IGF1Ec Expression in MG-63 Human Osteoblast-like Osteosarcoma Cells

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Abstract. *Aim: The insulin-like growth factor 1 (IGF1) gene gives rise to multiple transcripts, using an elaborate alternative splicing mechanism. The aim of this study was to shed light on the expression and role of the IGF1 system in human MG-63 osteoblast-like osteosarcoma cells. Materials and Methods: The expression of the IGF1Ea, IGF1Eb and IGF1Ec isoforms was characterized using reverse transcription polymerase chain reaction (RT-PCR), quantitative real time-PCR (qRT-PCR) and western blot analysis. Using trypan blue exclusion assays, we also examined the mitogenic effects of IGF1 and of a synthetic peptide related to the E domain of IGF1Ec (synthetic E peptide) on MG-63 cells, as well as on MG-63 cells which had been molecularly modified to restrain the expression of type I IGF receptor (IGF1R) and of insulin receptor (INSR) by siRNA techniques (IGF1R KO or INSR KO MG-63 cells). Results: MG-63 cells express only the IGF1Ea and IGF1Ec transcripts. Exogenous administration of dihydrotestosterone (DHT) significantly increased the expression of IGF1Ea and IGF1Ec mRNA and it induced the previously undetectable expression of IGF1Eb transcript. Exogenous administration of IGF1, insulin and the synthetic E peptide stimulated the growth of MG-63 cells, while only E peptide stimulated the growth of IGF1R KO and INSR KO MG-63 cells. Conclusion: These data suggest that the expression of all IGF1 isoforms is hormonally regulated in MG-63 cells and that the expression of IGF1Ec may be involved in osteosarcoma biology by generating the Ec peptide which acts via an IGF1R-independent and INSR-independent mechanism.*

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The expression of the insulin-like growth factor 1 (*IGF1*) gene is mainly regulated by growth hormone (GH) and sex steroid hormones, while its bioactive product, namely IGF1, originally described as 'the sulfation factor' (somatomedin C), is mostly produced by the liver, thus mediating the effects of GH on axial skeletal growth. Therefore, the GH/IGF1 axis controls skeletal growth, as well as bone modeling/remodeling (1). The biological actions of IGF1 are attributed to its binding and activation of type I IGF receptor (IGF1R), a tyrosine kinase receptor that signals and affects cell division, cell differentiation, apoptosis and DNA repair processes of practically all cell types, including osteosarcoma cells (2). In addition, IGF1 can also bind with lower affinity to type II IGF receptor (IGF2R), a non tyrosine kinase receptor that mostly mediates IGF1 internalization and metabolic processes (1, 2), and to the insulin receptor (INSR), another tyrosine kinase receptor which in its turn affects cell division and differentiation (2). Moreover, IGF1 binds and activates the hybrid receptors IGF1R/INSR that comprise an INSR hemi-receptor linked to an IGF1R hemi-receptor, thus producing cellular responses which are under intensive investigation, particularly in cancer biology (3). Since the IGF1 system has been implicated in cancer biology, including osteosarcoma, the IGF1Rs have become attractive candidates for targeted therapies. Currently, tyrosine kinase inhibitors and anti-IGF1R antibodies are being tested in several clinical settings (4-7).

Interestingly, *IGF1* gene (6 exons) gives rise to multiple heterogeneous transcripts, by alternative splicing mechanism (8-12). The three *IGF1* pre-transcripts in humans, namely, IGF1Ea, IGF1Eb and IGF1Ec, produce a common biologically active product, mature IGF1, however, they differ in the structure of the extension peptide (Epeptide) on the carboxy-terminal end (13, 14). The IGF1Ec splice variant was initially named 'mechano growth factor' for its putative specific induction/expression by skeletal muscle after mechanical loading and muscle injury (15, 16). Recently, we documented preferential expression of the IGF1Ec transcript in other

tissues, including injured myocardium (17). In addition, exogenous administration of a synthetic E peptide related to the E domain of the IGF1Ec transcript stimulated the growth of a variety of cell types (myoblasts, cardiomyocytes, endometrial-like cells and prostate cancer cells) *via* an apparently IGF1R-independent and INSR-independent mechanism (16-20).

