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SPECIAL ISSUE: 3D CELL BIOLOGY

SHORT REPORT

Epidermal growth factor suppresses intestinal epithelial cell shedding through a MAPK-dependent pathway

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ABSTRACT

Cell shedding from the intestinal villus is a key element of tissue turnover that is essential to maintain health and homeostasis. However, the signals regulating this process are not well understood. We asked whether shedding is controlled by epidermal growth factor receptor (EGFR), an important driver of intestinal growth and differentiation. In 3D ileal enteroid culture and cell culture models (MDCK, IEC-6 and IPEC-J2 cells), extrusion events were suppressed by EGF, as determined by direct counting of released cells or rhodamine-phalloidin labeling of condensed actin rings. Blockade of the MEK–ERK pathway, but not other downstream pathways such as phosphoinositide 3-kinase (PI3K) or protein kinase C (PKC), reversed EGF inhibition of shedding. These effects were not due to a change in cell viability. Furthermore, EGF-driven MAPK signaling inhibited both caspase-independent and -dependent shedding pathways. Similar results were found *in vivo*, in a novel zebrafish model for intestinal epithelial shedding. Taken together, the data show that EGF suppresses cell shedding in the intestinal epithelium through a selective MAPK-dependent pathway affecting multiple extrusion mechanisms. EGFR signaling might be a therapeutic target for disorders featuring excessive cell turnover, such as inflammatory bowel diseases.

KEY WORDS: Intestinal epithelium, Inflammatory bowel disease, Epidermal growth factor receptor, EGFR, MAP kinases, MAPKs, Epithelial cell, Cell shedding

INTRODUCTION

The intestinal epithelium, a monolayer of polarized cells separating the organism from luminal gut contents, is the most rapidly renewing tissue in adult mammals (Sancho et al., 2004). Routine turnover of this tissue without loss of the barrier requires coordination between stem cell proliferation and shedding (also called extrusion) of mature cells from the upper villus or colonic surface mucosa. Accelerated shedding, which can lead to infection and exacerbated immune responses (Hausmann, 2010; Knodler et al., 2010), is associated with inflammatory bowel diseases (IBD; Kiesslich et al., 2012; Liu et al., 2011) and endotoxemia (Assimakopoulos et al., 2012). This 'pathological' shedding can be induced by proinflammatory cytokines (Marchiando et al., 2011) or lipopolysaccharide (Williams et al., 2013). However, little is known about regulation of constitutive physiological shedding, and especially about signals that repress it. Understanding the mechanisms controlling normal physiological cell extrusion could identify targets for correcting pathological shedding in diseases such as IBD.

Physiological cell shedding occurs through at least two mechanisms. *In situ* apoptosis of a damaged cell can trigger extrusion (Andrade and Rosenblatt, 2011; Bullen et al., 2006; Marchiando et al., 2011). Alternatively, acute crowding promotes shedding of live cells through a sphingosine-1-phosphate- and Rho-kinase-dependent mechanism (Eisenhoffer et al., 2012), with apoptosis occurring after loss of attachment rather than as a cause. In either case, the process involves Rho-driven myosin ring formation and contraction by neighboring cells (Eisenhoffer et al., 2012) and remodeling of tight junctions (Guan et al., 2011; Marchiando et al., 2011). These mechanisms are conserved in several epithelial cell types (Madara, 1990; Rosenblatt et al., 2001) and presumably across most vertebrates.

Endogenous regulators of constitutive shedding, especially factors that restrain it, are not well understood. In this study, we tested whether epidermal growth factor (EGF) receptor (EGFR) is involved in this process. EGFR is a receptor tyrosine kinase that controls intestinal cell growth, repair and migration (Frey et al., 2006; Polk, 1998). The overlap in the mechanical forces and cytoskeletal alterations in cell migration and cell extrusion suggest a possible role for EGFR in shedding; furthermore, as EGF is an epithelial cell mitogen (Sheng et al., 2006), EGFR activation might be expected to induce crowding and thus increase shedding. We used coordinated *in vitro* (3D enteroids and cultured IEC-6, MDCK and IPEC-J2 cells) and *in vivo* (adult zebrafish gut) models to study the role of EGFR in constitutive non-pathological shedding. Our results show that, surprisingly, EGFR suppresses cell extrusion through a MAPK-dependent mechanism.

RESULTS AND DISCUSSION

EGF suppresses cell shedding *in vitro*

To model intestinal turnover, we first generated ileal epithelial enteroids (Sato et al., 2011a) from mice expressing the Lifeact–EGFP cytoskeleton-labeling construct (Riedl et al., 2010). Shedding events in these enteroids (Fig. 1A) show the characteristic early saccular and funnel morphologies described *in vivo* (Marchiando et al., 2011) and can be viewed in real time (Movie 1, the box encloses an event). Shed cells per unit distance of epithelial perimeter can be counted over time. Cultures treated with EGF showed a 40% decrease in shedding per unit distance versus control (Fig. 1A).

