Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6542-0

Generation of *Lactococcus lactis* capable of coexpressing epidermal growth factor and trefoil factor to enhance in vitro wound healing

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Received: 9 December 2014 / Revised: 11 March 2015 / Accepted: 13 March 2015

Abstract Epidermal growth factor (EGF) and trefoil factor 3 (TFF3) are peptides that actively support the restitution and repair of mucosal epithelial barriers. Previous studies have shown that TFF3 enhanced EGF effect in wound healing, suggesting that the combined application of the two factors may be advantageous in clinical tissue repair. Expression of multiple proteins in a single host is a desirable approach in a biotechnological process, allowing to reduce cost and increase production efficiency. The aim of the present study was to study the feasibility of coexpressing EGF and TFF3 in food grade bacteria, *Lactococcus lactis* (*L. lactis*). Using an expression construct allowing simultaneous translation of two separate recombinant peptides, we generated a *L. lactis* that coexpressed and secreted EGF and TFF3 dually (LL-ET). Western blot analysis revealed that LL-ET secreted 45–54 % more total recombinant peptides (EGF+TFF3) per flask fermentation and 21–37 % more total recombinant proteins in bioreactor fermentation compared to their single factor expressing *L. lactis* counterparts (LL-EGF and LL-TFF3, respectively). The resulted recombinant EGF and TFF3 showed enhancement in wound healing activity in vitro. Our data suggest that the dual expression and secretion of EGF and TFF3 by *L. lactis* effectively accelerated cell migration, demonstrating potential future oral application of *L. lactis* fermentation product containing dual factors or a cocktail of factors to potentially treat intestinal damage and inflammation.

Keywords TFF · EGF · Wound healing · Lactic acid bacteria · Coexpression · Intestine

Introduction

Upon tissue injury and insult, a variety of growth factors have been shown to be requisite in both the protection and repair of mucosal epithelia integrity by stimulating cell proliferation and migration. Tissue repair after insult is a dynamic process with precise orchestration of three phases that overlap—inflammation, cell migration, and cell proliferation involving soluble mediators and the extracellular matrix (Singer and Clark 1999). In particular, epidermal growth factor (EGF) and trefoil factor (TFF) are known potent regulators of epithelial restitution, particularly, for their respective mitogen and motogen effects (Carpenter and Cohen 1990; Hoffmann 2005).

TFF3 is a member of the TFF family. These trefoil factors are small secretory peptide products of mucin-producing cells such as those in the gastrointestinal, respiratory, and urinary tracts (Hoffmann and Jagla 2002). TFFs share a distinctive three-leafed secondary structure, the TFF domain, formed by three intermolecular disulfide bonds making them stable in low pH and enzyme-rich environments of the gastrointestinal tract (Thim et al. 1995; Wright et al. 1997). TFF3 is highly localized in the goblet cells of the small and large intestine and has an important role in regulating cell migration and maintaining gastrointestinal mucosal integrity [reviewed in (Taupin and Podolsky 2003)]. Expression of TFF3 is upregulated at sites of injury and in chronic inflammatory diseases (Chinery and Playford 1995; Kjellev 2009; Wright et al. 1992), and its

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6542-0) contains supplementary material, which is available to authorized users.

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Published online: 02 April 2015

activity has been shown to promote the mobility of epithelial cells without enhancing proliferation in damaged mouse intestinal and human bronchial epithelium to re-establish epithelial continuity in vivo and in vitro (Oertel et al. 2001; Vandenbroucke et al. 2004).

EGF is one of the most well-studied therapeutic peptides for the treatment of damaged or ulcerated mucosa. EGF is found in various tissues (Kajikawa et al. 1991), and maternal milk is the main source of intestinal EGF during the postnatal stage (Jaeger et al. 1990). Systemic and oral administration of EGF to weaned monogastric animals can increase enterocyte proliferation and digestive enzyme activities (Jaeger et al. 1990; Xu et al. 2000). Supplementing liquid diets with EGF has also been shown to facilitate rotavirus intestinal infection recovery in piglets (Donovan et al. 1994). The complementary roles of EGF and TFF3 in wound healing suggest a potential application of both peptides to simultaneously facilitate intestine repair during inflammation such as in Crohn's disease, ulcerative colitis, necrotizing enterocolitis, and early weaning. In a food-animal production setting, disturbed digestive function, the associated reduction in productivity immediately after weaning is one of the major problems in nursery pig (*Sus scrofa*) management, and oral supplementation of EGF and TFF3 may stimulate early-weaned pig intestinal development to improve their growth performance.

Combined EGF and TFF3 treatment has been shown to synergistically enhance the rate of wound healing in vitro and reduce indomethacin-induced gastric damage in rats (Chinery and Playford 1995). Upon tissue damage, multiple or a "cocktail" of biofactors are released to facilitate repair of the wounded tissue. Numerous studies have shown that biofactor combinations can enhance cellular function beyond that achieved with individual growth factors (Greenhalgh et al. 1993; Lee et al. 1995), suggesting that growth factor combination should be an important consideration for the treatment of tissue injuries.

The production of EGF and TFF3 peptides using current recombinant techniques and purification methods is too costly for routine therapeutic use, especially in an animal production setting. Using *Lactococcus lactis* (*L. lactis*), a generally regarded as safe (GRAS) bacteria, to coexpress these two bioactive factors may allow us to bypass the costly need for extensive purification for oral application of the recombinant proteins that are commonly associated with recombinant protein biotechnological processing.

The objective of the current study was to dually express recombinant EGF and TFF3 at a biologically active concentration and to assess the activity of the resulting EGF and TFF3 in enhancing wound repair using an in vitro porcine epithelial restitution model.

Materials and methods

Ethics statement

The experimental use of animals and procedures followed were approved by the University of Guelph's Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines.

