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Vitamin D inhibits the epithelial-mesenchymal transition by a negative feedback regulation of TGF- β activity

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Running title: TGF- β induces vitamin D receptor in epithelial-mesenchymal EMT transition.

Abbreviations: EMT, epithelial-mesenchymal EMT transition; ROS, reactive oxygen species;

COX2, Cytochrome C oxidase subunit 2.

Abstract

Vitamin D and TGF- β exert opposite effects on epithelial-mesenchymal EMT transition. Here we report a novel mechanism of action of TGF- β that promotes the counteracting activity of vitamin D; in two models of human epithelial-mesenchymal EMT transition we demonstrated for the first time that TGF- β strongly induced the expression of vitamin D receptor (VDR) and that 1,25(OH) $_2$ D $_3$ was able to contrast the TGF- β -driven EMT transition by transcriptional modulation. In human bronchial epithelial cells the effects of TGF- β on EMT transition markers (E-Cadherin expression and cell motility) were reversed by pre-treatment and co-treatment with 1,25(OH) $_2$ D $_3$, but not when the hormone was given later. Silencing experiments demonstrated that the inhibition of TGF- β activity was VDR-dependent. 1,25(OH) $_2$ D $_3$ abrogated the mitochondrial stimulation triggered by TGF- β . In fact we showed that 1,25(OH) $_2$ D $_3$ repressed the transcriptional induction of respiratory complex, limited the enhanced mitochondrial membrane potential and restrained the increased levels of mitochondrial ATP; 1,25(OH) $_2$ D $_3$ also decreased the production of reactive oxygen species promoted by TGF- β . Overall, our study suggests that the overexpression and activity of VDR may be a regulatory response to TGF- β signaling that could be exploited in clinical protocols, unraveling the therapeutic potentiality of 1,25(OH) $_2$ D $_3$ in the prevention of cancer metastasis.

Keywords: VDR; 1,25(OH) $_2$ D $_3$; TGF- β ; epithelial-mesenchymal EMT transition; mitochondrial respiratory activity

1. Introduction

The epithelial-mesenchymal EMT transition (EMT) is a complex process in which the epithelial cells undergo multiple biochemical changes and assume a mesenchymal phenotype, which includes the loss of cell adhesion and polarity, the enhanced migratory capacity and invasiveness, and the elevated resistance to apoptosis. In epithelial tumor progression, EMT is subsequent to the localized cancer proliferation and it is considered one of the hallmarks of malignancy [1,2]. Finding strategies able to contrast EMT and invasion should have high priority in the development of anti-metastatic protocols. TGF- β is one of the inflammatory cytokines that most potently trigger EMT. Its signaling is complex and mediated by the activation of several transcription factors such as Snail and the Smad family of proteins. The effects of TGF- β are multiple and vary from the regulation of proliferation to the increase of migration and even the control of apoptosis [3]. On the opposite, vitamin D is a negative modulator of EMT. The active form of vitamin D (1,25(OH) $_2$ D $_3$) inhibits proliferation, promotes differentiation and opposes EMT via the transcriptional activity of its receptor VDR, which controls the induction of several genes such as the invasion suppressor E-Cadherin and the tumor suppressor cystatin D, and acts by antagonizing the Wnt/ β -catenin pathway [4,5]. Several studies in vitro demonstrated the efficacy of 1,25(OH) $_2$ D $_3$ in contrasting the EMT triggered by TGF- β , due to the inhibited expression of EMT markers and the decreased migration and invasion of epithelial and ovarian cancer cells [6-10]. The clinical studies that have evaluated the efficacy of vitamin D supplementation have described a role merely preventive in cancer formation and progression, whereas there are no evidences for curative properties on malignant tumors [11-13].

The mechanisms responsible for the opposing activity of TGF- β and 1,25(OH) $_2$ D $_3$ are not completely clear and mostly limited to the investigation of transcriptional antagonism or cross-talk, such as the cross-talk demonstrated for VDR and Smad3 signaling. This effector of the TGF- β -Smad pathway is able to transactivate VDR [14] and it is consequently inhibited in its transcriptional activity [15]. In addition to the nuclear effects of 1,25(OH) $_2$ D $_3$, recently some new

metabolic effects of the hormone have been described; in fact a novel mitochondrial localization and function of VDR has been elucidated by works on platelets [16], keratinocytes [17-19], cancer cell lines [18] and brown adipocytes [20], which have demonstrated that 1,25(OH)₂D₃ reduces mitochondrial respiration. Also few metabolic effects of TGF-β have been described. TGF-β alters cellular lipid metabolism [21], promotes metabolic reprogramming [22,23] and affects mitochondrial function, although with contrasting evidences that seem to depend on the cellular context analysed [22-25]. The effects of TGF-β on cellular functions are often mediated by the increased production of reactive oxygen species (ROS) triggered by the cytokine [21,24-27]. The outcome of ROS modulation is variable, since TGF-β can lead to apoptotic death [28,29] or can promote cell survival via the recruitment of the antiapoptotic PTEN-induced putative kinase 1 (PINK1) protein, which supports the autophagic clearance of damaged mitochondria [24].

Interestingly, few reports have demonstrated that TGF-β can stimulate mitochondrial respiratory activity and oxidative phosphorylation [23,25]; the opposite effects of 1,25(OH)₂D₃ and TGF-β on mitochondrial activity prompted us to investigate whether the inhibitory effects of 1,25(OH)₂D₃ on TGF-β-driven EMT are partly mediated by a metabolic antagonism. In this work we evaluated the metabolic effects of TGF-β in the human bronchial epithelial cell line Beas-2B, which is a well characterized model of epithelium undergoing EMT as result of TGF-β activity [30,31] and is sensitive to EMT inhibition by 1,25(OH)₂D₃ [6]. The cross talk between the metabolic effects of VDR and TGF-β was evaluated by treating the cells with 1,25(OH)₂D₃ before (pre-treatment), together (co-treatment) or after (post-treatment) the exposure to TGF-β, in order to verify whether a timely administration of 1,25(OH)₂D₃ can make any difference on phenotype and metabolism of cells undergoing EMT.

In this study we demonstrate for the first time that TGF-β strongly induces the expression of VDR in two human models of EMT and we show that the vitamin D/VDR activity counteracts the effects of TGF-β on EMT phenotype, mitochondrial metabolism and production of ROS.

2. Materials and methods

2.1 Cell culture and treatments

The human bronchial epithelial cells Beas-2B and the human pleural mesothelial cell line MeT-5A were purchased from American Type Culture Collection (ATCC), USA, and were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% antibiotics [penicillin-streptomycin (Sigma-Aldrich)] at 37 °C in humidified 5% CO₂ atmosphere. When treated, cells were kept in RPMI supplemented with 1% fetal bovine serum and were subjected to stimulation with either 0.1% ethanol (vehicle) as control or 100 nM 1,25(OH)₂D₃ (Sigma-Aldrich, St. Louis, MO) or TGF-β1 (PeproTech, Rocky Hill, NJ). The treatments were carried out as shown by diagram in Fig. S1: 24 hours of pre-treatment in the indicated conditions, followed by 48 hours of single treatments or co-treatments.

