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miR-25 modulates the cross-talk between canonical and non-canonical Wnt signaling

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Abstract

Prostate cancer is considered the most common cancer and represents the second leading cause of death from cancer in men in the Western world. Once that the tumor has metastasized to the bone, no cure is available. To date, the molecular mechanisms responsible for cancer relapse and metastasis formation have not yet been elucidated. However, epithelial-to-mesenchymal transition (EMT) has been established as one of the key events that lead to cancer invasion, the occurrence of distant metastasis and therapy resistance. Cells that undergo EMT acquire a more motile phenotype and become highly migratory. The functional role of the so-called non-canonical WNT/planar cell polarity (WNT/PCP) pathway in the acquisition and maintenance of a motile phenotype has been firmly established.

In this paper we have studied a class of non-coding RNAs, the so-called microRNAs, that regulate gene expression and may play pivotal roles in carcinogenesis and tumor progression. We found that miR-25 that was shown previously to impair migration in a subpopulation of highly metastatic, cancer stem-like cells-, modulates the cross-talk between canonical and non-canonical WNT/PCP pathway. Our data show, for the first time, that miR-25 can modulate the expression of Dapper Homolog 1 (DACT1), an antagonist of β-catenin, thereby increasing canonical WNT signaling. TGF-β is considered to be one of the major drivers of EMT in various carcinomas, including those of the human prostate cancer. Our study, suggest that miR-25 might interfere with the cross-talk between WNT and TGF-β signaling and is capable to block the induction of migration produced by TGF-β in PC-3M-Pro4Luc2 human prostate cancer cells. Taken together, our observations suggest that targeting of non-canonical WNT/PCP pathway represents an interesting therapeutic strategy to block (or reverse) the acquisition of an invasive, stem/progenitor-like phenotype in human prostate cancer.

Introduction

Prostate cancer is the most common cancer in males and the second leading cause of death from cancer in the western male population (1). Occurrence of bone metastasis during the castration resistant phase represents one of the major problem for the patients, for which no therapeutic treatment is available at the moment. Despite the progresses in cancer biology, the mechanisms responsible for cancer relapse and metastasis formation have remained largely elusive. It is established that one of the critical event which precedes the formation of distant metastasis is the transition from an epithelial-like to mesenchymal-like state at the primary tumor (epithelial-to-mesenchymal transition, EMT) (2). Cells undergoing EMT become more motile and invasive and also display therapy resistance (3,4). One of the signaling pathways that are involved in the modulation of motility is represented by the so-called non-canonical WNT/planar cell polarity (WNT/PCP) pathway (5,6). The role for canonical WNT signaling during the initial phases of cancer initiation is indeed established. However, accumulating evidence suggests a critical role of the non-canonical WNT/PCP pathway during the second phase of the disease, when cancer progress, invades and metastasizes (5). microRNAs are a small class of non-coding RNA molecules that regulates gene expression and for which the role in pathogenesis and progression of prostate cancer is established (7). Several studies have also highlighted the predictive value of measuring the levels of microRNA in urine and blood to monitor the progression of the disease (8). However, there is a remarkable lack of information about the role of microRNA in aggressive subpopulation of cancer stem/progenitor like cells, characterized by high invasiveness and capable of forming metastasis. According to this scenario, the non-canonical WNT/PCP pathway represents one of the important player in the maintenance of high migration and invasion in these cells. Recently we have shown that miR-25 is strongly downregulated in a subpopulation of highly migratory and metastatic $ALDH^{high}$ cells in human prostate cancer (9).

In this study, we investigated whether miR-25 can interfere with the non-canonical WNT/PCP pathway in human prostate cancer cells. Our results indicate that miR-25 represents an interesting player in the maintenance of the balance between canonical and non-canonical WNT/PCP pathway. We show here -for the first time- that miR-25 modulates the expression of Dapper Homolog 1 (DACT1), an antagonist of β-catenin (10), thereby interfering with WNT signaling. Moreover we provide evidence that miR-25 interferes with the cross-talk between WNT and TGF-β signaling leading to attenuated TGF-β-induced migration of human prostate cancer cells. Together, our data highlight the role of miR-25 in prostate cancer progression, in particular the targeting of non-canonical WNT/PCP pathways as critical mediators of tumor invasiveness.

