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Production of functional active human growth factors in insects used as living biofactories

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ABSTRACT

Growth factors (GFs) are naturally signalling proteins, which bind to specific receptors on the cell surface. Numerous families of GFs have already been identified and remarkable progresses have been made in understanding the pathways that these proteins use to activate/regulate the complex signalling network involved in cell proliferation or wound healing processes. The bottleneck for a wider clinical and commercial application of these factors relay on their scalable cost-efficient production as bioactive molecules. The present work describes the capacity of *Trichoplusia ni* insect larvae used as living bioreactors in combination with the baculovirus vector expression system to produce three fully functional human GFs, the human epidermal growth factor (huEGF), the human fibroblast growth factor 2 (huFGF2) and the human keratinocyte growth factor 1 (huKGF1). The expression levels obtained per g of insect biomass were of 9.1, 2.6 and 3 mg for huEGF, huFGF2 and huKGF1, respectively. Attempts to increase the productivity of the insect/baculovirus system we have used different modifications to optimize their production. Additionally, recombinant proteins were expressed fused to different tags to facilitate their purification. Interestingly, the expression of huKGF1 was significantly improved when expressed fused to the fragment crystallizable region (Fc) of the human antibody IgG. The insect-derived recombinant GFs were finally characterized in terms of biological activity in keratinocytes and fibroblasts. The present work opens the possibility of a cost-efficient and scalable production of these highly valuable molecules in a system that favours its wide use in therapeutic or cosmetic applications.

1. Introduction

The immense social and economic impact of wounds worldwide is a consequence of their high rate of occurrence of diabetes globally and their increasing frequency in the ageing population (Natarajan et al., 2000). In addition, Cushing's syndrome, poor arterial perfusion, venous hypertension, poor nutrition or sepsis are also, directly or indirectly, involved in damage of cutaneous coverage (Robson et al., 2001). Therefore, increase in the occurrence and consistent development of cost-effective and clinically efficient technologies and products (compared to traditional offerings) are driving growth in the advanced wound care market.

The healing of an adult skin wound is a complex process requiring the collaborative effort of many different tissues, cells and molecules. Injury to the skin initiates a cascade of events, which can be temporally categorized into three major groups—inflammation, tissue formation and tissue remodelling (Singer and Clark, 1999). The three phases of wound repair are not mutually exclusive but rather overlapping in time for finally lead to at least partial reconstruction of the wound area (Martin, 1997). The repair process is initiated and controlled immediately after injury by the release of various growth factors (GFs), cytokines, and low-molecular weight compounds from the serum of injured blood vessels and from degranulated platelets (Velnar et al., 2009; Greenhalgh, 1996).

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Cytokines and GFs are a group of molecules that participate, as functional messenger between cells, in the complex regulatory network, which control cellular response and are related with recombinant protein therapies (Kim et al., 2010), vaccines (Jenq et al., 2009), regenerative medicine (Werner and Grose, 2003; Barrientos et al., 2008), biosimilars (Niederwieser and Schmitz, 2011) and stem cell medicine (Tenney and Discher, 2009; Eiselleova et al., 2009). Due to their wide activity, cytokines and GFs (and their related products) are highly valuables in research, diagnostics and biopharmaceuticals sectors.

Current researches in wound healing are being focused on GFs or/and human skin substitutes (Macri and Clark, 2009) optimizing both the cellular and molecular environment, thus decreasing healing time by modifying inflammation, and accelerating the proliferative phase. Multiple studies have demonstrated a beneficial effect of many GFs, *e.g.* epidermal growth factor (EGF) and fibroblast growth factors (FGFs) families on the healing process to attract cells into the wound stimulating their proliferation and having a profound influence on extracellular matrix deposition (Pastore et al., 2008; Wang et al., 2008).

EGF is one of the first GFs to be identified (Cohen, 1962). It is a single-chain acidic polypeptide of 53 amino acid residues containing three intramolecular disulfide bonds, which are required for his proper tertiary structure (Boonstra et al., 1995; Cohen and Carpenter, 1975). It is a member of the mitogenic family of EGF-like molecules which also includes TGFα, the poxvirus growth factors, and amphiregulin (Yates et al., 1991). Its function is exerted *via* binding to the epidermal growth factor receptor (EGFR), a transmembrane protein tyrosine kinase that is expressed on many different cell types, stimulating the proliferation and the migration of cells (Carpenter and Zendegui, 1986). A series of experimental and clinical studies have demonstrated a positive effect of EGF on wound repair (Hardwicke et al., 2008).

FGFs family comprise structurally related polypeptides, consisting of 22 members in human (Ormitz and Itoh, 2001). They transduce their signals through direct interaction with heparin or heparan sulfate proteoglycans, a characteristic feature of FGFs, which stabilizes FGFs to thermal denaturation and proteolysis, and which strongly limits their diffusibility (Burgess and Maciag, 1989; Dowd et al., 1999). The complex formed can binds specifically with one of a four high-affinity transmembrane protein tyrosine kinase, FGF receptors 1–4 (FGFR1-4) with different affinity (Johnson and Williams, 1993). Most members of the FGF family have a broad mitogenic spectrum. They stimulate proliferation of various cells of mesodermal, ectodermal, and also endodermal origin. In addition to their mitogenic effects, FGFs also regulate migration and differentiation of their target cells, and some FGFs have been shown to be cytoprotective and to support cell survival under stress conditions (Itoh and Ornitz, 2011; Kinoshita et al., 2012; Werner, 1998). All of FGFs *in vivo* effects suggest a role of these growth factors in wound repair. In particular, the basic fibroblast growth factor (bFGF) or fibroblast growth factor 2 (FGF2) was shown to stimulate angiogenesis in various assay systems (Risau, 1990). Other member of the FGF family, fibroblast growth factor 7 (FGF7), also known as keratinocyte growth factor (KGF1) has been found to have profound specific stimulatory effects on keratinocyte growth at least in the adult organism (Werner, 1998; Rubin et al., 1989) or in treatment of inflammation of the mucosal lining of the gastrointestinal tract (Amgen, US Patent 5965530). Thus these are clear candidates for implementing the wound healing response.

As previously mentioned, the production of these molecules in a cost-effective manner is being a relevant matter. The Baculovirus Expression Vector System (BEVS) allows high-level of heterologous proteins production by using strong baculoviral promoters (*polh, p10*). Furthermore, BEVS allows recombinant proteins to be producing very likely to their native conformation as they can be post-translationally modified efficiently. These characteristics make BEVS an important expression system for industrial applications. The expression of various GFs in insect cells are already successfully tested (Cronin et al., 1998; Lee et al., 2006). However, a more cost-effective alternative to produce recombinant GFs would be highly desirable for a more affordable application of these recombinant products.

The use of insect larvae as living biofactories for protein production has been explored as an alternative to fermentative technologies. The recombinant protein production by the combination of recombinant baculovirus and *Trichoplusia ni* (*T. ni*) insect larva has been denominated as Improved Baculovirus Expression System t(IBES) and represents one of the best production alternatives based on baculovirus vectors. The advantages of using insect larvae for protein production include the dramatic reduction in production costs with respect to insect cell cultures, an increase of recombinant protein yields, the absence of high-tech fermentation procedures, a reduced development times and an easy production scaling-up. These advantages make the use of insect larvae as biofactories as a real alternative to standard cell-culture fermentation systems.

