Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease

Bataille, F; Rohrmeier, C; Bates, R; Weber, A; Rieder, F; Brenmoehl, J; Strauch, U; Farkas, S; Fürst, A; Hofstädter, F; Schölmerich, J; Herfarth, H; Rogler, G

Postprint available at: http://www.zora.uzh.ch

Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease

Abstract

BACKGROUND: The pathogenesis of fistulae in Crohn's disease (CD) patients is barely understood. We recently showed that more than two-thirds of CD fistulae are covered with flat, mesenchymal-like cells (transitional cells [TC]) forming a patchy basement membrane. Epithelial-to-mesenchymal transition (EMT) is a process of reprogramming epithelial cells, allowing them to migrate more effectively and giving epithelial cells an "invasive" potential. EMT has been suggested to be crucial in fibrosis found in different tissues and diseases. We therefore investigated whether EMT could be involved in the pathogenesis of fistulae formation in CD. METHODS: In all, 18 perianal fistulae, 2 enteroenteric, and 1 enterovesical fistulae from 17 CD patients were analyzed. In addition 2 perianal fistulae of non-CD patients were studied. Hematoxylin and eosin staining, immunohistochemistry for the expression of cytokeratins 8 and 20, beta6-integrin, E-cadherin, beta-catenin, vimentin, and TGF-beta1 and 2 were performed according to standard techniques. RESULTS: The TC covering perianal or enteroenteric fistulae were strongly positive for cytokeratins 8 and 20 but negative for vimentin, indicating their epithelial origin. Beta6-Integrin and TGF-beta had the highest staining intensities in the transitional zone between the epithelium and the TC. Expression of junctional proteins such as E-cadherin was reduced in TC as compared to regular fistulae epithelium. In addition, a translocation of beta-catenin from the membrane to the cytoplasm was observed. CONCLUSIONS: Our data for the first time indicate an expression pattern of epithelial and mesenchymal markers in TC associated with fistulae formation that is characteristic for EMT. Studying the pathways of EMT during intestinal fistulae formation may help to develop new therapeutic strategies.
Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn’s disease

Frauke Bataille*, Christian Rohrmeier+, Richard Bates§, Achim Weber§, Florian Rieder+, Julia Brenmoehl+, Ulrike Strauch+, Stefan Farkas§, Alois Fürst#, Ferdinand Hofstädter*, Jürgen Schölmerich+, Hans Herfarth+*b, Gerhard Rogler+a

* Institute of Pathology
+ Department of Internal Medicine I
§ Department of Surgery
University of Regensburg
93042 Regensburg
Germany

# Department of Surgery
Krankenhaus St. Josef
93042 Regensburg
Germany

x Department of Cancer Biology
University of Massachusetts Medical School
Lazare Research Building - 470Y
364 Plantation Street
Worcester MA 01605
USA

$ Department of Pathology, Institute of Surgical Pathology
University Hospital of Zürich
CH-8091 Zürich
Switzerland

a present address:
Clinic of Gastroenterology and Hepatology
Department of Internal Medicine
University Hospital of Zürich
CH-8091 Zürich
Switzerland

b present address:
Department of Medicine
Division of Gastroenterology and Hepatology
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7555
USA
Word count: 3399

Key words: Crohn’s disease, fistulae, epithelial mesenchymal transition

Short title: EMT and fistula formation in CD

Address for correspondence:

Gerhard Rogler, MD, PhD
Clinic of Gastroenterology and Hepatology
Department of Internal Medicine
University Hospital of Zürich
CH-8091 Zürich
Switzerland
Tel. +41-44-255-9477
Fax. +41-44-255-4503
E-mail: gerhard.rogler@usz.ch

Word count: 3596 words

No conflicts of interest exist.

