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Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion

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ABSTRACT

TGF-β plays a dual role in cancer; in early stages it inhibits tumor growth, whereas later it promotes invasion and metastasis. TGF-β is thought to be pro-invasive by inducing epithelial-to-mesenchymal transition (EMT) via induction of transcriptional repressors, including Slug and Snail.

In this study, we investigated the role of Snail and Slug in TGF-β-induced invasion in an *in vitro* invasion assay and in an embryonic zebrafish xenograft model. Ectopic expression of Slug or Snail promoted invasion of single, rounded amoeboid cells *in vitro*. In an embryonic zebrafish xenograft model, forced expression of Slug and Snail promoted single cell invasion and metastasis. Slug and Snail are sufficient for the induction of single-cell invasion in an *in vitro* invasion assay and in an embryonic zebrafish xenograft model.

1. Introduction

Transforming Growth Factor-β (TGF-β) is a pleiotropic cytokine involved a multitude of biological processes. Deregulation 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 tumor 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 a heteromeric complex with Smad4. This complex translocates 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, such as Snail and Slug, are able to downregulate E-cadherin expression by binding to E-boxes in the E-cadherin promoter. Snail and Slug are zinc finger proteins of the Snail family that recognize E2 box type elements (CAGGTG/CACCTG). 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 regulators. Furthermore, Smad proteins also are able to 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 tumor [17]. Furthermore, cancer cells which have undergone TGF-β-induced EMT closely resemble cancer stem cells, of which a few cells can give rise to a whole new tumor [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.

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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 invasion.

2. Materials and methods

2.1. 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].

2.2. RNA isolation, cDNA synthesis and Q-PCR

2×10^5 cells were seeded per well into a six well plate and one day later starved for 16 h with DMEM/F12 (Gibco/Invitrogen) containing 2.5% horse serum (Gibco/Invitrogen), 10 ng/ml epidermal growth factor (EGF) (Upstate), 50 ng/ml cholera toxin (Calbiochem), 0.25 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 100 U/ml penicillin and 50 μg/ml streptomycin (Gibco/Invitrogen). After starvation, cells were stimulated for 24 h 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 ΔCt method using *ARP* or β-actin as reference and the non-stimulated condition was set to 1. Primers used are listed in Table 1.

2.3. Plasmids and transduction

For ectopic expression of Snail and Slug, mouse HA-Snail (mSnail) and mouse Slug (mSlug) were cloned into the lentiviral vector CMV-IRES-PURO. Transduction and selection of transduced cells was performed as described previously [24]. Expression in M-II cells was verified by Western blotting using antibodies C15D3 (Snail) and C19G7 (Slug), which were purchased from Cell Signaling Technology, Inc.

2.4. 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 min 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 Extended CS5.

2.5. 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), which contains a GFP marker in the endothelial cells, was crossed with the transparent casper mutant. Dechorionized 2 day old zebrafish embryos were anesthetized with 0.003% tricaine (Sigma) and positioned on a 10 cm Petridish coated with 1% agarose. M-II cells overexpressing mSnail, mSlug or the control vector were trypsinized into single cell suspensions, resuspended in phosphate buffered saline (PBS, Invitrogen), kept at room temperature before implantation and implanted within 3 h. 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 (1 mm O.D. × 0.78 mm 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 60 μm above the ventral end of the duct of Cuvier. Around 200 embryos were implanted per cell line. After implantation, zebrafish embryos (including non-implanted controls) were maintained at 34 °C [26].

Invasion and subsequent metastasis was monitored after 1, 3 and 5 days by imaging the zebrafish embryos with a confocal microscope. Invasion of perivascular tumor cells into the neighboring tail fin was quantified by counting the number of zebrafish embryos with at least three invading cells and is presented as the proportion of zebrafish embryos with invasion over all zebrafish embryos. Experimental micrometastasis in the a-vascular tail fin of extravasated cells (over 30 cells) was quantified by counting the number of zebrafish embryos in which experimental metastasis occurred and is presented as the proportion of metastasis positive embryos over total number of zebrafish embryos.

