Ulcer-associated cell lineage expresses genes involved in regeneration and is hallmarked by high neutrophil gelatinase-associated lipocalin (NGAL) levels

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Abstract

Neutrophil gelatinase-associated lipocalin (NGAL), also known as Lipocalin 2, is an antimicrobial protein, encoded by the gene LCN2, strongly upregulated in inflammatory bowel disease (IBD) and a promising biomarker for IBD. Here we demonstrate that NGAL is highly expressed in all parts of pyloric metaplasia, also known as the ulcer-associated cell lineage (UACL), a metaplastic cell lineage suggested to play a role in wound healing in Crohn’s disease (CD). We further show NGAL expression in regenerative intestinal crypts and in undifferentiated patient-derived colonoids. This indicates that NGAL is important in tissue regeneration process. The remarkable overexpression of NGAL in UACL led us to explore the pathobiology of these cells by transcriptome-wide RNA sequencing. This study is, to our knowledge, the first to characterize the UACL at this level. Biopsies with UACL and inflamed non-UACL epithelium from the terminal ileum of CD patients and epithelium from healthy controls were laser capture microdissected for RNA sequencing. Among the 180 genes differentially expressed between UACL and control epithelium, the ten most-upregulated genes specific for UACL were MUC5AC, PGC, MUC6, MUC5B, LCN2, POU2AF1, MUC1, SDC3, IGFBP5, and SLC7A5. PDX1 was among the most upregulated in both UACL and inflamed non-UACL epithelium. Immunohistochemistry and iDisco 3D visualization was used to characterize UACL histo-morphologically, and to validate protein expression of 11 selected differentially expressed genes. Among these genes, LCN2, NOTCH2, PHLD1A, IGFBP5, SDC3, BPIFB1, and RCN1 have previously not been linked to UACL. Gene expression results were analyzed for functional implications using MetaCore, showing that differentially expressed genes are enriched for genes involved in cell migration and motility, and for biomarkers of gastrointestinal neoplasia. These results support a role for UACL as part of the reepithelialization process during and after destructive intestinal inflammation.

Keywords: NGAL; IBD; CD; UACL; pyloric metaplasia; wound healing; regeneration

Introduction

The glycoprotein neutrophil gelatinase-associated lipocalin (NGAL) (also known as lipocalin 2 or siderocalin) has attracted interest due to its antimicrobial properties and expression in various cancer types and in inflammatory disorders. Nielsen et al [1] and our group [2] have found that the gene coding for NGAL, LCN2, is strongly upregulated in biopsies from patients with active ulcerative colitis (UC) and colitis in Crohn’s disease (CD). We have shown that fecal NGAL is massively increased in active inflammatory bowel disease (IBD) with properties making it a promising biomarker [3].

We recently reported that NGAL/LCN2 was markedly upregulated also in small intestinal CD [4]. In the healthy ileum, NGAL was expressed by Paneth cells and enteroendocrine cells, and the upregulation seen during inflammation was due to increased expression in epithelial cells. Notably, in contrast to the inflamed colonic epithelium where...
NGAL was evenly expressed, staining of the small intestinal epithelium showed a more patchy expression of NGAL.

In the present study we show that NGAL’s patchy appearance is due to expression in epithelium known as the ulcer-associated cell lineage (UACL). UACL can be found in the ulcer margins of patients with CD, and is believed to have a role in the wound healing process [5–7]. The UACL is also known as pyloric metaplasia due to its morphological appearance and content of gastric mucins. It expresses proteins such as members of the trefoil factor family (TFF) known to confer a protective effect on the intestinal mucosa [8], and growth factors including TGFβ and EGF [9]. The cell lineage seems to develop from crypts adjacent to ulcerations, forming a complex of apparently disorganized structures before maturing and developing a stem cell niche providing cells migrating to the gut lumen [5]. UACL is a common finding in ileal CD with prevalence of 22–87% [5,10,11], it is most often seen when examining inflamed, ulcerated mucosa. Similar crypts can be found in CD of the colon, adjacent to gastric- and duodenal ulcers, in cholecystitis and pancreatitis, and there are even reports of pyloric metaplasia in salpingitis and in the uterine cervix [12,13].

