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Human Eb Peptide: Not just a By-product of Pre-pro-IGF1b Processing?

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Abstract

Several physiological activities have been assigned to E-peptides derived from pre-pro-insulin-like growth factor (IGF1) processing; however, the whole range of the E-peptides' functions is still unknown. The objective of this study was to investigate human Eb peptide (hEb) in terms of its bioactivity, cellular localization, and intracellular trafficking using human cancer cells. Human Eb fused with red fluorescence protein (RFP) or green fluorescence protein (GFP) localizes strongly to nucleoli and to a lesser extent to nuclei of HeLa and U2-OS cells. Mutagenesis of hEb nucleolus localization sequence (NoLS) leads to its partial delocalization from nuclei and nucleoli to cytoplasm of transfected cells. Thus, NoLS is not sufficient for the hEb to be localized in nucleoli of the cells and a different mechanism may be involved in hEb targeting. A BrdU ELISA showed that the proliferation index of cells expressing hEb hybrid proteins increased up to 28 %. For comparison, the same assay was performed using HeLa cells treated extracellularly with synthetic hEb. A significant increase in the proliferation index was observed (41–58 % for concentrations ranging from 10–100 nM, respectively). Additionally, a cell migration assay was performed using stable U2-OS cell lines expressing hEb fused with RFP or RFP alone as a negative control. The migration index of hEb expressing cells was 38.3 % greater. The increase in cell proliferation index and in motile properties of hEb expressing cells demonstrate that hEb is more than a pre-pro-IGF1b processing product, and has intrinsic activity of biological significance.

Keywords

NoLS; nucleolus; protein trafficking; stable cell lines; proliferation and motility index

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Conflict of Interest

The authors do not have any conflict of interest.

Introduction

Insulin-like growth factor (IGF1) is a small protein with very potent actions. Despite its small size, the human *Igf1* gene, located at chromosome 12, extends over 85 kb. The gene comprises 6 exons separated with long introns. Exon 1 and 2 are differentially spliced to exon 3, producing alternative class 1 and class 2 transcripts. Exons 5 and 6 also demonstrate alternative splicing patterns. This gives rise to 6 IGF1 precursors: class 1A and 2A contain exons 3–4 and 6 of the transcript and form the IGF1-Ea isoform with C-terminal Ea extension peptide. Class 1B and 2B contain exons 3–5 (IGF1-hEb isoform – Fig. 1j) and C isoform (IGF1-Ec) arises from an internal splice site within exon 5, which joins 49 nucleotides of exon 5 with exon 6 [1]. All these propeptides undergo subsequent proteolytic processes and eventually result in one mature 70 amino acid long IGF1 protein encoded by exons 3 and 4, which is secreted from many tissues, and can be released into the bloodstream. The physiological role of alternative E peptides generated from IGF1-Ea, IGF1-Eb, and IGF1-Ec still needs more clarification, but they have been implicated in a variety of biological activities [2–4]. The longest of all human E-peptides is hEb, which is 77 amino acid long (it is even longer then IGF1 itself) and derives from IGF1 gene splicing pattern exon1/2-exon3-exon4-exon5. The first 16 residues are encoded by exon 4 (common to Ea and Ec domains) and the reminder by exon 5. It has been reported that this particular splice pattern with a long C-terminal extension overlapping exon 5 is only present in human and nonhuman primates [5]. It has been previously suggested that different E-peptides may have functions distinct from mature IGF1 [6, 7] and hEb can be further processed by protease cleavage to give rise to 2 distinct “sub-peptides” called IBE1 and IBE2. The former was shown to have mitogenic activity using synthetic analogue Y-23-R-NH_2 on normal and malignant bronchial epithelial cells [8]. On the other hand, it has also been shown that hEb inhibits growth of human breast cancer cells and invasion in vitro [9]. The aim of this study was to assess hEb in terms of its potential bioactivity (motogenic and mitogenic) and its cellular localization. We report here that hEb enhances cell growth of HeLa and U2-OS cells and increases motile properties of stable U2-OS cells.

Materials and Methods

Human Eb constructs

To study the role of the hEb, 2 cDNAs of different length were cloned into 3 mammalian expression vectors: pAcGFP1-C1, pAcGFP1-N3, and pDsRed2-C1 (Clontech, Montain View, CA, USA). These vectors allow for expression of 3 hybrid protein: RFP-C-hEb, GFP-C-hEb, and GFP-N-hEb (Fig. 1j). Two hEbs of different length were chosen for the current study, one composed of 61 amino acids (exon 5: 135Y to K195 of pre-7yopro-IGF1b) and the longer hEb consisting of 77 amino acids (encoded partly by exon 4 and exon 5: 119R to K195 of pre-pro-IGF1 (http://www.ncbi.nlm.nih.gov/protein/NP_001104755.1) [1]. The term hEb used in the current paper correspond to exon 4/5 splice variant, unless otherwise stated.

PCR reaction was performed in a final volume of 20 μl containing: 1 μM of each oligonucleotide (Table 1); 0.6 mM $MgCl_2$, 1 × KCl buffer for *Taq* polymerase, 0.4 U of *Taq* polymerase (Fermentas International, Burlington, Canada), 2 mM of each dNTP and about

10 ng of human cDNA. All PCR reactions were performed in Biometra T-gradient thermocycler under the following conditions: (1) predenaturation at 95 °C for 5 min, (2) denaturation at 95 °C for 30 s, (3) annealing at 55 °C for 30 s, (4) elongation at 72 °C for 30 s, and (5) final elongation at 72 °C for 7 min. Steps 2–4 were repeated 25 times. PCR products lengths were 183 bp for shorter and 231 bp for longer hEb version.

PCR products and vectors listed above were double-digested by *Kpn* I and *Bam* HI restriction enzymes as suggested by manufacturer (Promega, Fitchburg, WI, USA). The cloning procedure was performed in 12 μl in the following way: 6 μl of 2 × rapid ligation buffer for T4 DNA ligase, 1 μl of vector with sticky ends (50 ng), 4 μl of PCR product with sticky ends (25 ng) and 1 μl of T4 DNA ligase (3 Weiss units/μl) were incubated at room temperature for 1 h. All reagents for cloning were purchased from Promega. DNA sequencing of resulting genetic constructs was performed with BigDye Terminator v3.1 on a ABI Prism 3130XL analyzer (Applied Biosystems, Foster City, CA, USA). Sequence chromatograms were checked for accuracy, contigs edited, and then assembled using FinchTV 1.3.1 (Geospiza Inc., Seattle, WA, USA), and Gen-Doc 2.7.000 [10]. The nucleotide sequences were compared with GenBank sequences by using the BLAST program.