Generation of *L. lactis* strain expressing TFF3 and EGF

Total RNA was isolated from pig small intestinal tissue by using the Trizol (Life Technologies, Carlsbad CA) method according to the manufacturer's instruction. Reverse transcription and polymerase chain reactions were carried out by using forward (5′ CCAGCAGATCTGGGGAGTATGTGG-3′) and reverse (5′-CGTGGTCGAC**TCATCA**GAAGGTGCATTC-3′) primers to amplify the native porcine mature TFF3 sequence. Underlined letters indicated the addition of restriction enzyme sites *Bgl*II and *Sal*I in the forward and reverse primers, respectively. An additional stop codon was added to ensure efficient translation termination to the reverse primers indicated in bold letters. The following PCR protocol was applied: denaturation at 94 °C for 1 min and 35 cycles of three-step amplification including denaturation at 94 °C for 30 s, annealing at specified temperature at 53 °C for 30 s, extension at 72 °C for 50 s, final extension at 72 °C for 10 min. The resulting PCR TFF3 product (250 bp) was gel-purified using QIAEX Gel Extraction Kit (Qiagen, Germantown, MD) and ligated into pGEM-T Easy vector (Promega, Madison, WI) to generate pGEM-TFF3. After transforming into *Escherichia coli* DH5α (Life Technologies) and plasmid isolation, pGEM-TFF3 was digested with *Bgl*II and *Sal*I to release the TFF3 insert for subsequent cloning into the multiple cloning site of the *L. lactis* expression vector pAMJ399 (Bioneer, HØrsholm, Denmark) that was linearized with *Bgl*II and *Sal*I. The resulting construct is known as pAMJ399-TFF3 and transformed into electrocompetent *L. lactis* PSM565 (Bioneer), resulting in LL-TFF3. The original mature sequences of porcine TFF3 were codon-optimized to suit the codon preference bias of *L. lactis* generated by either DNA 2.0 (Menlo Park, CA) or by using PCR-based oligonucleotide assembly as described previously (Rydzanicz et al. 2005). The oligonucleotides were designed online using Johnson Lab (York University Canada) Assembly PCR Oligo Maker (http://startrek.ccs.yorku.ca/~pjohnson/AssemblyPCRoligomaker.html). The correctly assembled DNA molecule from PCR-based oligonucleotide assembly was inserted into the *L. lactis* expression vector pAMJ399, resulting in LL-TFFopt1 and verified by DNA sequencing. Optimized TFF3 sequence from DNA2.0 cloned into pAMJ399 was referred as pAMJ399-TFFopt2 (LL-TFFopt2 after *L. lactis* transformation). The pAMJ399 expression

construct containing the mature EGF pig-original codons (LL-EGF) was obtained from Cheung et al. (2009). Codon-optimized mature EGF sequence (DNA2.0) in the *L. lactis* pAMJ399 expression construct was obtained from Bedford et al. (2014) and is referred to as LL_oEGF. All resulting constructs contained a pH-regulated promoter (P170), a secretion signal peptide SP310mut2 and an erythromycin-resistance gene for selection. Each expression construct was transformed into *L. lactis* PSM565 via electroporation as previously described (Cheung et al. 2009). DNA sequencing verified the identity of each clone. The empty pAMJ399 vector without insert was transformed into *L. lactis* to serve as a negative control (LL-EV).

Generation of *L. lactis* coexpressing EGF and TFF3

The expression construct pAMJ399-TFFopt2, containing codon-optimized mature porcine TFF3 sequence, was used as a DNA template to amplify the construct composed of the internal ribosomal entry site (IRES; 5′-GAAAGGA-3′), secretion signal peptide sequence SP310mut2, and TFF3 sequence. Except the TFF3 sequence, all components are native to the pAMJ399 expression vector. PCR amplification was carried out using *Pfx* DNA polymerase (Invitrogen Life Technologies) according to manufacturer's instruction with 55 °C as the annealing temperature. Forward (5′-AGGATCTGCAGT CTAGATTAG-3′) and reverse (5′-CTAAGGATGATTTCTG GCAGGG-3′) primers were used to generate a 457-bp product, which was then ligated into pGEM-T Easy vector to generate pGEM-oTFFpst. The resulting plasmid was subsequently digested with *Pst*I to release a 317-bp product for cloning into pAMJ399-oEGF (Bedford et al. 2014) that has been digested with *Pst*I, which was downstream from the EGF-expressing cassette, to generate pAMJ399-ET. The pAMJ399-oEGF construct contains codon-optimized mature EGF sequence (DNA2.0). The resulting dual expression construct was transformed into *L. lactis* PSM565 via electroporation and DNA sequencing that confirmed the identity of the plasmid (LL-ET). The dual expression of EGF and TFF3 was controlled by the same promoter and transcription terminator (Fig. 2a).

Recombinant *L. lactis* flask fermentation

Recombinant *L. lactis* was streaked on M17 agar (Oxoid, Basingstoke, UK) supplemented with 1 % glucose and 1-μg/mL erythromycin. A single colony was inoculated into 10-mL M17 broth (Oxoid) supplemented with 1 % glucose and 1-μg/mL erythromycin (M17GE broth) and incubated at 30 °C overnight without shaking. The concentration of the cultures was measured by spectrophotometry (Fisher Scientific, Pittsburgh, PA) at 600 nm (OD_{600}), and the pH values were measured with a digital pH meter at different time-points from 0 to 48 h. Culture samples were centrifuged at 5000 rpm for 10 min at 4 °C, and both the supernatant and cell pellet were stored separately at −80 °C for analysis.

Recombinant *L. lactis* bioreactor fermentation

Recombinant *L. lactis* was fermented in M17GE broth for 48 h in a Winpact fermentation system (Montreal Biotech, Montreal, Canada) with 800-mL M17GE broth at 32 °C with continuous agitation (40 rpm). Each fermentation was inoculated with a 10-mL overnight preculture in M17GE broth. The culture pH was allowed to drop naturally to 5.8, after which it was maintained at pH 6.25 with 10-N NH_4OH and glucose-fed to maintain levels of 20-g/L glucose using 50 % glucose. Culture samples were centrifuged at 10,000 rpm for 10 min at 4 °C, and both the supernatant and cell pellet were stored separately at −80 °C for analysis.