In contrast to intestinal metaplasia in the esophagus and stomach, small intestinal UACL has not been related to an increased cancer risk. Recent reports have, however, pointed to an increased cancer risk also in small intestinal CD [14,15] and one report suggested a connection between UACL and CD enteritis-associated small bowel adenocarcinoma [16].

UACL is still not fully understood and the role of NGAL, other than as an antimicrobial peptide in an inflammatory gut environment, is far from clear. This study aimed therefore to study NGAL in the UACL, together with obtaining a molecular and histological characterization of UACL in the intestinal mucosa.

Methods

Patients and biopsies

Study subjects were recruited among patients with UC or CD coming to ileocolonoscopy or ileocecal resections at the Department of Gastroenterology or Department of Gastrointestinal Surgery, St Olav’s University Hospital, Trondheim, Norway. Control subjects were patients of Gastrointestinal Surgery, St Olav’s University Hospital as well as patients from the Region of North Trondelag with no known gastrointestinal disorders and with normal ileocolonoscopies. Biopsies were taken from colon, ileum or from neo-terminal ileum in operated patients. An experienced pathologist classified the biopsies into normal, chronic or active inflammation. Biopsies with UACL were identified using sections stained with H&E or Alcian Blue (AB)/PAS (Merck, Darmstadt, Germany). All participants gave written informed consent, and the study was approved by the Regional Committee for Medical and Health Research Ethics, approval nos. 5.2007.910 and 2013/212/REKmidt. Immunohistochemistry and in situ hybridization

Formalin-fixed paraffin-embedded (FFPE) biopsies were cut into 4 µm thick sections and mounted on glass slides. The following primary antibodies were used for immunohistochemistry (IHC): NGAL (Biporto, Hellerup, Denmark), Muc2, Muc5AC, PGC, Lysozyme, Notch2, SDC3 (all Abcam, Cambridge, UK), Ki-67, CgA, CK20 (all Dako, Glostrup, Denmark), RCN1, BPIFB1, FABP1 (Merck), IGFBP5 (R&D, Minneapolis, MN, USA) and PHLD1A (Santa Cruz, Dallas, TX, USA). All catalog-numbers, full names and dilutions are listed in supplementary material, Table S1. A primary antibody was applied for 1 h at room temperature or 4°C overnight. The secondary layer was rabbit/mouse EnVision-HRP (Dako) or anti-Goat (1:150) with Vectastain ABC HRP kit (Vector Laboratories, Burlingame, CA, USA, cat. no. BA-5000 and PK-4000, respectively) and visualization was accomplished using DAB+ (Dako, cat. no. K5007) before counterstaining with hematoxylin. Omitting the primary antibody or matching-isotype immunoglobulin was used as negative controls. Serial sections from at least eight biopsies from ileum with active CD and UACL, and six biopsies from controls were examined to verify gene expression results. For NGAL expression in colon, ten biopsies with inflamed CD or UC were examined, of which three contained UACL.

In situ hybridization (ISH) was performed using custom RNAscope probes (Advanced Cell Diagnostics, Hayward, CA, USA), according to the manufacturer’s protocol. Negative (DAPI) and positive (UBC) control probes were included.

diSto staining

Whole-mount immunostaining for 3D visualization of small intestinal mucosa was done according to the diSto+ protocol [17]. The protocol used is the version as of December 2016, available at the online resource https://idisco.info/, with n = 4 days. Tissue was stained using anti-NGAL as primary-, and Alexa Fluor 647-tagged Goat anti-mouse (1:2500, Thermo Fisher Scientific, Waltham, MA, USA, cat. no. A28181, RRID_AB_2536165) as secondary antibody. Hoechst 33342 (1 µg/ml, Thermo Fisher Scientific, cat. no. 62249) was used as a counterstain. Further details of the clearing and staining procedure and of the confocal imaging can be found in supplementary material, Supplementary materials and methods.

