snai1 nuclear accumulation under the form of polysumoylated proteins.

Keywords MDCK · Polyamines · EMT · MET · Snai1 · Sumo

Abbreviations

α-SMA	α-Smooth muscle actin
CDH1	E-cadherin
DFMO	Difluoromethylornithine
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HS	Horse serum
MDCK cells	Madin–Darby Canine Kidney cells
MET	Mesenchymal-to-epithelial transition
MW	Molecular weight
ODC	Ornithine decarboxylase
PAK-1	p21 activated kinase 1
PP-2A	Phosphoprotein phosphatases-2A
PUT	Putrescine
SF/HGF	Scattering factor/hepatocyte growth factor
SPD	Spermidine
TGF-β1	Transforming growth factor beta 1

Introduction

Epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) are complex biological processes responsible for conversion of epithelial cells into a

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mesenchymal phenotype or viceversa, respectively (Kalluri and Weinberg 2009). During embryo- and foetal-development, EMT and MET are critical in allowing the formation of the three-layered embryo through gastrulation as well as in the mesoderm formation, neural crest development, heart valve development, secondary palate formation, and male Mullerian duct regression, as well as in the formation of vertebrae, craniofacial structures, and neural derivatives, to name just a few. In the adult organism, these two processes are involved in wound healing as well as in the genesis and progression of organ fibrosis; moreover, it has been suggested that EMT and MET have a critical role in favouring increased invasiveness of epithelial cancer cells and formation of metastasis in experimental and human malignancies (Acloque et al. 2009).

EMT can be induced by several extracellular signals and the list includes: (1) growth factors such as TGF-β, WNT, FGF (fibroblast growth factor), EGF (epidermal growth factor) and SF/HGF (scattering factor/hepatocyte growth factor); (2) extracellular matrix (ECM) components, including collagen and hyaluronic acid. Within the cell, EMT is mediated through a number of transcription factors like Twist 1, Twist 2, Snai1, Snai2, ZEB 1, ZEB 2 and FOXC2 (Thiery and Sleeman 2006). Snai1 is a "zinc-finger protein" which targets several genes for proteins typically expressed by mesenchymal cells (fibronectin, vimentin and α-smooth muscle actin) as well as two E-boxes at E-cadherin (CDH1) promoter. E-cadherin is a membrane glycoprotein that has a crucial role in mediating cell-to-cell contacts in a Ca^{2+}-dependent manner and it has been suggested to play also an oncosuppressor role by negatively affecting invasive properties of cancer cells. Snai1, in particular, is able to recruit at least three co-repressor proteins like mSin-3A, histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2) in order to form a complex able to repress CDH1 gene expression (Peinado et al. 2004). Intracellular Snai1 levels are regulated not only through neosynthesis but also through three post-translational events: (a) *N*-acetyl-glycosylation of Ser^{112}, which leads to Snai1 stabilization and to the amplification of its repressor function on CDH1 gene (Park et al. 2010); (b) phosphorylation of Ser^{246} by p21 activated kinase 1 or Pak-1, leading to Snai1 nuclear accumulation and increased activity; (c) phosphorylation of Ser^{97} and Ser^{101} by GSK-3β that leads Snai1 to bind to β-Trcp and then to proteasomal degradation (Domínguez et al. 2003; Zhou et al. 2004). GSK-3β activity, in turn, is regulated through phosphorylation and de-phosphorylation steps by different kinases (ILK, FAK, AKT e PI3K) and phosphoprotein phosphatases (PP-2A) (Rayasam et al. 2009). Along these lines, kinase and phosphatase activities are known to be strictly dependent on the actual concentration of inorganic as well as organic ions. These include also polyamines which are ubiquitary polycations whose intracellular levels are carefully controlled by the activity of enzymes involved in their metabolism and by the activity of specific transporters able to either uptake them from or to extrude them into the extracellular environment (Bachrach 2004). As a matter of fact, polyamines are known to be able to regulate several cellular processes including differentiation (Pignatti et al. 2004). The aim of the present study has been to investigate whether the modulation of intracellular polyamine levels might affect EMT and MET in MDCK cells as well as to analyse molecular mechanisms involved. Modulation of polyamine levels was obtained by either inhibiting ornithine decarboxylase employing di-fluoromethyl-ornithine (DFMO), resulting in a decreased intracellular content of polyamines, or by combining DFMO treatment with addition of exogenous putrescine and spermidine to replete polyamine intracellular levels.