Herein we have attempted to characterize the expression of the *IGF1* mRNA isoforms in human MG-63 osteoblast-like osteosarcoma cells and the putative actions of this synthetic E peptide in human MG-63 cells *in vitro*.

Materials and Methods

Cell culture. The MG-63 cell line was obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA) and has been used by several laboratories as an *in vitro* model to investigate aspects of OS biology and osteoblasts pathophysiology (1, 21, 22). The MG-63 cells were maintained in Eagle's minimum essential medium (EMEM; Cambrex, Walkerville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom Berlin, Germany) and 100 U/ml penicillin/streptomycin (Cambrex) in a humidified chamber with 5% CO_2 at 37˚C. The cell density of the cultures was routinely maintained below 80% confluence. To evaluate the effects of the mitogens under investigation (namely, mature IGF1, synthetic E peptide of IGF1Ec, and insulin) in a dose-dependent manner, trypan blue exclusion assays were used to measure the number of viable cells as described elsewhere (19, 20). Briefly, MG-63 cells were plated at a cell density of about 2.3×10^4 cell/well in 6-well plates and grown with EMEM containing 10% FBS. Twenty-four hours after plating, the culture medium was changed to EMEM containing 0.5% FBS and MG-63 cells were treated with 0.5, 15 or 30 ng/ml of insulin (Novo Nordisk, Denmark), with 0.5, 25 or 50 ng/ml of mature IGF1 peptide (rhIGF1; Chemicon, Temecula, CA, USA), or with 0.5, 25 or 50 ng/ml of a synthetic E peptide related to the E domain of the IGF1Ec isoform. This synthetic E peptide corresponds with the last 24 amino acids of the C-terminal of the IGF1Ec isoform (parts of *exons 5* and *6*) (23) predicted from the cDNA sequence of the human Ec domain (12). It was synthesized by Eastern Quebec Proteomic Core Facility (Ste-Foy, Quebec, Canada), purified to >90% by high-performance liquid chromatography (HPLC) and its amino acid sequence was analysed by mass spectrometry and HPLC. The amino acid sequence of the synthetic E peptide was NH_2-YQPPSTNKNTKSQRRKGSTFEERK-COOH and its size was predicted to be 2,967 Da (23). Control MG-63 cells were treated with phosphate-buffered saline (PBS). The actual living cell number in the various MG-63 cell cultures was measured at different time intervals (24 and 48 h) using the trypan blue exclusion assay (19, 20). In addition, in order to evaluate the response in *IGF1* transcript expression, MG-63 cells were exposed to 100 nM of dihydrotestosterone (DHT) for 72 h.

INSR and IGF1R siRNA knock-out (KO) experiments. We used Stealth siRNA technology (Invitrogen Corp., Carlsbad, CA, USA) to create both *IGF1R* and *INSR* siRNAs. These sequences were obtained from the Invitrogen inventory and guaranteed more than 60% silencing. The siRNAs used for *IGF1R* were: UCUUCAAG GGCAAUUUGCUCAUUAA and for *INSR*: ACAAACUGCCC GUUGAUGACGGUGG. As a negative control, the universal negative control stealth siRNA (Invitrogen) was used. The siRNA transfection into MG-63 cells was carried out using Lipofectmine 2000 (Invitrogen) according to the manufacturer's instructions and as described previously (19, 20). Briefly, 200 nM siRNA was allowed to interact for 30 min and was administrated to MG-63 cells cultured in 24-well plates at 50-60% confluence. The KO efficiency was assessed 48 h after transfection, as previously reported (19, 20).

Table I. *The sequence of the specific sets of primers used in mRNA RT-PCR analyses*.

Target mRNA	PCR primer sequence	Product size (bp)
IGF1Ea	5'-GTGGAGACAGGGGCTTTTATTTC-3' 5'-CTTGTTTCCTGCACTCCCTCTACT-3'	251
IGF1Eb	5'-ATGTCCTCCTCGCATCTCT-3' 5'-CCTCCTTCTGTTCCCCTC-3'	411
IGF1Ec	5'-CGAAGTCTCAGAGAAGGAAAGG-3' 5'-ACAGGTAACTCGTGCAGAGC-3'	150
IGF1R	5'-ACCCGGAGTACTTCAGCGC-3' 5'-CACAGAAGCTTCGTTGAGAA-3'	230
INSR	5'-ACTCTCAGATCCTGAAGGAGCTGGA-3' 5'-AGTGTTGGGGAAAGCTGCCAC-3'	167

These sets of primers were designed to span two introns to avoid amplification of a similar-sized product from contaminating genomic DNA.