Similar results were observed in cell culture. MDCK cells on Transwell inserts were treated with EGF (10 ng/ml) or EGFR

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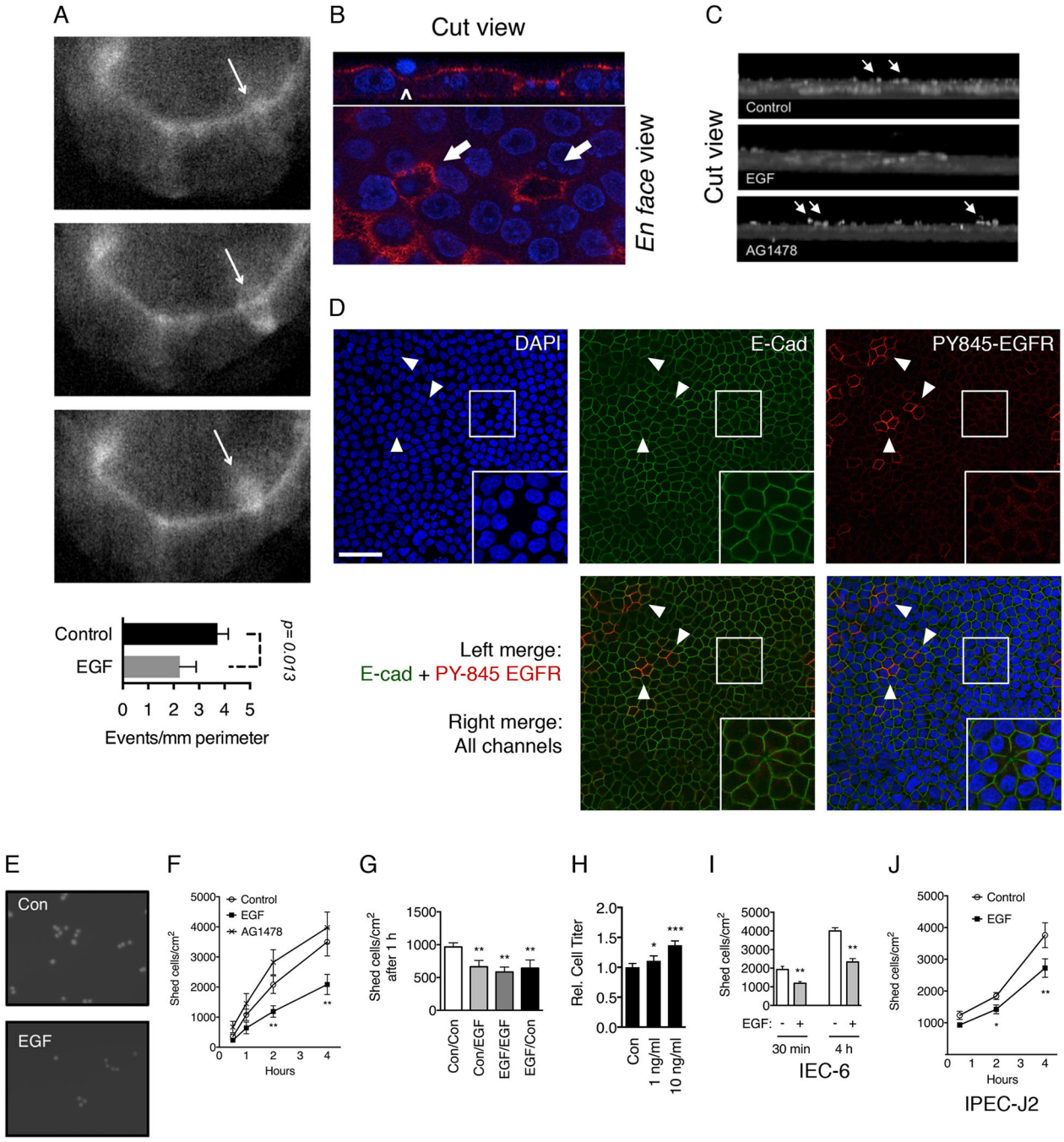