The use of such effective and inexpensive platform has been used to produce efficiently several recombinant antigens, including enzymes (Medin et al., 1990; Romero et al., 2011; Chazarra et al., 2010), antibodies (Reis and Blum, 1992; Gil et al., 2011; Gómez-Sebastián et al., 2012), hormones (Mathavan et al., 1995; Sumathy et al., 1996), vaccines (Gomez-Casado et al., 2011; Kuroda et al., 1989; Gil et al., 2001), cytokines (Pérez-Martín et al., 2010; Shi et al., 1996; Pham et al., 1999) and diagnostic proteins (Barderas et al., 2000; Pérez-Filgueira et al., 2006). In all these cases, proteins were processed correctly after synthesis, and their functional activities remained intact.

In the present work, we have evaluated the production of three recombinant human GFs, the human epidermal growth factor (huEGF), the human fibroblast growth factor 2 (huFGF2) and the human keratinocyte growth factor 1 (huKGF1) in *T. ni* larvae. We have used different modifications to optimize their production, the signal peptide from honeybee melittin (Mel) or the reticulum endoplasmic retention signal sequence KDEL (Lys-Asp-Glu-Leu). These sequences can facilitate an efficient translocation of recombinant proteins and their proper folding (Ruiz-Gonzalvo et al., 1996). We have also used one of the most used protein tag the polyhistidine tag (His-tag) (Terpe, 2003), that allows the purification of the tagged protein by metal affinity chromatography or a common fusion strategy in protein therapeutics, in which the fragment crystallizable region (Fc) of the human antibody IgG is used (Fc-tag) (Arnau et al., 2006). The recombinant GFs were finally characterized in terms of functionality by several biochemical assays including mitogen-activated protein kinase (MAPKs) activation and epithelial cells proliferation or migration experiments.

The results presented here show high production yields of the three human GFs analysed. These yields were around 9.1 mg (huEGF), 2.6 mg (huFGF) and 3 mg (huKGF) per gram of insect biomass, with similar functionality to their commercial counterparts in all the cases.

2. Materials and methods

2.1. Cell lines and cell culture

2.1.1. Insect cells

The insect ovarian cell line *Spodoptera frugiperda* (Sf21) was purchased from Invitrogen. Sf21 were routinely grown at 27 °C in *T. ni* Medium-Formulation Hink (TNM-FH) medium (PAN Biotech) supplemented with heat-inactivated 10% (v/v) Foetal Bovine Serum (PAN Biotech) and gentamycin sulphate 50 μg/ml (Lonza).

2.1.2. Mammalian cells

The immortal human keratinocyte line HaCaT were routinely grown at 37 °C with 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium) containing heat-inactivated 10% (v/v) Foetal Bovine Serum (Lonza) and 2 mM L-glutamine, 1 mM Hepes, 50 U/ml penicillin and 50 μg/ml streptomycin. Normal human dermal fibroblasts (NHDF) were purchased from ScienCell (San Diego, CA, USA) and mouse fibroblast cells NIH/3T3 from American Type Culture Collection (ATCC). Both were routinely grown at 37 °C with 5% CO_2 in supplemented DMEM following manufacturers' instructions.

2.2. Insect growth conditions and inoculation

T. ni (*T. ni*, Cabbage looper) larvae were reared under level-2 biosafety conditions following previously described methodology (Pérez-Filgueira et al., 2006). For all experiments, fifth-instar larvae (last instar larvae before pupation) of about 300 mg weight, were injected with the recombinant baculoviruses near the proleg (forward the body cavity) using different pfu/larva doses, as indicated on each experiment. Infected larvae were kept in growth chambers at 28 °C and collected at indicated times. Larvae were immediately frozen and kept at −20 °C until processed.

2.3. Recombinant baculoviruses

2.3.1. Clonings

The human epidermal growth factor (huEGF) (GENEBANK accession No: NP_001954, Asn971–Arg1023), the human fibroblast growth factor 2 (huFGF2) (GENEBANK accession No: M27968.1) and the human keratinocyte growth factor 1 (huKGF1) (GENEBANK accession No: NG_029159) sequences were synthesized by MrGene (http://blog.mrgene.com). The arthropods *Kozak sequence* (CAAATG) was also added at the 5′ end of each gene. The synthesized fragments flanked by *BamH*I and *Xba*I (huEGF and huKGF) or *Bgl*II and *Xba*I (huFGF2) were introduced into homologue restriction sites of modified pFastBac1™ vector (Invitrogen, USA) to generate the donor vectors. For each GF different versions were generated: (i) with an His-tag at the carboxy terminal site of the protein, (ii) with the honeybee melittin signal peptide at the amino terminal site and a His-tag at the carboxy terminal site and (iii) with a His-tag and the KDEL sequence at the carboxyl terminal site (Fig. 1A). For all GFs different versions fused to a modified Fc region of the human antibody IgG were generated: (iv) with the hinge region (Hinge$^+$), (v) with the Hinge$^+$ and the melittin signal peptide at the amino terminal site, (vi) with the Fc without hinge region (Hinge$^-$) or (vii) with the Hinge$^-$ and the melittin signal peptide at the amino terminal site (Fig. 4A). Additionally, in some of these constructs were added protease recognition sequences to falilitate the elimination of the Fc region fused to the GFs (factor *X* and furin cleavage sites) (Fig. 4A)

2.3.2. Recombinant baculoviruses

The resulting donor plasmids from every construct were denominated pEGFHis, pMelEGFHis and pEGFHisKDEL for huEGF; pFGF2His, pMelFGF2His and pFGF2HisKDEL for huFGF2; and pKGF1His, pMelKGF1His, pKGF1HisKDEL, pKGF1Hinge$^+$, pMelKGF1Hinge$^+$, pKGF1Hinge$^-$, pMelKGF1Hinge$^-$ for huKGF1. They were used to generate the corresponding recombinant baculoviruses using the Bac-to-Bac® Baculovirus system (Invitrogen) following the manufacturer's instructions. A baculovirus without any insert (BacNI) was used as a control baculovirus.

Recombinant baculoviruses were propagated and amplified in Sf21 insect cells to reach infective titres of around 10^7 plaquing forming units/ml (pfu/ml) (O'Reilly et al., 1994). Stocks were kept at 4 °C for daily use and −80 °C for long-term storage.

2.4. Insect protein extract preparation

Total protein (TP) fractions from frozen *T. ni* larvae infected by the recombinant baculoviruses were obtained by homogenization with 8 ml of extraction buffer - PBS 1X, Triton X-100 at 0.01%, complete protease inhibitor cocktail (Roche), and DTT 25 mM/g of biomass, using a Bag Mixer blender (Interscience). Total soluble protein (TSP) fractions were similarly obtained by centrifuging at 4000 rpm the TP fractions for 30 min. at 4 °C. Supernatants were then filtered through Miracloth papers (Calbiochem, USA) and aliquoted. TP and TSP samples were quantified by Bradford assay (Bradford, 1976).

