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Human Growth Factor Homologues, Detected in Externalised Secretions of Medicinal Larvae, Could be Responsible for Maggot-Induced Wound Healing

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ABSTRACT

Medicinal maggots (Lucilia sericata), are used for the treatment of sloughy, necrotic wounds. Well known for their ability to effectively debride a wound, clinical and laboratory observations also suggest that maggots may exert a range of potential secondary effects, including fibroblast migration and acceleration of wound healing. Previous studies attribute this migration mainly to the action of proteases present in externalised maggot secretions. In this study, we investigated the effect of native maggot excretions/ secretions on fibroblast migration in the absence of digestive proteases, and found significantly enhanced levels of fibroblast migration following exposure to protease-free L. sericata secretions, suggesting the presence of additional factors contributing to fibroblast migration. Since growth factors are believed to play an essential role in fibroblast migration, we analysed maggot secretions for any indication that these, or molecules like these, may be present. Here we report uniquely on the discovery and quantification of maggot-derived human growth factor homologues, detected by Western blot analysis and enzyme-linked immunosorbent assays in secretions of L. sericata larvae. We believe and propose that these growth factors may act inadvertently in the human wound, and be partly responsible for the observed and reported maggot -stimulated wound healing.

Keywords: *maggots, Lucilia sericata, larvae, wound healing, growth factors*

INTRODUCTION

Chronic wounds are characterised by aberrations in normal wound healing processes and a delay in wound healing (1). Often, when wounds fail to heal, epithelial cells become unresponsive to growth factor signals and proliferate around the wound margins, without migrating over the wounds surface (2).

Larval Debridement Therapy (LDT) or Maggot Therapy (MT) for chronic and necrotic wounds, including pressure sores (3) and diabetic foot ulcers (4), uses the medicinal maggot *Lucilia sericata*, and has been shown to debride wounds considerably quicker than other conventional treatments, including hydrogel therapy (5).

In addition, MT has recently been shown to have a wide range of positive healing effects *in vivo and in vitro:* Clinically, several reports claim that there is an improved healing outcome following treatments of patients with MT (6-8). To date, however, there has been only one major clinical trial which examined wound healing, and found there was no significant improvement in wound healing for patients following MT as opposed to conventional treatment (9). Future clinical trials, better designed to focus on healing rather than debridement as an endpoint (10), are warranted.

I*n vitro*, scientists have uncovered several enhanced effects of maggot secretions on wound healing pathways, including promotion of angiogenesis (8, 11-13) Zhang et al, 2010, 2017)), increasing microvascular epidermal cell migration (14), up regulating expression of wound healing related genes, including epidermal growth factor (EGF), transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF)-A (15).

Proteome sequencing has also shown the presence of 5 potentially therapeutic peptidases secreted by *L. sericata* (16), which have been suggested to contribute to wound healing by promoting remodelling of the extracellular matrix, maintaining cellular morphology within

the wound, promoting fibroblast migration (17) and promoting hepatocyte growth factor (HGF) secretion in fibroblasts (18).

Previous studies have attributed effects on fibroblaststo components present in larval extracts (19). More recently, reports suggested that maggot serine proteases, trypsin-like, chymotrypsin-like proteases and metalloproteinases present in externalised secretions were responsible for enhancing fibroblast migration (20-22). However, investigations into the mechanisms of fibroblast motility for wound healing have shown that several growth factors, including EGF, pDGF-BB and TGF Beta are vital for fibroblast migration (23-25). Recently, a report by Li *et al.*, (2015) showed that promotion of wound healing appears to be mediated via a TGF-β signalling pathway, which was activated in the presence of maggot extracts (26).

Maggots themselves grow into different developmental stages as they debride a wound, from tiny L1 stages, moulting twice on the wound to give rise to the final L3 stage. Larval growth of insects is still a puzzle, but is believed to be under environmental and physiological control, and dependent on the secretion of insect growth hormones (27). Conserved proteins showing homology to human growth factors have been demonstrated in a range of invertebrates and insects (28), including several in *Drosophila* (29). Decapentaplegic (Dpp), insulin-like peptides regulating the insulin receptor-signalling pathway (30), similar to the insulin receptor signalling-pathway in mammals, have been implicated in regulating growth and development in *Drosophila* (31). There have however no published reports to date of the presence of any human growth homologues in the ES of medicinal maggots.