Materials and methods

Materials

DMEM, RPMI 1640, monoclonal antibodies anti-β-Tubulin, anti-α-Smooth muscle actin and all other chemicals were from Sigma Chemical Co (St Louis, MO, USA). Peroxidase-conjugated secondary antibodies, ECL Plus Western Blotting detection reagents and PVDF membranes were from Amersham (Little Chalfont, Buckinghamshire, UK). Horse serum and foetal bovine serum from SPA (Milan, Italy). Monoclonal antibodies anti-Fibronectin, anti-β-Tubulin and polyclonal antibodies anti-SUMO 2/3, anti-Snai1, anti-E-cadherin and anti-Lamin A were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

MDCK cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum (FBS). Polyamine-related experiments were instead performed in RPMI 1640 supplemented with 10% horse serum (HS). Addition of HS to all experimental conditions instead of FBS is an established procedure aimed at minimizing cytotoxic effects due to high levels of amine-oxidases in FBS, which, on the contrary, is very low in HS. Cells were plated and pretreated, when scheduled, alternatively with 3 mM DFMO for 24 h, leading to the depletion of endocellular polyamines, or DFMO plus a mix of polyamines (10 μM putrescine and 10 μM spermidine) to validate the specific effect of DFMO. Medium was changed after 24 h, and cells were exposed to the following experimental conditions: control (RPMI + 10% HS), DFMO (RPMI + 10% HS + 3 mM DFMO), TGFβ1 (RPMI + 10% HS + 10 ng/ml

TGFβ1), DFMO + TGFβ1 (3 mM and 10 ng/ml, respectively, in RPMI + 10% HS) or DFMO + TGFβ1 + polyamine (3 mM, 10 ng/ml and polyamine mix, respectively in RPMI + 10% HS). Cells were allowed to grow up to 96 h. In some experiments, cells were seeded on rat tendon collagen gel prepared according to Strom and Michalopoulos (1982).

Western blot analysis

MDCK cells were washed with PBS and collected by boiling in Laemmli sample buffer. After sonication and centrifugation aliquots of the supernatant (10–20 μg protein) were resolved electrophoretically on SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to PVDF membranes by the standard procedure. After protein transfer, blots were incubated in blocking solution and then with primary antibody. Immunoreactive proteins were detected using an enhanced chemiluminescence system.

Nuclear extracts

Nuclear extracts were obtained by a slight modification of the procedure described by Lee et al. (1988). Briefly, cells were harvested in hypotonic buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF, pH 7.9), added with Triton X-100 (0.1% final concentration) and incubated for 15 min on ice. Cells were then disrupted by repeated passages into a syringe with a narrow-gauge (number 27) needle and cells lysis was checked by observation under phase-contrast microscope. Nuclear pellets, obtained by centrifugation at 11,000×g for 20 min, were rinsed once in hypotonic buffer, resuspended in hypertonic buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 420 mM NaCl, 0.5 mM DTT, 1 mM PSMF, 0.2 mM EDTA, pH 7.9), gently shaked for 30 min at 4°C and then centrifuged at 21,000×g for 7 min. The final supernatant was processed for Western blot analysis as described. Alternatively, nuclear pellets were treated for 30 min on ice with a solution containing Triton X-100 (1%, v/v) and 7.5 mM putrescine and then solubilized in Laemmli sample buffer.