Quantitative real time-PCR (qRT-PCR) analysis. Total RNA was extracted from MG-63 cells using Tri-Reagent RNA Isolation kit (RT-111; Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. The RNA samples were used for the determination of the mRNA of specific *IGF1* transcripts by reverse transcription (RT) and quantitative RT-PCR procedures. Each RT reaction was carried out in a reaction volume of 20 μl, including 2 μg of RNA mixed with 0.5 mM dNTPs (HT Biotechnology, Cambridge, UK), 3 μg/μl Random Hexamer Primers (Invitrogen Corp.), 40 U of human placental ribonuclease inhibitor (HT Biotechnology), reverse transcription reaction buffer (consisting of final concentrations of 10 mM DTT, 75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-HCl; Finnzymes, Espoo, Finland), 200 U of Murine Moloney Leukemia Virus Reverse Transcriptase (Finnzymes) and Nuclease-Free Water (Qiagen, Valencia, CA, USA). Samples were then incubated at 37˚C for 60 min and the reaction was inactivated by heating at 70˚C for 5 min. Samples were stored at –80˚C until subsequent analysis. The obtained cDNA was amplified by RT-PCR and quantitative RT-PCR. Different pairs of primers were designed using the Primer Select computer program (DNAStar; GIBCO, Madison, WI, USA) and prepared by Invitrogen; each set of primers was designed to include sequences from different exons to ensure the specific detection of only one of the *IGF1* transcripts and to avoid amplification of genomic DNA (Table I) (16). The expected sizes of the specific RT-PCR products were initially verified by electrophoretic separation in agarose gel; 10 μl of each PCR product (8 μl of the reaction mixture diluted with 2 μl of loading buffer) were separated in a 2% agarose gel by electrophoresis (80 V constant) for 45 to 60 min (depending on the size of each PCR product) and stained with ethidium bromide. Gels were run with molecular mass markers (100 bp DNA ladder; Invitrogen) to confirm the expected size of each of the target PCR products.

Images were captured under UV light using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290; Carestream Health, Inc. Rochester, NY, USA). Parameters for image development were kept consistent across all gels using predefined saturation criteria for the digital camera. Total exposure time was determined by the first point of saturation on the image for each gel. All target sequences were also identified by sequencing analysis to further verify each target mRNA. The obtained cDNA was then analysed using qRT-PCR procedures. Each qRT-PCR reaction was obtained in 25 μl using 12 μl SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 μg/ml oligo dTs (Fermentas, GmbH, Germany), 2 μl cDNA, and 0.3 μM primers for *INSR* and *IGF1R*. As internal controls in each case, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used. The cycling parameters in both cases were 95˚C for 30 s, and then 36 cycles of denaturation at 94˚C for 20 s, annealing at 60˚C for 30 s and extension at 72˚C for 30 s, with a final extension cycle at 72˚C for 5 min. The validation of the product identity was obtained by the melting curve and the threshold cycle values (Cts) were used to assess the mRNA expression of each transcript.

Western blot analysis. MG-63 cells treated with siRNAs or PBS were harvested with trypsin/EDTA, and PBS-washed cell pellets were then lysed with RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl) containing proteases and phosphatases inhibitors (Sigma, St. Louis, MO, USA). The extracts were analyzed for total protein concentration using the Bradford procedure (Bio-Rad Protein Assay; Bio-rad, Hercules, CA, USA). Equal amounts of protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [a 16% (w/v) polyacrylamide separating gel and a 4% (w/v) polyacrylamide stacking gel] and vertically electrophoresed at 200 V for 60 min. They were then transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Uppsala, Sweden) at 100 V for 90 min at 4˚C and incubated with the primary antibody at 4˚C overnight. A rabbit anti-human IGF1Ec polyclonal antibody (1:10,000 dilution), raised against a synthetic peptide corresponding to the last 24 amino acids of the E domain of the human IGF1Ec isoform, was used in this study as described previously (23). Membranes were then incubated with a horseradish peroxidase-conjugated secondary anti-rabbit IgG (goat anti-rabbit, 1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 1 h at room temperature. GAPDH was used as an internal control to correct for potential variation in the protein loading. A mouse monoclonal primary antibody was used for GAPDH (1:2,000 dilution; Santa Cruz Biotechnology), with a horseradish peroxidase-conjugated secondary anti-mouse IgG (goat anti-mouse, 1:2,000 dilution; Santa Cruz Biotechnology). Specific band(s) were visualized by exposure the membrane to x-ray film, after incubation with an enhanced chemiluminecent (ECL) substrate according to the manufacturer's protocol (SuperSignal; Pierce Biotechnology, Rockford, IL, USA). The films were captured under white light in the Kodak EDAS 290 imaging system, (Carestream Health, Inc.).

