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Activin A-mediated epithelial de-differentiation contributes to injury repair in an *in vitro* gastrointestinal reflux model

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ABSTRACT

Reflux esophagitis is a result of esophageal exposure to acid and bile during episodes of gastroesophageal reflux. Aside from chemical injury to the esophageal epithelium, it has been shown that acid and bile induce cytokine-mediated injury by stimulating the release of pro-inflammatory cytokines. During the repair and healing process following reflux injury, the squamous esophageal cells are replaced with a columnar epithelium causing Barrett's metaplasia, which predisposes patients to esophageal adenocarcinoma. We identified a novel player in gastroesophageal reflux injury, the TGFβ family member Activin A (ActA), which is a known regulator of inflammation and tissue repair. In this study, we show that in response to bile salt and acidified media (pH 4) exposure, emulating the milieu to which the distal esophagus is exposed during gastroesophageal reflux, long-term treated, tolerant esophageal keratinocytes exhibit increased ActA secretion and a pro-inflammatory cytokine signature. Furthermore, we noted increased motility and expression of the stem cell markers SOX9, LGR5 and DCLK1 supporting the notion that repair mechanisms were activated in the bile salt/acid-tolerant keratinocytes. Additionally, these experiments demonstrated that de-differentiation as characterized by the induction of YAP1, FOXO3 and KRT17 was altered by ActA/TGFβ signaling. Collectively, our results suggest a pivotal role for ActA in the inflammatory GERD environment by modulating esophageal tissue repair and de-differentiation.

1. Introduction and background

Gastroesophageal reflux disease (GERD) and its resulting consequences, such as esophagitis and Barrett's esophagus, affect millions of people in the United States [1], thereby leading to billions of dollars in health care costs [2]. The most adverse outcome associated with GERD and esophagitis is the formation of Barrett's esophagus (BE), which can progress to esophageal adenocarcinoma (EAC). BE is characterized by the metaplastic change of the squamous epithelial lining in the distal esophagus to a columnar epithelium as a result of acid and bile salt injury during reflux episodes [3]. The chronic exposure of intestinal bile and gastric acid affects the integrity of the esophageal epithelium [4] and induces an inflammatory response [5–7]. Stimulated by acid and bile salt, inflammatory mediators such as NF-κB are activated, which can be inhibited upon treatment with Aspirin, demonstrating the importance of inflammatory signaling to bile acid injury [8].

In vitro models to mimic GERD have been established to investigate the functional consequences of the repeated injury and repair cycles and to shed light onto the survival advantages which could ultimately lead to the initiation of esophageal adenocarcinoma. Long-term repeated acidified bile exposure using an hTERT-immortalized benign Barrett's cell line, BAR-T, was continued up to 65 weeks [9]. Progressive genomic instability upon long-term exposure phenocopied the metaplasia-dysplasia-carcinoma cascade observed in Barrett's esophagus and resulted in transformed cells as a direct consequence of chronic acid and bile salt exposure [10]. Similarly, a 30-week exposure of hTERT-immortalized normal esophageal squamous cells has shown that acid and bile salt can induce an incomplete columnar metaplasia through cellular reprogramming allowing the expression of columnar tissue-specific transcription factors CDX2, SOX9, and also columnar differentiation markers Villin and MUC2 [11]. Upon 90- week exposure to acidified bile acid media, HET-1A cells (immortalized with SV40 T antigen) showed activation of inflammatory signaling pathways such as STAT3, NF-κB p65, PTGS2 (COX-2) and IL6, but also pro-carcinogenic signaling such as EGFR and mTOR [12]. The acquisition of these

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Abbreviations: hTERT, telomerase reverse transcriptase; PTGS2, prostaglandin-endoperoxidase synthase (COX-2); CDX2, Caudal Type Homeobox 2; SOX9, sex determining region Y-box 9; MUC2, mucin 2; IL1β, interleukin-1β; TNFα, Tumor necrosis factor α; MAPK, mitogen-activated protein kinase; YAP1, yes-associated protein1

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expression patterns provided support that upon exposure to bile and acidified media, squamous cells can display characteristics found in Barrett's esophagus, and more so, the pre-malignant initiation phenocopies the activation of EGFR and other known cancer-associated pathways.

In vivo models have further demonstrated the pivotal role of inflammation in the Barrett's esophagus to cancer progression. Chronic inflammation in response to GERD-mediated injury is associated with increased levels of IL1β, a pro-inflammatory cytokine inducing IL6 and TNFα signaling cascades. A mouse model of IL1 β overexpression induces IL6-dependent Barrett's esophagus, but requires bile salts for full intestinal metaplasia and dysplasia [13]. Other cytokines found to be upregulated in this model of Barrett's esophagus were IL6 and IL8, which together with IL4 and IL10 are relevant to the human disease [14,15].

We and others have shown previously that INHBA, the Activin A gene, is upregulated during the initiation and progression from a normal squamous esophagus to Barrett's esophagus and adenocarcinoma [16,17], yet little is known about its role in Barrett's pathogenesis. ActA, a member of the TGFβ superfamily, has been considered a master regulator of homeostasis through its role not only in innate and adaptive immunity, but also in tissue repair [18]. In the gastrointestinal tract, increased levels of ActA have been detected in mouse models of inflammatory bowel disease [19] and in biopsies from patients with Crohn's disease and ulcerative colitis [20–22]. Prophylactic and therapeutic treatment with the ActA antagonist Follistatin improved epithelial cellular repair and increased barrier function in the colonic mucosa of a mouse model [21]. In acute pancreatitis, ActA is elevated and strongly correlated with disease severity *in vitro,* and it has been shown that high serum levels of ActA correlated with worse outcome in patients [23].

Based on its known association with inflammatory conditions in other organs, we surmised that ActA may be upregulated upon GERD-induced injury and confer autocrine pro-inflammatory signaling in the injured epithelium. In this study, we have established bile salt and acid-tolerant cells following repeated exposure to an equimolar mixture of glycocholate, taurocholate, glycodeoxycholate, glycochenodeoxycholate, and deoxycholate (20 μM final concentration each) in acidified media (pH 4), emulating the milieu to which the distal esophagus is ordinarily exposed during gastroesophageal reflux. We show here that in response to bile salt and acidified media (pH 4) exposure, esophageal keratinocytes exhibit increased ActA secretion and the induction of a pro-inflammatory cytokine signature. Furthermore, we noted increased motility and expression of stem cells markers supporting the notion that repair mechanisms were activated in the bile salt/acid-tolerant keratinocytes. Additionally, these experiments demonstrated that expression of the stem cell markers SOX9, LGR5 and DCLK1 can be altered by ActA/TGFβ signaling.

In summary, we defined the consequences of ActA signaling on esophageal inflammation and bile acid injury.

2. Materials and methods

2.1. Cell culture

Human esophageal epithelial cells, STR (EPC-h-TERT), which have functional p53 and no alterations in p16^{INK4a}, were cultured as previously described [24]. In brief, Keratinocyte-SFM medium was supplemented with 1 ng/ml Epidermal Growth Factor, 0.05 mg/ml Bovine Pituitary Extract, and 1% penicillin streptomycin antibiotics (Gibco™, for Life Technologies, Inc., Carlsbad, CA). BAR-T cells, a human Barrett's Esophagus cells line (kind gift from Dr. Rhonda Souza, Baylor Scott White) were cultured in Epithelial Cell Medium-2 supplemented with 5% epithelial cell growth supplement-2 (EpiCGS-2) and 1% penicillin/streptomycin antibiotics (ScienceCell™ Research Laboratories, Carlsbad, CA). All cells were incubated at 37 °C with 5% CO_2. Treatment mimicking gastroesophageal reflux consisted of repeated weekly exposure to either acidified medium (pH 4 – using 6 N hydrochloric acid) and a 100 μM of cocktail of bile salts alone or the combination thereof. The cocktail included 20 μM of each, deoxycholic acid (MP Biomedicals, Santa Ana, CA), glycocholic acid, taurocholic acid, sodium glycodeoxycholate and sodium glycochenodeoxycholate (All Sigma, St. Louis, MO). To establish treatment-tolerant STR cell lines, each treatment was performed for 4 min in a 37 °C incubator with 5% CO_2, and then cells were allowed to recover for up to three days and the surviving cells were propagated before the next treatment. A83-01, a selective ActA/TGFβ signaling inhibitor (Tocris, Bristol, UK) was added to the culture medium at a concentration of 1 μM. Recombinant Activin A (R&D Systems, Minneapolis, MN) was used at 10 ng/ml. Both Activin A and A83-01 treatments were performed continuously for up to 10 days with media change every 2 days.