Laser capture microdissection

Ileal biopsies from six patients with active CD with UACL and seven controls were used for microdissection and gene expression analysis. Clinical and demographic characteristics of the study subjects are given in supplementary material, Table S2. The FFPE biopsies were cut into 10 µm sections, mounted on glass slides and stained with AB-PAS in order to identify UACL. The sections were treated in 100%
ethanol and xylene in order to remove all traces of H₂O. Sections were then air-dried in a desiccator (30 min) before laser capture microdissection (LCM) (MMI CellCut on Olympus IX71, Eching, Germany). UACL glands, inflamed non-UACL epithelium (crypts and villi) from the same biopsies and epithelium from controls were isolated. In sections with ample amounts of epithelium available, a maximum area of 500,000 μm² (approx. 5000 cells) was isolated. For some sections, particularly for the UACL structures, significantly smaller areas could be isolated. Details of isolated area for each sample can be found in supplementary material, Table S3. Supplementary material, Figure S1 shows an example of the microdissection process. After LCM, the isolated cells were immediately transferred to buffer from the RNA extraction kit and stored at −80 °C.

RNA isolation, library preparation and sequencing

Total RNA was extracted from LCM material, using an RNeasy FFPE kit (Qiagen, Hilden, Germany, cat. no. 73504) according to the manufacturer’s instructions. RNA integrity was assessed using an Agilent RNA 6000 Pico Kit on a 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). The DV200 value (percentage of RNA fragments >200 nucleotides) was used as a measure of RNA quality. The mean DV200 values for the samples were 72.0 ± 12.6% (details in Supplementary material, Table S3). RNA-sequencing libraries are described in supplementary materials, Supplementary materials and methods.

Libraries were normalized and pooled before denaturation, final concentration of the library was 2.7 pm for clustering on NextSeq 500 high output flowcells. Single read sequencing was performed for 75 cycles on a NextSeq 500 instrument (Illumina Inc., San Diego, CA, USA), according to the manufacturer’s instructions. Base calling was done by RTA 2.4.6. FASTQ files were generated using bcl2fastq2 Conversion Software v2.17 (Illumina Inc.).

For RNA-sequencing analysis the gene abundance quantification was done with featureCounts [18] after alignment of the reads to the genome (GRCh38, release 7) using STAR v2.4.1b. Gene models were defined by protein-coding genes in the Ensembl GTF file for the GRCh38 genome. Genes that did not meet the criterion of a gene count above three in more than five samples were filtered out. The matrix of filtered read counts were imported to R, normalized using trimmed mean of M-values (TMM) and subsequently voom normalized [19]. Differential expression was identified by linear models using the edgeR/limma packages and significance decided by Benjamini–Hochberg false discovery rate adjusted P values ≤0.05. RNA sequencing data were deposited in the Gene Expression Omnibus ( GEO) repository and assigned the GEO accession number GSE126299.

Pathway analysis

Gene ontology (GO) processes of the genes in the UACL gene signature were analyzed using the MetaCore analysis suite (GeneGo Inc., http://www.genego.com). Genes over-expressed with p < 0.1 in UACL versus controls, and not regulated in inflamed non-UACL epithelium versus controls (p > 0.2), were entered into the analysis program. Very unspecific GO processes containing >1500 genes like e.g. ‘positive regulation of cellular process’ were excluded. Diseases (by biomarkers) were ranked after ‘MetaCore disease association analysis’.

Human colonoids

We used a protocol [20] based on that of Sato et al [21] to isolate intestinal crypts from colon pinch biopsies from three healthy subjects. After culturing in basement membrane matrix (Matrigel, Corning Life Sciences, Tewksbury, MA, USA; cat. no. 734-1101) and medium containing factors critical for stem cell growth (e.g. Wnt3a, R-Spondin and Noggin), the crypts reestablished themselves as three-dimensional spheroids. For differentiation, the medium was changed 5–7 days after splitting, by diminishing Wnt3a concentration, removing nicotinamide and the MAPK inhibitor SB202190 and adding the pan-Notch inhibitor DAPT to the medium. After 4 or 5 days of differentiation the matrigel was depolymerized by cell recovery solution (Corning, Corning, NY, USA, cat. no. 354253), and the colonoids centrifuged (500 × g). Five microliters of plasma and 1 μl thrombin solution was added to colonoid-pellets and allowed to coagulate before formalin fixation for 48 h. The colonoids were then paraffinized, and after deparaffinization IHC and ISH was performed according to their respective protocols. IHC for the markers LGR5 (stem cells), FABP1 (absorptive cells), MUC2 (goblet cells), and CgA (enteroendocrine cells) was done to confirm successful differentiation with four known colonic cell types represented (data not shown).