2.6. 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.

Table 1
Sequences of primers used in this study.

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′

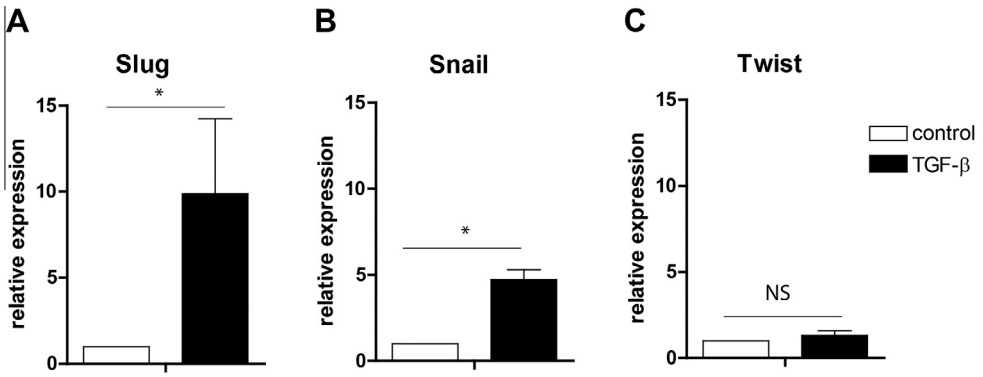

Fig. 1. Induction of Slug, Snail and Twist by TGF-β in M-II cells. M-II spheroids were stimulated with TGF-β3 (5 ng/ml) or mock treated. RNA was isolated after 24 h and analyzed by Q-PCR for Snail (A), Slug (B) or Twist (C) expression. Data shown is the mean of two independent experiments ± SEM. Significance $^*p < 0.05$, NS not significant.

3. Results

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

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, M-II spheroids were stimulated with TGF-β3 (5 ng/ml) or mock treated. After 24 h, we analyzed expression of Snail, Slug and Twist by quantitative real time PCR. TGF-β3 induced Slug mRNA 10-fold, whereas Snail mRNA was induced 5-fold (Fig. 1A and B). No effect of TGF-β3 treatment on Twist mRNA could be detected (Fig. 1C). Thus, TGF-β3 induces expression of Snail and Slug, but not Twist in M-II cells.

3.2. Ectopic expression 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-β3 on Slug and Snail expression by inducing ectopic expression of mSlug and mSnail. This was achieved by transduction of M-II cells with lentiviral vectors containing either no insert (M-II-vector), mSnail (M-II-Snail) or mSlug (M-II-Slug). Ectopic expression was verified by Western blotting (Fig. 2A). To test whether mSnail and mSlug expression was functional, we performed real-time quantitative PCR for epithelial markers. EPCAM and keratin 18 (KRT18) were both downregulated by expression of mSnail or mSlug to a similar level as which was obtained after a 24 h treatment of ML-II-vector cells with TGF-β3 (Fig. 2B and C). Expression of the mesenchymal marker vimentin was increased by expression of mSnail or mSlug compared to control as detected by Western blotting (Fig. 2D). Expression of mSnail or mSlug did not directly affect TGF-β/Smad signaling, since the induction of the typical Smad target gene PAI-1 was equal in all cells (Fig. 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-β3. Invasion into the collagen was measured after 24 h. Expression of mSnail or mSlug enhanced basal invasion of M-II cells compared to vector control cells, indicating that Snail and Slug mimic the effect of TGF-β3 on invasion (Fig. 3A and B). In addition, invasion of M-II-Snail or M-II-Slug cells could be further enhanced by TGF-β3, 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 expressing mSnail or mSlug invaded in a different mode compared to control cells. Control cells invaded in a manner resembling collective cell migration, keeping cell–cell contacts intact and appearing rather epithelial (Fig. 3C), whereas mSlug or mSnail expressing cells invaded as single cells with a more rounded, amoeboid morphology (Fig. 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.