2.2. Spheroid formation

In brief, cells were resuspended in 2% Matrigel in Corning™ Costar™ Ultra-Low attachment microplates (Corning, NY). Cells were fixed in 4% paraformaldehyde (Fisher Scientific, Hampton, NJ) overnight at room temperature.

2.3. Organotypic cultures

Organotypic cultures were grown similar as described previously [25]. In brief, the matrix for the organotypic culture was made by initially seeding 7.5×10^4 esophageal fibroblasts on Corning insert plates (Corning, NY) and culturing them in DMEM with 10% BSA, 1% antibiotics and the presence of 50 μg/ml of ascorbic acid, the medium was renewed every two days. Repeated fibroblast layers were seeded for secretion and deposition of an extracellular matrix. On day 28, 5×10^5 epithelial cells were seeded on top of the fibroblasts layer and the culture was grown as previously described in [25]. Cultures were air lifted on day 39 to induce differentiation and harvested on day 43. The tissue was fixed in 4% paraformaldehyde (Fisher, Pittsburg, PA) overnight and paraffin embedded.

2.4. qRT-PCR

Total RNA was extracted and purified from STR and BAR-T cells after each treatment via phenol and guanidine thiocyanate extraction using the miRNeasy® Mini Kit (Qiagen, Santa Clara, CA) according to the manufacturer's protocol. Reverse transcription was performed using QuantiTect® Reverse Transcription Kit (Qiagen, Santa Clara, CA), the subsequent Real time qRT-PCR was then performed using the SYBR® Green PCR kit by Qiagen. The genes Rpl13A and Eif3d were used as internal controls. Primer sequences were selected as followed (Integrated DNA Technologies, Coralville, IA): TNFα, forward GAA TTC GGA CAC CAT GAG C AC TG, reverse GGA TCC ACC GGT TCA CAG GGC AAT GAT CCC; IL1β, forward ATG ATG GCT TAT TAC AGT GGC AA, reverse GTC GGA GAT TCG TAG CTG GA; PTGS2 (COX-2), forward TGA GCA TCT ACG GTT TGC TG, reverse TGC TTG TCT GGA ACA ACT GC; SOX9 forward CAT GAG CGA GGT GCA CTC C, reverse TCG CTT CAG GTC AGC CTT G; LGR5, forward AAC AGT CCT GTG ACT CAA CTC AAG, reverse TTA GAG ACA TGG GAC AAA TGC CAC; DCLK1, forward AGT CTT CCG ATT CCG AGT TGA G, reverse CAG CAA CCA GGA ATG TAT TGG A. KRT17, forward AAC AAG ATC CTC ACA GCC AC, reverse CCA TTG ATG TCG GCC TCC; FOXO3, forward CGT GCC CTA CTT CAA GGA TA, reverse CTA TGC AGT GAC AGG TTG TG. The Quantitect YAP1 primer set (QT0008022) was purchased from Qiagen (Santa Clara, CA). Additionally, KRT8 (QT00091350) and KRT18 (QT01003562) were purchased from Qiagen.

2.5. ELISA

ELISA Assays were performed on conditioned media using the Activin A DuoSet® ELISA assay by R&D Systems (Minneapolis, MN) and according to the manufacturer's protocol.

2.6. Cytokine array

Cytokine Arrays were performed using RayBio® C-Series Human Cytokine Antibody Array C6 and according to the manufacturer's protocol (RayBiotech, Peachtree Corners, GA).

2.7. Western blotting

Cells were lysed 30 min post treatment using IP Lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton x-100, 1% NP-40) supplemented with a cOmplete™ EDTA-free protease Inhibitor Cocktail tablet. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Subsequently, protein samples were separated on a 10% acrylamide gel via SDS PAGE. Proteins were transferred to a PVDF membrane and visualized using primary antibodies and HRP-conjugated secondary antibodies using Imager from ThermoFisher.

2.8. Antibodies

Antibody against COX-2/PTGS2, cleaved PARP, cleaved Caspase 9, phospho-SMAD2/3 (Ser423/425), phospho-ERK ½ (Thr202/Tyr204), as well as their corresponding total antibodies and NF-κB (p65, clone D14E12) were purchased from Cell Signaling Technologies (Danvers, MA). Phospho-STAT1 pY701 and phospho-STAT3 pY705 and their respective total antibodies were purchased from BD Transduction Laboratories (Franklin Lakes, NJ). SOX9 was purchased from EMD Millipore (Burlington, MA). The antibodies listed above were all diluted at 1:1000. The β-Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO) and α-Tubulin was purchased from Abcam (Cambridge, United Kingdom), both were used as loading controls at a 1:5000 dilution. Antibodies used for immunofluorescence stainings are LGR5 (R& D Systems, Minneapolis, MN), ACVR1B (Abcam, Cambridge, MA), KRT8 (Santa Cruz Biotechnologies, Dallas, TX), KRT14 (Thermo Fisher, Waltham, MA) and KRT19 (Cell Signaling Technologies, Danvers, MA).

2.9. Immunofluorescence

Organotypic culture tissue, previously fixed in formalin for 24 h and embedded in paraffin, was sectioned at 5 µm, deparaffinized, and heated in 1X TE buffer in a pressure cooker for 12 min for antigen retrieval. Samples were blocked in 1X PBS with 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO) for 1 h before incubation with primary antibody in 1x PBS 5% BSA overnight at 4 °C. Tissues were then rinsed three times in PBS and incubated with secondary antibodies in 1x PBS 5% BSA for 1 h at room temperature. After additional rinses with 1x PBS, the sections were mounted with SouthernBiotech mounting medium containing DAPI (SouthernBiotech, Birmingham, AL). Images were taken on a Zeiss microscope, using Axiocam and Axiovision Zen blue 2.0 software (Carl Zeiss Microscopy, Thornwood, NY) or a Leica sp5 confocal microscope.

2.10. Cell invasion

The ability of cells to migrate through a reconstituted extracellular matrix (ECM) was measured using BioCoat™ Matrigel® Invasion Chambers (Corning, Corning, NY) and according to the manufacturer's protocol. For analysis, the number of cells that had migrated through the ECM was counted in four microscopic fields per insert (10)X magnification), with three replicate inserts per condition. The role of Activin A signaling was evaluated using A83-01 and recombinant Activin A.

2.11. Dataset analysis

Dataset GDS1321 was used to query the gene expression of inflammatory cytokines and stem cell markers during the induction of Barrett's esophagus, publicly available from GEO Datasets (http://www.ncbi.nlm.nih.gov/gds/). The collected information was analyzed and visualized in Prism version 6.00 for Mac (GraphPad software, La Jolla, California).

2.12. Biostatistical analysis

Biostatistical analysis was performed using Prism version 6.00 for Mac. *In vitro* and *in vivo* experiments were analyzed using unpaired Student's *t*-test or one-way ANOVA. Statistical significance was set at $P < 0.05$. All experiments were done in triplicates with at least three biological replicates.