Synthetic hEb

Human Eb (based from GenBank: X56774.1) was synthesized using a Thuramed TETRAS 106 peptide synthesizer, and purified via HPLC >95 % (Shimadzu LC-10AT pump). The final product was confirmed by MALDI mass spectrometry (WATERS ZQ 2000 Mass Spectrometer & Shimadzu LCMS-2010EV). All these steps were performed by Novazym Polska (Poznan, Poland). The scramble peptide was created as described earlier [2].

Cell lines and cell transfections

HeLa and U2-OS cells were transfected with constructs expressing hEb tagged with RFP or GFP protein using FugeneHD transfection reagent (Promega). HeLa are epithelial adenocarcinoma HPV-18 positive cells from cervix, whereas U2-OS cell line derives from bone cancer cells (osteosarcoma). Co-transfections were performed with 1:1 weight/molar ratio of constructs. Usage of fluorescent hybrid protein allows for observation of hybrid protein cellular localization. For hEb localization assays, after trypsinization cells were counted using hemocytometer and 2×10^5 cells were seeded into the wells of the 6-well plate. The next day, a mixture of 2 μg and 8 μl of FugeneHD reagent in 100 μl of DMEM medium was added to the cells in DMEM medium supplemented with 10 % FBS and 100 U of ampicillin/streptomycin. All these cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA). After 24 h, cells were washed 3 × times with PBS, fixed with 3.8 % paraformaldehyde, washed again 3 × times with PBS, and stained with DAPI for laser confocal microscope observation (Zeiss LSM 510, Faculty of Biology, A. Mickiewicz University, Poznan, Poland) or fluorescence microscope (X-CITE SERIES 120PC or Leitz DM RBE, University of Pennsylvania, Philadelphia, USA).

Co-localization assays of hEb and nuclear structures

For co-localization assays of hEb and nuclear bodies 2 strategies were followed: co-transfection of constructs (in case of hEb, human SC-35 splicing factor and human nucleolin) and immunostaining of transfected cells (in case of promyelocytic leukemia and Cajal nuclear bodies, PMLs and CBs, respectively). Co-transfections were carried out in the same manner as single transfections, for details see previous section. For staining the Cajal bodies, rabbit anti-coilin antibody at 1:500 dilution was used as a primary antibody (a kind gift from Prof. G. Eric Blair), and to stain promyelocytic leukemia protein (PML speckles), a mouse monoclonal antibody anti-PML at 1:500 dilution was used as a primary antibody (Santa Cruz Biotechnology, CA, USA). Secondary anti-mouse and anti-rabbit antibodies were coupled to Alexa Fluor 488 and contained in SFX Kit (Invitrogen, Carlsbad, CA, USA), supplemented also with Image-iT FX signal enhancer. All antibodies were diluted in PBS with 1 % w/v BSA. Otherwise, sample preparations for microscopy were the same as described in the previous section.

Site-directed mutagenesis of nucleolar localization sequence

In order to verify to what extent hEb nucleolar localization sequence (NoLS) is required for the hEb hybrid protein to be solely localized within nucleolus and nucleus, wild-type NoLS sequence KKGK was mutagenized to KGGK (single hEb mutant) and to GGGK (double hEb mutant). The set of oligonucleotides used can be found in Table 2. The PCR amplification and bacteria cells transformation were performed following the instruction manual (QuickChange Site-Directed Mutagensis Kit, Agilent Technologies, Santa Clara, CA, USA). PCR reaction was carried in Verti 96 Well thermal cycler (Applied Biosystems, Foster City, CA, USA). Plasmid extractions were performed using Qiagen Plasmid Mini and Qiagen Plasmid Midi kits (Valencia, CA, USA). The same methodology for cell transfection and observation was used for localization assays of human wild-type and mutagenized hEb as described in the previous section.

BrdU ELISA proliferation assay

In order to carry out BrdU enzyme-linked immunosorbent proliferation assay (Cell Proliferation ELISA, BrdU; Roche Applied Science, Penzberg, Upper Bavaria, Germany) HeLa and U2-OS cells were transfected with genetic constructs (pAcGFP1-C1-hEb, pDsRed2-C1-hEb, pAcGFP1-N3-hEb) and control plasmids (pAcGFP1-C1, pDsRed2-C1, pAcGFP1-N3). Background controls were untransfected cells and cells not treated with BrdU, for which OD values did not exceed 0.2 as recommended in manufacturer's guidelines. Briefly, 5×10^3 cells were seeded per well in a 96-well plate. The next day the cells were transfected as described in Cell Transfection section with minor modifications: 2 μg of DNA and 8 μl of FugeneHD were mixed with 100 μl of DMEM medium and only 5 μl of the final mixture was used to transfect cells in each well. At least 8 wells of a 96-well plate were used for each assay of background control cells, negative control cells, and study cells transfected with constructs containing hEb cDNA sequence. BrdU assays were continued only if approximately 80–90 % of transfected cells were observed by fluorescence microscopy 24-h post-transfection, then the cells were incubated with BrdU for 6 h

following the instructions of the manufacturer. Each BrdU assay was performed 4–6 times: both for HeLa and U2-OS cells, and for all 3 different constructs generated for this study.

The same BrdU ELISA proliferation assay was performed using HeLa cells treated with synthetic hEb generated especially for this study, with a scramble peptide as a negative control [2], and a human recombinant IGF1 (Gemini Bio-Products, West Sacramento, CA, USA) as a positive control. Briefly, 5×10^3 cells were seeded per well in a 96-well plate, and the next day cells were starved for 6 h in serum-free DMEM media and treated with peptides or recombinant IGF1 overnight. Three different concentrations of each compound were tested: 10, 30, and 100 nM. Each assay was carried out 3 times, 8 replicates for each condition.

The principle of the Cell Proliferation ELISA assay is incorporation of BrdU into genomic DNA, then the quantity of incorporated BrdU is measured using an anti-BrdU antibody coupled to horse-radish peroxidase. Thus, the proliferation index of cells is expressed by absorbance values in colorimetric assay using an ELISA reader (Tecan Sunrise Basic, AMU, Poznan, Poland or Spectra Max M5, Molecular Devices, University of Pennsylvania, Philadelphia, USA).