Abbreviations: 5-ASA: 5-aminosalicylic acid; CD: Crohn’s disease; DAB: dianinobenzidine chromogen; EMT: epithelial-to-mesenchymal transition; IBD: inflammatory bowel disease; H&E: haematoxylin and eosin; TC: transitional cells

Acknowledgements:

This work was supported by the Bundesministerium für Bildung und Forschung, BMBF (Kompetenznetz CED), and the Deutsche Forschungsgemeinschaft (DFG Ro1236/15-1).
We thank Paul Weinreb and Shelia Violette at Biogen-Idec Inc. Cambridge MA, USA, for providing the integrin beta6 antibody.
Abstract

BACKGROUND: The pathogenesis of fistulae in Crohn’s disease (CD) patients is barely understood. We could recently show that more than 2/3 of CD fistulae are covered with flat, mesenchymal-like cells (transitional cells; TC) forming a patchy basement membrane. Epithelial-to-mesenchymal transition (EMT) is a process of re-programming epithelial cells allowing them to migrate more effectively and giving epithelial cells an “invasive” potential. EMT has been suggested to be crucial in fibrosis found in different tissues and diseases. We therefore investigated, whether EMT could be involved in the pathogenesis of fistulae formation in CD.

METHODS: 18 perianal fistulae, two entero-enteric and one entero-vesical fistulae from 17 CD patients were analysed. In addition 2 perianal fistulae of non-CD patients were studied. H&E staining, immunohistochemistry for the expression of cytokeratins 8 and 20, β6-integrin, E-cadherin, β-catenin, vimentin and TGF-β1 and 2 were performed according to standard techniques.

RESULTS: The TC covering perianal or entero-enteric fistulae were strongly positive for cytokeratins 8 and 20, but negative for vimentin indicating their epithelial origin. β6-integrin and TGF-β had highest staining intensities in the transitional zone between the epithelium and the TC. Expression of junctional proteins such as E-cadherin was reduced in TC as compared to regular fistulae epithelium. In addition a translocation of β-catenin from the membrane to the cytoplasm was observed.

CONCLUSION: Our data for the first time indicate an expression pattern of epithelial and mesenchymal markers in TC associated with fistulae formation which is characteristic for EMT. Studying the pathways of EMT during intestinal fistulae formation may help to develop new therapeutic strategies.
Introduction

Fistulae are a frequent and serious complication of patients with Crohn’s disease (CD). The cumulative incidence in population based studies ranges from 17 up to 50 % \(^1\text{-}^4\). The clinical management of CD-fistulae remains a major therapeutic challenge. Medical treatment is mainly based on antibiotics and immunosuppressants such as azathioprine, cyclosporine A, tacrolimus or infliximab \(^3\text{-}^8\). However, an initially successful closure of fistulae can only be maintained in about 30 to 40 % of patients after 12 months. Despite the clinical importance of the problem the pathogenesis of fistulae-formation is poorly understood and investigations focussed on the etiology of fistulae are sparse. A better knowledge of the pathophysiology of fistulae-formation would be crucial for the development of new and more effective therapeutic options.

It is generally assumed that tissue destruction is the first step in the pathogenesis of fistula formation. To gain further insights into fistula formation we histomorphologically characterized fistulae resected from CD patients and patients without the diagnosis of inflammatory bowel disease (IBD) \(^9\). Ninety-seven fistulae of an unselected patient cohort of 78 patients were investigated. Eighty-four fistula specimens were derived from 67 CD patients. The results were compared to 13 fistulae of 11 patients without IBD. Histologically all fistulae showed a central fissure penetrating through the lamina propria and the muscularis mucosae into the deeper layers of the underlying tissue. All fistulae were surrounded by a granulation tissue with histiocytes and a tight network of capillaries. In patients with CD, the interior wall of the studied fistulae usually was infiltrated by CD45RO positive T cells, followed by a small band of CD68 positive macrophages. At the outer fistulae wall, there was a dense infiltrate of CD20 positive B cells.
31 % of the „control fistulae“ (i.e. non-IBD) and 27 % of the CD-fistulae showed a layer with easily recognizable epithelial cells. Those cells showed tight junctions and a basement membrane. Interestingly the „non-epithelialised“ CD-fistulae - present in more than 2/3 of all cases - were covered by a thin layer of myofibroblast-like “transitional cells” (TC) with gap junctions. These cells also formed a basement membrane-like structure. In a subgroup of specimens a region could be identified in which the mucosal epithelial cells seemed to continuously transform into the TC-layer.