2.2 Extracts preparation and Western blotting analysis

Subcellular fractionation and Western blotting analysis was carried out as previously described [16]. Lysates were subjected to differential centrifugation to isolate the nuclear and mitochondrial fraction. Proteins were extracted by incubation in boiling sample buffer followed by sonication. Fifty µg of total lysates or thirty µg of nuclear or mitochondrial fractions were separated by 10% SDS-PAGE and analysed by Western blotting. Mouse monoclonal antibodies anti-VDR (sc-13133), anti E-Cadherin (sc-21791) and goat antibody anti-UCP2 (sc-6525) were from Santa Cruz, CA, USA. Rabbit anti-UCP1 was obtained from Sigma (U6382). Our previous works [17,18] have shown that the antibody against VDR gives some unspecific signal. The correct band was identified in past studies by molecular weight and silencing experiments in HaCaT, MCF7 and HeLa cells [17,18], and corresponds to the lower band when a doublet band is present. The signal that detects VDR is indicated in all figures. The loading controls were carried out on the same membranes and

detected by antibodies anti-VDAC (monoclonal anti-porin 31HL, Calbiochem), anti-actin (mouse monoclonal sc-8432 Santa Cruz) and rabbit antibody anti-PARP (sc-7150, Santa Cruz).

2.3 Lentiviral-mediated shRNA targeting

Silencing of VDR was carried out as previously described [18]. Briefly, PLKO.1 lentiviral shRNA clone 3 targeting the human VDR and a scrambled non-targeting control were purchased from Sigma (Sigma Mission shRNA). Lentiviral transduction particles were delivered by overnight incubation and infected Beas-2B cells were selected by puromycin. Four days after infection, the cells were seeded for experimental assays and harvested for protein analyses.

2.4 Cell proliferation assay, Migration assay, Wound healing assay, and Real-Time PCR analysis.

The protocols used in this study are described in Supplementary Materials and Methods.

2.5 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), a mitochondrial dye staining mitochondria in living cells in a membrane potential-dependent fashion, was used to determine $\Delta\Psi_m$, as previously reported [18]. JC-1 is a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green (530 nm) to red (590 nm). Briefly, cells were incubated with JC-1 (2 $\mu\text{g/ml}$ final concentration) at 37°C for 30 minutes. The amount of JC-1 retained by 10,000 cells per sample was measured at 530 nm (FL-1 green fluorescence) and 590 nm (FL-2 red fluorescence) with a flow cytometer and analyzed with Cell Quest Alias software. The ratio FL2/FL1 was evaluated to determine $\Delta\Psi_m$. Experiments were performed in triplicates and repeated three times.

2.6 Evaluation of mitochondrial ATP levels

After treatments, the amount of ATP in mitochondria, prepared by subcellular fractionation, was measured with the ATP Bioluminescent Assay Kit (FL-AA, Sigma), using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA). ATP was quantified as

relative light units (RLU); data were converted into nmol ATP/mg mitochondrial proteins and were expressed in comparison to control values (relative ATP). Experiments were performed in triplicates and repeated three times.

2.7 Measurement of intracellular ROS production

After treatment, cells were harvested and were loaded for 15 min with 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). DCFH-DA is a cell-permeable probe that is cleaved intracellularly by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound dichlorofluorescein (DCF) [32]. DCF fluorescence was determined at an excitation wavelength of 504 nm and an emission wavelength of 529 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The fluorescence values were normalized to the protein content and expressed as values relative to control. Experiments were performed in triplicates and repeated three times.

2.8 Bands quantification and statistical analysis

Bands from protein electrophoresis were quantified by scanning digital densitometry using an ImageJ software analysis (ImageJ version 1.29, Sun Microsystems Inc., Palo Alto, CA). Statistical analysis of data was performed using ANOVA test with Tukey's post-hoc correction. p values <0.05 were considered significant and indicated. All data were expressed as mean \pm S.D of three independent experiments.

3. Results

3.1 TGF- β and 1,25(OH) $_2$ D $_3$ exert opposite effects on proliferation and differentiation of human epithelial cells.

With the aim of dissecting the metabolic effects of both 1,25(OH) $_2$ D $_3$ and TGF- β on EMT, first of all we tested the responsiveness of our cellular model, the Beas-2B cell line, to the two agents. This

cell line is the epithelial model most utilized in studies of EMT [6,33,34]; the modulation of the markers of EMT transition by TGF- β and 1,25(OH) $_2$ D $_3$ has been extensively characterized in previous work [6]. The concentration of the two drugs is the standard dose employed in vitro when their effect is investigated in EMT [6,33,35]. As expected, the treatment with 1,25(OH) $_2$ D $_3$ reduced the proliferation rate (Fig. 1A) and increased the expression of the epithelial marker E-Cadherin (Fig. 1B, quantified in Fig. 1C). TGF- β apparently had no significant effect on proliferation, and in co-treatment the inhibitory effect of vitamin D prevailed. The EMT transition toward a mesenchymal phenotype in Beas-2B incubated with TGF- β was demonstrated by the suppressed expression of E-Cadherin (Fig. 1B, quantified in Fig. 1C). Surprisingly, we found that TGF- β promoted a remarkable induction of VDR, since VDR levels were strikingly higher in whole lysates of TGF- β treated cells compared to the modest expression detected in control cells; the increase triggered by TGF- β was much more intense than the effect exerted by vitamin D on its own receptor (Fig. 1B and 1C). VDR can be up-regulated at both the transcriptional and post-translational levels, the latter through a ligand-dependent stabilization [38-40]. We measured the VDR transcript in untreated and treated cells (Fig. 1D) by real time PCR analysis and we found a two-fold induction of VDR messenger by its ligand, and a much stronger transcriptional induction exerted by TGF- β on VDR, which accounted for the observed sharp increase in protein expression. The induction of VDR was evident also in another human lung cell line, the mesothelial cells MeT-5A. Also in this model TGF- β was able to modulate the epithelial marker E-Cadherin and was effective in upregulating VDR expression (Fig. 1E), demonstrating that this effect was not cell-specific. We decided to inquire into this novel consequence of TGF- β activity and we set forth to investigate the influence of 1,25(OH) $_2$ D $_3$, TGF- β and their possible synergic action on VDR expression in Beas-2B cells, the best characterized model of EMT.