3.3. Expression of mSlug and mSnail in M-II cells enhances the invasion and metastatic behaviour in zebrafish embryos

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, located in the yolk sac of zebrafish embryos, which then disperse through the bloodstream, and subsequently can invade, and/or form metastasis [26]. This model system has the advantage over other model systems, that it is a rapid and reproducible system where the whole process of invasion and metastasis can be easily followed. When we injected M-II-Snail and M-II-Slug cells, we observed a dramatic increase in the amount of embryos in which cells had invaded compared to control cells (Fig. 4A and B). This increase was evident from day 3 after injection, with only 13% of control embryos showing invasion, whereas 31% and 39% of the embryos injected with M-II-Snail and M-II-Slug, respectively, showed invasion. After 5 days, 38% of the control embryos showed invasion whereas 70% and 55% of the embryos injected with M-II-Snail and M-II-Slug cells, respectively, showed invasion. Also, the amount of embryos displaying metastasis (>30 cells) was increased in cells expressing mSnail or mSlug (Fig. 4C). While metastasis in embryos injected with controls cells was rare after 3 days (0.29%) or 5 days (0.58%), embryos injected with M-II-Snail or M-II-Slug expressing cells showed a more than tenfold increase in metastasis after 3 days (2.2% and 4.1%, respectively). This dramatic increase was maintained after 5 days (6.4% and 5.1%, respectively). Furthermore, M-II with Snail or Slug also showed a difference in invasive morphology. Whereas, M-II cells normally form clusters of cells exclusively in the area between the circulatory loop at the posterior ventral end of the caudal hematopoietic tissue [26], the expression of Snail or Slug caused some of the cells to move into the collagen fibres of the tail fin as individual cells. This suggests an important role for Slug and Snail in invasion and subsequent metastasis *in vivo*.