Statistical analysis. Changes in cell numbers were assessed using one-way analysis of variance (ANOVA) (SPSS v. 11 statistical package; SPSS Inc., Chicago, IL, USA). Where significant F ratios were found for main effects or interaction ($p<0.05$), the means were compared using Tukey's *post-hoc* tests. All data are presented as the mean±standard error of the mean (S.E.M). The level of acceptable significance was set at $p<0.05$.

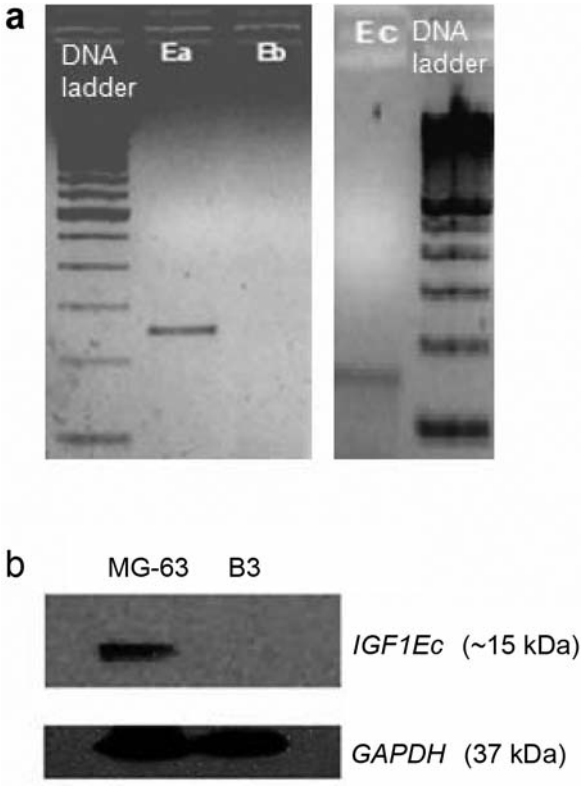

Figure 1. *An example of the pattern of the IGF1 transcripts detected in MG-63 cells. Detection of the IGF1Ea and IGF1Ec isoforms by RT-PCR analysis (a) and by Western blot analysis (b). Under our experimental conditions, the IGF1Eb isoform was not expressed in MG-63 cells. In Western blot analysis, HLE-B3 (B3) cell line was used as negative control, since it does not express the IGF1Ec isoform (24).*

Results

RT-PCR analysis documented that under our experimental conditions, MG-63 cells express the *IGF1Ea* and *IGF1Ec* mRNA isoforms while they do not express the *IGF1Eb* transcript (Figure 1a). Western blot analysis confirmed the expression of IGF1Ec at the protein level (Figure 1b). The pattern of *IGF1* transcripts and the lack of IGF1Eb expression in MG-63 cells were re-confirmed using qRT-PCR (Figure 2). Exposure of MG-63 cells to DHT for 72 h enhanced significantly the expression of *IGF1Ea* and *IGF1Ec* transcripts and, more importantly, induced the expression of *IGF1Eb* transcript (Figure 2). This effect was not seen in human lens epithelial (HLE)-B3 cells which do not express *IGF1Eb* and *IGF1Ec* transcripts (24). These data indicate that the expression of *IGF1* transcripts is hormonally regulated in MG-63 osteosarcoma cells.

