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Trefoil factor 2 activation of CXCR4 requires calcium mobilization to drive epithelial repair in gastric organoids

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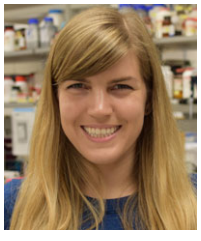
Key points

- Determining the signalling cascade of epithelial repair, using murine gastric organoids, allows definition of regulatory processes intrinsic to epithelial cells, at the same time as validating and dissecting the signalling cascade with more precision than is possible *in vivo*
- Following single cell damage, intracellular calcium selectively increases within cells adjacent to the damage site and is essential for promoting repair.
- Trefoil factor 2 (TFF2) acts via chemokine C-X-C receptor 4 and epidermal growth factor receptor signalling, including extracellular signal-regulated kinase activation, to drive calcium mobilization and promote gastric repair.
- Sodium hydrogen exchanger 2, although essential for repair, acts downstream of TFF2 and calcium mobilization.

Abstract The gastric mucosa of the stomach is continually exposed to environmental and physiological stress factors that can cause local epithelial damage. Although much is known about the complex nature of gastric wound repair, the stepwise process that characterizes epithelial restitution remains poorly defined. The present study aimed to determine the effectors that drive gastric epithelial repair using a reductionist culture model. To determine the role of trefoil factor 2 (TFF2) and intracellular calcium (Ca^{2+}) mobilization in gastric restitution, gastric organoids were derived from TFF2 knockout (KO) mice and yellow Cameleon-Nano15 (fluorescent calcium reporter) transgenic mice, respectively. Inhibitors and recombinant protein were used to determine the upstream and downstream effectors of gastric restitution following photodamage (PD) to single cells within the gastric organoids. Single cell PD resulted in parallel events of dead cell exfoliation and migration of intact neighbouring cells to restore a continuous epithelium in the damage site. Under normal conditions following PD, Ca^{2+} levels increased

Kristen Engevik is a PhD candidate at the University of Cincinnati College of Medicine. Kristen's research focuses on investigating the mechanism behind gastric restitution. Her expertise lies in culturing three-dimensional gastric organoids and the use of two-photon light microscopy to induce photodamage with respect to examining the pathways involved in gastric repair.



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within neighbour migrating cells, peaking at ∼1 min, suggesting localized Ca^{2+} mobilization at the site of cell protrusion/migration. TFF2 KO organoids exhibit delayed repair; however, this delay can be rescued by the addition of exogenous TFF2. Inhibition of epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK)1/2 or a TFF2 receptor, chemokine C-X-C receptor 4 (CXCR4), resulted in significant delay and dampened Ca^{2+} mobilization. Inhibition of sodium hydrogen exchanger 2 (NHE2) caused significant delay but did not affect Ca^{2+} mobilization. A similar delay was observed in NHE2 KO organoids. In TFF2 KO gastric organoids, the addition of exogenous TFF2 in the presence of EGFR or CXCR4 inhibition was unable to rescue repair. The present study demonstrates that intracellular Ca^{2+} mobilization occurs within gastric epithelial cells adjacent to the damage site to promote repair by mechanisms that involve TFF2 signalling via CXCR4, as well as activation of EGFR and ERK1/2. Furthermore NHE2 is shown to be important for efficient repair and to operate via a mechanism either downstream or independent of calcium mobilization.

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Introduction

Gastric epithelial barrier integrity and proper repair of a disrupted barrier are essential functions to sustain this primary barrier that protects the inner body from noxious contents of the stomach (Niv & Banic, 2014). When encountering either small or extensive epithelial damage, cell migration promotes rapid re-establishment of epithelial integrity as the initial response of epithelial repair. In the presence of more severe damage, this restitution event occurs before regeneration (cell proliferation) or inflammatory responses. During gastric restitution, dead or damaged cells are expelled into the stomach lumen and adjacent viable cells release bioactive peptides that act as motogens to signal cells to cover the denuded mucosa without proliferation (Svanes *et al.* 1982; Lacy & Ito, 1984). The mechanism of restitution is speculated to be intrinsic to the epithelial cells themselves because the process can be demonstrated *in vitro* in cell lines (Rutten & Ito, 1983; Svanes *et al.* 1983; Kim *et al.* 2012; Wang *et al.* 2012), although the regulation and co-ordination of this multicellular process is poorly understood both *in vivo* and *in vitro*. Various factors have been shown *in vivo* and/or *in vitro* to influence gastric epithelial restitution, including Ca^{2+}, trefoil factor peptides (TFFs) and epidermal growth factor (EGF) (Hansson *et al.* 1990; Furukawa *et al.* 1999; Nie *et al.* 2003; Yang *et al.* 2006; Xue *et al.* 2010; Xue *et al.* 2011; Aihara *et al.* 2013; Aihara *et al.* 2018).

Ca^{2+} is a ubiquitous second messenger that influences multiple cellular processes, including mucus secretion and cell migration in various cell types (Belkacemi *et al.* 2005; Schreiber, 2005; Wei *et al.* 2008; Aihara & Montrose, 2014; Xie *et al.* 2017). *In vivo*, gastric damage elicits increased intracellular and extracellular Ca^{2+} (Takeuchi *et al.* 1985; Koo, 1994; Takeuchi *et al.* 1999) and both are necessary for proper gastric wound repair (Aihara *et al.* 2013). Inhibition of intracellular Ca^{2+} release or uptake significantly prevents cell migration following wounding in cultured rabbit gastric cells (Ranta-Knuuttila *et al.* 2002). Although studies indicate the overarching role of endogenous Ca^{2+} in gastric epithelial repair, little is known about upstream signalling to regulate Ca^{2+} mobilization.

Another known factor involved in gastric restitution is the motogenic TFF peptide family. TFFs play an important role within the gastrointestinal (GI) mucosal barrier throughout the GI tract (Lefebvre *et al.* 1993; Nie *et al.* 2003; Aihara *et al.* 2017). In epithelial cell culture models, TFFs have been shown to promote cell migratory and anti-apoptotic activities (Kinoshita *et al.* 2000; Taupin & Podolsky, 2003; Hoffmann, 2005), therefore identifying potential roles in mediating mucosal repair. In the stomach of both rodents and humans, TFF2 is abundantly secreted from the stomach mucous neck cells (Hoffmann, 2005; Aihara *et al.* 2017). TFF2 deficient ($TFF2^{-/-}$) mice exhibit delayed gastric repair *in vivo* (Xue *et al.* 2010; Xue *et al.* 2011; Aihara *et al.* 2016); functional assays suggest that C-X-C chemokine receptor 4 (CXCR4) acts as a TFF receptor both *in vitro* and *in vivo* (Farrell *et al.* 2002; Dubeykovskaya *et al.* 2009; Xue *et al.* 2011). During healing, *in vivo* epithelial levels of CXCR4 and TFF2 are increased and the addition of exogenous TFF accelerates the healing process (Poulsen *et al.* 1999; Xu *et al.* 2013). TFF2 is also increased in response to *Helicobacter pylori* infection or severe damage caused by repetitive administration of non-steroidal anti-inflammatory drugs and such TFF2 upregulation can precede changes in other growth factors, including EGF (Konturek *et al.* 1998; Chen *et al.* 2018).

EGF is another peptide produced by the gastric mucosa (Wright *et al.* 1990) and EGF receptor (EGFR) is present in gastric epithelial cells (Mori *et al.* 1987; Menard & Pothier,

1991; Chen *et al.* 2001). EGF stimulates gastric epithelial cell migration and accelerates wound healing acting via EGFR and extracellular signal-regulated kinase (ERK)1/2 signalling in both *in vivo* and *in vitro* models (Tarnawski & Jones, 1998; LI *et al.* 2003; Tarnawski & Ahluwalia, 2012). There is a potential link between TFF2/CXCR4 and EGFR. In gastric cancer cell lines, CXCR4-EGFR cross-talk has been shown to promote cell migration (Guo *et al.* 2007; Cheng *et al.* 2017). Additionally, it has also been reported that TFF2 can trigger phosphorylation of EGFR in HT29 colon cancer cells (Kinoshita *et al.* 2000; Rodrigues *et al.* 2003; Kosriwong *et al.* 2011). However, it is not known whether such interactions between TFF2/CXCR4 and EGFR occur outside the setting of cancer cell lines.

Evaluating the epithelial signalling cascade associated with gastric restitution *in vivo* is difficult. Only a limited number of inhibitors and agonists are suitable for *in vivo* studies, and the tools for manipulating and monitoring intracellular calcium are less precise *in vivo*. The organoid culture system allows for the growth and differentiation of primary, normal epithelial cells from mouse tissue (Bartfeld *et al.* 2015; Schlaermann *et al.* 2016; Aihara *et al.* 2018). Gastric organoids contain all epithelial cell types of native tissues (Bartfeld *et al.* 2015; Schumacher *et al.* 2015*a*). We have previously shown gastric organoids provide a unique reductionist model system for examining the molecular mechanisms of restitution in the gastric epithelium (Aihara *et al.* 2018). Using gastric organoids from normal and mutant mice, we aim to evaluate involvement of TFF2, CXCR4 and EGFR in calcium-dependent restitution of gastric damage. The results of the present study demonstrate that a novel convergence of the TFF2, EGFR and Ca^{2+} signalling pathways is essential for gastric epithelial restitution.

Methods

Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati (protocol 04-03-08-01). The investigators' work complies with the ethical principles under which the *Journal of Physiology* operates, as described in Grundy (2012).

