ORIGINAL ARTICLE

Beyond Epithelial to Mesenchymal Transition: A Novel Role for the Transcription Factor Snail in Inflammation and Wound Healing

Birgit Hotz · Alexander Visekruna · Heinz-Johannes Buhr · Hubert Georg Hotz

Abstract

Introduction Snail, a transcription factor linked to epithelial to mesenchymal transition (EMT) during embryonic development and tumor progression, is associated with migration of cells. During inflammation and tissue injury, cell movement is also observed to provide the first line of defense against bacteria and to promote wound healing. Therefore, we studied the function of Snail in activated macrophages in a variety of inflammatory processes.

Materials and Methods In this study, we examined the expression and localization of Snail during inflammation and tissue injury in rats and human tissue specimens, by immunohistochemistry, Western blot, and real-time PCR. We investigated Snail expression after stimulation of macrophages with TGF-β1, LPS, Interleukin-8, and MMP-3 in vitro. To further understand the role of Snail in activated macrophages, we used Stealth siRNA against Snail, transfected the human macrophage cell line THP-1, and measured migration of cells in an in vitro invasion assay.

Results and Discussion We found a strong, transient, and time-dependent activation of Snail in migrating macrophages at the sites of injury in vivo and in vitro, as well as in patients with inflammatory bowel disease. Furthermore, we showed that induction of Snail in macrophages is dependent on TGF-β1 signaling pathway. Downregulation of Snail by Stealth siRNA led to impaired migration of THP-1 cells in an invasion assay after stimulation with TGF-β1.

Conclusion We conclude that TGF-β1 induced migration of activated macrophages during inflammation and wound healing is mediated by snail. These results give insights in a novel EMT-like mechanism present in immune cell movement during tissue injury.

Introduction

The transcription factor Snail, a zinc finger protein, has been shown to be implicated in triggering epithelial to mesenchymal transition (EMT) which convert epithelial cells into mesenchymal cells with migratory properties.[1] The central event during the loss of epithelial phenotype is the repression of the adhesion molecule E-cadherin by Snail.[2] Snail-induced EMT is believed to play a crucial role in both embryonic development and pathological circumstances such as tumor progression and fibrosis.[3,4] In addition, several lines of evidence point to a role for Snail in the cell survival contributing to the protection of cell death. Interestingly, recent evidence shows that the members of the Snail family (including Snail itself) participate in the regulation of cell adhesion and migration independently of the induction of EMT such as mesoderm formation in *Drosophila*.[5] The idea that Snail is involved in cell movement that does not require a full EMT arises the question whether triggering of EMT would be just one of the mechanisms used by this transcription factor to induce loss of cell adhesion and to increase cell migration.[5]

Birgit Hotz and Alexander Visekruna contributed equally to this work.

B. Hotz · A. Visekruna · H.-J. Buhr · H. G. Hotz (✉)
Department of Surgery I, Charité School of Medicine,
Campus Benjamin Franklin,
Hindenburgdamm 30,
12203 Berlin, Germany
e-mail: hubert.hotz@charite.de

Tissue injury triggers an organized and complex cascade of cellular and biochemical events that result in a healed wound. This wound healing response can be divided into three distinct but overlapping phases: (1) hemostasis and inflammation, (2) proliferation, and (3) remodeling.[6] The inflammatory phase is an essential phase of healing, characterized by increased vascular permeability, chemotaxis of neutrophils from the circulation into the wound milieu, local release of cytokines and growth factors, and activation of migrating cells, especially macrophages.[6]

Here, we report for the first time the transient and time-dependent activation of Snail in migrating macrophages during inflammation and wound healing. We have investigated gastrointestinal anastomoses as well as wound healing of the skin in rats, and have found an induction of Snail expression mostly in macrophages, and to a less amount in neutrophils at the wound site. In order to assign these results to human specimens we examined sites of wound healing in the human skin and in addition in acute appendicitis, and found an increased expression of Snail in macrophages only at the wound site.

Inflammation occurs also during chronic inflammatory bowel disease (IBD) like Crohn's disease (CD) and ulcerative colitis (UC)[7,8] and therefore we examined tissue samples of patients with these diseases. Snail expression was also found in macrophages in affected colon tissues in contrast to healthy ones.

Although the mechanisms that modulate the function of Snail in migrating macrophages remain incomplete, we examined four pathways which are activated during inflammation, transforming growth factor beta (TGF-β1),[9–12] lipopolysaccharide (LPS),[8] interleukin-8 (IL-8),[13] and matrix metalloproteinase-3 (MMP-3),[14,15] and found that only TGF-β1 and to a less extent LPS are able to increase the induction of Snail. The other examined pathways seem to play minor roles in the regulation of Snail. To verify the role of Snail in the mechanism of TGF-beta-1-induced migration, we generated siRNA-Snail transiently transfected human macrophages (THP-1), measured cell migration in vitro in the presence of TGF-β1, and found an impaired movement of transfected THP-1 cells. Based on these findings, we propose a novel function of Snail in macrophage movement that is an unexpected and EMT-independent mechanism of this transcription factor.

Material and Methods

Patients and Paraffin-Embedded Tissue Sample

Ten tissue samples of inflamed wounds, acute appendicitis, 20 intestinal tissue samples of both Crohn's disease and ulcerative colitis, and 15 tissue samples of healthy intestine were obtained from patients, who underwent surgery at the Charité, CBF. Tissues were divided in two parts and either fixed in 4% formalin and embedded in paraffin, or shock frozen and stored at −80°C.

