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Snail regulates cell survival and inhibits cellular senescence in human metastatic prostate cancer cell lines

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Abstract

The epithelial-mesenchymal transition (EMT) is regarded as an important step in cancer metastasis. Snail, a master regulator of EMT, has been recently proposed to act additionally as a cell survival factor and inducer of motility. We have investigated the function of Snail (*SNAI1*) in prostate cancer cells by down-regulating its expression via siRNA and measuring the consequences on EMT markers, cell viability, death, cell cycle, senescence, attachment and invasivity. Of eight carcinoma cell lines, the prostate carcinoma cell lines LNCaP and PC-3 showed the highest and moderate expression of *SNAI1* mRNA, respectively, as measured by quantitative RT-PCR. Long-term knockdown of Snail induced a severe decline in cell numbers in LNCaP and PC-3 and caspase activity was accordingly enhanced in both cell lines. In addition, suppression of Snail expression induced senescence in LNCaP cells. SNAI1-siRNA treated cells did not tolerate detachment from the extracellular matrix, probably due to downregulation of integrin α6. Expression of E-cadherin, vimentin and fibronectin was also affected. Invasiveness of PC-3 cells was not significantly diminished by Snail knockdown. Our data suggest that Snail acts primarily as a survival factor and inhibitor of cellular senescence in prostate cancer cell lines. We therefore propose that Snail can act an early driver of prostate cancer progression.

Introduction

Prostate cancer (PCa), the leading cause of non-cutaneous malignancy in men in Western industrialized countries, originates almost always from the glandular epithelia of the organ (Jemal et al., 2007). With current treatment options, the 5-year survival rate for men diagnosed with organ-confined PCa is approaching 100%; however, the prognosis becomes worse once the cancer extends beyond the prostatic capsule (Jemal et al., 2007). PCa is clinically heterogeneous because many tumors take an indolent course that does not significantly affect an individual patient's lifetime. In contrast, once cancers metastasize to other organs, a majority of patients die from their tumors as opposed to other causes (Scher and Heller, 2000). Therefore, metastatic PCa remains the main cause of prostate cancer–related death in men. A mechanism that may facilitate this progression to a more aggressive phenotype is epithelial-mesenchymal transition (EMT), during which epithelial cell layers lose polarity and cell–cell contacts and undergo remodeling of the cytoskeleton thus permitting cell migration (Lawrence et al., 2007). These changes are accompanied by downregulation of epithelium-specific genes, such as E-cadherin (*CDH1*), and increased expression of genes encoding mesenchymal markers like vimentin (Lee et al., 2006). Snail (*SNAI1*), located on chromosome 20q13, is a master regulator of EMT during embryonic development and tumor progression (Nieto, 2002). The genomic region harboring the gene is reported to show copy number gain in PCa (Torring et al., 2007). Of note, Heeboll et al recently showed that gene-amplification in the region leads to Snail overexpression in PCa (Heebøll S et al., 2009). Interestingly, comparative genomic hybridization showed that gains of chromosomes 5q, 8q and 20 in human prostate epithelial cells (HPEC) are nonrandomly associated with bypassing senescence (Jarrard et al., 1999).

Snail-induced EMT converts epithelial cells into mesenchymal cells with migratory properties. In this fashion Snail contributes to the formation of many tissues during embryonic development and to the acquisition of invasive properties in epithelial tumors (Barrallo-Gimeno and Nieto, 2005). Snail family members have also been reported to exert additional functions beyond EMT induction (Barrallo-Gimeno and Nieto, 2005; Thiery et al., 2009). Snail can behave as a cell cycle regulator through repression of cyclin D2 and induction of $p21^{CIP1}$ expression (Vega et al., 2004), albeit in a cell type and context-dependent manner (Olmeda et al., 2007a). Moreover, Snail has been characterized as an antiapoptotic factor. Cells expressing Snail show an increased ability to survive serum deprivation and resist apoptosis induced by genotoxic agents and other proapoptotic stimuli (Kajita et al., 2004; Vega et al., 2004).Thus, it has been proposed that the most pertinent function of Snail might be to regulate cell adhesion rather than to induce frank EMT (Barrallo-Gimeno and Nieto, 2005; Thiery et al., 2009).