Our study aimed to investigate if components of *L. sericata* nES, other than endogenous proteases, played a role in fibroblast migration, and in light of the above, to determine if we could detect the presence of other proteinaceous factors which could be contributing to the observed enhancement of wound healing following treatment with LDT.

METHODS

Collection of L. Sericata Secretions

Sterilised late larval stage 2/3 *L. sericata* larvae were supplied by Biomonde®, Bridgend, UK with nES collected as previously described (32). Briefly, larvae were incubated with 200 µl of sterile ultrapure water per gram of larvae in darkness, for one hour, at 30^{O}C. Secretions were then collected, centrifuged at 10,000 x *g* for 5 minutes and the supernatant retained for testing. Where specified, PI cocktail (AEBSF at 104 mM, Aprotinin at 80 µM, Bestatin at 4 mM, E-64 at 1.4 mM, Leupeptin at 2 mM and Pepstatin A at 1.5 mM, Sigma Aldrich, Dorset, UK) was added to maggot ES at a concentration of 1µl per 1000 µl of nES. Following collection of nES, larvae were frozen to -20^{O}C.

Preparation of L. Sericatalysate

For lysate preparation, frozen larvae were thawed at 37^{O}C and 2 g of maggots placed in a glass homogeniser with 2 ml of RIPA buffer (Sigma Aldrich) and 20 µl of PI cocktail. Maggots were fully homogenized by hand and incubated at 4^{O}C for 5 minutes before being centrifuged at 6,000 rpm for 15 minutes at 4^{O}C. The supernatant was collected, passed through a 0.2 µm filter to remove any particulates and stored at -20^{O}C until required for testing.

Cell Culture Conditions

Human foreskin fibroblasts (HFF-1) were cultured throughout at 37^{o}C and 5% CO_2 in 250cm^2 flasks (Corning B.V. Life Sciences, Amsterdam, The Netherlands), in Dulbecco's modified eagle medium (DMEM) (Life Technologies, Paisley, UK) containing 15% foetal bovine serum (FBS), penicillin, streptomycin and further supplemented with 6ml glutamine. Cultured HFF-1 cells were split when fully confluent and trypsinised for 5 minutes at 37^{o}C and 5% CO_2. Trypsinisation was halted upon addition of 2ml DMEM (15% FBS). Prior to any experiment taking place, cultured HFF-1 cells were counted and viability assessed using the trypan blue exclusion method.

Fibroblast Migration in the Presence of L. Sericatanes and nES with Added PI

A stock solution of 3x10^5 HFF-1 cells/ml was prepared in DMEM 1% FBS. One hundred µl of the stock solution were aliquoted into the wells of a 24 well plate, supplemented with 400 µl of DMEM 1% FCS and incubated at 37^{O}C and 5% CO_2 for 24 hours for the cells to become fully confluent and adherent. A 20-200 µl pipette tip was used to scratch a “wound” down the centre of each well and the media aspirated and replaced with either with DMEM 1% FBS (vehicle control), 5 µg/ml nES with or without

PI in DMEM 1% FBS. The scratch was immediately photographed using Zen lite 2011 software on a Zeiss Axiocam ERc5s camera attached to a Zeiss primovert microscope (Carl Zeiss LTD. Cambridgeshire, UK) at x200 magnification to document the size of the wound at *t*0. As this study was only interested in the time taken for the wound to close, only the later stages of the assay were photographed from 12 hours onwards. The plate was then incubated for a further 12 hours at 5% CO_2 at 37^{O}C before the wells were again photographed at x200 magnification at 12, 14 and 16 hours. The experiment was stopped after the 16 hour time point as, following observation, cells in wells exposed to nES with added PI had fully closed the wound. Cell migration was measured using ImageJ software (national Institutes of Health).

Western Blot Analysis

As this was an exploratory analysis, nES was concentrated by a factor of 10 by centrifugation under reduced pressure. Five hundred microliters of nES were then retained at a concentration of x 10 with dilutions made by addition of ultrapure water.