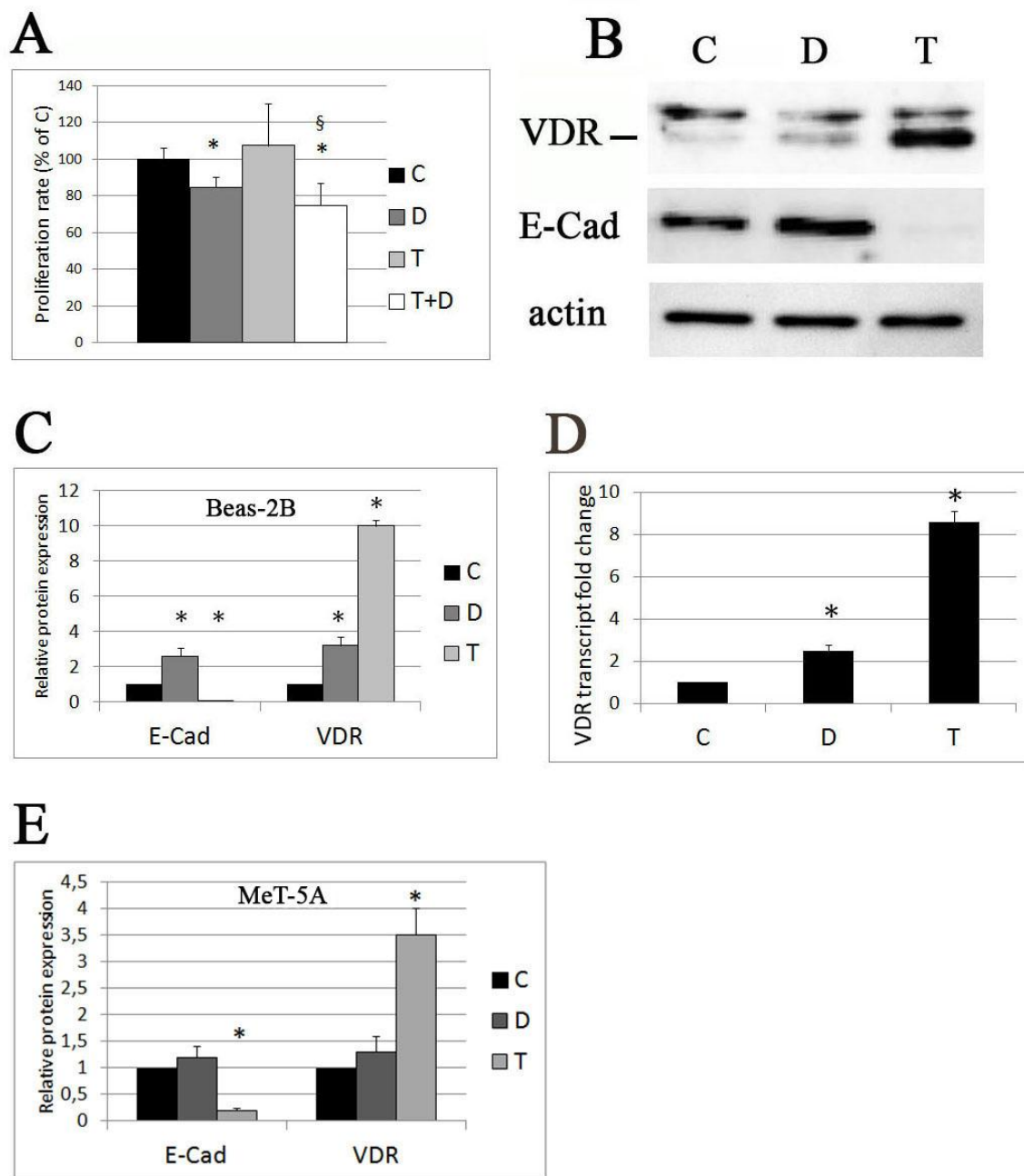


Fig. 1. The effects of TGF- β and 1,25(OH)₂D₃ on proliferation and differentiation of human epithelial cells. (A) After five days of treatment with 1,25(OH)₂D₃ (D), TGF- β (T) or both (T+D), Beas-2B cell growth was evaluated by crystal violet staining and values expressed as percentage of the untreated cells (control, C). (B) After 48 hours of incubation the expression of E-Cadherin, VDR, and the loading control actin was analysed by Western blotting in whole lysates from Beas-2B cells. VDR protein is indicated as the lowest band of the double band, as explained in Methods. (C) Bands from three independent experiments were quantified, normalized for loading as a ratio to

actin expression and data plotted on graph relative to control. (D) At the end of the same incubation mRNAs from Beas-2B cells were purified and assayed by real time PCR for VDR transcript expression. The values plotted on the graph represent the fold change in transcript expression in treated versus untreated cells. (E) The same protein analysis was carried out on MeT-5A cells treated as in (B). In all graphs data are displayed as the means \pm SD of three independent experiments. * $P < 0.05$ compared to the untreated cells. § $P < 0.05$ compared to the cells treated with TGF- β (T).

3.2 The expression of both nuclear and mitochondrial VDR is induced by TGF- β and this modulation is not affected by the addition of 1,25(OH) $_2$ D $_3$.

Our previous studies described the intracellular distribution of VDR [16-18] and demonstrated that the receptor is abundant in the mitochondrial compartment of several cell lines [16-18]. In this work by Western blot analysis we investigated the induction of VDR in total extracts, mitochondrial and nuclear fractions of cells treated with the two molecules alone or in combination. The increased expression of VDR was evident in all fractions when the cells were treated with TGF- β alone, and the co-treatment did not change the effect (Fig. 2A). Also when the co-treatment was preceded by the incubation with 1,25(OH) $_2$ D $_3$ or TGF- β the levels of VDR remained very high (Fig. 2B).

Representative blots of these experiments are shown in Fig. 2C. Based on these observations we concluded that TGF- β was able to enhance the expression of the receptor in all the subcellular compartments, independently from its ligand, and the induction was particularly evident in mitochondrial VDR. With the aim of investigating whether the stimulation was exerted both on VDR expression and VDR activity on gene targets, we tested the mRNA levels of CYP24A1 as a read-out of the transcriptional activity of the induced VDR. As expected, the elevated amount of VDR produced by TGF- β signaling potently induced the transcription of the CYP24A1 enzyme in a ligand-dependent modality; interestingly, the addition of 1,25(OH) $_2$ D $_3$ before or after TGF- β

treatment made no difference on the high VDR transcriptional activity (Fig. 2D). We concluded that TGF- β induced the expression of a vitamin D receptor sensitive to ligand activation. The reported efficacy of 1,25(OH) $_2$ D $_3$ in reducing EMT triggered by the cytokine could be mediated by the increase of VDR expression and activity. Therefore we evaluated the effect of 1,25(OH) $_2$ D $_3$ and TGF- β alone or in combination on the EMT phenotype and on metabolism of cells undergoing EMT.