Enzyme-linked immunosorbent assay

NGAL levels in supernatant from colonoid culture were measured using human LCN2/NGAL ELISA (R&D Systems, Minneapolis, MN, USA, cat. no. DY1757), according to the manufacturer’s protocol. Supernatant from each technical replicate was assayed in duplicate, after dilution 1:40 in PBS + 0.1% BSA.

Statistics

Statistical analysis was performed using the Prism 5 software program (GraphPad Software, San Diego, CA, USA). A Wilcoxon matched-pairs signed rank test was used for the comparison of secreted NGAL from differentiated and undifferentiated colonoids from seven individual experiments, and a Kruskal–Wallis test for comparison of secreted NGAL after stimulation with various factors (based on results from three independent biological replicates). Significant values were defined as p < 0.05 (two-sided) if not otherwise stated.
UACL expresses genes engaged in regeneration and is hallmarked by NGAL

Figure 1. NGAL/LCN2 is expressed in all stages of small intestinal UACL and in colonic regenerative crypts. (A) Serial sections (from left to right) showing H&E, AB-PAS, IHC for NGAL and ISH for LCN2 with positivity in UACL-crypts. (B) IHC NGAL (left) and AB-PAS (right) showing scattered NGAL and AB/PAS positive cells possibly part of the early formation of UACL buds. (C) Serial sections with ISH LCN2, ISH LGR5 and IHC Ki-67 showing LCN2 in both proliferating (black arrows) and nonproliferating (white arrows) parts of the UACL. LGR5-positive stem cell area in adjunction with UACL marked with white arrow-head. (D) Serial sections with IHC for NGAL (top) and MUC5AC (bottom) showing NGAL expression in all parts of UACL, while MUC5AC was expressed mainly toward the lumen of the bowel. (E) IHC for NGAL in colonic non-UACL inflamed epithelium with crypt abscesses and branching. NGAL expression is intense in the reactive and branching epithelium (arrows). NGAL is also expressed in granulocytes and in cell debris.

Results

NGAL is strongly expressed in UACL and regenerative colonic epithelium

The UACL differs in cellular composition from its early basal buds to the surface epithelium [22]. We used IHC and ISH to explore NGAL’s expression in the various parts of UACL in ileum and colon from patients with CD. NGAL was strongly expressed in UACL in all biopsies examined. Figure 1A–D shows positive staining for NGAL/LCN2 in all parts of UACL from the early buds to the ductular- and surface cells. Some single or confluent AB/PAS positive cells at the bottom of the crypts also strongly expressed NGAL and could be part of the early UACL-process (Figure 1B). NGAL was expressed in both proliferating and nonproliferating parts of UACL as assessed by Ki-67-staining of serial sections (Figure 1C). Here we also observed positive staining for the stem cell
marker **LGR5** in a Ki-67-positive crypt bottom directly adjacent to UACL, while **LGR5** was absent in the UACL. NGAL was strongly expressed in regenerative, branching crypts in active UC and CD in colon (Figure 1E).

**UACL** shows a distinct gene expression signature compared to control epithelium

The prominent staining of NGAL in UACL led us to perform a transcriptome-wide RNA sequencing of microdissected UACL. We found 180 genes to be differentially expressed (*p* < 0.05) between UACL and control epithelium (158 up-/+22 downregulated). Of these, 31 genes were also differentially expressed (*p* < 0.2) in inflamed non-UACL epithelium compared to controls, among them **DUOX2**, **CLL28**, **PDX1**, and **OLFM4**. These were excluded in the enrichment analysis in order to identify genes specific for UACL. Excluded genes are listed in supplementary material, Table S4. A list of the 30 most-upregulated genes specific for UACL is presented in Table 1. Gastric mucins **MUC5AC**, **MUC6**, and **MUC5B** as well as **PGC** (the gene coding for Gastricsin, also known as Pepsinogen C) were the most-upregulated genes in UACL, confirming a gastric phenotype of UACL.