Animal Model

Male Wistar rats were anesthetized with isoflurane, followed by intraperitoneal injection of xylazinhydrochloride (Rompun, 12 mg/kg BW; Bayer, Leverkusen, Germany) and esketaminhydrochloride (Ketanest S, 40 mg/kg BW; Parke-Davis/Pfizer, Karlsruhe, Germany). A 3-cm median laparotomy was made and the descendent colon carefully mobilized. The colon was divided by a scissor and subsequently reanastomosed using 6–0 prolene by one layer continuous sutures. The laparotomy was closed in two layers with 3–0 absorbable suture (Vicryl, Ethicon, Germany). For pain relief, a subcutaneous injection of Carprofen (Rimadyl, 4 mg/kg BW; Pfizer) was given after surgery and the animals had food and water ad libitum; 6, 24, 48, and 96 h and 2 weeks after surgery, the animals were sacrificed by a lethal dose of isoflurane and the anastomoses was excised. The edge 5-mm oral and aboral the suture line was considered "anastomoses" and was harvested for the following experiments. The anastomoses were divided longitudinal in two parts and either fixed in 4% formalin and embedded in paraffin, or shock frozen and stored at −80°C.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin-embedded tissue. Three-micrometer-thick sections were cut, using a rotation microtom (Leica, RM2125RT). The sections were deparaffinized in xylene (2×5 min) and rehydrated in graded alcohols (100–70%, 5 min each) and distilled water. After antigen retrieval with 0.01% EDTA pH 8.0 (10 min boiling in a microwave), endogenous peroxidase activity was blocked with 1% hydrogen peroxide in distilled water for 25 min followed by washing with distilled water and finally phosphate-buffered saline (PBS) +0.1% Tween for 5 min. To bind nonspecific antigens, the sections were incubated with 1× Power Block (BioGenex, San Ramon, Ca) for 5 min. The primary antibodies for Snail, and CD68 were either polyclonal rabbit anti-Snail (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or polyclonal mouse anti-CD68 (Thermo Scientific, Fremont, CA, USA). Antibody dilution ranges from 1:50 to 1:150 in antibody diluent (DCS, Hamburg, Germany) for 30 min at 37°C. As negative control, sections were incubated with antibody diluent instead of the primary antibody. This was followed by incubation with biotinylated anti-rabbit/anti-mouse immunoglobulin G (1:200, Santa Cruz) for 30 min at 37°C and after washing with PBS+Tween by peroxidase-

conjugated avidin–biotin complexes (KPL, Gaithersburg, MD, USA) and 3,3`-diaminobenzidine (Sigma, DE, USA). The sections were then counterstained with Mayer's hematoxylin for 2 min, upgraded alcohols (70–100%, 2 min each), mounted and analyzed by standard light microscopy. Cells stained positive for Snail were counted in ten random fields of view at ×100 magnification and expressed as the average number of cells/field of view.

Cell Lines and Stimulation with TGF-β1, LPS, Interleukin-8, and MMP-3

The human monocyte lymphoma cell lines THP-1 as well as Jurkat T cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, USA). The cells were cultured in RPMI-1640 medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS-Gold, PAA), Penicillin G (100 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (0.25 mg/ml). The cells were incubated at 37°C in humidified air with 5% CO_2. Macrophages were generated from undifferentiated THP-1 using 100 nM phorbol 12-myristate 13-acetate (PMA)/ml medium (Sigma Aldrich). After incubation for 14 h with PMA, media was replaced by fresh RPMI medium and cells were maintained in medium for further 72 h. Differentiation was determined by increased cell attachment to the flasks and by changes in cell morphology. Recombinant TGF-β1 (10 ng/ml), LPS (1 µg/ml), interleukin-8 (10 nM/ml), and MMP-3 (40 nM/ml; Sigma Aldrich) were added to the culture medium. At 24 h and 48 h of stimulation, cells were harvested for western blotting and real-time polymerase chain reaction (PCR).

siRNA

After overnight culture of differentiated THP-1 cells in a six-well dish, the medium was replaced with 2 ml new medium (RPMI-1640 without FBS and antibiotics) and the cells were transiently transfected with Stealth/siRNA-Snail (100 pmol, Invitrogen, Carlsbad, CA, USA) or control-siRNA using Lipofectamine 2000 (Invitrogen) for an additional 48 h (using the manufacturer's protocol). Cells were then treated with TGF-β1 as described above and were harvested for Western blotting and reverse transcriptase (RT)-PCR. Stealth/siRNA-Snail Primer 1: 5′-UGGCACUGGUACUU CUUGACAUCUG-3′; Primer 2: 5′-CAGAUGUCAAGAA GUACCAGUGCCA-3′. Control-Stealth/siRNA Primer 1: 5′-UACCGUCAUGAUACAGUCACCGAGG-3′; Primer 2: 5′-CCUCGGUGACUGUAUCAUGACCGUA-3′.

Reverse Transcriptase Polymerase Chain Reaction

Total cellular RNA was extracted from cell cultures using the NucleoSpin RNA II-Kit (Macherey & Nagel, Düren,

Germany) according to the manufacturer's instructions and resuspended in 50 µl of DMPC-treated distilled water. RNA concentration was determined using a BioPhotometer (Eppendorf Scientific, Hamburg, Germany). Total RNA (2 µg) was primed with an oligo(dT) oligonucleotide and reverse-transcribed with M-MLV reverse transcriptase (Promega, Mannheim, Germany) and dNTPs (Sigma-Aldrich, Seelze, Germany) according to the manufacturer's instructions. First-strand cDNA was amplified with transcript-specific oligonucleotides using Ready-Mix Taq PCR Reaction Mix (Sigma-Aldrich, Seelze, Germany).

Quantitative Real-Time PCR

Real-time quantitative RT-PCR analyses for Snail (NM_005985.2), and PMM-1 (NM_002676) were performed by using the Light cycler^R with the Light cycler software 3.5^R (Roche, Mannheim, Germany). Primers were designed using the Light cycler Probe Design Software 2.0^R (Roche), each amplifying an approximately 150 bp product. Primers were manufactured by TIBMolbiol (Berlin, Germany). PMM-1 was included as a housekeeping gene control to correct for equal RNA amounts. We then calculated relative amounts of mRNA with the Relative Quantification Software^R (Roche). The Light cycler runs were done in duplicates. The principle of real-time RT-PCR has been described in detail before. Briefly, real-time RT-PCR is based on fluorescence emission by a sequence-specific primer pair. PCR was performed using the Light cycler Faststart DNA SYBR Green I-Kit (Roche, Mannheim, Germany) according to manufacturer's instructions. SYBR Green I binds to the minor groove of the DNA double helix. In solution, the unbound dye exhibits very little fluorescence; however, fluorescence (wavelength, 530 nm) is greatly enhanced upon DNA binding. Therefore, during PCR, the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated. Five microliters of diluted cDNA (1:5 with PCR-grade water), 0.5 µl primers (10 pmol/µl), and 0.8 µl $MgCl_2$ (25 mM) in a 10 µl final reaction mixture were used. After a 15-min incubation at 95°C for activation of the polymerase, each of the 35 cycles consisted of 15 s of denaturation at 95°C and amplification of primers for 30 s at 64°C. The melting curve analysis was performed in one cycle of 95°C for 1 s and 65°C for 10 s, each with a temperature transition rate of 20°C/s and then ramping to 95°C at 0.1°C/s.