SDS PAGE and Western blot analysis

Proteins in the bacterial supernatant and cell pellets were separated by 12 % SDS-PAGE and then transferred to PVDF membranes for 1 h at 100 V. Protein bands in SDS-PAGE gels were visualized with 0.5 % Coomassie blue G-250 (Fisher Scientific). The blots were blocked at room temperature for 1 h in 5 % skim milk phosphate-buffered saline (PBS) and then incubated with polyclonal rabbit primary antibody against pig EGF (1:1000 dilution; Biomatik, Cambridge, Canada) or pig TFF3 (1:1000 dilution; Biomatik) at room temperature for 1 h. Membranes were washed in PBS-0.2 % Tween 20 and then blotted in goat antirabbit IgG horseradish peroxidase-linked antibody (1: 1000; Cell Signaling Technology, Beverly, MA) at room temperature for 1 h. After washing, ECL Plus Western Blotting Detection System kit (Amersham, Piscataway, NJ) was used to detect EGF or TFF3 protein band according to manufacturer's instructions. Bands were quantified by densitometry using the public domain program ImageJ software (available at http://rsb.info.nih.gov/ij/). Recombinant protein secretion efficiency calculations were performed as reviewed by Le Loir et al. (2005). The ratio of the secreted and cytoplasmic proteins was determined for each recombinant *L. lactis* strain.

Indirect ELISA

EGF and TFF3 levels were measured by Express ELISA kit for rabbit primary antibodies (GenScript, Piscataway NJ) according to manufacturer's instructions. Recombinant human EGF (Sigma Chemical Co., St. Louis, MO) and TFF3 (Cedarlane Lab Ltd., Hornby ON) were used as standards for this indirect ELISA detection method (Supplementary Fig. S1a, b). Briefly, bacterial supernatant samples were diluted with autoclaved MilliQ water to 50 μl, and 50 μl of 2×

coating buffer was added to each sample to a final volume of 100 μl in Nunc-Immuno Maxi-Sorb 96-well plates (Nunc, Roskilde, Denmark). For the detection of EGF and TFF3, polyclonal rabbit primary antibody against pig EGF (1:1500) and pig TFF (1:1500) was used, respectively. Development of the substrate with TMB was carried out at room temperature for 10 min. The provided stop solution was added, and the absorbance was measured at 450 nm. Data were analyzed with Prism version 5.0 analysis software (Graphpad Software, La Jolla, CA) using the four-parameter fit algorithm.

In vitro migration assay

Using a scratch assay, in vitro wound modeling was performed as described by FitzGerald et al. and Storesund et al. (FitzGerald et al. 2004; Storesund et al. 2008). Briefly, confluent layers of IPEC-J2 cells (Schierack et al. 2006) grown in six-well plates (Corning, NY USA) in medium consisting of Dulbecco's modified Eagle's medium (DMEM) F-12 (Life Technologies) containing 5 % fetal bovine serum at 37 °C in 5 % CO_2 were serum starved for 24 h. The confluent layers were then scratched with a 200-μl plastic pipette tip to create a cell-free area. Wells were gently washed with PBS to remove loose cells. The cells were then incubated with serum-free DMEM F-12 in the presence of 10-μl filter-sterilized bacterial supernatant from overnight 24-h flask cultures from LL-oEGF, LL-oTFF3, LL-ET, and LL-EV. To ensure that the same regions are examined at each time point, the base of the plates was premarked to facilitate alignment. Each scratch field area was photographed (0 to 5 h), and the cell-free area was determined using ImageJ.

In vitro cell proliferation assay

IPEC-J2 cells were grown in six-well plates (Corning, NY USA) until 50 % confluent in medium consisting of DMEM F-12 containing 5 % fetal bovine serum at 37 °C in 5 % CO_2 and were then serum starved for 24 h. The cells were then incubated with serum-free DMEM F-12 in the presence of 10-μl filter-sterilized bacterial supernatant from overnight 24-h flask cultures from LL-oEGF, LL-oTFF3, LL-ET, and LL-EV cultured for 24 h. Cells were then trypsinized and enumerated using a hemocytometer.

Transwell assay

To analyze migration of IPEC-J2 cells, eight-micron pore sized millicell cell culture transwell inserts were used (Millipore Inc, Temecula, CA). A total of 1×10^5 cells were plated in the upper inserts and the lower chamber contained serum-free DMEM F-12 in the presence of 10-μl filter-sterilized bacterial supernatant from overnight 24-h flask cultures from LL-oEGF, LL-oTFF3, LL-ET, and LL-EV. After incubation for 16 h, the cells were fixed with 4 % (w/v) paraformaldehyde. Cells that did not migrate into the membrane were gently scraped off the upper surface of the transwell with a cotton swab. Migration was quantified by cell enumeration through Hoechst 33342 staining of cell nuclei (Life Technologies).

Folding of RNA structure

The UNAFold MFold© program with default values for parameters (T=37 °C) (Zuker 2003) was used to compute secondary structures from RNA sequences. For each RNA sequence, the free energy of the minimum free energy (most negative ΔG) was used as a measure for free energy change in folding. A low ΔG corresponds to a strongly folded RNA transcript, while a high ΔG corresponds to weaker folding.

GenBank accession numbers

The accession number for porcine mature TFF3 and EGF is NM_001243483 and NM_001243483, respectively; the accession number of the codon-optimized TFF3 sequence in LL-TFFopt1 is KP289205; the accession number of the codon-optimized TFF3 sequence in LL-TFFopt2 (also referred to as LL-oTFF3) is KP289204; the accession number of codon-optimized EGF (used in LL-oEGF and LL-ET) is KP314254.

Statistical analysis

Results are expressed as mean±standard error mean (SEM). The data were analyzed by two-factor analysis of variance (ANOVA) using Prism version 5.0 analysis software (Graphpad Software). Data sets were analyzed by Tukey's test for multiple comparisons to determine statistical differences between groups. The results were considered significant at a P value of <0.05.