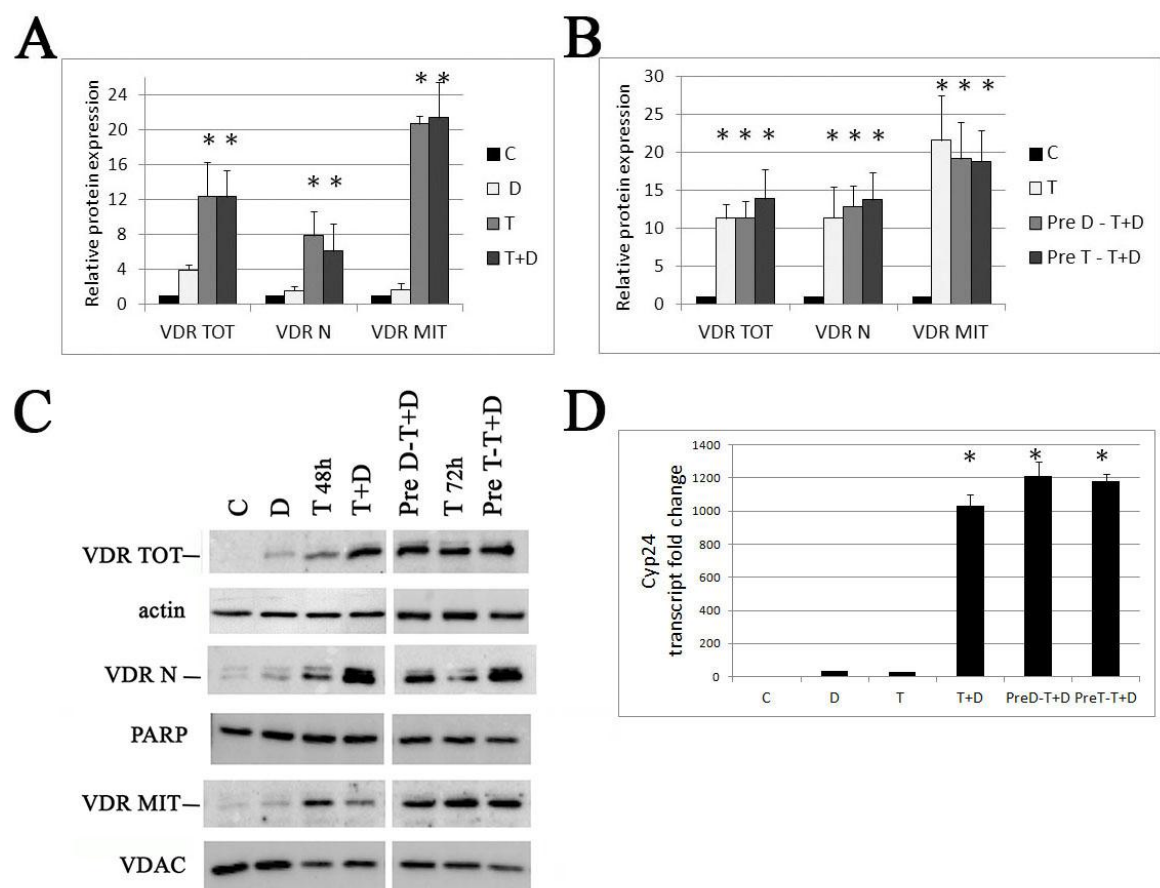


Fig. 2. Analysis of VDR expression in subcellular fractions and VDR activity in Beas-2B cells treated with different combinations of TGF- β and 1,25(OH) $_2$ D $_3$. (A) The cells were subjected for 48 hour to single (D, 1,25(OH) $_2$ D $_3$; T, TGF- β) or combined treatment (T+D). (B) In a second experimental setting the cells were pre-treated for 24 hours with 1,25(OH) $_2$ D $_3$ (preD-T+D) or TGF- β (preT-T+D) before 48 hours of co-treatment, and for 72 hours with TGF- β alone. VDR expression was evaluated by Western blotting on total lysates (VDR TOT), nuclear fractions (VDR N) and mitochondrial extracts (VDR MIT). Bands were quantified and normalized to loading

control bands on the same blot (actin, PARP, VDAC for total, nuclear and mitochondrial extracts respectively); data were expressed relative to control. (C) The blots are representative of the experiments quantified in (A) and (B). VDR protein is indicated as the lowest band of the double band, as explained in Methods. (D) Under the same experimental conditions the transcriptional activity of VDR was evaluated as modulation of CYP24A1 mRNA by real time PCR. The graphs display the means \pm SD of three independent experiments. * $P < 0.05$ compared to the untreated cells.

3.3 1,25(OH)₂D₃ is efficient in opposing EMT phenotype only when administered together with TGF- β , but not when its activity is exerted at a later stage.

We expected that the increased expression of VDR could mediate the activity of vitamin D and could inhibit the EMT transition, but we wondered whether vitamin D/VDR could hamper EMT not only in pre-treatment or co-treatment with TGF- β , but also when administered later on. This different protocol of incubation in vitro could give contrasting results and could mimic two different clinical situations: the case in which the oncologic patient shows adequate levels of vitamin D or the condition found in a subject where the insufficiency of vitamin D is corrected when EMT has already developed.

To settle this issue, we propose the same experimental paradigm throughout our analysis of phenotype and metabolic assessment of EMT. The experimental protocol of exposure is shown in supplementary Fig. S1. First, we tested two hallmarks of EMT: the expression of the epithelial marker E-Cadherin (Fig. 3A) and the migrating potential of the cells (Fig. 3B and 3C).

The expression of E-Cadherin was repressed by TGF- β but it was partially restored by 1,25(OH)₂D₃ in co-treatment and reverted to control levels when vitamin D was administered before and together TGF- β (preD-T+D). On the opposite, the treatment with 1,25(OH)₂D₃ was not effective when was initiated after TGF- β (preT-T+D) (Fig. 3A). Representative blots of these experiments are shown in

supplementary Fig. S2. Cellular movement typically increasing during EMT was investigated by migration tests (Fig. 3B) and wound healing assays (Fig. 3C). In both the analysis, cell motility was enhanced by TGF- β , and 1,25(OH) $_2$ D $_3$ reverted the effect when the treatment started before or together with TGF- β , but not later (preT-T+D). These data consistently demonstrated that 1,25(OH) $_2$ D $_3$ must be present before or at the beginning of TGF- β signaling to be effective in reverting the EMT phenotype. On the opposite, the pre-treatment with TGF- β elicits a cellular response that is not suppressible by 1,25(OH) $_2$ D $_3$.

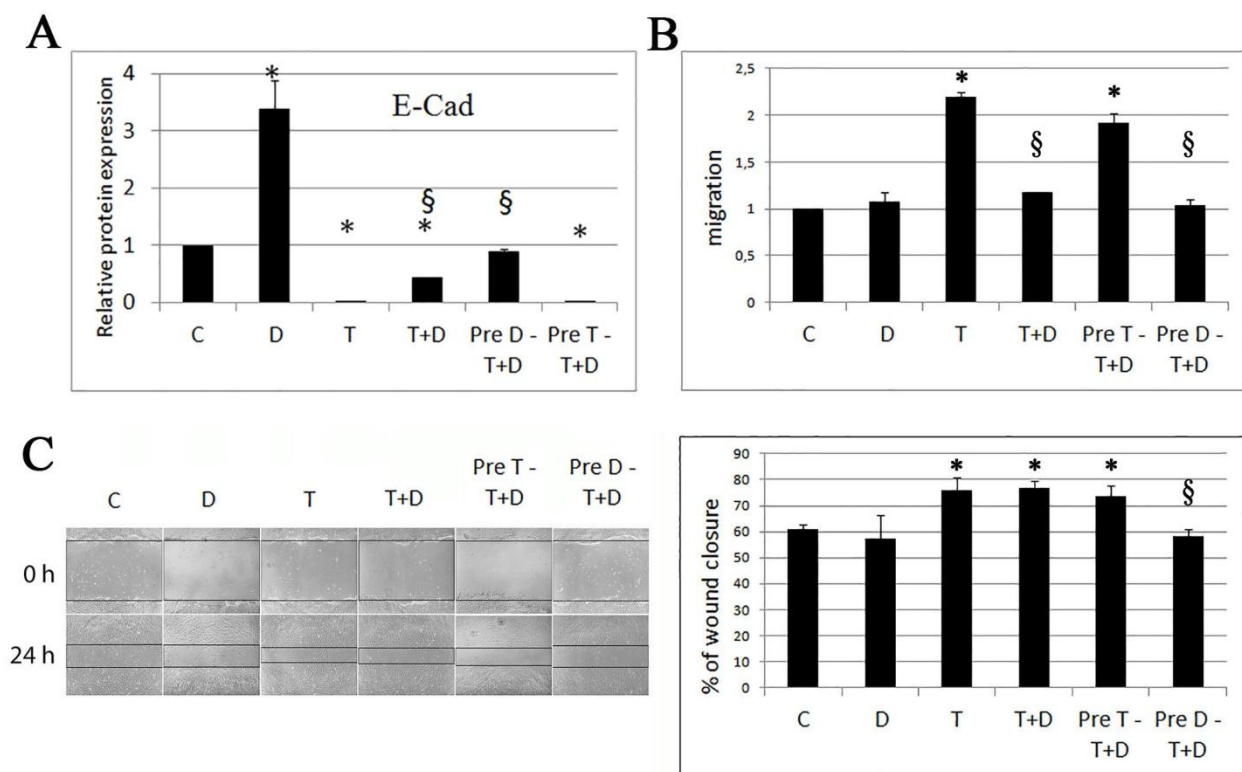


Fig. 3. The effects of 1,25(OH) $_2$ D $_3$ on EMT phenotype. Beas-2B cells were treated as schematized in fig. S1 and the markers of EMT were tested. (A) The expression of E-Cadherin was evaluated in whole lysates by Western blotting, bands were quantified, normalized for loading and data expressed relative to control. (B) The effects of 1,25(OH) $_2$ D $_3$ on the TGF- β -induced cell motility of Beas-2B cells at 24 hours were quantified using a transwell assay. Data are expressed relative to control. (C) The effects on cell migration at 0 and 24 hours were evaluated using a wound-closure assay. The figure presents the empty areas in the wound-closure assay under different experimental

conditions; these areas were measured and expressed on graph as percentage of wound closure as described in methods. The graphs display the means \pm SD of three independent experiments. * $P < 0.05$ compared to the untreated cells; § $P < 0.05$ compared to the cells treated with TGF- β alone.