Animal husbandry

Experiments used C57BL/6J mice (IMSR catalogue no. JAX:000664, RRID:IMSR_JAX:000664), in-house bred TFF2 knockout (KO) (backcrossed onto a C57BL/6 background until >90% of genomic microsatellite markers were from C57BL/6J) mice (Xue *et al.* 2011), in-bred sodium hydrogen exchanger 2 (NHE2) KO (FVB/N background) mice (Xue *et al.* 2011) or transgenic mice (C57BL/6 background) expressing the yellow Cameleon-Nano15 (YC-Nano) Ca^{2+} sensor fluorescent proteins (Oshima *et al.* 2014). We are grateful to Dr Y. Oshima for supplying the animals from the YC-Nano colony. For experiments examining TFF2 KO (–/–) and NHE2 KO (–/–) genotypes, wild-type (WT) controls were composed of +/+ genotypes from the same colony. Pups were genotyped by genomic PCR as described previously (Schultheis *et al.* 1998; Bell *et al.* 1999; Farrell *et al.* 2002) and male and female mice were used for experimentation at 2–4 months of age. Animals were given standard rodent chow diet and water, both available *ad libitum*.

Primary culture of gastric organoids

Gastric organoids were generated from mouse gastric corpus as described previously (Mahe *et al.* 2013; Schumacher *et al.* 2015*a*; Engevik *et al.* 2018). Mice were killed by isoflurane inhalation, immediately followed by cervical dislocation. Isolated gastric epithelium from the corpus was cultured in Matrigel (Corning, New York, NY, USA) diluted 1:1 in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} in an eight-well or two-well Lab-Tek chamber with coverglass (Thermo Scientific, Waltham, MSA, USA) for growth of gastric organoids. Gastric organoids were cultured in a 5% CO_2 incubator at 37°C for 3–4 days prior to the experiments.

Induction of two-photon laser-induced photodamage

Experiments were performed in organoid culture medium under conditions of 5% CO_2/37°C in a microscope incubation chamber (PeCon, Erbach, Germany) on an inverted confocal microscope (LSM 510 NLO; Carl Zeiss, Oberkochen, Germany) and imaged with a C-Achroplan NIR 40× objective lens. In some experiments, gastric organoids were pre-incubated for 30 min with the DNA stain Hoechst 33342 (10 μg mL^{-1}; Invitrogen, Carlsbad, CA, USA) to visualize cellular nuclei. In experiments intended for analysis of damage area and cell exfoliation in YC-Nano gastric organoids, images of Hoechst 33342 (titanium-sapphire laser; Ti-Sa; excitation 730 nm, emission 435–485 nm) and YFP (excitation 514 nm, emission 535–590 nm) in the gastric organoid were collected simultaneously with transmitted light and a confocal reflectance image (reflecting 730 nm light to show cell/tissue structure). In TFF2 and NHE2 WT and KO gastric organoids, images of Hoechst 33342 were collected simultaneously with transmitted light and confocal reflectance images, using the wavelengths reported above. For assessing intracellular Ca^{2+} changes in YC-Nano gastric organoids, images of YFP-Förster resonance energy

transfer (FRET) (Ti-Sa; excitation 840 nm, emission 535–590 nm) and CFP (Ti-Sa; excitation 840 nm, emission 500–530 nm) were collected simultaneously with a transmitted light image. Wavelength selections for Ca^{2+} imaging were guided by previous work with YC sensors (Horikawa *et al.* 2010; Oshima *et al.* 2014). In all photodamage experiments, after collecting a set of control images, a small rectangular region (~5 μm^2) of a single cell was repetitively scanned at high Ti-Sa laser power (730 or 840 nm: 630 mW average) for 500 iterations (requiring ~3 s).

Experiments examined gastric organoids embedded in Matrigel, located ~100–300 μm from the cover glass. In some cases, BAPTA/AM (50 μM; Calbiochem, San Diego, CA, USA) was applied to medium and incubated for at least 30 min prior to experiments. Inhibitors were pre-incubated at least 1 h prior to experimentation to ensure equilibration in Matrigel, and were kept in the medium during experiments. Inhibitory reagents included: AMD3100 (1 μM; Sigma, St Louis, MO, USA), AG1478 (200 nM; Cayman Chemical Company, Ann Arbor, MI, USA), FR180204 (10 μM; Tocris Bioscience, St Louis, MO, USA) and Hoechst 694 (100 μM; a gift from Dr H. J. Lang, Sanofi-Aventis, Frankfurt, Germany). The final DMSO concentration in the experiments was <0.1%. Solvent control groups contained 0.1% DMSO added to medium. Concentrations were determined based upon prior *in vitro* studies (Chen *et al.* 2002; Hurst *et al.* 2008; Aihara *et al.* 2018) or were shown in preliminary experiments to have no observed significant toxicity in intact organoids, as measured by changes in epithelial permeability or cell death (data not shown). Vehicle control groups contained either 0.1% DMSO, ddH_2O or dPBS added to the medium; vehicle was dependent on the solution that the inhibitors used were constituted in.

Damage-repair cycle was measured independently once per gastric organoid and the outcomes from at least four different gastric organoids (derived from at least three animals) were compiled for each experimental protocol.

Microinjection

For rescue experiments in TFF2 and NHE2 KO gastric organoids, recombinant human TFF2 (rTFF2; 40 μM stock; R&D Systems, Minneapolis, MN, USA) was microinjected as described previously (Engevik *et al.* 2018). Gastric organoids (~400–500 μm in diameter) were injected with 9 nL of rTFF2 40 μM stock for an estimated final rTFF2 concentration of 400 nM. In rescue experiments utilizing inhibitors, rTFF2 was microinjected following 1 h of pre-incubation with inhibitors. Control vehicle TFF2 KO or NHE2 KO gastric organoids were microinjected with 9 nL of dPBS.

Image analysis

Damaged area (units of μm^2) was quantified from the time course of images as described previously (Xue *et al.* 2010; Xue *et al.* 2011; Aihara *et al.* 2013; Aihara *et al.* 2018) using ImageJ (NIH, Bethesda, MD, USA; RRID:SCR_003070) and/or Metamorph, version 6.3 (Molecular Devices, Sunnyvale, CA, USA; RRID:SCR_002368). The damaged area was measured as the region of cellular loss of YFP fluorescence in YC-Nano gastric organoids. In each experiment of YC-Nano gastric organoids, we determined the time point displaying maximal damage area and estimated rates of epithelial restitution starting from this time with a single exponential curve fit to the size of damage area over time (Xue *et al.* 2010; Aihara *et al.* 2018). Best fit values of the rate constant were used as estimates of the rate of repair (units of min^{-1}). Additionally, movement of nuclei of the damaged cell was traced and exfoliation (units of μm) was measured as the maximum distance of the dead cell nuclear movement at 20 min following photodamage. This time point was selected because it allowed for observation of delayed exfoliation after the addition of inhibitors. Changes in intracellular Ca^{2+} were measured as FRET/CFP ratio using YC-Nano gastric organoids. Background images were subtracted from FRET-YFP and CFP images, the resultant images were divided on a pixel-by-pixel basis to calculate the FRET/CFP ratio image. All time course ratio images were then normalized to the averaged pre-damage baseline images. Regions of interest were determined by transmitted light and 514 nm excited YFP images to define cellular structures for whole cell and lateral region measurements.

Statistical analysis

All values are reported as the mean ± SEM from '*n*' organoid experiments. Statistical significance was determined using an unpaired Student's *t* test or one-way ANOVA with Dunnett's multiple comparison *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Organoids as a model of gastric restitution

Our laboratory has previously used two-photon photodamage *in vivo* and *in vitro* as a method for targeting individual gastric cells (Xue *et al.* 2010; Xue *et al.* 2011; Aihara *et al.* 2013; Aihara *et al.* 2018) and optically monitoring repair by measuring cell exfoliation and/or the restoration of an intact epithelium caused by migration of neighbouring cells. Recently, we have introduced this approach to gastric organoids (Aihara *et al.* 2018). In YC-Nano gastric organoids, localized photodamage to part of a single cell nucleus (stained by Hoechst

33342) caused prompt loss of cytosolic YFP fluorescence (Fig. 1*A*). Over time, this damage area diminished as neighbouring cells migrated into the damaged region (Fig. 1 *A*). Consistent with our recent findings (Aihara *et al.* 2018), both exfoliation of damaged cell(s) and closure of damaged area are essential for complete repair (Aihara *et al.* 2018). As described in the Methods, these parallel events can be quantified by measuring the damage area size and/or the distance of the damaged cell nuclei movement (exfoliation) over time ($n = 7$) (Fig. 1*B*). Within ~10 min, the damage area repairs fully (rate of repair 0.42 ± 0.07 min^{-1}, $n = 7$) and maximal nucleus exfoliation is observed (exfoliation distance 9.17 ± 1.45 μm, $n = 7$). The results demonstrate that restitution of the gastric organoid is completed promptly following single cell damage and that multiple measurements can report the progression of this event.

Calcium is required for epithelial wound repair in gastric organoids

To assess intracellular Ca^{2+} mobilization during the epithelial repair process, gastric organoids were generated from transgenic YC-Nano mice that ubiquitously express a sensitive fluorescent Ca^{2+} reporter (Oshima *et al.* 2014), allowing the use of FRET to measure intracellular Ca^{2+}