Western Blotting

For isolation of total protein, the tissue was homogenized in lysis buffer containing 10 mM Tris (pH 6.8), 2 mM EDTA (pH 8.0), 0.15 M NaCL, 0.1% Brij 96, 0.1% NP-40, 2 mM PMSF, and 1× Protease inhibitor cocktail (Sigma-Aldrich).

Protein was estimated using QuantiPro BCA assay kit (Sigma-Aldrich) according to manufacturer's instructions. Thirty micrograms proteins were denatured at 95°C with sample buffer (0.125 M Tris (pH 6.8), 4% SDS, 20% glycerol, 2% mercaptoethanol, and 0.03 mM bromphenol blue) for 5 min and were separated by electrophoresis in 12% SDS-PAGE gels according to their molecular weight. Proteins were transferred onto a PVDF membrane (PerkinElmer, Zaventem, Belgium), blocked 2 h in blocking solution (5% non-fat dry milk in TBS) at room temperature on a rotating plate for 2 h. The membrane was then exposed to the primary antibody overnight at 4°C. The primary antibodies were Snail (anti-rabbit, Biozol, Eching, Germany), and β-actin (anti-mouse, LabVision, Fremont, USA), and the dilution range was from 1:1,000 to 1:10,000. After washing with TBS, the membranes were incubated for 1.5 h at room temperature with peroxidase-linked secondary antibody (Roche, Mannheim, Germany), and signals were detected using Lumilight Plus Western Blotting Kit reagents (Roche, Mannheim, Germany) according to the manufacturer's instructions and luminescence imaging (LAS-1000, Fujifilm).

In Vitro Invasion Assay

For in vitro invasion assay, 1.5×10^5 cells of differentiated macrophages were plated in the top chamber of Matrigel-coated PET membranes (24-well insert, pore size 8 μm; Becton Dickinson). Medium with 20% FCS and 1% BSA was used as a chemoattractant in the lower chamber. The cells were incubated for 48 h in a humified tissue culture incubator, at 37°C and 5% CO_2 atmosphere. Those cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were fixed with methanol and stained with hematoxylin. Cells in ten random fields of view at ×100 magnification were counted and expressed as the average number of cells/field of view. All experiments were performed in triplicates. The data were represented as the average of the three experiments with the SD of the average indicated.

Results

Expression of Snail in Activated Macrophages

Recent findings in our group revealed that Snail is highly induced in the invasive front of ductal adenocarcinomas of the pancreas, where tumor cells begin their migration to distant tissues due to EMT.[16] During inflammation a comparable migrating process occurs in immune cells which move to the affected region.[17–19] It is well known that macrophages exhibit similar behavior by infiltrating inflamed tissues.[20–22] Based on this evidence our idea was to look for a possible role of Snail in these migrating macrophages which may be not related to EMT. We used inflamed tissue derived from an intestinal anastomotic model and wounded skin of rats and performed immunohistochemistry. As shown in Fig. 1, Snail was highly induced in inflamed areas of colon, whereas low expression of this transcription factor in normal tissue was observed. To prove which cells express Snail, we performed double immunostaining with a marker for macrophages (CD68). Interestingly, induction of Snail expression was identified in macrophages (Fig. 2c), only at sites of inflammation and injury.

### Expression of Snail is Time- and Distance-Dependent in Inflamed Tissues

In inflammation and injury neutrophils are recruited from the blood followed by monocytes which locally differentiate into macrophages.[23] During the resolution of inflammation and wound healing, active macrophages are downregulated by specific signals, promoting apoptosis.[24] We hypothesized that Snail positive macrophages are recruited cells that migrate to the wound. Therefore, we examined the time-course of intestinal wound healing and several tissue samples of increasing distance to the local wound site, by cell count, immunohistochemistry, and real-time PCR in rats. Our results from the cell count at the anastomotic site at different time points showed an increase of Snail positive macrophages mostly in the lamina propria (Fig. 2c). The expression of Snail was elevated up to 18-fold at 48 h after surgery and then to 7.3-fold at 2 weeks after surgery (Fig. 2a). Real-time PCR results showed the increased expression of Snail at the m-RNA level in a similar way (Fig. 2b). In a second experiment, we examined the expression of Snail depending on the distance to the wound site. With increasing distance to the wound, the expression of Snail decreased on the mRNA and protein level (Fig. 3c). Cell counting revealed a 21.7-fold increased level of Snail positive macrophages at the anastomoses compared to normal colon tissue whereas the amount of Snail positive cells decreased to 14.3-fold at 1 cm distant to the wound and to 4.8-fold at 4 cm to the wound (Fig. 3a). Real-time PCR data confirmed these results (Fig. 3b).

Based on these results, we concluded that Snail positive macrophages, which are only located at the inflamed wound site, are recruited macrophages activated to defend inflammation.

Induction of Snail in Macrophages During Acute and Chronic Inflammation in Humans

Having shown activated macrophages expressing Snail in acute inflammation in rat tissues, we further examined a