3. Results

3.1. Initial exposure to acidified media and bile salts induces apoptosis in esophageal keratinocytes

Gastric and bile acids are the major components of acid reflux and confer an increased risk for Barrett's esophagus in patients with chronic GERD. The combination of bile salts and acid has been shown to synergistically induce DNA damage, leading to apoptosis in the majority of the exposed cells, with few cells activating stress responses and survival signaling [26–28]. The resistant cells may be at the heart of bile salt/acid wound repair and pre-malignant metaplastic transformation. To validate that the conditions used in this study induce tolerant cell populations, we analyzed epithelial cell apoptosis over a time course of the initial six treatments. We modeled long-term risk factor exposure using normal hTERT-immortalized esophageal epithelial cells (STR) treated with acidified (pH 4) bile salts in an equimolar mixture of glycocholate, taurocholate, glycodeoxycholate, glycochenodeoxycholate, and deoxycholate (20 µM final concentration), reflecting the juices to which the distal esophagus is ordinarily exposed during gastroesophageal reflux. After the first three treatments, we observed numerous cells undergoing apoptosis as shown by Western Blot (Fig. 1). Cleaved PARP and cleaved caspase 9 signals were detected within 5 h of treatment and increased from the first treatment to the third. Upon the third treatment, apoptosis levels were reduced, and the exposed cells appeared to be tolerant or resistant to further repeated exposure to acidified media containing bile salts. Three independent tolerant cell lines were established and used for further experimentation.

3.2. Inflammatory signaling is activated upon long-term acid/bile salt treatment of esophageal keratinocytes

We established long-term treated (up to 33 weekly 4 min exposures) normal hTERT-immortalized human esophageal primary epithelial cells, after apoptosis induction in the majority of the cell population during the first 3 treatments. Upon three treatments, cells were tolerant to bile salt/acid exposure as shown in Fig. 1, and considered long-term upon 9 repeated treatments. Damage to the esophageal mucosa and epithelium are major factors in gastroesophageal reflux disease (GERD) and eventually result in inflammation and changes in tissue architecture. Recent reports suggest that the pathogenesis of reflux esophagitis is cytokine-mediated and not only a result of the chemical injury [6,7,29,30].

Tolerant esophageal keratinocytes were analyzed for the induction of inflammatory signaling through signal pathway activation and the expression and secretion of cytokines known to be relevant to GERD-

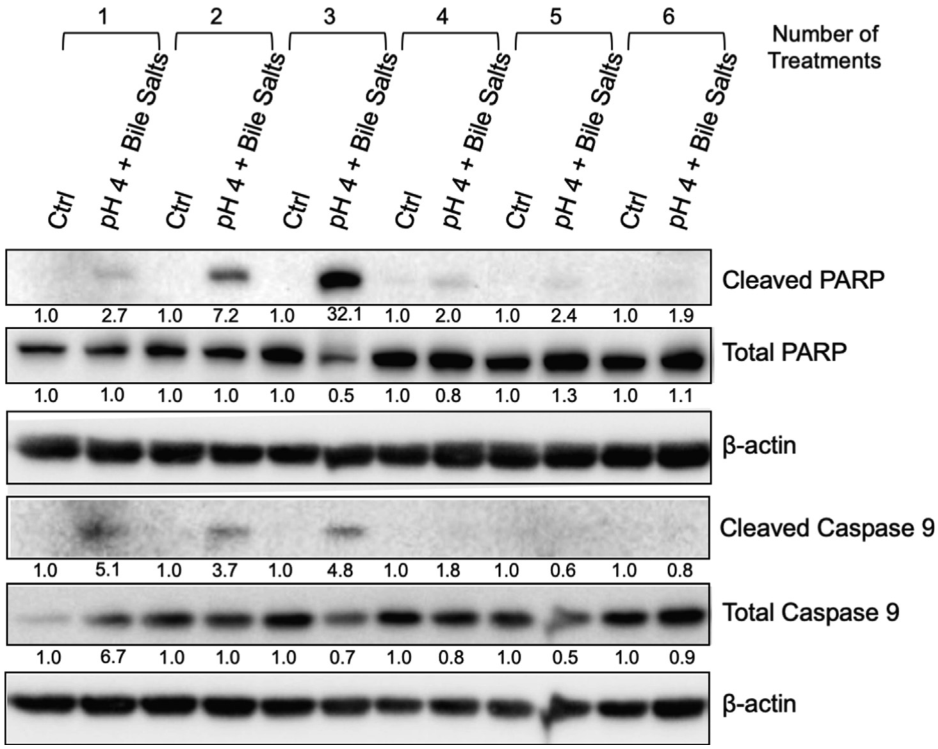


Fig. 1. pH 4-bile salt-tolerant cell populations emerge upon initial induction of apoptosis. Cell lysates were collected 5 h upon repeated treatment with pH4-acidified and bile salt containing media, and analyzed for the cleavage of PARP and Caspase 9 using specific antibodies by Western blotting. β-actin was used as a loading control. The signal intensity was calculated in each lane, normalized according to β-actin and compared to untreated control.

mediated esophagitis. We showed that STAT1 was activated by phosphorylation of residue pY701upon long-term repeated exposure to acidified, bile salt-containing medium compared to pH 4 media alone or bile salt addition alone using Western blotting, demonstrating a synergistic effect of the combination treatment. STAT1 activation can lead to NF-κB induction, which was increased in the combination treatment only, not in the controls (Fig. 2A, Supplemental Fig. 1A). STAT3 signaling was induced as seen by phosphorylation on pY705 in pH4 media alone and the combination with bile salt. A cytokine array demonstrated the secretion of various pro-inflammatory interleukins, with a main focus on mediators of the inflammatory response in esophagitis [31] such as IL1β, IL6 and TNFα compared to pH 4 and bile salt alone (Fig. 2B). We confirmed the increase of IL1β and TNFα on the mRNA level by qRT-PCR (Fig. 2C), and also the upregulation of the NF-κB-target PTGS2 (COX-2) by qRT-PCR and Western blotting (Fig. 2D). As these cytokines and their downstream targets are known to be implicated in Barrett's pathogenesis, we perused a publicly available database containing gene expression results from normal esophageal and Barrett's esophagus specimen (GDS1321) demonstrating the relevance of IL1β and PTGS2 upregulation during esophagitis and GERD-induced injury (Fig. 2E). The IL1β signaling cascade via the transcription factor NF-κB can regulate cell proliferation, inflammation, and survival and their activity plays a role in the progression to dysplastic BE and EAC [31].