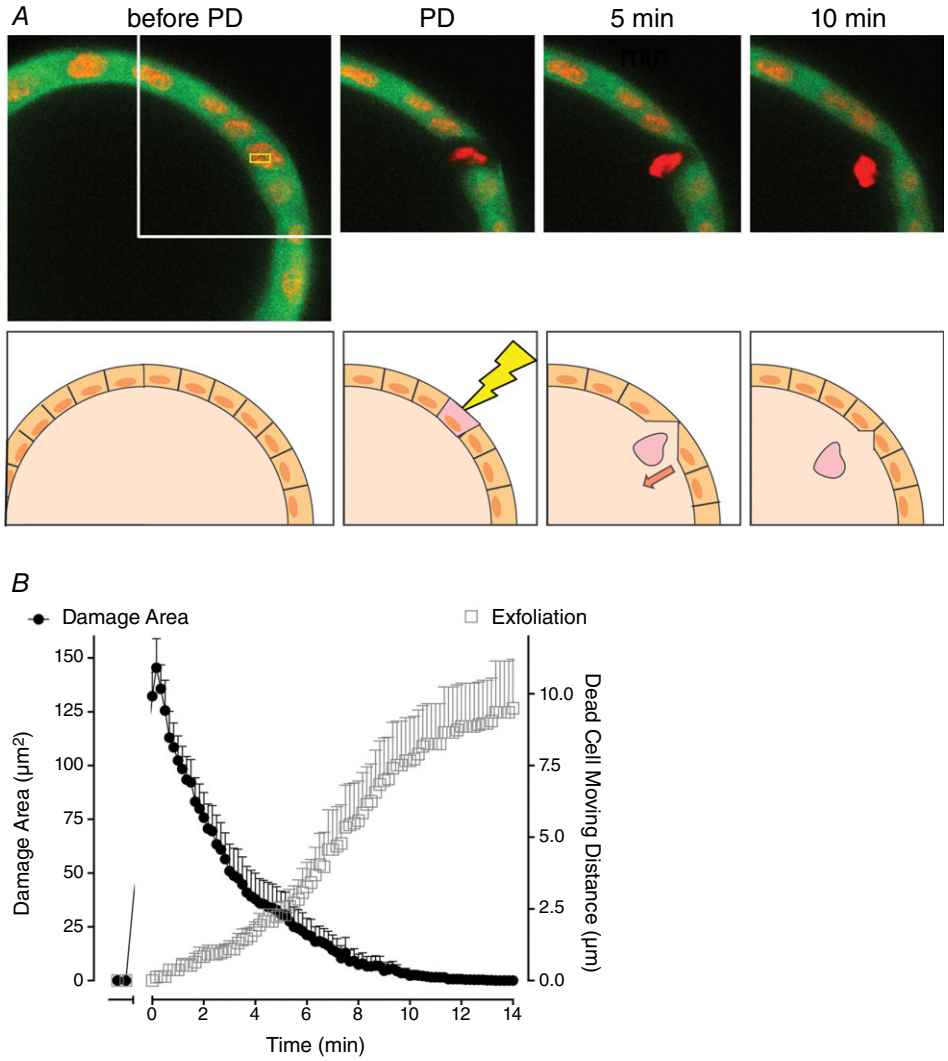

Figure 1. Assessment of repair in photodamage organoid model
A, top: series of confocal images of YC-Nano gastric organoid with Hoechst 33342 (red) stained nuclei and gastric organoid structure (YFP, green) before and up to 10 min following single cell photodamage (PD). Single cell PD occurs in the rectangular region (shown in yellow). Below: series of representative illustrations demonstrate measurements taken over time of the damage area and exfoliation of damaged nuclei. Following PD, the damaged cell exfoliates into the lumen coinciding with the closure of damaged area by neighbouring cells filling in the gap left by the departing cell. *B*, measurement of repair based on damage area (black) and exfoliation (grey) in YC-Nano gastric organoids following PD at $t = 0$ min ($n = 7$).

via ratiometric imaging. Based upon transmitted light and YFP images, cellular boundaries were determined and used for assessment of Ca^{2+} mobilization within intact cells (Fig. 2*A*). Upon photodamage to single cells in YC-Nano organoids (Fig. 1), cells adjacent to the damage site demonstrate Ca^{2+} mobilization as indicated by the FRET/CFP ratio (Fig. 2*B*, neighbour). Ca^{2+} mobilization peaked at 0.75 ± 0.30 min and dissipated by 4.4 ± 1.0 min ($n = 7$). As shown in Fig. 2*B*, maximal Ca^{2+} mobilization was greater within the cells directly neighbouring the damage site *vs.* cells one or two cell positions away from the damage site ($P < 0.05$). We also tested for subcellular heterogeneity of Ca^{2+} mobilization within the cells neighbouring the damage. In addition to the whole cell measurement shown in Fig. 2*B*, the subcellular lateral membrane region directly adjacent to the damage site was measured separately to assess changes in the FRET/CFP ratio (Fig. 2*C*). In cells neighbouring the damage, Ca^{2+}

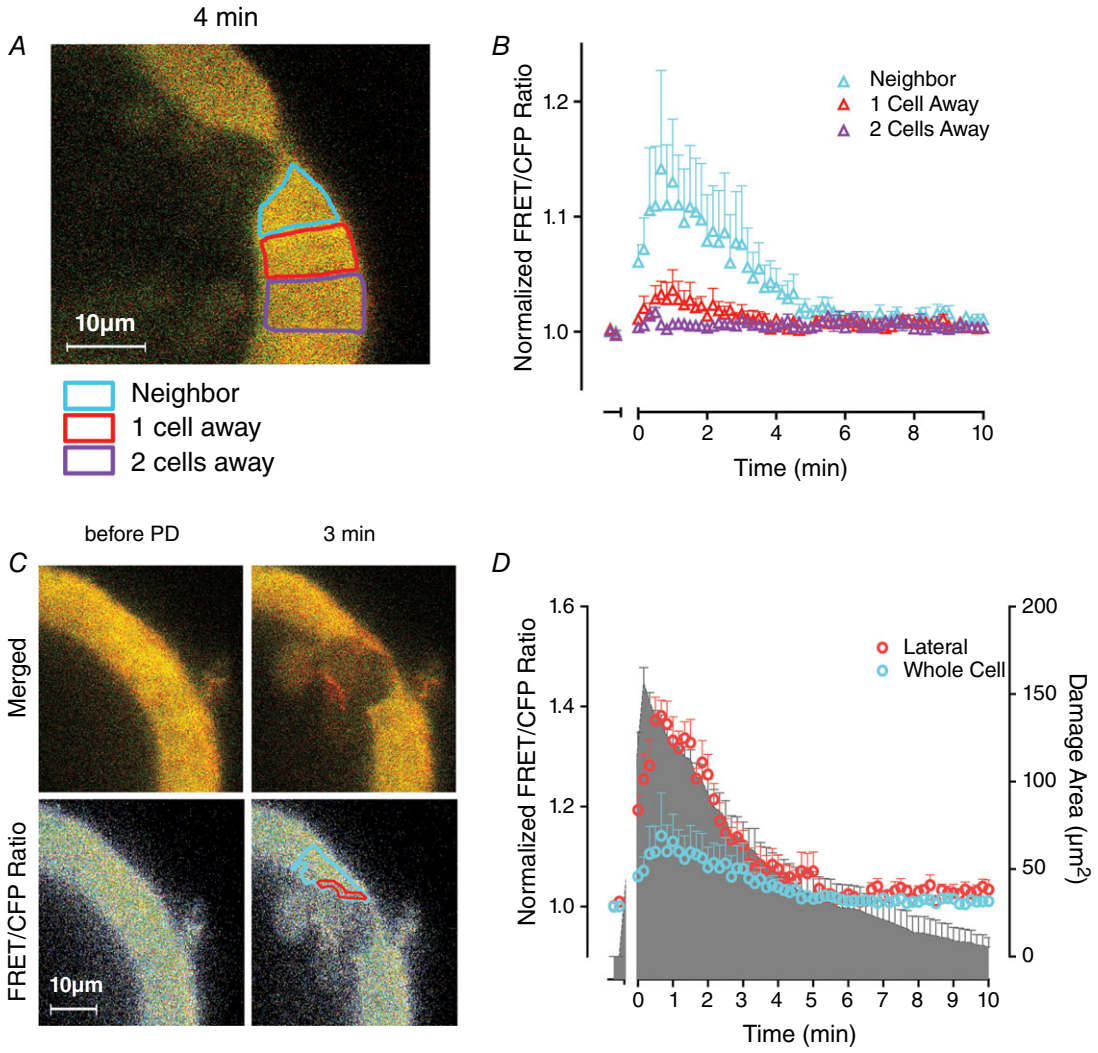

Figure 2. Comparison of intracellular calcium mobilization in cells near the site of damage
Fluorescence of YC-Nano gastric organoids imaged over time, before and after photodamage (PD). In time courses, PD occurred at $t = 0$ min. *A*, confocal FRET (red)/CFP (green) fluorescence merged image of YC-Nano gastric organoid at $t = 4$ min after PD. Representative colour outlines refer to the area used to measure whole cellular calcium levels in intact cells adjacent to damage (neighbour, blue), intact cells one cell space away from damage (1 cell away, red) and intact cells two cell spaces away from damage site (two cells away, purple). Both sides of the damage site were measured and averaged. *B*, time course measurement of the normalized FRET/CFP ratio from the three cellular regions indicated in (*A*) ($n = 4$). Cells adjacent to the damage site show the greatest calcium mobilization after damage. *C*, confocal FRET/CFP ratio fluorescence merged image and FRET/CFP ratio image of YC-Nano gastric organoid before and 3 min after PD. Representative colour outlines refer to the area used to measure intracellular calcium levels in lateral membrane region in intact cell adjacent to the damage site (red) and intact whole cell adjacent to damage site (blue). *D*, time course measurement of the normalized FRET/CFP ratio data from regions indicated in (*C*) ($n = 4$). The measurement of damage area (grey) is also shown to report time course of repair.

within the lateral membrane region mobilized with a similar time course as the whole cell (Fig. 2*D*). However, the lateral membrane region showed a significantly greater maximal FRET/CFP ratio change (1.43 ± 0.04, $n = 4$) *vs.* the whole cell measurement (1.18 ± 0.07, $n = 4$, $P < 0.05$). Therefore, the lateral membrane region was measured routinely as a more sensitive indicator of Ca^{2+} mobilization in all subsequent experiments.