Stable cell lines production and cell migration assay

For the cell migration assay stable cell lines expressing hEb were generated. Only pDsRed2-C1 vector was used as it had been suggested that GFP protein exhibited cytotoxic activity while expressed in a long-term manner [11]. After transfection of U2-OS and HeLa cells with pDsRed2-C1-hEb construct or pDsRed2-C1 alone as described in Cell Transfection section, cells were grown in DMEM medium containing geneticin (G-418). After massive death of cells (2 weeks), the surviving cells were diluted and seeded in a 96-well plate in the way to obtain 1 cell per well. Single cells were cultured for a couple of weeks and transferred onto bigger dishes for subsequent experiments. The cell migration assay was performed as described previously [12, 13] with modifications in 24-well Transwell plates (Transwell Permeable Support, 8 μm polycarbonate membrane, 3 422, Costar, Corning Incorporated, Acton, MA, USA). Cells overexpressing RFP-C1-hEb hybrid protein in a stable manner and cells expressing RFP alone were counted using hemocytometer and 2.5×10^4 cells were loaded onto the upper chamber in serum free DMEM medium and DMEM supplemented with 10 % FBS was added to the lower chamber as a chemoattractant. After 16 h of incubation, the cells on the upper side of the well were wiped with Q-Tips and the cells on the lower side were fixed with 4 % formaldehyde for 5 min, and washed 3 times with PBS. The membranes were cut out of the wells, transferred onto the glass slides cell side submerged in DAPI liquid (VectaShield mounting medium for fluorescence H-1200, Vector Laboratories, Burlingame, CA, USA) and covered with cover slips. Nuclei and cells were photographed from 20 nonoverlapping microscopic fields from 3 separate replicates per condition using a 20 × objective (X-CITE Series 120PC or Leitz DM RBE, University of Pennsylvania, Philadelphia, USA).

Calculations, statistics, and bioinformatics

All calculations of SD (standard deviation) were performed using Excel (Microsoft Office 2010). For BrdU ELISA proliferation assays performed on HeLa cells treated with synthetic peptides and recombinant IGF1, a 2-way ANOVA followed by a Bonferroni posthoc test were applied. As for BrdU ELISA assays performed on cells transiently transfected, a 2-way ANOVA for cell line and hEb construct was utilized. A Bonferroni post-hoc test was performed for comparisons between the hEb constructs within each cell line, and between cell lines for each construct. For the migration assay, a paired *t*-test between each of 3 replicates of hEb expressing and vector only U2-OS stable cell lines was performed (20 fields for each replicate). Finally, for all BrdU ELISA assays and migration assays normalization to 1.0 was performed. All error bars represent SD. Significance was set to $p < 0.05$ for all comparisons.

All alignments and analysis of DNA sequences were performed using Align Sequences Nucleotide BLAST available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi and Chromas Lite software. In order to assess biophysical properties of hEb a Protein Calculator from http://www.molbiol-tools.ca was applied. For fluorescence profile comparison of cells transfected with mutagenized and wild-type hEb encoding constructs vs. original plasmid encoding only, fluorescent protein data were exported from the OpenLab software (3.5.1 version) as pdf files and replotted in Excel file.

Results

Cellular localization of hEb

To determine the localization of hEb within cells, we generated fusion constructs of hEb labeled either at the N- or C-terminus. The rationale for preparing both C- and N-fusions of GFP and C-fusion of RFP was first to track the hEb in cells and be able to co-stain the same cells with a different fluorescent dye in search of cellular structures harboring hEb and second, to capture differential localization of the potential sub-fragments in case of hEb proteolysis as there are 2 putative cleavage sites (RRK and KKK) within the hEb giving rise to “sub” peptides [1, 8]. Also, to determine if cellular localization included the entire hEb or only one portion, we generated a series of constructs allowing for expression of 2 hEbs of different length. Constructs presented in details in Fig. 1j show exon 4/5 only version, similar constructs were also prepared containing exon 5 only encoded hEb. It has been previously shown that human IGF1 fused with GFP was localized to nucleoli of transfected cells if the GFP-IGF1 chimeras contained *IGF1* exon 5 [14]. We focused our attention on hEb in the absence of mature IGF1 to determine if nucleolar localization was dependent upon the whole sequence or only hEb. The hEb tagged with RFP protein was localized to several round/oval structures present in nuclei in HeLa and U2-OS transfected cells, where it was highly concentrated compared to the much fainter signal observed within the whole nucleus (Fig. 1e, h). As controls, the same cells were also transfected with vectors allowing only for expression of fluorescent proteins RFP and GFP. We observed red or green fluorescent signal dispersed in the whole cell for all control plasmids used in this study (Fig. 1b). The same localization pattern was observed while hEb was fused with GFP-C (Fig. 1a, d) and GFP-N (Fig. 1g). Further, when cells were co-transfected with constructs containing

hEb tagged with RFP-C and GFP-N, the proteins were completely co-localized. Expression of the shorter Eb fragment (encoded only by exon 5) fused with RFP-C, GFP-N, or GFP-C displayed similar cellular localization patterns to the large hEb (data not shown). In many cases the labeled structures harboring hEb were numerous in transfected cells (up to 7–8) and had a range of sizes. Therefore, we decided to clarify if other small nuclear structures such as promyelocytic leukemia nuclear bodies (PML) or Cajal bodies (CB) harbored hEb. A series of immunostaining assays with specific antibodies to PMLs and CBs were performed using transfected cells expressing hEb fused with RFP. No co-localization of RFP-hEb and CBs, or PMLs stained in green was observed in HeLa and U2-OS cells, and structures harboring human RFP-hEb were larger than CBs and PMLs (Fig. 2a–c, d–f). In the same cells, RFP-hEb and SC-35 splicing factor fused to GFP were co-expressed. SC-35 is known to be localized to very small SC-35 speckles distributed in the entire nucleus, but not to nucleoli [15]. We observed some weak co-localization for RFP-hEb and SC-35 in the entire nuclei (Fig. 2g–i), but there was no overlap between hEB and SC-35 in nucleoli. Finally, to specify the localization of the RFP-hEb within the nucleus, HeLa and U2-OS cells were co-transfected with a construct encoding human nucleolin tagged with GFP protein and a construct encoding hEb fused with RFP (Fig. 1j–l). We confirmed that hEb localization was nucleolar. Thus, the entire hEb was localized to the nucleolus. Further, fragments potentially generated by proteolysis were not detectable inside the cells because of the complete overlap between the N- and C- terminal fusion proteins in terms of cellular localization.