Figure 1 Immunofluorescence staining of control colon (*left column*) and inflamed colonic tissue (*right column*) from rats. Tissue was stained for CD68 (marker for macrophages, *green*) and Snail (*red*). Overlay of CD68 and Snail show that macrophages display positive Snail expression. DAPI was used to visualize the nucleus (*blue*).
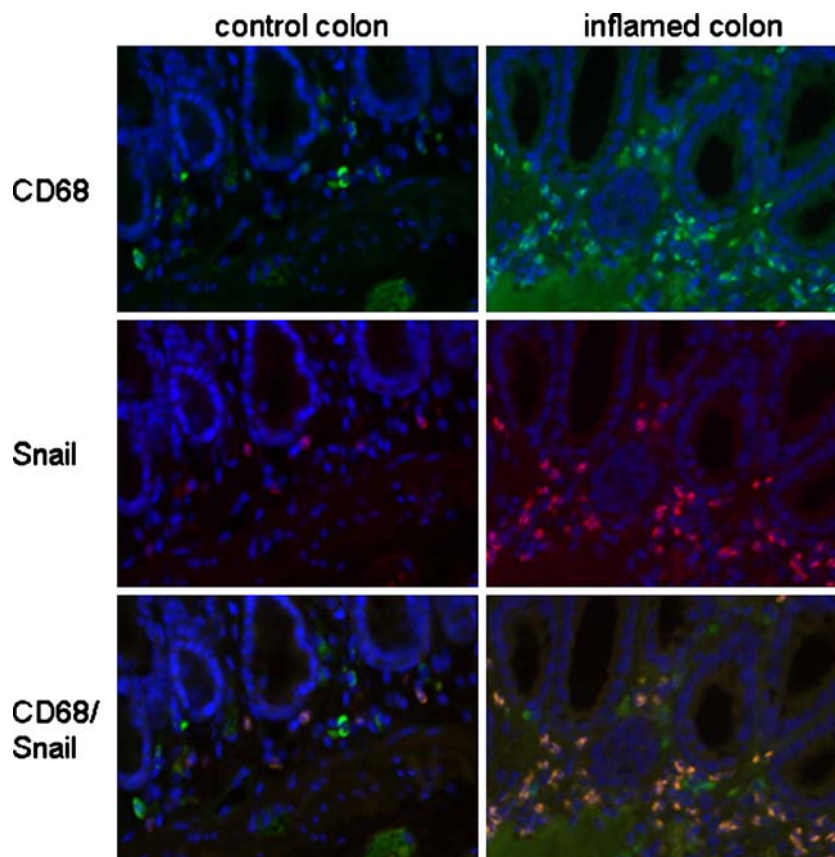
possible correlation in human tissues. Therefore we immunostained acute inflamed tissue from patients with wound infects after surgery and acute appendicitis and found an increased number of Snail positive macrophages at the wound site (Fig. 4).

Beside acute inflammation which occurs after injury, chronic inflammation, characterized by a permanent activation of immune cells, is present in some diseases like inflammatory bowel disease.[7] Two main forms of IBD are Crohn's disease and ulcerative colitis. To determine the expression of Snail during these chronic gut inflammations, we examined Snail mRNA levels in inflamed intestinal tissue of CD and UC patients as well as in normal gut by using real-time PCR. Snail mRNA expression was found to be 12- and fivefold elevated in inflamed intestinal tissue of CD and UC patients as compared to the normal colon, respectively (Fig. 5a). Immunohistological analysis was used for detection of protein expression of Snail and its localization in the colon. Whereas normal colonic tissue does not seem to express Snail constitutively (Fig. 5b), the amount of this transcription factor was markedly elevated in the mucosal lamina propria of CD and UC (Fig. 5c, d). Thus, the protein abundance of Snail in inflamed gut is related to its mRNA expression. Immunohistological staining revealed that the infiltrated macrophages were the predominant producers of Snail. This finding correlates

with our results obtained in the model of anastomotic wound healing in rats and human acute inflammation.

Induction of Snail by TGF-β1 in the Human Macrophage Cell Line THP-1

TGF-β1 has been shown to induce Snail in an EMT-dependent process during various stages of embryonic development.[2] Based on this observation, we next asked whether stimulation of THP-1 macrophages with this cytokine can affect the expression of Snail. In addition, we determined whether other signaling pathways might play a role in the regulation of this transcription factor. We therefore analyzed the induction of Snail in differentiated THP-1 cells after stimulation with TGF-β1, LPS, IL-8, and MMP-3 using real time-PCR and western blotting (Fig. 6). Only stimulation with TGF-β1 led to the induction of Snail in a time-dependent manner. Real-time PCR analysis revealed a threefold induction of Snail within 48 h after stimulation of THP-1 cells with TGF-β1. For LPS and interleukin-8 we observed a slight increase in Snail expression after 48 h. Stimulation of MMP-3 showed no increase in Snail expression (Fig. 6a). Similarly, Western blotting data showed a significant increase of Snail abundance in THP-1 macrophages during stimulation with TGF-β1, but not with LPS, IL-8, and MMP-3 (Fig. 6b).

Figure 2 Expression of Snail in a time-dependent manner during anastomotic wound healing in rats. **a** Cell count of Snail positive immune cells at different time points showed an increase of invading macrophages up to 48 h after surgery. **b** The analysis of the relative mRNA intensity of Snail from the anastomotic site confirmed the results from the cell counts. **c** Immunostaining of tissue from the anastomotic site revealed expression levels of Snail protein (brown color reaction) in macrophages peaking at 48 h. Magnification ×400. All sections were additionally stained with CD68 to confirm the specific expression of Snail in macrophages. (*$P<0.05$ was considered as significant).
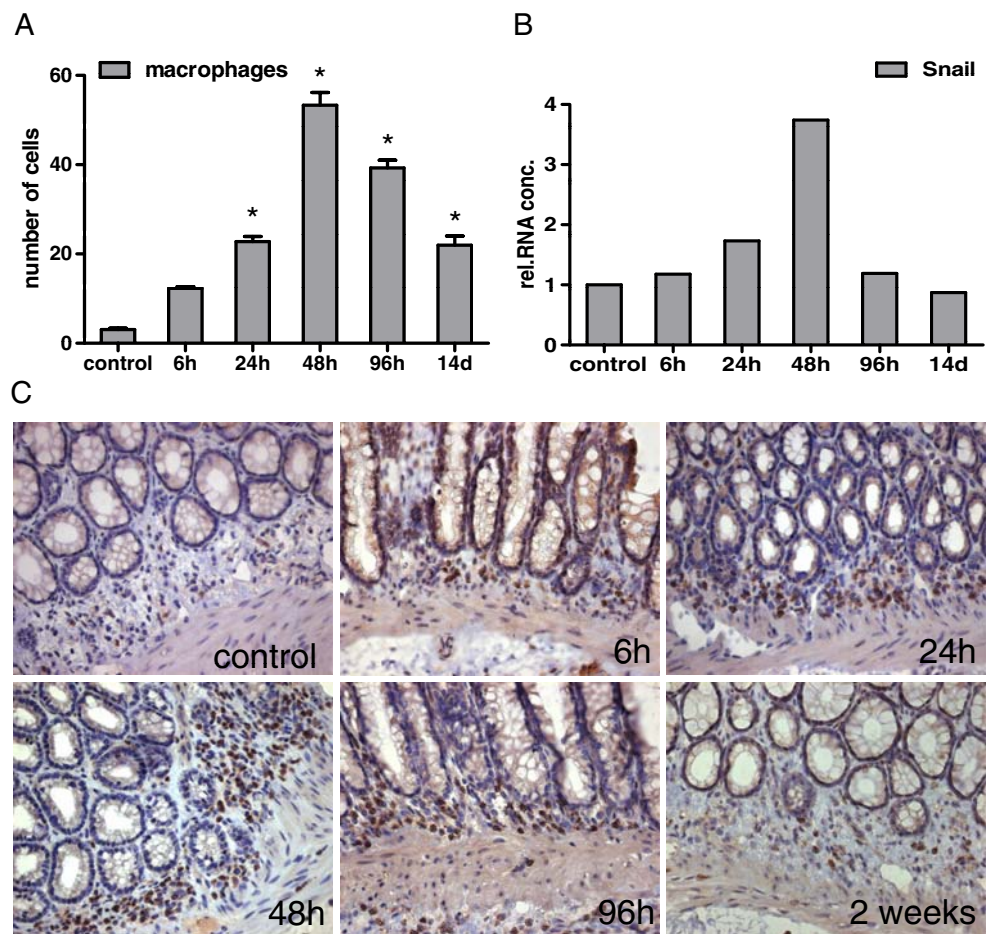
Jurkat T cells were used as a control showing no influence on induction of Snail by all four mediators (Fig. 6c). These results demonstrated that only the TGF-β1 pathway can activate Snail in macrophages during inflammation.