Maggot nES, at protein concentrations ranging from 30 - 3.13 µg/ml were run on 8%, 10%, 12% or 20% tris-glycine PAGE gels (Bio-Rad Laboratories, Hemel Hempstead, UK) depending on the size of the protein of interest. For TGF-β and VEGF positive controls, lysates of the prostate cell lines PC3, LNCaP and PNT2 cells were prepared in RIPA buffer (Sigma-Aldrich) as described by the assay protocol. Materials for Western blot analysis were obtained from the following manufacturers: Primary antibodies for TGF-β and VEGF, Abcam (Cambridge, UK); recombinant human EGF and its primary antibody, R&D systems Inc. (Oxon, UK); recombinant human PDGF and its primary antibody, Cell Signalling technologies (Leiden, The Netherlands); recombinant human HGF, EMD Millipore (MA, USA) with the primary antibody obtained from Abcam; recombinant human FGF and its primary antibody, EMD Millipore; secondary mouse anti-rabbit-HRP conjugated antibody, Cell Signalling technologies; anti β-actin antibody, Abcam; secondary rabbit anti-mouse-HRP conjugated antibodies, Abcam; biotinylated molecular weight (MW) ladder and antibiotin-HRP conjugated antibody, Cell Signalling technologies; rainbow MW ladder, Thermo Scientific (Loughborough, UK); skimmed milk powder, Sigma-Aldrich. All reagents were stored as per the manufacturer's guidelines. For VEGF and TGF-β positive controls, protein obtained from the prostate cell lines PC3, LnCAP and PNT2 were used since these cells secrete TGF-β and VEGF (33-34).

Positive controls and nES samples, along with 10 µl of MW ladder were suspended 1:1 in 2x concentrated Laemmli sample buffer (10% SDS, 4 ml; 10% glycerol, 2 ml; 1M Tris-Cl, pH 6.8, 1.2 ml, β-mercaptoethanol, 1 ml; Milli-Q water, 1 ml) and spun in a bench top mini-centrifuge for 1 minute to mix all sample constituents and remove any air bubbles before being heated to 95^{O}C for 5 minutes. All samples along with 10 µl of rainbow ladder were loaded into the wells of an SDS-polyacrylamide gel. Any empty wells were filled with loading buffer to promote even migration of the proteins and allow any non-specific binding to be identified. A 4% polyacrylamide stacking gel was used throughout, with the following polyacrylamide resolving gel concentrations being used for each assay: EGF, 20%; TGF-β, VEGF and PDGF, 12%; FGF, 10% HGF, 8%. All gels were run at 60V through the stacking gel and then 140V through the resolving gel. Electrophoresis was stopped once the loading front neared the bottom of the gel to assure adequate separation of the protein bands on the resolving gel. Proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Amersham, UK). Membranes were blocked for 30 mins with 5% non-fat dry milk in 1%tween/ trid buffered saline to inhibit nonspecific binding (all Sigma Aldrich) and incubated overnight at 4°C using primary antibodies to, HGF (1:1000), FGFb (1:1000), EGF (1:1000), PDGF-BB (1:1000), VEGF (1:1000) and TGF-β (1:1000). Membranes were washed free of primary antibody and incubated with horseradish peroxidase conjugated secondary anti-mouse/anti-rabbit antibodies (1:1000) for 1 hour at room temperature. Proteins were visualized using the Immun-Star Western chemiluminescene detection kit (Bio-Rad) and viewed using a Chemidoc XRS system (Bio-Rad). Western blots were performed at least in triplicate. As he VEGF protein in all positive control samples was present at the predicted 45 kDa in all three cell lines. Given that VEGF and β-actin are of similar sizes (45 kDa and 41 kDa respectively), once VEGF was detected, the membrane was re-probed for β-actin. As a

result, β-actin was not present in the results. For negative controls, one well was filled with Laemmli buffer minus maggot secretions, prior to electrophoresis.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits for human TGF-β1, VEGF, PDGF-BB, EGF and FGFb were purchased from R&D systems (R&D systems, Oxon, UK). Maggot lysate was tested to confirm that these growth factors were being produced endogenously by *L. sericata*, with three independent batches of nES with added PI (concentrated by a factor of 6 to achieve a detectable concentration of growth factors during ELISA (<4 pg/ml), with the exception of PDGF-BB (15 pg/ml)) tested to investigate whether these proteins were being actively secreted by the maggots. Assays were carried out as specified by the assay protocol using maggot nES and maggot lysate with added PI. For the detection of PDGF-BB and EGF, samples were incubated with conjugate for the maximum recommended time of 2 hours, suggested in the assay protocol. All plates were read spectrophotometrically at 450 nm with correction at 540 nm. The growth factor homologue concentrations detectable in nES were then corrected to the mean protein concentration of the three batches of nES (2519.2 ± 0.1 µg/ml) and expressed as pg/ml.