Results

As shown in Fig. 1, MDCK cells acquired a fibroblastoid phenotype following treatment with TGF-β1. These EMT-like morphological changes were emphasized in cells that were also exposed to DFMO (D) to decrease the intracellular levels of polyamines. Accordingly, addition of exogenous polyamines (putrescine plus spermidine, 10 μM each) almost completely abolished the combined action of DFMO and TGF-β1 (T) then substantiating a specific role for these polycations in modulating the transition from epithelial-to-mesenchymal phenotype. Morphological changes were associated with significant changes in the expression of specific markers of epithelial and mesenchymal lineage (Fig. 2). Exposure of cells to the combined treatment D + T resulted in decreased protein levels of the epithelial marker E-cadherin and increased levels of mesenchymal proteins like fibronectin and α-smooth muscle actin (α-SMA). The relevance of decreasing polyamine levels was underlined by the fact that the exposure to TGF-β1 alone (used here as positive pro-EMT signal) resulted only in very modest changes for the same parameters. Moreover, the concomitant addition to the system of exogenous polyamines (D + T + PA treatment) fully counteracted changes in E-cadherin and α-SMA levels and, partially, those related to fibronectin induced by D + T treatment. It is well known that myofibroblasts (i.e., a typical example of mesenchymal-like cell) have an intrinsic ability to produce and secrete matrix metalloproteases as well as to adhere to fibrillar collagen of ECM and then to contract by exerting traction on ECM fibres. In our experiments, MDCK cells cultured on a collagen gel, when exposed to putative pro-EMT conditions, i.e., D + T treatment, were found to be able to partially degrade collagen and to detach the whole gel from the Petri dish (Fig. 3). The concomitant addition of polyamines (D + T + PA) was able to significantly prevent the detachment of the collagen gel and its degradation induced by the D + T treatment, whereas, as expected, no change was observed for untreated MDCK cells. The putative modulatory role of polyamines in TGF-β_1-induced EMT has been further confirmed by experiments designed to investigate whether the addition of exogenous polyamines may result in the induction of the reverse process, i.e., MET. In these experiments, the role of polyamines was tested in MDCK cells exposed to the following protocol of culture conditions:

1. MDCK cells cultured for 5 days in the putative pro-EMT medium containing DFMO + TGF-β1 (used here as a positive control to obtain a mesenchymal phenotype);
2. MDCK cells cultured for 10 days in normal medium (negative control);
3. MDCK cells cultured for 5 days in a medium containing DFMO + TGFβ1 and subsequently cultured for additional 5 days in normal culture medium;
4. MDCK cells cultured for 5 days in a medium containing DFMO + TGFβ1 and subsequently cultured for additional 5 days in a medium containing putrescine (10 μM) and spermidine (10 μM).

Under these experimental conditions, Western blot analysis (Fig. 4) revealed that, as expected, EMT induction

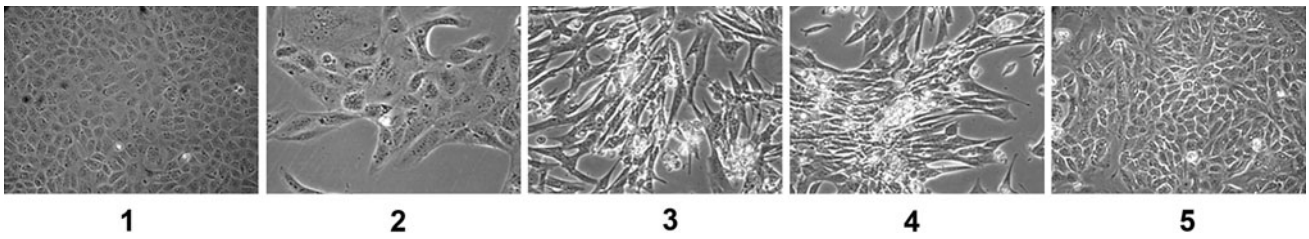

Fig. 1 Phase contrast of MDCK cells. Cultures were untreated (*1*) or treated with DFMO (*2*), TGF-β1 (*3*), DFMO + TGF-β1 (*4*) or DFMO + TGF-β1 + polyamine mix (*5*) as described in "Materials and methods". Originalmagnification × 200