To confirm the importance of intracellular Ca^{2+} mobilization in gastric restitution, BAPTA/AM was applied 30 min prior to photodamage to chelate intracellular Ca^{2+} (Fig. 3). BAPTA/AM significantly blocked repair (Fig. 3*A* and *B*); the damage area remaining at 10 min in BAPTA-treated organoids (76.27 ± 24.79 μm^2, $n = 4$) was significantly larger than in control organoids (2.3 ± 1.7 μm^2, $n = 4$, $P < 0.05$) and the corresponding repair rate of 0.11 ± 0.04 min^{-1} for BAPTA-treated organoids was significantly reduced compared to 0.36 ± 0.04 min^{-1} as observed in control gastric organoids (both $n = 4$, $P < 0.05$). The addition of BAPTA/AM significantly blunted Ca^{2+} signalling within cells adjacent to the damage site in YC-Nano organoids, where the control FRET/CFP ratio peak was 1.49 ± 0.04 compared to a FRET/CFP ratio peak of 1.14 ± 0.01 in the presence of BAPTA/AM (both $n = 4$, $P < 0.05$) (Fig. 3*C* and *D*). These data indicate that the FRET/CFP ratio measurements reflect intracellular Ca^{2+} levels because incubation with BAPTA/AM effectively

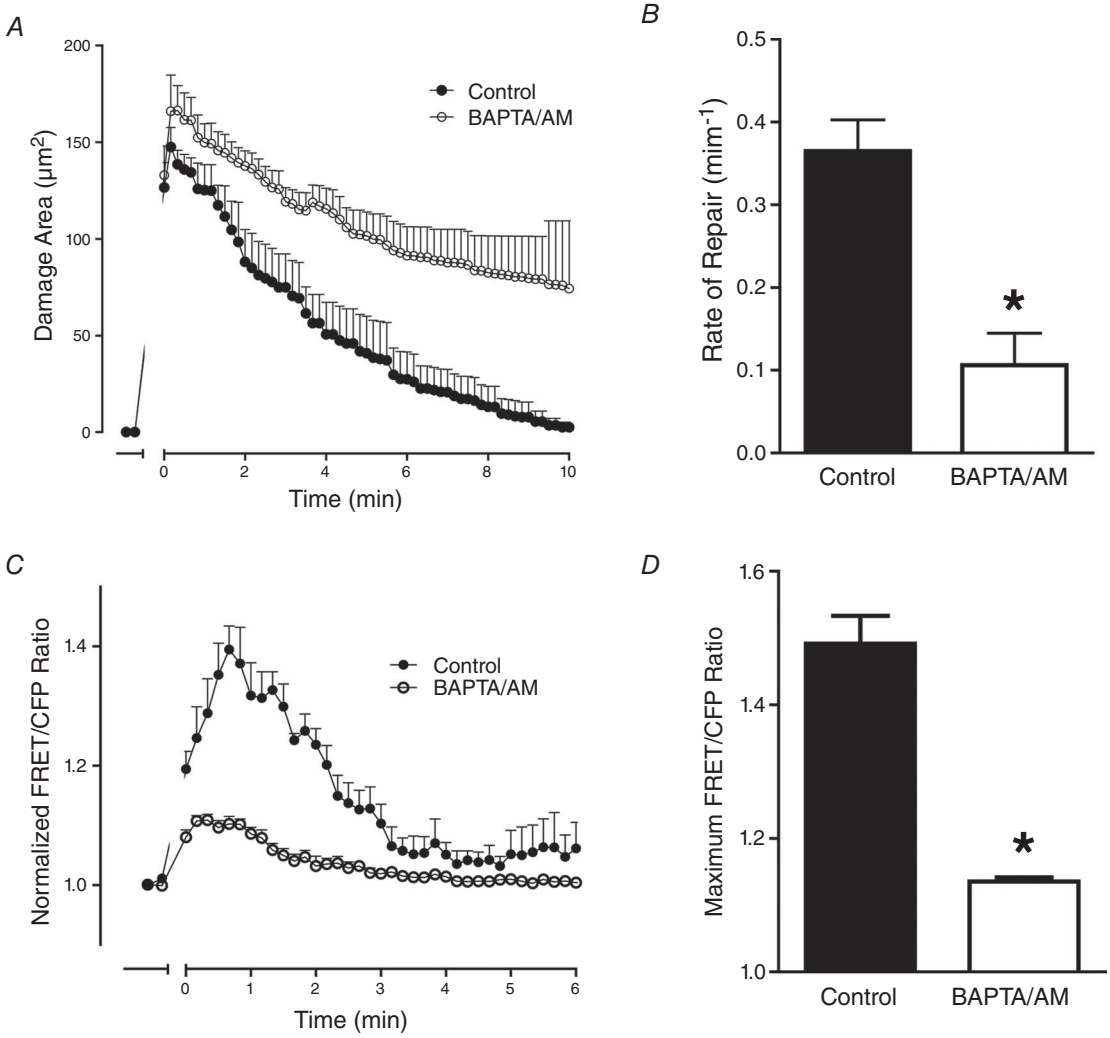

Figure 3. Effect of intracellular calcium chelation on repair and calcium mobilization
Fluorescence of YC-Nano gastric organoids imaged over time. BAPTA/AM (50 μM) was added to organoid medium 30 min prior to experimentation. In time courses, PD occurred at $t = 0$ min. *A*, damage area measured in control (black) and BAPTA/AM supplemented gastric organoids (white) over time ($n = 4$). *B*, comparison of the rate of repair between control (black) and BAPTA/AM supplemented gastric organoids (white) ($n = 4$, $^*P < 0.05$). *C*, measurement of the normalized FRET/CFP ratio of the lateral membrane region of cells adjacent to the damage site comparing control (black) and BAPTA/AM supplemented gastric organoids (white). *D*, comparison of the maximum FRET/CFP ratio from (*C*) between control (black) and BAPTA/AM (white) gastric organoids ($n = 4$, $^*P < 0.05$).

diminishes the mobilization of free Ca^{2+} after damage. These results further demonstrate that intracellular Ca^{2+} mobilization is necessary for repair within the gastric organoid model.

The TFF2 receptor CXCR4 acts upstream of Ca^{2+} mobilization and is involved in gastric restitution

Epithelial damage is known to elicit the release of TFF2, which acts via CXCR4 within the gastric epithelium (Xue *et al.* 2010) and in immune cells (Dubeykovskaya *et al.* 2009). To determine whether epithelial CXCR4 was involved in gastric organoid restitution, the CXCR4 inhibitor AMD3100 was added to YC-Nano gastric organoids. At 10 min, control gastric organoids exhibited a $5.8 \pm 3.9\ \mu m^2$ damage area and a repair rate of $0.41 \pm 0.05\ min^{-1}$, whereas organoids treated with 1 μM AMD3100 displayed a $56 \pm 18\ \mu m^2$ damage area and significantly delayed repair rate of $0.20 \pm 0.07\ min^{-1}$ ($n = 4$, $P < 0.05$) (Fig. 4*A* and *B*). A parallel examination of Ca^{2+} mobilization revealed that CXCR4 inhibition significantly blunted Ca^{2+} mobilization from 1.43 ± 0.04 FRET/CFP ratio peak in control to a 1.17 ± 0.03 FRET/CFP ratio peak in cells adjacent to the damage site ($n = 4$, $P < 0.05$) (Fig. 4*C* and *D*). The results indicated that CXCR signalling and CXCR4-mediated repair involve Ca^{2+} mobilization.

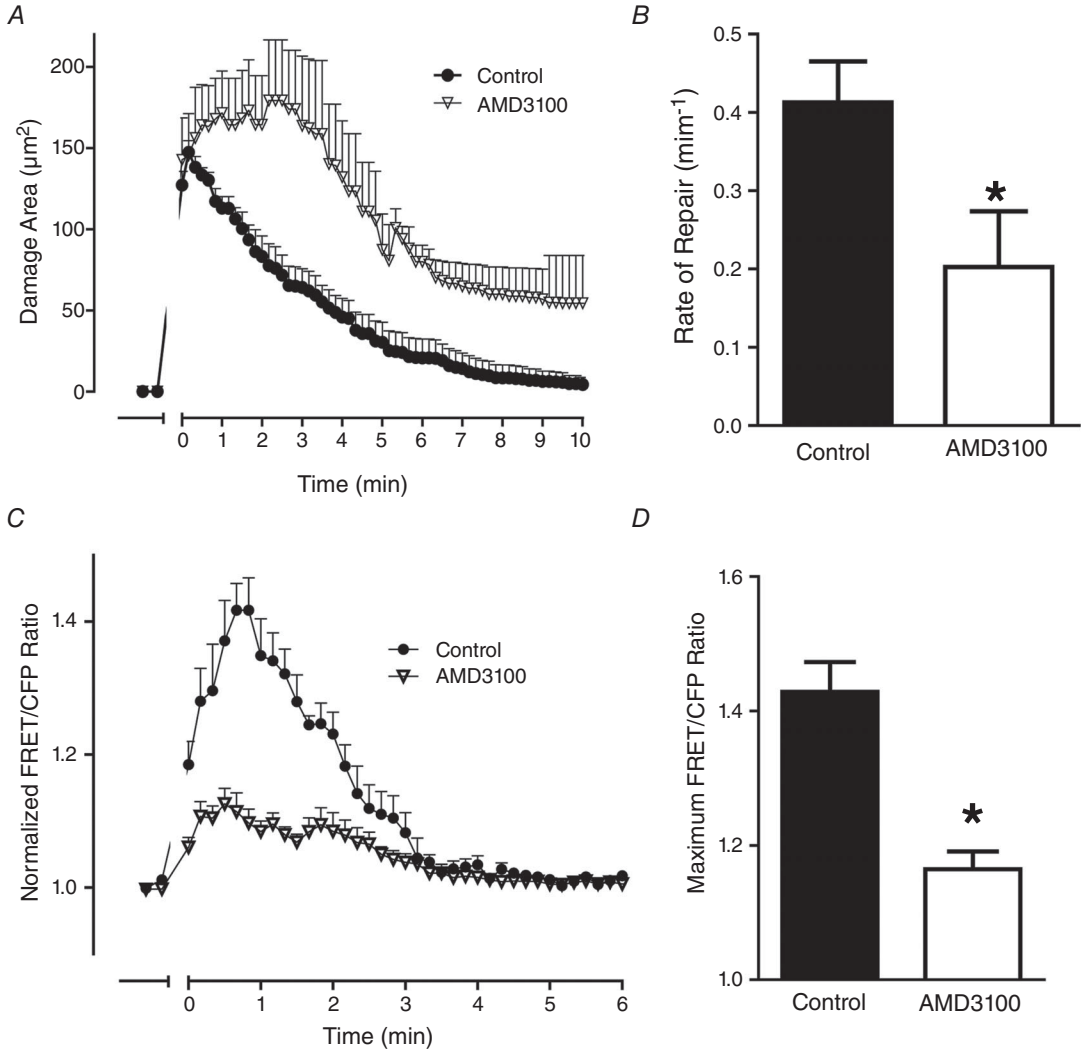

Figure 4. Effect of CXCR4 inhibition on repair and calcium mobilization
Fluorescence of YC-Nano gastric organoids imaged over time. Where indicated, AMD3100 (1 μM) was added to organoid medium 1 h prior to experimentation. In time courses, PD occurred at $t = 0$ min. *A*, damage area measured in control (black) and AMD3100 supplemented gastric organoids (white) ($n = 4$). *B*, comparison of the rate of repair between control (black) and AMD3100 supplemented gastric organoids (white) ($n = 4$, $^*P < 0.05$). *C*, measurement of the normalized FRET/CFP ratio of the lateral membrane region of cells adjacent to the damage site comparing control (black) and AMD3100 supplemented gastric organoids (white). *D*, comparison of the maximum FRET/CFP ratio from (*C*) between control (black) and AMD3100 (white) gastric organoids ($n = 4$, $^*P < 0.05$).

