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HEPARIN-BINDING EGF–LIKE GROWTH FACTOR AS A PROSPECTIVE MEDIATOR OF TISSUE REPAIR AND REGENERATION

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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family. It contains EGF-like and heparin-binding domains allowing interactions with heparin and cell-surface heparin sulfate. [1]

It can activate human epidermal growth factor receptors 1 and 4 resulting in a multitude of cell responses, including cellular proliferation, migration, adhesion, and differentiation. HB-EGF levels can increase in response to different forms of injuries as well as extracellular stimuli, such as lysophosphatidic acid, retinoic acid, and 17β-estradiol [2].

HB-EGF plays a critical role in tissue repair and regeneration. It promotes cutaneous wound healing, hepatocyte proliferation after partial hepatectomy, intestinal anastomosis strength, alveolar regeneration after pneumonectomy, neurogenesis after ischemic injury, bladder wall thickening in response to urinary tract obstruction, and protects against ischemia/reperfusion injury in many cell types [1, 3, 4].

Aim. To obtain bioactive recombinant human HB-EGF and investigate its proliferation capacity.

Methods. p*ET*32(a)-HB-EGF cloning plasmid was extracted from *E. coli* DH10B cells by GeneJet Plasmid miniprep kit. *E. coli* BL 21 (DE3) Rosetta were transformed with p*ET*32(a)-HB-EGF by electroporation.

Clone-producer of recombinant protein was cultivated in LB medium with ampicillin at 37 °C with intense aeration. The expression of HB-EGF was induced under 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and intense aeration at 30 °C for 3-4 h.

The HB-EGF was purified by metal-affinity chromatography with Co^{2+}-NTA. Recombinant 6His-TRX-HB-EGF was eluted with a Wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, pH 8,0) that contained 250 mM imidazole. Purified HB-EGF was analyzed using 10% SDS–PAGE. Proteins in the gel were detected by staining it with Coomassie Brilliant Blue G-250.

Interaction of HB-EGF with receptors was analysed by conducting flow cytometry. A431 cells were grown to confluence and were detached from Petri dishes with 20 mM EDTA in phosphate-buffered saline (PBS). Cells were incubated with 10 μg EGFP and 10 μg EGFP-HB-EGF for 30 min at 4 °C. After incubation, cells were washed and resuspended in PBS with 1% BSA and 0.01% NaN_3. The fluorescence intensities on FITC-A channels were measured with DxFLEX Flow Cytometer.

The effect of HB-EGF on the proliferation capacity of the 3T3 and L929 fibroblast cell lines was tested using the MTT assay. Cells' suspension was added to a 96-well plate with a concentration of 25000 cells/well in RPMI-1640 containing 10% fetal bovine serum. After 24 hours, the medium was replaced with the fresh fetal bovine serum-free culture medium containing 50, 500 and 1000 ng/mL of HB-EGF. After 48 or 72 hours, MTT reagent (0.5 mg/mL) was added for 3–4 h to evaluate cell proliferative activity.

Results and Discussion. To obtain high-quality therapeutically applicable HB-EGF, an optimized culturing and processing protocol for protein yielding and purification was required. For this

purpose, several technologies have been combined in this study: plasmid purification and *E.coli* strain transformation, optimizing the process of affinity chromatography for His-tag recombinant protein purification.

Firstly, we optimized the protein extraction protocol to obtain a high amount of bioactive HB-EGF. Several adjustments were performed to improve the protocol of affinity chromatography purification. These steps include choosing Co^{2+}-NTA instead Ni^{2+}-NTA due to the level of concentration HB-EGF in eluate, modifying Wash Buffer, and preparing the sample for chromatography using additional enzymes and protease inhibitors. These optimization steps provided us with a high amount of HB-EGF of high purity, which was further validated with SDS-PAGE (Fig. 1).

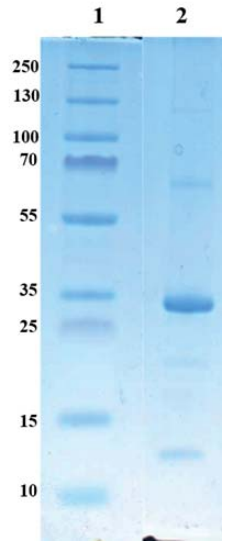

Fig. 1. **SDS–PAGE of the purified recombinant HB-EGF**
Lane 1: Molecular weight markers, kDa. Lane 2: 6His-TRX-HB-EGF

Fig. 2. **HB-EGF interaction with receptors in A431, analyzed with flow cytometry:**
A — A431 cells incubated with EGFP; *B* — A431 cells incubated with EGFP-HB-EGF

The activity of the obtained protein was further tested in the mouse embryonic fibroblast cell line (3T3), mouse fibroblast established from the subcutaneous areolar and adipose tissue (L929) and human epidermoid carcinoma cell line (A431) using MTT assay and flow cytometry.