Statistical Analysis

Significance was determined throughout using Students two-tailed t-test to compare significance with *P* determined to be significant at <0.05, highly significant at <0.005 and very highly significant at <0.001.

Results

Fibroblast Migration

Results showed that at all time periods analysed (12, 14 and 16 hours) the migration of fibroblasts was higher in the presence of protease-free nES than in nES or vehicle control (figure 1). For example, 14 hours after treatment, fibroblast migration was significantly higher in the presence of nES with added PI (96.4% ± 1.3%, $P<0.001$, $n = 6$) compared to the vehicle control (84.4% ± 3.1%, $n = 6$) and a significantly higher ($P<0.05$) compared to fibroblasts to which nES with nES (80.4% ± 3.0%, $n = 6$), (Fig. 1). Furthermore, fibroblast migration over 16 hours resulted in >95% wound closure in five replicates and >98% closure in three replicates, significantly greater than wound closure in the vehicle control ($P<0.05$). In contrast, fibroblast migration after 16 hours following treatment with nES only increased wound closure to 90.0% ± 3.0% ($n=6$), with only two replicates achieving closure of >95%. After 16 hours, there was almost complete closure of the wound (96.7%) in the presence of protease-free nES (Fig 1), compared to 82.7% and 90.0% in the vehicle control and nES wounds respectively, although the difference between the wound space in fibroblasts treated with nES and nES with added PI was not significant ($P>0.05$).

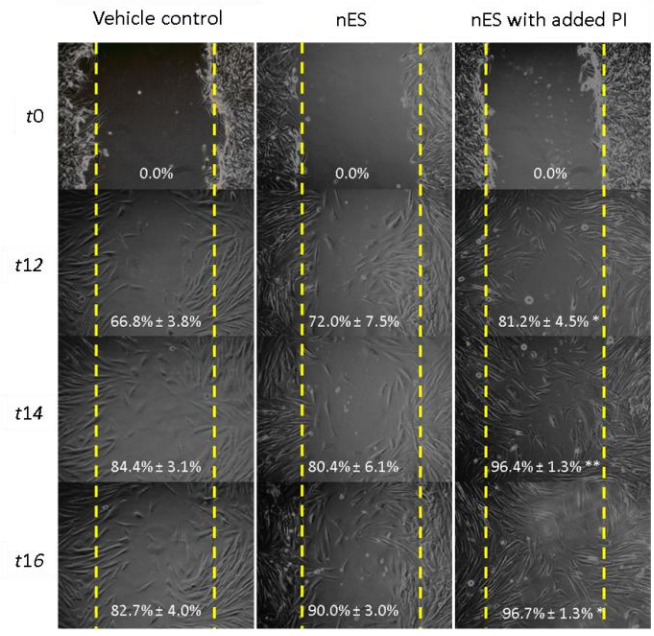

Figure1. *Fibroblast migration following treatment with nES and protease-free nES*

A uniform wound was made in confluent HFF-1 cells using a 20-200 µl pipette tip and the images taken at x200 magnification at 0, 12, 14, 16 and 18 hours. The wound space was defined as the space between the two leading edges of fibroblasts, with the whole field of the image being measured to ensure consistency between samples. The space within the scratch was quantified by counting the number of pixels highlighted within the space between the two populations of fibroblasts. The mean number of pixels within the wound space was converted into a percentage with the mean number of pixels at *t*0 representing 0% wound closure. Percentage of wound closure at 12, 14 and 16 hours was then calculated as a percentage by comparing the number of pixels between the two population of fibroblasts at each time point to the number of pixels present in the scratch within the wound space at time 0. Data represents mean closure of the scratch compared to the time at *t*0 ± S.E.M. * = P <0.05, ** = P <0.001. n = 6 for all replicates: nES = native excretions/secretions; t = time; PI = protease inhibitor.