Materials and Methods

Cell lines and culture conditions

Human osteotropic prostate cancer cell lines PC-3M-Pro4 and PC-3M-Pro4Luc2 cells were maintained in DMEM with 10% FCII, 1% Penicillin-Streptomycin (Life Technologies) and 0.8 mg/ml Neomycin (Santacruz, USA) for cells expressing Luciferase 2. Human embryonic kidney HEK293T cells were maintained in DMEM with 10% FCS and 1% Penicillin-Streptomycin (Life Technologies, USA). Cells were maintained at 37°C with 5% CO_2.

Transient transfection with pre-miR-25 or pre-negative control and luciferase reporter assay

For microRNA overexpression, transfection was performed with Lipofectamine® 2000 (Invitrogen, USA) according to manufacturer's protocol with Pre-miR-25 (ID: PM10584; Life Technologies) and pre-miRNA negative control (scramble) (ID: AM17110; Life Technologies). Total RNA was collected after 72 hours to assess positive overexpression and target gene down-regulation.

For luciferase reporter assay, PC-3M-Pro4, HEK293T or HEK293T knock-down cells were seeded 10,000 cells in 500 µL medium in a 24-wells plate and Lipofectamine® 2000 used according to manufacturer's protocol. For experiments in combination with miR-25 overexpression, for each condition, 100 ng of BAT-luciferase (reporter for canonical WNT signaling (11)) or ATF2-luciferase (reporter for non-canonical WNT signaling (12-14)) or CAGA-Luc (TGF-β reporter) and 10 ng CAGGS-renilla were used. After 24 hours, medium was replaced and cells were treated with 0.6, 1.8, 3.0 µM SB216763 (Sigma-Aldrich, The Netherlands); 5, 12.5, 20 mM LiCl; 50, 75, 100 mM GIN (11) or 100 µM PNU-74654 (Sigma-Aldrich) or 0.1, 0.5, 1, 5, 10 ng/mL TGF-β for 24 hours before assessment of Luciferase activity. The *Photonis pyralis* (firefly) luciferase (Fluc) and *Renilla reniformis* luciferase (Rluc) activities in the lysates were measured with Dual Luciferase Assay (Promega, USA). Data are shown as Relative Light Units (RLU, Fluc normalized for Rluc levels).

RNA extraction and qRT-PCR

RNA was extracted using Trizol (Invitrogen) and cDNA synthesized by reverse transcription (Promega, USA) according to manufacturer's protocol. qRT-PCR was performed with Biorad CFX96 system (Biorad, The Netharlands). DACT1 (FW: GGCGACCTTGAGTCTCTCAG; RV: CTGAGGCCTGGTCTTCACAG) expression was normalized to GAPDH (FW: GACAGTCAGCCGCATCTTC; RV: GCAACAATATCCACTTTACCAGAG) and/or HPRT (FW: AGACTTTGCTTTCCTTGGTCAGG; RV: GTCTGGCTTATATCCAACACTTCG).

miRNA target prediction and bioinformatic analysis of cluster of genes

Targetscan v6.2 and microT-CDS (15) were used to identify miR-25 predicted targets. Functional annotation was performed using DAVID Bioinformatics Resources 6.7 (16,17) and KEGG database (18).

ALDEFLUOR® assay

Aldehyde Dehydrogenase (ALDH) activity was measured using the ALDEFLUOR® assay kit (StemCell Technologies, USA) according to the manufacturer's protocol (19). ALDH substrate was added to the cells and fluorescent product measured by flowcytometry. For sorting, FACS ARIA cell sorter (BD Bioscience) was used. After sorting, RNA was collected from $ALDH^{high}$= highest 10% and gene expression compared to $ALDH^{low}$ = lowest 10%).