Snail is overexpressed in PCa, but since overexpression correlates neither with propensity to metastasis nor recurrence, it has been suggested that Snail may play a role in the early progression of the disease (Heebøll S et al., 2009). However, its biological functions in the progression of PCa have not been systematically investigated. Therefore, we have performed loss of function studies by transient *SNAII* interference in two metastatic prostate cancer cell lines, LNCaP and PC-3, which differ with respect to androgen responsiveness and tumorigenicity (Stewart et al., 2004) and their genetic background (Jarrard et al., 1999). We present evidence that Snail knock-down significantly reduces the viability of both cell lines, but does not alter the cell cycle distribution of either cell line even at minimal serum concentration. The most dramatic effect observed was that SNAI1-siRNA-treated

LNCaP cells did not tolerate detachment from the extracellular matrix and became rapidly unable to reattach to the surface, an effect we have traced to regulation of integrin α6. In addition, suppression of Snail expression induced senescence in LNCaP cells. Together, our data suggest that Snail primarily acts as a survival factor and inhibitor of cellular senescence, general prerequisites for malignant conversion. This could indicate that Snail may constitute an early driver of cancer progression in the prostate.

Materials and methods

Cell lines and cell cultivation. The prostate carcinoma cell lines LNCaP, PC-3 and its three derivatives PC3-13, PC3-16 and PC3-24 (Lensch et al., 2002), 22Rv1, and DU145 were cultured in RPMI-1640 (Gibco Life Technologies, Karlsruhe, Germany), supplemented with 10% fetal calf serum and 100 U/ml penicillin, 10 µg/ml streptomycin. MDA-PCA-2b was cultured as described (Wlazlinski et al., 2007). Normal prostate epithelial cells (PrECs) were cultured in Clonetics PrEGM basal medium with recommended supplements (Cambrex, Verviers, Belgium).

RNA interference. Double-stranded, short (21-mer) interfering RNA (siRNA) corresponding to *SNAI1* mRNA and an irrelevant siRNA were designed with the following sense and antisense sequences (Jafarnejad et al., 2008) and purchased from MWG-Biotech (Ebersberg, Germany):

SNAI1:

Sense: CACGAGGUGUGACUAACUAdTdT

Antisense: UAGUUAGUCACACCUCGUGdGdA

Irrelevant:

Sense: CUGAUGCAGGUAAUCGCGUdTdT

Antisense: ACGCGAUUACCUGCAUCAGdTdT

Cells were harvested with 0.25% trypsin, 1 mM EDTA in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} and plated in six-well plates at 15×10^4 cells per well. The next day, when the cultures were 30–50% confluent, Cells were transfected with 25 nM siRNA using Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany) transfection reagent according to the manufacturers' instruction. Forty-eight and 72 hours after transfection, cells were used for further experiments. For determining the long-term effect of Snail knock-down, the cells were transfected again after 3 days and cultured for two further days before harvesting for different assays.

RNA extraction. Total RNA was isolated from sub-confluent cell cultures and cell lines using Qiazol reagent (Qiagen, Hilden, Germany) and purified via RNeasy columns (Qiagen, Hilden, Germany) and was further purified by in-column DNase I digestion (Qiagen, Hilden, Germany). Synthesis of cDNA was performed using SuperScriptII reverse transcriptase (Invitrogen, Karlsruhe, Germany) with oligo-dT and random primers, according to the manufacturer's protocol.

RT-PCR. Real-time RT-PCR assays were performed using the LightCycler II apparatus (Roche, Mannheim, Germany). Real-time RT-PCR for Integrin α6 (ITGA6), Integrin β4 (ITGB4), Fibronectin (FN1,) GAPDH and TBP mRNAs was performed using specific Quantitect primer assays (Qiagen, Hilden, Germany) with the *QuantiTect SYBR Green PCR Kit* (Qiagen, Hilden, Germany). Real-time RT-PCR

for Snail (SNAI1), Slug (SNAI2), E-cadherin (CDH1), Keratin-18 (KRT18), β-glucuronidase (GUSB) and Vimentin (VIM) mRNAs was done using specific primers (Table 1) with the LightCycler-FastStart DNA Master PLUS SYBR Green I kit (Roche, Mannheim, Germany). The PCR for the five latter genes included an initial denaturation step at 95°C for 10 mins, followed by 45 amplification cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. All measurements were performed in at least duplicates and the calculated final values regularly exhibited a less than 10% difference.