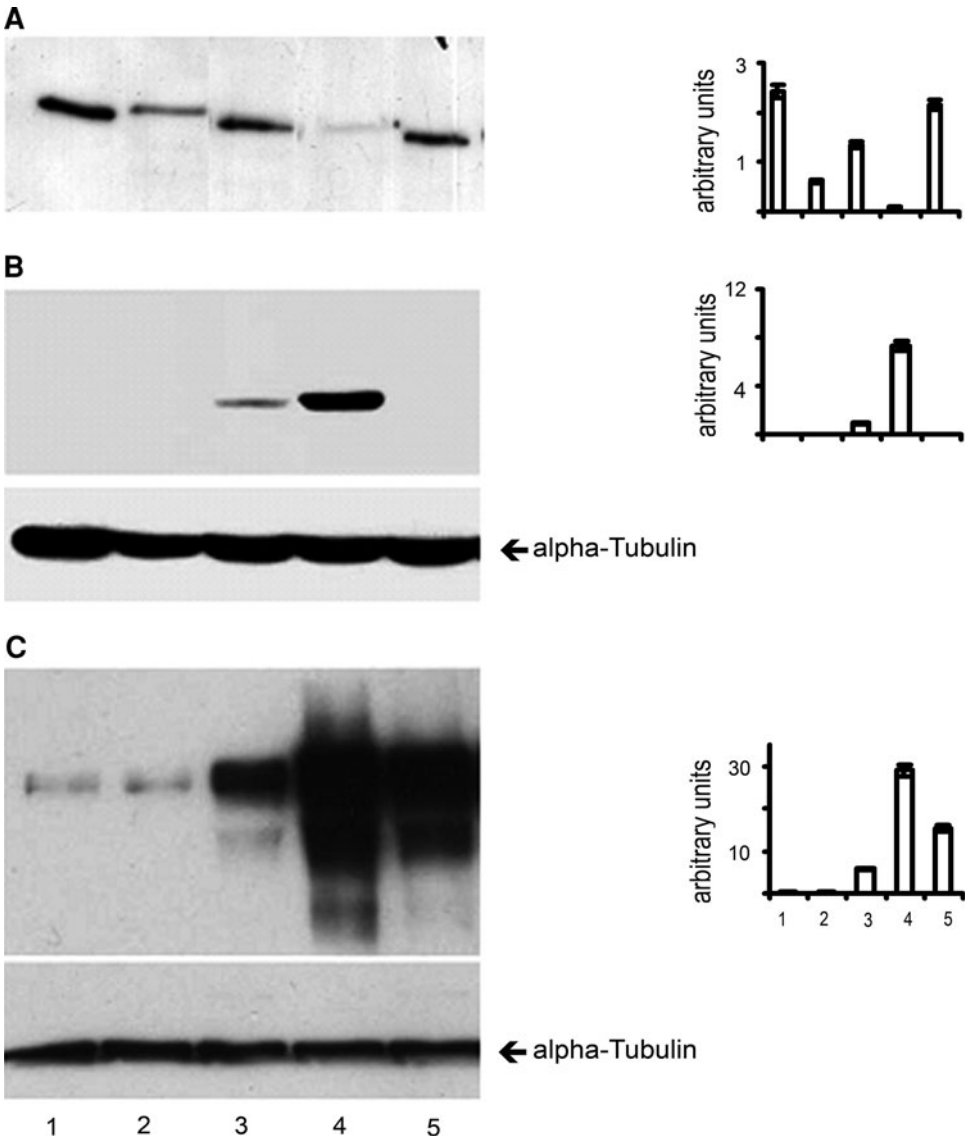

Fig. 2 Representative Western blots for E-cadherin, α-SMA or fibronectin of MDCK cells. Cultures were untreated (*lane 1*) or treated with DFMO (*lane 2*), TGF-β1 (*lane 3*), DFMO + TGF-β1 (*lane 4*) or DFMO + TGF-β1 + polyamine mix (*lane 5*) as described in "Materials and methods". The expression levels of E-cadherin (**a**), α-SMA (**b**) and fibronectin (**c**) were detected by Western blotting. Western blot scanning densitometry for five independent experiments was reported on the right. Blots were probed for α-Tubulin to ensure equal protein loading

by D + T resulted in increased protein levels of both fibronectin and α-SMA (lane 1) versus negative controls (lane 2). Up-regulation of fibronectin and α-SMA was unaffected by culturing MDCK cells for additional 5 days in a normal medium (i.e., devoid of stimuli) (lane 3), whereas the addition of a medium containing exogenous polyamines in this second experimental phase (i.e., following induction of EMT) resulted in a dramatic disappearance of both fibronectin and α-SMA, an event suggesting the occurrence of MET-like changes (line 4). As recalled in the introductory remarks, Snai1 is a transcription factor known to play a critical role in controlling the EMT process, with intracellular levels being also regulated through post-translational modifications involving