Migration assay

To assess migration, cells were starved overnight in medium containing 0.3% serum and 60,000 cells seeded the day after in medium containing 0.3% serum in Transwell (8-µm) upper-chamber (Corning, The Netherlands). The lower chamber was filled with medium containing 10% serum. After 16-18 hours of incubation, cells on the upper side of the filters were removed and cells on the lower side were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min at RT (Sigma-Aldrich, USA). Cell migrated were subsequently counted.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad software, USA). T-test was used for comparison between two groups. Data is presented as mean ± SEM. P-values ≤ 0.05 are considered to be statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

miR-25 induces canonical-WNT signaling via a WNT-dependent mechanism

To investigate the effect of miR-25 on WNT signaling pathway, we employed a canonical WNT signaling bioluminescent reporter, BAT-Firefly Luciferase (Fluc) (20,21) and CAGGS-Renilla luciferase (Rluc) as control for transfection efficiency. miR-25 overexpression in PC-3M-Pro4 human prostate cancer cell line resulted in significant increase in BAT-Fluc signaling indicating up-regulation of canonical WNT signaling (**Fig. 1 A, B and C,** Control=vehicle condition). Our group has previously shown that inhibition of GSK3-β produced increase in canonical WNT signaling in human prostate cancer (11). Interestingly, we found that treatment with different GSK3-β inhibitors (SB216763 (22), LiCl (11) and GIN (23)) combined with miR-25 overexpression resulted in significant enhancement of canonical WNT signaling, suggesting an additive effect of miR-25 on GSK3-β inhibition (**Fig. 1 A, B and C** respectively). To assess whether the observed effect of miR-25 was WNT-dependent, we used a downstream inhibitor of canonical WNT signaling, PNU-74654, and measured the activity of canonical WNT reporter upon miR-25 overexpression alone or in combination with PNU-74654. We confirmed that miR-25 was capable of inducing canonical-WNT signaling and found that simultaneous incubation with 100 μM of PNU-74654 led to complete reversal of the induction of WNT signaling in PC-3M-Pro4 cells (**Fig. 1 D**). Together, these suggest that the effect of miR-25 on canonical WNT signaling is direct and can be reversed upon treatment with downstream WNT inhibitors.

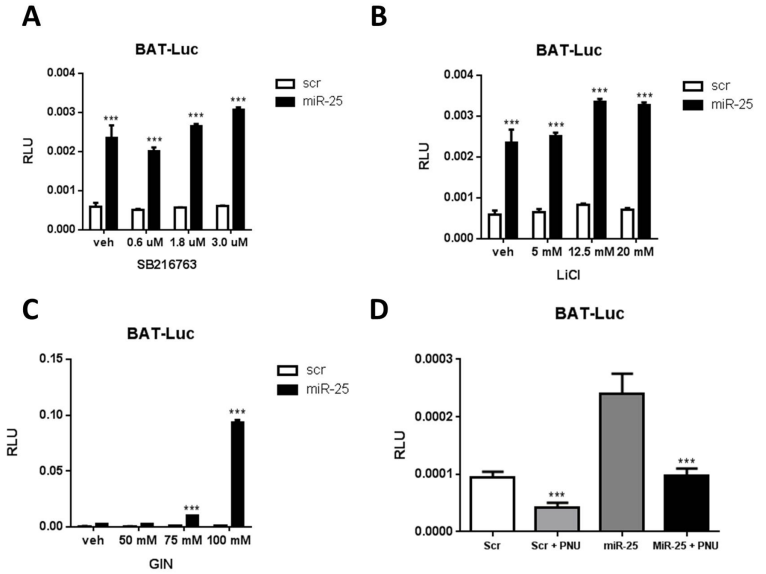

Figure 1. miR-25 overexpression directly induces canonical WNT signaling. **A-C**) Treatment with different concentration of GSK3-β inhibitor SB216763 (**A**), LiCl (**B**) and GIN (**C**) in combination with miR-25 overexpression produced additive effect on canonical WNT signaling induction. **D**) Administration of downstream inhibitor of WNT signaling PNU-74654 (PNU) can reverse the induction of WNT signaling produced by overexpression of miR-25. Error Bars ± SEM. $p < 0.05$ (*), $p < 0.001$ (***).