Snail is Necessary for TGF-β-Induced Movement of Macrophages In Vitro

To investigate the invasion-promoting effect of Snail in human macrophages, differentiated THP-1 cells were stimulated with TGF-β1 (10 ng/ml) for 48 h and loaded into artificial basement matrix coated Transwell™ chambers. Numbers of invasive cells in TGF-β1-stimulated macrophages were markedly increased up to 50% compared to untreated cells (Fig. 7b). Jurkat T cells were used as a control for the specific regulation of Snail and showed no increased invasion of cells through the Transwell chambers after stimulation with TGF-β1 (data not shown). To prove the relevance of Snail during cell movement we treated transiently transfected THP-1 cells (Stealth/siRNA-Snail and control-siRNA) with TGF-β1 and observed no significant change in migrating activity in THP-1 cells silenced for Snail in contrast to control siRNA transfected THP-1 cells (Fig. 7b). Therefore, we

conclude that Snail mediates the migrating properties of activated macrophages.

Induction of Snail During Inflammation is not Accompanied by Other EMT-Regulators

Snail, the related transcription factor Slug and the basic helix-loop-helix transcription factor twist are known to be master regulators of EMT, which initiate a complex program of gene regulation controlling this process.[25] Our findings that Snail is upregulated in activated macrophages led us to investigate whether Slug and Twist are also involved in this process. Therefore, we examined the expression of Slug and Twist in tissue specimens of acute and chronic inflammation in rats and humans by immunohistochemistry and Western blot as well as real-time PCR and found no constitutive expression of these transcription factors (Data not shown).

Discussion

The transcription factor Snail is involved in processes that imply pronounced cell movement, both during embryonic

Figure 3 Expression of Snail in a distance-dependent manner during anastomotic wound healing in rats. **a** Cell count as well as **b** real-time PCR showed a decrease of Snail positive macrophages and Snail mRNA depending on the distance to the anastomotic wound. **c** Immunostaining of different tissue regions 48 h after surgery revealed highest expression levels of Snail protein (*brown*) in macrophages of the anastomotic region (in comparison to 1 and 4 cm distance). All sections were additionally stained with CD68 to confirm the specific expression of Snail in macrophages. Magnification ×400. (*$P<0.05$ was considered as significant).
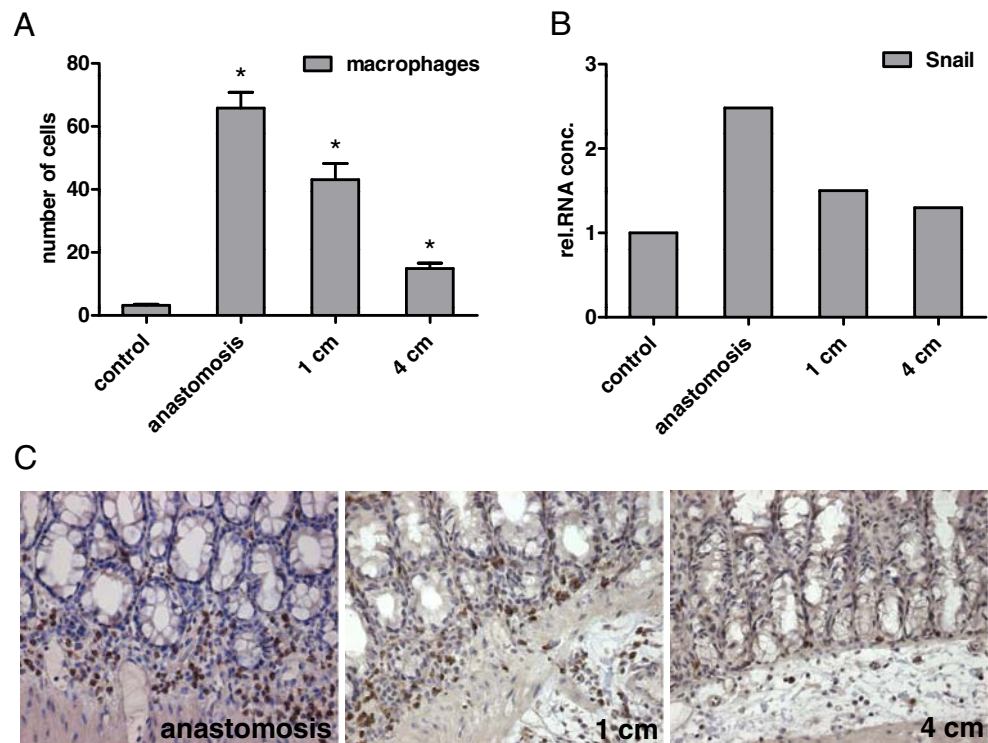
development and in the acquisition of invasive and migratory properties during tumor progression.[2,4,5] This role in promoting cell movement has been extended and one of the best studied mechanisms is the induction of EMT.[26,27] Snail-induced EMT converts epithelial cells into mesenchymal cells by direct repression of E-cadherin.[1] Although Snail seems to be required for all processes of EMT that have been examined, this does not necessarily mean that the induction of EMT is the prevalent role of Snail genes. They also have additional cellular functions that may occur independently of the induction of EMT such as protection of cells from apoptosis.[28]
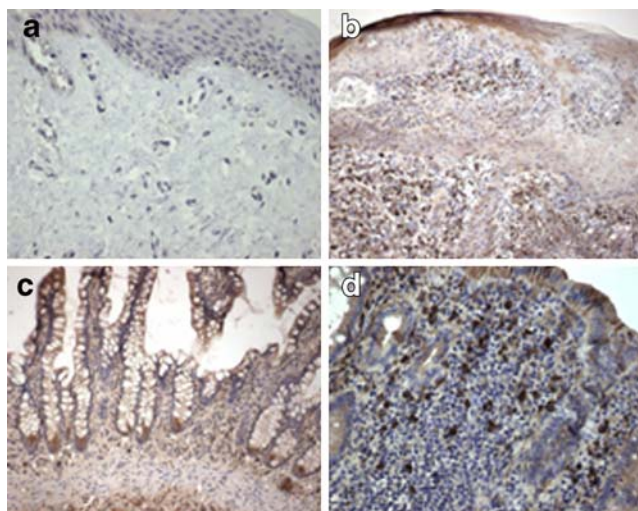