4. Discussion

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

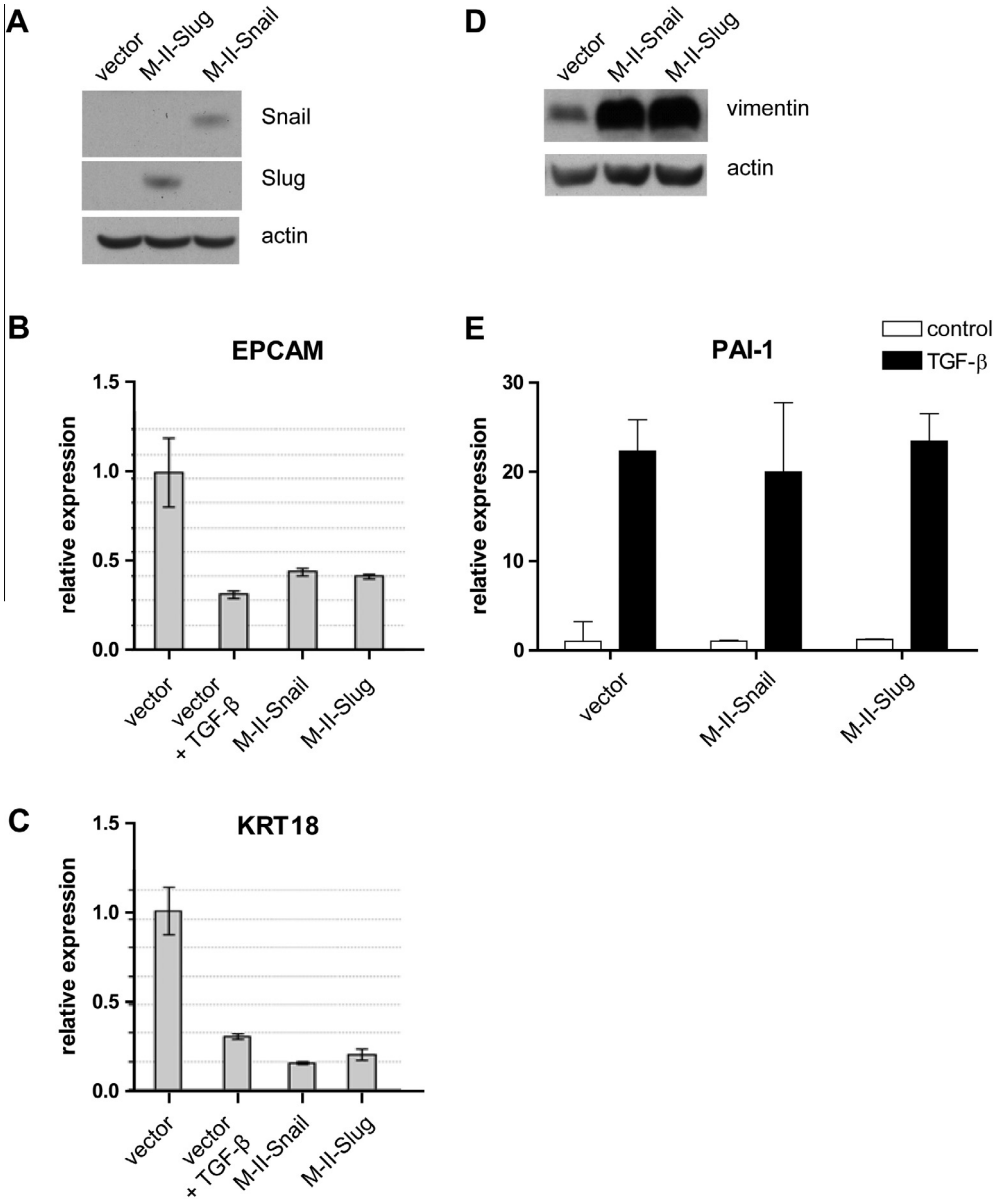

Fig. 2. Functional expression of mSnail and mSlug. M-II cells were transduced with control virus, mSlug expressing virus or mSnail expressing virus. Cells were plated for RNA isolation and cell lysates. Lysates of cells were analyzed by SDS–PAGE & Western blotting for mSnail and mSlug expression (A) and Vimentin expression (D). Actin was used as a loading control. M-II-vector cells were mock-treated or incubated with TGF-β3 (5 ng/ml) for 24 h. Expression of human EPCAM (B) and human keratin 18 (KRT18) (C) mRNA was determined by Q-PCR and the expression levels were compared to the expression levels in M-II-Snail and M-II-Slug cells (B and C). Cells were plated, serum starved and stimulated for 2 h without ligand or with TGF-β3 (5 ng/ml). RNA was isolated and human PAI-1 expression was analyzed by Q-PCR (E).

invasion. TGF-β induced the expression of Slug and Snail mRNA in *H-Ras*-transformed MCF10AT (M-II) cells. Ectopic expression of mSlug or mSnail induced an EMT-like phenotype in M-II cells, with decreased expression of the epithelial markers EPCAM and KRT18 and an increased expression of the mesenchymal marker Vimentin.

M-II-Snail and M-II-Slug cells showed an 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 mSlug and mSnail 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 mSlug or mSnail 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.