2.5. Analysis of growth factors production in insect larvae

2.5.1. Baculovirus dose assays

Groups of 60 larvae were infected with 10^5, 10^4 or 10^3 pfu of each baculovirus and processed at 72 hpi. TSP and/or TP of huFGF2 and huKGF1 were resolved in 15% or 12% SDS-PAGE and then stained by Coomassie Blue or transferred onto nitrocellulose membranes following standad conditions. Specific conditions were used in case of huEGF protein extracts. Those conditions consisted on polyacrylamide gels electrophoresis (PAGE) performed using glycine gels (resolving gels - Bis/acrylamide 18%; 1,5 M TrisHCl pH 8,45; SDS 0,1%; and stacking gels - Bis/acrylamide 4%; 2 M TrisHCl pH 8,45; SDS 0,1%; ammonium persulfate 0,1%). Electrophoresis was run during 120 min. at 100 V using a specific electrophoretic solution (Tris 0,5 M; Tricine 0,5 M; SDS 0,1%) and then transferred onto nitrocellulose membranes during 25 minu (100 V).

The biomass and number of larvae collected were analysed for each dose of each recombinant baculovirus. Production data were expressed as an average of three independent experiments.

2.5.2. Western blot

Membranes were blocked in PBS-0.05% Tween 20 (PBST) 4% skim milk (blocking buffer, BF) overnight (ON) at 4 °C. They were then incubated at room temperature (RT) for 1 h with an anti-His monoclonal antibody (mAb) (Clontech) at 1:2000 in BF. After three washes of 10 min. with PBST, a second incubation was made using an anti-mouse IgG, horse radish peroxidase labelled (HRP) (GE Healthcare) at 1:2000 dilution in BF for 1 h. Protein extracts of Fc-tagged proteins were assayed using an anti-IgG1-HRP mAb (Promega) at 1:2000 dilution in BF. All membranes were developed using ECL (Enhance Chemiluminescence) reagent. Images were capture by Chemidoc (Bio-Rad) and analysed using the Image lab™ Software (Bio-Rad, version 4.1).

2.6. Purification

Recombinant His-tagged proteins were purified from infected larva extracts using Co^{2+}-based immobilized metal affinity chromatography (IMAC) resins TALON® (Clontech) as previously described (Gomez-Casado et al., 2011).

The quantification of Coomassie blue stained His-tagged purified proteins was done by densitometry of specific bands in SDS-PAGE and analysed using the Image lab™ Software (Bio-Rad, version 4.1) and a BSA standard curve as a reference protein. In addition, for analysing the purity of the proteins, the specific purified samples were loaded in Pro260 chips (Bio-Rad) and analysed by capillary electrophoresis using the Experion system (Bio-Rad), following the manufacturer's instructions. The Experion system resolved and quantified protein samples around 10 to 260 kDa in size with high sensitivity comparable to colloidal Coomassie blue SDS-PAGE gels staining.

Recombinant Fc-tagged proteins were purified from infected larva extracts using protein A agarose resin (ABT) following

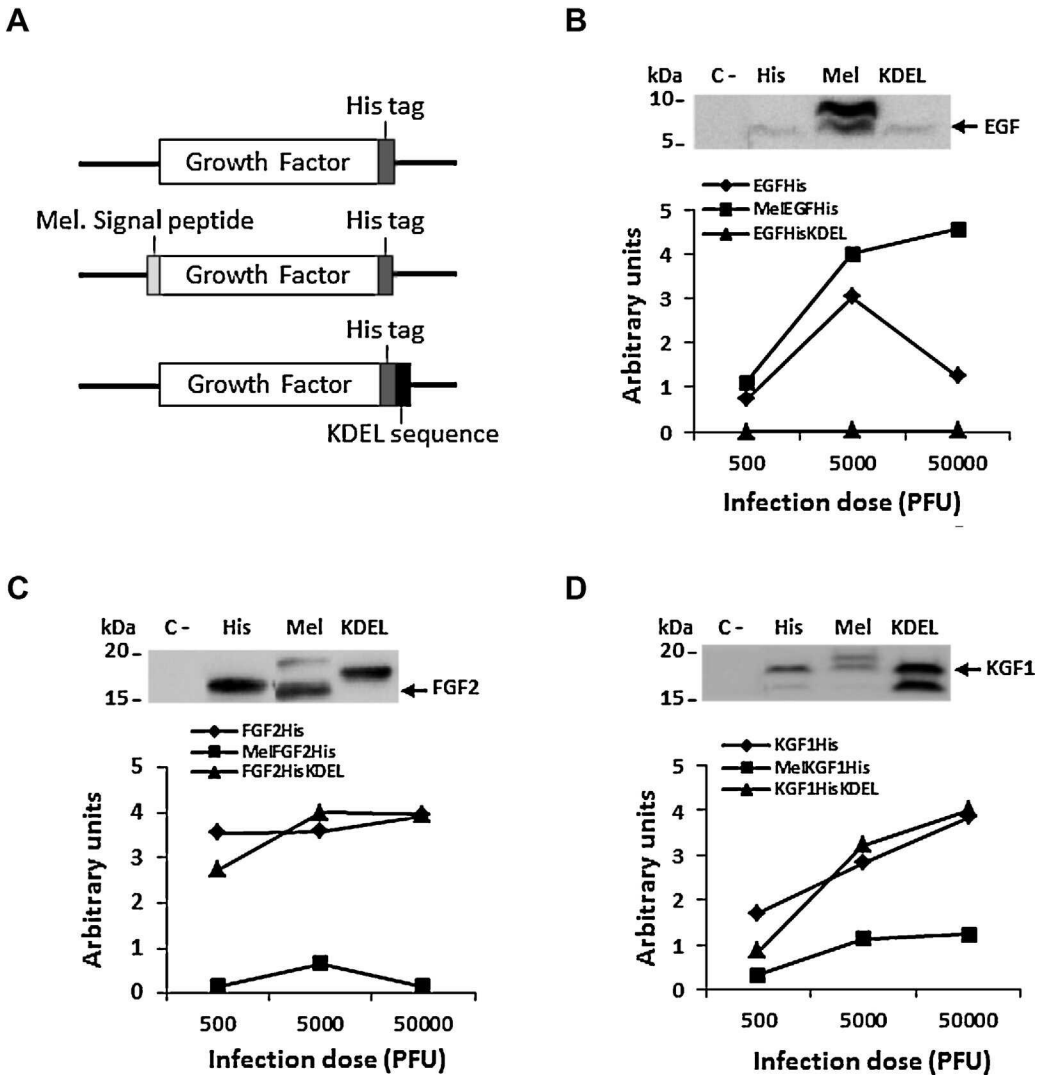

Fig. 1. Expression of human growth factors in *Trichoplusia ni* (*T.ni*) insect larvae used as living biofactories. (A) Schematic representation of three different constructs used for the generation of recombinant baculoviruses expressing the His-tagged huEGF, huFGF2 and huKGF2. (B) Expression of huEGF in larva detected by Western blot using an anti-His tag antibody and the expression levels obtained using different baculovirus infection doses to produce the protein. Expression levels were quantified from Western blots and expressed as arbitrary units. (C) and (D) represents the same experiments described in B but for huFGF2 and huKGF2, respectively. These experiments were used to determine the optimal conditions for expression of every recombinant growth factor.