Figure 4 Immunodetection of Snail (brown color reaction) in human tissues. Snail is highly expressed in macrophages during wound healing, the difference demonstrated between normal (**a**) and inflamed skin (**b**). Sections of normal terminal ileum (**c**), and appendicitis (**d**) also depict the induction of Snail during acute inflammation. Magnification ×200.
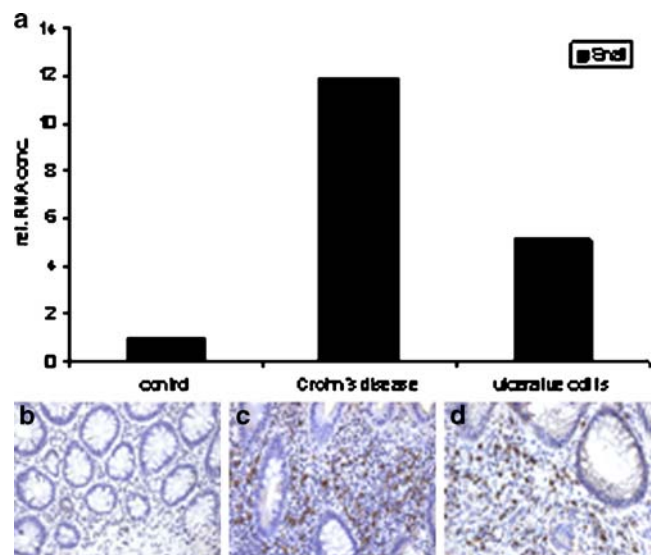

Figure 5 Expression of Snail in chronic inflammatory bowel disease (IBD). **a** Snail mRNA in normal colon, CD, and UC from pooled human tissue material. Immunohistochemistry of normal colonic tissue (**b**), Crohn's disease (**c**), and ulcerative colitis (**d**) from representative patient tissues shows increased expression levels of Snail (*brown*) in macrophages during IBD. Comparison of the mRNA and protein levels of Snail in CD and UC revealed significantly higher expression of Snail in CD than UC. Staining of the macrophage marker CD68 served as a control. Magnification ×400.
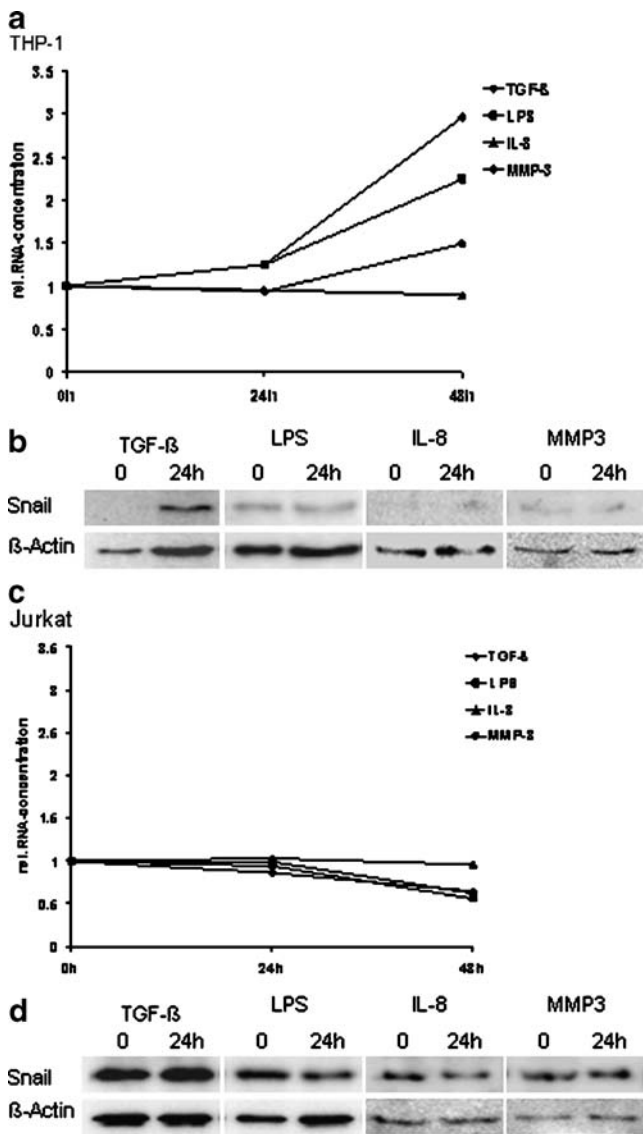
Figure 6 Induction of Snail in human macrophage cell line THP-1. **a** Stimulation of THP-1 cells with TGF-β resulted in an increase of Snail mRNA expression up to threefold by real-time PCR. **b** Western blot analysis confirmed the real-time PCR data indicating TGF-β-mediated induction of Snail protein in macrophages. **c** Stimulation of the control T cell line Jurkat with TGF-β showed no significant differences of Snail expression by real time PCR. **d** Western blot of the control T cell line Jurkat confirmed the real-time PCR data showing no differences of Snail protein expression after treatment with all four stimuli.