The C-terminus of hEb contains a putative nucleolar localization sequence [14]. To determine if this sequence directed hEb localization, HeLa and U2-OS cells were transiently transfected with hEb fusion proteins bearing one (KKGK → KGGK) or 2 mutations (KKGK → GGGK) of the putative nucleolar localization sequence of hEb (Table 2 and Fig. 1j). Site-directed mutagenesis of this sequence resulted in partial delocalization of hEb from its primary nucleolar site to both the nucleus and the cytoplasm (Fig. 3a–d vs. e, f). This delocalization was observed for both single and double NoLS mutants (Fig. 3a, b and c, d respectively). The extent of delocalization is represented in Fig. 3i, where the fluorescence intensity was plotted for comparison from different compartments of cells expressing wild-type hEb fused with RFP, single-mutagenized hEb fused with RFP, and RFP alone. However, for both single and double NoLS mutants, a significant portion of hEb was still retained in the nucleolus. Therefore, while the putative NoLS modulated the extent of nucleolar retention, it is not the sole regulator, and additional sequences are also important.

Bioactivity of hEb expressed in cells

In the present study, a BrdU proliferation assay was performed on transiently transfected HeLa and U2-OS cells expressing hEb. The same naïve cells were also treated with the synthetic hEb in extracellular manner. Results of proliferation assays are presented in Fig. 4a, c. For transient transfection studies, both cell lines exhibited significantly more proliferation with hEb expression (3–5 replicates, and n = 8 samples per condition). Specifically, transfection of pDsRed-C1-hEb caused a 28 % (1.28±0.17) increase for HeLa and a 21 % (1.21±0.09) increase for U2-OS cells. Effects on proliferation were observed to a lesser extent following transfection of pAc-GFP-N3-hEb, where HeLa cells still showed

significantly higher proliferation index (1.21±0.08) than untreated cells, yet U2-OS cells did not reach statistical significance. Finally, transfection of pAcGFP1-C1-hEb failed to cause any change in proliferation in either cell line. Thus, even though this construct displays similar cellular localization to the others, the construct design may have impaired the proliferative activity of hEb.

In a complementary experiment, HeLa cells were treated exogenously with a synthetic hEb. This strategy eliminated the presence of fluorescence protein, which seemed to alternate hEb activity, especially in case of GFP constructs. Moreover it helped us see if hEb could act from outside of cell. A significantly increased proliferation index was observed: 1.41±0.03; 1.51±0.06; 1.59±0.15 as compared to control for each of the following concentrations: 10, 30, and 100 nM, respectively. At 1 nM hEb there was no significant increase in optical density values, so this concentration was not pursued further. The effect of synthetic hEb on HeLa cells proliferation index was stronger as compared to hEb transiently expressed in the same cells. This effect was also dose-dependent within the given concentration range (Fig. 4c). These results confirm that hEb can act in extracellular manner and shows that its potency to stimulate cells to divide is stronger than the one observed in intracellular expression experiments.

To determine the effects of hEb on cell migration, stable cell lines were generated using the U2-OS cells expressing hEb fused with RFP. Stable HeLa cells lines could not be achieved, and so they were not evaluated in migration assays. In transwell assays there were 1.38±0.17 times more migrated U2-OS stable cells expressing hEb fused with RFP protein (U2-OS-DsRed-C1-hEb) than U2-OS cells expressing RFP alone (U2-OS-DsRed-C1) on the opposite side of the membrane (Fig. 4b).

Discussion

In a number of studies, the presence of growth factors has been identified in the nucleolus, which is a primary site of ribosomesubunit biogenesis, as well as many other biochemical processes [16–19]. Nucleolar Localization Sequences (NoLSs) are emerging as a predominant mechanism in the targeting of proteins to this nuclear site [20]. Earlier it was widely believed that protein localization in the nucleolus may be a result of interaction by high affinity binding to nucleolar core components such as ribosomal DNA, RNA or major protein components [21]. Our study shows that all hybrid proteins consisting of hEb and a fluorescent protein are localized to nucleoli of HeLa and U2-OS cells. While studying nuclear structures their dynamics has to be taken into consideration. Initially, we based our knowledge on the eukaryotic cells containing a single nucleolus, which may be misleading. After cell division, up to 10 smaller nucleolus-like structures may be observed and they can be easily mistaken for other smaller nuclear bodies abundant in nucleus. Certain cancer cells may also have multiple nucleoli. It can also be concluded from the current study that the observed nucleolar localization of the hEb is neither influenced by the type or fusion site of the fluorescent protein nor by the cell lines used. If hEb can be cleaved by proteases, this process could have been followed in cells co-transfected with 2 constructs, in which hEb is fused with GFP at N-terminus and RFP at C-terminus. This was observed neither in HeLa nor in U2-OS cells. All hybrid proteins generated for this study were always localized and

co-localized within the same nuclear structures, confirmed later to be nucleoli. This observation implies that there are no intracellular proteases able to cleave RRK or KKK sequences in HeLa or U2-OS cells or they are not good cleavage sites. If it is true for other cell lines and tissues remains an open question. Similar observations were made previously [14]. Tan and co-workers also suggested that a short sequence in the C-terminal part (KKGK) of the exon 5-encoded domain contained only in IGF1-b isoform is responsible for nucleolar targeting [14]. The KKGK sequence of hEb was mutagenized in our study and a partial delocalization from nuclear and nucleolar sites of the peptide was observed. Because hEb was not completely dispersed from the nucleoli, it suggests that additional mechanisms for hEb targeting exist. This is the case of proinsulin C-peptide, which lacks a classic NLS and deviates from many intracrine factors by its low pI, ~3.5. It has been reported that this 31-amino acid long peptide upon nuclear entry in the target cell, is trafficked to nucleoli, where ribosomal DNA (rDNA) generates ribosomal RNA (rRNA) [22]. Human Eb and insulin C-peptide have no sequence homology, though they are products of closely related pro-IGF1b and proinsulin processing, and they also have different chemical properties since hEb is very basic (pI = 11.5). It suggests that localization of these 2 sequences must occur through different mechanisms. Thus, it can be concluded that NoLS of certain nucleolar proteins is not sufficient for a protein/peptide to be directed to nucleoli. Other factors can also contribute to nucleolar localization such as a low pI (C-peptide case) allowing for interaction with histones, or quite on the contrary, it might be also a high pI (hEb case) allowing for a number of interactions with negatively charged molecules such as nucleic acids. This issue still needs to be studied more extensively. We did not expect that human Eb peptide would have any specific function/activity in the cell lines used in this study. There have been no previous reports concerning hEb expression in normal human cells or tissues. HaLa and U2OS cells were good candidates as they were of different origin, but shared a low IGF1 expression level. Therefore they could have been transfected with constructs encoding IGF-derived hEb. In future it would be interesting to test more cell lines of cancerous and noncancerous origin.