manufacturers' instructions. Brieftly, TSP fractions from larva extracts were filtered by a 22 μm filter (Miracloth, Calbiochem®, Merck) and mixed in binding buffer (at a 1:1 ratio) with previously equilibrated resin. After 3 h of shaking at 4 °C, samples were washed twice with binding buffer (NaH_2PO_4, 20 mM, pH 7). Fc-tagged proteins were then eluted in elution buffer (Glycine 100 mM, pH 2,6) by shaking for 20 min. They were finally centrifuged at 2500 rpm and 4 °C and supernatants were neutralized by adding neutralizing buffer (Tris–HCl 1 M, pH 9) at ratio 1:10 to neutralize the pH of eluted fraction.

Fc tagged proteins were also quantified by densitometry using a standard curve of known quantities of purified Fc protein known quantities as a pattern.

2.7. Biological assays

2.7.1. Immunodetection of activated ERK 1+2

The HaCaT, NIH-3T3 or NHDF cells (10^5/ml) were incubated in serum free medium for 24 h and then stimulated for 30 min with purified recombinant growth factors for 30 min at the indicated concentrations. Cells were then washed with PBS and proteins extracted in 50 μl of lysis buffer (20 mM Hepes pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaF, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA) and 30 μg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 Å at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 °C, and immunodetection of the phosphorylated MAPK ERK1+2 was carried out with using the anti-p-ERK 1+2 mAb sc-7383 (Santa Cruz Biotechnology, San Diego, CA, USA). The membranes were re-blotted with the anti-ERK 1+2 mAb M5670 (Sigma Co, St. Louis, MO, USA) that recognized the non-phosphorylated form of this MAPK.

2.7.2. Cell proliferation assay

The HaCaT, NIH-3T3 or NHSF cells were seeded in 96-well plates at 5×10^3 cells/well, and 24 h later incubated in serum-starved for

16 h to inactivated cell proliferation. Then the cells were stimulated with increasing concentrations of purified growth factors for additional 48 h. Cellular proliferation was assessed by the MTT method. Briefly, 50 μl of a mixture containing MTT (5 mg/ml) was added to each well and incubated for 3–4 h. The medium was then removed and dimethyl sulfoxide (100 μl/well) was added to dissolve insoluble formazan crystals. Finally the optical density of each well was measured using a microplate spectrophotometer TriStar LB 941 (Berthold Technologies, GmbH & Co. KG) at a 550 nm wavelength. The absorbance of untreated cells was taken as 100% proliferation, and the percentage of proliferation was calculated as follow:

$$\%\text{proliferation} = 100 \times \frac{(\text{Treated cells} - \text{Blank wells})}{(\text{untreated cells} - \text{Blank wells})}$$

2.7.3. Wound healing assay

NIH3T3 cells were seeded into 24-well plates and grown to 100% confluence. Cells were serum-starved for 16 h and wounded by scratching the surface with a sterile pipet tips and cellular debris removed by washing with PBS. Cells were then stimulated with purified growth factors. Initial wounding and the movement of the cells in the scratched area were photographically monitored using a Leica DFC300FX microscope (10×).

3. Results

3.1. Production of recombinant human growth factors in Trichoplusia ni larvae

Different constructs of the recombinant human epidermal growth factor (huEGF), the human basic fibroblast growth factor (huFGF2) and the human keratinocyte growth factor (huKGF1) sequences (Fig. 1A) were synthesized and cloned into a pFastBac1™ baculovirus transfer vector. The plasmids obtained were named pEGFHis, pMelEGFHis, pEGFHisKDEL; pFGF2His, pMelFGF2His, pFGF2HisKDEL; pKGF1His, pMelKGF1His and pKGF1HisKDEL. These plasmids were used to obtain the corresponding recombinant baculoviruses (bacEGFHis, bacMelEGFHis, bacEGFHisKDEL, bacFGF2His, bacMelFGF2His, bacFGF2HisKDEL, bacKGF1His, bacMelKGF1His and bacKGF1HisKDEL) as described in the methods section. These baculoviruses were tested for their productivity in the baculovirus/insect larva system. For this purpose, 50,000 pfu of each virus were inoculated into the insect larvae. After 72 h postinfection, TP extracts from infected larvae were obtained and analysed by SDS-PAGE and Western blot using an anti-His monoclonal antibody. Recombinant baculoviruses harboring the huEGF, huFGF2 and huKGF1 encoding genes expressed in larvae tissues reactive proteins with the anti-His antibody of around 7.5 kDa, 18.3 kDa and 20.1 kDa, respectively (Fig. 1B, C and D). However, additional bands of a slightly higher or lower mobility were also observed in the extracts containing some of the versions of the GFs generated. Baculoviruses containing the constructs MelEGFHis, MelFGF2His and MelKGF1His (with the honeybee melittin signal peptide at the 5′end of the gene) were more productive for huEGF and huFGF but not for huKGF (Fig. 1B, C and D). In the case of huEGF, Melittin signal peptide increased expression levels in around 30 folds (Fig. 1B). In contrast, the introduction of the KDEL endoplasmic reticulum retention signal in the constructs of the different GFs made more efficient only the baculovirus expressing the huKGF, increasing productivities in about 3 times with respect to the other constructs (Fig. 1D). Recombinant baculoviruses bacMelEGFHis, bacFGF2His, bacFGF2HisKDEL, bacKGF1His and bacKGF1HisKDEL expressed the GFs in a way that could be solubilized from TP fractions by a treatment with a mild extraction buffer to keep functional the recombinant molecules. Percentages of GFs solubility obtained by the most productive baculoviruses are shown in Table 1.

Table 1
Comparison of recombinant huGFs quantities found in the TP and in the TSP larvae extracts using different expression constructs.

	MelEGFHis	FGF2His	KGF1HisKDEL
Biomass (TP) (mg/g)	9.1 (±1.9)	2.6 (±0.3)	0.8 (±0.2)
Biomass (TSP) (mg/g)	4.1 (±0.9)	1.9 (±0.2)	0.4 (±0.1)
Solubilization (%)	45%	74%	49%
Purity (%)	96.9%	98.7%	95%
	MelEGFMFcS	FGF2MFcD	MelKGF1MFcS
Biomass (TP) (mg/g)	5.61 (±0.73)	1.45 (±0.41)	3.05 (±0.31)
Biomass (TSP) (mg/g)	2.78 (±1.00)	1.22 (±0.42)	1.67 (±0.20)
Solubilization (%)	49% (±14%)	85% (±15%)	55% (±2%)

Next step was the optimization of the soluble GFs production using different baculovirus infectious doses to infect the insect larvae (500, 5000 or 50,000 pfu). Most baculoviruses expressing the three GFs presented a peak of productivity when the maximum virus dose was used (50,000 pfu). The only exceptions were bacEGFHis and bacFGF2HisKDEL which showed the maximum productivity when 5000 pfu was used to infect the insects (Fig. 1B). Taken together the production yields obtained after infection with the different doses assayed and the larvae mortality rates shown in each case (not shown), we selected a inoculation dose of 5000 pfu/larvae to infect the larvae with the baculoviruses bacMelEGFHis, bacFGF2His and bacKG1HisKDEL to produce the proteins to be purified for functionality experiments.