3.3. Activin A signaling is induced in response to pH 4 and bile salt exposure

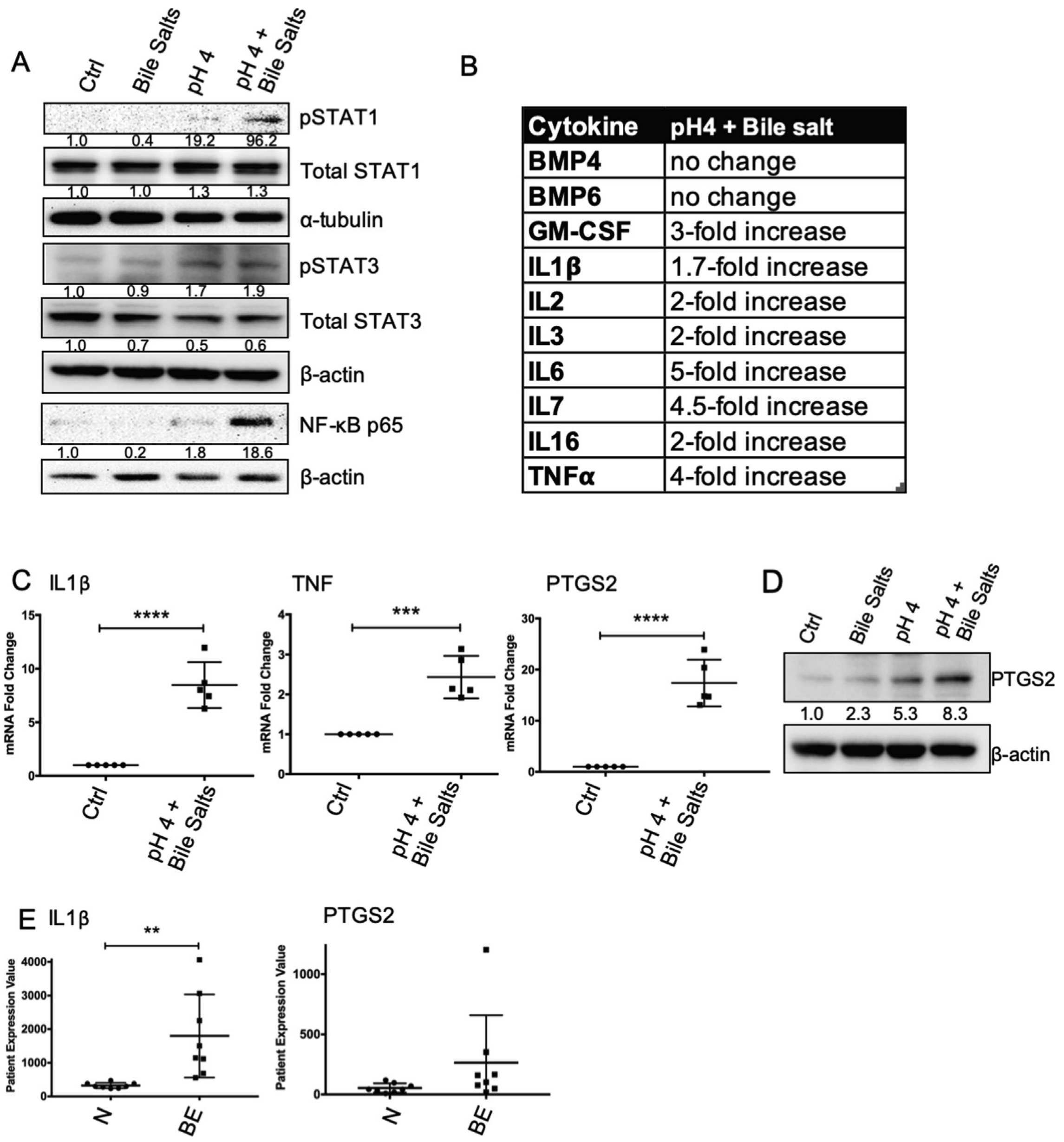
ActA is involved in stress responses, *e.g.,* DNA damage drives an ActA-dependent PTGS2 (COX-2) induction in premalignant lesions [32]. We and others have shown previously that INHBA, the Activin A gene, is upregulated during the initiation and progression from a normal squamous esophagus to Barrett's esophagus and adenocarcinoma [16,17]. Here, we analyzed the secretion and signaling activation of ActA of esophageal keratinocytes in response to acidified media (pH4) and bile salt and exposure. Upon repeated exposure, tolerant esophageal epithelial cells exhibited sustained increased ActA secretion as shown by ELISA using conditioned media from acute and long-term treatments (Fig. 3A). TGFβ, on the contrary, was not secreted in any of the conditions (data not shown). The ActA gene, INHBA, and the downstream canonical mediator SMAD3 were upregulated upon exposure to acidified media (pH4) and bile salt as shown by qRT-PCR (Fig. 3B). We surmise that the increase in expression and secretion leads to autocrine signaling activation, as we observed activation of SMAD2/3 by phosphorylation of Ser423/425 residues upon pH 4 and combined treatments compared to untreated-control and bile salt only (Fig. 3C, Supplemental Fig. 1B). Furthermore, non-canonical ActA signaling involving ERK1/2 was induced specifically in the combination of acidified media/bile salt resulting in increased phosphorylation of Thr202/Tyr204, possibly as a stress response.

3.4. Upon bile-acid injury esophageal keratinocytes show ActA-dependent repair features

Damage to the esophageal squamous epithelium is a major consequence of gastroesophageal reflux disease (GERD) and eventually results in inflammation and changes in tissue architecture. In the skin, ActA signaling is critical for normal wound healing and dramatically upregulated in chronic wounds [33–35].

We show that pH4 and bile salt exposure induced an increase in cell motility on a basement membrane (Fig. 4A). BAR-T cells, a Barrett's esophagus cell line, presented with a higher motility which could be recapitulated with the addition of recombinant ActA (Fig. 4B). Inhibition of ActA and TGFβ signaling using the chemical compound A83-01 (Supplemental Fig. 2) reversed the effect of pH4 and bile salt treatment by reducing the motility (Fig. 4B). As TGFβ was secreted at low levels (data not shown), the A83-01 inhibitor may contribute to this phenotype by suppressing preferably ActA-mediated signaling. Motility was not affected by proliferation (Supplemental Fig. 3). Together, these data demonstrate an ActA-dependent motility phenocopying the effect of the acidified medium with bile salts.

A key cell type during wounding and inflammation in self-renewing tissues, such as the squamous epithelium of the esophagus, is the stem cell. To determine the long-term effects of esophageal epithelial injury and repair, during which metaplasia occurs, it is necessary to focus on



Cytokine	pH4 + Bile salt
BMP4	no change
BMP6	no change
GM-CSF	3-fold increase
IL1β	1.7-fold increase
IL2	2-fold increase
IL3	2-fold increase
IL6	5-fold increase
IL7	4.5-fold increase
IL16	2-fold increase
TNFα	4-fold increase

Fig. 2. Inflammatory signaling is activated upon long-term pH4-bile salt exposure of esophageal epithelial cells. A. Activation of inflammatory signaling pathways such as STAT1/3 is shown by Western Blot analysis with phospho-specific antibodies, pY701 and pY 705, compared to total protein control and NF-κB p65 using cell lysates from long-term treated EPC-hTERT cells and controls. The signal intensity was calculated in each lane normalized according to β-actin and compared to untreated control. Pro-inflammatory cytokines relevant to Barrett's tumorigenesis are upregulated as shown B. using a cytokine antibody array and C. validated by qRT-PCR using RNA isolates from pooled independent long-term treated EPC-hTERT cell lines compared to no treatment controls, unpaired *t*-test. D. Selective upregulation of COX-2 (PTGS2) after long-term combination treatment; the signal intensity was calculated in each lane normalized according to β-actin, and compared to untreated control. E. Comparison of IL1β and PTGS2 arbitrary expression values in human patients was based on a publicly available GEO dataset (accession number GDS1321), N = normal, BE = Barrett's esophagus; unpaired *t*-test. Statistical significance: ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

the cells that reside in a tissue over long periods of time.

We therefore queried the expression of progenitor markers described to be involved in the progression to Barrett's esophagus, such as SOX9, LGR5 and DCLK1 [36–38]. The long-term insult with bile salt containing acidified media induced the expression of these stem cell markers (Fig. 4C and D). To demonstrate the role of ActA in the regulation of these markers, we treated cells with recombinant ActA and with the ActA and TGFβ inhibitor, A83-01. While use of the inhibitor returned the expression close to control baseline, suggesting ActA-dependence, stimulation with recombinant ActA alone could not induce a similar increase as observed with pH4 and bile salts, other than for LGR5. We speculate that this could be either due to the short incubation time compared to long-term exposure in the GERD model or that ActA alone cannot induce the full spectrum of activation compared to bile acid, indicating that additional factors are required. Nonetheless, we conclude that the repair of bile/acid injury is associated with an Activin A-dependent increase in cell invasion with the increased expression of stem cell markers. SOX9 and LGR5 were found to be upregulated in tissues from Barrett's esophagus patients compared to normal in a publicly available dataset (GDS1321), yet LGR5 due to the low number of specimen not significantly and DCLK1 remained unchanged between normal and Barrett's esophagus tissues (Fig. 4E).