Table 1

Western blotting. The cytoplasmic and nuclear proteins from cells were prepared using a commercially available nuclear extraction kit (Active Motif, Carlsbad, CA, USA). The nuclear proteins were resolved in a 12% Tris–HCl gel (Biorad, Hercules, CA, USA) and electrotransferred onto Immobilon-P membranes (Millipore, Hamburg, Germany). Blots were blocked for 1 h with 5% milk powder in PBST. Samples were then probed with polyclonal antibody against Snail (1:1000; Santa Cruz, CA), monoclonal antibody against TBP (1:2000; Abcam, UK), mouse monoclonal antibody against E-cadherin (1:5000, Santa Cruz, CA), goat polyclonal antibody against α-tubulin (1:10000, Santa Cruz, CA) and subsequently with the horseradish peroxidase (HRP)-conjugated anti-rabbit (1:5000; Santa Cruz, CA) or HRP-conjugated secondary anti-mouse (1:5000; Santa Cruz, CA) antibodies. Staining was visualized using a chemiluminescence kit (Amersham Biosciences, Freiburg, Germany). Quantitation of western blot results was performed by Labimage software (version 2.6; Kapelan, Germany).

Viability assay. Cell viability was determined by the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) according to the manufacturer's instruction. Results were based on four different experiments.

Caspase assays. Apoptosis was quantified by measurement of caspase-3 and caspase-7 activities with the Caspase-Glo 3/7 reagents (Promega, Mannheim, Germany). Briefly, after adding Caspase-Glo 3/7 reagents to the cell suspension and incubating at room temperature for 1 h, luminescence of each sample was measured by a plate reader luminometer (Victor2, Perkin-Elmer). Following subtraction of blanks, values were normalized to cell numbers. Each sample was measured in quadriplicate.

Cell cycle analysis. For FACS analysis, cells were harvested by trypsinization 72 h after the first transfection or 48 h after the second transfection, washed with PBS, then stained with 50 µg/ml propidium iodide solution containing 0.1% triton X-100 and sodium citrate as described (Janssen et al., 2007) and were analyzed for cell cycle distribution using a FACSCalibur instrument (Becton Dickinson; Heidelberg, Germany). Cell cycle profiles were analyzed using CellQuestPro software.

Detachment-reattachment assay. Cells were harvested by trypsinization 48 h after first transfection, and then suspended in a bacterial plate for a maximum of 7 h. At indicated times (1 h intervals), treated and untreated cells were transferred to a new 6-well cell culture plate and allowed them to grow until the mock-treated cells became confluent. Then cells were stained with Giemsa.

Cell surface staining and flow cytometry. Cells (15×10^5) were incubated with monoclonal antibody against integrin α6 antibody (1:1000; Abcam, UK) for 15 min at 4°C, washed with FACS buffer (PBS with 2% BSA), incubated for another 15 min with Alexa Fluor 488-conjugated goat anti-mouse IgG (1/1000, Invitrogen, Karlsruhe, Germany), and after further washing analyzed in a FACScan cytometer (Becton Dickinson; Heidelberg, Germany).

Senescence associated (SA)-β-galactosidase assay. Staining for SA-β-galactosidase was done according to a previously published protocol (Dimri et al., 1995).

Migration Assay. 72 h after transfection, the experiment was started by scratching a gap into the monolayer and following the closure of the gap by microscopy, taking photographs at regular intervals.

Matrigel Invasion Assay. In vitro invasiveness was determined by a Matrigel invasion assay, performed essentially as described (Engers et al., 2001).

Statistical analyses. All experiments were replicated two or three times, and statistical significance was measured by using the students t-test. *P* values of < 0.05 were considered as statistically significant.

Results:

Expression of Snail mRNA in normal prostate epithelial cells and carcinoma cell lines. We investigated expression of *SNAI1 (*Snail*)* mRNA by qRT-PCR in Fig 1

established prostate carcinoma cell lines as well as normal prostate epithelial cells (Fig. 1). Among several house-keeping genes (TBP, GUSB and GAPDH), TBP was found to show the lowest variation during different experimental conditions and used as a reference gene throughout. Of eight carcinoma cell lines, LNCaP showed the highest *SNAI1* expression. In contrast, *SNAI1* expression was very low in normal epithelial cells (PrECs) and low in the MDA-PCA-2b, Du145 and 22Rv1 carcinoma cell lines (Fig. 1). For knock-down experiments, we selected the LNCaP and PC-3 cell lines, with the highest and moderate expression of *SNAI1*.

Specific downregulation of Snail expression by siRNA treatment. Analysis by RT-PCR demonstrated that application of SNAI1-siRNA resulted in a dramatic reduction in *SNAI1* mRNA (average 80%, $p<0.05$), compared to IR-siRNA treated cells (Fig. 2a). The designed siRNA against *SNAI1* specifically interfered with human *SNAI1* mRNA without affecting the expression of the closely related *SNAI2* (Slug) gene, except in PC-3 cells, where an about 30% reduction was observed (Fig. 2b). To assess whether the observed decrease in mRNA is followed by a similar diminution of the protein level, western blotting was performed. As evident in figure 2c and d, Snail protein was successfully down-regulated after treating LNCaP cells with specific SNAI1-siRNA, but only weakly in PC-3. Quantitation with TBP as a standard showed the protein levels were reduced by 40% in LNCaP and 22% in PC-3 cells.