3.4 The inhibition exerted by 1,25(OH) $_2$ D $_3$ on TGF- β activity is VDR-dependent.

In order to verify the most reasonable assumption of our work, that the increased expression of VDR could mediate the effects of vitamin D on EMT transition, we suppressed the receptor by genetic silencing and we tested the effects of TGF- β and 1,25(OH) $_2$ D $_3$ on wild type and silenced Beas-2B cells. The results of this approach are shown in Fig. 4. As previously published [18] our silencing tools were very effective in abating VDR expression; as expected, also the levels of E-Cadherin were very low in VDR KO cells and the protein expression did not increase after treatment with 1,25(OH) $_2$ D $_3$. When we investigated the EMT transition phenotype, we found that in silenced cells TGF- β retained the ability of repressing E-Cadherin and inducing cell motility, but the pre-treatment with 1,25(OH) $_2$ D $_3$ could no longer revert the effects of the cytokine. We therefore demonstrated that the influence of vitamin D activity on EMT and its antagonism with TGF- β described in our experiments were mediated by the increased expression of VDR.

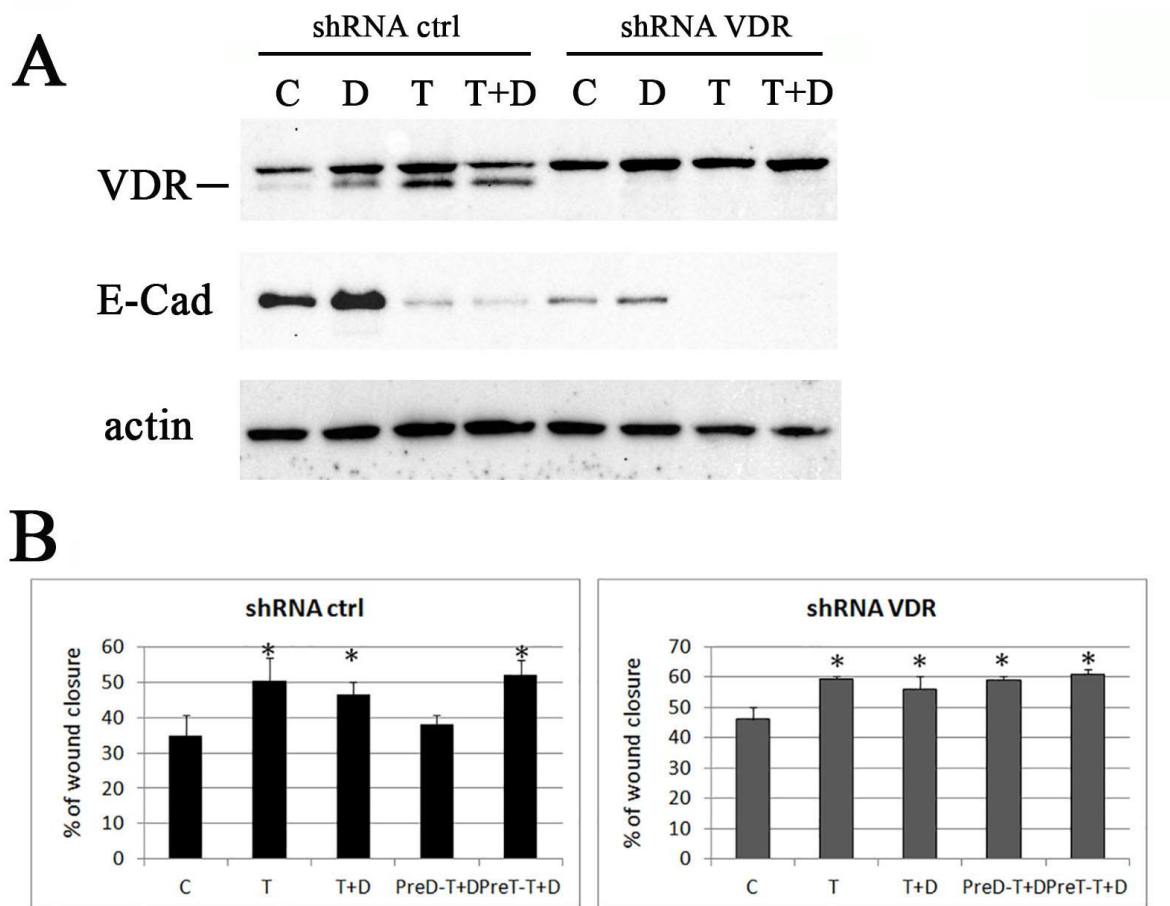


Figure 4. VDR silencing abolishes the effects of 1,25(OH)₂D₃ on EMT transition markers. (A) Beas-2B cells were infected with lentiviral VDR shRNA (shRNA VDR) or shRNA control (shRNA ctrl) and then subjected for 48 hour to single (D, 1,25(OH)₂D₃; T, TGF- β) or combined treatment (T+D); the silencing efficacy was examined in the total extracts by Western blotting. The identity of VDR protein indicated as the lowest band of the double band was confirmed by its disappearance in silenced cells. On the same blot the expression of the EMT transition marker E-Cadherin was investigated, and actin was detected as internal control for protein loading. (B) The effect of silencing on cell migration was evaluated by a wound-closure assay under the different experimental conditions. The data plotted on graph display the means \pm SD of three independent experiments. * P<0.05 compared to the untreated cells.

3.5 1,25(OH)₂D₃ and TGF-β modulate the mitochondrial respiratory chain with opposite effects.

After the analysis of the phenotype switch triggered by TGF-β and modulated by vitamin D, we set forth to evaluate the outcome of the contrasting effects of 1,25(OH)₂D₃ and TGF-β on mitochondrial respiration. We measured the mitochondrial transcript of one of the respiratory complexes, the subunit 2 of cytochrome C oxidase (COX2) (Fig. 5A), and we tested the respiratory activity as mitochondrial membrane potential (Fig. 5B). TGF-β induced the transcription and activity of the respiratory chain and 1,25(OH)₂D₃ reverted the effect, both in co-treatment and when the incubation started 24 hours before TGF-β (preD-T+D). However, in line with the observations made on EMT markers, also the experiments on respiratory modulation demonstrated that the administration of vitamin D in a later phase (preT-T+D) lost its efficacy in contrasting the effects of TGF-β. 1,25(OH)₂D₃ alone had no effect on mitochondrial respiration, probably because of the very low levels of VDR found in control cells, which are enhanced only by TGF-β treatment.

3.6 The results of metabolic control affect coupled respiration and not UCP-mediated uncoupling.

The augmented proton gradient created by an increased respiratory chain activity can be coupled with oxidative phosphorylation and can result in a boosted ATP synthesis and partial dispersion of the gradient. Alternatively, protons can cross back the mitochondrial inner membrane via leakage or uncoupling proteins. We evaluated these two possible routes of utilization of that mitochondrial gradient we found increased upon TGF-β treatment. We carried out the analysis of mitochondrial ATP synthesis (Fig. 5C) and we found that TGF-β augmented the mitochondrial ATP levels; the effect was reverted by the treatment with 1,25(OH)₂D₃, both in co-treatment and pre-treatment. Again, after a pre-incubation with TGF-β, vitamin D was not able to oppose the effects of the cytokine. The treatment with 1,25(OH)₂D₃ alone did not change the synthesis of ATP, as observed for respiratory activity, due to the low levels of VDR found in these cells. On the other hand, we

found no differences in the mitochondrial levels of the two most commonly expressed uncoupling proteins, UCP1 and UCP2 (Fig. 5D), which suggested that the uncoupling process was not modulated by TGF- β , although we cannot rule out the involvement of other minor uncoupling processes such as proton leak.