With flow cytometry, we validated that EGFP-HB-EGF binds to surface receptors of A431 cells (Fig. 2, *A, B*).

HB-EGF ability to promote cell proliferation was analyzed with 3T3 and L929 cell lines using MTT assay. Cell treatment with HB-EGF noticeably affected the growth of 3T3 cells. Exposure of these cells to 500 ng/mL HB-EGF for 48 h induced a 67% increase in optical density in wells, indicating a higher cell proliferation rate (Fig. 3). A higher concentration of HB-EGF of 1000 ng/mL further augmented cell proliferation resulting in a 112% increase in cell population (Fig. 3, *A*). These results indicate that the recombinant HB-EGF stimulates the proliferation of the 3T3 fibroblast cells in a dose-dependent manner. As a control, we used the L929 cell line, which does not express excess

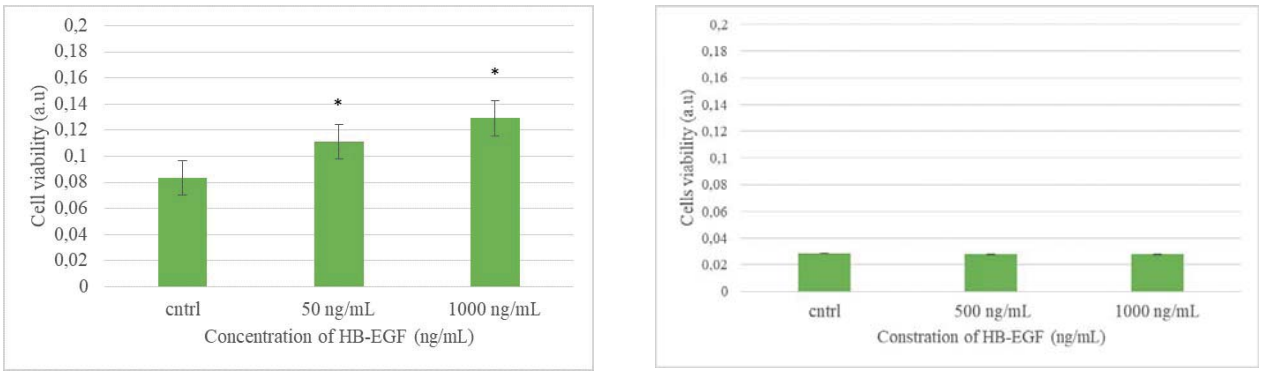

Fig. 3. **hb-egf promoted the proliferation of 3t3 fibroblast cells (*A*) but not l929 fibroblast cells (*B*):** *A* — 3t3 cells were treated with 500 and 1000 ng/ml hb-egf; *B* — l929 cells were treated with 500 and 1000 ng/ml hb-egf. Cell proliferation was measured as a change in the optical density of the cell population using mtt assay.
* $P < 0,05$ compared to control

receptors to HB-EGF as much as the 3T3 cell line. As expected, we did not observe any changes in the cell proliferation of L929 when they were treated with 500 and 1000 ng/mL of HB-EGF for 48 hours (Fig. 3, *B*).

It provides further pieces of evidence that cell HB-EGF actively regulates a wide array of biological functions, including cell proliferation, thus, providing an excellent potency for therapeutic use in wound healing.

Conclusions. We have optimized protocol of obtaining and purification a high yield of biologically active HB-EGF in *E. coli*. Its bioactivity was further validated in the A431 and 3T3 cell lines, where HB-EGF could interact with receptors and strongly increase cell proliferation. This biologically active HB-EGF holds potential for further studies as a prospective mediator of tissue repair and regeneration.

Key word: HB-EGF, recombinant protein, proliferation, tissue repair.

Authors' contribution. AD conducted plasmid purification and transformation, protein expression, purification, samples preparation for flow cytometry, performed data analysis, and wrote manuscript; AS cultivated cell culture, flow cytometry analysis and edited the manuscript; DK suggested the study and reviewed the manuscript.

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