Fig 2

Varied changes in EMT markers after Snail RNAi treatment. Because Snail is an EMT inducer (6), the effect of SNAI1 knock-down on the epithelial markers (E-cadherin (*CDH1*) and keratin 18 (*KRT18*) and the mesenchymal markers (vimentin (*VIM*) and fibronectin (*FN1*)) was analyzed. *CDH1* expression increased mainly in

LNCaP cells (Fig. 3a and 3e); while *KRT18* (Fig. 3b) and *VIM* (Fig. 3c) showed the expected upregulation and downregulation, respectively, only in the PC-3 cell line, despite the weaker decrease in Snail protein in that line. *FN1* expression increased significantly in LNCaP cells ($p<0.05$), whereas no significant change was observed in the PC-3 cell line (Fig. 3d).

Fig 3

Reduced cell viability after Snail suppression. The effect of Snail knock-down on cell viability was measured by an assay, which determines the number of viable cells based on overall ATP amounts. There was a significant 30% - 79% reduction in the relative ATP amount of *SNAI1*-siRNA treated cells compared to cells treated with IR-siRNA after long-term knockdown of Snail for 5 days (Fig. 4).

Fig 4

Increased apoptosis after repeated Snail siRNA treatment. To explore the cause of reduced cell numbers after the second SNAI1-siRNA treatment, caspase-3 and caspase-7 activities were measured by a highly quantitative luminescence-based assay. SNAI1-siRNA-treated PC-3 and LNCaP cells showed a doubling of caspase-3/7 activities compared to cells treated with IR-siRNA (Fig. 5).

Fig 5

Unchanged cell cycle distribution after Snail knock-down. Potential alterations of the cell cycle after treatment with *SNAI1*- and IR-siRNAs were investigated by flow cytometry. Fig. 6 shows that Snail siRNA treatment did not alter the cell cycle of the cell lines 72 h after transfection.

Fig 6

Reduced viability of LNCaP cells maintained in low serum after SNAI1-siRNA treatment. To analyze the response to serum deprivation, 72 h after transfection cells

were placed into a medium containing minimal FCS (0.5%). After 48 h, there was a small, but significant reduction in the ATP amounts in SNAI1-siRNA treated LNCaP cells compared to the cells treated with IR-siRNA ($17 \pm 2 \times 10^3$ versus $23 \pm 6 \times 10^3$, $p<0.05$). PC-3 cells did not show a similar effect. We also looked for potential changes in cell cycle distribution under these circumstances, but did not detect any significant effect on the cell cycle of PC-3 and LNCaP cells (data not shown).

Blocked re-adhesion of prostate cancer cells by Snail knock-down. To analyze the response to detachment from extracellular matrix, transfected cells were kept in suspension and at 1 h intervals *SNAI1*-siRNA-treated and control cells (IR-siRNA-treated or mock-treated) were transferred to a new 6-well plate and allowed to grow until the mock-treated cells became confluent. *SNAI1*-siRNA-treated LNCaP cells did not tolerate detachment from the extracellular matrix and immediately after detachment became unable to reattach to the surface, resulting in a lack of cell colonies (Fig. 7a). The effect on PC-3 cells was not as potent as the effect on the LNCaP cells, although the cell number decreased considerably in SNAI1-siRNA-treated PC-3 cells (Fig. 7b).

Fig 7

Downregulation of integrin α6 expression after suppression of Snail. To determine a plausible mechanism for the inability to reattach after Snail knock-down, we measured the expression of integrins α6 (*ITGA6*) and β4 (*ITGB4*) at the mRNA level, before and after the cells were detached from the extracellular matrix. Expression of *ITGA6* was considerably diminished by SNAI1-siRNA in both cell lines ($p>0.05$) (Fig. 8a), whereas expression of *ITGB4* increased significantly in LNCaP cells while decreasing in PC-3 cells ($p<0.05$) (Fig. 8b). We also measured the

Fig 8

expression of integrin α6 at the protein level by cell surface staining. Fig. 9 shows that integrin α6 became downregulated after Snail knockdown in both LNCaP and PC-3 cells.