Another mechanism in the human organism that requires directed cell movement without involving EMT is the recruitment of neutrophils and macrophages during injury and inflammation.[23,29] Macrophage differentiation from monocytes recruited to inflammatory foci plays a critical role in defense mechanisms against pathogens and in inflammatory diseases.[30]

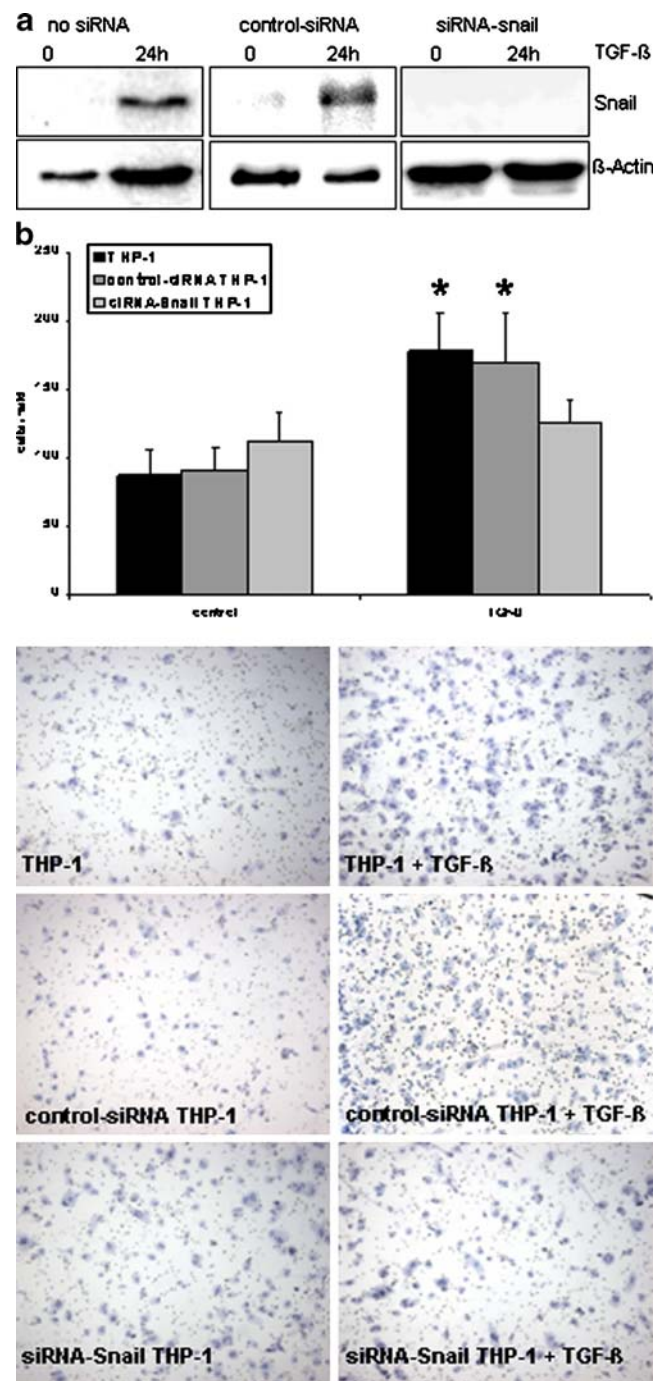Here, we demonstrate for the first time, that Snail expression is induced in invading macrophages at sites of

Figure 7 a Western blot analysis of THP-1 cells and control-siRNA transfected THP-1 cells compared to Snail gene silenced THP-1 cells (siRNA-Snail THP-1) after stimulation with TGF-β 1. β-actin was used as a loading control. **b** Invasion assay of unstimulated THP-1, control-siRNA THP-1, and siRNA-Snail transfected THP-1 cells versus stimulated with TGF-β 1 (10 ng/ml). After 48 h of stimulation with TGF-β, 50% more THP-1 cells (*blue*) invaded through the membrane of the Transwell chambers compared to untreated cells. The same effect was observed in control-siRNA transfected THP-1 cells after stimulation. In contrast, siRNA-Snail THP-1 cells showed no significant changes in migration after stimulation with TGF-β 1. Magnification ×100. (*$P < 0.05$ was considered as significant).

injury in a time- and distance-dependent manner. We examined different sites of wound healing like skin and anastomoses in the colon of rats and compared the results with specimens from patients with wound healing sites and acute inflammation. Snail expression was only detectable in macrophages at the affected wound site.

Although both processes, EMT and the recruitment of macrophages, share some molecular and cellular characteristics like motile cell behavior, invasion of distinct tissues, and transmigration across endothelial cells, the classical definition of EMT is the transformation of epithelial to mesenchymal and motile cells.[27] Macrophages have no epithelial characteristics like adhesive junctions between cells which enables even undifferentiated monocytes to migrate. During differentiation of macrophages, they have the ability to modulate an inflammatory phenotype and several molecular changes contribute to a coordinated transmigration towards injured tissue.[22]

Our experiments in an anastomotic wound healing model in rats revealed that the number of Snail positive macrophages increased during 48 h after surgery and decreased within the observation period of 2 weeks. We also examined the number of Snail positive macrophages dependent on the distance to the anastomotic site and found a decrease of these cells with increasing distance to the wound. In control tissue, we found only a few Snail-positive macrophages probably due to occasional invasion of bacteria through the mucosa of the colon. These results imply that the activated macrophages express high levels of Snail, whereas the same cell type, more distant to the wound and not involved in wound healing or inflammation, exhibits no expression of Snail.

Thus, we postulate that expression of Snail is a potent marker for migrating macrophages during acute inflammation and early wound healing.

Another question of interest is whether this transcription factor is also involved in chronic inflammation. The impact of Snail on immunologically mediated chronic inflammatory disorders such as Crohn's disease and ulcerative colitis remains poorly understood. Both conditions are heterogeneous diseases with an increased risk for developing colon cancer.[7,31] CD and UC are characterized by an abnormal mucosal immune response.[32] In this study we found a strong induction of Snail in the inflamed gut of both CD and UC patients. The constitutive expression of Snail was rarely detectable in control colonic tissue, whereas the abundance of this transcription factor was markedly elevated in infiltrating macrophages in CD and UC patients. These rare Snail positive macrophages in the normal colon tissue might result from some invading bacteria bearing pathogen-associated molecular patterns which are physiologically present in the normal gastrointestinal tract.[33,34] Thus, we show here for the first time that migrating

macrophages of the mucosal immune system in chronically inflamed tissue exhibit high expression of Snail, which is normally not expressed in adult organs.

