Chapter 6

Key regulators of EMT are essential for TGF-β-induced invasion by promoting single cell invasion

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Abstract

TGF-β plays a dual role in cancer; in early stages it inhibits tumour growth, whereas in later stages it promotes invasion and metastasis. Recent data indicate that TGF-β promotes invasion of single cells, which move faster and are able to enter the bloodstream, in contrast to their collectively invading counterparts. TGF-β is thought to be pro-invasive by inducing epithelial-to-mesenchymal transition (EMT). Key players in EMT are transcriptional repressors, such as Slug, Snail and Twist. In this study, we investigated the role of these transcription factors in TGF-β-induced invasion. TGF-β induced the expression of the transcription factors Snail and Slug, but not of Twist in the premalignant H-Ras transformed breast cell line MCF10AT. In consequence, overexpression of Slug or Snail promoted invasion of breast cancer cells in an *in vitro* invasion assay. These cells invaded more as single, rounded amoeboid (cells compared to control cells. Knockdown of Slug inhibited TGF-β-induced invasion of these cells. Furthermore, in an embryonic zebrafish xenograft model, overexpression of Slug and Snail promoted invasion and metastasis. Also here, cells invaded predominantly as single cells. Thus, Slug and Snail appear to be critical mediators of TGF-β-induced invasion *in vitro* and *in vivo*.

Introduction

Transforming Growth Factor-β (TGF-β) is a pleiotropic cytokine involved a multitude of biological processes. Dysregulation of TGF-β signaling has been observed in several diseases, such as fibrosis and cancer [1]. In cancer, TGF-β plays a dual role; in early stages it inhibits tumour growth, whereas in later stages it promotes invasion and metastasis [2]. In line with its oncogenic role, TGF-β is frequently overexpressed in breast cancer [3-5]. Furthermore, inhibition of TGF-β signaling in breast cancer reduces metastasis in several mouse models of breast cancer [6-9].

TGF-β signals through a heteromeric receptor complex, composed of the TGF-β type I receptor Activin-receptor like kinase (ALK) 5 and the TGF-β type II receptor (TGF-βRII).

Within this complex, the TGF-βRII phosphorylates ALK5, which on its turn phosphorylates

Smad2 and Smad3. Phosphorylation of Smad2 and Smad3 induces a conformational change, which allows these Smad proteins to form heteromeric complexes with Smad4. This complex transclocates to the nucleus, where it affects transcription of target genes [2,10-12]. In addition, receptor activation also results in non-Smad signaling, such as the mitogen activated protein kinases (MAPK) pathway [13].

TGF-β is thought to be pro-invasive by inducing epithelial-to-mesenchymal transition (EMT). During EMT, carcinoma cells acquire a more motile, mesenchymal phenotype. This process is marked by the loss of epithelial markers, such as E-cadherin, zona occludens (ZO)-1, EPCAM and keratin 18 (KRT18), and induction of mesenchymal markers, such as α-smooth muscle actin (α-SMA), vimentin and N-cadherin. Several transcription factors are able to downregulate E-cadherin expression by binding to E-boxes in the E-cadherin promotor, such as Snail and Slug. Snail and Slug are zinc finger proteins of the Snail family that recognize E2 box type elements C/A (CAGGTG. Furthermore, the bHLH factor Twist is also able to induce EMT, although it does not directly regulate E-cadherin [14]. Besides E-cadherin, Snail and Slug transcription factors downregulate components of the adherens junctions: desmosomes, polarity proteins and miRNAs [15]. TGF-β regulates EMT by inducing these transcription factors. Furthermore, Smad proteins also are able of interact with these transcription factors and jointly regulate target genes [16].

Although studies *in vitro* clearly have demonstrated that cancer cells are able to undergo EMT, the question if EMT also occurs in cancer *in vivo* has been subject of debate. The presence of more mesenchymal cells at the invasive edge suggested that EMT might occur in this area of the tumour [17]. Furthermore, cancer cells which have undergone TGF-β-induced EMT closely resemble cancer stem cells, a few cells of which can give rise to a whole new tumour [18]. Further studies revealed that Snail is partially responsible for the TGF-β-induced stem cell phenotype [19]. These studies strongly suggest the occurrence of EMT in cancer.

