Oncogenes and Tumor Suppressors

Snail Cooperates with Kras\textsuperscript{G12D} \textit{In Vivo} to Increase Stem Cell Factor and Enhance Mast Cell Infiltration

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is associated with a pronounced fibro-inflammatory stromal reaction that contributes to tumor progression. A critical step in invasion and metastasis is the epithelial-to-mesenchymal transition (EMT), which can be regulated by the Snail family of transcription factors. Overexpression of Snail (Snai1) and mutant Kras\textsuperscript{G12D} in the pancreas of transgenic mice, using an elastase (EL) promoter, resulted in fibrosis. To identify how Snail modulates inflammation in the pancreas, we examined the effect of expressing Snail in EL-Kras\textsuperscript{G12D} mice (Kras\textsuperscript{G12D}/Snail) on mast cell infiltration, which has been linked to PDAC progression. Using this animal model system, it was demonstrated that there are increased numbers of mast cells in the pancreas of Kras\textsuperscript{G12D}/Snail mice compared with control Kras\textsuperscript{G12D} mice. In addition, it was revealed that human primary PDAC tumors with increased Snail expression are associated with increased mast cell infiltration, and that Snail expression in these clinical specimens positively correlated with the expression of stem cell factor (SCF/KITLG), a cytokine known to regulate mast cell migration. Concomitantly, SCF levels are increased in the Kras\textsuperscript{G12D}/Snail mice than in control mice. Moreover, overexpression of Snail in PDAC cells increased SCF levels, and the media conditioned by Snail-expressing PDAC cells promoted mast cell migration. Finally, inhibition of SCF using a neutralizing antibody significantly attenuated Snail-induced migration of mast cells.

Implications: Together, these results elucidate how the EMT regulator Snail contributes to inflammation associated with PDAC tumors. \textit{Mol Cancer Res; 12(10); 1440–8. ©2014 AACR.}

Introduction

Pancreatic cancer, the fourth leading cause of cancer-related death in the United States (1, 2), is associated with a pronounced stromal reaction composed of collagen-rich extracellular matrix, pancreatic stellate cells, and inflammatory cells (3–5). It has also been shown that there are dynamic changes in the number and function of these inflammatory cells during pancreatic cancer progression (6, 7). We previously published that mast cells, which have been extensively studied in allergic reaction and autoimmunity (8), are increased in human pancreatic ductal adenocarcinoma (PDAC) tumors and contribute to pancreatic cancer progression (9). A recent report showed that mast cells modulate proliferation and activation of pancreatic stellate cells (10), which are key mediators of pancreatic fibrosis \textit{in vivo} (11, 12). Significantly, chronic pancreatitis, which is associated with ongoing inflammation and fibrosis (13), is a risk factor for pancreatic cancer in humans and facilitates pancreatic cancer development in transgenic mouse models (14, 15).

Epithelial-to-mesenchymal transition (EMT) has been identified as a key step in tumor invasion and metastasis, enabling cells to disrupt normal tissue architecture and invade surrounding structures (16, 17). One of the key regulators of EMT is the transcription factor Snail (Snai1), which is associated with higher-grade PDAC tumors and is upregulated in >75% of human PDAC tumors (18, 19). Significantly, Snail also promotes fibrosis \textit{in vivo} (20, 21). Transgenic overexpression of Snail in the kidney was sufficient to induce fibrosis in mice (20), whereas ablation of Snail in the liver attenuated chemical-induced fibrosis (21). We also recently showed that overexpression of Snail with mutant Kras\textsuperscript{G12D} (Kras\textsuperscript{G12D}/Snail) in mouse pancreas promoted fibrosis (22). Importantly, Snail can also modulate inflammatory signaling \textit{in vivo} through upregulation of chemokines and cytokines (21, 23, 24). Snail overexpression in keratinocytes increased cytokines and chemokines \textit{in vitro} (23), whereas Snail overexpression in epidermal keratinocytes in a transgenic mouse model promoted cutaneous...
inflammation that was associated with increased cytokine production (24).