| No. | Gene symbol | Gene name | Log FC | Adj. P value |
|-----|-------------|-----------|--------|--------------|
| 1   | MUC5AC      | Mucin 5 AC, oligomeric mucus/gel-forming | 15.51  | 0.0004       |
| 2   | PGC         | Pepsinogen C | 14.48  | < 0.0001     |
| 3   | MUC6        | Mucin 6, oligomeric mucus/gel-forming | 12.60  | 0.01         |
| 4   | MUC5B       | Mucin 5B, oligomeric mucus/gel-forming | 12.08  | 0.0097       |
| 5   | LCN2        | Lipocalin 2 | 10.95  | 0.0016       |
| 6   | POU2AF1     | POU class 2 associating factor 1 | 10.32  | 0.0014       |
| 7   | MUC1        | Mucin 1, cell surface associated | 10.17  | < 0.0001     |
| 8   | SDC3        | Syndecan 3 | 10.03  | 0.0008       |
| 9   | IGFBP5      | Insulin-like growth factor binding protein 5 | 9.98  | 0.0009       |
| 10  | SLC7A5      | Solute carrier family 7 member 5 | 9.96  | 0.0037       |
| 11  | RCN1        | Reticulocalbin 1 | 9.35  | 0.0070       |
| 12  | PROM2       | Prominin 2 | 9.31  | 0.0027       |
| 13  | SOAT1       | Sterol O-acetyltransferase 1 | 8.92  | 0.0070       |
| 14  | RAB11A      | RAB11A, member RAS oncogene family | 8.81  | 0.0064       |
| 15  | COL18A1     | Collagen type XVIII alpha 1 chain | 8.54  | 0.0037       |
| 16  | GSPT1       | G1 to S phase transition 1 | 8.51  | 0.0142       |
| 17  | AURKB       | Aurora kinase B | 8.46  | 0.0117       |
| 18  | MID1IP1     | MID1 interacting protein 1 | 8.41  | 0.0112       |
| 19  | LYZ         | Lysozyme | 8.35  | 0.0003       |
| 20  | RBFOX2      | RNA binding protein fox-1 homolog 2 | 8.35  | 0.0066       |
| 21  | PDE4A       | Phosphodiesterase 4A | 8.29  | 0.0190       |
| 22  | AP0BEC3C    | Apolipoprotein B mRNA editing enzyme catalytic subunit 3C | 8.28  | 0.0170       |
| 23  | RBPMS       | RNA binding protein, mRNA processing factor | 8.25  | 0.0066       |
| 24  | MRPS30      | 38S ribosomal protein S30, mitochondrial | 8.20  | 0.0118       |
| 25  | RUNX1       | Runt-related transcription factor 1 | 8.11  | 0.0118       |
| 26  | BPIFB1      | BPI fold-containing family B member 1 | 8.08  | 0.0112       |
| 27  | UBAP2       | Ubiquitin-associated protein 2 | 7.82  | 0.0097       |
| 28  | RCC2        | Regulator of chromosome condensation 2 | 7.77  | 0.0112       |
| 29  | CLDN2       | Claudin 2 | 7.77  | 0.0112       |
| 30  | PACS1       | Phosphofructin acidic cluster sorting protein 1 | 7.75  | 0.0112       |

| ... | ... | ... | ... | ... |
| 35  | TFF1        | Trefoil factor family 1 | 7.66  | 0.0118       |
| 77  | PHLD1A      | Pleckstrin homology-like domain family A member 1 | 6.97  | 0.0371       |
| 95  | NOTCH2      | Notch 2 | 6.61  | 0.0085       |

**Table 1.** Top 30 protein-coding genes significantly upregulated in UACL versus control epithelium and other genes of interest

Genes verified by IHC are written in bold. Genes upregulated (*p* < 0.2) in inflamed non-UACL epithelium versus control were excluded and are listed in supplementary material, Table S4. UACL; ulcer-associated cell lineage, Log FC; Log2 fold-change, Adj.; adjusted.