Whether EMT is necessary for invasion remains to be established. Cancer cells expressing E-cadherin have been observed to invade as a sheet of cells [20]. However, intravital imaging studies have shown that single breast cancer cells move faster than these sheets

and these individually moving cells had active TGF-β signaling [21]. Thus, EMT might be important in TGF-β-induced invasion. Therefore, we studied the role of key players of EMT, namely Snail, Slug and Twist, in TGF-β-induced invasion.

Materials & Methods

Cell culture

H-Ras transformed MCF10AT (M-II) were obtained from Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, USA) cultured and maintained as described before [22].

RNA isolation, cDNA synthesis and Q-PCR

2 x 10^5 cells were seeded onto a six well plate and one day later starved for 16 hr DMEM/F12 (Gibco/Invitrogen) containing 2.5% horse serum (Gibco/Invitrogen), 10ng/ml epidermal growth factor (EGF) (Upstate), 50ng/ml cholera toxin (Calbiochem), 0.25μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 100U/ml penicillin and 50μg/ml streptomycin (Gibco/Invitrogen). After starvation, cells were stimulated for 24 hr with no ligand or recombinant human TGF-β3 (generous gift of Dr. K. Iwata, OSI Pharmaceuticals, Inc, New York, USA). RNA isolation was performed using a RNA extraction kit (Macherey-Nagel) according to manufacturer's instructions. cDNA synthesis and quantitative real time PCR were performed as previously described [23], on a StepOne Plus (Applied Biosystems) instrument and Sybr Green (Roche) as detection reagent. All samples were analyzed in triplicate for each primer set. Gene expression levels were determined with the comparative Δ Ct method using *ARP* or β-actin as reference and the non-stimulated condition was set to 1. Primers used are listed below.

Gene	Forward	Reverse
hARP	5'-CACCATTGAAATCCTGAGTGATGT -3'	5'-TGACCAGCCGAAAGGAGAAG -3'
hβ-actin	5'- AATGTCGCGGAGGACTTTGATTGC -3'	5'-AGGATGGCAAGGGACTTCCTGTAA-3'
hSLUG	5'-ATGAGGAATCTGGCTGCTGT-3'	5'-CAGGAGAAAATGCCTTTGGA -3'
hSNAIL	5'-GCTGCAGGACTCTAATCCAGAGTT-3'	5'-GACAGAGTCCCAGATGAGCATTG-3'
hTWIST	5'-GACAGAGTCCCAGATGAGCATTG-3'	5'-TTCTCTGGAAACAATGACATCTAGGT-3'
mSnail	5'- TCCAAACCCACTCGGATGTGAAGA-3'	5'- TTGGTGCTTGTGGAGCAAGGACAT-3'
mSlug	5'-CACATTCGAACCCACACATTGCCT-3'	5'- TGTGCCCTCAGGTTTGATCTGTCT-3'
hEPCAM	5'-CTTTATGATCCTGACTGCGATGAG-3'	5'-TCAGTGTCCTTGTCTGTTCTTCTGA -3'
hKRT18	5'-TGGCGAGGACTTTAATCTTGGT-3'	5'- ACCACTTTGCCATCCACTATCC-3'

Plasmids and transduction

For overexpression of Snail and Slug, mouse HA-Snail and mouse Slug were cloned into the lentiviral vector CMV-IRES-PURO. Lentiviral constructs expressing shRNA against human Slug and human Snail were from the Mission library (Sigma). The empty vector was used as a negative control. Transduction and selection of transduced cells was performed as described previously [24]. Knockdown and overexpression in M-II cells was verified by Q-PCR.