TFF2 action requires CXCR4 and calcium mobilization acting downstream during gastric restitution

Prior data show the close correlation of damage area and dead cell exfoliation as two independent measurements of gastric repair (Aihara *et al.* 2018). As a result of the lack of intrinsic fluorescence probes, only exfoliation was measured to assess repair in subsequent experiments using TFF2 WT and KO organoids. By contrast to WT organoids, which exhibited an exfoliation distance of 8.81 ± 0.70 μm ($n = 7$) at 20 min post-injury, exfoliation was significantly diminished in WT organoids treated with AMD3100 (1.33 ± 0.35 μm, $n = 6$) or BAPTA/AM (1.52 ± 0.24 μm, $n = 4$) ($P < 0.05$) (Fig. 5). Compared to WT, TFF2 KO organoids also exhibited a significant reduction of exfoliation at 2.11 ± 0.27 μm ($n = 10$, $P < 0.05$). Treatment of TFF2 KO organoids with AMD3100 (1.33 ± .35 μm, $n = 6$) or BAPTA/AM (1.31 ± 0.31 μm, $n = 4$) did not alter the already compromised exfoliation. However, the delayed exfoliation observed in TFF2 KO was rescued by microinjection of exogenous rTFF2 into the organoid lumen (8.55 ± 0.94 μm, $n = 10$, $P < 0.05$) (Fig. 5). This rescue was not significantly different regardless of whether exogenous rTFF2 was added to the organoid medium or microinjected (7.96 ± 0.68 μm, $n = 4$). As a result of limited availability of rTFF2, gastric organoids were microinjected in the present study. Exogenous rTFF2 was unable to rescue the exfoliation in the presence of AMD3100 (2.22 ± 0.29 μm, $n = 5$) (Fig. 5).

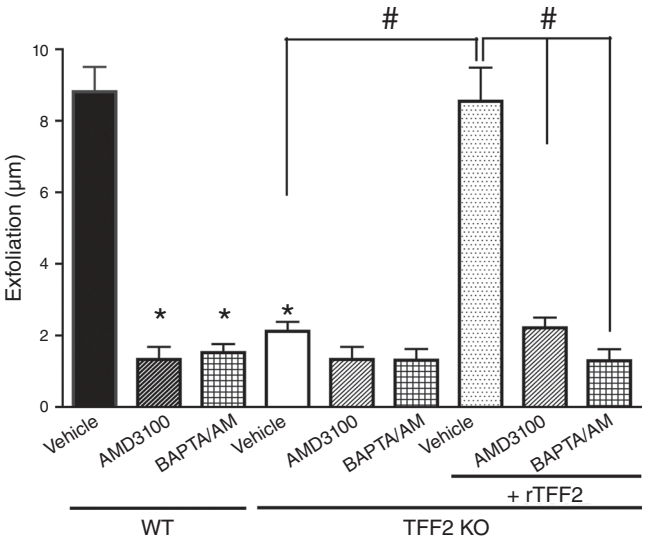

Figure 5. Comparison of exfoliation within WT and TFF2 KO organoids with and without treatments
Results from WT and TFF2 KO gastric organoids imaging over time, measuring the movement of fluorescent nuclei (Hoechst 33342 stain) after PD. PD occurred at $t = 0$ min. WT and TFF2 KO gastric organoids were treated with AMD3100 (1 μM) for 1 h or BAPTA/AM (50 μM) for 30 min before PD as indicated. rTFF2 was microinjected into the lumen of organoids 30 min before the study (see Methods). Exfoliation was determined based on the maximum distance of damaged nuclei into gastric organoid lumen over 20 min. Vehicle (WT control, $n = 7$; TFF2 KO control, $n = 10$; TFF2 + rTFF2 Control, $n = 10$); AMD3100 (WT, $n = 6$; TFF2 KO, $n = 6$; TFF2 KO + rTFF2, $n = 5$); BAPTA/AM (WT, $n = 4$; TFF2 KO, $n = 4$; TFF2 KO + rTFF2, $n = 8$). *$P < 0.05$ *vs.* WT vehicle, #$P < 0.05$ *vs.* rTFF2 treatment in TFF2 KO.

CXCR4 inhibition altered Ca^{2+} mobilization (as shown in Fig. 3) and separately prevented rTFF2 action during repair, although the link between the two outcomes is unclear. To directly test whether TFF2 action is dependent upon Ca^{2+} mobilization, BAPTA/AM was added to the media in the presence of gastric organoids microinjected with rTFF2. Incubation with BAPTA/AM prevented the rTFF2 rescue (1.30 ± 0.32 μm, $n = 8$, $P < 0.05$) (Fig. 5). These results indicate that TFF2 action requires Ca^{2+} mobilization to promote the repair process.

EGFR acts upstream of Ca^{2+} mobilization and is involved in gastric restitution

In renal, ovarian and colonic cancer cells, TFF2 and/or CXCR4 have been shown to interact with or activate EGFR (Rodrigues *et al.* 2003; Guo *et al.* 2007; Kosriwong *et al.* 2011). Because EGFR has been implicated separately in epithelial wound healing (Hansson *et al.* 1990), YC-Nano organoids were treated with an EGFR inhibitor (AG1478; 200 nM) to test the role of EGFR in gastric restitution (Fig. 6). At 10 min, compared to control gastric organoids that exhibited a damage area of 1.0 ± 0.5 μm^2 and a repair rate of 0.43 ± 0.05 min^{-1}, EGFR inhibition significantly delayed epithelial repair with a damage area of 42 ± 26 μm^2 ($P < 0.05$) and a repair rate of 0.25 ± 0.02 min^{-1} ($P < 0.05$ (Fig. 6*A* and *B*). Furthermore, EGFR blockage significantly blunted the maximal FRET/CFP ratio peak from 1.32 ± 0.02 in control to 1.04 ± 0.01 ($n = 4$, $P < 0.05$) (Fig. 6*C* and *D*). These results suggest that EGFR promotes Ca^{2+} mobilization and gastric restitution.

TFF2 KO organoids were then used to test whether the EGFR is a potential downstream effector of TFF2/CXCR4. The addition of AG1478 caused a significant delay in exfoliation (1.76 ± 0.41 μm *vs.* 8.18 ± 0.35 μm in control, $n = 6$, $P < 0.05$) (Fig. 7). However, in TFF2 KO organoids, the addition of AG1478 (1.56 ± 0.89 μm, $n = 5$) had no additive effect on exfoliation compared to vehicle (1.56 ± 0.31 μm, $n = 8$). Furthermore, the addition of rTFF2 significantly rescued exfoliation in TFF2 KO (8.87 ± 0.82 μm, $n = 8$, $P < 0.05$). However, in the presence of AG1478, rescue by rTFF2 was significantly prevented (2.30 ± 0.55 μm, $n = 5$, $P < 0.05$). Taken together, these results suggest that EGFR acts downstream of TFF2/CXCR4 in the repair pathway. Because both receptors are necessary to stimulate Ca^{2+} mobilization and promote gastric restitution, these data suggest that

both CXCR4 and EGFR may be acting via the same Ca^{2+} mobilizing signalling pathway during repair.

ERK1/2 is necessary for the repair process, acting upstream of calcium mobilization

CXCR4 and EGFR both act via ERK1/2 signalling in various systems (LI *et al.* 2003; Billadeau *et al.* 2006; Zimmerman *et al.* 2011). To test for a role of ERK1/2 within our organoid model, an ERK1/2 inhibitor (10 μM FR180204) was added to YC-Nano gastric organoids. By contrast to control gastric organoids that exhibited a fully repaired space of 0 μm^2 damage area at 10 min and a repair rate of 0.45 $\pm$ 0.04 min^{-1}, organoids treated with FR180204 displayed a 34.9 $\pm$ 2.6 μm^2 damage area at 10 min and a repair rate of 0.21 $\pm$ 0.03 min^{-1} (Fig. 8*A* and *B*) (both $n = 4$, $P < 0.05$). Examination of Ca^{2+} mobilization by FRET/CFP ratio in gastric organoids revealed that FR180204 dampened Ca^{2+} mobilization in