In this report, we examined the effect of Snail expression in epithelial cells on mast cell infiltration in vivo and in vitro. We show that there are increased numbers of mast cells in the pancreas of Kras\textsuperscript{G12D}/Snail mice compared with control mice (Kras\textsuperscript{G12D}). We also show that human pancreatic tumors with increased Snail expression are associated with increased mast cell infiltration. To further understand how Snail increases mast cell infiltration, we examined in human PDAC tumor samples the association between Snail levels and cytokines previously shown to regulate mast cell migration. We show that Snail expression in human PDAC tumors positively correlates with expression of stem cell factor (SCF). In addition, we show that SCF levels are increased in the Kras\textsuperscript{G12D}/Snail mice compared with Kras\textsuperscript{G12D} mice. Moreover, we demonstrate that in vitro overexpressing Snail in PDAC cells increases SCF levels and that the media conditioned by Snail-expressing PDAC cells increase mast cell migration. Significantly, inhibiting SCF using neutralizing antibody blocks Snail-induced migration of mast cells. Overall, these results increase our understanding of how the EMT regulator Snail contributes to pancreatic cancer progression through inflammatory cell infiltration.

Materials and Methods
Antibodies/reagents
Reagents for trichrome, chloroacetate esterase (CAE), and toluidine blue (TB) stains were purchased from Sigma. The TβRI inhibitor SB431542 was obtained from Calbiochem. FOXP3 (FJK-16a) antibody was from eBioscience, Snail (C15D3) and Slug (C19G7) antibodies were purchased from Cell Signaling Technology, F4/80 (123105) antibody was from BioLegend, Gr-1 (Ly6C/Ly6C clone RB6-8C5) and CD45 (30-F11) antibodies were purchased from BD Bioscience, and CD3 (ab 5690) antibody was purchased from Abcam. Tryptase (sc-33676) and α-tubulin (sc-8035) antibodies were purchased from Santa Cruz Biotechnology. Secondary antibodies were purchased from Sigma. Function-blocking antibody against SCF (AF-255-NA) and isotype-matched IgG antibody were purchased from R&D Systems. The SCF Quantikine ELISA Kit (DCK00) was obtained from R&D Systems.

Transgenic mice
All animal work was conducted in compliance with the Northwestern University Institutional Animal Care and Use Committee (IACUC) guidelines. TRE-Snail transgenic mice were created by the Transgenic Core Facility at Northwestern University (Illinois, IL) and have been described previously (22). The TRE-Snail mice were crossed with EL-tTA mice, kindly provided by Dr. Eric Sandgren (University of Wisconsin, Madison) (25), to generate EL-tTA/TRE-Snail (Snail) bimanic mice. In EL-tTA mice, the transactivator tTA is expressed downstream of elastase (EL) promoter, thus enabling targeting of Snail to pancreatic acinar and centroacinar cells (25, 15). The bimanic mice were further crossed with EL-Kras\textsuperscript{G12D} mice, which express constitutively active mutant Kras in the pancreatic acinar cells (15, 25, 26), to generate Kras\textsuperscript{G12D}/Snail trigenic mice (22). Notably, these mice at the point of histologic examination at 3 to 4 months of age do not develop overt pancreatic neoplasms but instead demonstrate increased acinar-ductal metaplasia, proliferation, and increased fibrosis (22).

Immunohistochemistry
Pancreatic tissue specimens from Kras\textsuperscript{G12D}/Snail and control Kras\textsuperscript{G12D} were stained for leukocytes using CD45 antibody, T cells using CD3 antibody, T regulatory cells using FOXP3 antibody, and macrophages using F4/80 antibody. The tissue sections were also stained with anti-Gr-1 antibody, which identifies both granulocytes and myeloid-derived suppressor cells (27). Mast cells were identified with CAE, TB, or tryptase stains (8–10). Antigen retrieval was performed as previously described (22, 26, 28). Photographic for quantitative comparison were taken using a Carl Zeiss Axioskop 40 microscope and camera (22, 26).

Human PDAC tissue analysis
Pancreatic tissue was obtained from patients with pancreatic adenocarcinoma on an Institutional Review Board (IRB)—approved protocol and de-identified. The specimens were stained for mast cells using CAE and tryptase staining. In addition, RNA was isolated from human PDAC tumors previously stored in RNAlater and analyzed for Snail, tryptase, and cytokines by quantitative RT PCR (29–31).