Based on these findings, the next step to understand the role of Snail was to investigate regulatory pathways activated during inflammation, especially TGF-β, LPS, IL-8, and MMP-3. TGF-β1 is an important cytokine involved in the mucosal immune response displaying a broad spectrum of activities during inflammation and production of this cytokine is increased in patients with Crohn's disease and ulcerative colitis.[7] Since TGF-β1 is important in regulating lymphocyte infiltration in the intestine as well as in tumor progression, this pathway was the first candidate for a possible regulation of Snail. After exposure to TGF-β1, the differentiated human macrophage cell line THP-1 revealed increasing levels of Snail on the mRNA and protein level in a time-dependent manner.

In contrast, exposure of the differentiated human macrophage cell line THP-1 to LPS did not increase expression of Snail. A key chemokine involved in immune cell recruitment is interleukin-8, which is a strong chemo-attractant for monocytes, albeit less potent than TGF-β1.[13] Stimulation of differentiated human macrophages with IL-8 led to no change of Snail expression. Matrix metalloproteinases contribute to tissue injury and inflammation,[14] and stimulate the production of cytokines in macrophages at the site of the developing inflammation. It has been shown that MMP-3 can also trigger EMT[35] and induce Snail expression in murine mammary cells.[1] Treatment of differentiated THP-1 macrophages with MMP-3 showed no increased Snail expression on the mRNA and protein level.

Based on our observation that TGF-β1 induces the expression of Snail in differentiated macrophages, we performed invasion assays to study possible changes of migrating behavior in human macrophages after stimulation with TGF-β1. Interestingly, we observed an increased migration of macrophages through a matrigel-coated membrane compared to untreated cells, suggesting that activation of Snail is regulated through this specific pathway and that Snail might be involved in the recruitment of macrophages at sites of tissue injury and inflammation.

To prove this hypothesis we generated human macrophages with Snail gene silencing using siRNA technique. After stimulation of these cells with TGF-β1 we observed no induction of Snail at the RNA and protein level and notably no significant increase in migratory properties in performed invasion assays.

Since Snail is not the only potent regulator of EMT, we further asked whether other important EMT-inducers like Slug and Twist are also expressed in activated macrophages, but we found no expression of these transcription

factors in macrophages during acute inflammation and wound healing (data not shown).

In conclusion, our results provide a novel mechanism in the recruitment of macrophages by induction of Snail and offer a basis for investigating a new role of this transcription factor, which may be involved in directed cell movement beyond EMT.

References

1. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2006;7(2):131–142.
2. Acloque H, Thiery JP, Nieto MA. The physiology and pathology of the EMT. Meeting on the epithelial-mesenchymal transition. EMBO Rep 2008;9(4):322–326.
3. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 2003;15(6):740–746.
4. Nieto MA. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 2002;3(3):155–166.
5. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 2005;132(14):3151–3161.
6. Witte MB, Barbul A. General principles of wound healing. Surg Clin North Am 1997;77(3):509–528.
7. MacDonald TT, Di Sabatino A, Gordon JN. Immunopathogenesis of Crohn's disease. JPEN J Parenter Enteral Nutr 2005;29(4 Suppl):S118–S124. discussion S124–S125, S184–S188.
8. Macdonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. Science 2005;307(5717):1920–1925.
9. Roberts AB. Transforming growth factor-beta: activity and efficacy in animal models of wound healing. Wound Repair Regen 1995;3(4):408–418.
10. Wahl SM et al. Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 1987;84(16):5788–5792.
11. Del Zotto B et al. TGF-beta1 production in inflammatory bowel disease: differing production patterns in Crohn's disease and ulcerative colitis. Clin Exp Immunol 2003;134(1):120–126.
12. Ohtsuka Y, Sanderson IR. Transforming growth factor-beta: an important cytokine in the mucosal immune response. Curr Opin Gastroenterol 2000;16(6):541–545.
13. Smythies LE et al. Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. J Leukoc Biol 2006;80(3):492–499.
14. Nerusu KC et al. Matrix metalloproteinase-3 (stromelysin-1) in acute inflammatory tissue injury. Exp Mol Pathol 2007;83(2):169–176.
15. Shima I et al. Expression of matrix metalloproteinases in wound healing after glaucoma filtration surgery in rabbits. Ophthalmic Res 2007;39(6):315–324.
16. Hotz B et al. Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer. Clin Cancer Res 2007;13(16):4769–4776.
17. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol 2005;6(12):1182–1190.
18. Granger DN, Kubes P. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. J Leukoc Biol 1994;55(5):662–675.
19. Diacovo TG et al. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood 1996;88(1):146–157.
20. Albelda SM, Smith CW, Ward PA. Adhesion molecules and inflammatory injury. Faseb J 1994;8(8):504–512.
21. Goerdt S et al. Alternative versus classical activation of macrophages. Pathobiology 1999;67(5–6):222–226.
22. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5(12):953–964.
23. Godaly G et al. Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. J Leukoc Biol 2001;69(6):899–906.
24. Smythies LE et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 2005;115(1):66–75.
25. Teng Y, Zeisberg M, Kalluri R. Transcriptional regulation of epithelial-mesenchymal transition. J Clin Invest 2007;117(2):304–306.
26. Arias AM. Epithelial mesenchymal interactions in cancer and development. Cell 2001;105(4):425–431.
27. Gavert N, Ben-Ze'ev A. Epithelial-mesenchymal transition and the invasive potential of tumors. Trends Mol Med 2008;14(5):199–209.
28. Metzstein MM, Horvitz HR. The *C. elegans* cell death specification gene ces-1 encodes a snail family zinc finger protein. Mol Cell 1999;4(3):309–319.
29. Mahida YR et al. Migration of human intestinal lamina propria lymphocytes, macrophages and eosinophils following the loss of surface epithelial cells. Clin Exp Immunol 1997;109(2):377–386.
30. Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. Clin Sci (Lond) 2003;104(1):27–38.
31. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001;48(4):526–535.
32. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448(7152):427–434.
33. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol 2003;3(7):521–533.
34. Neurath MF et al. Regulation of T-cell apoptosis in inflammatory bowel disease: to die or not to die, that is the mucosal question. Trends Immunol 2001;22(1):21–26.
35. Radisky DC et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature 2005;436(7047):123–127.