3.2. Purification and quantification of His-tagged growth factors

Recombinant His-tagged GFs were purified in a single step by immobilized metal affinity chromatography (IMAC) from total soluble protein (TSP) extracts. Purified GFs were characterized by Coomassie blue staining and Western Blot assay using an anti-His mAb. In both cases, bands of the expected molecular weight for each recombinant GFs were detected (Fig. 2).

In the case of the huEGF, a band of higher molecular weight was also detected in the TSP (Fig. 2A). However, only one band was shown after protein purification. In contrast, the purified huFGF2 also showed an additional band with higher molecular weight (around 35 kDa), not detected previously either in the TP or in the TSP containing this factor (Fig. 2B). Finally, the purified huKGF1 showed several bands of lower electrophoretic mobility, probably corresponding to proteolytic forms (Fig. 2C).

The resulting purified proteins were also analysed by microfluidic chips using the Experion™ Automated Electrophoresis System. It determined a purity level of 97%, 99% and 95% for huEGF, huFGF2 and huKGF1, respectively. Purified GF, quantified using standard BSA curves in SDS-PAGE, were used to determine the expression yields of huEGF, huFGF2 and huKGF in the TSP fractions and expressed in relation to 1 gram of insect biomass. These were determined as 4,1, 1,9 and 0,5 mg, respectively. Quantities corresponded to the arithmetic mean of three independent production batches (Table 1).

3.3. Bioactivity of larvae-expressed GFs

3.3.1. Recombinant GFs increased ERK phosphorylation and cellular proliferation

The MAPK extracellular signal-regulated kinase (ERK) is one of the main downstream signalling cascade components in response to different growth factors. Accordingly ERK 1 + 2 is phosphorylated in response to huEGF (Abdull Razis et al., 2008), huFGF2 (Mu et al., 2008) and huKGF1 (Ron et al., 1993). To demonstrate that our GFs produced in larva were fully functional we stimulated three different types of dermal cells (HaCaT, NIH3T3 and HNDFs) with those factors and we analysed the steady state of total and

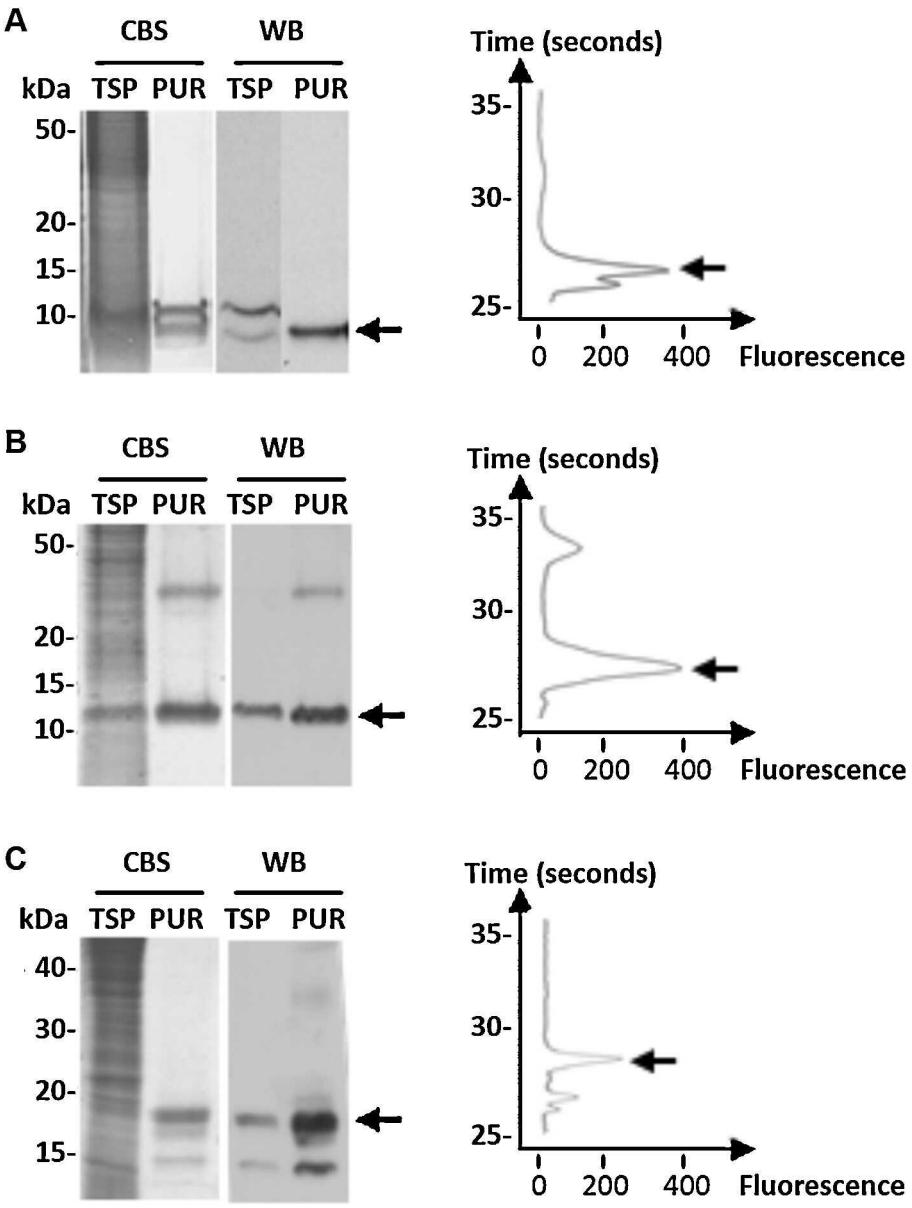

Fig. 2. Purification of different human growth factors produced in insect larvae. The figure shows Coomassie blue staining (CBS) or Western blot (WB) of 60 μg of total soluble protein extracts (TSP) from larvae or 10 μl of purified (PUR) fractions obtained from TSP extracts using cobalt IMAC affinity chromatography. (A) MelEGFHis; (B) FGF2His; (C) KGF1HisKDEL. All purified GFs were analysed by microfluidic electrophoresis chips using Experion™ (Automated Electrophoresis System). Arrows indicate the purified bands of every recombinant protein. Homodimers were detected in the purified huFGF2.

phosphorylated ERK 1 + 2 by immunoblots. As depicted in Fig. 3A EGF, FGF2 and KGF1 induced ERK 1 + 2 phosphorylation in HaCaT, NIH-3T3 and HNDFs, respectively.