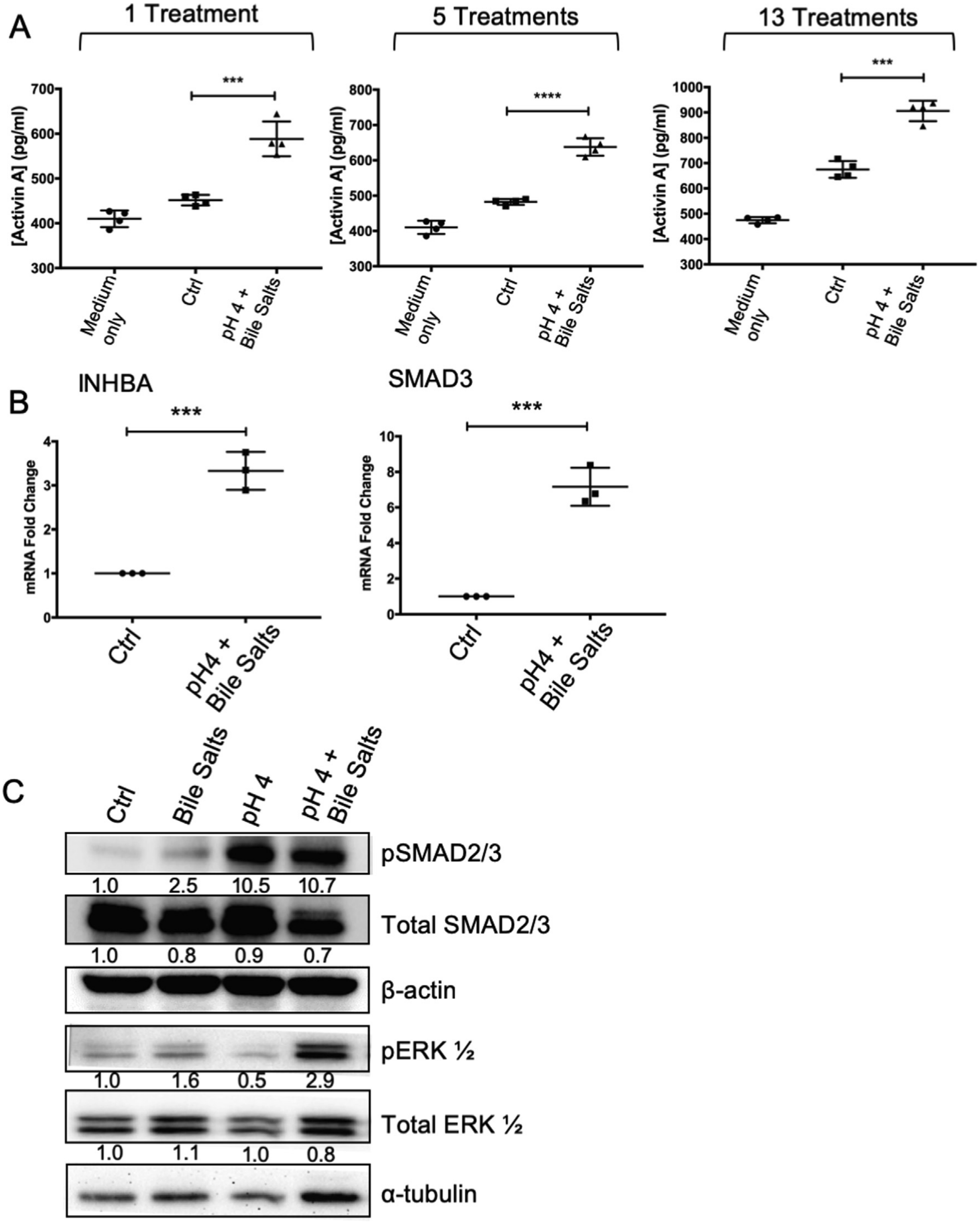


Fig. 3. Activin A expression and secretion is induced in response to bile/acid treatment in esophageal epithelial cells. A. ActA concentration in conditioned media is increased with bile salt-containing pH4 media treatments compared to media only and media from control untreated cells (collected at the indicated time points) as determined by ELISA. n = 4. B. The ActA gene, INHBA, and SMAD3 are upregulated as shown by qRT-PCR from RNA isolated from controls compared to bile acid treatments. C. Downstream canonical SMAD-mediated and non-canonical ERK1/2- induced signaling is activated based on phosphorylation of pSMAD2/3 on residues Ser423/425 and pERK1/2 (Thr202/Tyr204) shown in Western Blot. The signal intensity was calculated in each lane normalized according to β-actin, and compared to untreated control. Unpaired *t*-test, statistical significance: *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.5. Regulators of de-differentiation are upregulated in this model of acidic bile flux

More recently, it has been reported that during regeneration of an injured epithelium, the repopulation of different epithelia can be facilitated by "reserve stem cells" which can contribute to epithelial homeostasis upon de-differentiation [39–42]. Consequently, we analyzed long-term pH4 and bile salt-treated cells for the expression of de-differentiation markers such as YAP1, FOXO3, KRT19 and KRT17. Our data show significantly increased expression of YAP1, FOXO3, KRT19 and KRT17 in long-term bile acid-treated cells compared to controls as determined by qRT-PCR (Fig. 5A, Supplemental Fig. 4). Furthermore, expression of YAP1, FOXO3 and KRT17 was inhibited following treatment with A83-01, implying an association with ActA/