miR-25 downregulates Dapper, an antagonist of β-catenin, Homolog 1 (DACT1)

To study whether novel miR-25 predicted target gene(s) involved in WNT signaling could be identified, we predicted gene targets of miR-25 by publically-available tools *in silico*. Our analysis found that Dapper, an antagonist of β-catenin, homolog 1 (DACT1) was shown as miR-25 predicted target gene in two independent online bioinformatic tools, TargetScan (24) and microTCDS (15).

Previously we have shown that miR-25 is strongly downregulated in a subpopulation of highly tumorigenic and metastatic, stem-like $ALDH^{high}$ cells compared to non-tumorigenic and non-metastatic $ALDH^{low}$ subpopulation isolated from PC-3M-Pro4Luc2 human prostate cancer cell line (9). Therefore, we hypothesized that miR-25 and DACT1 expression in $ALDH^{high}$ vs. $ALDH^{low}$ subpopulation could be inversely correlated. To test this hypothesis, we measured the expression of DACT1 mRNA in subpopulation of $ALDH^{high}$ compared to $ALDH^{low}$, isolated after viable cell sorting from PC-3M-Pro4Luc2 human prostate cancer cells with ALDEFLUOR kit (19). Our mRNA analysis showed that DACT1 mRNA is, indeed, significantly higher in $ALDH^{high}$ vs. $ALDH^{low}$ subpopulation (**Fig. 2A**). Next we evaluated whether transient overexpression of miR-25 could reduce the

mRNA level of its predicted target gene DACT1 in two independent cell lines. Interestingly, we found that overexpression of miR-25 could significantly decrease mRNA expression of DACT1 in PC-3M-Pro4Luc2 cells ($p<0.01$) and similar trend was observed in HEK293T cells (**Fig. 2B and C** respectively).

Taken together, miR-25 and DACT1 expression are inversely correlated in $ALDH^{high}$ compared to $ALDH^{low}$ subpopulation as we hypothesized. Moreover, miR-25 overexpression may directly inhibit the predicted target gene DACT1 as was found from the *in silico* analysis described above.

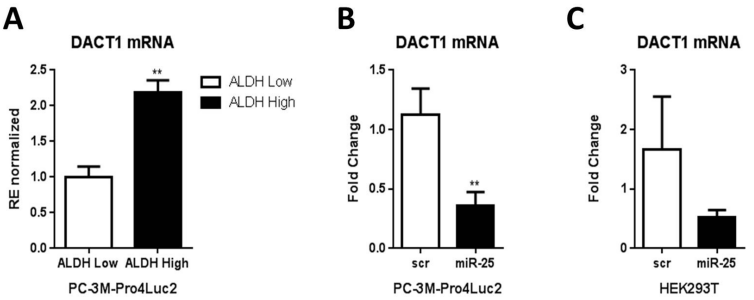

Figure 2. DACT1 is elevated in $ALDH^{high}$ vs $ALDH^{low}$ subpopulation and miR-25 overexpression downregulate its mRNA expression. A) qRT-PCR in $ALDH^{high}$ vs. $ALDH^{low}$ subpopulation isolated from PC-3M-Pro4Luc2 human prostate cancer cell line show increased expression of DACT1 in $ALDH^{high}$ cells. **B**) mRNA analysis after 72 hours following miR-25 overexpression shows significant reduction in the level of DACT1 mRNA in PC-3M-Pro4Luc2 cells. **C**) mRNA analysis after 72 hours following miR-25 overexpression shows reduction in the level of DACT1 mRNA in HEK293T cells. Error Bars ± SEM. $p < 0.01$ (**).