Under physiologic conditions fibroblasts are recruited to the sites of tissue injury. However, we recently showed that the migratory potential of CD mucosal fibroblasts is reduced compared to controls. An even stronger reduction of migratory ability was found in fibroblasts derived from CD fistula tissue (unpublished data) indicating a disturbance in wound healing. Epithelial cell migration is another hallmark of the attempt of the intestinal mucosa to rapidly close defects of the intestinal barrier. It is induced by a number of different growth factors. However, epithelial cells migrate slowly. If a superficial tissue defect of the mucosa cannot be closed by intestinal fibroblasts epithelial cells might migrate towards the defect.

Recent evidence suggests that epithelial cells do not represent a final and irreversible state of differentiation. Conversion of an epithelial cell to a mesenchymal cell has been shown to be critical during embryogenesis and is a structural feature of organ development. Current interest in this process – called epithelial–to-mesenchymal transition (EMT) - has increased due to its involvement in adult pathologies. It has been proposed that epithelial tumours undergo EMT, facilitating their invasion.
EMT is also an essential component of tissue remodelling, and wound repair $^{23-27}$. During this transition, the epithelial cells - characterized by strong cell-cell junctions and polarity - are replaced by a mesenchymal phenotype, with reduced cell-cell adhesions, a fibroblast morphology and function $^{22, 23, 28}$. There are several molecular markers for the detection of EMT in vivo $^{22, 23}$. These include decreased E-cadherin and β-catenin expression, and increased expression of β6 integrin $^{29, 30}$. During EMT β-catenin translocates to the nucleus. TGF-β has been shown to be an inducer of EMT.

We hypothesized that EMT could be important in the pathophysiology of fistula development in CD. Adhesions between mesenchymal cells are less tight than between epithelial cells facilitating an increased migratory capacity. Epithelial cells undergoing EMT have an increased migratory potential independently of cell-cell contacts $^{22}$. To test our hypothesis we used fistula specimens of patients with CD for immunohistochemical staining of EMT markers. In addition we established primary fibroblast cultures from CD patients with and without fistulae. Here we present first time evidence for EMT in intestinal wound healing and the pathogenesis of fistulae in CD.
**Materials and Methods**

**Patients**

18 entero-cutaneous fistula specimens from 15 patients were examined retrospectively. In addition we studied 2 perianal fistulae from patients without CD. This series was obtained from unselected cases at the Institute of Pathology of the University of Regensburg. Specimens had been surgically removed between August 1993 and May 2003. 14 CD fistula specimens showed flat TC covering the fistula walls, 4 fistulae had a squamous cell layer. The two non-CD fistulae also showed flat TC cells covering the fistula tract. In addition, two entero-enteric and one entero-vesical fistulae from 2 patients were investigated. Those two patients underwent surgery in December 2008 at the University Hospital of Zurich. Those fistulae had a columnar epithelium at the opening and flat mesenchymal like cells along the fistula tracts.

The diagnosis of CD was based on established clinical, endoscopic, histological and radiological parameters \(^{31, 32}\). Details on fistula distribution, patient age at presentation and patient gender are shown in **table 1**.

The degree of inflammation was graded microscopically by determination of the inflammatory infiltrate of neutrophils, eosinophils and lymphocytes: 0 = no inflammation, 1 = low degree of inflammation, 2 = severe inflammation.
This study was approved by the Ethics Committee of the University of Regensburg and performed according to the declaration of Helsinki.

**Specimen preparation**

Tissue specimens were fixed in 4% buffered formalin for at least 24 h and embedded in paraffin. Sections of approximate 2 – 3 µm thickness were cut from tissue blocks and stained with H&E (haematoxylin and eosin) according to standard protocols.