The UACL gene expression signature was confirmed at protein level

To verify RNA-sequencing results by protein expression we performed IHC for 11 selected proteins coded by differentially expressed genes. The genes were selected based on high expression or if considered interesting in the context of wound healing. **MUC5AC**, **PGC**, **LCN2**, **SDC3**, and **IGFBP5** were all among the ten most-upregulated genes. Other genes considered to be of particular pathobiological interests were **LYZ**, **BPIFB1**, **RCN1**, **PHLD1A**, **NOTCH2**, and **MUC2**. **LYZ**, like **LCN2**, is known to be expressed in Paneth cells, and **BPIFB1** and **RCN1** were chosen due to possible expression in Paneth cells based on literature and a search in Human Protein Atlas (www.proteinatlas.org), respectively. **PHLD1A** was the most upregulated known stem cell marker. **NOTCH2** is known to play an important role in epithelial homeostasis and regeneration. **MUC2** is the main intestinal mucin, and the only downregulated among the chosen genes (Log fold-change −2.54, *p* = 0.054).
Expression of the 11 proteins selected for IHC are shown in Figure 2. Insulin-like growth factor-binding protein 5 (gene IGFBP5) showed membrane staining in all parts of UACL, and staining of stem cells and villus epithelium in both controls and inflamed epithelium (Figure 2B). Mucin 5AC (gene MUC5AC) was seen in distal ductular- and surface cells only and was absent in controls (Figure 2C). Mucin 2 (gene MUC2) was absent in UACL but expressed in goblet cells of control and inflamed epithelium (Figure 2D). Reticulocalbin 1 (gene RCN1) was expressed in a subset of UACL, but also showed staining of non-UACL inflamed crypts with strong staining of Paneth cells in controls and inflamed epithelium (Figure 2E). Pleckstrin homology-like domain family A member 1 (gene PHLDA1) showed staining in stem cells and membrane staining in the UACL, most pronounced in acinar and proximal ductular parts. In addition there was prominent PHLDA1 staining of cells morphologically resembling myofibroblasts in ulcers and in the edges of damaged crypt epithelium (Figure 2F). Pepsinogen C (gene PGC) and lysozyme (gene LYZ) showed cytoplasmic staining in all parts of the UACL, and PGC was also present in single cells in the crypt bases most probably being part of the UACL, while lysozyme was expressed in Paneth cells and inflammatory cells (Figure 2G,H). BPI fold-containing family B member 1, also known as LPLUNC (gene BPIFB1) stained a small subset of mature UACL-crypts in addition to a subset of Paneth cells in controls and inflamed epithelium (Figure 2I). Notch2 and syndecan 3 (gene SDC3) also had most prominent staining in the distal parts of UACL and the two proteins showed a high grade of overlapping pattern with strong perinuclear staining corresponding the ER/Golgi in the distal crypts in addition to weaker membrane staining. NOTCH2-positive membranes were present in the proximal ductular parts (Figure 2K,L).

Three-dimensional visualization underlines the abundance of UACL glands

The organization of UACL in the mucosa can be difficult to visualize in a planar histological section in the microscope. Their crypts are often seen in cross-section as they make a 90° angle with the normal crypts. To further understand the structure of UACLs, we used the iDisco method for visualization of UACLs in a 3D-format. A surgical specimen from ileum with inflamed and erosive CD was...
made transparent and stained for NGAL to identify the UACL-crypts. The 3D-image (Figure 3) unveiled numerous NGAL-positive UACLs as continuous complex glands with several budings along the whole gland length. A video showing multiple UACL-crypts in a 3D format is available supplementary material, Movie S1.

**Enrichment analysis of the UACL gene expression signature is compatible with a role in wound healing**

We used the analysis program MetaCore to make an enrichment analysis of GO processes and significant networks associated with the genes overexpressed in UACL. Due to a high variance in the gene expression within the UACL group we chose to include genes with \( P \) values up to 0.1. Thus, a total of 279 genes were included in the analysis. Among the six most significant GO processes (when general processes with >1500 genes were excluded), five were related to regulation of migration/motility and gland development (Table 2). The genes implicated in the GO processes are listed in supplementary material, Table S5. Among the five most significant disease groups were colonic neoplasms, intestinal polyposis and stomach neoplasms, all conditions associated with aspects of cell growth (Table 2).