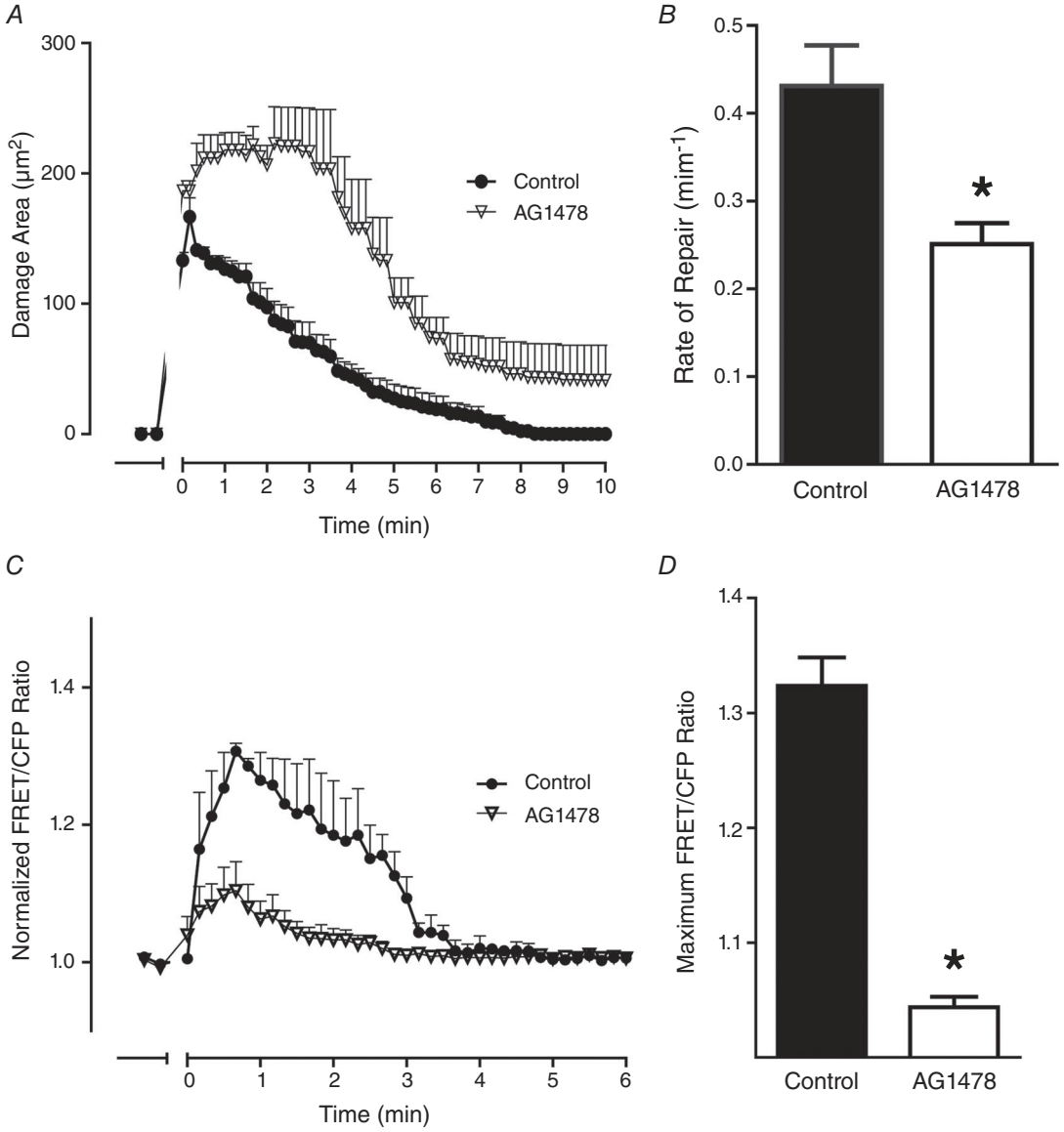

Figure 6. Effect of EGFR inhibition upon repair and calcium mobilization
Fluorescence of YC-Nano gastric organoids imaged over time. AG1478 (200 nM) was added to organoid medium 1 h prior to experimentation. In time courses, PD occurred at $t = 0$ min. *A*, damage area measured in control (black) and AG1478 supplemented gastric organoids (white) ($n = 4$). *B*, comparison of the rate of repair between control (black) and AG1478 supplemented gastric organoids (white) (*$P < 0.05$).*C*, measurement of the normalized FRET/CFP ratio of the lateral membrane region of cells adjacent to the damage site comparing control (black) and AG1478 supplemented gastric organoids (white). *D*, comparison of the maximum FRET/CFP ratio from (*C*) between control (black) and AG1478 supplemented gastric organoids (white) ($n = 4$, *$P < 0.05$).

the cell adjacent to the damage site (1.13 ± 0.02 compared to control 1.33 ± 0.03, both $n = 4$, $P < 0.05$) (Fig. 8*C* and *D*). These results indicate that ERK1/2 operates upstream of Ca^{2+} mobilization pathways during repair.

NHE2 acts downstream of calcium mobilization in TFF2-driven repair

NHE2 has been previously implicated as acting downstream of TFF2 action, in an unknown manner, to promote gastric repair *in vivo* (Xue *et al.* 2011). To determine whether NHE2 was necessary to repair within the *in vitro* gastric organoid model and investigate whether it affected Ca^{2+} mobilization, the selective NHE1/2 inhibitor Hoechst 694 (Hoe 694, 100 μM) was pre-incubated in YC-Nano gastric organoids prior to photodamage. At 10 min following damage Hoe 694 delayed epithelial repair, with a damage area of 32.03 ± 7.53 μm^2 and a repair rate of 0.28 ± 0.04 min^{-1} *vs.* a damage area of 3.50 ± 2 μm^2 and a repair rate of 0.49 ± 0.05 min^{-1} in control (Fig. 9*A* and *B*) (both $n = 4$, $P < 0.05$). Interestingly, Hoe 694 did not significantly alter the Ca^{2+} mobilization following damage(Fig. 9*C* and *D*) (control 1.37 ± 0.04 *vs.* Hoe 694 1.30 ± 0.02, both $n = 4$). These results show that, although NHE2 action is important for repair, it does not affect Ca^{2+} mobilization during the repair process, suggesting that NHE2 acts downstream of Ca^{2+} mobilization during repair.

Consistent with these findings, as well as prior *in vivo* work (Xue *et al.* 2011), NHE2 KO gastric organoids exhibited a significantly delayed exfoliation of 4.21 ± 0.66 μm compared to the WT gastric organoid exfoliation of 8.81 ± 0.70 μm (Fig. 9*E*) ($n = 5$, $P < 0.05$). To confirm that NHE2 acts downstream of TFF2 action during repair, as suggested by the previous *in vivo* studies (Xue *et al.* 2011), rTFF2 was microinjected into NHE2 KO organoids and monitored over time. Microinjection of rTFF2 into NHE2 KO organoids did not stimulate exfoliation (4.61 ± 0.41 μm, $n = 6$, $P < 0.05$) (Fig. 9*E*). To confirm that NHE2 is involved in TFF2-driven repair, TFF2 KO organoids were used to test whether Hoe 694 would affect rTFF2 rescue action (Fig. 9*F*). Similar to earlier results measuring damage area (Fig 9*A*), WT control exfoliation (9.66 ± 1.05 μm, $n = 5$) was significantly inhibited by the addition of Hoe 694 (1.90 ± 0.56 μm, $n = 5$, $P < 0.05$). Reduced exfoliation was again observed in TFF2 KO (1.26 ± 0.32 μm, $n = 6$) and the addition of Hoe 694 did not inhibit exfoliation further (1.33 ± 0.24 μm, $n = 4$). Microinjection of rTFF2 rescued exfoliation (9.0 ± 0.71 μm, $n = 4$); however, the presence of Hoe 694 prevented the rescue effect of rTFF2 (1.42 ± 0.23 μm, $n = 4$, $P < 0.05$). These data further support NHE2 being necessary for the repair process and as a probable downstream target of TFF2 action during repair.

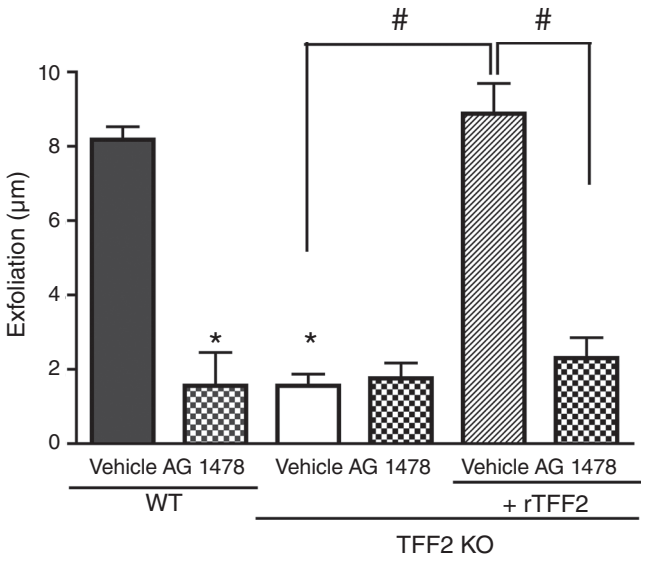

Figure 7. Comparison of exfoliation within EGFR inhibited WT and TFF2 KO organoids
Results from imaging of WT and TFF2 KO organoids over time; measuring the movement of fluorescent nuclei (Hoechst 33342 stain) after PD. Some organoids were treated with AG1478 (200 nM) as indicated. rTFF2 was microinjected into the lumen of organoids before the study. Exfoliation was determined based on the maximum distance of damaged nuclei into gastric organoid lumen over 20 min. Vehicle (WT control, $n = 6$; TFF2 KO control, $n = 8$; TFF2 KO + rTFF2, $n = 8$); AG1478 (WT, $n = 6$; TFF2 KO, $n = 5$; TFF2 KO + rTFF2, $n = 5$). *$P < 0.05$ *vs.* WT vehicle, #$P < 0.05$ *vs.* rTFF2 treatment in TFF2 KO.

Discussion

Restitution is the initiating event in epithelial repair and involves cell migration, not proliferation, for the rapid re-establishment of epithelial integrity following injury. In the present study, we focused upon gastric epithelial restitution in response to microscopic photodamage, using a reductionist gastric epithelium model comprising gastric organoids. This model allows us to investigate the innate epithelial response separate from the complexities of native tissue because the organoid system is devoid of other tissue cell types (immune cells, mesenchymal cells, smooth muscle, neurons, etc.).