Fig 9

Induction of senescence by knockdown of Snail in LNCaP cells. Morphological inspection of the three cell lines transfected with *SNAI1*-siRNA showed that many LNCaP cells became large and flat and exhibited enlarged nuclei. These features are hallmarks of senescence. This effect was not seen in the other two cell lines. To verify this finding, we stained the cells for senescence-associated β-galactosidase (SA-β-gal), a specific marker for senescent cells. In IR-siRNA treated LNCaP cells, less than 5% stained positive for SA-β-gal, as compared to up to 30% of *SNAI1*-siRNA-treated LNCaP cells in some wells ($p<0.05$) (Fig. 10). As expected from the morphology, only a few PC-3 cells were positive for SA-β-gal, independent of siRNA treatment (data not shown).

Fig 10

Lack of effect of Snail suppression on invasive behavior of PC-3 cells. To determine the effect of Snail knockdown on invasiveness of PC-3 cells, we performed a Matrigel invasion assay. Invasiveness of PC-3 cells was not significantly affected and in fact, a slightly higher number of cells treated with SNAI1-siRNA invaded the gel (Fig. 11). To substantiate this somewhat unexpected result, we investigated the effect of Snail knockdown on the migration of LNCaP and PC-3 cells in a 'scratch' assay. Over a period of two days, we did not observe substantial differences in the ability of cells pretreated with SNAI1 siRNA for 3 d to close a gap in the monolayer compared to cells treated with IR siRNA or mock-treated. Instead, as shown in the

Fig 11

above experiments, we observed a higher number of cells with morphological signs of senescence or apoptosis in the siRNA-treated cultures (data not shown).

Discussion

Snail has been identified as a master regulator of EMT and it functions as a prime promoter of metastasis in a variety of cancer types (Barrallo-Gimeno and Nieto, 2005; Becker et al., 2007). Snail/*SNAI1* expression has been observed in breast (Blanco et al., 2002), ovarian (Elloul et al., 2005), endometrial (Blechschmidt et al., 2007), hepatocellular (Miyoshi et al., 2005), colorectal (Roy et al., 2005), esophageal (Takeno et al., 2004), adrenocortical (Waldmann et al., 2008), thyroid (Hardy et al., 2007), skin (Olmeda et al., 2008) and head and neck (Yang et al., 2007) malignancies and is often associated with progression towards metastasis. Recently, Heebøll et al (Heebøll S et al., 2009) also demonstrated Snail overexpression in prostate cancer: Interestingly, Snail overexpression was not correlated to cancer progression or prognosis, but rather appeared to represent a relatively early event in the progression of the disease. As detailed below, our present study, the first one on the function of Snail in prostate cancer, provides an explanation for this unexpected observation. We used two metastatic prostate cancer cell lines, LNCaP and PC-3, differing in the level of Snail expression, androgen responsiveness and tumorigenicity and genetic background (Jarrard et al., 1999; Stewart et al., 2004), to investigate the effect of Snail knockdown on various cellular properties associated with tumorigenicity.

One major result of our study is that knock-down of Snail induced senescence in LNCaP cells. It seems therefore that Snail is sufficient to prevent stress-induced premature senescence (as defined by (Hornsby, 2007)) in the LNCaP cell line. This function has previously been reported to be exerted by other master regulators of EMT, Twist1 and 2 (Ansieau et al., 2008) and Zeb1 (Liu et al., 2008), which inhibit

premature senescence in both murine and human cells, but this is the first instance of this observation for Snail. Unexpectedly, induction of senescence markers in LNCaP cells was not associated with a prominent cell cycle arrest. On the other hand and in contrast to LNCaP cells, knock-down of Snail in PC-3 cells did not lead to premature senescence. These two cell lines differ in respect to their p16, retinoblastoma (Rb) and p53 status, crucial gatekeepers of cellular senescence (Jarrard et al., 1999). In comparison between the two pathways (p16/pRb and p14/p53/p21) governing cellular senescence (Caino et al., 2009; Ohtani et al., 2009), p16 may often be most important in human cells (Ohtani et al., 2009). In particular, cellular senescence in human prostate epithelial cells is accompanied by increasing p16 levels (Jarrard et al., 1999; Sandhu et al., 2000) and p16 is the primary factor limiting the proliferative capacity of these cells (Bhatia et al., 2008). Introduction of p16 induces cell cycle arrest only in cells retaining functional pRb (Lukas et al., 1995). Therefore, since LNCaP cells lack pRb despite functional p16 (Jarrard et al., 1999), induction of senescence markers in these cells may be uncoupled from a prominent cell cycle arrest. On the other hand, the lack of induction of senescence in PC-3 cells which lack p16 expression (Jarrard et al., 1999) may be due to the inactivation of cellular components necessary for inducing senescence in PC-3 cells, especially p16 and also p53, which contains a frameshift mutation in this line. Alternatively, it could be due to the weaker expression of Snail protein in PC-3 cells overall and a more limited decrease after siRNA treatment at the protein level. Specifically, in PC-3 cells, we could detect Snail protein only in nuclear extracts, and the sensitivity of western blotting was not sufficient for whole cell lysates.