Since the ERK 1-2 pathway plays a key role in GFs-induced keratinocyte and fibroblast proliferation we stimulated serum-starved HaCaT, NIH-3T3 and HNDFs with increasing concentrations of EGF, KGF1 and FGF2, respectively, and proliferative measured after 48 h. In Fig. 3B we show that the three GFs induced cellular proliferation in a concentration dependent manner.

3.3.2. *Recombinant growth factors increased fibroblast motility*

Wound healing assay was performed and analysed to investigate the effect of huFGF2 and huEGF on epithelial cell migration. The chemotactic motility of fibroblasts treated with 10 ng/ml of each GF is shown in Fig. 3C. Recombinant purified huFGF2 and huEGF as well as 10% FCS clearly promoted cell motility since epithelial cells migrated towards the gap and an almost complete closure of the healing in 48 h.

3.4. *Fusions of growth factors to Fc immunoglobulin fragments*

In order to further optimize the yields of the recombinant human GFs production in insect larvae, the encoding sequences of huEGF, huFGF2 and huKG1 molecules were cloned in-frame to the crystallizable fragment (Fc) of the human antibody IgG. Different baculoviruses were obtained as described in the methods section to assay the optimal construct.

From all baculovirus tested, only those expressing the huKGF1 presented an increase in productivity with respect to the above mentioned baculoviruses (data not shown). The recombinant Fc-fused huKGF1 constructs were clearly identified in a specific western blot assays using larvae extracts and an anti-Fc from IgG1

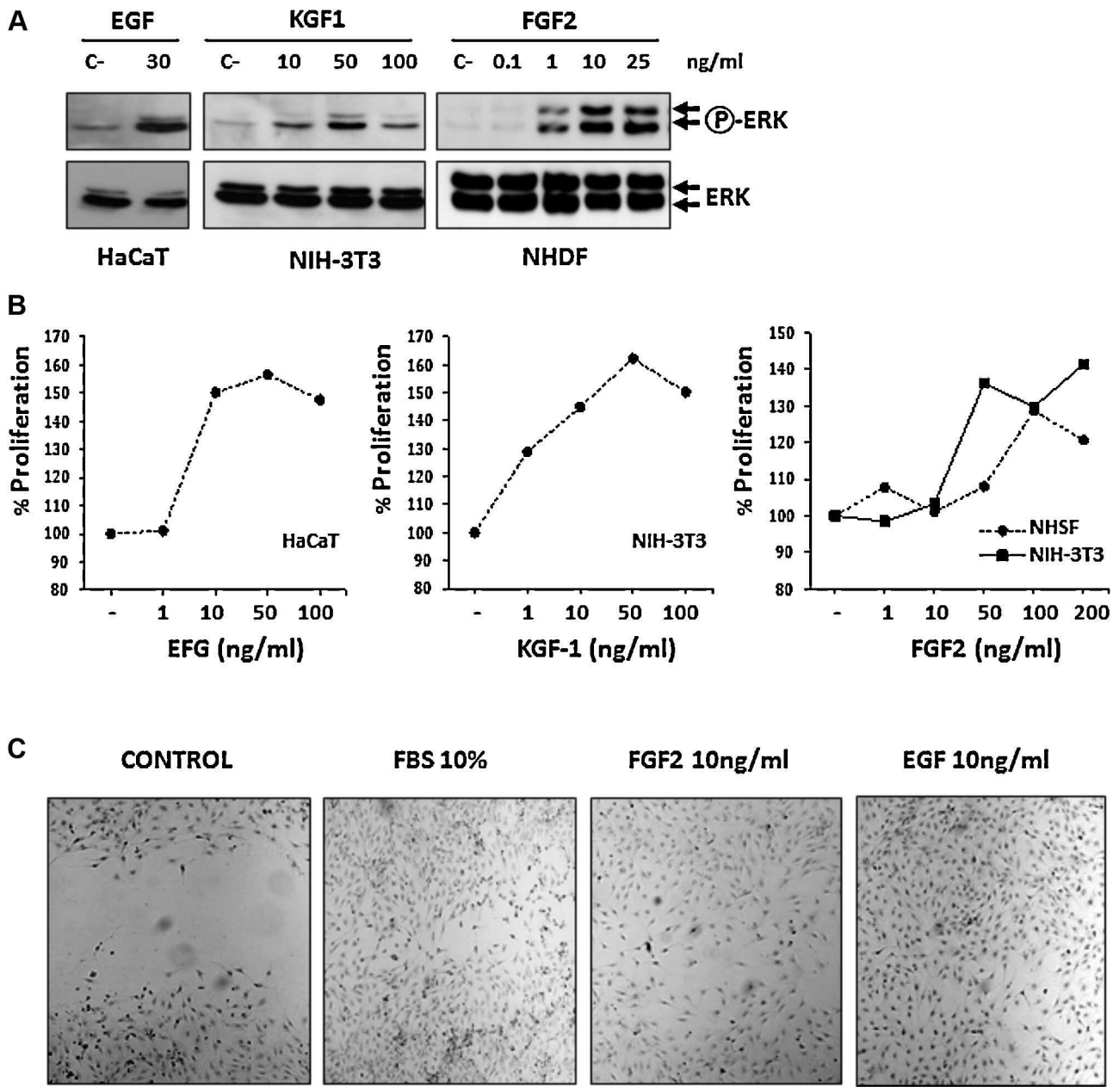

Fig. 3. Bioactivity assay of growth factors produced in insect larvae. (A) Serum-starved HaCaT, NIH3T3 or NHSF cells were incubated with the indicated concentration of recombinant huEGF, huFGF2 or huKGF1 for 30 min and the phosphorylation of ERK 1 and 2 analysed by Western blots (indicated by arrows). BacNI (baculovirus vector without any inserted gene) was used as negative control (C−). (B) The cells were stimulated with increasing concentrations of the recombinant growth factors and the proliferative activity measured by the MTT assay. (C) Scratched NIH3T3 monolayers were treated with serum free medium (control), medium containing 10% FCS, huFGF2 or huEGF and migration into the wound was photographed after 48 h of culture (10× amplification). Results are representative of three independent experiments.

antibody, showing the expected electrophoretic moiety of around 47 kDa and 45 kDa for the KGF1FcHinge^{+} and for the KGF1FcHinge^{-}, respectively (Fig. 4). Bands of higher molecular weight were also detected when they were expressed with the Mel signal peptide, probably due to post-translational modifications of the fusion protein. Finally, several minor bands of lower electrophoretic mobility were also observed probably corresponding to proteolytic forms of the protein.

Good expression yields were observed in the TP fractions from infected larvae inoculated with 5000 pfu of every recombinant baculovirus and analysed at 72 hpi. However, the recombinant bacMelKG1Hinge^{-} was the most productive in terms of expression and solubilization of this GF (Fig. 4).