**NGAL is strongly expressed in undifferentiated human colonoids**

With increased expression of LCN2 in UACL and in regenerative crypts in colitis, we next studied if NGAL was expressed in developing, undifferentiated human colonoids, which may be considered a form of regenerative process from single stem cells. While differentiated colonoids contain mature cells like absorptive, goblet and neuroendocrine cells mimicking the in vivo normal epithelial layer, undifferentiated colonoids mainly consist of stem cells and immature epithelial cells. ISH and IHC indeed showed increasing LCN2/NGAL in undifferentiated cultures from cultures of undifferentiated and differentiated colonoids (first two columns), and colonoids differentiated for 2 days before restimulation with Wnt3a, Notch (by removing the notch-inhibitor DAPT), MAP kinase inhibitor (SB202190), nicotinamide, or all four factors for 2 days (last five columns). Mean and SEM from \( n = 3 \) biological replicates. (D) Serial sections of undifferentiated (left) and differentiated (right) colonoids. ISH for LCN2 (top) and IHC for CK20 (bottom) shows LCN2 expression mainly in CK20-negative, undifferentiated colonoids. Scale bar 100 μm.
or 72 h before addition of Wnt3A, nicotinamide, MAPK inhibitor (SB202190) and removal of the pan-NOTCH inhibitor DAPT, either concomitantly or each factor alone. However, none of the factors could significantly increase NGAL in differentiated epithelium (Figure 4C). Serial sections stained for the proliferation marker Ki-67 (data not shown) and differentiation marker CK20 showed that LCN2 expression was confined mainly to the undifferentiated, CK20-negative, Ki-67-positive colonoids (Figure 4D). This suggests that NGAL is expressed during the proliferative phase of the colonoids, and disappears during the differentiation process.

Discussion

In line with our initial studies, we found NGAL/LCN2 to be strongly expressed in UACL, present in all parts from the early buds to the surface. The 3D immunofluorescence image of NGAL-positive UACL shows the extent and morphology of UACL-crests in the mucosa. We further observed strong NGAL staining in colonic, branching, regenerative crypts. In patient-derived colonoids we found a remarkable overexpression of NGAL/LCN2 in undifferentiated epithelium, disappearing during the differentiation process. This indicates that NGAL is important in tissue regeneration. Although not established for IBD, such a role for NGAL has been suggested in various studies, but the exact function of the protein is still unknown. Some studies have shown that NGAL increases migration of epithelial cells [24–26], others have shown a role in differentiation [27]. NGAL is also known as an antibacterial protein [28,29] that may serve a function in shaping the microbiota and protecting the intestinal barrier. However, the antibacterial activity seems an unlikely explanation for a potent upregulation in undifferentiated colonoids which is then switched off during maturation.

The remarkable overexpression of NGAL in UACL led us to explore the pathology of these cells by transcriptome-wide RNA sequencing. This study is, to our knowledge, the first to characterize UACL at this level. Although RNA sequencing was performed on small samples yielding very little RNA, confirmation with IHC of 11 proteins encoded by mRNAs with various fold changes and P values shows that the results are highly reliable for genes identified as differentially expressed. Some genes coding for proteins known to be differentially expressed in UACL, such as TFF2, CDX2 and E-cadherin [30,31], are not among our differentially expressed genes although, e.g. E-cadherin seems clearly downregulated by IHC. These genes were most probably not detected due to small sample sizes with little RNA extracted, reflecting that this transcriptome assay may have been conducted close to the limitations of the method.

Our analysis of the UACL transcriptome shows that the most significant networks based on an enrichment analysis are compatible with a role for UACL in wound healing. We chose to focus on GO process networks with less than 1500 objects in our results, as this opens for the more specific categories to become prominent. The analysis shows that the differentially expressed genes are enriched for genes involved in cell migration and motility, and for biomarkers of gastrointestinal neoplasia, supporting the understanding of a role for UACL as part of a reepithelialization or reparative process. The prominent upregulation of NGAL in UACL, together with the findings in our organoid preparations, make it likely that NGAL is a protein relevant for these processes although its precise role cannot be determined based on these results.