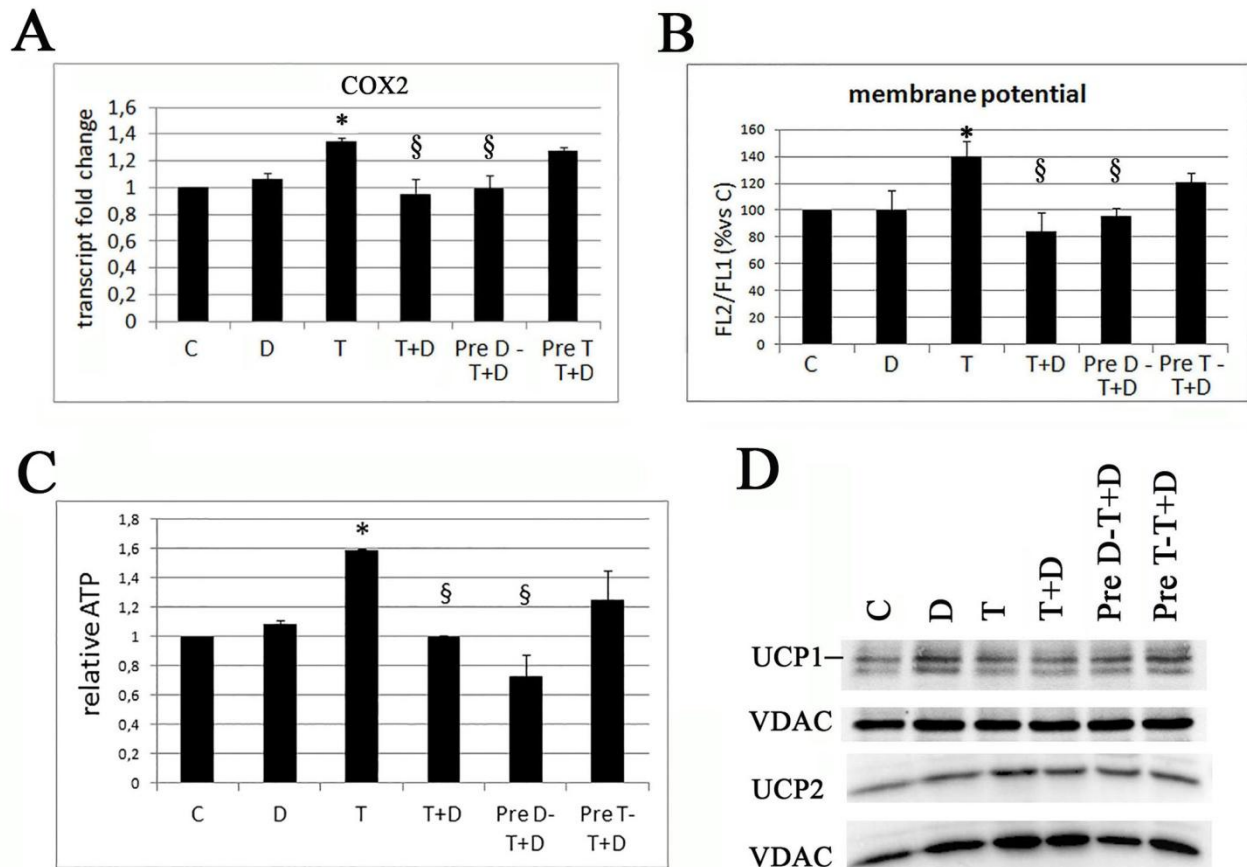


Fig. 5. 1,25(OH) $_2$ D $_3$ and TGF- β exert opposite effects on mitochondrial respiratory chain of Beas-2B cells. (A) After the indicated treatments, mRNAs were purified and assayed by real time PCR for cytochrome C oxidase subunit 2 (COX2) transcript expression. (B) The mitochondrial membrane potential was examined using JC-1 cytofluorimetric evaluation. For each experimental condition the FL-2/FL-1 ratio was calculated and expressed as a percentage of the value obtained for untreated cells.

(C) After the indicated treatments the levels of mitochondrial ATP were measured by a chemiluminescence-based assay. (D) The expression of the two main uncoupling proteins (UCP1 and UCP2) was analysed in mitochondrial fractions by Western blotting, and VDAC was used as

loading control. The blots are representative of three independent experiments. The values plotted on the graphs represent the fold change in treated versus untreated cells and are displayed as the means \pm SD of three independent experiments. * $P < 0.05$ compared to the untreated cells. § $P < 0.05$ compared to TGF- β treated cells.

3.7 The treatment with 1,25(OH) $_2$ D $_3$ counteracts the intracellular production of ROS triggered by TGF- β .

Many effects of TGF- β activity are mediated by ROS. In particular, in Beas-2B cells TGF- β is able to stimulate the production of ROS [24]. We measured the intracellular levels of ROS in cells treated for 24 hours with TGF- β and we evaluated the effect of 1,25(OH) $_2$ D $_3$ on this signaling pathway (Fig. 6). We found that the increase of ROS caused by TGF- β activity was reversed by the presence of 1,25(OH) $_2$ D $_3$ in all experimental conditions.

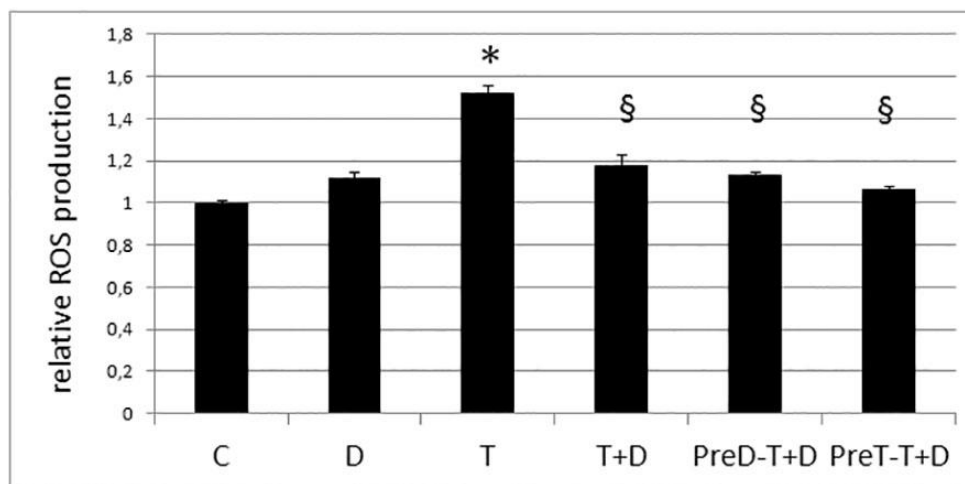


Fig. 6. 1,25(OH) $_2$ D $_3$ and TGF- β control intracellular ROS formation. After 24 hours of single treatments or pre-treatments followed by 24 hours of co-treatments, the levels of intracellular ROS in Beas-2B cells were evaluated by fluorescence assay and the results expressed relative to control. Values are reported as means \pm SD of three independent experiments. * $P < 0.05$ compared to the untreated cells. § $P < 0.05$ compared to TGF- β treated cells.