Prostate epithelial cells undergo cellular senescence *in vitro* (Jarrard et al., 1999; Sandhu et al., 2000) and *in vivo* (Castro et al., 2003; Choi et al., 2000).

Occurrence of cellular senescence may act as a tumor suppressor mechanism that inhibits progression of benign to malignant lesions (Ohtani et al., 2009). In this regard, bypassing the barrier of senescence is an early event during cancer progression. In accord with this notion, Heebøll et al (Heebøll S et al., 2009) recently suggested that increased expression of Snail is a relatively early event in the progress of the prostate cancer. Their results indicate that Snail expression is upregulated at an early stage of prostate cancer development. Taken together, our findings strengthen the idea that by controlling two safeguard mechanisms against cancer (cellular senescence and apoptosis) in addition to EMT proteins like Snail, Twist and Zeb1 can exert a double driving effect on tumor progression (Smit and Peeper, 2008; Thiery et al., 2009). Specifically, it has been proposed (Ansieau et al., 2008) that the dissemination of primary tumor cells undergoing EMT at the invasive edge may commence early in response to environmental cues, coupling metastatic spread to the bypass of senescence.

Loss of E-cadherin is found in many cancers and a main phenotypical characteristic of the EMT (Guarino et al., 2007). In cultured stomach, liver, colon, ovarian, and breast cancer cell lines, expression of Snail has been shown to downregulate E-cadherin and to induce an invasive, mesenchymal phenotype (Barrallo-Gimeno and Nieto, 2005; Peinado et al., 2004; Thiery, 2002). In keeping with these data, our results show that suppression of Snail expression in both cell lines led to an elevation of *CDH1*/E-cadherin at both transcript and protein levels. The effects on other EMT markers, e.g. vimentin, were more variable. In this respect, it has to be considered that EMT can be regulated by several factors which may be differently expressed between the cell lines so that Snail is not a decisive factor for the expression of each individual gene involved. Specifically, Slug is expressed at a

much higher level in PC-3 cells than in LNCaP cells (Emadi-Baygi et al, unpublished data). Moreover, Snail can upregulate Slug (Aybar et al., 2003; Barrallo-Gimeno and Nieto, 2005; Olmeda et al., 2007b) in line with our observation that Slug mRNA is decreased after SNAI1 depletion in PC-3 cells. Finally, it has recently been proposed that Snail proteins may require the cooperation of tissue specific binding proteins (Thiery et al., 2009), which should also differ between the two lines. Of note, knocking-down of Snail did not affect migratory ability of the prostate cancer cells and in the same vein did not abolish invasive behavior of metastatic hormone-refractory PC-3 cells. A limited function of Snail in invasiveness and metastasis of prostate cancer would account for the lack of relation between Snail expression and tumour metastasis or recurrence in prostate cancer observed by Heebøll et al (Heebøll S et al., 2009). In a very recent study, Baritaki et al (Baritaki et al., 2009) found that overexpression of Snail, especially in its mutant form CMV-f-SnailS6A, promoted EMT in LNCaP cells complementing our siRNA results. They also observed that downregulation of Snail diminished several EMT markers in the Du145 cell line. Although Du145 is an untypical prostate cancer cell line lacking recognizable prostate markers and derived from a brain metastasis, this finding suggests that the function of Snail in individual prostate cancers could vary, likely depending again on the modulating factors discussed above.

Our data, however, implicated an important function of Snail in regulating adhesion of prostate cancer cells. Transient suppression of Snail resulted in reduced expression of integrin α6, elevated expression of integrin β4 (especially in LNCaP cells) and a dramatic decrease in the ability of cells to reattach to the ECM. The *α6β4* integrin is an adhesion receptor for most of the known laminins and expressed