Spheroid invasion assay

The invasion spheroid assay was performed essentially as described [25]. Briefly, 10^3 cells were allowed to form spheroids in DMEM/F12 containing 5% horse serum and 20% methylcellulose in a 96 well round bottom plate and spheroids were embedded in a 1:1 collagen:methocel mixture onto a collagen coated plate. Recombinant human TGF-β3 was added directly into the collagen/methocel mixture at 4 °C. After 30 minutes DMEM/F12 containing 1.6 % horse serum was added on top of the collagen. Invasion was monitored during the next two days and quantified by measuring the area using Adobe Photoshop.

Zebrafish embryonic xenograft model

Zebrafish and embryos were raised, staged and maintained according to standard procedures in compliance with the local animal welfare regulations. The transgenic line Tg (fli1:GFP) was crossed with the transparent casper mutant. Dechorionized 2d day old zebrafish embryos were anesthetized with 0.003% tricaine (Sigma) and positioned on a 10cm Petridish coated with 1% agarose. M-II cells overexpressing HA-Snail, Slug or the control vector were trypsinized into single cell suspensions, resuspended in PBS (Invitrogen), kept at room temperature before implantation and implanted within 3 hours. Cells were labeled with the fluorescent cell tracker CM-DiI (Invitrogen) according to the manufacturer's instructions. The cell suspension was loaded into borosilicate glass capillary needles (1mm O.D. x 0.78mm I.D.; Harvard Apparatus) and the injections were performed using a Pneumatic Pico pump and a manipulator (WPI). 50~400 cells, manually counted, were injected at approximately 60µm above the ventral end of the duct of Cuvier. Around 200 embryo's were implanted per cell line. After implantation, zebrafish

embryos (including non-implanted controls) were maintained at 34°C.

Invasion and subsequent metastasis was monitored after 1,3 and 5 days by imaging the zebrafish embryos with a confocal microscope. Invasion of perivascular tumour cells into the neighboring tail fin was quantified by counting the number of zebrafish embryo's with invading cells and is presented as the proportion of zebrafish embryo's with invasion over all zebrafish embryo's. Experimental micrometastasis in the tail fin of extravasated cells was quantified by counting the number of zebrafish embryo's in which experimental metastasis occurred and is presented as the proportion of metastasis positive embryo's over total number of zebrafish embryo's.

Statistical analysis

Representative results of single experiments are presented as the mean ± SD. Data shown from multiple independent experiments are shown as the mean ± SEM. Statistical differences were examined by one-way ANOVA followed by Bonferroni's multiple comparison test. $p<0.05$ was considered as statistically significant.

Results

TGF-β induces mRNA expression of Slug and Snail in M-II

To identify which EMT regulators are of importance for TGF-β-induced invasion, we first tested which regulators are induced by TGF-β in this cell line. To this end, we stimulated MII cells with TGF-β (5 ng/ml) and after 24h we analyzed expression of Snail, Slug and Twist by quantitative real time PCR. TGF-β induced Slug mRNA 10 fold, whereas Snail mRNA was induced 5 fold (Figure 1A, B). No effect of TGF-β treatment on Twist mRNA could be detected (figure 1C). Thus, TGF-β induces expression of Snail and Slug, but not Twist in M-II cells.

Overexpression of Slug and Snail enhances invasion by promoting single cell motility

To study the function of Slug and Snail in invasion, we decided to mimic the effect of TGF-β on Slug and Snail expression by overexpression of Slug and Snail. This was achieved by