DACT1 knock-down partially recapitulates miR-25 overexpression phenotype

To functionally evaluate the biological role of DACT1 we used two shRNAs direct against DACT1 and one shRNA control to perform stable knock-down of this gene in the two independent cell lines PC-3M-Pro4Luc2 and HEK293T cells.

We found that DACT1 knock-down affected cell morphology and cellular density in both cell lines employed with both shRNA constructs (**Fig. 3A**). ShRNA#63 drastically reduced cellular density and ShRNA#64 induced a spindle-shape phenotype (**Fig. 3A**). Specificity of knock-down was confirmed by qRT-PCR for both shRNA and showed significant decreased DACT1 expression with shRNA#63 (ShDACT1_#63) and partial knock-down was achieved with shRNA#64 (ShDACT1_#64) (**Fig. 3B**). As expected, we measured a

significant increase in BAT luciferase activity with both shRNAs construct against DACT1 used to perform the knock-down ($p<0.001$ for both shRNAs) (**Fig. 3C**).

Given the stimulatory effect on canonical WNT signaling upon miR-25 overexpression, these findings all together reinforce the hypothesis that miR-25 interferes with WNT signaling via DACT1.

Previously we have demonstrated that miR-25 overexpression strongly inhibits cell migration in human prostate cancer (9). This led us to hypothesize that DACT1 knock-down could result in a similar functional phenotype. We used transwell boyden chambers to measure migration in PC-3M-Pro4Luc2 and HEK293T cells with DACT1 knock-down (**Fig. 3D**). Interestingly, we found a strong and significant inhibition of migration in HEK293T cells ($p<0.001$ for both shRNAs) (**Fig. 3E**). However, no decrease in migration was detected in PC-3M-Pro4Luc2 cells which showed a surprising increase in motility with shRNA#63 ($p<0.001$) and no effect with shRNA#64 (**Fig. 3F**). These data suggest that miR-25 overexpression and DACT1 knock-down result in a similar effect on WNT signaling activity. However DACT1 knock-down can only partially recapitulate, in the human prostate cancer cell line, the phenotype produced by miR-25 overexpression.

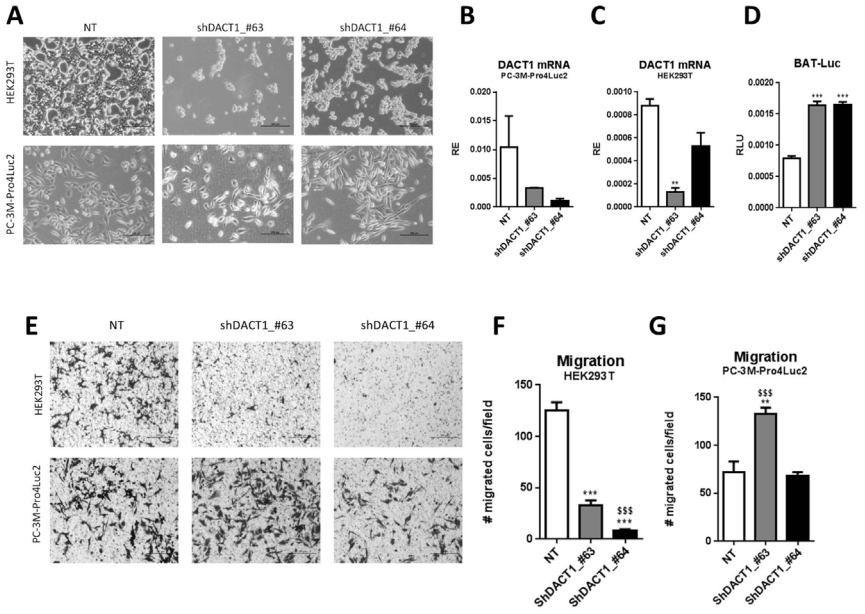

Figure 3. DACT1 knock-down affects cell morphology and partially recapitulates the miR-25 overexpression phenotype in human prostate cancer cells. **A**) DACT1 knock-down induces changes in cell morphology compared to shRNA control (NT, left panels, top = HEK293T and bottom = PC-3M-Pro4Luc2 cells); effect on cellular density is observed for both shRNAs in both cell lines (central and right