To investigate the putative role of the *IGF1Ec* expression in MG-63 cells, we tested the mitogenic activity of insulin, IGF1 and that of a synthetic E peptide, designed to contain the last

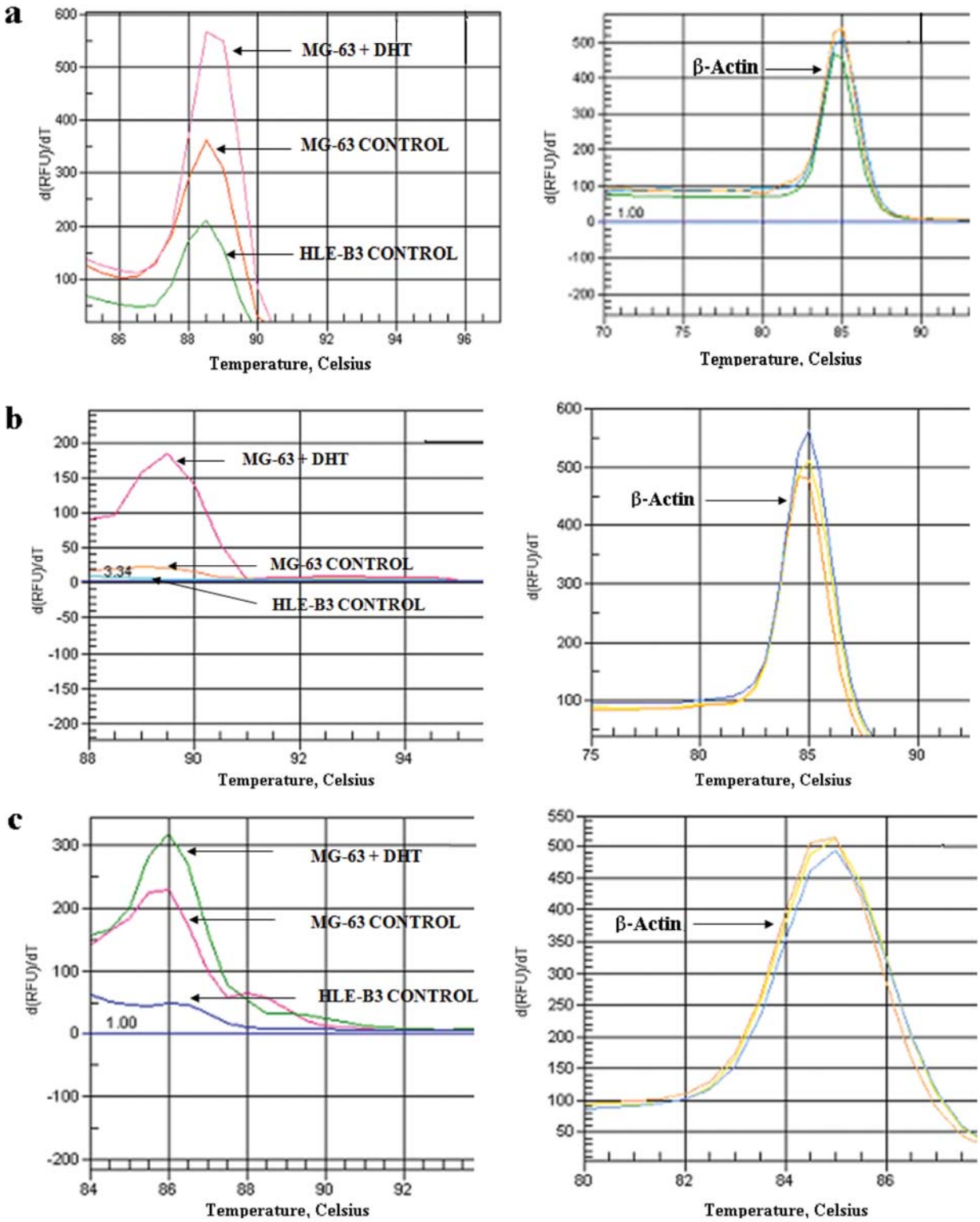

Figure 2. *An example of the quantitative analysis of the IGF1 transcripts using qRT-PCR. Melting curves of the expression of the IGF1 isoforms were assessed before and after the administration of DHT (100 nM for 72 h). MG-63 cells expressed the IGF1Ea (a), and IGF1Ec (c) but they did not express IGF1Eb (b). Administration of exogenous DHT increased the expression of all the IGF1 isoforms after 72 h, suggesting that their expression is hormone-dependent. HLE-B3 was used as a negative control, a cell line of human lens epithelium which has been previously described as not expressing the IGF1Eb and IGF1Ec isoforms (24). Normalization in all cases was carried out using β-actin.*