primarily on the basal surface of most epithelia (Kikkawa et al., 2000; Mercurio, 1995; Pouliot et al., 2000). A primary function of *α6β4* is to maintain the integrity of epithelia, especially the epidermis (Dowling et al., 1996; van der Neut et al., 1996). This critical role for *α6β4* derives from its ability to mediate the formation of hemidesmosomes on the basal cell surface that link the intermediate filament cytoskeleton with laminins in the basement membrane (Borradori and Sonnenberg, 1999; Green and Jones, 1996). These stable hemidesmosomal cell attachment sites appear to restrict cell migration (Edlund et al., 2001). Consequently, it seems possible that in PC-3 cells downregulation of Snail leads to downregulation of *α6β4*, accounting for the observed slight increase in the invasiveness of the transfected cells. The elevated expression of integrin β4 in LNCaP cells could reflect a compensatory response to down-regulation of α6, since α6 preferentially partners with β4 (Edlund et al., 2001). Our data suggest that integrin α6 is a target of Snail and in the light of the reports discussed above it seems very likely that down-regulation of *α6* integrin after Snail knockdown is a decisive factor influencing cell attachment and survival. Analysis of the genes encoding integrin α2 (Zutter et al., 1994), α3 (Kato et al., 2002), α6 (Lin et al., 1997; Nishida et al., 1997) as well as β1 (Cervella et al., 1993) and β4 (Takaoka et al., 1998) has revealed the presence of potential E-boxes in all five promoters, suggesting that they might represent Snail targets. However, in contrast to our data, Haraguchi et al (Haraguchi et al., 2008) recently reported that basement membrane (BM) proteins such as laminin α3, β3 and γ2 (Laminin-5/LN-5) and receptors for LN-5 such as integrins α3, α6 or β4 are down-regulated in Snail-overexpressing canine MDCK kidney or human A431 lung carcinoma cells. These authors also reported that Snail did not confer resistance to anoikis induced by loss of contact to substrate but instead enhanced cell attachment to extracellular matrices,

especially fibronectin (Haraguchi et al., 2008). These apparent discrepancies in relation to Snail function may reflect the specific contribution of different cellular contexts or the complexity in its regulation and action (Becker et al., 2007; Thiery et al., 2009). Not least, overexpression experiments with a pleiotropic factor like Snail may yield different results from knockdown experiments achieving reduction of physiological levels. Obviously, the increased propensity to undergo apoptosis as a consequence of Snail downregulation may also contribute to the decreased adhesion and clone formation ability in our experiments, the more so as integrin downregulation is expected to influence both adhesion and survival signaling.

Finally, our data demonstrate that knockdown of Snail significantly reduced the viability, as measured by total ATP, of both prostate cancer cell lines (LNCaP and PC-3) in the presence of serum, but did not alter the cell cycle of either cell line. At a minimal serum concentration, the viability of LNCaP cells was further reduced. Accordingly, long-term *Snail* knockdown induced an increase in caspase activities in PC3 and LNCaP cells. However, despite the decrease in total ATP and increased caspase activity, we did not observe an increase in the sub-G1 fraction of LNCaP and PC-3 cells after long-term knockdown of *Snail*. This apparent discrepancy most likely reflects loss of apoptotic cell fragments and cells through a major effect on decreased adherence. Overall, these data are in accord with previous findings according to which cell cycle regulatory effect of Snail take place in a cell type/context dependent manner (Olmeda et al., 2007a) and Snail protects cells from caspase-mediated programmed cell death induced either by the loss of survival factors or by direct apoptotic stimuli (Kajita et al., 2004; Vega et al., 2004).

In summary, our data support the contention that Snail acts primarily as a survival factor and inhibitor of cellular senescence in prostate cancer, contributing

general prerequisites for malignant conversion, in addition to influencing specific aspects of the EMT. Therefore we suggest that in the context of prostate cancer Snail should be investigated more closely as an early driver of tumor progression.

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Figures Legend:

Fig. 1: Expression of *SNAI1* mRNA in normal prostate epithelial cells and carcinoma cell lines. Histograms comparing the relative gene expression of *SNAI1* to *TBP* as determined by quantitative RT-PCR in normal prostate epithelial cells and carcinoma cell lines. Values shown represent the mean ± SEM.

Fig. 2: Efficiency of siRNA-mediated *SNAI1*/Snail suppression

a) Effect of *SNAI1* siRNA treatment on *SNAI1* mRNA in LNCaP and PC-3 cell lines as determined by quantitative RT-PCR 48 h after transfection. For each cell line, the relative expression of *SNAI1* in IR-siRNA treated controls was adjusted to 100. b) Effect of *SNAI1* knock-down on expression of the closely related *SNAI2* gene as determined by quantitative RT-PCR 48 h after transfection. For each cell line, the relative expression of *SNAI2* in IR-siRNA treated controls was adjusted to 100. c and d) Effect of *SNAI1* siRNA on Snail protein levels after 72 h, as determined by western blotting on nuclear extracts. TBP was used as a loading control for western blotting. Values shown represent the mean ± SEM. Statistically significant differences ($p<0.05$) are indicated by an asterisk.