Results

Generation of TFF3 expressing *L. lactis* and expression enhancement via codon optimization of native sequence

Since heterologous proteins are expressed outside of their original context in a foreign host, the genes may contain codons that are rarely used in the foreign host or contain regulatory elements such as GC-rich followed by AT-rich regions that could terminate transcription within their coding sequence, resulting in suboptimal recombinant protein yield. Modifying the gene to be expressed so that it reflects the host's frequent codon usage without affecting the final amino acid sequence is a common strategy to improve heterologous

protein expression (Gustafsson et al. 2004). In general, there are two common strategies used to optimize genes for expression. The codon adaptation index (CAI) is a classically used method for analyzing codon usage bias as it measures the deviation of a given protein-coding gene sequence with respect to a reference set of genes in the expression host of interest (Sharp and Li 1987). Based on the index, codons in the gene sequence can be replaced with the most common synonymous codon (high CAI) for each amino acid. The other strategy combines codon usage bias with the use of complex algorithms that take a variety of critical factors involved in different stages of protein expression into account. In this second strategy, systemic analysis of sequence variables that can affect heterologous expression of recombinant proteins such as translation initiation regions, mRNA structural elements, and expression-limiting regulatory elements within coding sequence is assessed and modified to further enhance recombinant expression (Angov 2011; Gustafsson et al. 2004; Tessier et al. 1984). We optimized the porcine TFF3 codons using both strategies to compare the resulting recombinant expression level of TFF3. In the first construct, the original codons were replaced with highly used codons by *L. lactis,* and the resulting synthetic gene (LL-TFFopt1) was produced by PCR assembly (Gupta et al. 2004; Rydzanicz et al. 2005). In the second construct, TFF3 was codon optimized by DNA2.0 (LL-TFFopt2) using their proprietary GeneGPS™ algorithm, basing the second strategy described above. As shown in Fig. 1a, c, codon optimization increased recombinant TFF3 expression in *L. lactis*. The expression of TFF3 with pig original codons (LL-TFF3) was 1.2±1.3 ng/μl in flask cultures. Codon optimization in LL-TFFopt1 and LL-TFFopt2 resulted in an enhancement of recombinant TFF3 expression at 6.5±0.6 ng/μl and 7.3±0.7 ng/μl, respectively in flask cultures. No difference was observed in the growth curve of recombinant *L. lactis* clones with original or optimized codons. Thus, codon optimization using the two strategies, respectively, resulted in a ~5 fold increase of TFF3 secretion *by L. lactis*, and the efficacy difference between the two optimization methods was insignificant. A positive *L. lactis* clone, LL-TFFopt2, was used for downstream cell culture studies, hereon referred as LL-oTFF3. Codon optimization also resulted in a ~3-fold increase in expression level of EGF from 2.0±0.7 ng/μl (LL-EGF) to 5.9±0.4 ng/μl (LL-oEGF) using DNA2.0 GeneGPS™algorithm (Fig. 1b, d). EGF produced by LL-oEGF was used for subsequent cell culture studies.

Folding free energies of optimized and nonoptimized TFF3 and EGF mRNA transcripts

To investigate if the increased expression is corresponding to free energy changes for folding (ΔG), which is associated with the secondary structure in RNA, UNAFold MFold © software was used. Strongly folded secondary structure is associated with lower ΔG. Using the full-length sequence of the mature mRNA, the ΔG was −73.3±1.2 kcal/mole for nonoptimized (pig-original) codons (LL-TFF3). Replacing the original codons with *L. lactis* highly used codons (LL-TFFopt1) resulted in increased ΔG at −36.6±0.8 kcal/mole, and the second optimized construct, LL-TFFopt2, also had

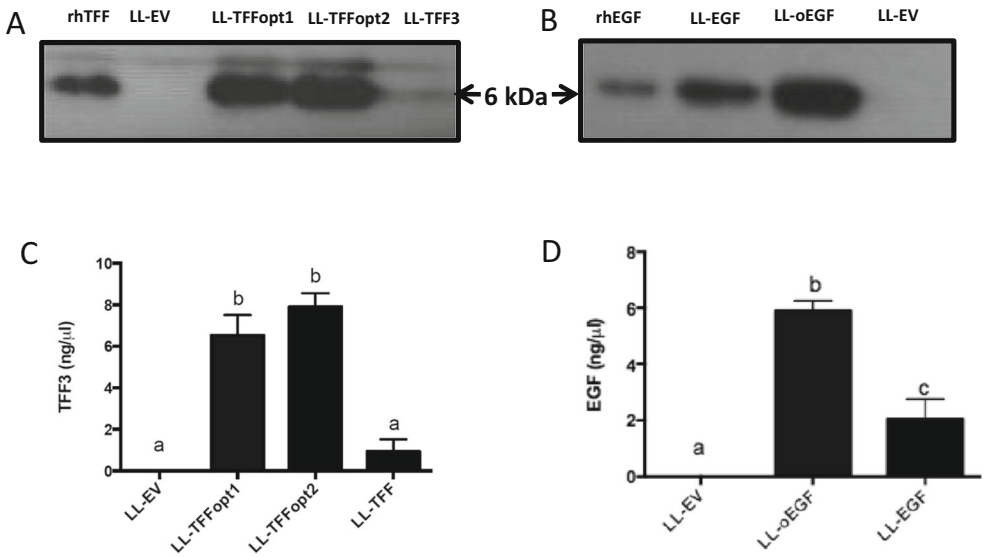

Fig. 1 Codon optimization enhances TFF3 and EGF expression. In-house codon-optimized TFF3 and EGF gene (LL-TFFopt1 and LL-oEGF, respectively), outsourced optimized TFF3 (LL-TFFopt2), and nonoptimized TFF3 and EGF (LL-TFF3 and LL-EGF, respectively) were expressed by *L. lactis* in 24-h flask fermentations. Resulting recombinant TFF3 and EGF were detected by Western blot (**a** and **b**, respectively) and indirect ELISA (**c** and **d**, respectively). *L. lactis* PSM565 with empty vector served as a negative control (LL-EV), and recombinant human TFF3 (rhTFF) served as a positive control. Data represent the mean± SEM of three independent experiments. *Letter subscripts* denote significant difference between groups ($p<0.05$)

increased ΔG at −47.8±1.0 kcal/mole compared to the nonoptimized construct. Replacing the EGF original codons (LL-EGF) with optimized codons (LL-oEGF) also results in a significant increase in ΔG from −50.2±0.7 kcal/mole to −40.6±0.8 kcal/mole.