Cell culture
AsPC1 and CD18/HPAF-II cells were obtained from the American Type Culture Collection, while human mast cell line HMC-1 has been described previously (32, 33). AsPC1 cells were last authenticated by STR profiling at the Johns Hopkins Genetic Resources Core Facility in 2010, while CD18 cells were authenticated by STR profiling in 2013. AsPC1 and CD18 cells expressing Snail or Slug were generated as detailed previously (30, 34). AsPC1-vector, AsPC1-Snail, AsPC1-Slug, CD18-vector, and CD18-Snail cells have not been previously authenticated.

Conditioned media
Cells expressing control vector or Snail were allowed to condition the media for 72 hours to generate Vector- and Snail-conditioned media (V\textsuperscript{CM} and Sn\textsuperscript{CM}, respectively).

Immunoblotting
Immunoblotting for Snail, Slug, and α-tubulin was done as previously described (26, 30).

Quantitative real-time PCR analysis
Reverse transcription of mRNA to cDNA was performed using TaqMan Reverse Transcription reagents from Applied Biosystems. Quantitative gene expression was performed with gene-specific TaqMan probes, TaqMan Universal PCR Master Mix, and the 7500 Fast Real-time PCR System from Applied Biosystems. The data were then quantified with the comparative \(C_T\) method for relative gene expression (28).
Cytokine protein expression
SCF in the media conditioned by control cells or cells expressing Snail or Slug was measured using the Quantikine ELISA Kit purchased from R&D systems.

Mast cell migration assay
HMC-1 mast cells (2 x 10^5) were added to the upper chamber of an 8-µm uncoated Boyden chamber with media conditioned by control cells or Snail-expressing cells in the lower chamber. The HMC-1 cells were allowed to migrate over 18 hours into the lower chamber, collected, and counted (10). Neutralizing antibody or isotype-matched control antibody was added to the lower chamber. All migration assays were performed in triplicate and repeated a minimum of three times.

Statistical analysis
In vivo and in vitro results were compared using t test analysis. Error bars represent standard error of the mean. All analyses were performed on GraphPad Prism 5 for Mac OS X.

Results
Snail expression in EL-Kras^{G12D} mice increases inflammation and promotes mast cell infiltration
Recently, we showed that overexpression of Snail and mutant Kras (Kras^{G12D}) in mouse pancreas using an elastase (EL) promoter enhanced collagen-rich stromal reaction without causing overt pancreatic cancer (Fig. 1A; ref. 22).