**Immunohistochemistry**

Immunohistochemical studies for the expression of cytokeratins 8 and 20, β6-integrin, TGF-β, E-cadherin, β-catenin and vimentin were performed on 17 fistulae of 15 patients with CD by using an avidin-biotin peroxidase method with diaminobenzidine (DAB) chromogen. After antigen retrieval (microwave treatment of formalin-fixed, paraffin-embedded 2-3 µm tissue sections for 40 min at 240 W in citrate buffer, pH 6.0) immunohistochemistry was carried out in a NEXES immunostainer (Ventana Medical System, Tucson, AZ) according to the manufacturer’s instructions. As primary antibodies mouse monoclonal and rabbit polyclonal antibodies were used (β-catenin [Santa Cruz, SC-7963, mouse monoclonal (E-5), dilution 1:50]; E-cadherin [Santa Cruz, SC-8426, mouse monoclonal (G-10), dilution 1:75]; TGF-β2 [Santa Cruz (SC-90), rabbit polyclonal, dilution 1:30]; TGF-β1 [Acris (DM1047), mouse (TB21) monoclonal, dilution 1:200]; CK8 [Dako M0631], mouse (35βH11) monoclonal, dilution 1:50], CK20 [Progen (61026), mouse (ITKS20.8) monoclonal, dilution 1:10]). After incubation for 24 min at 37° C, the slides were rinsed in PBS, and incubated with the secondary antibody (rabbit-anti-mouse; Ventana Medical System, 1:500 dilution in PBS) for 2 h at room temperature.
Antibody binding was visualized with 0.05% DAB (Ventana Medical System), and 0.01% hydrogen peroxide. The material was rinsed in PBS and counterstained with haematoxylin. For integrin β6 staining, antigen retrieval was performed using incubation in pepsin solution [Zymed Laboratories Inc.] at 37°C, prior to overnight incubation with the monoclonal antibody 2G2 (Biogen-Idec, 2 ug/ml).

Three different pathologists (F. Bataille, R. Bates, A. Weber) evaluated the specimens according to subjective criteria and graded the expression into three different categories (0 = no expression; 1 = weak expression and 2 = strong expression). The results of this evaluation were consistent.
Results

We evaluated differences in the expression of specific antigens between the cells of origin (intestinal columnar epithelium or squamous cell epithelium) and the TC by immunohistochemistry. 14 perianal fistulae with intestinal epithelium, two entero-enteric, one entero-vesical fistulae, four perianal fistulae with squamous cell epithelium from CD patients and two perianal fistulae from non-IBD patients were investigated. The expression of the respective antigen staining intensity was semi-quantitatively rated as “absent” (0), “weak” (1) or “strong” (2). A median was calculated and used for the comparison of the two fistula areas.

E-Cadherin

During the process of EMT a loss or reduction of E-cadherin expression is well known to occur. E-cadherin is involved in homophilic interactions between epithelial cells and is necessary for the formation of zonulae adherentes. In the normal intestinal epithelial cells E-cadherin staining was found at the lateral cell membrane at the cell-cell contact sites. In the fistulae lining cells a decrease in the intensity of staining was found (Figure 1). In 64.3% of the specimens additionally a redistribution of the membranous E-cadherin was found giving the staining at the cell wall a scattered appearance. Comparable observations were made in fistulae containing a squamous cell layer. A decrease in the median from weak staining to virtually absent staining was noted. With higher magnification it became evident that the pattern of E-cadherin expression in the fistula lining cells was dependent on the distance of the cells from the origin of the fistula in the gut lumen. Figure 2 clearly shows that cells closer to the mucosa (Figure 2A and B) have a more regular
expression of E-cadherin than cells from deeper areas of the fistula (Figure 2C and D). In Figure 2E this change in localization of E-cadherin can be seen in a typical “transition zone”.

**β-Catenin**

The process of EMT is characterized by a re-distribution of β-catenin \(^{38-42}\). Initially during EMT β-catenin expression is increased, however, the protein is no longer membrane associated but localized in the cytoplasm or even in the nucleus. In the later stages of EMT the synthesis of β-catenin is reduced. As expected we found a strong staining of the lateral cell membrane of the columnar normal mucosal intestinal epithelial cells (Figure 3 A, B and D). In the TC a diffuse and much weaker expression was found, which was localized cytoplasmatically (Figure 3 B, C and D). Comparable findings were obtained in the four fistulae with a squamous cell epithelial layer (Figure 3 E). The staining was much stronger in the regular epithelia compared to TC (Figure 3 G and H).