panels, top for HEK293T and bottom for PC-3M-Pro4Luc2); in PC-3M-Pro4Luc2 shDACT1_64 induces a spindle cell shape phenotype (left bottom panel) compared to a more epithelial-like morphology induced by shDACT1_63 (central bottom panel). **B-C**) qRT-PCR confirms DACT1 knock-down with both shRNAs in both the cell lines used **D**) Bioluminescent reporter for canonical WNT signaling (BAT-Luc) in HEK293T cells support knock-down of antagonist of β-catenin (DACT1) and shows increased bioluminescent activity for both shRNAs used. **E**) Representative pictures of HEK293T and PC-3M-Pro4Luc2 cells with knock-down for DACT1 with two shRNAs. **F**) Both shRNAs for DACT1 significantly reduce migration in HEK293T cells. *** vs NT (ShRNA control) and $$$ vs shDACT1_#64. **G**) shDACT1_#63 induce migration in PC-3M-Pro4Luc2 cells (*** vs NT, ShRNA control and $$$ vs shDACT1_#64). Error Bars ± SEM. p< 0.01 (**), p<0.001 (***, $$$).

TGF-β modulates the cross-talk between canonical- and non-canonical WNT signaling and miR-25 interferes with TGF-β signaling

One of the critical events during prostate cancer progression is represented by epithelial-to-mesenchymal transition (EMT) (25). In this process, TGF-β represents one of the major drivers of EMT and promotes migration (26). The notion that Wnt/PCP and canonical-Wnt signaling are both part of a negative feedback-loop where Wnt/PCP negatively regulates canonical-Wnt signaling and *vice versa* (27) led us to test whether TGF-β could modulate canonical and non-canonical WNT signaling in our model. To this aim, we transfected PC-3M-Pro4 human prostate cancer cells with canonical (BAT-Luc (11)) and non-canonical (ATF2 Luc (12)) WNT bioluminescent reporter. Treatment with different concentrations of TGF-β (1, 5, 10 ng/mL) induced a significant, dose-dependent decrease in both canonical WNT signaling (**Fig. 4A**) and non-canonical WNT signaling (**Fig. 4B**).

Given the stimulatory role of miR-25 on canonical WNT signaling, and its inhibitory effect on cell migration, we hypothesized that the modulation of canonical and non-canonical WNT signaling produced by TGF-β could be modulated by miR-25. To test this, we transfected PC-3M-Pro cells with a bioluminescent Smad-dependent TGF-β reporter (CAGA-Luc) and simultaneously overexpressed miR-25 and a scramble negative control. 24h after transfection, cells were treated with TGF-β (0.1, 0.5, 1 ng/mL) and assessment of bioluminescent activity revealed that miR-25 was capable of blocking TGF-β signaling (**Fig. 4C**). Interestingly, we found that overexpression of miR-25 abolished the pro-migratory effect of TGF-β compared so scramble negative control (**Fig. 4D**). Taken together our results suggest that miR-25 might represent an interesting player in the modulation that TGF-β exerts on canonical and non-canonical WNT signaling.