![Figure 1](image-url). Snail expression in EL-Kras^{G12D} mice increases inflammation and promotes mast cell infiltration. The TRE-Snail mice were crossed with EL-tTA mice to generate Snail (EL-tTA+/TRE-Snail^+) mice. The Snail mice were crossed with EL-Kras^{G12D} mice to generate mice expressing both Snail and Kras^{G12D} in the pancreas (EL-Kras/EL-tTA/TRE-Snail = Kras^{G12D}/Snail) or littermate control mice that expressed only Kras^{G12D} (EL-Kras/EL-tTA/TRE-Snail = Kras^{G12D}). A, pancreas from Kras^{G12D} and Kras^{G12D}/Snail mice were analyzed for Snail expression by immunofluorescence using DAPI to counterstain nuclei. Representative pancreatic sections from 3-month-old Kras^{G12D} and Kras^{G12D}/Snail mice were analyzed for fibrosis using trichrome staining (blue, fibrosis). B, pancreatic tissue samples from 3-month-old Kras^{G12D} and Kras^{G12D}/Snail mice were collected and stained for leukocytes using CD45 antibody, and the number of leukocytes averaged over 8 high-power fields were counted. C and D, pancreatic tissue samples from 3-month-old Kras^{G12D} and Kras^{G12D}/Snail mice were collected and stained for mast cells using CAE (C) and TB (D) and the total number of mast cells per section was counted. The P values were calculated using an unpaired t test. Scale bars, 50 µm.
Because inflammation plays a significant role in PDAC development and progression (15, 35), we evaluated the effect of Snail on inflammatory cell infiltration in our EL-Kras<sup>G12D</sup> mouse model by staining for leukocytes using CD45 antibody. As shown in Fig. 1B, there was a significant increase in leukocyte infiltration, which were primarily located in the pancreatic stroma, in the Kras<sup>G12D</sup>/Snail mice compared with the Kras<sup>G12D</sup> mice. Previously, we published that increased infiltration of mast cells in human PDAC tumors was associated with worse prognosis (9). Because increased Snail expression in human PDAC tumors is also associated with more advanced disease (18, 19), we examined the effect of Snail expression in the EL-Kras<sup>G12D</sup> mice on mast cell infiltration using CAE and TB stains to identify mast cells. As shown in Fig. 1C, there were increased numbers of CAE<sup>+</sup> cells in pancreas from Kras<sup>G12D</sup>/Snail mice compared with Kras<sup>G12D</sup> mice, indicating that Snail promotes infiltration of mast cells in the EL-Kras<sup>G12D</sup> mice. Consistent with the CAE stain, TB staining also showed that there were increased numbers of mast cells in the Kras<sup>G12D</sup>/Snail mice compared with the Kras<sup>G12D</sup> (Fig. 1D). Similar to the leukocytes, the mast cells were also primarily located in the pancreatic stroma. We also examined the extent to which Snail affected mast cell infiltration following induction of pancreatitis using cerulein treatment. As shown in Supplementary Fig. S1, there was a trend toward increased infiltration of mast cells in Kras<sup>G12D</sup>/Snail mice compared with Kras<sup>G12D</sup> mice following induction of pancreatitis.

**Effect of Snail expression in EL-Kras<sup>G12D</sup> mice on infiltration of CD3<sup>+</sup> cells, FOXP3<sup>+</sup> cells, Gr-1<sup>+</sup> cells, and F4/80<sup>+</sup> cells**

In an effort to further characterize the inflammatory cell infiltration into the pancreatic microenvironment, we evaluated the effect of Snail expression in the EL-Kras<sup>G12D</sup> mice on CD3<sup>+</sup> cells to identify T cells; FOXP3<sup>+</sup> cells to identify T regulatory cells; Gr-1<sup>+</sup> cells to identify granulocytes and also myeloid-derived suppressor cells; and F4/80<sup>+</sup> cells to identify macrophages. There was no significant difference in the overall number of T cells (Fig. 2A) or in the number of T regulatory cells (Fig. 2B) in pancreas from Kras<sup>G12D</sup>/Snail mice compared with Kras<sup>G12D</sup> mice. However, there was a statistically significant increase in the number of Gr-1<sup>+</sup> cells and macrophages in the pancreas of Kras<sup>G12D</sup>/Snail mice compared with Kras<sup>G12D</sup> mice (Fig. 2C and D).

**Snail expression in human PDAC tumors correlates with mast cell activation marker tryptase and with the cytokine SCF**

To understand how Snail expression increases mast cell infiltration in the EL-Kras<sup>G12D</sup> mice, we examined the effect of Snail expression on cytokine expression using human PDAC tissue samples. Initially, we examined the relationship between Snail expression in human PDAC tumors and tryptase, which is primarily expressed in mast cells (36). We show that both CAE and tryptase stain for mast cells in human PDAC tumors (Fig. 3A). Because of lack of a commercial antibody to detect endogenous Snail protein, we examined the relationship between Snail at the mRNA level and mast cells using tryptase mRNA as a surrogate for mast cells (36). As shown in Fig. 3B, tumor samples with increased Snail mRNA demonstrate increased tryptase mRNA levels. We next examined the relationship between Snail in PDAC tumor samples and cytokines known to be involved in mast cell migration (8). Specifically, we examined the relationship between Snail mRNA expression and expression of SCF, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-8 (IL8), and chemokine (C–C motif) ligand 5 (CCL5) mRNA levels. There was very little GM-CSF mRNA expression in our human tumor samples (data not shown). Although there was no correlation between expression of Snail mRNA and CCL5 mRNA or between expression of Snail mRNA and IL8 mRNA levels, there was a significant correlation between Snail mRNA and SCF mRNA expression in human PDAC samples (Fig. 3C).