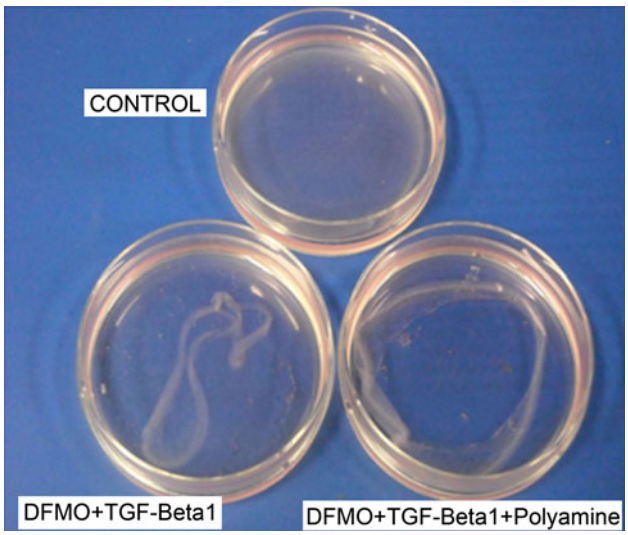

Fig. 3 DFMO plus TGF-β1 treatment induces myofibroblast-properties in epithelial MDCK cells. MDCK cells were seeded on rat tendon collagen gel and untreated or treated for 96 h with DFMO + TGF-β1 or with DFMO + TGF-β1 + polyamine mix as described in "Materials and methods"

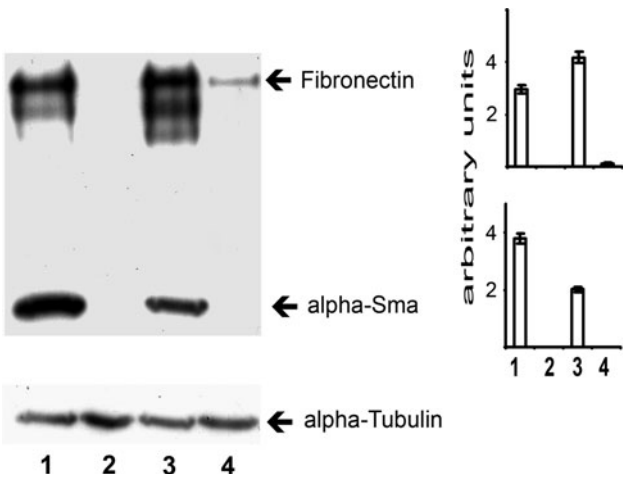

Fig. 4 Exogenous polyamines revert MDCK cells previously transformed into mesenchymal phenotype to epithelial phenotype. Representative Western blot for fibronectin and α-SMA. MDCK cells were cultured for 5 days in medium containing DFMO + TGF-β1 (*lane 1*; positive control of mesenchymal phenotype); for 10 days in normal medium (*lane 2*; negative control); for 5 days in a medium containing DFMO + TGFβ1 and subsequently cultured for additional 5 days in normal culture medium (*lane 3*); for 5 days in a medium containing DFMO + TGFβ1 and subsequently cultured for additional 5 days in a medium containing putrescine (10 μM) and spermidine (10 μM) (*lane 4*). Western blot scanning densitometry for three independent experiments was reported on the right**.** Blots were probed for α-Tubulin to ensure equal protein loading

phosphorylation/dephosphorylation. Along these lines, it has been previously reported that polyamine levels may affect the enzymatic activity of either protein kinases and phosphoprotein phosphatases. In order to verify the