The results of the present study provide a deeper validation regarding the gastric organoid model maintaining fidelity for major features of gastric restitution compared to the *in vivo* photodamage results (Xue *et al.* 2010; Xue *et al.* 2011; Demitrack *et al.* 2012; Aihara *et al.* 2013; Aihara & Montrose, 2014). We have recently demonstrated that the gastric organoid system is comparable to native tissue *in vivo* with respect to demonstrating the shedding of dead cells into the gastric

lumen with an epithelial repair time course of ∼10 min (Aihara *et al.* 2018). In the present study, gastric organoids are also found to be similar to native tissue because they demonstrate (i) increased intracellular Ca^{2+} mobilization during repair (Aihara *et al.* 2013); (ii) dependence on TFF2, CXCR4 and NHE2 for repair (Xue *et al.* 2010; Xue *et al.* 2011); and (iii) placement of NHE2 as the most downstream effector identified in the TFF2/CXCR4 repair pathway (Xue *et al.* 2011). These results identify features intrinsic to the epithelium, which operate in the absence of normal tissue architecture and accessory cell types. Furthermore, the present study delineates the relationship among known components of wound healing and links them within a signalling pathway, using an *in vitro* culture that more closely reflects native tissue. Through the gastric organoid system, we have been able to determine upstream and downstream effectors of gastric restitution, which had been previously difficult to achieve *in vivo*. The present study now identifies specific pathways that can be tested *in vivo* in future investigations.

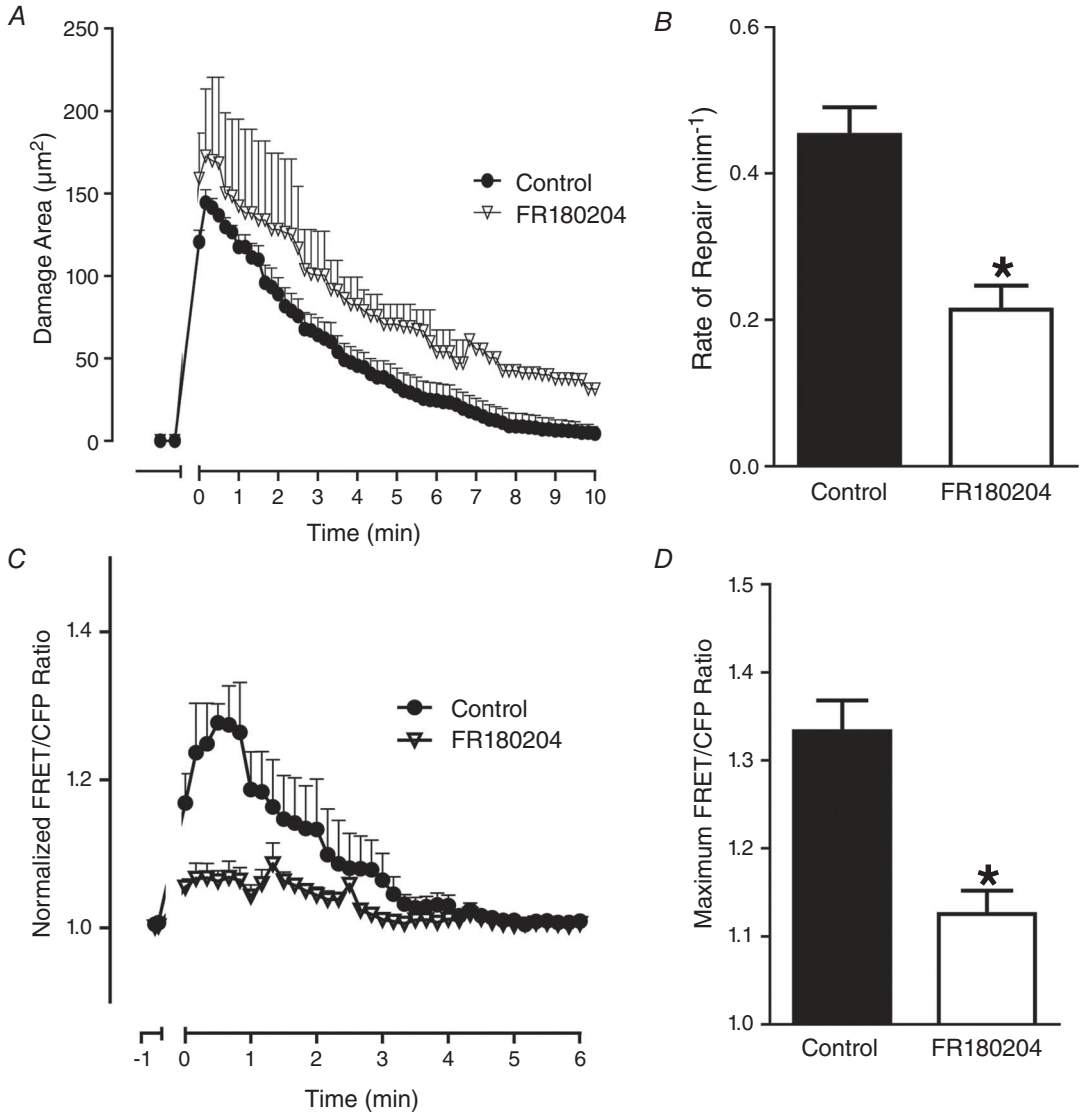

Figure 8. Effect of ERK1/2 inhibition on repair and calcium mobilization
Fluorescence of YC-Nano gastric organoids imaged over time. Where indicated, FR180204 (10 μM) was added to organoid medium 1 h prior to experimentation. In time courses, PD occurred at $t = 0$ min. *A*, damage area measured in control (black) and FR180204 supplemented gastric organoids (white) ($n = 4$). *B*, comparison of the rate of repair between control (black) and FR180204 supplemented gastric organoids (white) ($^*P < 0.05$). *C*, measurement of the normalized FRET/CFP ratio of the lateral membrane region of cells adjacent to the damage site comparing control (black) and FR180204 supplemented gastric organoids (white). *D*, comparison of the maximum FRET/CFP ratio from (*C*) between control (black) and FR180204 supplemented gastric organoids (white) ($n = 4$, $^*P < 0.05$).

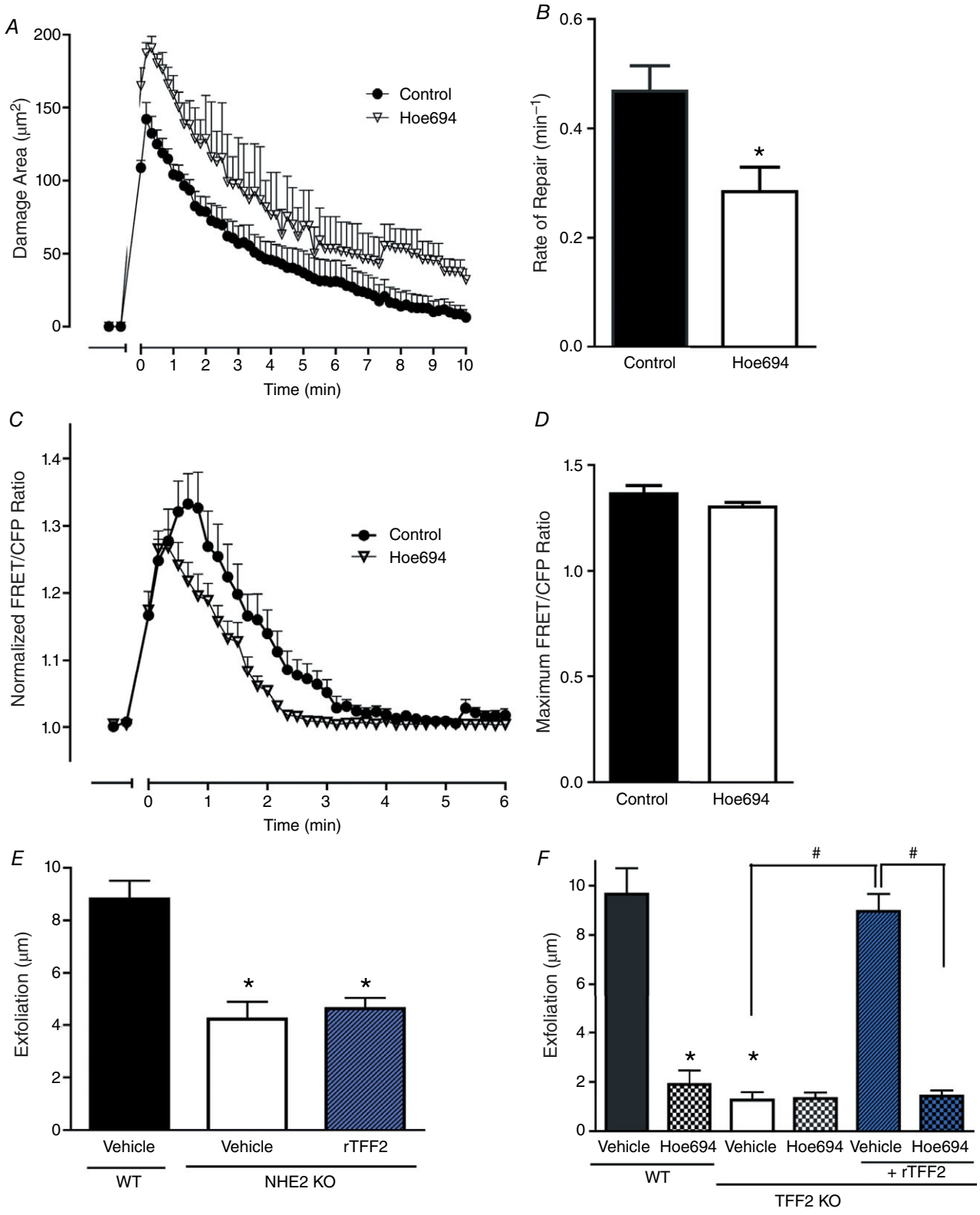

Figure 9. Effect of NHE1/2 inhibition and loss of NHE2 function on calcium mobilization and repair
Fluorescence of YC-Nano gastric organoids imaged over time in (*A*) to (*D*) and cell exfoliation measured over time in (*E*) to (*F*). Where indicated, Hoe 694 (100 μM) was added to organoid medium 1 h prior to experimentation. In time courses, PD occurred at $t = 0$ min. *A*, damage area measured in YC-Nano control (black) and Hoe 694 supplemented gastric organoids (white) ($n = 4$). *B*, comparison of rate of repair between YC-Nano control (black)

The gastric organoid system is reported to contain various cell types as seen *in vivo*, as well as to exhibit responses similar to infection and damage in native tissue (Schumacher *et al.* 2015*a*; Schumacher *et al.* 2015*b*; Aihara *et al.* 2018). Despite having a diversity of cell types within the organoid system, damage repair has not been shown to be a heterogenous response either in the present study or previous work (Aihara *et al.* 2018). Different cell types were not morphologically identifiable and no criteria were applied during the selection of cells to undergo damage, beyond their physical location in a site amenable to optical tracking of repair and cell extrusion. This observed homogeneity in the cellular response to damage suggests that this function is not dependent on cell type but, instead, comprises a highly conserved response to prevent the loss of epithelial barrier function.