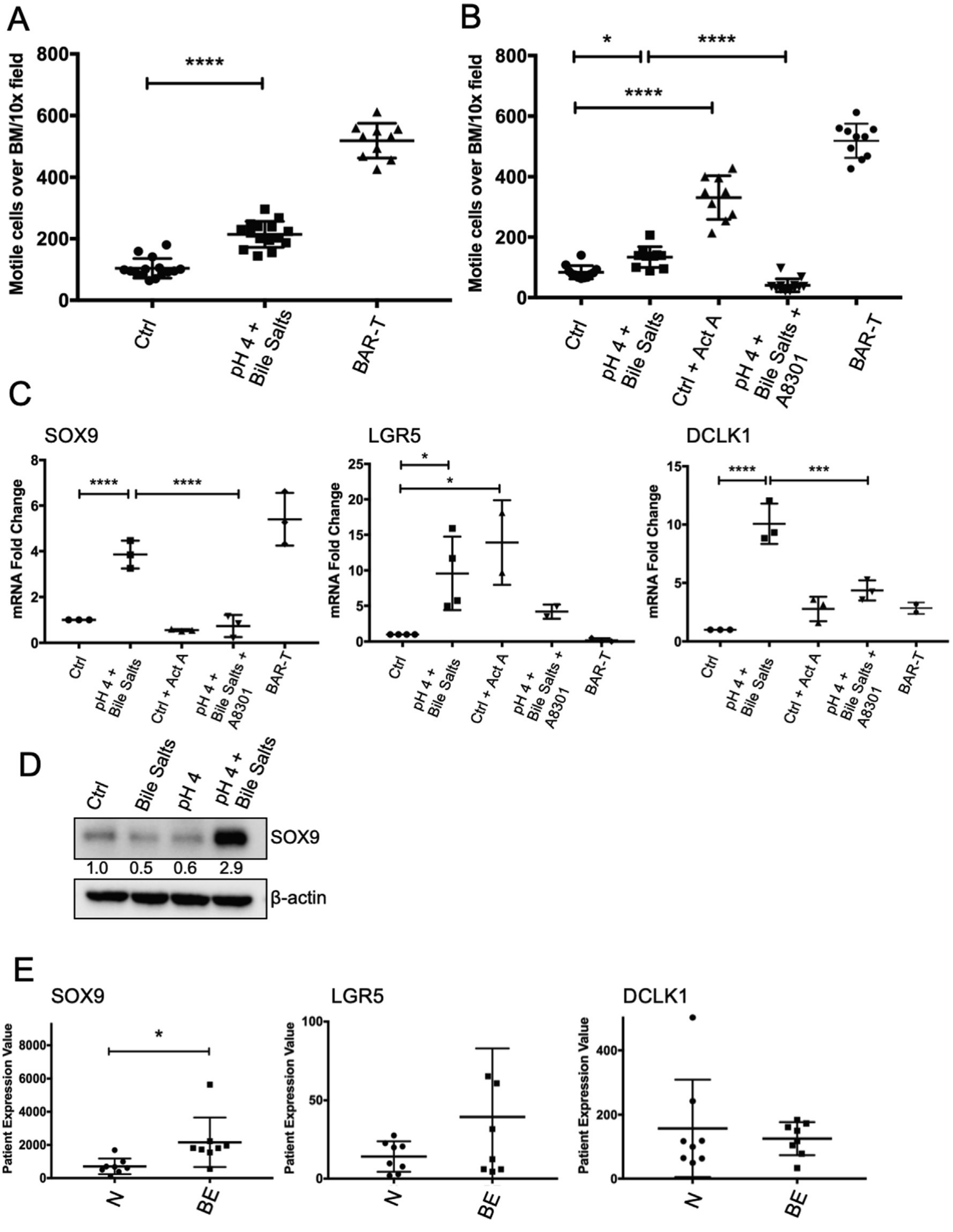


Fig. 4. (A-B) The repair of bile/acid injury is associated with an Activin A-dependent increase in cell invasion and associated with (C-E) Increased expression of stem cell markers. A. Motility over a basement membrane (BM) is increased upon bile acid exposure , comparable to the Barrett's esophageal cell line BART. B. The increased motility is ActA dependent as shown using recombinant ActA and the TGFβ/ActA chemical compound inhibitor A83-01 (one-way ANOVA). Stem cell markers, such as SOX9, LGR5 and DCLK1, were upregulated in an ActA-dependent bile acid-induced manner as shown by C. qRT-PCR ($n = 3$, one-way ANOVA) and D. Western Blot for SOX9 using RNA isolates from long-term treated EPC-hTERT cells compared to no treatment controls. E. Comparison of SOX9, LGR5 and DCLK1 expression was based on a publicly available GEO dataset (accession number GDS1321). N = normal, BE = Barrett's esophagus; unpaired *t*-test. Statistical significance: * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$, **** $p \le 0.0001$.

TGFβ signaling (Fig. 5A). To evaluate whether there is a change in the expression of keratins associated with undifferentiated basal cells or simple embryonal esophageal surface epithelium *in vivo*, we evaluated the expression of KRT8, KRT17, KRT18 and KRT19

in the publicly available GDS1321 dataset. We found a significant increase in expression values for KRT8 and KRT18 between tissue from normal and Barrett's esophagus patients (Fig. 5B). We did not observe a statistical increase for KRT19 and KRT17 based on this small dataset, indicating that larger numbers of human specimen are needed for a reliable expression analysis.

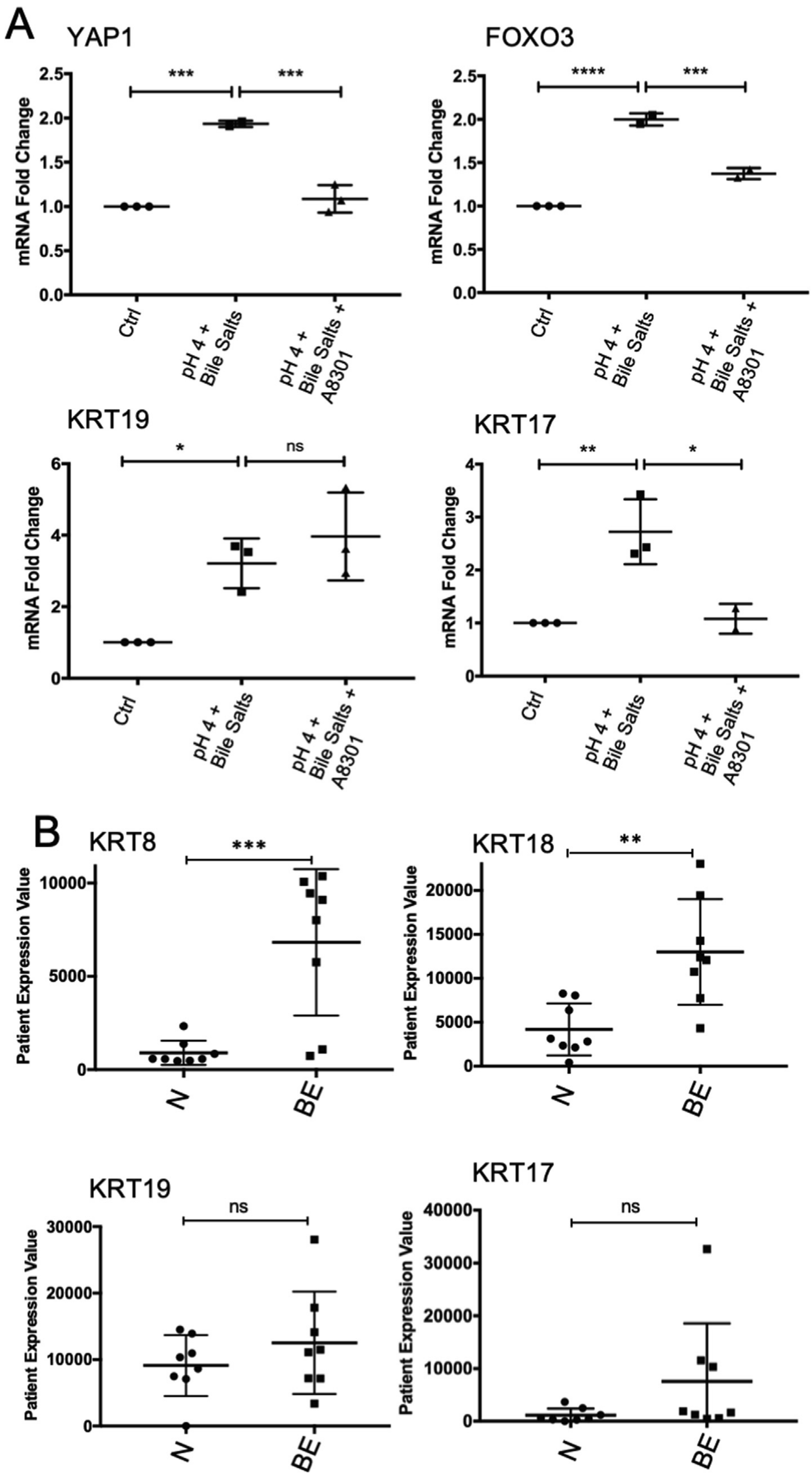


Fig. 5. Long term pH4-bile acid treatments results in the de-differentiation of esophageal epithelial cells. A. Expression of potential de-differentiation markers, YAP1, FOXO3, KRT19 and KRT17, was increased in long-term pH4 and bile salt-treated esophageal cells, and suppressed in the presence of the A83-01 inhibitor for al markers other than KRT19 as shown by qRT-PCR, n = 3, statistical analysis using one-way ANOVA. B. Expression of KRT8, KRT18, KRT19 and KRT17 was evaluated based on a publicly available GEO dataset (accession number GDS1321). N = normal, BE = Barrett's esophagus; unpaired *t*-test. Statistical significance: ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.6. Expression of select stem cell markers and keratins in three-dimensional culture models

For in situ analysis, we grew control and pH4 + bile salt-tolerant cells in three-dimensional organotypic reconstruct cultures (Fig. 6A), which represent a physiological environment containing an extracellular matrix deposited by esophageal fibroblasts. We detected an overall stronger immunofluorescent signal for LGR5 in treated cells