Growth Factor Homology

A number of proteins with homology to human growth factors were detected in *L. sericata* nES by Western blot analysis. Homologues for TGF-β and VEGF (Figs 2A and B) were found to be present in *L. sericata* nES at a molecular weight approximately 10 kDa lower than human TGF-β or VEGF. Conversely, protein homologues of EGF and FGF (Figs 2C and D) were detected in *L. sericata* nES at MWs of approximately 25 kDa and 29 kDa respectively, much larger than those of human EGF (6 kDa) and FGF (18 kDa). The homologues of human FGF, however, appeared to be present at a lower concentration than the other growth factors detected, as indicated by the fainter bands. Furthermore, some faint bands were observed at ~30 kDa and ~60 kDa. Interestingly, the homologues of human PDGF-BB detected were similar in MW to that of the recombinant human PDGF protein control (Fig. 2 E). However, homologues of HGF were not detectable (Fig. 2 F). Interestingly, with the exception of Figure2A, there appears to be no dose-dependency found in the western blots. This could be due to the low concentration of proteins present in each of the Western blots.

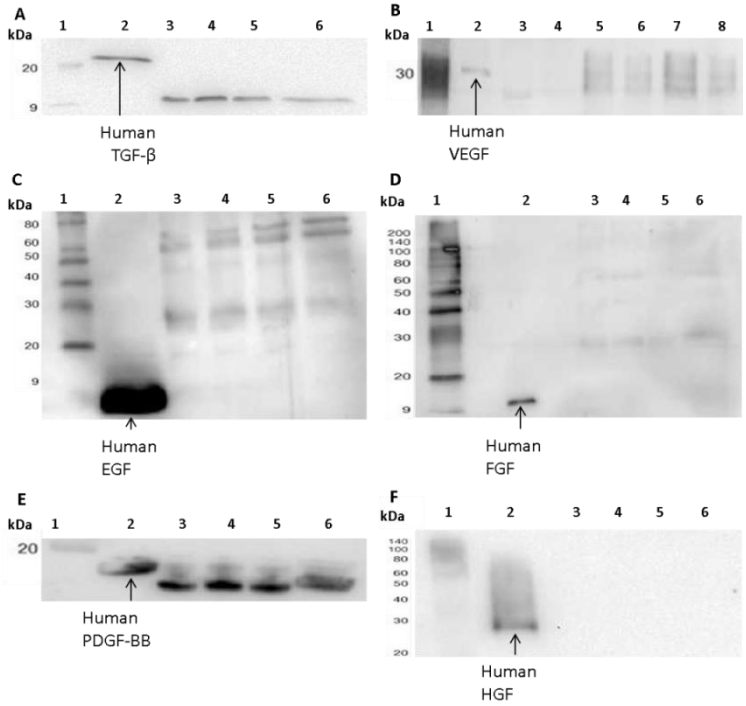

Figure2. *Representative Western blots demonstrating the presence of TGF-β (A), VEGF (B), EGF (C), FGF (D), PDGF (E) human growth factor homologues and the absence of HGF (F) human growth factor homologues in L. Sericata nES.*

Figures 1A, C-F: lane 2, positive control; Lane 3-6 nES (30, 15, 7.5 and 3.75 µg or 25, 12.5, 6.25 and 3.13 µg for B). Figures 1B: lane 2-4, positive cell control lysates (PC-3, PNT-2, LnCAP); Lane 5-8 nES. n = >4 for all replicates.

Human growth factor homologues to TGF-β, VEGF, PDGF, FGF and EGF were also detectable in both *L. sericata* lysate (Fig. 3A) and in nES (Fig. 3B) by ELISA, confirming the finding of Western blot analysis. Following quantification in 6x concentrated nES at concentrations above the minimum detection limit for each assay kit, and correction back to original mean protein concentration of the nES samples (2519.2 ± 0.1 µg/ml), the mean growth factor homologue concentrations per ml of nES was found to be; 51.3 ± 5.9 pg/ml (TGF-β2), 24.9 ± 0.9 pg/ml (VEGF), 2.4 ± 0.7 pg/ml (PDGF-BB), 1.8 ± 0.4 pg/ml (FGFb) and 1.0 ± 0.2 pg/ml (EGF).