4. Discussion

In this work we describe a new mechanism by which TGF- β operates during EMT. For the first time we demonstrated in two human models of EMT that the cytokine induces a massive expression of VDR that mediates the opposing effects of 1,25(OH) $_2$ D $_3$ on EMT transition, measured as epithelial E-Cadherin expression and cellular migration. The effects of 1,25(OH) $_2$ D $_3$ are mediated by VDR, as demonstrated by silencing experiments. Past studies demonstrated a link between vitamin D and TGF- β signaling via Smad3 [14,15]. In this work we unveil a novel molecular detail of this cross-talk that doubles the effect of TGF- β on vitamin D signaling: TGF- β induces the expression of VDR and through Smad3 enhances VDR activity [14]. The mechanism responsible for the increased levels of VDR requires further investigation. However, our findings are in line with the observations made in a human colon cancer cell line, which show the involvement of the TGF- β signaling pathway in the induction of VDR expression in response to butyrate [41]. In CaCo-2 cells the increase of VDR induced by butyrate is mediated by Smad3 phosphorylation [41]; a similar signaling can be envisaged in our model of EMT and will be the subject of future studies. An interesting observation emerging from this study is that 1,25(OH) $_2$ D $_3$ becomes ineffective if its activity starts after the commitment of epithelial cells into the EMT program evoked by TGF- β signaling. We tested the transcriptional activity of VDR and we found a ligand-dependent strong induction of CYP24A1 messenger, but no difference was found whether 1,25(OH) $_2$ D $_3$ was added prior to or after TGF- β treatment. Because CYP24A1 is both a transcriptional target of VDR and a catabolic enzyme of 1,25(OH) $_2$ D $_3$, our data suggest that the levels of active hormone must be similar in all experimental conditions, notwithstanding the contrasting effects of pre and post-treatment with 1,25(OH) $_2$ D $_3$ on EMT transition phenotype. Based on these considerations we exclude that the inefficacy of post-treatment can be due to some inhibitory mechanism exerted on vitamin D levels or transcriptional activity of its receptor; rather, one could hypothesize that the EMT process is irreversible and thus 1,25(OH) $_2$ D $_3$ cannot undo TGF- β 's effects. This time-

dependent efficacy of $1,25(\text{OH})_2\text{D}_3$ in contrasting the EMT transition could be crucial if vitamin D was proposed as anti-metastatic agent and brings forth at least two considerations. First, the individual levels of $1,25(\text{OH})_2\text{D}_3$ or the functionality of its receptor (for example due to polymorphisms) could be among the factors that protect some subjects in those categories exposed to cancer risk. Several epidemiologic investigations [42-48] suggest a protective effect for vitamin D against cancer. Few recent studies have reported the association between plasma $25(\text{OH})\text{D}_3$ and risk of colorectal cancer [49] and breast cancer [50] and several published or ongoing studies have considered or are testing the efficacy of vitamin D supplementation in cancer prevention [51,52].

Second, based on our experimental data we suggest that vitamin D supplementation can represent a good preventive approach in subjects exposed to the risk of EMT, but it is not useful when the invasion process has started. The discrepant properties of vitamin D dietary intake in prevention and cure of cancer have been debated in several studies, and in this work we found the experimental evidences of a temporal window of efficacy in using vitamin D against cancer EMT.

The other important findings of this study emerge from the analysis of the metabolic switch promoted by TGF- β and reverted by $1,25(\text{OH})_2\text{D}_3$. In fact we demonstrated that in our model of EMT TGF- β potentiates the respiratory activity coupled to ATP synthesis, thus it favors the oxidative catabolism and the production of the energy currency of the cell. Our results are in agreement with the data described in recent studies, which show the increase in intracellular ATP content and oxygen consumption in TGF- β -treated A549 non-small cell lung cancer cells [23].

Moreover, it has been reported that TGF- β increases mitochondrial membrane potential, mitochondrial oxygen consumption and ATP generation in cultured mouse podocytes [25]. In addition to these experimental studies, the analysis of the free energy changes during the TGF- β -induced EMT for lung cancer cells demonstrated the metabolic shift resulting in increased cytosolic ATP levels [53]. The increased ATP synthesis is of paramount importance for migration taking place in EMT, actually several studies have demonstrated that mitochondrial ATP production is crucial for cancer cell motility [54-56]. In this study we described for the first time in human

bronchial epithelial cells a metabolic effect of TGF- β that supports the cellular invasion seen in EMT transition; 1,25(OH) $_2$ D $_3$ exerts the opposite effect on mitochondrial respiration and is able to prevent the surplus production of ATP triggered by TGF- β . Therefore we identified a novel signaling pathway by which 1,25(OH) $_2$ D $_3$ opposes the effect of TGF- β in EMT: in addition to the previously described transcriptional activity such as the induction of E-Cadherin, 1,25(OH) $_2$ D $_3$ restrains mitochondrial respiration and reduces the production of energy required for cell motility. Taken together our observations suggest that the increased expression of VDR might represent a regulatory negative feedback exerted by TGF- β on its own signaling. Negative regulation plays an important role in restriction and termination of TGF- β signaling; for example, the occurrence of a transcriptional negative feedback loops has been demonstrated for SnoN and Smad7 [57-58], which are targets of TGF- β activity and act in a negative feedback loop to inhibit TGF- β transcriptional effects. Interestingly the work by Ding and coll. has shown that TGF- β modifies VDR cistrome and facilitates the binding of VDR to SMAD3-targeted genes [59]. The resultant genomic antagonism attenuates TGF- β nuclear signaling and leads to the reduction of fibrosis. In other words, the study has provided the evidences of the negative feedback exerted by VDR on TGF- β activity at the cistromic level. Our is the first report of a negative feedback on TGF- β signaling mediated by a metabolic antagonism and our non-genomic mitochondrial data complete the paradigm of the regulatory feedback that controls the activity of TGF- β .

Moreover, the results of many studies [60] have suggested the existence of a double negative feedback loop operating between 1,25(OH) $_2$ D $_3$ and EMT inducers, which may contribute to the acquisition of the epithelial versus mesothelial phenotype dictated by the extracellular cues. Among the transcription factors involved in such loops, SNAIL and ZEB are the most investigated; we believe that in our experimental setting ZEB could mediate the transcriptional cross-talk between Vitamin D and TGF- β , because it has been described as a cell- and context-dependent positive regulator of VDR [61], whereas SNAIL proteins seem to be generally negative modulators of VDR levels, thus more involved in those cancer models where the expression of the receptor is silenced.

It is reasonable to suppose that the metabolic negative feedback regulation demonstrated in our study could be necessary to balance the excess of mitochondrial stimulation that could lead to stress and apoptosis. This hypothesis led us to investigate the effect of $1,25(\text{OH})_2\text{D}_3$ on the intracellular production of reactive oxygen species. ROS are often elevated in inflammation and cancer microenvironment [62]. It has been established that ROS may regulate TGF- β expression in epithelial cells [63] and increment TGF- β bioavailability [64]. In turn, TGF- β regulates ROS levels, not only by inducing their production, but also by downregulating the expression of antioxidant enzymes. [65,66]. Several studies have demonstrated that ROS mediate many effects of TGF- β during tumorigenesis, since they regulate the effectors of TGF β signal transduction, such as Smads, MAPKs and NF- κ B, and they modulate the increase of cell motility [65,67,68]. In this study we show that the intracellular production of ROS triggered by TGF β was hampered by the co-treatment with $1,25(\text{OH})_2\text{D}_3$. This observation suggests that the negative feedback exerted by $1,25(\text{OH})_2\text{D}_3$ could be mediated at least in part by the control over ROS production. ROS can induce cell damage and lead to apoptosis, therefore a strict regulation of this positive feedback between TGF- β and oxidative stress/ROS must be established in order to favor tumor progression without damaging the cell. The negative feedback exerted by $1,25(\text{OH})_2\text{D}_3$ could be necessary to maintain a balance in metabolism and avoid the excessive production of ROS. A local controlled biosynthesis of $1,25(\text{OH})_2\text{D}_3$ could produce an alternance of opposed metabolic signaling that could be optimal for cancer cell survival and spreading; intriguingly, this mechanism could be exploited for therapeutic benefit, because the elevated levels of $1,25(\text{OH})_2\text{D}_3$ could restrain the metabolic shift evoked by TGF- β and could limit or even prevent cancer migration and metastasis, as schematically depicted in Figure 7.