Fig. 1. EGF suppresses cell shedding *in vitro*. (A) Ileal enteroids isolated from Lifeact–EGFP-expressing mice were starved of growth factors for 24 h, then treated with vehicle (PBS) or 10 ng/ml EGF and live-imaged for 24 h. Characteristic morphological stages of apical cell shedding (arrows) and the number of events per µm of the epithelial perimeter per 24 h are shown. (B,C) MDCK cells were treated with vehicle (Control), EGF (10 ng/ml) or EGFR inhibitor AG1478 (150 nM) for 4 h, fixed and stained with Rhodamine–phalloidin (red) and DAPI (blue). See text for quantification. (B) Example image showing cell 'rosettes' (arrows) indicating shedding events. Arrowhead in top panel, nucleus from shedding cell. n=6 independent experiments. (C) Orthogonal projections of confocal z-stacks; arrows, shedding nuclei above the monolayer. n=6. (D) MDCK cells stained with anti-E-cadherin, anti-PY-845-EGFR and DAPI. Cell rosettes are sites of shedding. An example is shown in the magnification boxes. Arrowheads, cells positive for PY-845-EGFR. Scale bar: 50 µm. (E,F) MDCK (n=8) cells were labeled with DAPI and treated with vehicle, EGF or AG1478; shed cells were collected and counted; representative images in D. (G) MDCK cultures were exposed to EGF for 4 h, EGF was washed out, and then cells were cultured either with or without EGF and shed cells were counted at 4 h. (H) Relative cell numbers after 48 h in control and EGF-treated cultures, determined by resazurin reduction assay (n=4). (I) IEC-6 and (J) IPEC-J2 cells were cultured with or without EGF and shed cells at 30 min or 4 h were counted (n=4). Quantitative results are mean±s.e.m. *P<0.05 vs control; **P<0.01 vs control ; ***P<0.001 vs control (one-way ANOVA with Tukey's post-test analysis).

inhibitor (AG1478, 150 nM) for 4 h and stained with Rhodamine–phalloidin. EGF reduced the number of shedding events (0.55 versus 3.0 per field in control; P<0.01), identified as a cell surrounded by a condensed actin ring or funnel with neighboring cells assembled in the characteristic 'rosette' pattern [(Rosenblatt et al., 2001) and Fig. 1B]. By counting the number of nuclei

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displaced above the plane of the monolayer in orthogonal projection (Fig. 1C), we also observed that EGF reduced and AG1478 induced shedding (4.5 or 19.8 displaced nuclei per field versus 12.5 in control; $P<0.01$). Interestingly, phosphorylated (activated) EGFR in unstimulated MDCK monolayers was only found in cells distant to a shedding event (Fig. 1D), consistent with the notion that EGFR activation restrains shedding.

To develop a convenient model for mechanistic studies, we live-labeled cells with DAPI (Daniel and DeCoster, 2004), washed away debris and cultured in fresh medium for up to 4 h. Over time, extruded cells were collected and counted by fluorescence microscopy (example images, Fig. 1E). Results from this method were consistent with enteroids and Transwell cultures; EGF reduced shedding, whereas AG1478 caused a consistent but nonsignificant trend towards more cell extrusion (Fig. 1F). Suppression appears to be an early event in the shedding process, as an EGF pulse followed by wash-out and chase did not provoke a synchronized wave of shedding (Fig. 1G). Thus, EGF is likely blocking the onset of the process rather than arresting it midway. Consistent with decreased extrusion and the known mitogenic effects of EGF, 48 h exposure (Fig. 1H) resulted in increased cell density, although the effects of suppressed shedding and increased proliferation cannot be separated over this longer period. *In vivo*, increased mucosal area as a response to EGF (Berlanga-Acosta et al., 2001) likely relieves the compressive pressure of the resulting increased cellularity. EGF also reduced shedding of IEC-6 rat intestinal and IPEC-J2 pig jejunal epithelial cells (Fig. 1I,J). Overall, these results show that EGFR-mediated suppression of cell extrusion is conserved in cell culture models.

MEK–ERK signaling is required for EGFR suppression of shedding

To examine the molecular mechanisms of this effect, we used inhibitors to signaling intermediates which might impact caspase or Rho-kinase activity. Labeled cells were treated with EGF with or without inhibitors to MEK1 and MEK2 (MEK1/2, also known as MAP2K1 and MAP2K2, respectively; 5 µM U0126), protein kinase C (PKC; 1 µM BisI) or phosphoinositide 3-kinase (PI3K; 5 µM LY294002), and shed cells were collected and counted. MEK–ERK inhibition reversed the suppression of shedding mediated by EGF in both MDCK and IEC-6 cells (Fig. 2A–C). In contrast, neither PKC nor PI3K inhibition had any effect. Consistent with a role for MAPK signaling, constitutively shed cells showed low basal ERK1 and ERK2 (ERK1/2, also known as MAPK3 and MAPK1, respectively) activation versus attached cells (Fig. 2D,E). Furthermore, treatment with neuregulin-1β (NRG1β) or fibroblast growth factor 10 (FGF10), both of which stimulate ERK1/2 (Frey et al., 2010; Yamada et al., 2016), suppressed shedding (Fig. 2F,G). Taken together, these data suggest a model in which overall MAPK activation, which can be stimulated by multiple growth factors, regulates shedding. Although off-target effects of U0126 are a possibility, identical results were obtained with a second inhibitor