To quantify the recombinant huKGF1 produced after fusion with the Fc fragment, TP fractions from larvae infected with each baculovirus were analysed by 12% SDS-PAGE and Western Blot using an anti-IgG1 mAb. A standard curve of known quantities of purified Fc protein fragment was used. An analysis of reactive bands by densitometry allowed us to estimate that productivities of around 0.7 mg, 0.1 mg, 1.6 mg or 3.1 mg of recombinant huKGF1 protein per gram of insect biomass were obtained with bacKGF1FcHinge^{+}, bacKGF1Hinge^{-}, bacMelKGF1Hinge^{+} and bacMelKGF1Hinge^{-}, respectively. Quantities corresponded to the arithmetic media of three independent experiments. This result indicated that the fusion of huKGF1 to the Fc fraction was able to increase 3 times its productivity with respect to the non-fused

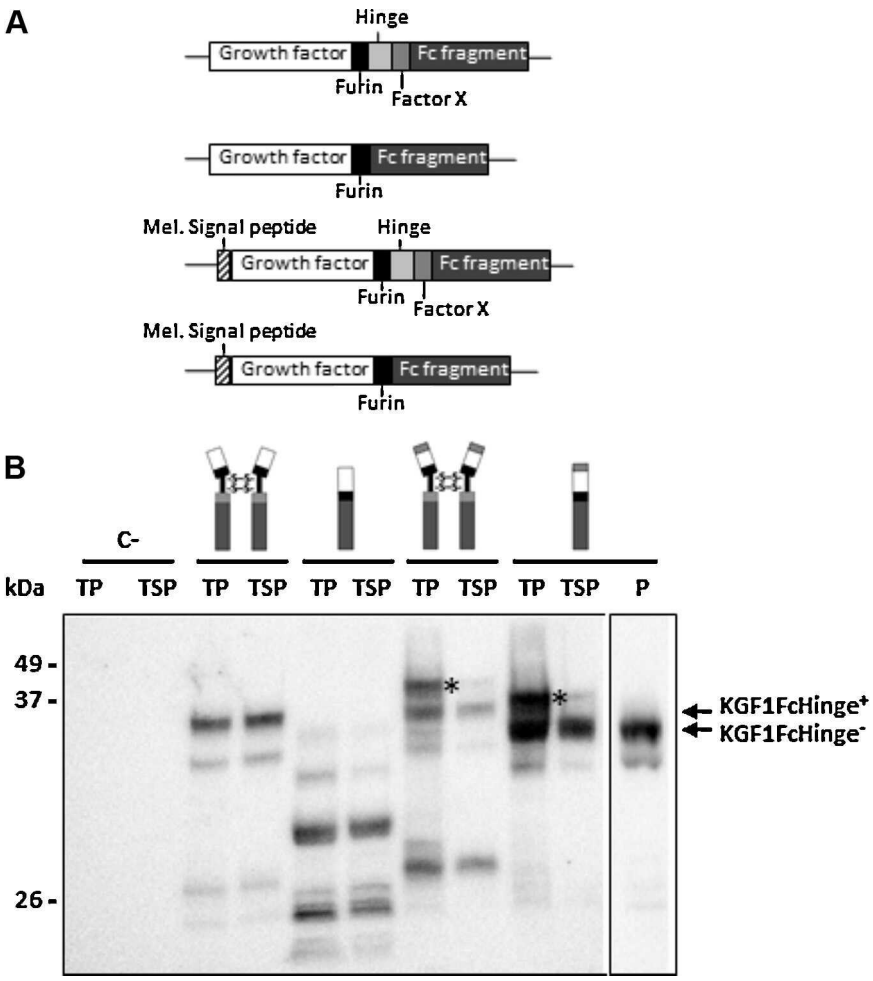

Fig. 4. Comparison of the chimeric huKGF1 Fc-tagged expression using different constructs. (A) Schematic representation of four different constructs used for the generation of recombinant baculoviruses expressing the huKGF1 Fc-tagged protein. (B) Ten μg of TP or total soluble protein (TSP) fractions from infected larvae with the recombinant baculoviruses bacKGF1FcHinge$^+$, bacKGF1FcHinge$^-$, bacMelKGF1FcHinge$^+$ or bacMelKGF1FcHinge$^-$ were resolved in 12% glycine SDS-PAGE gels and revealed using a human anti-IgG1 monoclonal antibody. This figure also shows the reactive pattern of 10 μg of MelKGF1Hinge$^-$ purified recombinant protein by using Protein A agarose beads (P), and obtained from a TSP extract from infected larvae. Arrows indicate the signal peptide processed and non-processed forms of each construct. Extracts from BacNI-infected larvae were used as negative control (C−).

factor (Table 1). The fusion protein KG1Hinge$^+$ was the most soluble (in about 90%), while the other fusion proteins were soluble in lower percentages (43% for KG1Hinge$^-$, 28% for MelKG1Hinge$^+$ and 48% for MelKG1Hinge$^-$).

Considering production yields, solubility ratios and larvae mortality after infection with every baculovirus (data not shown), all together, the recombinant baculovirus bacMelKG1FcHinge$^-$, used at 5000 pfu/larvae, was selected for further production of this recombinant Fc-tagged GF. To carry out the purification of the MelKG1Hinge$^-$ recombinant protein produced in larva (TSP fraction), protein A agarose beads were used in a single step process. The Fc-tagged KGF1 purified protein was detected by Western Blot assay using an anti-IgG1 monoclonal antibody and showed a major band of the expected molecular weight (45 kDa; Fig. 4).

4. Discussion

Growth factors are determinant molecules for a normal control of the cellular proliferation and differentiation. During the past decade, various studies have evaluated the role of a single or multiple growth factors combinations in a controlled cellular environment. These studies demonstrated the importance of growth factors as therapeutic agents (Morito et al., 2009), for damaged tissues repairing (Pastore et al., 2008) or in reducing the signs of facial aging skin (Mehta and Fitzpatrick, 2007; Sundaram et al., 2009). However, cost production of growth factors has limited a widely market expansion of these compounds.

Living biofactories such as plants, transgenic mammalians and insects represent feasible alternatives to the fermentation systems to produce recombinant proteins, providing excellent results (Gil et al., 2007). Taking into account the efficiency and production costs, the technology based on the use of baculovirus in combination with insect larvae represents a very good alternative (32, 35, 39, 42, 45, 49). Some comparative estimations among baculovirus-based production systems rendered about 10-fold reduction in fixed investments and cost of goods when insect larvae are used as biofactories with respect to the use of insect cells in bioreactors (unpublished results). Additionally, this technology also facilitates the production scaling-up and reduces the development times of the molecules (Gil et al., 2001). In the present work, we have demonstrated the successful expression in insect larvae of three growth factors, (huEGF, huFGF2 and huKGF1) using *T. ni* insects as living biofactories. Their bioactivity has been also demonstrated by analysing their ability to induce dose-dependent responses in fibroblast or keratinocyte cells.