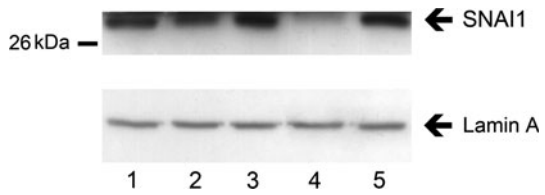

Fig. 5 Representative Western blot for Snai1 in nuclear hypertonic solution extracts of MDCK cells. Cultures were untreated (*lane 1*) or treated with DFMO (*lane 2*), TGF-β1 (*lane 3*), DFMO + TGF-β1 (*lane 4*) or DFMO + TGF-β1 + polyamine mix (*lane 5*) as described in "Materials and methods". Protein extracts obtained resuspending nuclear pellets in hypertonic buffer were analyzed. Blots were probed for Lamin A to ensure equal protein loading

hypothesis that a decreased intracellular level of polyamines may exacerbate EMT induced by TGF-β1 through Snai1 stabilization, we evaluated Snai1 levels in nuclear extracts that have been specifically obtained (see "Materials and methods" for more details) by solubilizing the nuclear matrix with a hypertonic salt solution. Unexpectedly, immunodetectable Snai1 nuclear levels under these conditions were significantly lower in extracts obtained from cells treated for 96 h with D + T (lane 3, Fig. 5) versus cells treated with TGF-β1 alone (lane 2). However, literature data suggest that transcriptional factors are often assembled in the nucleus into high molecular weight- and DNA-bound complexes. Previous studies (Peinado et al. 2004; Hou et al. 2008) showed that Snai1 is often associated with other transcriptional factors that contribute to the modulation of gene expression. On these basis, our hypothesis was that a decrease of polyamine intracellular levels may result in an effective challenge to the solubilization of these macromolecular complexes. We then decided to treat nuclear suspension with a solution containing Triton X-100 (1%, v/v) and 7.5 mM putrescine. By applying this procedure, we found that extracts from cells treated with D + T, as compared with extracts from control cells (Fig. 6a, lane 1) or cells exposed to DFMO (lane 2) or to TGF-β1 (lane 3) or to D + T + PA (lane 5), revealed upregulation of Snai1 immunopositive bands in a range of molecular weight between 55 and 72 kDa, with additional positive bands being detected at MW higher than 170 kDa (lane 4). Moreover, extracts obtained from cells treated with D + T were also showing an increase of the signal at the predicted MW (about 26.5 kDa) of Snai1. These results strongly suggested that under the experimental conditions employed in the present study, Snai1 may undergo additional post-translational modification(s) other than phosphorylation and dephosphorylation. Taking into consideration the fact that Snai1 operates within the nucleus, we decided to analyze whether Snai1 may covalently bind to a SUMO isoform; then, after removal of Snai1 primary antibodies and of secondary peroxidase-linked antibodies, we exposed the same Western blot membrane to antibodies raised against

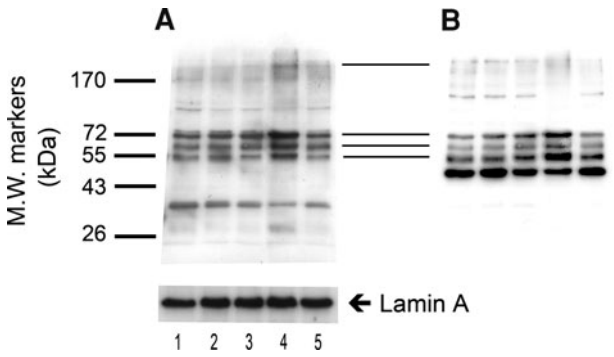

Fig. 6 Representative Western blot for Snai1 in nuclear Triton/Putrescine extracts of MDCK cells and overlapping of Sumo 2/3 signal. Cultures were untreated (*lane 1*) or treated with DFMO (*lane 2*), TGF-β1 (*lane 3*), DFMO + TGF-β1 (*lane 4*) or DFMO + TGF-β1 + polyamine mix (*lane 5*) as described in "Materials and methods". Protein extracts obtained resuspending nuclear pellets in Triton/Putrescine solution were analyzed with antibody anti-Snai1 (**a**). After removal of Snai1 primary antibodies and of secondary peroxidase-linked antibodies, the same membrane was exposed to antibodies against Sumo 2/3 (**b**). Blots were probed for Lamin A to ensure equal protein loading

Sumo 2/3. As one can appreciate from Fig. 6b, we observed an overlapping of positive immunostaining for both proteins (SUMO 2/3 and Snai1).