In the current study, we observed that intracellular expression of hEb fused with a fluorescence protein augments a cell proliferation index, especially in HeLa cells and to a lesser extent in U2-OS cells. Part of the difference lies in the slower doubling time of naïve U2-OS cells (30 h vs. 24 h for HeLa), and so over the course of a 6-h assay, the effects of hEb were more apparent in cells that divide more rapidly during the assay. The enhanced proliferation effects are consistent with observations made by Siegfried and co-workers [8], who reported a dose-dependent increase in growth of normal and malignant bronchial epithelial cells treated with synthetic Y-23-R-NH_2 peptide (IBE-1), although the peptide constitutes only a small portion of the full length hEb used in our study. These results are consistent with observations of naïve HeLa cells treated with a synthetic hEb in 3 different concentrations (10, 30, and 100 nM). Dose-dependent growth was also observed reaching 58 % at 100 nM of hEb as compared to nontreated HeLa cells. On the other hand, there has also been one report, in which the authors stated that a synthetic hEb inhibited anchorage-independent growth of established human cancer cells such as MDA-MB-231, HT-29, SK-N-F-1 and Hep-2 [9]. It can be concluded from these data that hEb can have different actions depending on the type of cells it is expressed in or targeted to. However, there are more data

supporting that hEb increases proliferation index. It was shown that the dose-response neurite outgrowth induced by hEb was produced via phospho-ERK activation (MAPK pathway) [23]. Here, interesting questions arise: is the human pro-IGF1-hEb secreted out of cell, processed while interacting with IGF1R of the target cell in order to release hEb, and then it can be directed to nuclear compartments as it is the case of C-peptide derived from proinsulin [19]? Or, does hEb never leaves the cell, in which it is originally produced? Or, is pre-pro-IGF1b processing is even more complex and both scenarios are legitimate? NoLS and a very high positive charge allowing for strong trafficking of hEb to nucleoli might support the second concept. That was also the rationale for intracellular expression of hEb to test its bioactivity. The current work also shows that hybrid proteins containing a fluorescent component and the study peptide may be a handy tool for cell proliferation BrdU assays; though it is crucial to test more than one combination of fluorescent protein vs. C- and N-terminus fusion as these factors may interfere with properties of the studied protein/peptide.

The cell migration assay was performed in a number of studies in order to compare changing motile properties of cells treated with chemicals of interest. It was also used to test motogenic properties of human and murine E-peptides. In the study by Mills and co-workers, it was shown that synthetic human 24 amino acid MGF (Mechano Growth Factor from IGF1c) acts as a motogenic factor for human precursor myogenic cells and may increase the migration index of these cells up to 100 % (for 25 ng/ml concentration) [24]. Our study showed that the hEb overexpression in osteosarcoma cells leads to an augmented cell motility of 38.3 %. Similar observations were made using murine Eb (not related to hEb) for treatment of C2C12 cells, the migration index was increased of over 50 % as compared to control [2]. To our knowledge the presence of 77 amino acid hEb was never demonstrated in vivo by a Western blot or any other immunotechnique. In one study it was reported that using a polyclonal antibody to Y-23-R-NH_2, an IBE_1-like immunoreactivity in human lung cancer cell lines by immunoblot analysis had been identified [8]. In a different study IGF1, most probably IGF1b as the only IGF1 isoform containing NoLS, was detected in nuclei of human cervical tissues in different stage of HPV-related cancer development by immunohistochemical analysis using an anti-IGF1 antibody [25]. It can be assumed from these 2 studies that hEb might be also involved in development of certain type of cancers; however further extensive research is required. In conclusion, the data cited above together with findings described herein support that hEb promotes cell division (HeLa, U2-OS) and increases cell motility rate (U2-OS). Nuclear/nucleolar localization of this peptide may support a concept that it is an independent factor having more functions to pre-pro-IGF1b processing (similar to C-peptide having an independent activity from insulin). Moreover, putative NoLS present in hEb has to be complemented by other mechanisms, so hEb can be efficiently targeted to nucleoli.

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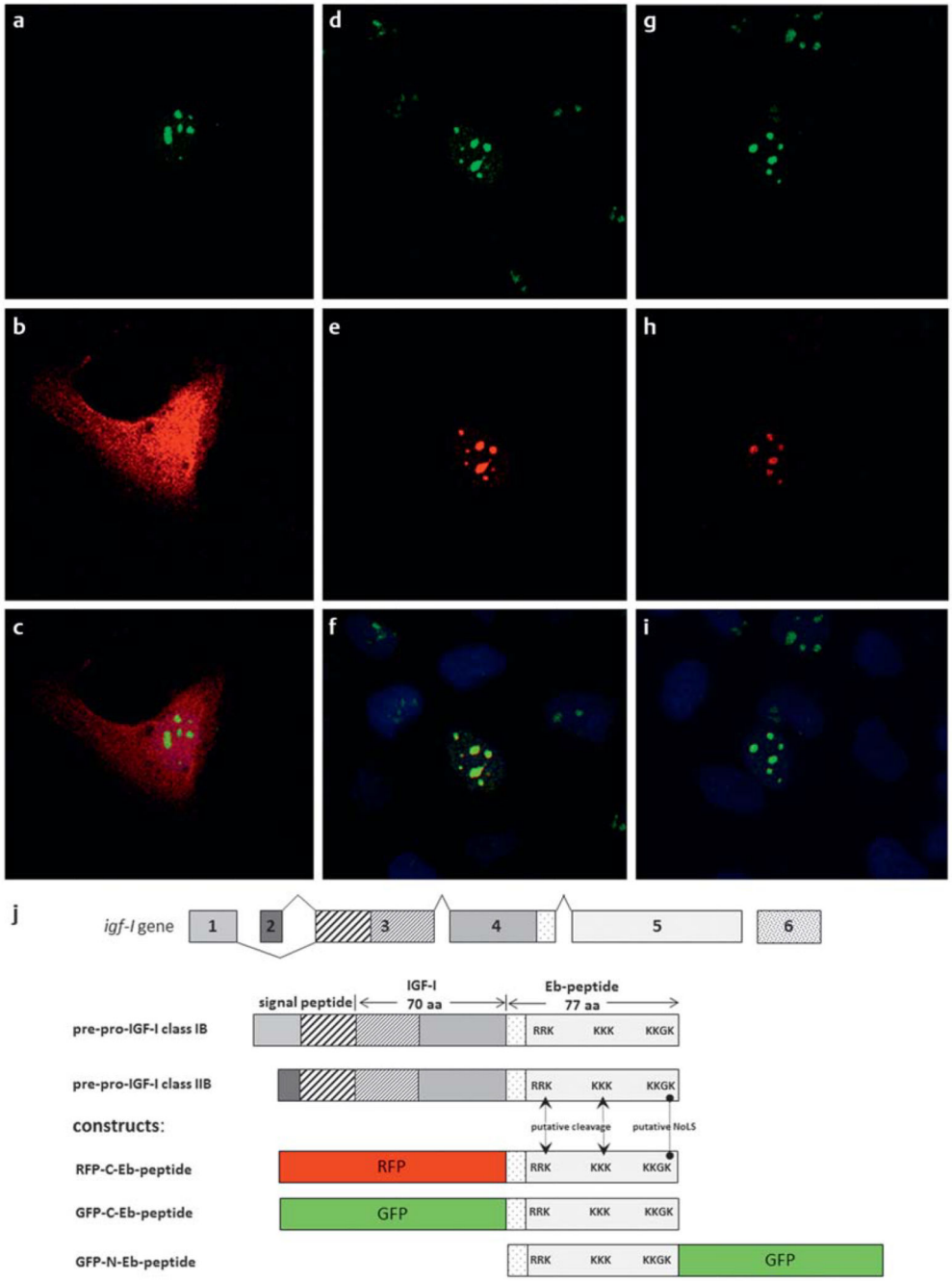