The present study is the first to utilize the genetically encoded YC-Nano Ca^{2+} reporter in studies of gastric tissues. Previously, our laboratory used YC 3.0 transgenic mice to show that endogenous Ca^{2+} mobilization is required for proper wound repair *in vivo* (Aihara *et al.* 2013). The gastric organoid model, utilizing the more sensitive YC-Nano Ca^{2+} indicator, offers a significant improvement over *in vivo* techniques because Ca^{2+} levels in individual cells can be resolved using a greater dynamic range of FRET/CFP ratio change, and a brighter overall signal (data not shown). Using YC-Nano gastric organoids, we show that intracellular Ca^{2+} mobilization is a downstream event stimulated by TFF2, CXCR4 and EGFR activity during the repair process. Using the enhanced imaging resolution of organoids, we determined that Ca^{2+} mobilization was largely restricted to the cells directly adjacent to the wound site. Furthermore, within these cells, the lateral membrane region adjacent to damage was a proverbial hot spot of Ca^{2+} mobilization. Recently, we demonstrated that actin increases in the lateral membrane to initiate restitution and that this action requires calcium and CXCR4 (Aihara *et al.* 2018). We speculate that this subcellular region may be a localized area optimized to stimulate Ca^{2+}-dependent biochemical events, such as actin dynamics, in the part of the cell mediating cell motility. Although Ca^{2+} mobilization is demonstrated to be important in repair, further studies are necessary to understand the source of this raised cytosolic Ca^{2+}, as well as whether it is the direct result of Ca^{2+} released from intracellular stores and/or from activation of Ca^{2+} flux across the plasma membrane.

Several lines of evidence support a link between TFF2 and Ca^{2+} mobilization. In Jurkat cells, TFF2 activates Ca^{2+} signalling via the CXCR4 chemokine receptor (Dubeykovskaya *et al.* 2009). In colonic Caco-2 epithelial cells, activation of CXCR4 stimulated the release of intracellular Ca^{2+} and enhanced intestinal epithelial restitution via reorganization of the actin cytoskeleton (Agle *et al.* 2010). It has also been reported that gastric epithelial damage is associated with intracellular and extracellular Ca^{2+} mobilization *in vivo* and that this flux of Ca^{2+} is required to mediate tissue repair (Aihara *et al.* 2013).

Although it has been shown previously in separate studies (and distinct cell types) that TFF can promote Ca^{2+} mobilization or Ca^{2+} can modulate repair, the present study is the first to provide direct evidence that causally links and extends these observations. We show that Ca^{2+} mobilization is required for TFF2-mediated repair, which occurs via CXCR4. The key observations are that inhibition of CXCR4 impedes Ca^{2+} mobilization and slows repair in normal tissue, and CXCR4 is also required when exogenous rTFF2 is added to rescue repair in TFF2-KO cells. Normal repair, rTFF2-rescued repair and damage-induced Ca^{2+} mobilization can all be blocked by the Ca^{2+} chelator BAPTA/AM. This demonstrates the calcium dependence of the repair process and provides strong evidence indicating that Ca^{2+} mobilization is an essential downstream effector of TFF2/CXCR4 action during repair.

Evidence obtained outside of the GI tract, as well as studies with GI cancer cell lines, suggests that CXCR4 and EGFR may act via the same repair pathway, introducing the concept of CXCR4 activating EGFR during the repair process (Billadeau *et al.* 2006; Guo *et al.* 2007; Cheng *et al.* 2017). There is also evidence suggesting the ability of TFF2 to either directly or indirectly activate EGFR in colonic cancer cells during cell invasion (Rodrigues *et al.* 2003; Kosriwong *et al.* 2011). Furthermore, ERK is an integration point for multiple receptor-mediated pathways. There is also evidence obtained *in vitro* indicating that TFF2 treatment causes activation of ERK1/2 via the CXCR4 receptor in gastric cancer epithelial AGS cells and lymphocytic cancer Jurak cells (Dubeykovskaya *et al.* 2009), suggesting that TFF2 activation of CXCR4 mediates ERK signalling. Studies

and Hoe 694 supplemented gastric organoids (white) (*$P < 0.05$). *C*, measurement of the normalized FRET/CFP ratio of the lateral membrane region of cells adjacent to the damage site comparing control (black) and Hoe 694 supplemented gastric organoids (white). *D*, comparison of the maximum FRET/CFP ratio from (*C*) between control (black) and Hoe 694 supplemented gastric organoids (white) ($n = 4$, *$P < 0.05$). *E*, comparison of exfoliation in WT ($n = 5$) and NHE2 KO vehicle ($n = 5$) and rTFF2 injected organoids ($n = 6$) (*$P < 0.05$). *F*, comparison of exfoliation in WT and TFF2 KO gastric organoids treated with Hoe 694 and/or microinjection of rTFF2. Vehicle (WT Control, $n = 5$; TFF2 KO, $n = 6$; TFF2 KO + rTFF2, $n = 4$); Hoe 694 (WT, $n = 5$; TFF2 KO, $n = 4$; TFF2 KO + rTFF2, $n = 4$). *$P < 0.05$ *vs*. WT vehicle, #$P < 0.05$ *vs*. rTFF2 treatment in TFF2 KO.

in Caco2 cells show that ERK phosphorylation during repair is attenuated by EGFR inhibition, indicating that ERK phosphorylation is triggered via a pathway involving EGFR activation (Buffin-Meyer *et al.* 2007). Stimulation of EGFR and subsequent activation of ERK1/2 have been demonstrated to be present in healing gut mucosa (Hansson *et al.* 1990), although MEK/ERK signalling is not always essential for restitution (Frey *et al.* 2004), possibly as a result of to region- or tissue-specific effects. There is additional evidence that ERK1/2 activation is primarily responsible for TFF mediated initiation of healing. Yu *et al.* (2010) reported that TFF2 enhanced cell migration and wound healing in the gastric cell line AGS and rat small intestine cell line IEC-6 in an ERK1/2 activation-dependent manner.

Our data suggest that EGFR potentially acts downstream of CXCR4 and as a necessary component during TFF2-driven repair; however, further research is needed to determine whether this is by transactivation or whether EGFR acts independently of CXCR4. Furthermore, our results indicate that ERK1/2 activity is a necessary component for proper repair in the epithelium, although it has not been formally addressed as to whether phosphorylation of ERK1/2 in this cascade is the direct effect of either CXCR4 or EGFR activation. Our data show that ERK1/2 acts upstream of intracellular Ca^{2+} mobilization during the repair process. Evidence from previous studies and the current literature suggests that ERK1/2 may be the primary pathway of EGFR action during repair. Future studies are needed to confirm whether ERK is acting in the same pathway as TFF2 (or EGFR) during repair in the gastric epithelium.

Previously, our laboratory has shown that, *in vivo*, NHE2 is necessary during the repair process and probably acts downstream of TFF2 during repair (Xue *et al.* 2011). The results of the present study have extended these findings because the addition of exogenous rTFF2 to NHE2 KO organoids did not alter delayed repair, with NHE1/2 inhibition slowing the repair of normal organoids. EGF contribution to restitution has been shown to be mediated in part by stimulation of NHE in gastric epithelial cells (Yanaka *et al.* 2002). EGF is involved in acute regulation of cytoskeletal elements and NHE activity (Iwatsubo *et al.* 1989; Ghishan *et al.* 1992; Furukawa & Okabe, 1997; Furukawa *et al.* 1999) and the ERK pathway was also shown to be a critical component of NHE activation (Yoo *et al.* 2011; Muthusamy *et al.* 2012). We demonstrate that inhibition of NHE2 does not affect Ca^{2+} mobilization, suggesting that Ca^{2+} acts upstream of NHE2 or (less probable) that NHE2 action is regulated via a parallel Ca^{2+} independent pathway. The role of NHE2 in promoting repair remains unknown but, based upon the literature, we hypothesize that NHE2 may regulate actin polymerization during repair and a necessary component for cytoskeletal structural rearrangements during migration (Vexler *et al.* 1996; Denker & Barber, 2002).

Conclusions

The present study demonstrates that TFF2 acts via CXCR4 and EGFR signalling, including ERK activation, to drive Ca^{2+} mobilization and promote gastric repair. This work expands upon knowledge concerning the TFF2 signalling pathway (Dubeykovskaya *et al.* 2009; Xue *et al.* 2010) and points to TFF2 and its activation of CXCR4 and EGFR as potential targets for promoting restitution. Additionally, these studies validate gastric organoids as a platform for studying repair and identifying potential future therapeutic targets.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

KAE, EA and MHM were responsible for conceiving the study. KAE and EA were responsible for study methodology. KAE was responsible for formal analysis. KAE, EA, HH and ALM were responsible for conducting investigations. YO and MRF were responsible for study resources. KAE and MHM were responsible for writing the original draft. EA and MHM were responsible for supervision. KAE, EA and MHM were responsible for project administration. KAE, EA, MHM and MRF were responsible for acquisition of funding.

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