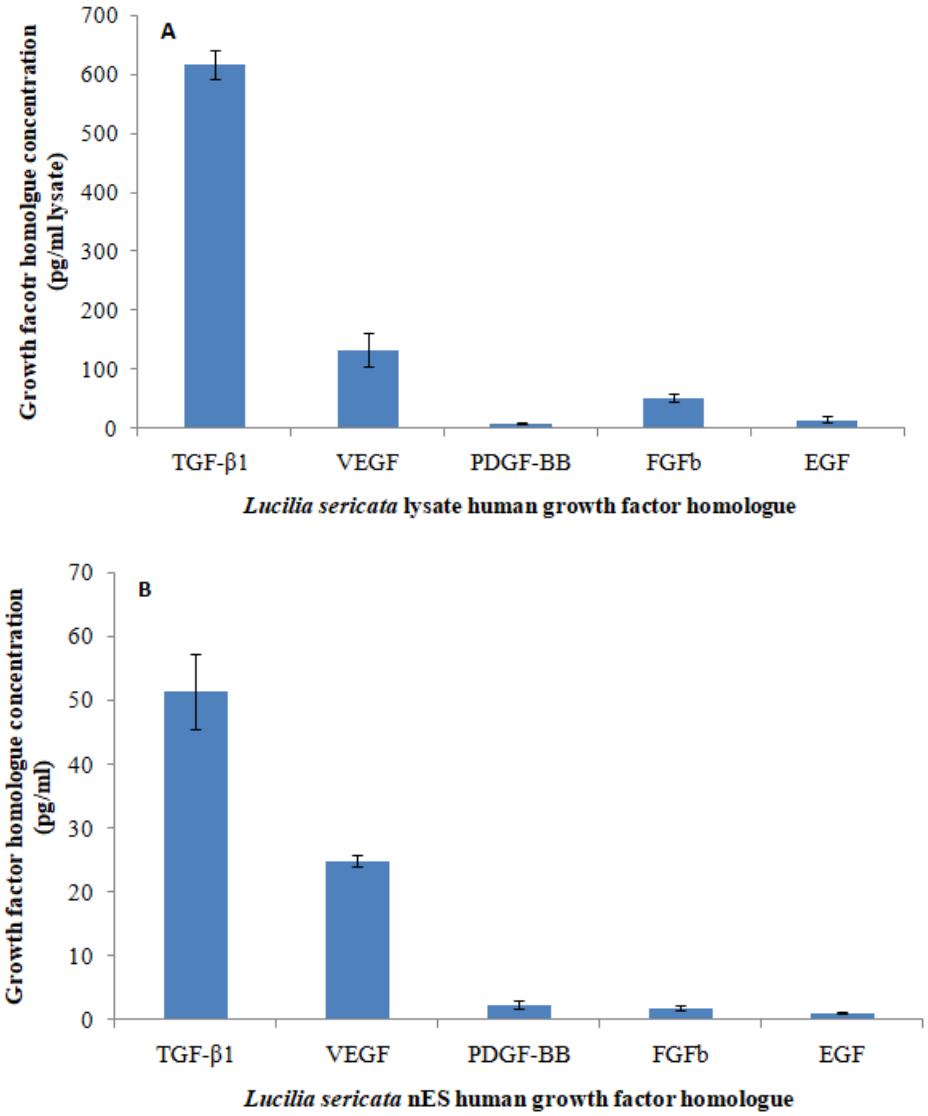

Figure3. *Concentration of human growth factor homologues present in L. sericata lysate and nES*

Luciliasericata lysate (A) and nES (B) were examined using the enzyme linked immunosorbent assay to measure the production (lysate) and excretion/secretions (nES) of 5 human growth factor homologues. Data represents the mean concentration of human growth factor homologues detected in L. Sericata lysate and nES (pg/ml) with n = 6 for each homologue tested. Error bars represent standard error of the mean

DISCUSSION

Our investigation has shown that protease-free nES was able to significantly enhance fibroblast migration. The increase in migration was higher than observed for fibroblasts in the presence of native secretions containing proteases. Previous *in vitro* investigations attributed the enhanced fibroblast migration to endogenous maggot proteases (20-22), and whilst this has been partially confirmed in this study, as nES (without proteases inhibitors added) was able to significantly enhance fibroblast migration above the vehicle control, our results suggest that maggot-enhanced fibroblast migration may be

due to some other or additional molecules present in larval secretions.