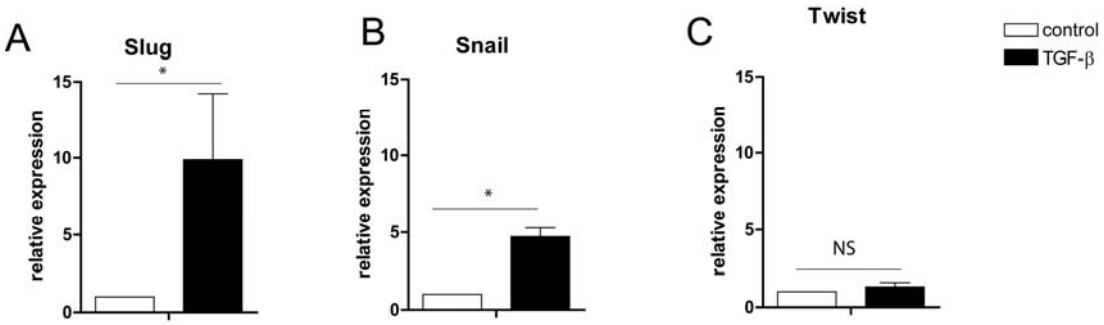

Figure 1 *Induction of Slug, Snail and Twist by TGF-β in M-II.* Spheroids of M-II were embedded with no ligand or TGF-β and RNA was isolated after 24 hr. RNA was analyzed by Q-PCR for Snail (A), Slug (B) or Twist (C). Data shown is the mean of two independent experiments ± SEM. Significance * $p < 0.05$ NS not significant

transduction of M-II cells with vectors containing either no insert (vector), mouse HA-Snail or mouse Slug. Overexpression was verified by quantitative real-time PCR for mouse Snail or Slug (Figure 2A,B). To test whether HA-Snail or Slug overexpression was functional, we performed real-time quantitave PCR for epithelial markers. As expected, EPCAM and keratin 18 (KRT18) were both downregulated by overexpression of HA-Snail or Slug (Figure 2C,D). Overexpression of HA-Snail or Slug did not affect directly TGF-β/Smad signaling, since the induction of the typical Smad target gene PAI-1 was equal in all cells (Figure 2E).

These cells were used in a spheroid *in vitro* invasion assay. Cells were allowed to form spheroids which were subsequently embedded in a collagen matrix in the presence or absence of TGF-β. Invasion into the collagen was measured after 24 hr. Overexpression of HA-Snail or Slug enhanced basal invasion of M-II cells compared to vector control cells, indicating that Snail and Slug mimic the effect of TGF-β on invasion (Figure 3A, B). In addition, invasion of HA-Snail or Slug overexpressing MII cells could be further enhanced by TGF-β, suggesting that other factors than Snail and Slug may also play a role in TGF-β-induced invasion.

When observing the invading spheroids more closely, we noticed that cells overexpressing -HA-Snail or Slug invaded in a different mode compared to control cells. Control cells remained invaded in a manner resembling collective cell migration, keeping cell-cell contacts intact and appearing rather epithelial (Figure 3C), whereas Slug or HA-Snail overexpressing cells invaded as single cells with a more rounded, amoeboid morphology (Figure