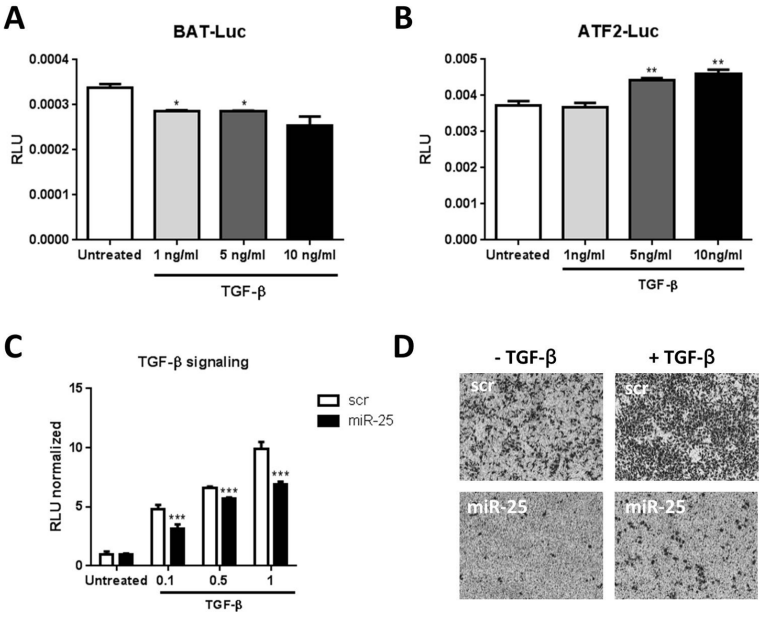

Figure 4. TGF-β modulates canonical and non-canonical WNT signaling in a different manner (A,B) and miR-25 interferes with TGF-β signaling and cell migration under basal- and TGF-β stimulated conditions (C,D). **A**) Canonical WNT signaling activity measured by bioluminescent reporter (BAT-Luc) in presence of an increasing dose range of TGF-β (1, 5, 10 ng/mL) (* p<0.05 vs. Untreated). **B**) Non-canonical WNT signaling activity measured by bioluminescent reporter (ATF2-Luc) in presence of an increasing dose range of TGF-β (1, 5, 10 ng/mL) (** p<0.01 vs. Untreated). **C**) miR-25 reduces the activity of TGF-β signaling and is capable of blocking the stimulation with an increasing dose range of TGF-β (0.1, 0.5, 1 ng/mL). (***, p<0.001 with two way ANOVA). **D**) miR-25 overexpression can block the induction of migration produced by 1ng/mL of TGF-β compared to scramble negative control.

Discussion

The role of the canonical WNT developmental pathway in tumorigenesis has been firmly established in several types of cancers (28). However, more recent oncological research has highlighted the critical contribution of the so-called non-canonical WNT/planar cell polarity (PCP) pathway in the progression phase of the disease, typically characterized by cell migration, invasion and formation of metastasis (5).
Previously we have shown that miR-25 is downregulated in a subpopulation of highly migratory and metastatic cells ($ALDH^{high}$) and this miR appears to be a critical player in the modulation of cell motility, migration and differentiation of human prostate cancer cells (9). Here we investigated the role of miR-25 in the modulation of canonical and non-canonical WNT signaling and identified Dapper, Antagonist of β-catenin, homolog 1 (DACT1) as a miR-25 predicted target gene. Wnt/PCP and canonical-Wnt signaling are part of a negative feedback-loop where Wnt/PCP negatively regulates canonical-Wnt signaling and *vice versa* (27). One of the important mediator of this feedback mechanism is represented by Dishevelled (Dvl) which is considered to be the "Hub of Wnt signaling" (29). In this context, when canonical WNT signaling is active, the recruitment of Dvl by Frizzled prevents the constitutive destruction of β-catenin and results in its accumulation and subsequent nuclear translocation (29). DACT1 has been shown to negatively regulate canonical WNT signaling by promoting lysosomal degradation of Dvl, leading to degradation of β-catenin (30). Real-time measure of canonical WNT signaling by bioluminescent reporter in prostate cancer cells in which DACT1 was knocked down supported this notion. The WNT/PCP pathway is critically involved in cytoskeletal remodeling and cell motility (30). Interestingly, loss of DACT1 has been shown to disrupt WNT/PCP pathway, altering Dvl activity and leading to malformation in mice (30). Here we found that miR-25 might target directly DACT1 mRNA, suggesting that a regulatory role for this miR in the WNT/PCP pathway. Previously, we demonstrated that miR-25 can disrupt cell migration and identified several predicted target genes involved in the modulation of F-actin assembly and in the remodeling of the cytoskeleton (9). Our data show that miR-25 expression is capable of stimulating the canonical WNT signaling pathway. Given the negative feedback-loop between canonical and non-canonical WNT/PCP signaling, we speculated that miR-25-induced activation of canonical WNT signaling will result in the attenuation of the non-canonical WNT/PCP pathway leading to a reduction in cell migration. In support of this observation, transcriptional analysis revealed that DACT1 is significantly up-regulated in invasive, metastatic $ALDH^{high}$ vs sessile, non-metastatic $ALDH^{low}$ subpopulation of prostate cancer cells. Moreover, we have previously demonstrated that miR-25 is strongly downregulated in $ALDH^{high}$ compared to $ALDH^{low}$ subpopulation of PC-3M-Pro4Luc2