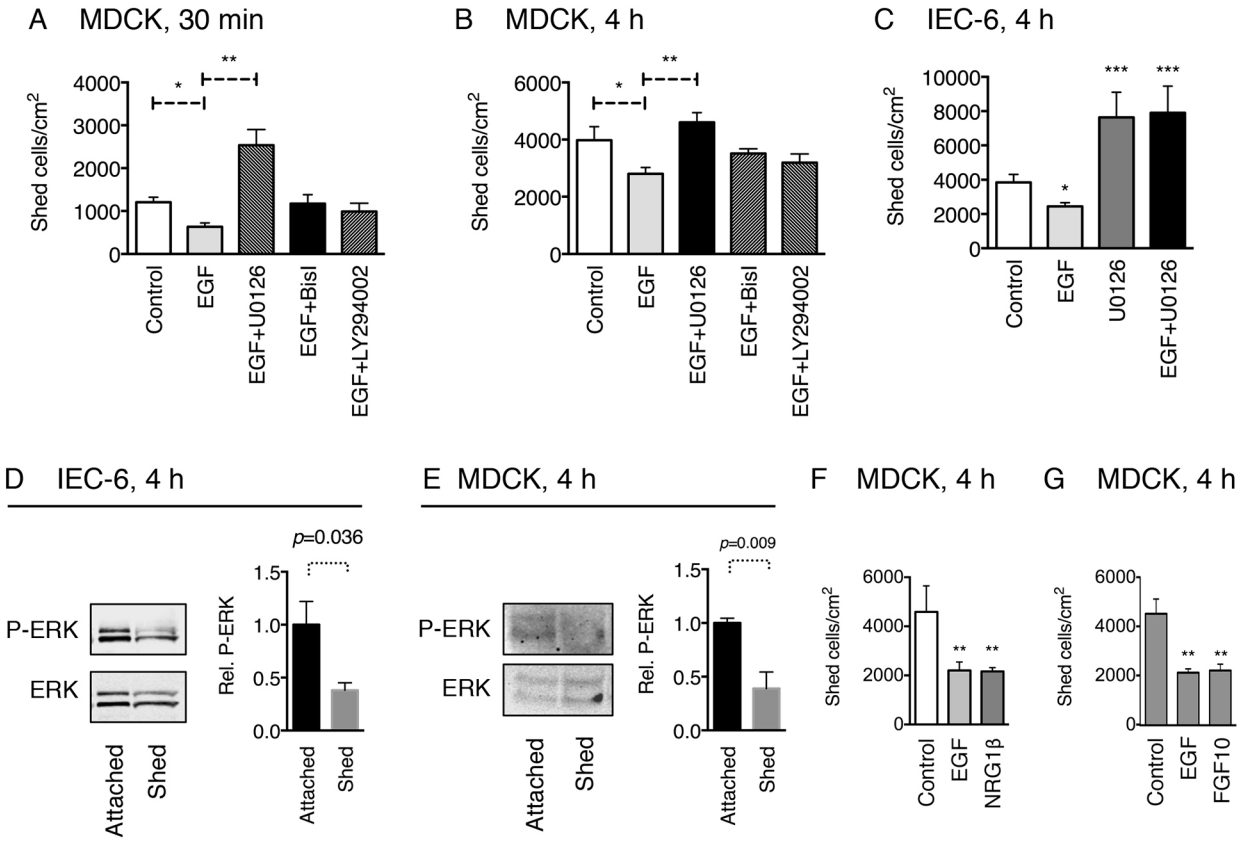

Fig. 2. MEK1/2 activity is required for EGF suppression of epithelial cell shedding *in vitro*. (A,B) MDCK (n=6) and (C) IEC-6 (n=5) cells were labeled and then treated with vehicle (Control) or EGF, with or without 5 µM U0126 (a MEK1/2 inhibitor), 5 µM LY294002 (a PI3K inhibitor) or 1 µM BisI (a PKC inhibitor) for 30 min or 4 h. Shed cells were collected and counted. (D,E) Attached and shed cells from control cultures (no EGF) collected over 4 h were subjected to western blotting for phosphorylated ERK (P-ERK) (n=4). (F,G) MDCK cells (n=4) were treated with 10 ng/ml NRG1β or 2.5 ng/ml FGF10 and shed cells were counted.. *P<0.05; **P<0.01; ***P<0.001 (one-way ANOVA with Tukey's post-test analysis).