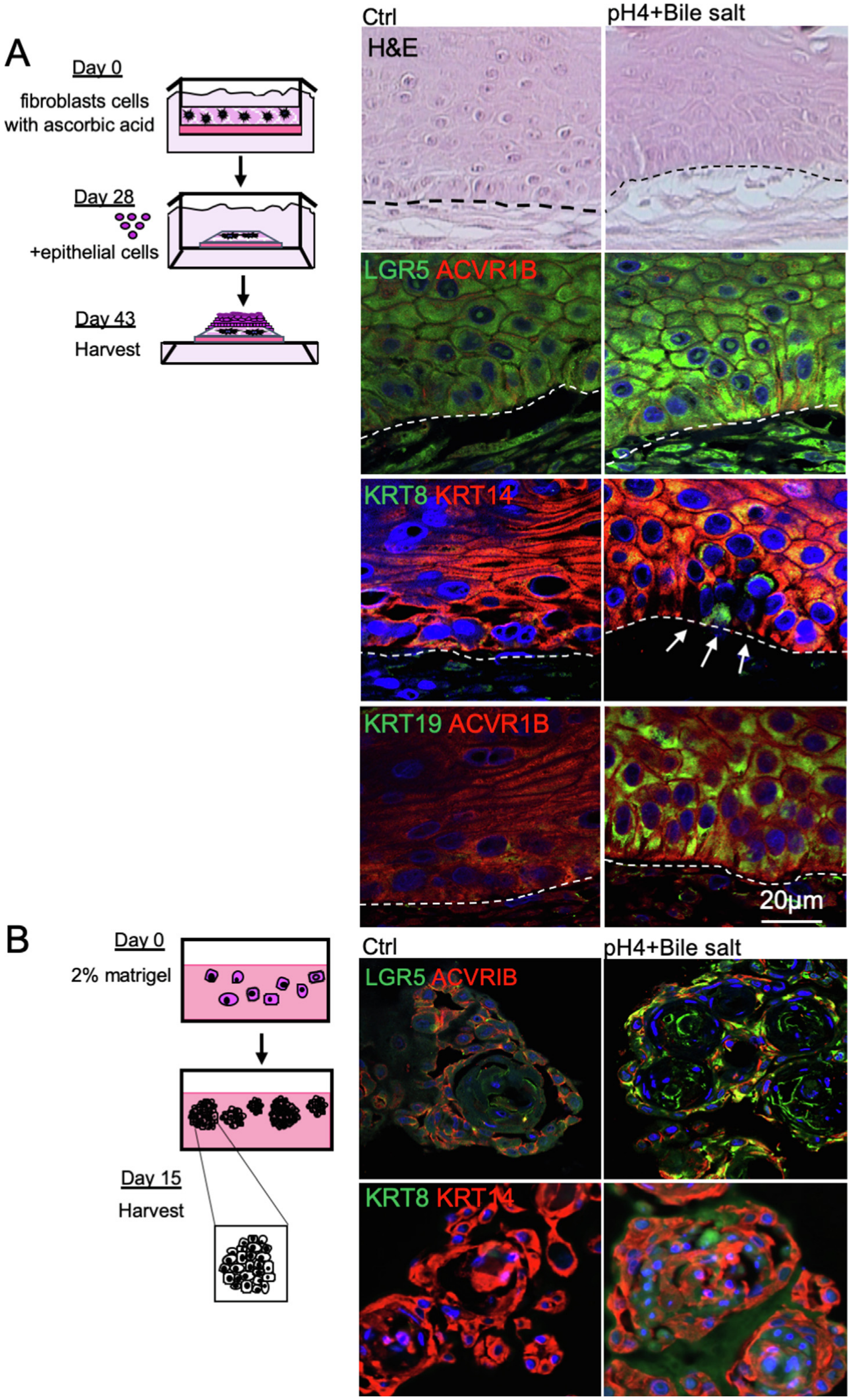


Fig. 6. In situ expression of select markers is elevated upon long-term bile/acid exposure. A. Increased signal for LGR5, KRT8, KRT19 and ACVR1B was detected using double immunofluorescence staining of organotypic reconstruct cultures for long-term treated tolerant and control cells were grown on an extracellular matrix deposited by human esophageal fibroblasts. KRT14 signal is lost in KRT8-positive cells, arrows. H&E is hematoxylin & eosin staining of the paraffin embedded organotypic culture tissue. B. Double immunofluorescence with antibody against LGR5 and ACVR1B showed an increase in pH4 + bile salt-exposed cells grown suspended 2% Matrigel to induce spheroid formation. The number of KRT8-positive, KRT14-negative cells is increased in tolerant-cells compared to control.

supporting the upregulation shown by qRT-PCR on RNA extracted from monolayer cultures (Fig. 4). This increase was also observed in long-term pH4 and bile salt-exposed cells suspended in matrigel to induce a three-dimensional spheroid compared to control (Fig. 6B). Furthermore, pH4, bile salt-tolerant cells in three-dimensional cultures were characterized by a focal loss of KRT14-positive cells correlating with a concomitant increase in KRT8 (Fig. 6A, arrows, 6B). Additionally, we show KRT19 expression and distribution together with the activin receptor 1B, ACVRIB, to be enhanced in bile acid-treated esophageal cells compared to controls by immunofluorescence staining of organotypic reconstruct cultures (Fig. 6A).

Together these data suggest the activation of stem cells and a cellular de-differentiation program as a response to bile salt and acid-mediated injury to the esophageal squamous epithelium (Fig. 7).

4. Discussion

Activin A participates in inflammation via NF-κB and MDM2

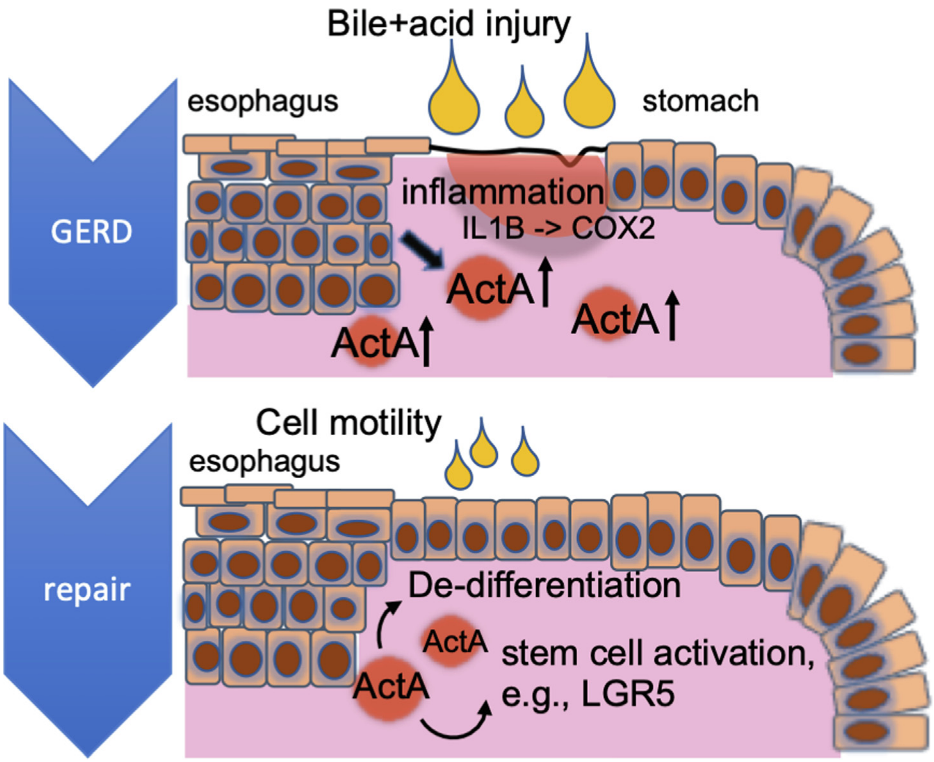


Fig. 7. Model. Increased ActA expression and secretion as a result of bile and acid epithelial injury induce stem cell activation and de-differentiation to facilitate tissue repair.