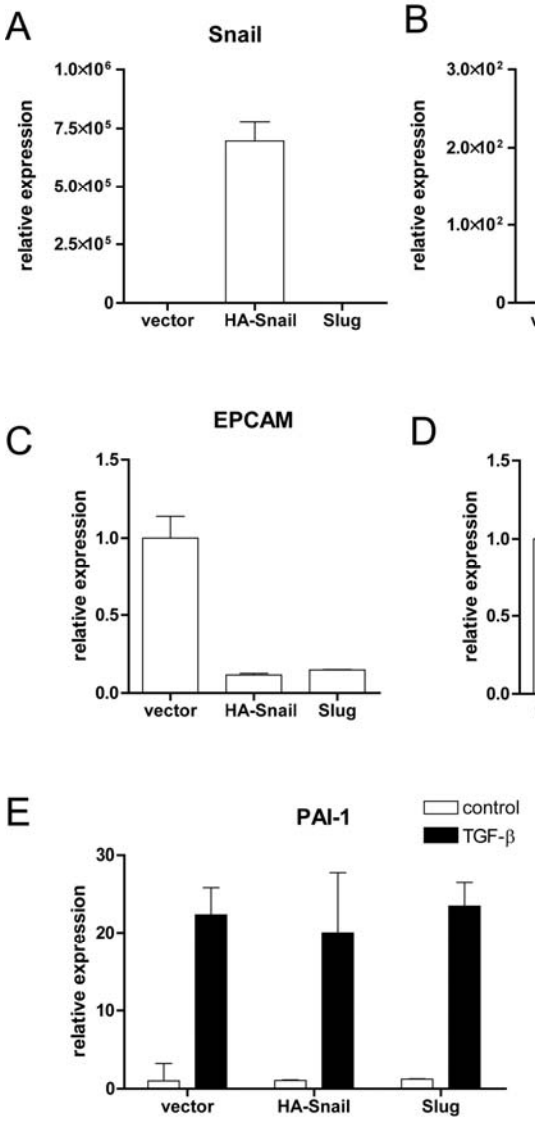

Figure 2 *Functional overexpression of HA-Snail and Slug* M-II cells were transduced with control plasmid, mouse Slug overexpression plasmid or mouse HA-Snail overexpression plasmid. After selection, cells were plated for RNA isolation. mRNA expression was analysed by Q-PCR for A mouse Snail B. mouse Slug C. human EPCAM D. human keratin 18 (KRT18) E. After selection cells were plated, serum starved and stimulated for 2 hr with no ligand or TGF-β (5 ng/ml). RNA was was isolated and human PAI-1 expression was analysed by Q-PCR.

3C). This resembles the individually moving cells with active TGF-β signaling observed previously *in vivo*. These cells moved faster than their collectively invading counterparts [21]. Thus Slug and Snail may promote invasion by inducing single cell motility.

Slug is necessary for TGF-β-induced invasion

To confirm the importance of Slug for TGF-β-induced invasion, we performed shRNA-mediated knockdown experiments. MII cells were infected with a pool of lentiviral viruses expressing five different shRNA constructs for Snail or Slug respectively. Slug knockdown of MII cells was confirmed by quantitative real time PCR (figure 4A). Unfortunately,