Fig. 1.

Transiently co-transfected HeLa cells observed under a confocal microscope. **a** cell expressing GFP-C1-hEb; **b** RFP-C1; **c** merge of A + B and DAPI staining; **d** cell expressing GFP-C1-hEb; **e** RFP-C1-hEb; **f** merge of D + E and DAPI staining; **g** GFP-N3-hEb; **h** RFP-C1-hEb; **i** merge of G + H and DAPI staining; and **j** schematic representation of *IGF1* gene composed of 6 exons, 5 of which are spliced to be translated into pre-pro-IGF1b. Approximate localization of putative cleavage sites as well as NoLS sequence are shown within hEb fragment. Also, hybrid proteins used for the study are drawn.

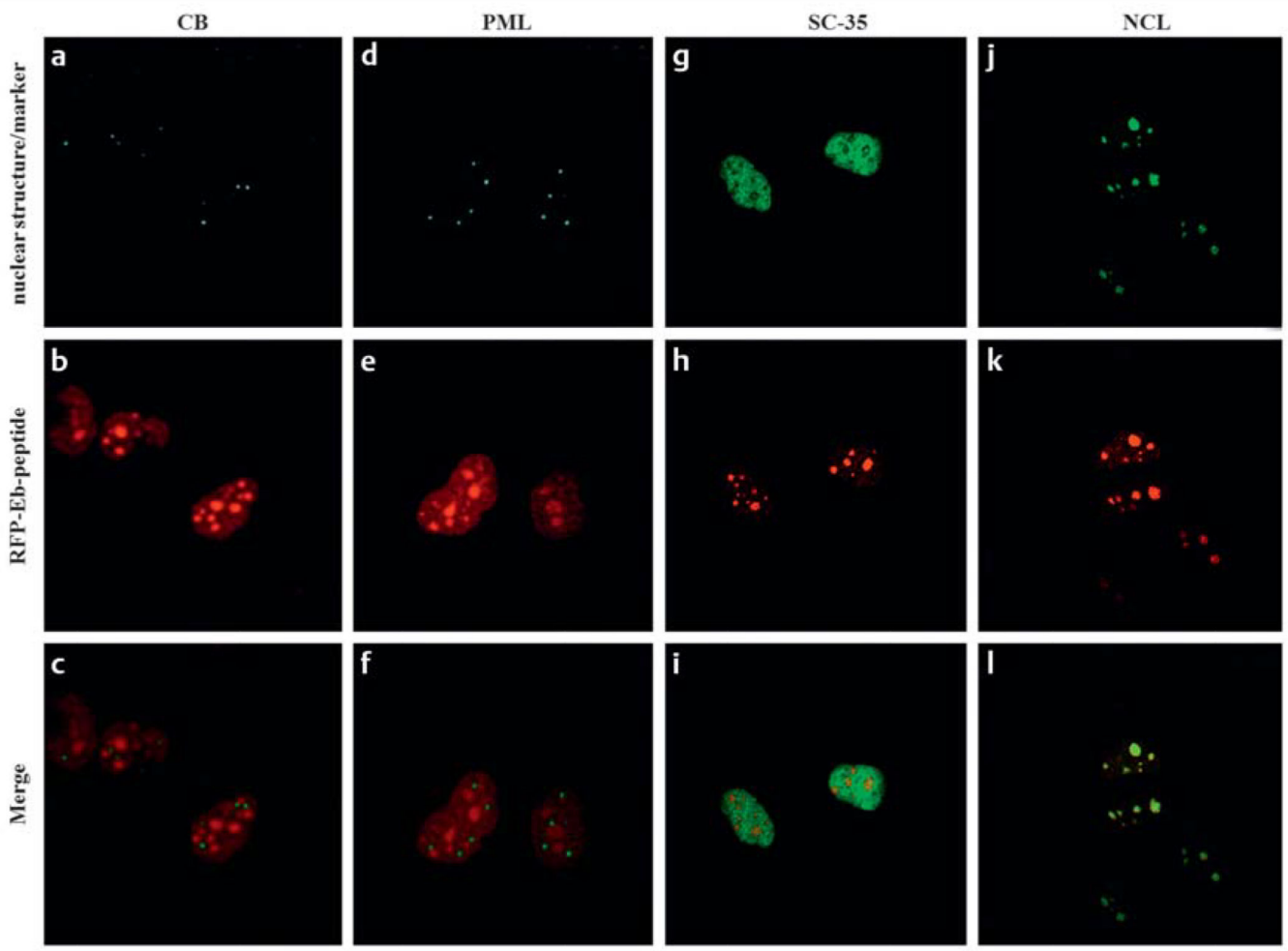

Fig. 2.

Transiently transfected HeLa cells observed under a confocal microscope. **a–c** cells expressing RFP-hEb stained for Cajal bodies (in green); **d–f** cells expressing RFP-hEb stained for PML bodies (in green); **g–i** co-transfected cells overexpressing RFP-hEb and human SC-35 splicing factor fused with GFP; and **j–l** co-transfected cells overexpressing RFP-hEb and human nucleolin fused with GFP.