Discussion

Epithelial-to-mesenchymal transition is a fundamental process, paradigmatic of the concept of cell plasticity that has been originally described in embryonic development where cell migration and tissue remodelling have a primary role in regulating morphogenesis in multicellular organisms. According to the current literature, the original definition of EMT is focused on the formation of mesenchymal cells from epithelial and involves the loss of epithelial cell polarization as a result of disappearance of specialized junctional structures, cytoskeleton reorganization, and organelle redistribution and then the gradual acquisition of typical EMT-related mesenchymal features and behaviour. The peculiarity of the EMT process relies on the fact that the mesenchymal cells can eventually undergo a reverse transition process, known as MET, leading them to regain a fully differentiated epithelial phenotype. In the previous decade, the EMT process (and then possibly MET) was identified in at least two other well-defined pathophysiological conditions including organ fibrosis and cancer progression and metastasis (Cannito et al. 2010).

The main message conveyed by the present study is that a significant decrease of intracellular polyamine content, as obtained by treating cells with the ODC inhibitor DFMO, is a specific signal able to strongly exacerbate EMT induction by TGF-β1 in MDCK cells, which are a widely accepted non-tumoral cell model used to investigate EMT process and related molecular mechanisms. This concept is unequivocally supported by a number of critical EMT-related findings obtained in MDCK cells treated with both DFMO and TGF-β1 (D + T): (a) morphological changes towards a full mesenchymal and myofibroblast-like phenotype; (b) very significant E-cadherin down-regulation; (c) dramatic over-expression of both fibronectin and α-SMA; (d) all these EMT-related features were almost completely counteracted and/or abolished when exogenous putrescine and spermidine were added together with DFMO and TGF-β1; (e) moreover, addition of exogenous polyamines to MDCK cells that underwent EMT was able to revert the mesenchymal phenotype to the epithelial one.

As for the polyamine mechanism of action, it is known that their availability may affect transcription of c-myc, matrix metalloproteinase (MMP1, MMP2), TGF-β1, and collagen type I mRNA (Stabellini et al. 2005). Moreover, we recently showed in a previous study that the decrease of polyamines may rely, at least in part, on endoplasmic reticulum (ER) stress, as indicated by the fact that polyamine-depleted cells showed a twofold increased expression of the ER-stress proteins GRP78, GRP94 and HSP90 α/β (Prunotto et al. 2010). Results obtained in the present study suggest that the polyamine-related role in EMT modulation relies more likely on polyamine depletion-dependent stabilization of Snai1, a major EMT-related transcription factor. In particular, we provide evidence that a low level of polyamines favours Snai1 nuclear accumulation under the form of polysumoylated proteins. Protein sumoylation consists in the formation of covalent binding on lysine residues of proteins belonging to the SUMO ("small ubiquitin-like modifier") family, with four characterized SUMO isoforms in mammalians. Sumoylation can be envisaged as a post-translational modification which affects several proteins involved in critical cellular processes including regulation of gene expression, nuclear import and signal transduction (Johnson 2004). Along these lines, a major function of sumoylation consists in the regulation of the activity of numerous transcription factors. In some instances sumoylation represses the activity, as in the case of p53 (Wu and Chiang 2009) and p66 (Gong et al. 2006), whereas sumoylation becomes necessary for transcription factors to operate, as it has been described for "zinc fingers" transcription factors like ZNF451 (Karvonen et al. 2008) and ZXDC (Jambunathan and Fontes 2007). Protein sumoylation is a potentially reversible process, and de-sumoylation is catalyzed by specific proteases defined as sentrin proteases or SENPs. Six different isoforms of SENPs have been described that differ for their nuclear localization and substrate specificity (i.e., the different SUMO isoforms). At present, knowledge on the regulation of SENPs activity is very limited (Kim and Baek

2009), with no mediator (whether positive or negative) characterized so far, and then we can only speculate that polyamines may represent putative regulators of these proteases.

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