**Cytokeratin 8 and 20**

Cytokeratin 8 (CK 8) is an intermediate filament and part of the cytoskeleton of intestinal epithelial cells \(^{43}\). We used this typical epithelial marker to evaluate its expression in the flat TC covering the deeper areas of fistulae. In the 14 fistulae containing intestinal epithelial cells and TC no difference between the two cell populations with respect to CK 8 expression was observed (Figure 4). In the four specimens that contained a squamous cell layer a difference was noted. The squamous epithelial cells did not express CK 8 whereas in the fistula lining cells a median staining intensity of 1.5 was found.
Comparable results were found for cytokeratin 20 (CK 20). Intestinal epithelial cells as well as TC were strongly positive for CK 20 (data not shown).

**Vimentin**

Vimentin is an intermediate filament and one major component of the cytoskeleton. It is abundantly expressed in fibroblasts and endothelial cells. We used this typical mesenchymal marker to evaluate its expression in the flat TC covering the deeper areas of fistulae. In our staining all epithelia – columnar and squamous epithelia as well as TC – were negative for vimentin (data not shown).

**Integrin β6**

The β6 integrin-chain is restricted to epithelial cells and expressed during embryonic development and organogenesis. Re-induction of αvβ6 indicates an important role of this receptor during intestinal EMT. Six fistulae were stained with a specific antibody for β6 integrin. A clear difference in the intensity of staining between normal epithelial and fistulae covering cells could be noted. As expected virtually no protein expression of β6 integrin could be found in normal intestinal epithelial cells. Especially in the „transitional zones“, where a stepwise flattening of the epithelial cells occurs, a strong staining pattern (Figure 5) was detected.

**TGFβ1 and TGFβ2**

TGF is known to play a major role in the induction of EMT and its induction is linked to β6 integrin-expression. Immunohistochemistry for TGFβ1 showed a weak staining in the fistula lining cells whereas normal mucosal intestinal epithelial cells were negative for TGFβ1. This indicates an induction of TGFβ1 expression in the...
fistula tract. A clear difference of the medians of expression was found. In specimens containing squamous cells a comparable observation was made (data not shown).

Expression of TGFβ2 was weak in normal intestinal epithelial cells. The expression in the TC was stronger (Figure 6). Similar observations were made in the four specimens containing squamous cells.

**Entero-enteric fistulae**

To investigate whether the described features of EMT are generally found in “mesenchymal cell” covered CD fistula of specific for perianal fistula we investigated two enter-enteric fistulae obtained by surgical resection. The first patient suffered from ileo-sigmoidal and ileo-vesical fistulae and the second patient from ileo-ileal fistula formation. The Immunohistochemical results in these two fistula patients were identical to entero-cutaneous fistulae described above (Figure 7). Cytokeratin 8 and cytokeratin 20 were found to be expressed in all fistula lining cells independent of whether they had epithelial or mesenchymal morphology (Figure 7B). E-Cadherin expression clearly was associated to cell membranes and cell-cell contacts in the beginning of the entero-enteric fistulae tracts. However in the transition zone E-cadherin expression clearly was reduced or almost absent (Figure 7C, D and E). Whereas β-catenin expression was located at cell borders and cell membranes at the luminal end of the fistulae (Figure 7F and G) in the transition zone β-catenin was localized in the cytoplasm or in the nucleus (Figure 7H) as described to be typical for EMT. These data are indicative for a role of EMT in the formation of entero-enteric and entero-vesical fistulae.
Non-CD fistulae.

Two specimens from patients without CD were investigated by immunohistochemistry with the same antibodies as used above (with the exception of β6 integrin). Very similar to the findings indicated above we found evidence for EMT in these specimens (data not shown).
Discussion

In the present manuscript we show clear first time evidence for EMT in the epithelial transitional zone close to the luminal origin of perianal, entero-cutaneous, entero-enteric as well as entero-vesical fistulae tracts in CD patients. We provide data that the flat TC, that used to be morphologically classified as fibroblasts retain epithelial markers such as CK 8 and CK 20, are vimentin negative, show a redistribution of E-cadherin and β-catenin as described earlier to be typical for EMT and illustrate a re-induction of markers of early morphogenesis such as β6-integrin. TGF-β is highly expressed in the transitional zone between normal intestinal epithelium and fistula lining “mesenchymal cells” indicating a potential role during fistula-EMT. We therefore hypothesize, that CD fistula form via the TGF driven initiation of EMT which so far has only been described in cancer metastasis and tissue fibrosis.