**Snail expression in EL-Kras<sup>G12D</sup> mice increases SCF expression**

Because Snail mRNA levels in human PDAC tumors correlates with SCF mRNA levels, we examined the effect of Snail expression in EL-Kras<sup>G12D</sup> mice on SCF levels. As shown in Fig. 4A, there was a significant increase in SCF expression in the Kras<sup>G12D</sup>/Snail mice compared with Kras<sup>G12D</sup> mice. We also examined the effect of Snail expression in the EL-Kras<sup>G12D</sup> mice on CCL5 and CXCL1, the mouse homolog for IL8. Consistent with our human PDAC tumor samples, there was not a significant increase in CCL5 (Fig. 4B) or CXCL1 (Fig. 4C) mRNA expression in the pancreas of Kras<sup>G12D</sup>/Snail mice compared with Kras<sup>G12D</sup> mice.

**Snail expression in human PDAC cells in vitro increases SCF production and mast cell migration**

We next examined whether Snail expression in vitro also results in increased SCF levels. Snail expression in the PDAC cell lines AsPC1 (Fig. 5) and CD18 (Supplementary Fig. S2) significantly increased SCF mRNA levels. Moreover, the media conditioned by Snail-expressing PDAC cells (SnCM) demonstrated increased levels of SCF protein (Fig. 5B and Supplementary Fig. S2B). Because we have previously reported that Snail expression in AsPC1 cells increases TGFβ signaling (22), we examined the effect of blocking TGFβ signaling on Snail-induced SCF expression. As shown in Fig. 5C, treatment with the TGFβ type 1 receptor inhibitor SB431542 significantly attenuated Snail-induced SCF mRNA levels.

We next examined the effect of Snail expression in PDAC cells on migration of the human mast cell line HMC-1 cells using a Transwell migration assay (9, 10) and media conditioned by PDAC-V cells (VCM) and PDAC-Sn cells (SnCM). Relative to migration toward media conditioned by PDAC-V cells, there was increased migration of mast cells toward media conditioned by Snail-expressing PDAC cells.
Finally, we examined the effect of blocking SCF on the ability of HMC-1 mast cells to migrate toward media conditioned by control cells or Snail-expressing cells. As shown in Fig. 5E and Supplementary Fig. SD, treatment with the function-blocking anti-SCF antibody decreased migration of HMC-1 cells toward media conditioned by Snail-expressing PDAC cells without affecting migration of HMC-1 cells toward media conditioned by control PDAC cells. Overall, these results demonstrate that Snail increases expression of SCF to regulate mast cell migration in vitro and suggest that the increased infiltration of mast cells in Snail-expressing lesions is mediated by SCF.

Discussion

There is significant interplay between EMT and inflammation in regulating cancer progression. It was previously shown that inflammatory signaling could induce EMT. For example, Snail activity is increased via stabilization at the protein level in response to TNFα-driven NF-κB signaling (37). Conversely, Snail can also modulate inflammatory signaling in vitro through upregulation of chemokines and cytokines (21, 23, 24). Significantly, Snail-related protein Slug (Snai2) can also modulate inflammatory signaling in vitro through upregulation of chemokines and cytokines (23, 38). Snail and Slug overexpression in keratinocytes increases production of cytokines IL6, IL8, and the...
chemokine CXCL1 (23). Snail overexpression in epidermal keratinocytes in a transgenic mouse model promotes cutaneous inflammation that is associated with increased IL6 production (24). Snail ablation in turn attenuates the inflammatory response in a chemical-induced liver fibrosis model (21), whereas Slug ablation attenuates cutaneous inflammatory response following ultraviolet radiation (38). In this report, we show that Snail expression in the mouse pancreas also enhances inflammation. Snail expression in mouse pancreas increases infiltration of mast cells, and Snail expression in pancreatic cancer cells induces expression of the cytokine SCF to promote mast cell migration. In addition, we show that Snail expression in human tumors correlates with the mast cell marker tryptase and SCF, suggesting that Snail may also promote infiltration of mast cells in human PDAC tumors by increasing SCF expression.