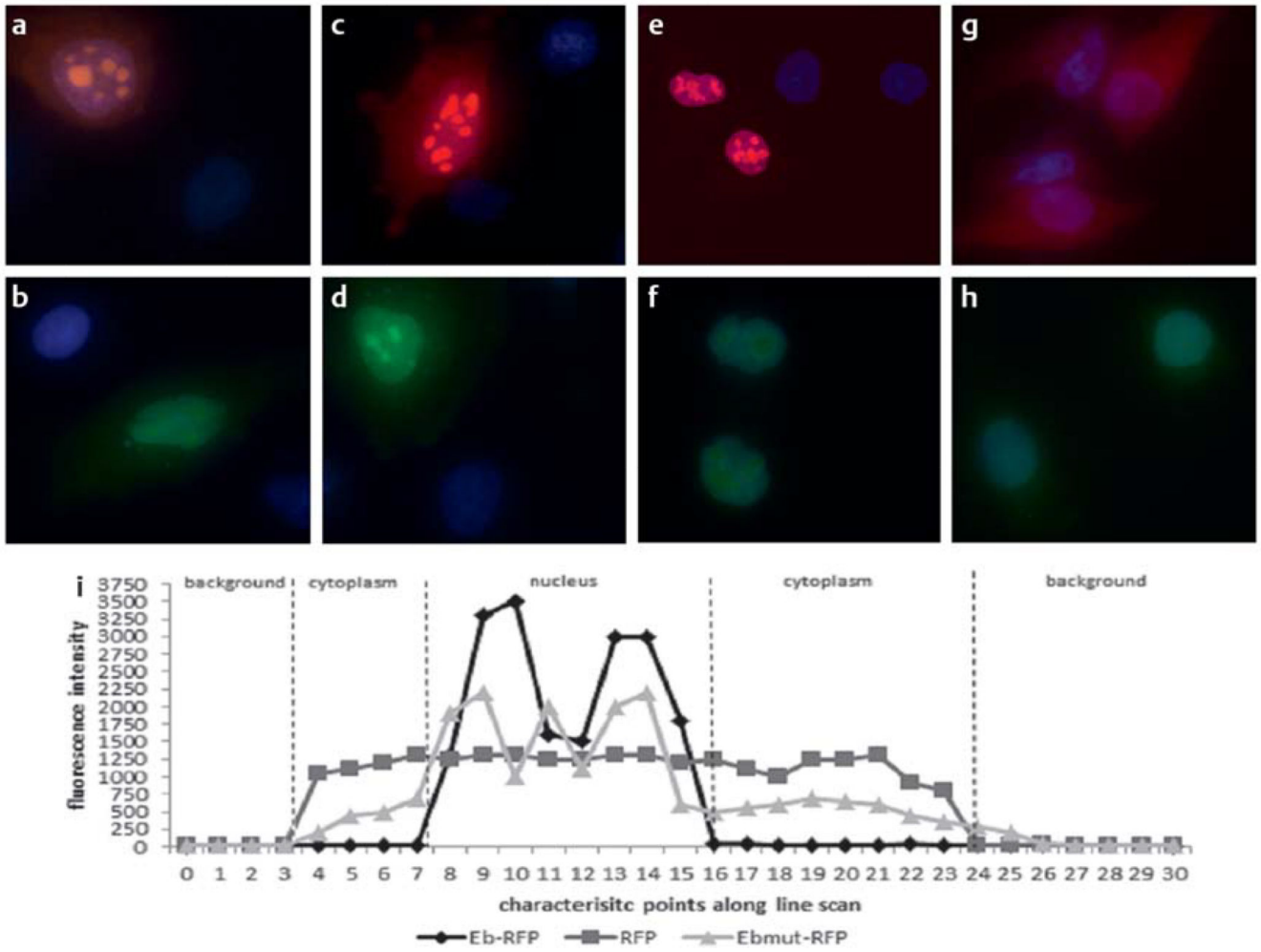

Fig. 3.

Transiently transfected HeLa cells observed under a fluorescence microscope. **a** single mutant of nucleolus localization sequence (NoLS) in RFP-C1-hEb, merged with DAPI; **b** single NoLS mutant of GFP1-C1-hEb, merged with DAPI; **c** double NoLS mutant of RFP-C1-hEb, merged with DAPI; **d** double NoLS mutant of RFP-C1-hEb, merged with DAPI; **e** wild-type RFP-hEb expressing cells, merged with DAPI; **f** wild-type GFP-hEb expressing cells merged with DAPI; **g** cells transfected with control pDsRed-C1 vector; **h** cells transfected with control pAcGFP1-C1 vector; **i** cell fluorescence intensity along line scan obtained from OpenLab software: HeLa cells transfected with pDsRed2-C1-hEb (hEb-RFP), single mutant of pDsRed2-C1-hEb (Ebmut-RFP) and pDsRed2-C1 (RFP). Due to different cell sizes the same number of measurements was taken from each cell compartment, 30 points in total for each line scan.