Generation of *L. lactis* coexpressing EGF and TFF3

In order to generate an *L. lactis* that is capable of dually producing and secreting EGF and TFF3, we generated an expression construct to coexpress the swine-derived EGF and TFF3 gene under the control of the pH-inducible promoter, P170 (Fig. 2a). An internal ribosome entry site coding sequence was placed between the bacterium-preferred cDNA sequence of codon-optimized porcine EGF and TFF3, allowing simultaneous translation of EGF and TFF3. *L. lactis* growth peaked at 24 h, and no significant differences were observed in the growth profile of recombinant *L. lactis* clones expressing single (LL-oEGF and LL-oTFF3) or dual factors (LL-ET) in both flask and bioreactor fermentation systems (Fig. 2b). Western blot analysis revealed that both EGF (Fig. 2c) and TFF3 (Fig. 2d) were produced and secreted by the EGF-TFF3 dual-expressing *L. lactis* (LL-ET). As controls, we also generated single factor expressing *L. lactis*. The secretion of EGF and TFF3 by single factor expressing *L. lactis* LL-oEGF, LL-oTFF3, and dual factor expressing LL-ET, respectively, is shown in Fig. 2c, d. An empty expression vector transformed *L. lactis* control was also generated and served as a negative control (LL-EV). As shown in Fig. 2e, in small-scale flask fermentations, the level of recombinant EGF from LL-ET and LL-oEGF secretion was observed at 7.4 ±1.3 ng/μl and 8.7±0.9 ng/μl, respectively. They did not differ statistically. Recombinant TFF3 from LL-ET and

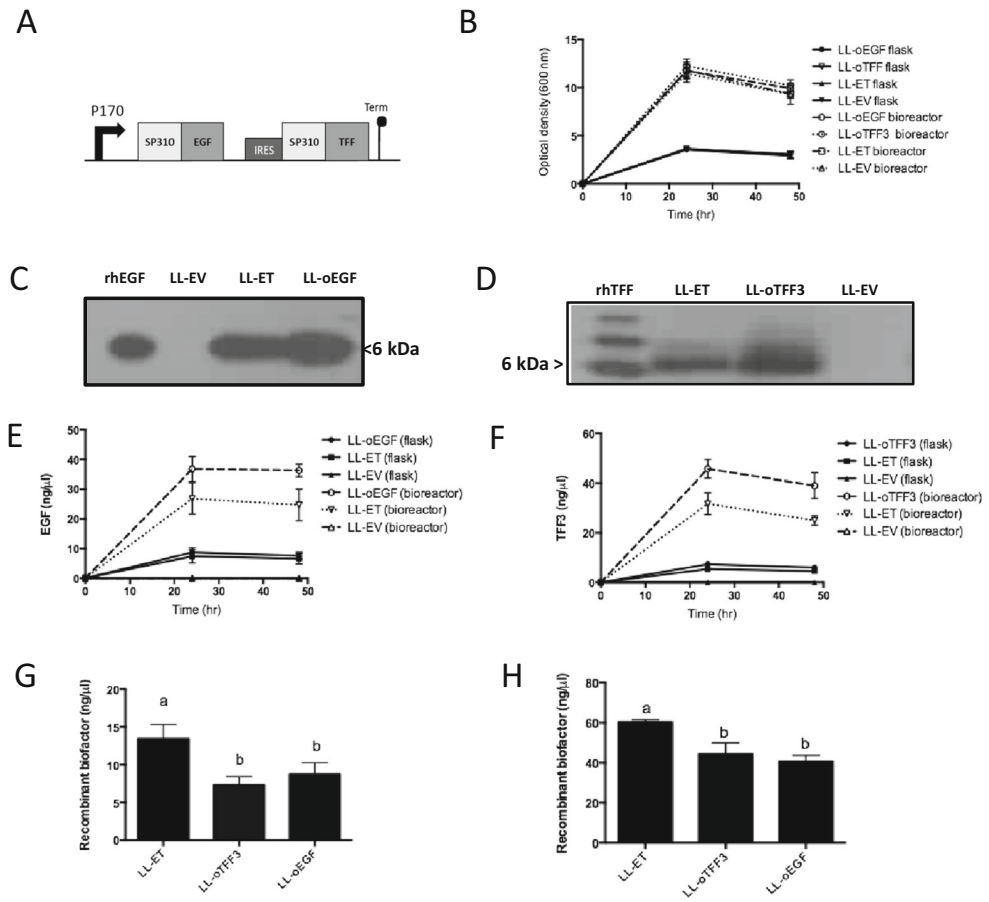

Fig. 2 Coexpression of recombinant TFF3 and EGF by *L. lactis* containing dual-expression constructs. TFF3 and EGF were successfully coexpressed and secreted into the medium by *L. lactis* after 24-h fermentation (**a**). Schematic representation of the dual-expression *L. lactis* plasmid construct. P170, a pH-inducible promoter; IRES, internal ribosomal-binding site; SP310, a secretion signal of SP310mut2 protein; Term, transcription terminator. EGF and TFF are the genes encoded, respectively. Growth optical density time profile during flask and bioreactor fermentations (**b**). Western blot detection of *L. lactis* supernatant containing EGF (**c**) and TFF3 (**d**) from bioreactor fermentations. Time profile quantification of EGF (**e**) and TFF3 (**f**) concentration in flask and bioreactor fermentations by indirect ELISA. Comparison of overall functional protein concentration in *L. lactis* flask (**g**) and bioreactor (**h**) fermentations expressing either single or both EGF and TFF recombinant proteins. *Bars* represent the mean±SEM of three independent experiments. *Different lowercase letters* denote significant difference between groups ($p<0.05$)