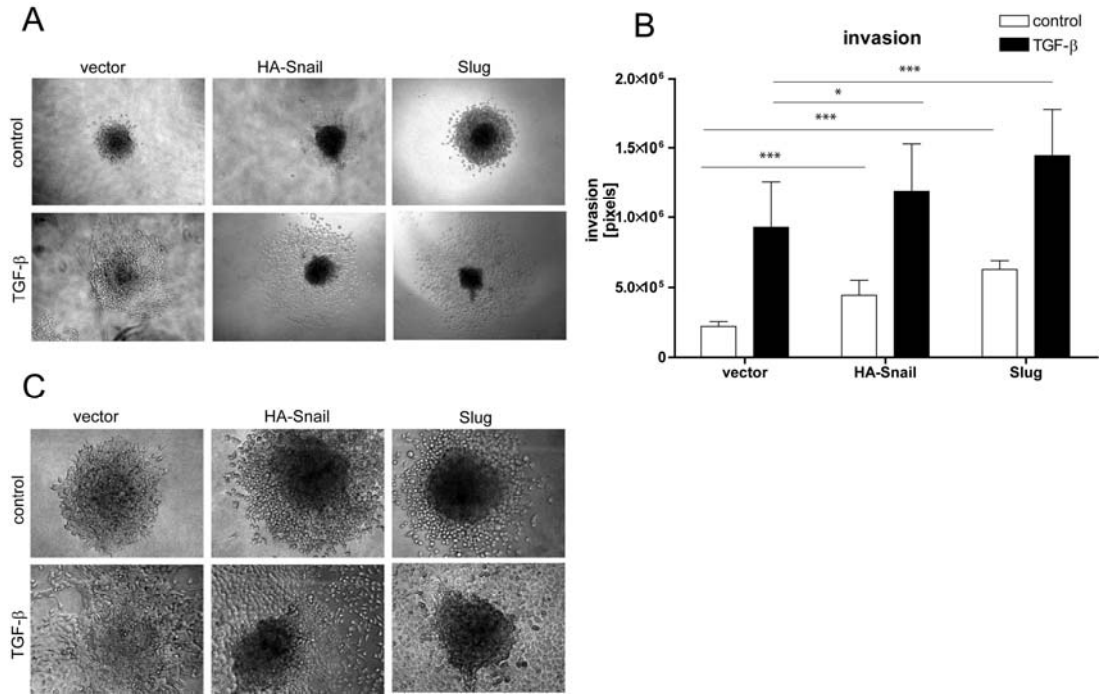

Figure 3 *Overexpression of HA-Snail and Slug enhances invasion* A. Cells overexpressing vector, mouse HA-Snail or mouse Slug were plated 8 hrs after transduction for spheroid culture and selection. These spheroids were embedded and allowed to invade into collagen. C. Quantification of invasion D. Higher magnification (100x) of spheroids after. Data shown is a representative of two independent experiments ± SD. Significance *** $p < 0.001$ * $p < 0.05$

we were not able to achieve sufficient knockdown of Snail (not shown).

To determine if knockdown of Slug affects TGF-β-induced invasion, we used the Slug knockdown cells in the spheroid invasion assay. Slug knockdown reduced basal invasion by 41%. (figure 4B,C). However, this did not reach statistical significance. Slug knockdown did significantly inhibited TGF-β-induced invasion by 66%. Thus, Slug is necessary for TGF-β-induced invasion.

Overexpression of Slug and Snail in M-II cells enhances their invasion and metastatic behaviour in zebrafish embryo's

Having demonstrated a role for Slug and Snail in *in vitro* invasion, we decided to validate the role of Snail and Slug in invasion *in vivo*. To this end, we made use of a zebrafish embryo xenograft model in which cells are injected in the ducts of Cuvier in the yolk sac of zebrafish embryo's, which then disperse through the bloodstream, invade the embryo