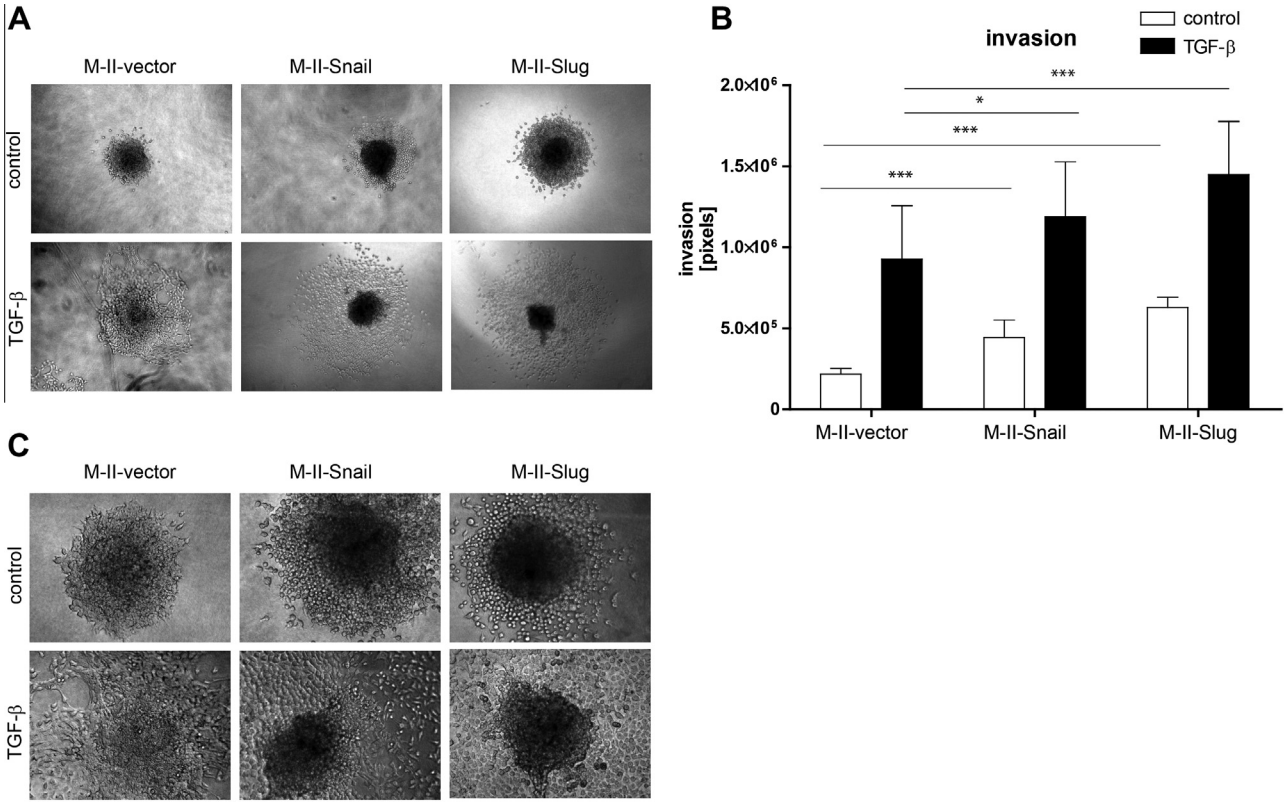

Fig. 3. Expression of mSnail and mSlug enhances invasion. M-II-vector, M-II-Snail and M-II-Slug cells were plated 8 h after transduction for spheroid culture and selection. These spheroids were embedded and allowed to invade into collagen. Photographs show 40× magnification (A). Quantification of invasion (B). Higher magnification (100×) of spheroids after 16 h incubation (C). Data shown is a representative of two independent experiments ± SD. Significance $^{***}p < 0.001$, $^{*}p < 0.05$.