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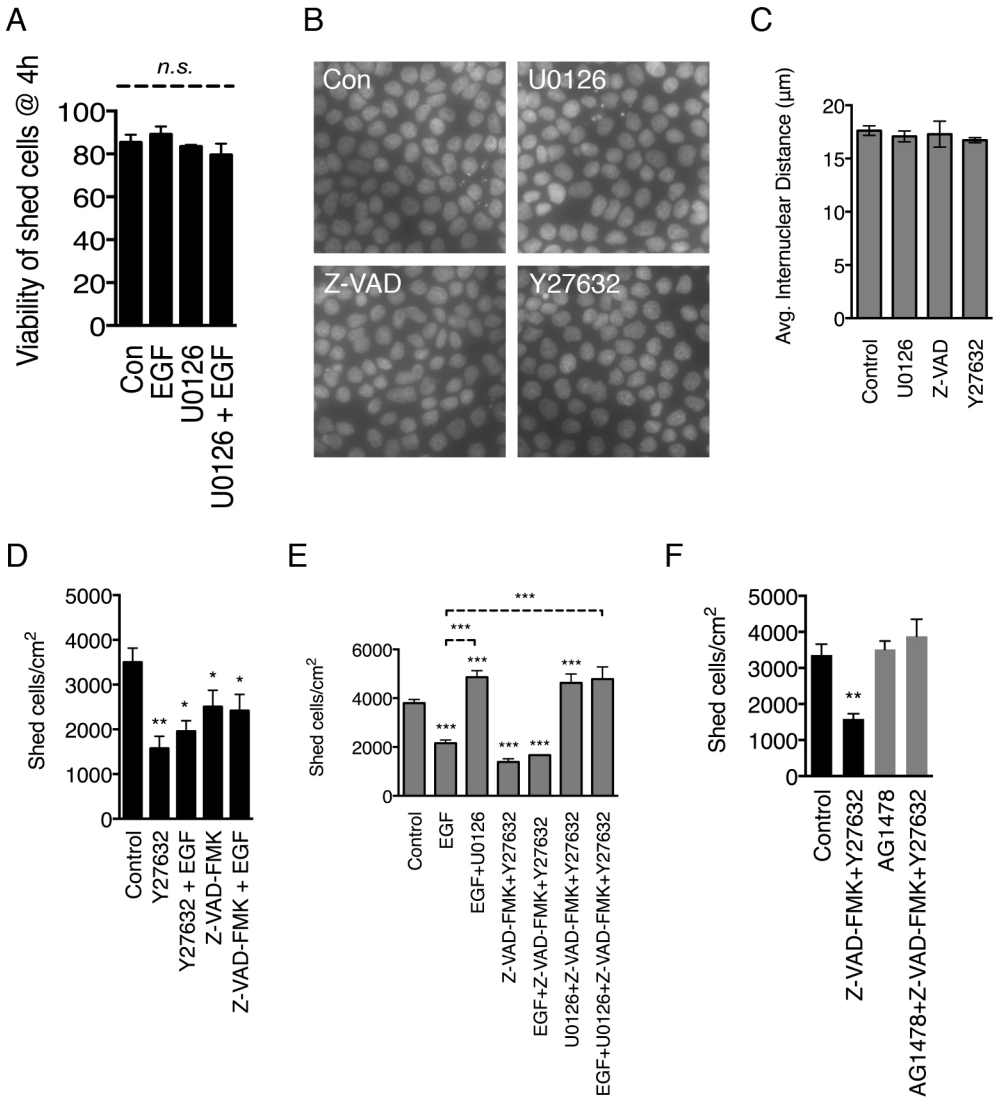

Fig. 3. MEK inhibition reverses suppression of cell shedding by EGF, caspase inhibitor or Rho-kinase inhibitor. (A) Shed MDCK cells in vehicle (Control), EGF- and U0126-treated cultures were collected after 4 h, and viability was assessed by Trypan Blue exclusion (n=4). (B) MDCK cells were treated as indicated for 4 h and DAPI-stained to show density. (C) Delaunay analysis of mean internuclear distance on cultures. (D) MDCK cells (n=6) were labeled then treated with vehicle, EGF, Z-VAD-FMK (caspase inhibitor; 1 μM) or Y27632 (Rho-kinase inhibitor; 20 μM) for 4 h; shed cells were collected and counted. *P<0.05 versus control (one-way ANOVA with Tukey's post-test analysis). (E,F) MDCK (n=5) cells were labeled then treated as indicated for 4 h. Shed cells were collected and counted. Quantitative results are mean±s.e.m. ***P<0.001 vs control; *** brackets, P<0.001 between columns indicated (one-way ANOVA with Tukey's post-test analysis).

(PD98059, data not shown) and the apparent increase in shedding beyond baseline in the presence of inhibitor is likely due to loss of the basal MAPK activity in untreated cells.