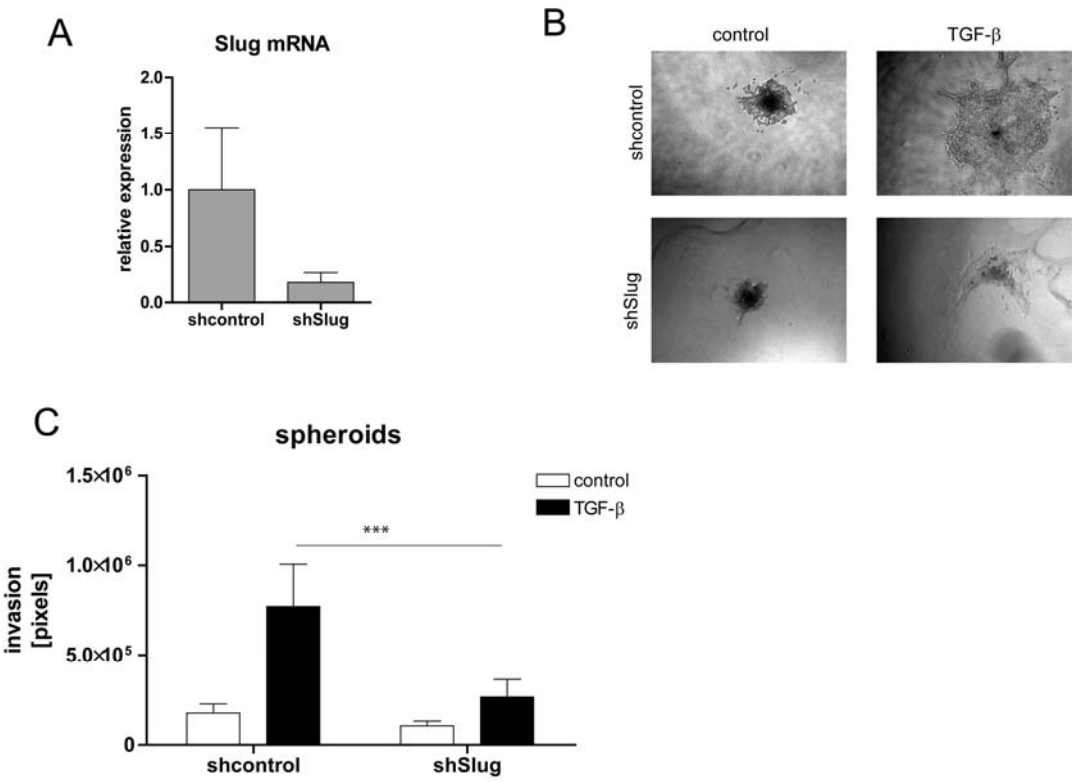

Figure 4 *Slug is necessary for TGF-β-induced invasion in M-II.* A. M-II was transduced with control shRNA or a combination of 5 shRNAs against Slug. After selection, knockdown efficiency was determined by Q-PCR. B. These cells were used for the spheroid invasion assay. C. Quantification of spheroids. Data shown is a representative of two independent experiments ± SD. Significance *** $p < 0.001$

an subsequently grow into metastasis [26]. When we injected M-II cells that overexpresses HA-Snail or Slug, we observed a dramatic increase in the amount of embryo's in which cells were invading (Figure 5A). This increase was evident from day 3 after injection, with only 13% of control embryo's showing invasion, whereas 31% and 39% of the embryos injected with HA-Snail and Slug, respectively, showed invasion. After 5 days, 38% of the control embryos showed invasion whereas 70% and 55% of the embryos injected with HA-Snail and Slug cells, respectively, showed invasion. Furthermore, the amount of embryo's displaying metastasis was also increased in cells overexpressing HA-Snail or Slug. While metastasis in embryo's injected with controls cells was rare after 3 days (0.29%) or 5 days (0.58%), embryo's injected with HA-Snail or Slug showed a more than tenfold increase in metastasis after 3 days (2.2% and 4.1%, respectively). This dramatic increase was