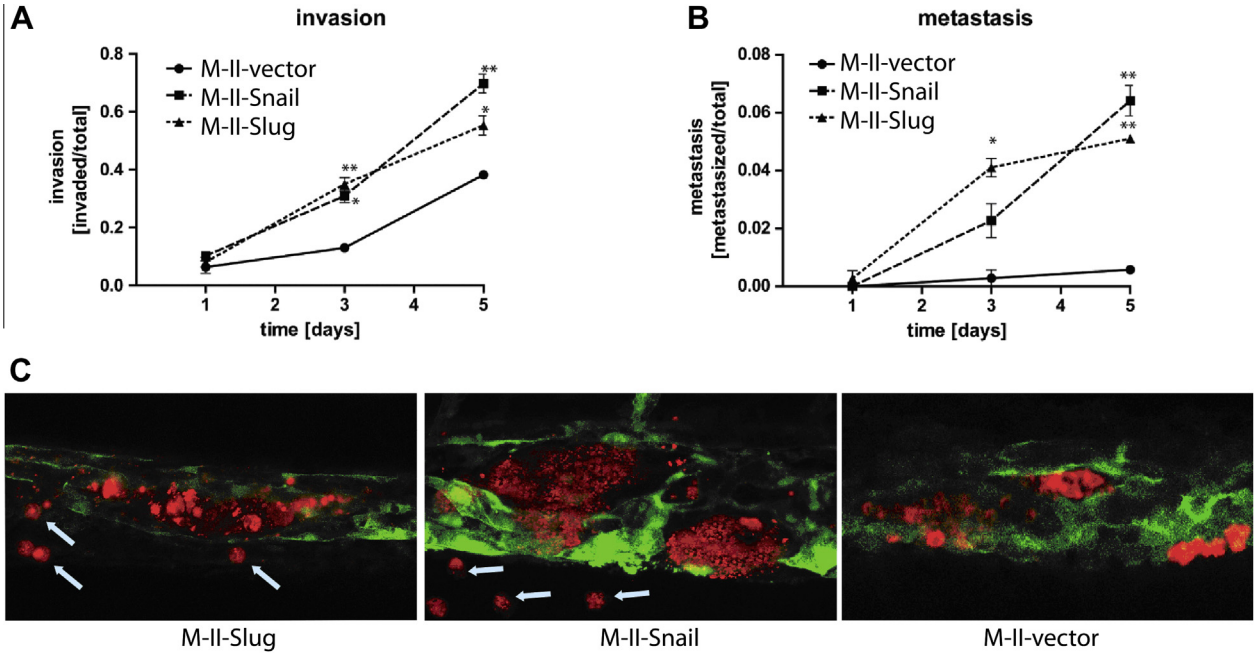

Fig. 4. Expression of mSnail and mSlug enhances invasion and metastasis *in vivo*. M-II-vector, M-II-Snail and M-II-Slug cells were dyed with CmDiI and injected into the ducts of Cuvier of 2 days old Fli-GFPxCasper zebrafish embryos. Cells were monitored after 1, 3 and 5 days by imaging the zebrafish embryos with a confocal microscope. At least 170 embryos were analysed for each condition on each day. Data shown is the mean of two independent experiments ± SEM. Significance $^{**}p < 0.01$, $^{*}p < 0.05$. Invasion was quantified by counting the number of zebrafish embryos where invading cells were observed and is expressed as the proportion of zebrafish embryos with invasion over all zebrafish embryos (A). Metastasis was quantified by counting the number of zebrafish embryos in which metastasis occurred and is expressed as the proportion over total zebrafish embryos (B). M-II cells (red) expressing mSlug or mSnail or vector displayed highly metastatic ability in zebrafish when compared to control. Furthermore mSlug and mSnail expression caused a change in morphology (C). Individual cell invasion was seen in M-II-Snail and M-II-Slug cells (arrows), which is not seen in control. These are representative images from at least 170 examined embryos (magnification 63×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 M-II-Snail or M-II-Slug cells also acquired some stem cell traits. Recent studies in SW480 cancer cells also suggest that Snail induction by TGF-β is involved in restoration of cancer stem cell properties, such as expression of CD133 [36]. However, we found that CD133 is downregulated by Snail and Slug in M-II cells (data not shown).

Taken together, we have shown that ectopic expression of the transcription factors mSlug and mSnail is sufficient for single cell invasion of breast cancer cells. This further reinforces the role of these EMT mediators in metastasis.

Acknowledgments

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