5 Conclusions

In conclusion, in this study we identified a novel signaling pathway by which $1,25(\text{OH})_2\text{D}_3$ opposes the effects of TGF- β in EMT; we found that in human bronchial epithelial cells TGF- β induces the expression of VDR, which opposes EMT via a transcriptional and metabolic signaling. The metabolic control is exerted by $1,25(\text{OH})_2\text{D}_3$ on mitochondrial respiration, synthesis of ATP and on the production of intracellular ROS. Vitamin D/VDR activity serves as a negative control mechanism to curb the effects of TGF- β and probably aims to avoid the excessive stimulation that could lead to cellular damage. The preventive treatment with $1,25(\text{OH})_2\text{D}_3$ could be effective in reducing the undesirable effects of TGF β activity, such as the induction of EMT found in cancer.

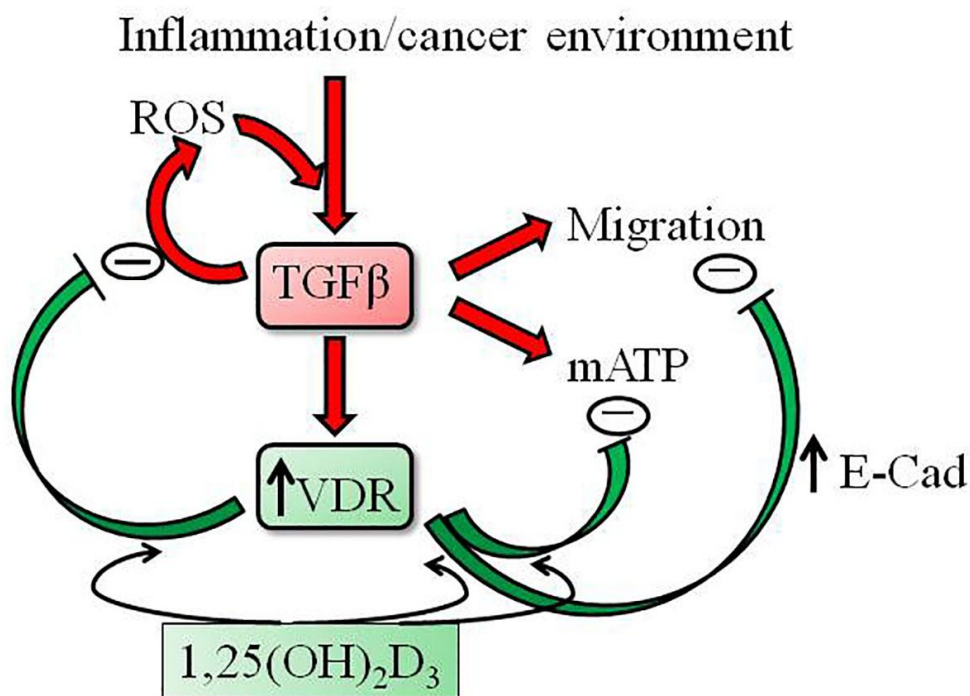


Fig. 7. A working model of the molecular mechanisms underlying the effects of $1,25(\text{OH})_2\text{D}_3$ on TGF- β signaling. The induction of VDR promoted by TGF- β potentiates the regulatory effects of $1,25(\text{OH})_2\text{D}_3$; the negative feedback is exerted on mitochondrial energy metabolism

(mitochondrial ATP, mATP), cell migration is inhibited also by the transcriptional induction of E-Cadherin (E-Cad), and the autocrine loop of ROS production is curtailed.

Conflict of interest

All authors declared there are no competing financial interests.

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Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Supplementary Materials and Methods

Cell proliferation assay

2000 cells were seeded on 96-multiwell plates and cultured for 5 days with 100 nM 1,25(OH)₂D₃ or 10 ng/ml TGF-β, alone or in combination. At the end of this period, cells were fixed for 15 min with 11% glutaraldehyde, plates were washed three times, air-dried and stained for 20 min with 0.1% crystal violet solution. The plates were then extensively washed and air-dried prior to solubilization of the bound dye with 10% acetic acid solution. Absorbance was determined at 595 nm. Data from twelve wells were averaged for each experimental condition and the experiment was repeated three times.

Migration assay

The migration assay was performed with 24 well trans-well filters with 8.0 μm pores (Corning, USA) as described previously [36]. Cells were pre-treated for 24 hours, resuspended in 100 μl serum free medium, added to the upper chamber and incubated for 24 hours with co-treatment. RPMI medium with 10% FBS was added to the lower chamber. The cells that migrated to the opposite side of the membrane were fixed and stained with crystal violet, the bound dye was solubilized with 10% acetic acid solution and absorbance was determined at 595 nm. Data from three wells were

averaged for each experimental condition and expressed relative to control. The experiment was repeated three times.

Wound healing assay

The assay was carried out as previously described [37]. Cells were seeded in a 24 well plate and when they reached 80% confluency they were starved over night and then incubated in pre-treatment for 24 hours. A wound line was generated with a sterile pipette tip, followed by co-treatment for 24 hours. Images were obtained at 0 and 24 hours using a light microscope at 20x magnification with a digital camera under bright field illumination. The area of the wound was measured in the central part of each well using ImageJ software. The measurements were then converted into a percentage of wound closure: $100 - [(area\ at\ t_{24}/area\ at\ t_0) \times 100]$.

RNA Extraction and Real-Time PCR

RNA was extracted using TRIzol (Invitrogen) and then 1 µg of total RNA, treated with DNase (Roche), was used for reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR was performed with iQ SYBR Green (Bio-Rad) with the following primers:

VDR fwd 5'-ACTTGTGGGGTGTGTGGAGAC-3', rev 5'-GGCGTCGGTTGTCCTTCG-3',
COX2 fwd 5'-CGACTACGGCGGACTAATCT-3', rev 5'-TCGATTGTCAACGTCAAGGA-3',
CYP24, fwd 5'-CGTTTGGACGATGATGGTCAC, rev 5'-TTTCTTGAAGCCGATTCTGGTG;
S14 fwd 5'-AGGTGCAAGGAGCTGGGTAT-3'; rev 5'-TCCAGGGGTCTTGGTCCTATTT-3'.

The housekeeping gene ribosomal subunit protein S14 was used as internal control. Real-time PCR parameters were as follows: cycle 1, 50°C for 2 minutes; cycle 2, 95°C for 10 minutes, followed by 45 cycles at 95°C 15 seconds and then 60°C for 1 minute. The $2^{-\Delta\Delta CT}$ method was used to analyze the data.

Supplementary Figure legends

Fig. S1. Design of treatments. The figure schematizes the protocol of incubation: 24 hours of pre-treatment in the indicated conditions, followed by 48 hours of single treatments or co-treatments.

Empty boxes represent the incubation medium with vehicle .

Fig. S2. Analysis of E-Cadherin expression in whole lysates. The blots are representative of three independent experiments quantified in figure 3A. Actin was used as loading control.