LL-oTFF3 at 24-h fermentation was secreted at 5.3±0.37 ng/μl and 7.3±0.63 ng/μl ($P<0.05$, Fig. 2e, right panel), respectively. Recombinant protein expression peaked at 24 h in bioreactor fermentations, where recombinant EGF from LL-ET and LL-oEGF group was secreted at 26.8±3.0 ng/μl and 36±2.4 ng/μl ($P<0.05$, Fig. 2e), respectively. Recombinant TFF3 from LL-ET and LL-oTFF3 was secreted at 31.7±2.4 ng/μl and 45.7±2.2 ng/μl ($P<0.01$, Fig. 2f). Despite the decrease in TFF3 and EGF level in the LL-ET group, the dual factor expressing *L. lactis* produced and secreted 45–54 % more total recombinant proteins (EGF+TFF3) than their single factor expressing *L. lactis* counterpart per flask fermentation (12.7 ng/μl vs. 8.7 or 7.3 ng/μl; $P<0.05$) and 21–37 % more total protein in bioreactor fermentation (58.6 ng/μl vs. 36.7 or 45.7 ng/μl ($P<0.05$, Fig. 2g, h). To examine if the recombinant protein, secretion efficiency was affected due to increased heavy loading of the secretion pathway in the dual-expression system. Western blot was performed to reveal the secreted- and intracellular-recombinant protein concentrations (Fig. 3a, b). Densitometry analysis of the Western blot results showed that EGF was secreted ~17 % higher in LL-oEGF than in LL-ET group, while the secretion efficiency for TFF3 is ~3 % higher in LL-oTFF than in LL-ET groups. To investigate the possibility of increased stress in *L. lactis* during protein coexpression that could lead to cell lysis, contamination of cellular proteins in the culture supernatant was examined. Using *L. lactis* cell lysates as a protein control, no obvious bands were detected in the culture supernatant in either dual-, or single-recombinant protein expression (Fig. 3c). This suggested that the higher content of secreted target recombinant proteins in the dual expression group may not be due to cell lysis.

EGF and TFF3 protein coexpressed by *L. lactis* enhanced wound closure in vitro

To confirm that the recombinant EGF and TFF3 are functional, an in vitro wounding scratch assay was performed. After wound induction (Fig. 4a; 0 h), cells were then cultured with the addition of 10 μl of supernatant from LL-EV, LL-oEGF, LL-oTFF3, or LL-ET from flask fermentations. Cells treated with supernatant from LL-EV had no EGF or TFF3. ELISA analysis revealed that groups treated with this volume of LL-oEGF or LL-ET supernatant had EGF final media concentration of 30 ng/mL and 25 ng/mL, respectively. Groups treated with LL-oTFF3 or LL-ET had TFF3 final concentration of 24 ng/mL and 18 ng/mL, respectively. As shown in Fig. 4a, partial wound closure was observed in wounded culture in the presence of LL-oEGF or LL-oTFF3 at 5 h, respectively. This repair appears more completed when cultured with LL-ET supernatant, which contains both EGF and TFF3 (Fig. 4a). Quantitative analysis revealed that cells treated with both EGF and TFF3 (LL-ET) resulted in 92±5.8 % wound closure at 5 h, significantly greater than single LL-oEGF or LL-oTFF3-treated cells and the negative control LL-EV with wound closure at 76±7.2 %, 74±10.3 %, and 45±9.4 %, respectively ($P<0.05$, Fig. 4b). TFF3-only (LL-oTFF3) and EGF-only (LL-oEGF) enhanced wound closure compared to LL-EV, but they are not significantly different from each other. To study if the increased wound healing is due to cell migration, proliferation or both transwell cell migration study was performed using IPEC-J2 cells, a cell line derived from porcine intestine epithelium. As shown in Fig. 5a, cell migration in the groups treated with LL-oEGF, LL-oTFF3, and LL-ET was enhanced. Migrated cell count analysis revealed that

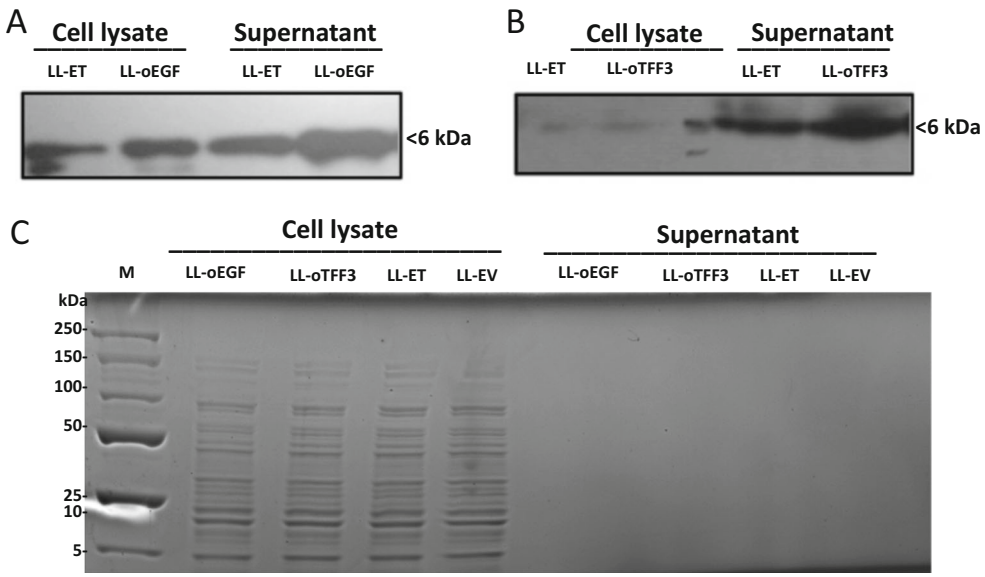

Fig. 3 Cellular proteins were not detected in the culture supernatant in single- and dual-factor expressing *L. lactis*. Western blot detection of recombinant EGF (**a**) and TFF3 (**b**) in cytoplasmic fractions (cell lysate) and culture supernatant. Representative image of Coomassie stained SDS-PAGE to detect proteins in cytoplasmic fractions and supernatant (**c**)