EGFR regulates both caspase- and Rho-dependent shedding

Physiological shedding from epithelial monolayers includes both apoptotic cells (caspase-dependent mechanism) and Rho-kinase driven extrusion of live cells (Eisenhoffer et al., 2012; Marchiando et al., 2011). Trypan Blue staining of shed MDCK cells at 4 h showed no difference in cell viability with EGF or U0126 (Fig. 3A; control 85.3±3.6% viable; EGF 89.2±1.0%; U0126 83.4±1.3%; U0126+EGF 79.5±9.2%; mean±s.e.m., P=0.31, ANOVA), suggesting that the effects of EGF and MAPK are not simply due to blocking cell death. To further explore which pathways are impacted, we performed shedding experiments using caspase (Z-VAD-FMK, 1 μM) or Rho-kinase (Y27632, 20 μM) inhibitors in the presence or absence of EGF and/or U0126. None of the inhibitors affected the bulk density of cultures in the short term (Fig. 3B,C), showing that shedding changes are not simply due to altered cell numbers. Both caspase and Rho-kinase inhibitors reduced MDCK shedding, as expected (Fig. 3D,E). However, EGF did not augment this reduction. These results suggest that EGFR is blocking both the apoptotic caspase and the Rho-kinase-dependent myosin contraction shedding pathways. Interestingly, MEK inhibition stimulated shedding even at baseline or in the presence of caspase plus Rho-kinase inhibitors (Fig. 3E), suggesting that EGF→MEK→ERK signaling blocks shedding downstream of both of these pathways. Similarly, the EGFR inhibitor AG1478 reversed suppression of baseline shedding by Z-VAD-FMK+Y27632 (Fig. 3F). As MEK→ERK signals are known to contribute to tight junction maintenance in the intestine (Kinugasa et al., 2000), it is possible that an initial step in the mechanical shedding process requires loss of MAPK activity in the target cell to dissolve cell–cell junctions.

EGFR suppresses constitutive intestinal epithelial cell shedding *in vivo* in zebrafish through the MEK–ERK pathway

To test our findings *in vivo*, we established the adult zebrafish intestine as a shedding model. On Rhodamine–phalloidin-stained sections of adult zebrafish midgut, all stages of the shedding process – including actin 'funnels' characteristic of cytoskeletal rearrangements in epithelial cells preparing to undergo extrusion – are clearly visible (Fig. 4A). We injected adult fish intraperitoneally with 20 μl vehicle (0.01% DMSO), EGF (1 μg/ml; final 60 μg/kg) or AG1478 (200 nM; final 388 μg/kg). After 4 h, intestines were collected and stained with Rhodamine–phalloidin to detect F-actin-

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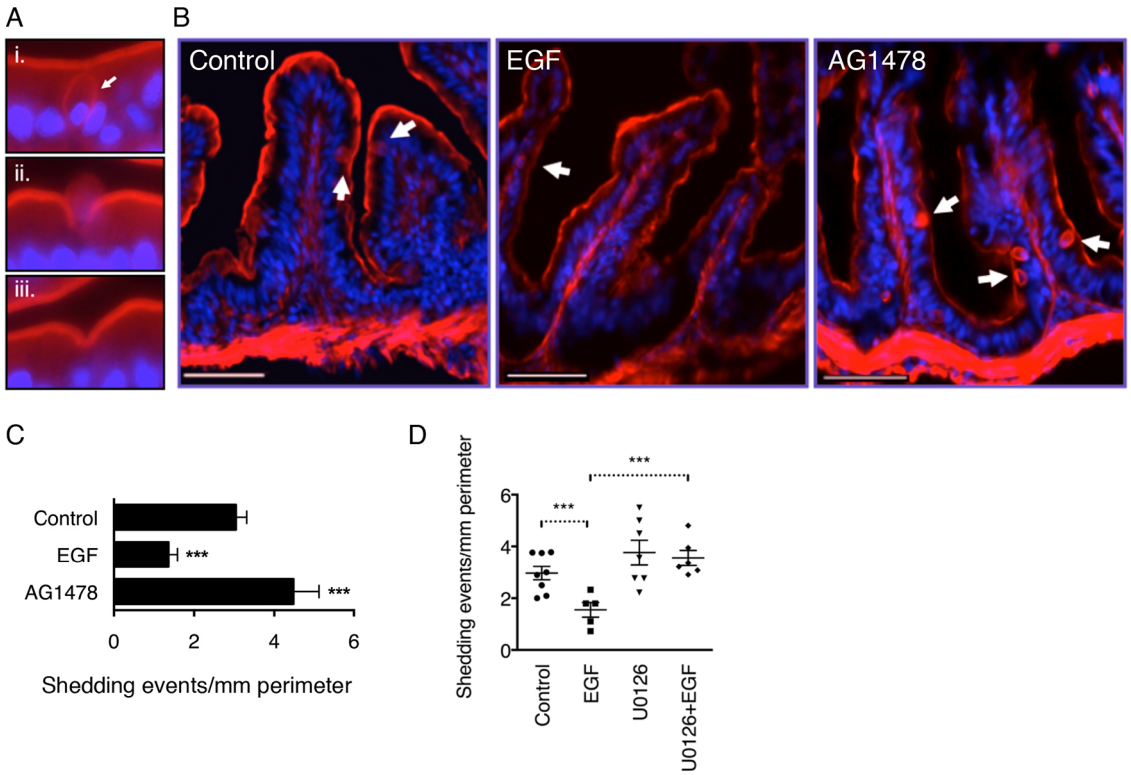