human prostate cancer cells (9). Taken together, our data show an inverse correlation between miR-25 and DACT1 expression in $ALDH^{high}$ cells, suggesting that WNT/PCP pathway might be directly involved in the maintenance of an invasive phenotype in this subpopulation of cells. DACT1 knock-down in HEK293T cells resulted in a complete loss of migratory properties and recapitulated the miR-25 overexpression phenotype. However, the fact that the knock-down of DACT1, in PC-3M-Pro4Luc2 cells, failed to interfere with migration, suggests the presence of additional mechanisms that might determine their malignant phenotype. For example, PC-3M-Pro4Luc2 cells express high levels of α_6 and α_v integrins, that are directly targeted by miR-25 and that are functionally involved in the activation of latent TGF-β, motility, invasion and metastasis (9). Moreover, one single microRNA can downregulate multiple genes at the same time, leading to a global functional effect that logically cannot be entirely reproduced by the downregulation of a single gene.
Our group described previously that TGF-β increases the size of $ALDH^{high}$ stem-like subpopulation of human prostate cancer cells (31). Here we show that TGF-β negatively regulates canonical WNT signaling and positively regulates non-canonical WNT/PCP pathway. This reinforces the tumor supportive role of TGF-β and its involvement in EMT and cell migration. In the perspective of a reciprocal feedback-loop between canonical and non-canonical WNT/PCP signaling, DACT family proteins have been identified in mammals as multi-adapter molecules with the ability to modulate and integrate WNT and TGF-β signaling (10). Here we showed that miR-25 inhibits TGF-β signaling and strongly reduced the migratory effect induced by TGF-β. These data, combined with the notion in literature, suggest that DACT1 might represent an important player in the modulation between the cross-talk between TGF-β and WNT signaling and that miR-25 might interfere with this balance.

Finally, although additional experiments are required to elucidate the exact role of miR-25 in this process, we hypothesized a model in which non-canonical WNT/PCP pathway and TGF-β signaling maintain the highly migratory phenotype in PC-3M-Pro4Luc2 human prostate cancer cells. It is remarkable indeed that our bioluminescent measurement of WNT reporters revealed that the order of magnitude difference between non-canonical WNT/PCP compared to canonical WNT signaling is approximately a factor of 10. This suggests that there is a basal disbalance between canonical and non-canonical WNT/PCP signaling that result in a shift toward WNT/PCP pathway and that functionally sustain motility and migration. PC-3M-Pro4Luc2 cells are indeed highly migratory and metastatic upon inoculation in mice. Furthermore, the fact that overexpression of miR-25 leads to downregulation of DACT1, coinciding with loss of migration and strong induction of canonical WNT signaling, indicates that DACT1 might

regulate this process. Moreover, administration of TGF-β reduces canonical WNT signaling and augments non-canonical WNT/PCP signaling, which support the notion that TGF-β strongly induces the acquisition of an invasive phenotype.

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