Different approaches were evaluated for optimizing the recombinant protein production. The GF sequences were expressed with the signal peptide of Honeybee melittin (Mel) which can enable

efficient translocation of proteins into the endoplasmic reticulum and could increase the amount of soluble protein in the cytosol (Tessier et al., 1991). We also studied how the fusion of the molecules to a endoplasmic reticulum retention signal (KDEL), which, by the retention of the recombinant proteins into the endoplasmic reticulum reduces its degradation, may improve the GFs production yields (Pichon et al., 1997). The use of Mel and KDEL to express recombinant proteins in *T. ni* larvae has been previously reported benefits in their production yields (Gómez-Sebastián et al., 2012; Gomez-Casado et al., 2011). Additionally, two types of tags were used in the present work, and were preselected based on their potential use for protein detection and/or purification. Those corresponded to His-tag and the Fc fragment of human IgG1. These systems can also lead to an increase in solubility and yield of the recombinant protein expressed (Muraki and Honda, 2010).

The recombinant huEGF, huFGF2 and huKGF1 proteins were expressed in larvae as protein forms of around 7.5 kDa, 18.3 kDa and 20.1 kDa, respectively, as it was expected. However, in some constructs, protein forms of higher electrophoretical moiety (around 10.2 kDa, 21.1 kDa or 23.0 kDa, respectively) were also detected, specially when the *Honeybee* melittin signal peptide was used. These other bands could be explained by the glycosylation level of the protein as it has be shown in other studies (Gómez-Sebastián et al., 2012) or to a lack of signal peptide processing in part of the recombinant protein expressed. In the case of the huFGF2 homodimer formation as a result of the protein concentration during the purification process (Fig. 3B), has been previously described (Kwan et al., 2001). Interestingly, the addition of the KDEL sequence to FGF2 increased its electrophoretic mobility more than in the other GFs, probably by the modification of its moiety.

In terms of solubility of each GF produced in larvae (presence of the recombinant protein in the TSP fraction), every protein presented different results (Fig. 2). In the case of the huEGF the use of Mel signal peptide was clearly the best option with a solubilization ratio around 45%. Regarding huFGF2 and huKGF1 constructs solubilization ratios were around 84% (FGF2His) and 65% (KGF1HisKDEL). Considering the protein solubility and mortality ratios of infected larvae (data not shown) obtained with every construct, the most productive baculoviruses in larvae were bacMelEGFHis, bacFGF2His and bacKGF1HisKDEL and the optimal inoculation dose was fixed for all constructs in 5000 pfu/larvae. With these constructs we obtained approximately a total productivity of 9.1 mg, 2.6 mg or 0.8 mg per gram of insect biomass, respectively. From these productivities, the soluble fraction of each protein was approximately of 4.1 mg, 1.9 mg or 0.5 mg per gram of insect biomass (Table 1). These data can be translated to 1.2 g, 0.3 g or 0.1 g (from TP) or 0.5 g, 0,2 mg or 0.06 g (from TSP) per liter of insect extract in order to facilitate the comparison with conventional expression systems based on bioreactors.

Previous expression studies of huEGF produced in *Escherichia coli* (Smith et al., 1982; Oka et al., 1985), *Bacillus subtilis* (Flock et al., 1984), *Bacillus brevis* (Yamagata et al., 1989) or yeast (Clare et al., 1991; Urdea et al., 1983) rendered maximum productivities of recombinant huEGF of 250 mg per liter. Recombinant huFGF2 was produced in bacteria (Ron et al., 1993) or in yeast (Mu et al., 2008) with a maximum yield of around 150 mg/l. Less information is available from the huKGF1 production, probably due to the difficulties found with its efficient expression. Maximum productivities reported were of around 17 mg/l (Luo et al., 2004). In the present work, we increased productivities by using insect larvae of around 2-folds for huEGF and huFGF2 or 3-folds for huKGF1 with respect to current production systems.

The analysis of the biological activities of the larva-derived GFs revealed that those were identical to that found in these molecules obtained by other methodologies. The larva-derived GFs retained their capacity of inducing the phosphorylation of mitogen-activated protein kinase (MAPKs), especially the extracellular signal-related kinase 1/2 (ERK 1/2) which has been reported to play a pivotal role in a range of cellular functions, including cell growth, proliferation, migration, protein synthesis and transcription procedure. As shown in Fig. 3, ERK 1/2 phosphorylation was enhanced by all of the recombinant purified-growth factors at a concentration of 30 ng/ml in the case of the huEGF, or at concentrations higher than 10 ng/ml in the case of huFGF2 or huKGF1. These results are comparable with other studies (Abdull Razis et al., 2008; Mu et al., 2008; Ron et al., 1993). Moreover, cell proliferation measured by MTT assay confirmed the biological activity of each growth factor at concentrations of 10 ng/ml or less (including 1 ng/ml for the KDEL construction of huKGF1) (Fig. 3B). Finally, a wound-healing *in vitro* assay confirmed the induction of cell proliferation and repair of cell-induced physical damage in epithelial cell cultures by all the three purified growth factors (Fig. 3C).

With the aim of incrementing the huKGF1 production, the GF poorly expressed by the other construct strategies, we designed other expression strategy based on the fusion to a Fc immunoglobulin fragment. The human IgG1 Fc region has been successfully used as a fusion polypeptide to improve the expression of a GF maintaining its functionality (Heo et al., 2002). Four expression vectors coding for various versions of Fc fragment transcriptionally fused to the huKGF1 encoding gene were generated. The first one, consisted on the use of the entire human Fc sequence (Hinge$^+$). The second one, fused to the Fc sequence without Hinge region (Hinge$^-$) for avoiding the natural homodimere formation. Both constructs were expressed fused or not with the *Honeybee* melittin (Mel) signal peptide. The use of this signal peptide could somehow improve the expression of the Fc-fused protein as a result of a better folding of the Fc that includes some glycosylation sites into its sequence.

The Fc-tagged huKGF1 without Hinge region, expressed with the Mel signal peptide, had expression yields of around 3 folds higher than the His-tagged huKGF1 protein (1.6 mg of soluble protein per gram of larva biomass). This means around 0.2 g of soluble huKGF1 per litre of insect protein extracts. This represents a significant improvement (10-folds) of huKGF1 with respect to previously reported productivities (Luo et al., 2004).

Furthermore, the protein produced by the baculovirus expressing the MelKGF1Hinge$^-$ construct was easily purified by one-step protein A-Sepharose affinity chromatography (Fig. 4), as described for other Fc-fused GFs (Ogiwara et al., 2005). This construct included a specific protease cleavage sites, Furin and Factor *X* (Aurell et al., 1977; Esmon et al., 1974), to facilitate, in case that it was necessary, the liberation of the huKGF1 from the Fc fragment.

In conclusion, we have demonstrated the successful expression of three functionally active human growth factors (huEGF, huFGF2 and huKGF1) in insect larvae of *T. ni*. at levels never reached previously by conventional means. Furthermore, in the case of huKGF1, a GF more difficult to be produced, we improved in around 10-folds its production yield by its fusion to a Fc immunoglobulin fragment. Then, we have demonstrated that the use of insects (*T. ni*) as living biofactories in combination with baculovirus expression vectors provide an alternative non-fermentative system for the efficient production of human growth factors efficiently, open the possibility of this technology for their large scale production needed in some applications like regenerative medicine or cosmetics.

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