Fig. 4. EGF inhibits cell shedding in zebrafish intestine via MAPK signaling. (A) Zebrafish midgut tissue was fixed and stained with Rhodamine–phalloidin (red, F-actin) and DAPI (blue, nuclei). Examples of (i) early, (ii) late and (iii) completion of shedding process shown. Arrow, example saccular stage event. (B) Fish were treated intraperitoneally with vehicle (Control), EGF or AG1478 for 4 h. Early-stage shedding funnels (arrows) per mm of tissue perimeter were counted (C). Scale bars: 50 µm. ***P<0.001 vs control, n=5 per condition. (D) Fish were treated intraperitoneally with vehicle (Control), EGF or U0126 for 4 h. Tissue was fixed, stained and shedding funnels counted; n=6–8 per condition as shown. Quantitative results are mean±s.e.m. ***P<0.001 (one-way ANOVA with Tukey's post-test analysis).

rich shedding funnels. EGF suppressed and AG1478 induced shedding compared to control (1.7 or 4.3 shedding events/mm tissue perimeter versus 2.5 in control; P<0.05, Fig. 4B,C). Comparing the location of shedding events, we found that a greater proportion of events in AG1478-treated fish were in the lower half of the villus folds or in the inter-fold region (61.4±10.2% in AG1478 versus 45.1±12.1% in control; mean±s.e.m.) suggesting altered localization with EGFR blockade. As the MEK–ERK MAPK cascade was essential for EGF-mediated suppression of cell shedding *in vitro* (Fig. 2), we tested the effect of this pathway in zebrafish using the pharmacological MEK inhibitor U0126 (10 µM; final 228 µg/kg). Similar to our *in vitro* results, MEK inhibition abrogated EGF-induced reduction in detectable shedding events on intestinal villus folds of the fish (Fig. 4D).

Taken together, these data indicate that EGFR suppresses constitutive intestinal epithelial cell shedding and position EGFR ligands as a suite of soluble factors potentially used by the tissue to regulate its own turnover. The relevant physiological ligands for controlling shedding have not yet been defined, but as discussed above, any ligand that stimulates MAPK is potentially important. Endogenous EGF from salivary and Brunner's glands is present in the intestinal lumen (Playford and Wright, 1996; Scheving et al., 1989; Thompson et al., 1994), but its availability to EGFR on the basolateral membranes of enterocytes (Playford et al., 1996) might be limited and thus, for example, TGF-α released from basolateral surfaces (Dempsey et al., 2003) might be more relevant under homeostatic conditions. Ligands are also produced by subepithelial myofibroblasts (Shao and Sheng, 2010) and Paneth cells (Poulsen et al., 1986; Sato et al., 2011b), possibly creating growth factor gradients along the crypt–villus axis. This could explain why extrusion is normally restricted to the upper villi. Consistent with this notion, weaning pigs exhibit increased intestinal epithelial turnover (Skrzypek et al., 2005) coincident with a loss of EGFR expression on the villi (Schweiger et al., 2003). Studies in *Drosophila* gut indicate that intestinal stem cells and enterocytes communicate to coordinate generation of new cells with loss of old ones (O'Brien et al., 2011) and that EGF plays an important role in maintaining the intestinal stem cell compartment (Xu et al., 2011). Thus, EGFR ligand gradients within the epithelium could provide a mechanism for coordination between stem cells and the shedding zone.

In summary, we have shown that EGF helps regulate epithelial homeostasis by suppression of constitutive cell extrusion through a MEK–ERK signaling mechanism. Ongoing work is focused on understanding the fundamental cellular mechanisms targeted by this signaling pathway, and on determining whether EGFR also regulates pathologic cell shedding under inflammatory conditions. Several investigators have reported reduced EGFR ligand expression in IBD (Alexander et al., 1995; Hormi et al., 2000). Furthermore, cytokines involved in Crohn's disease, which promote shedding and the formation of epithelial gaps in the mouse small intestine (Marchiando et al., 2011, 2010), can also inhibit EGFR activation in intestinal epithelial cells *in vitro* (Kaiser and Polk, 1997; McElroy et al., 2008) and *in vivo* (Feng and Teitelbaum, 2012). Thus, understanding the mechanism regulating constitutive shedding might lead to insight into pathological processes as well.