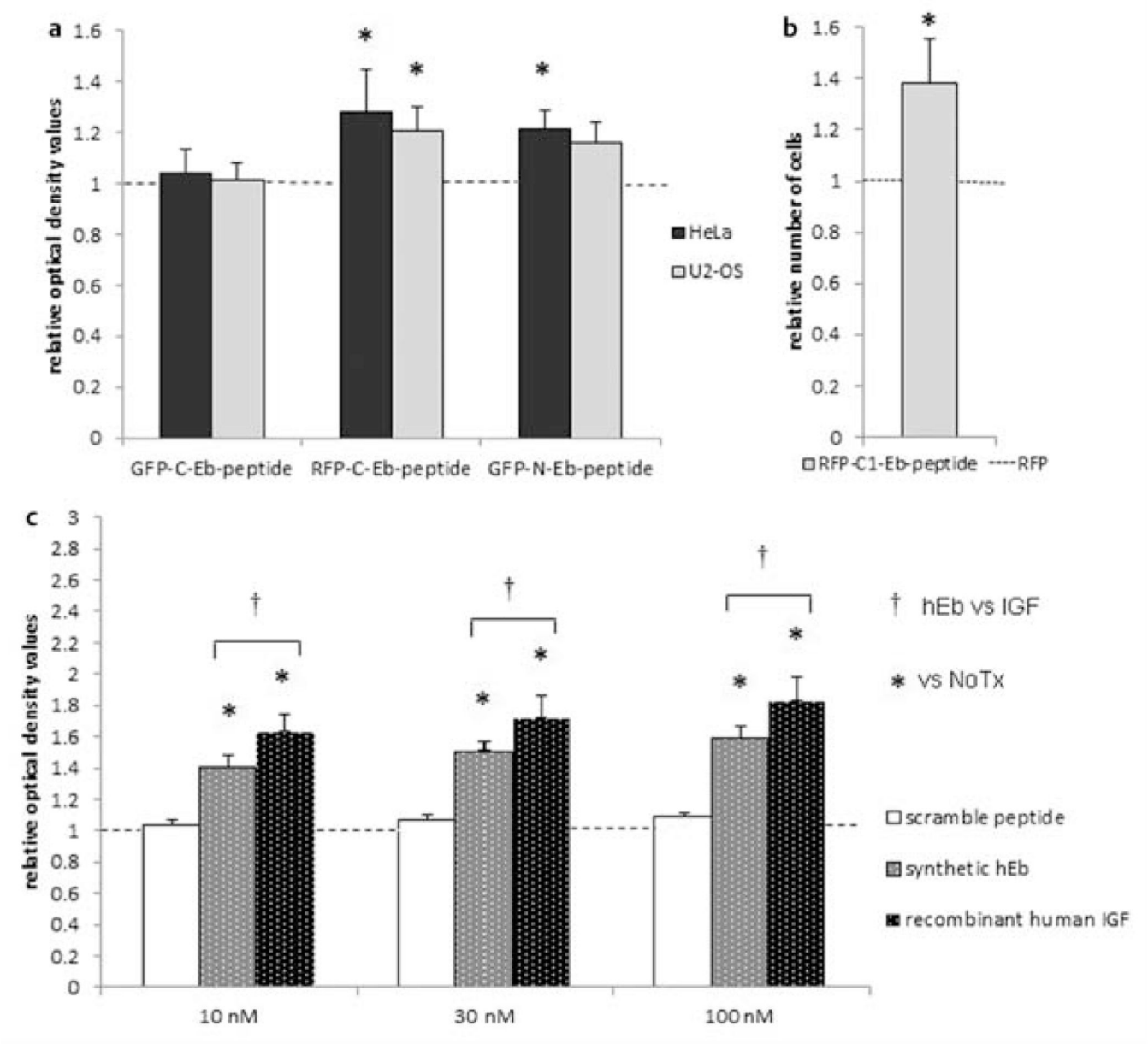

Fig. 4.

Bioactivity of hEb. **a** proliferation index of HeLa and U2-OS cells expressing hEb; HeLa (dark grey bars) and U2-OS cells (light gray bars) were transiently transfected with 3 different hEb encoding constructs: pAc-GFP-C1-hEb, pDsRed2-C1-hEb, and pAcGFP1-N3-hEb (from left to right). HeLa and U2-OS cells were also transiently transfected with control plasmids pAcGFP1-C1, pDsRed2-C1and pAcGFP-N3. Control mean values were normalized to 1.0 and represented as a dashed line. Calculations were performed by a 2-way ANOVA with Bonferroni post-hoc test and statistical significance was set at $p < 0.05$ (denoted by *); **b** cell migration index of stable U2-OS cells expressing hEb. Two stable U2-OS cell lines were compared in the migration assay: U2-OS expressing hEb fused with RFP and expressing RFP alone. Results were calculated by a Student's *t*-test. Number of control

migrated cells was normalized to 1.0 and represented by a dashed line. Statistical significance was set at $p < 0.05$ (denoted by *); **c** proliferation index of HeLa cells treated with synthetic hEb. Scramble peptide, hEb, and recombinant IGF1 were applied onto serum starved cells in 3 different concentrations: 10 nM, 30 nM, and 100 nM. For control, HeLa cells were not treated with any peptide (NoTx). All results were calculated by a 2-way ANOVA with Bonferroni post-hoc test, dashed line represents relative optical density values normalized to 1.0 for each concentration. It is shown that hEb treatment of HeLa cells is statistically different than IGF1 one at each concentration. Statistical significance was set at $p < 0.05$ (significant comparisons are denoted either by * or †).

Table 1

Oligonucleotides used for cloning of human Eb-peptide cDNA.

Name of oligonucleotide	Sequence 5′→3′	Cloning plasmid for expression
Kpn-ex5-for	ATT GGT ACC TAT CAG CCC CCA TCT ACC AAC	pAcGFP1-C1, pDsRed2-C1, pAcGFP1-N3
K pn-ex4/5-for	ATT GGT ACC CGC TCT GTC CGT GCC CAG CGC C	
Bam-rev	GCC GGA TCC TCA TTT TCC TTT TTT GCC TCT GCA TTC	pAcGFP1-C1, pDsRed2-C1
Bam-rev-bis	GCC GGA TCCTTT TCC TTT TTT GCC TCT GCA TTC	pAcGFP1-N3

Exon 5 and exons 4/5 portions of IGF1-b cDNA were used as template. In the Bamrev-bis oligonucleotide the stop codon was deleted

Table 2

Oligonucleotides used for site-directed mutagenesis of nucleolar localization signal of human Eb-peptide.

Name of oligonucleotide	Sequence 5′→3′	Genetic construct template	Genetic construct product	amino acid change
C1mutNS-1	GCTGAATGCAGAGGCAAAGGAGGAAAATGAGGATCC	pAcGFP1-C1-Eb pDsRed2-C1-Eb	pAcGFP1-C1-Eb-1mut pDsRed2-C1-Eb-1mut	KKGK →KGGK
C1mutNS-1rev	GGATCCTCATTTTCCTCCTTGCCTCTGCATTCAGC			
N3mutNS-1	GCTGAATGCAGAGGCAAAGGAGGAAAAGGATCCATC	pAcGFP1-N3-Eb	pAcGFP1-N3-Eb-1mut	
N3mutNS-1rev	GATGGATCCTTTTCCTCCTTTGCCTCTGCATTCAGC			
C1mutNS	GCTGAATGCAGAGGCGGAGGAGGAAAATGAGGATCC	pAcGFP1-C1-Eb, pDsRed2-C1-Eb	pAcGFP1-C1-Eb-2mut pDsRed2-C1-Eb-2mut	KKGK → GGGK
C1mutNSrev	GGATCCTCATTTTCCTCCTCCGCCTCTGCATTCAGC			
N3mutNS	GCTGAATGCAGAGGCGGAGGAGGAAAAGGATCCATC	pAcGFP1-N3-Eb	pAcGFP1-N3-Eb-2mut	
N3mutNSrev	GATGGATCCTTTTCCTCCTCCGCCTCTGCATTCAGC			
ex4seq	CGCTCTGTCCGTGCCCAGCGCC	Sequencing of all		
ex5seq	CAGCCCCCATCTACCAACAAG	constructs		