What may be the reason for epithelial cells to undergo EMT during fistula formation? Normally regularly spaced cell–cell junctions and adhesions between neighbouring epithelial cells hold them together in a tight formation preventing the movement of individual cells away from the epithelial monolayer. In contrast, mesenchymal cells lack tight intercellular adhesions making them much more mobile followed by an increased migratory capacity. Whereas epithelial cells usually move as a sheet en block, mesenchymal migration is much more dynamic. Cells move individually and can leave part of the trailing region behind 54.

Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, downregulation of cell-cell adhesion systems, and migratory capacity. Commonly used molecular markers for EMT include β6-integrin, vimentin
and cytokeratin expression, cadherins and nuclear localization of β-catenin.

A defining feature of EMT is a reduction in E-cadherin levels (E for epithelial cadherin). Cadherins are transmembrane proteins whose homotypic interaction between neighbouring cells creates adherens junctions. Alteration of cadherin-based adhesion has a key role in modulating development, organogenesis or turnover of rapidly growing tissues. At the cell membrane, cadherin proteins are found as homodimers tethered to the actin cytoskeleton by a multiprotein complex that includes α-catenin, β-catenin, and p120-catenin. The presence of E-cadherin in epithelial cells allows for greater cell–cell adhesive strength compared with that of the N-cadherin–expressing mesenchyme.

β-catenin is the direct physiological link between cadherins and the actin cytoskeleton at the adherens junctions. In addition β-catenin has a role as a signal-transducing molecule influencing the state of the actin cytoskeleton. Catenins directly modulate the adhesive state of the cadherin extracellular homophilic adhesive binding domain and therefore control epithelial adhesion and junctional formation in a way similar to integrins. The nuclear translocation of β-catenin from the cytoplasm is considered a key molecular step in EMT. E-cadherin as well as β-catenin was down regulated in TC compared to mucosal epithelial cells and squamous cells. In addition in 2/3 of the specimens a redistribution of E-cadherin with a scattered appearance was detected. β-catenin in TC showed a diffuse cytoplasmatic localization in comparison to localization to the lateral cytoplasmic cell membrane in squamous epithelial or mucosal epithelial cells. This is clear evidence for a disaggregation of the epithelial units and a reshaping for movement.
The integrin complexes represent the major receptors that mediate attachment to the extracellular matrix, with ligand occupancy triggering intracellular signalling pathways. The expression of \( \beta_6 \)-integrin is restricted to epithelial cells. The \( \beta_6 \)-chain only associates with the \( \alpha V \) subunit. Among the ligands of the \( \alpha V \beta_6 \) complex are fibronectin, tenascin, and "TGF-\( \beta \) latency-associated peptide" (LAP). \( \alpha V \beta_6 \) is expressed during embryonic development and organogenesis. In adults \( \alpha V \beta_6 \) expression is only found in few epithelial tissues. Expression of \( \alpha V \beta_6 \) can be re-induced during specific morphogenetic processes such as inflammation and wound healing. Re-induction of \( \alpha V \beta_6 \) during intestinal EMT indicates an important role of this receptor in that process. It could be shown that the re-expression of \( \alpha V \beta_6 \) in colon carcinoma cell lines is correlated with aggressiveness of metastases and the extent of EMT. In CD fistulae \( \alpha V \beta_6 \) was solely expressed in TC with the highest staining intensity in the transitional zone between regular intestinal epithelium and TC. The invasive potential of the EMT cells could explain why fistulae similar to carcinoma cells are relatively aggressive. EMT in the context of fistula formation, however, differs from EMT found during tumour metastasis as in the case of fistulae normal, non-transformed cells undergo this change in differentiation.

TGF-\( \beta \) has been shown to be a key inducer and an important regulator of EMT. The TGF-\( \beta \) effect on EMT activation depends on \( \beta \)-integrin transduction. The effect of TGF\( \beta \) typically is either mediated via a Smad3-dependent regulation of transcription or a Smad3-independent, p38MAP-kinase-activation and GTPase-mediated signalling. The induction of \( \beta 6 \) integrin expression is likely to be involved in the induction of autocrine TGF secretion. In concordance with this TGF was stronger expressed in the TC versus squamous cells or mucosal epithelial cells.
Usually an induction of vimentin expression has been associated with EMT. Thus, the characteristics of the TCs fit most but not all criteria for EMT. However, there is no clear consensus in the literature defining the use of the term EMT. Therefore, we suggest that our lesser stringent definition of EMT is appropriate.