Inflammation plays a critical role in pancreatic cancer development and progression. Acute pancreatitis can accelerate the progression of precursor pancreatic intraepithelial neoplastic lesions to PDAC in mutant K-ras–driven mouse models of pancreatic cancer (39, 40). Importantly, although

Figure 3. Snail expression in human PDAC tumors correlates with mast cell activation marker tryptase and with the cytokine SCF. De-identified human PDAC samples were procured on an IRB-approved protocol. A, formalin-fixed paraffin-embedded tumors were stained with CAE and immunostained with anti-tryptase antibody to identify mast cells. Scale bars, 50 μm. B and C, the mRNA samples from human PDAC tumors were processed for Snail, tryptase, CCL5, IL8, and SCF mRNA by qRT-PCR and normalized using GAPDH mRNA levels and the relationship between Snail and tryptase, CCL5, IL8, or SCF was analyzed using Pearson correlation coefficient.

Figure 4. Snail expression in EL-Kras^{G12D} mice increases SCF expression. A–C, the mRNA samples from pancreatic tissue of 3-month-old Kras^{G12D} and Kras^{G12D}/Snail mice were processed for SCF (A), CCL5 (B), and the mouse functional homolog to IL8 chemokine (C-X-C motif) ligand 1 (CXCL1; C) mRNA by qRT-PCR and normalized using GAPDH mRNA levels. The P values were calculated using an unpaired t test.
expression of embryonic mutant K-ras is sufficient for tumor initiation in various mouse models of pancreatic cancer (41, 42), expression of mutant K-ras in adult mouse pancreas does not result in any obvious phenotypic changes (15). However, induction of chronic pancreatitis promotes PDAC development in adult mice expressing mutant K-ras (14, 15).

We have recently published that Snail expression in EL-KrasG12D mice resulted in more advanced acinar-ductal metaplasia that was associated with increased proliferation and fibrosis (22). It is possible that some of the phenotypic changes that we recently reported in the KrasG12D/Snail mice may have been due to increased infiltration of inflammatory cells. Significantly, it is increasingly recognized that mast cells contribute to pancreatic cancer progression. Increased numbers of mast cells in human PDAC tumors are associated with poor prognosis (9, 10). Moreover, there is significant cross-talk between cancer cells and mast cells, resulting in pancreatic cancer progression. Pancreatic cancer cells in vitro can increase migration and activation of mast cells that can in turn increase proliferation of pancreatic cancer cells (9).

Recently, it was also shown that blocking mast cell migration and function in vivo reduces PDAC growth and increases survival of mice bearing PDAC tumors (10).

We also show that Snail-induced migration of mast cells was mediated by SCF. We have also found that Slug can induce SCF expression in PDAC cells (Supplementary Fig. S3). Although we are still in the process of determining the mechanism by which Snail and Slug increase SCF, our preliminary studies indicate that Snail increases SCF through TGFβ signaling. Significantly, we have recently shown that TGFβ2 expression is induced by Snail in PDAC cells and that increased TGFβ signaling in KrasG12D/Snail is associated with increased fibrosis and stellate cell activation (22). Notably, mast cells also promote proliferation and activation of stellate cells (10), which are key regulators of fibrosis in vivo (11, 12). The increased activation of stellate cells was shown to be due to increased TGFβ2 production (10). Thus, it is possible that the increased number of mast cells in our Snail-expressing mice may also have contributed to the stellate...
cell activation and fibrosis that we have previously reported to be present in the Kras $^{G12D}$/Snail mice (22). Overall, we demonstrate that Snail in the presence of the oncogenic Kras $^{G12D}$ induces inflammation that is associated with increased mast cell infiltration. Moreover, we show that Snail expression in human PDAC tumors is also associated with evidence of increased mast cell infiltration. Mechanistically, Snail increases SCF production to promote mast cell migration. These results increase our understanding of the interplay between the EMT regulator Snail and inflammation in pancreatic cancer progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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