activation, which can be mediated through the degradation of p21, an important tumor suppression protein as observed in advanced colorectal cancer [43]. ActA has been shown to be released early during acute inflammation, followed by TNFα and then IL6 [44]. Yet, interestingly, reports to date suggest ActA can exert pro- as well as anti-inflammatory effects depending on the cellular context [45]. ActA has been considered a master regulator of homeostasis through its role not only in innate and adaptive immunity, but also in tissue repair or even fibrosis [18,46,47]. ActA mediates several aspects of normal wound healing in squamous epithelia, *e.g.*, overexpression of the soluble ActA antagonist Follistatin in the epidermis of transgenic mice reveals the importance for ActA signaling in wound closure; after injury, a severe delay in wound healing and reduced granulation tissue formation was observed with inhibition of ActA [34]. Surprisingly, transgenic mice overexpressing ActA in epidermal keratinocytes display enhanced granulation tissue formation after wounding as well as epidermal hyperplasia and dermal fibrosis in normal skin [33]. Bamberger et al. [35] surmised, based on a mouse model with expression of dominant-negative ACVRIB in keratinocytes, that the observed morphogenic and repair phenotypes are dependent upon ActA effects on both stromal cells and keratinocytes in normal and wounded skin *in vivo*. These data strongly suggest that ActA signaling is critical for normal skin wound healing and dramatically upregulated in chronic skin wounds [33–35]. A role for ActA in the repair of reflux injury in the esophagus has not yet been described. Prolonged exposure to acidification and bile salts during chronic occurrences of GERD results in three distinct histological phenotypes: non-erosive reflux disease (NERD), erosive esophagitis and Barrett's esophagus. The cell injury and often subsequent necrosis require repair of the epithelium, which can be mediated by migratory cells adjacent to the disrupted epithelium or in a slower form of repair through increased cell proliferation and regeneration to preserve the integrity of the epithelium [4]. More recently, epithelial-mesenchymal transition has been reported to contribute to the re-epithelialization of the esophagus [48,49]. We have identified that ActA contributes to the motility of esophageal epithelial cells upon acid and bile salt injury and therefore is central to promoting epithelial repair and tissue regeneration in the context of GERD.

Epithelial wound healing occurs in stages of inflammation, proliferation and remodeling [50]. Any long-term effect of inflammation during esophageal injury will manifest itself in the stem cell compartment and can alter the behavior of these cells, *e.g.,* increase their proliferative, migratory and invasive capacity [43]. ActA plays a role in maintaining the expression of pluripotency factors, thereby inhibiting differentiation [51] and promoting self-renewal in adult stem cells [52–54]. It has been suggested that signaling via SMAD2/3 could allow rapid cell fate decisions when priming for the expression of tissue-specific genes [55]. We have shown that the slowly proliferating esophageal basal cells exhibit a TGFβ gene superfamily expression signature [56], suggesting a role of TGFβ/Activin A in the regulation of stem cell markers. Moreover, TGFβ signaling is linked to the expression of stem cell markers associated with Barrett's tumorigenesis [57–60] *e.g.,* SOX9, LGR5, and DCLK1 that were induced in our model of experimental bile salt/acid injury [13,61]. Our data support a role for ActA as a regulator of stem cell marker expression in the phase of regeneration after repeated acid-mediated injury, such as GERD.

Inflammation has been recognized as a driver of the initiation of Barrett's esophagus in the setting of chronic reflux disease, yet the cell of origin for Barrett's esophagus is still the center of active investigation as a number of theories exist. Based on murine models such as the IL-1β transgenic model of chronic esophagitis [13] and a model by Wang [62], in which damage at the squamous columnar junction lead to an expansion of KRT7+ progenitors cells in the cardia, it is thought that the migration of precursor cells from the neighboring gastric cardia or the squamous columnar junction is responsible for the replacement of the injured squamous epithelium with a metaplastic columnar epithelium. Other potential sources of a stem or progenitor cell as an initiator of Barrett's metaplasia have been considered including cells from submucosal glands or circulating bone marrow-derived cells [63,64]. Additionally, there is increasing evidence that progenitor cells through molecular reprogramming in a process of transcommitment can differentiate into columnar cells giving rise to Barrett's esophagus [64]. At the same time, the reactivation of developmental signaling pathways such as Hedgehog can induce the expression of genes associated with Barrett's metaplasia [65]. Our data support the activation of signaling pathways responsible for a de-differentiation with marker expression indicative of the reactivation of embryonal programs.

Recently, a growing number of studies describe examples of cellular reprogramming in different settings such as injury, regeneration but also tumorigenesis [66]. In the stomach and pancreas, cellular and molecular alterations induced by injury have been shown to confer the ability to de-differentiate cells that then can participate in repair [67]. This process has been coined paligenosis, which supports injury repair and regeneration but can also introduce an increased risk for tumorigenesis, as mutations can accumulate and propagate during phases of de-and re-differentiation [68]. The stomach displays remarkable plasticity as an adaptation to chronic cellular stress by acid through surface epithelial cell migration and proliferation of repair acid-induced erosion, but also by glandular changes known as spasmolytic polypeptide-expressing metaplasia, SPEM [69].

YAP1 signaling plays a role in cellular polarity and inflammation [70], and has been demonstrated to contribute to intestinal regeneration [71], by mediating extracellular matrix remodeling during wound repair and allowing cellular reprogramming [72]. During this process, the repairing epithelium showed elevated ITGB1 and YAP1 expression, two markers that were increased in our injury model.

Dedifferentiation of keratinocytes is often suspected to accompany tissue repair and tumorigenesis. In the case of the esophagus, this can mean the reactivation of embryonal programs associated with the original epithelial lining, i.e., a simple columnar single cell layer epithelium expressing KRT8, 18 and 19 [73]. Expression of these keratins can be detected in the squamous epithelium of the adult esophagus but frequently in inconsistent patterns [74], yet their distribution becomes widespread in esophageal pre-neoplasia and malignancy indicating a role of these keratins in the response towards stress such as local inflammation and proliferative stimuli. That these keratins are not mere bystanders of the activation process has become evident when inhibiting them: Loss of these keratins results in diminished malignancy [75,76]. Our observation that there is a change in the expression of keratins associated with undifferentiated basal cells or simple embryonal esophageal surface epithelium implies an important process in esophageal tissue repair. Frequently, the repair mechanism to chronic bile acid injury involves Barrett's metaplasia, the replacement of a fully differentiated squamous tissue with a columnar tissue, which is characterized by the induction of KRT8/18 [5], KRT19 [77] loss of KRT14 and also the expression of SOX9 [5]. All changes we observed in response to bile acid injury in our model and associated with ActA signaling highlighting the potential important role of ActA. Furthermore, we found FOXO3 to be upregulated, which so far has only been linked to esophageal adenocarcinoma [78], but has a function in cell cycle and autophagy [79], two pathways related to paligenosis. Based on our data, we believe that ActA has a novel function in the de-differentiation of esophageal epithelial cells participating in repair and possibly pathogenesis of reflux esophagitis.

CRediT authorship contribution statement

Cedric Roudebush: Data curation, Formal analysis, Methodology, Validation, Visualization. **Alma Catala-Valentin:** Data curation, Formal analysis, Methodology, Supervision, Validation. **Thomas Andl:** Conceptualization, Data curation, Formal analysis, Supervision. **Gregoire F. Le Bras:** Data curation, Formal analysis, Methodology, Visualization. **Claudia Andl:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Validation.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154782.

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