The specific pathogenesis of fistulae in CD is not known. There is a body of data suggesting a persistent functional change of intestinal myofibroblasts isolated from inflamed CD compared to controls. This is accompanied by a reduction of FAK protein and FAK phosphorylation, which may be responsible for the reduced migratory capacity of CD-myofibroblasts and TNF/IFN-γ treated cells. The reduced ability of inflammation-modified CD myofibroblast to close ulcers and deep tissue defects may force epithelial cells to undergo EMT and become rapidly moving and “invasive” to re-establish an epithelial layer and consecutively the mucosal barrier.

In summary our data indicate a change in phenotype of intestinal mucosal and squamous epithelial cells into TC. Staining for markers of EMT provides evidence for this mechanism in perianal, entero-cutaneous, entero-enteric and entero-vesical fistulae. The functional characteristics of EMT, such as increased capacity for migration and three-dimensional invasion by downregulation of molecules, which promote epithelial integrity, might be an attempt of the intestinal non-immune cells to close the mucosal defects, however in this case with a detrimental effect. The presence of similar results in non-CD fistulae implies that EMT may not only be involved in fistula formation in CD. This indicates that EMT may be a general feature of histologically similar fistulae, regardless of their etiology.
It will be important to further understand the mechanisms of EMT in the pathophysiology of fistula formation. This study opens a new area of research. Interfering with EMT may finally provide new therapy targets in the treatment of CD fistulae.
Figure legends

Figure 1. Expression of E-cadherin in CD fistulae
The regular mucosal epithelial cells (black arrow) showed a stronger expression of E-cadherin, compared to the transitional zone and the TC (red arrow; A, B). 2/3 of the specimens showed a scattered appearance of the fistula epithelium (B). In the TC originating from the squamous epithelial cells no E-cadherin expression was noted (C). The negative control is shown in figure D. Semi-quantitative analysis of the staining intensity revealed a clearly higher expression of E-cadherin in the mucosal epithelial cells versus the TC (E). Epithelial cells from the specimen with the squamous epithelial lining expressed E-cadherin with a lower intensity, which was even lower in TC (F).

Figure 2. Change of the pattern of expression of E-cadherin in CD fistulae
The pattern of E-cadherin expression in the fistula lining cells was dependent on the distance of the cells from the origin of the fistula in the gut lumen. Cells closer to the mucosal surface show a more regular expression of E-cadherin (A and B) than cells from deeper areas of the fistula (C and D). E: typical “transition zone” showing this change of localization of E-cadherin staining. (A, C, D and E: magnification x 200; C: magnification x 400).

Figure 3. Expression of β-catenin in CD fistulae
The regular mucosal epithelial cells (black arrow) showed a strong expression of β-catenin, which was located at the cell membrane. The TC however (red arrow) express lower amount of β-catenin, which was mainly fragmented in the cytoplasm.
(A – D). The same observation could be made for fistulae with a squamous cell epithelial origin (E). The negative control is shown in figure F. Semi-quantitative analysis of the staining intensity revealed a higher expression of β-catenin in the mucosal epithelial cells versus the TC (G). The same observation could be made for fistulae with a squamous cell epithelial origin (H).

**Figure 4.** Expression of cytokeratin 8 in CD fistulae

The mucosal epithelial cells (black arrow) as well as the TC lining the fistula lumen (red arrow) are strongly positive for cytokeratin 8 (A - D; C: same specimen as shown in Figure 3C). In D the transition zone with more flat cells to the lower right corner is clearly visible without reduction of CK 8 expression. The TC, which originated from squamous epithelial cells were also positive for cytokeratin 8 (E). The negative control is shown in figure F. Squamous epithelial cells did not express cytokeratin 8 versus a median expression of 1.5 in TC.