In addition to LCN2/NGAL, several of the genes upregulated in UACL were verified using IHC. Genes were chosen either because they are relevant in the context of wound healing, or had highly statistically significant differential expression between UACL and healthy control tissue. Among the interesting genes expressed in UACL and confirmed by IHC was NOTCH2. In combination with NOTCH1, NOTCH2 is known to play an important role in intestinal homeostasis [32]. One study suggested that while NOTCH1 is the primary receptor in intestinal stem cell regulation, the combination of the two are important in cell fate

### Table 2. Most significant GO processes and diseases associated with the 279 genes upregulated in UACL versus controls

| GO processes (processes containing > 1500 genes excluded) | P      | FDR       | Network objects from active data |
|----------------------------------------------------------|--------|-----------|---------------------------------|
| 1. Positive regulation of cell migration                 | 2.62e-9 | 6.95e-7   | 28/727                          |
| 2. Positive regulation of cellular component movement    | 3.31e-9 | 7.96e-7   | 29/784                          |
| 3. Positive regulation of cell motility                  | 6.32e-9 | 1.28e-6   | 28/757                          |
| 4. Gland development                                     | 7.44e-9 | 1.43e-6   | 29/813                          |
| 5. Negative regulation of protein phosphorylation        | 9.08e-9 | 1.58e-6   | 25/624                          |
| 6. Regulation of cell migration                          | 1.2e-8  | 1.91e-6   | 35/1154                         |

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determination and wound healing [33]. NOTCH2 has also been found to be important for goblet cell metaplasia in the lung [34]. In our IHC analysis we found an overlapping pattern between NOTCH2 and syndecan 3 (SDC3) in UACL. NOTCH and SDC3 have been shown to co-localize and cooperate in homeostasis of the satellite cells and myofiber size in muscle. One study showed that SDC3 was required for NOTCH processing by ADAM 17/TNFα-converting enzyme (TACE) and signal transduction [35]. Whether such a cooperation exists in the intestine is yet to be explored. Another interesting gene confirmed with IHC was the highest upregulated stem cell marker PHLDA1, which has also been shown to play a role in migration [36].

UACL induction occurs in the proximity of Paneth cells and stem cells in crypt bases at ulcer margins, but the mechanisms are not known. In a recent publication, Goldenring [7] suggests that Paneth cells can be the origin of UACL due to UACL’s origin in the crypt bases. Paneth cells express several proteins important for stem cell maintenance and cell growth like WNT, EGF, TGFβ, and the Notch ligand DLL4 [37]. Moreover, recent studies have suggested Paneth cells acquire stem-like features during inflammation-related loss of stem cells [38]. In an earlier study we found NGAL to be expressed by a subset of Paneth cells [4]. In addition to NGAL, among the regulated genes to be verified by IHC, we chose to include the highly upregulated Paneth cell marker Lysozyme (LYZ) and both were expressed in UACL. Moreover, IHC showed the two UACL-expressed proteins BPIF1 and reticulocalbin1 to be specifically expressed in Paneth cells of both healthy controls and inflamed ileal mucosa. To our knowledge these are novel observations.

Interestingly, in the present study we found the homeobox transcription factor PDX1 to be among the most-upregulated genes in both UACL and inflamed non-UACL epithelium compared to control epithelium. PDX1 has been shown to decrease CDX2 [39]. A previous study found increased expression of PDX1 and decreased expression of CDX2 in UACL [31]. Simmini et al [40] showed that murine intestinal LGR5+ stem cells converted cell-autonomously into pyloric stem cells in the absence of CDX2. If this is true also in human intestinal stem cells, it suggests that inhibition of CDX2 can be an important factor for the induction of UACL.

In conclusion, several UACL genes are implicated in migration and gland development supporting a role for UACL in wound healing. We find that NGAL is highly expressed in UACL, regenerative colonic crypts and in undifferentiated colonoids, strongly suggesting that NGAL plays a role in the regeneration process in IBD.

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Author contributions statement

ST, AvBG, IB, JKD, AEØ, TB, and AKS contributed to the conception and design of different parts of the study. ST, AvBG, TDS, IB, and TB performed the experiments. ST, AvBG, IB, ESR, THF, JKD, AEØ, TB, and AKS contributed to the assembly, analysis and interpretation of data. ST, AvBG, and AKS wrote the manuscript. All authors were involved in final revision and approval.

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