MATERIALS AND METHODS

Zebrafish

Animal use was approved and monitored by the Children's Hospital Los Angeles IACUC and designed to follow ARRIVE guidelines. Adult zebrafish were maintained under standard conditions with a 14-h-dark–10-h-light cycle at 28.5°C, anesthetized with tricaine (Sigma-Aldrich), injected intraperitoneally with vehicle (DMSO), EGF, AG1478 or U0126, and killed after 4 h. Intestines were collected and cryosectioned (4 µm). Midgut was stained with Rhodamine–phalloidin (Molecular Probes) and mounted in Vectashield with DAPI (Vector Laboratories, Inc.). Condensed actin funnels surrounding cells in early stages of shedding (Marchiando et al., 2011) were identified, imaging at least 50 villi per fish. Shedding events per mm epithelial perimeter were traced using ImageJ (National Institutes of Health).

Enteroid cultures

Ileal epithelial crypts from LifeAct–EGFP mice (Riedl et al., 2010) were isolated by Ca^{2+} chelation and shaking, then grown in Matrigel plus R-spondin (500 ng/ml), noggin (100 ng/ml) and EGF (20 ng/ml) as previously described (Sato et al., 2011a). Growth factors were removed for 24 h before experiments. Confocal *z*-stacks of control and EGF-treated cultures were recorded every 5 min for 24 h.

Growth factors and inhibitors

Recombinant EGF was from Peprotech. Recombinant NRG1β, FGF10, R-spondin and noggin were from R&D Systems. AG1478 was from Cayman Chemical. U0126, LY294002 and BisI were from Calbiochem. Z-VAD-FMK was from G Biosciences. Y27632 was from Enzo Life Sciences.

Cell culture

IEC-6 (ATCC#CRL-1592) and MDCK (ATCC#CCL-34) cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro 10-013-CV; Mediatech Inc.) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin, and (IEC-6 only) insulin, transferrin and selenium (BD Biosciences) at 37°C in a 5% CO_2 atmosphere. IPEC-J2 cells (Rhoads et al., 1994), a kind gift of Anthony Blikslager (North Carolina State University, Raleigh, NC), were cultured in DMEM/F12 with 5% FBS, 100 U/ml penicillin and streptomycin, and insulin, transferrin and selenium. Cells were banked on receipt and used within 10 passages of original stock; lack of cross-contamination was confirmed with species-specific PCR. Density of some cultures was confirmed by a resazurin reduction assay (Promega) or Delaunay analysis of mean internuclear distance.

Confocal analysis of shedding

MDCK cells seeded on 0.4-µm-pore polycarbonate tissue culture inserts (Transwells; Corning Biosciences) were treated, fixed, stained with DAPI and Rhodamine–phalloidin, and mounted on slides. Confocal *z*-stacks of 15–20 consecutive 0.4 µm slices from four regions per sample were acquired using a Zeiss LSM700 microscope. Shedding cells were counted in two ways: by viewing condensed F-actin 'rosettes' surrounding a cell in *en face* (*xy* plane view) view (Rosenblatt et al., 2001) or by viewing nuclei leaving the plane of the monolayer in an orthogonal projection.

Immunostaining and immunoblotting

Cultures fixed in 4% formaldehyde were immunostained using anti-E-cadherin (1:100; RR1; Developmental Studies Hybridoma Bank, University of Iowa) and antibody against EGFR phosphorylated on Y845 (PY-845-EGFR; 1:100; Cell Signaling cat. no. 2231); secondary antibodies were anti-mouse-IgG conjugated to Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 (Invitrogen). Western blotting for MAPK activation in cells was as previously described (Bernard et al., 2012), using Cell Signaling antibodies cat. no. 9101 and cat. no. 4696 (1:1000).

Direct counting of shed cells

Cells were seeded in 24-well cell culture plates (250,000 cells/well) and grown for 24 h. The next day, cultures were labeled for 15 min with DAPI (0.1 µg/ml; Sigma-Aldrich), washed with PBS and supplemented with fresh medium. Medium was sampled at defined times. Shed cells were collected by centrifugation and counted by wide-field fluorescence. A minimum of four images were captured and analyzed from each well.

Statistical analysis

Data are representative of at least five individual zebrafish per treatment or three independent cell culture experiments. Quantification was performed by an investigator blinded to experimental conditions. Statistical analyses used Prism (GraphPad, Inc.). Significance of differences from controls was assessed by one-way ANOVA with Tukey's post-test analysis. Error bars represent the s.e.m.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.C.M., D.B.P., C.-L.L., A.J.M.W. and M.R.F. conceived and designed the experiments. J.C.M., A.A.M., J.J.H., D.A.A., L.C.H. and M.R.F. performed the experiments and analyzed the data. J.C.M., A.A.M., C.-L.L., A.J.M.W. and M.R.F. wrote the manuscript.

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Supplementary information

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Movie 1. Ileal enteroids isolated from Lifeact-EGFP-expressing mice were live-imaged for 24 h to view cell shedding in real time. Box encloses an area in which an example shedding event occurs.

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