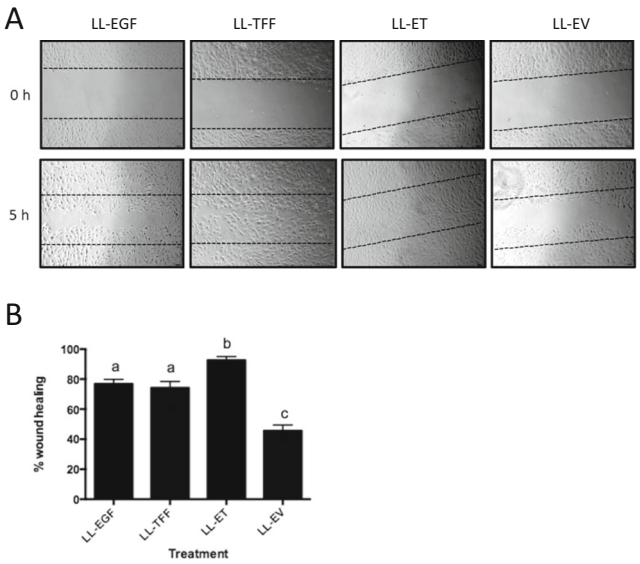

Fig. 4 *L. lactis* supernatant EGF and TFF enhance cell restitution in an in vitro wound healing model. **a** Scratched cell monolayer images at 0 h and 5 h during incubation with *L. lactis* flask-fermented supernatant from LL-EV (no EGF or TFF3), LL-oEGF, LL-oTFF3, and LL-ET (EGF and TFF3). **b** Data are expressed as percentage of wound closure at 5 h. *Bars* represent the mean±SEM of three experiments. *Different lowercase letters* denote significant difference between groups ($p<0.05$)

there was a 2.3-fold (LL-oEGF), 2.4-fold (LL-oTFF3), and 3.5-fold (LL-ET) significant increase in cell migration when compared to the LL-EV negative control ($P< 0.05$, Fig. 5b). To study if the wound closure is associated with increase in cell growth, cell-counting proliferation assays were performed. As shown in Fig. 5c, the proliferation of cells was significantly stimulated by the supernatant of LL-oEGF and LL-ET cultures ($P<0.05$), when compared to the negative control LL-EV group. Treatment with LL-oTFF3, however, resulted in no change in cell proliferation.

Discussion

Simultaneous coexpression can reduce the amount of media and resources required to produce recombinant biologically active protein. However, it is generally accepted that transformants containing two or more plasmids with the same replication of origin may not be stably maintained for long-term (Austin and Nordstrom 1990). This may result in inconsistent expression of each respective recombinant protein. It is thus desirable to use a single plasmid to express two recombinant proteins, allowing the coexpression of recombinant proteins to occur at a consistent and controlled level. In addition, coexpression of both proteins from the same vector will require only one antibiotic-resistant marker for stable maintenance of the plasmid in the cell. The use of two separate plasmids will require compatible replicons and different antibiotic-resistant markers for stable maintenance of both plasmids. Exposure to multiple antibiotics can increase cellular stress level and energy requirement for the bacterium to confer resistance. As a result, reduced target protein yield has been observed in cells harboring multiple plasmids (Glück et al. 2010). Furthermore, when stress responses are induced, cellular activity can shift to readjust metabolic fluxes and enzyme composition, which can decrease rates of transcription and translation, thereby decreasing target protein yield (Hoffmann and Rinas 2004). At the protein level, induction of cellular stress response can also lead to protein misfolding, and recombinant proteins that fail to reach correct conformation can undergo proteolytic degradation or form inclusion bodies (Baneyx and Mujacic 2004). To enhance overall productivity and sustainability of the dual recombinant protein expression process, the use of a biscistronic vector instead of two plasmids may be more favorable and energy efficient. Furthermore, the use of an auxotrophic marker can further enhance the biosafety of the selection system.

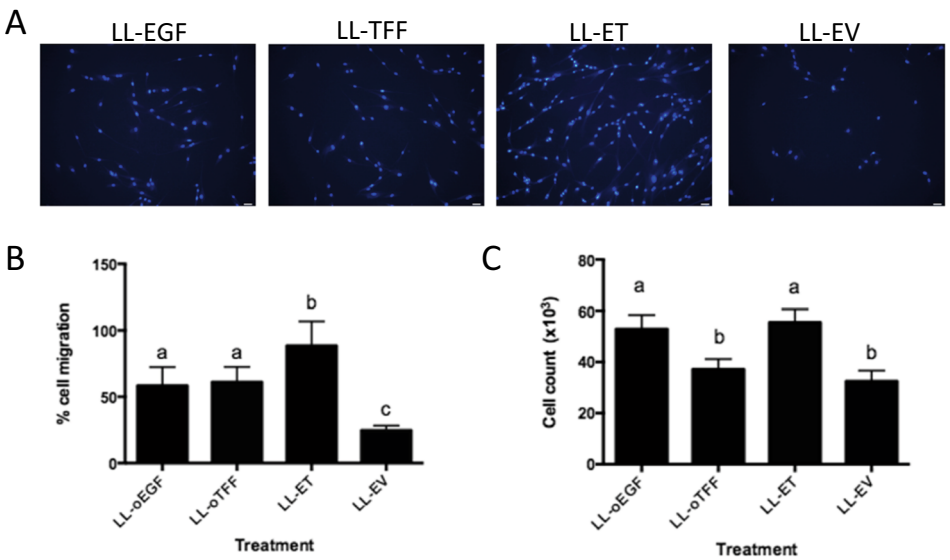

Fig. 5 Effect of EGF and TFF3 from *L. Lactis*-fermented supernatant on cell migration and proliferation. **a** Representative images of transwell migrated IPEC-J2 cells stained with Hoechst 33342. **b** Quantification of cell migration from transwell migration assay. **c** For cell proliferation study, cells were treated with *L. lactis* flask-fermented supernatant for 24 h and enumerated. *Bars* represent the mean± SEM of three experiments. *Different lowercase letters* denote significant difference between groups ($p<0.05$)