**Figure 5.** Expression of β6 integrin in CD fistulae

The regular mucosal epithelial cells (black arrow) did not express β6 integrin, whereas the staining in the transitional zone (red arrow) is strongly positive (A - D). A higher magnification (areas from panel D) clearly shows the difference in staining intensity between TC cells (left) and normal epithelium (right). The isotype control did not show any staining (data not shown). Semi-quantitative analysis of the staining intensity revealed a clearly higher expression of β6 integrin at the transitional zone versus the regular mucosal epithelium (F).
**Figure 6.** Expression of TGFβ2 in CD fistulae

The regular mucosal epithelial cells (black arrow) showed a weak expression of TGFβ2, whereas the staining of the TC (red arrow) was strongly positive for TGFβ2 (A). In panel B a higher magnification of the fistula lining cells and in C of a normal crypt is shown to clearly demonstrate the difference in immunohistochemical staining. The same observation was made for fistulae with a squamous cell epithelial origin (D). Semi-quantitative analysis of the staining intensity revealed a higher expression of TGFβ2 at the transitional zone versus the regular mucosal epithelium and the squamous epithelium (E, F).

**Figure 7.** Evidence for EMT in an entero-enteric fistula

A: H&E staining of an entero-enteric fistula. The fistula origin is to the upper left. B: Cytokeratin 8 is expressed in all fistula lining cells, even in regions where they are flat and no longer columnar with a more mesenchymal-like morphology. E-Cadherin expression was associated to cell membranes and cell-cell contacts in the beginning of the fistula. However in the transition zone E-cadherin expression clearly was reduced (C). Higher magnifications (D and E) indicate this difference. Whereas β-catenin expression also could be located to cell borders and cell membranes at the beginning of the fistula (F and higher magnification in G) in the transition zone β-catenin was localized in the cytoplasm or even in the nucleus (H) as described to be typical for EMT.
### Tables

**Table 1**

Characteristics of the fistulae investigated in this study.

<table>
<thead>
<tr>
<th>Age of patient at presentation</th>
<th>gender</th>
<th>localisation of fistula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entero-cutaneous, CD patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 m</td>
<td>f</td>
<td>ileum</td>
</tr>
<tr>
<td>67 f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 m</td>
<td></td>
<td>ileum</td>
</tr>
<tr>
<td>24 m</td>
<td></td>
<td>left sided colon</td>
</tr>
<tr>
<td>37 m</td>
<td></td>
<td>terminal ileum</td>
</tr>
<tr>
<td>23 f</td>
<td></td>
<td>terminal ileum</td>
</tr>
<tr>
<td>20 m</td>
<td></td>
<td>cecum</td>
</tr>
<tr>
<td>20 m</td>
<td></td>
<td>sigmoid colon</td>
</tr>
<tr>
<td>48 f</td>
<td></td>
<td>sigmoid colon</td>
</tr>
<tr>
<td>42 f</td>
<td></td>
<td>colon</td>
</tr>
<tr>
<td>24 m</td>
<td></td>
<td>ileum</td>
</tr>
<tr>
<td>40 m</td>
<td></td>
<td>ileum</td>
</tr>
<tr>
<td>37 f</td>
<td></td>
<td>perianal</td>
</tr>
<tr>
<td>27 f</td>
<td></td>
<td>ileum</td>
</tr>
<tr>
<td>71 m</td>
<td></td>
<td>perianal</td>
</tr>
<tr>
<td>39 f</td>
<td></td>
<td>perianal</td>
</tr>
<tr>
<td>24 f</td>
<td></td>
<td>perianal</td>
</tr>
<tr>
<td>35 m</td>
<td></td>
<td>perianal</td>
</tr>
<tr>
<td>Entero-enteric and entero-vesical, CD patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52 m</td>
<td></td>
<td>Ileum – sigmoid colon (entero-enteric)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and ileum-bladder (entero-vesical)</td>
</tr>
<tr>
<td>19 m</td>
<td></td>
<td>Ileum-ileum (entero-enteric)</td>
</tr>
<tr>
<td>Entero-cutaenous, non IBD patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 m</td>
<td></td>
<td>perianal</td>
</tr>
<tr>
<td>56 m</td>
<td></td>
<td>perianal</td>
</tr>
</tbody>
</table>
References


59. Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an epithelial to mesenchymal transition in human immortal and malignant