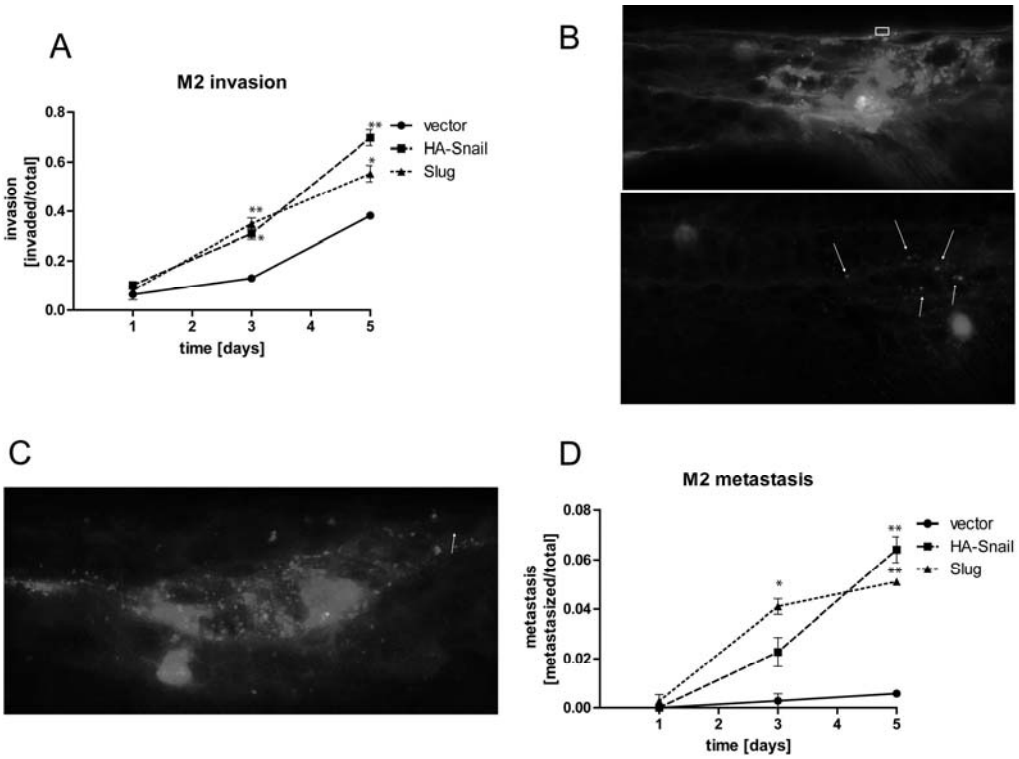

Figure 5 *Overexpression of HA-Snail and Slug enhances invasion and metastasis in vivo* M-II cells overexpressing HA-Snail, Slug or the control vector were dyed with CmDil and injected into the ducts of Cuvier of 2 days old Fli-GFPxCasper zebrafish embryo's. Cells were monitored after 1,3 and 5 days by imaging the zebrafish embryos with a confocal microscope. A. Invasion was quantified by counting the number of zebrafish embryo's where invading cells were observed and is expressed as the proportion of zebrafish embryo's with invasion over all zebrafish embryo's. B. Upper panel: Picture of a whole zebrafish embryo (green) with invading M2 cells overexpressing HA-Snail (red). Lower panel: larger magnification showing single invading cells (arrows) C. Picture of a whole zebrafish embryo (green) with invading M2 cells overexpressing Slug (red) D. Metastasis was quantified by counting the number of zebrafish embryo's in which metastasis occurred and is expressed as the proportion over total zebrafish embryo's. Data shown is the mean of two independent experiments ± SEM. Significance ** $p < 0.01$ * $p < 0.05$

maintained after 5 days (6,4% and 5.1% respectively). This suggests an important role for Slug and Snail in invasion and subsequent metastasis *in vivo*.

Discussion

In this study, we investigated if Snail and Slug, two key players in EMT, also have an important role during TGF-β-induced invasion. TGF-β induced the expression of Slug and

Snail mRNA in *H-Ras*-transformed MCF10AT (M-II) cells. Overexpression of Slug or Snail enhanced invasion in an *in vitro* invasion model. Most notably, these cells invaded as single cells. Using a zebrafish embryo xenograft model, we were able to observe the same effects *in vivo*. This study thus provides a link between TGF-β-induced EMT and single cell invasion and formation of experimental metastasis in zebrafish embryos.

Both Slug and Snail have been reported to induce MMP9 expression [27,28]. Since we have demonstrated a critical role of MMP9 in invasion [25], it is tempting to speculate that Slug and/or Snail are involved in TGF-β-induced MMP9 expression. However, MMP9 expression was not induced by Snail or Slug in our cells (data not shown).

Single cell invasion promoted by Slug and Snail resembles the amoeboid type of movement which occurs in the presence of MMP-inhibitors, without pericellular proteolysis [29,30]. Instead, cells generate enough actomyosin force to deform the extracellular matrix [31]. This type of movement is promoted by Rho-ROCK signaling [29]. Interestingly, RhoA is reported to be a Snail target gene [32], whereas RhoB is reported as a Slug target gene [33]. Furthermore, the RhoA-ROCK signaling axis has been implied in TGF-β-induced EMT and actin fiber rearrangements [34,35]. Thus, Snail and Slug might act via induction of Rho on TGF-β-induced invasion.

The single cell movement induced by Slug or Snail resembles cells invading *in vivo* which have active TGF-β-signaling. These cells invaded rapidly [21]. Since our data also implies Slug and Snail in single cell invasion *in vivo*, we speculate that Slug and Snail are mediators of TGF-β-induced single cell invasion, thus promoting metastasis.

TGF-β-induced EMT is also linked to the transition of cancers cells into cancer stem cells [18]. Snail has recently been implied in this transition [19]. Since a few of these cancer stem cells are able to establish a metastasis, it is likely that the Snail or Slug overexpressing cells also acquire some stem cell traits. It would be interesting to further investigate if these stem cell traits contribute to the invasive behaviour of these cells.

Taken together, we have shown a crucial role for the transcription factors Slug and Snail in TGF-β-induced single cell invasion of breast cancer cells. This further reinforces the role of these EMT mediators in metastasis.

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