Investigating this further, we found that Western blot analysis using monoclonal antibodies, with no other species cross reactivity, revealed the presence of molecules with homology to human growth factors FGF, TGF-β, VEGF, EGF and PDGF-BB, growth factors integral to the wound healing process. The enzyme-linked immunosorbent assay confirmed our findings of the Western blot analysis and demonstrated that *L. sericata*larvae are able to produce and secrete proteins, homologous to TGF-β2, VEGF, PDGF, FGF and EGF.

The *L. sericata* FGF homologue was detected in *L. sericata* nES by both ELISA and Western blot analysis, although at a different MW to that of human FGF. Differences in MW between growth factors in humans and insects are however well documented in FGF (35): Fibroblast growth factor specifically in humans varies from 17 – 34 kDa, but is present as the 84 kDa protein branchless (Bnl) in *D. Melanogaster* (35) with these proteins being structurally similar to one another (36). All members of the FGF family have also been shown to share a conserved amino acid sequence of 120 residues that express 16% - 65% identity (35). Three FGF genes have also been detected in *D. melanogaster* and two in *Caenorhabditiselegans* (37) (genes *elg-17*(38) and *let-756*(39) which encode for an FGF-like ligand). It may therefore be likely that human FGF homologues are present in *L. sericata*nES. Furthermore, as FGF has been found to be essential for *D. melanogaster* development, but is structurally similar to vertebrate FGFs (36), it may also play a significant role in *L. sericata* development.

Transforming growth factor-β (TGF-β) homologues have also been detected in invertebrates. Members of the Bone Morphogenic Protein (BMP) super family of proteins for example are also present in *D. melanogaster*, including Dpp, 60A and screw (40). Furthermore, 60A in *D. melanogaster* shows greater homology to human BMP 5, 6 and 7 than to Dpp (41). Members of the TGF-β family have also been described in other invertebrates including sea urchins (42). Considering its abundance in both vertebrates and invertebrates (notably *D. melanogaster*), it may be that the protein homologue for human TGF-β detected in this investigation was *L. sericata* TGF-β, which appears to show homology to *H. sapiens* TGF-β, but has a different molecular weight. *Lucilia sericata* TGF-β did also appear to be present at a relatively higher concentration than other proteins analysed. This may suggest that *L. sericata* TGF-β plays an important role in larval growth and/or development, as it does in *D. melanogaster* development (43).

The VEGF homologue detected in *L. sericata* nES was found to be approximately 10 kDa smaller than human VEGF, suggesting that a protein present within *L. sericata* nES shares homology with human VEGF. A number of VEGF proteins and VEGF receptors with homology to human VEGF proteins and receptors have been reported in *D. melanogaster*, with predicted signal peptide and central domains which are common to VEGF super family members (44). These include a VEGF receptor with homology to human VEGF receptor *Pvr*, which has been demonstrated in *D. melanogaster* gene GC8222 (cytological region 29A), with predicted signal peptide and central domains which are common to VEGF super family members (38). *Caenorhabditis elegans* PDGF/VEGF-1 has also been shown to bind and activate human VEGF receptors -1 and -2 in human umbilical vein endothelial cells inducing angiogenic responses (45). This evidence again supports the hypothesis that the proteins detected in *L. sericata* nES may be homologous to human growth factors and able to elicit a response in human cells.

The presence of EGF and PDGF-BB homologues in *L. sericata*nESIs also supported by the presence of these proteins in the *D. melanogaster* genome (44), including the EGF homologue genes *Notch* and *Delta* (Dl) (46). The results do appear to suggest that maggots produce proteins homologous to human EGF and PDGF-BB. However, no proteins homologues to human HGF were detected in *L. sericata* nES and no literature is available on the presence of HGF in insects. It may be that HGF plays no functional role in insect development was therefore not present within *L. sericata* nES.

In conclusion, this study has shown, upon inhibition of their endogenous proteases, *L. sericata* larval nES are still able to stimulate fibroblast migration, to a greater extent than nES with proteases, which were originally thought to be the main promoters of fibroblast

migration *in vitro*. We suggest that the increase in fibroblast migration may, in part, be due to the presence of other proteins, possibly growth factor-like proteins that we have detected for the first time in maggot secretions, and which show homology to five human growth factors. Further investigations are now required to determine what concentrations of these growth factors are secreted into the wound during MT and how the concentration of growth factors secreted in the wound are able to affect fibroblast, keratinocyte proliferation and migrations, as well as any effect on angiogenesis.

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