Fig. 3: Expression of different EMT markers after knocking down of *SNAI1* expression

mRNA levels of the genes a) *CDH1*, b) *KRT18,* c) *VIM* and d) *FN1* were measured 48 h after transfection by qRT-PCR and adjusted to *TBP*. For each cell line, the relative expression of the genes in IR-siRNA treated controls was set as 100. Values shown represent the mean ± SEM. Statistically significant differences ($p<0.05$) are indicated

by an asterisk. (e) E-cadherin protein levels 72 h after transfection of the indicated siRNAs in the prostate cancer cells as measured by western blotting; α-tubulin was used as a loading control.

Fig. 4: Effect of Snail knockdown on cell viability in LNCaP and PC-3 cancer cell lines

Suppression of Snail caused a significant reduction in number of viable LNCaP (a) or PC-3 (b) cells as assessed by a luminescence based ATP measurements represented in relative luminescence unit (RLU). Values shown represent the mean ± STD. Statistically significant differences ($p<0.05$) are indicated by an asterisk.

Fig. 5: Elevation of apoptosis after Snail suppression in LNCaP and PC-3 cancer cell lines

Elevation in caspase3/7 relative activities in response to the Snail knockdown in LNCaP (a) and PC-3 (b) cells were determined with Caspase-Glo 3/7 kit as a representative of apoptosis rate. Values were normalized to the viable cell numbers as reported in figure 4 and represented as the mean ± STD.

Fig. 6: Effect of Snail knock-down on cell cycle distribution

The upper panels show the distribution and percentage of cells in the G0/1, S and G2/M phases and the lower panels show the sub-G1 population of cells as depicted by the FL3 filter of the FACS instrument in LNCaP (a) and PC-3 (b) cells 72 h after transfection with SNAI1-siRNA.

Fig. 7: Effect of Snail knock-down on cell survival after detachment of siRNA-treated cells

LNCaP (a) and PC-3 (b) cells treated with SNAI1-siRNA, IR-siRNA or mock-treated were detached from the ECM and kept in suspension for 0 and 3 h before reseeding in culture plates. When the mock-treated cells became confluent, cells were fixed and stained with Giemsa.

Fig. 8: Expression of *INTGA6* and *INTGB4* in prostate cancer cell lines treated with *SNAI1* siRNA

In the cell lines, before and after detachment of siRNA-treated cells from the extracellular matrix, *INTGA6* and *INTGB4* expression was determined by quantitative RT-PCR. For each cell line, the relative expression of the genes in IR-siRNA treated controls was adjusted to 100. Values shown represent the mean ± SEM. Statistically significant differences ($p<0.05$) are indicated by an asterisk.

Fig. 9: Downregulation of integrin α6 after SNAI1-siRNA treatment

Expression of integrin α6 as determined by cell surface staining: Mean fluorescence intensity (MIF) for IR- and SNAI1-siRNA treated cells was calculated by subtracting the MIF of isotype control from each sample.

Fig. 10: Detection of senescence of LNCaP cells after knocking-down of SNAI1 using SA-β-gal as a marker.

a) IR-siRNA treated LNCaP cells and b) SNAI1-siRNA treated LNCaP cells. Photographs were taken at a 400x magnification.

Fig. 11: Effect of Snail knock-down on invasiveness of PC-3 cells

Number of cells having invaded the ECM substrate after siRNA treatment as indicated. The data shown are representative for three independent experiments. Values shown represent the mean ± SEM.

Tables:

Table1: Sequences of RT-PCR primers

Designation	Sequence	Product size (bp)
Snail fw	TTGGATACAGCTGCTTTGAG	150
Snail rv	ATTGCATAGTTAGTCACACCTC	
Slug fw	GAGTCTGTAATAGGATTTCCCATAG	122
Slug rv	CTTTAGTTCAACAATGGCAAC	
E-cadherin fw	ACCAGAATAAAGACCAAGTGACCA	171
E-cadherin rv	AGCAAGAGCAGCAGAATCAGAAT	
Keratin-18 fw	ACACAGTCTGCTGAGGTTGGAG	160
Keratin-18 rv	TGCTCCATCTGTAGGGCGTAG	
Vimentin fw	AATGGCTCGTCACCTTCGTGAAT	159
Vimentin rv	CAGATTAGTTTCCCTCAGGTTCAG	
Beta-glucuronidase fw	CACGACACCCACCACCTACATC	121
Beta-glucuronidase rv	GACGCACTTCCAACTTGAACAG	