Using the internal ribosome-binding site, plasmids were constructed for the dual expression of TFF3 and EGF. From our study, the dual-protein producing *L. lactis* (LL-ET) produces significantly more total recombinant proteins than its single-expressing *L. lactis* counterparts, thereby increasing the amount of total recombinant biofactors per bioreactor fermentation and flask fermentation. Not only does this strategy allow the production and codelivery of the two different bioactive factors to enhance tissue repair, it is also more cost effective than producing them separately in two different single fermentations. In addition to equipment and maintenance costs, media is a major expense for large-scale microbial fermentation, and simultaneous expression of both proteins in a single bioreactor can reduce media cost significantly. In production, two man-days were considered per fermentation. With dual-expression, *L. lactis* cultivation costs can be reduced by 50 %. During coexpression, both EGF and TFF3 proteins were consistently secreted with the aid of the N-terminal SP310mut2 secretion signal. The P170 promoter utilized in our system is a pH-inducible promoter. The promoter is regulated by pH and *L. lactis* growth phase, preventing the need for the addition of an exogenous inducer (Bredmose et al. 2001; Madsen et al. 1999). The promoter activity is strongly upregulated at pH below 6.5 in the transition to stationary growth phase. To our knowledge, recombinant protein coexpression under the control of this promoter has not been reported. Similar to the dual-expression of recombinant proteins under the nisin-inducible promoter in *L. lactis* (Bahey-El-Din et al. 2010), the level of EGF and TFF3 production, respectively, was lower in the dual-expressing strain in comparison to the single-protein expressing *L. lactis* counterparts. Potential translational stress during protein dual expression may reduce recombinant protein yield. In addition, the level of expression of the downstream second recombinant gene located furthest from the promoter (e.g. TFF3) appears to be more reduced in comparison to the first recombinant gene that is located closest to the promoter (e.g. EGF, Fig. 2a). This is also in agreement with a previous report using nisin-inducible promoter as well (Bahey-El-Din et al. 2010), further suggesting that higher production of recombinant protein can potentially be obtained by decreasing the distance between the promoter and the cloned gene (Remaut et al. 1981). For future applications, the generation of tandem head-to-tail expression cassettes may be ideal where the expression of each protein is driven by its own promoter to achieve high coexpression of proteins. Similar strategy has been used in *Pichia pastoris* where increased expression level correlates to increased copy number of integrated expression cassettes, in which each has its own promoter (Cereghino and Cregg 2000). The future direction of the dual-factor expressing *L. lactis* includes manipulating the proportions of EGF and TFF3 expression to result in optimal ratios that can further improve restitution and repair of mucosal epithelial cells. Potentially optimal ratios of EGF and TFF3 have been reported, and these ratios can serve as a baseline target to attain the desirable EGF and TFF3 expression level in the dual-factor expressing *L. lactis* (Chinery and Playford 1995; Chwieralski et al. 2004; Oertel et al. 2001). Manipulations such as increasing or decreasing gene-promoter distances and flipping gene order can be considered to obtain desired proportions of the two protein factors.

The expression of porcine TFF3 and EGF was demonstrated to be enhanced by codon optimization of the native sequence for expression in *L. lactis*. In addition, although the *L. lactis* codon usage bias parameter was the main focus, the transcript from the optimized coding sequence also resulted in increasing mRNA folding energy (ΔG). It is conceivable that this may also have a part in increasing recombinant protein expression, which is in line with previous findings (Reeve et al. 2014; Ringnér and Krogh 2005; Tuller et al. 2010). For a given mRNA length, lower folding free energy (ΔG) corresponds to a more stable secondary structure, where thermodynamically stable mRNA secondary structures are associated with low translation rates as these molecules will generally form longer stems that could potentially interfere with ribosome binding (Tuller et al. 2010). In relation to optimizing recombinant protein expression, codon usage bias and folding free energy parameters should be further considered to enhance overall translation efficiency. If the two parameters conflict, codon usage bias is more important as there is a significant association between codon bias and translation efficiency but less association between folding energy and translation (Tuller et al. 2010).

Using the supernatant from *L. lactis* fermentation containing both EGF and TFF3 (LL-ET) for in vitro wound-healing assays, we observed enhanced effect of the two factors on enhancing wound healing of intestinal epithelial cells. This finding is consistent with the previous report on human EGF enhancing TFF effect on cell migration in an in vitro wounding colorectal epithelial cell line model (Chinery and Playford 1995). Our data on cell migration and proliferation suggest that the wound healing effect of TFF3 was mainly via its enhancement of cell migration without affecting cell number, while EGF was perhaps via both its stimulation on cell proliferation and migration. Using time-lapse video microscopy analysis, it was previously reported that EGF and TFF3 enhanced cell migration to the wounded area in different fashions: EGF treated cells repopulated the wounded faster but with less full coverage, while TFF3 triggered a slower but more precise coverage (Durer et al. 2007). Taken together, the combined application of EGF and TFF3 (LL-ET) from *L. lactis* fermented supernatant would likely not only be complementary (proliferation vs. migration) but also be additive with the dual advantage of both speed and precision in cell migration in wound healing during tissue repair. Our finding on cotreatment with both factors synergistically enhancing

cell proliferation and migration for wound repair is consistent with this notion.

In summary, it is feasible to dually express functional EGF and TFF3 in food grade *L. lactis* bacteria. The combination of the resulted two bioactive factors in the fermentation supernatant showed synergy enhancement in the repair of induced wound healing in vitro. Since the recombinant EGF-TFF3 proteins may potentially be applied as a feed additive, the use of unpurified culture supernatant in the tests was a stringent way to examine the activity of the peptides in the potential presence of inhibitory factors in the *L. lactis* culture supernatant. Purified and nonpurified proteins may also exhibit different activity level and stability. The use of these purified proteins for cell restitution has also been well-reported in literature (Chinery and Playford. 1995; Oertel et al. 2001; Chwieralski et al. 2004). Since the target proteins were produced in *L. lactis*, a safe food-grade bacterium, the use of unpurified proteins in the culture supernatant can mimic the potential application setting.

Our results suggest that coexpression of recombinant EGF and using *L. lactis* could potentially be used to improve intestinal health in the porcine/food-animal industry. This strategy may lay the foundation for future application of *L. lactis* fermentation product containing dual factors to treat intestinal damage and inflammation.

Acknowledgments This project has been funded by Ontario Pork, Natural Sciences and Engineering Research Council of Canada (NSERC), and Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA).

Conflict of interest The authors have declared that no competing interests exist.

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