24 amino acids of the translation product of the E domain of IGF1Ec, *in vitro*. Under our experimental conditions, insulin, IGF1 and synthetic E peptide stimulated the proliferation of MG-63 cells in a time-dependent (24 and 48 h) and dose-dependent manner (0.5 ng/ml to 50 ng/ml) (Figure 3).

To evaluate whether the synthetic E peptide action is mediated by IGF1R and/or INSR, we silenced the IGF1R and INSR expression in MG-63 cells, thus producing *IGF1R* KO MG-63 cells and *INSR* KO MG-63 cells, respectively. The effectiveness of our silencing methodology was confirmed by qRT-PCR as previously described (19, 20). We documented that such silencing methods suppressed the *IGF1R* and *INSR* expression in KO MG-63 cells by 80% and 90% of that reported in parental MG-63 cells and controlled MG-63 transfectants, respectively (Figure 4). Although the mitogenic actions of insulin and IGF1 was blocked in *INSR* KO and *IGF1R* KO MG-63 cells, respectively (Figure 3a and b), the exogenous administration of the synthetic E peptide continued to stimulate the growth of *IGF1R* KO MG-63 cells and of *INSR* KO MG-63 cells (Figure 3c). These data suggest that the actions of E peptide are probably not mediated *via* IGF1R or INSR in MG-63 cells.

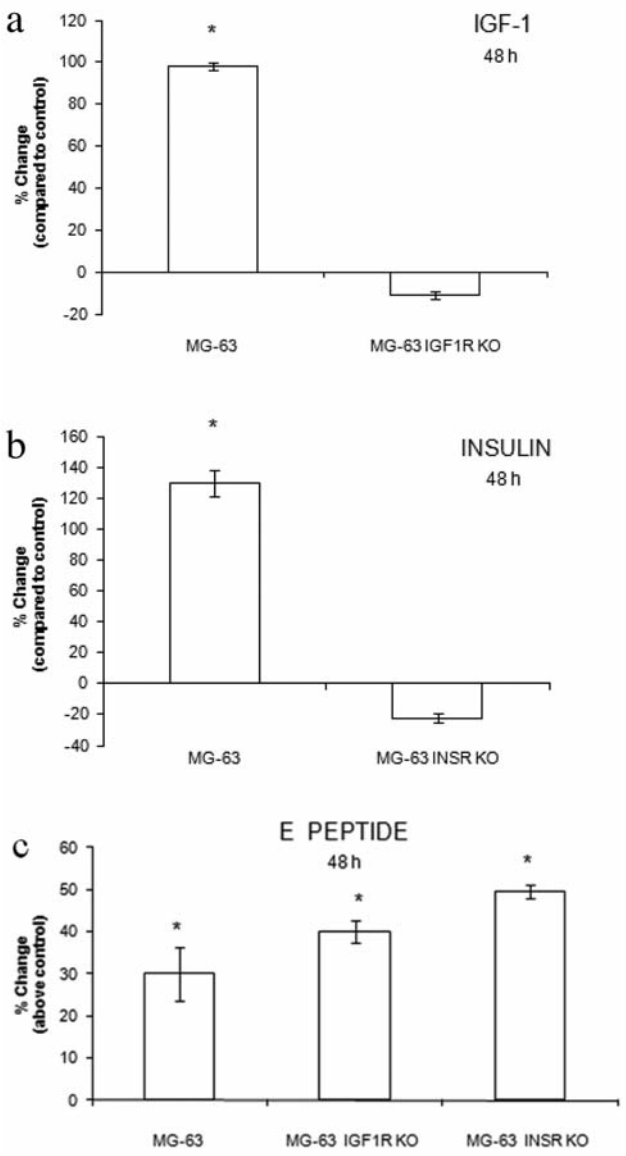

Figure 3. *An example of the mitogenic activity of IGF1, insulin and of the synthetic E peptide of the IGF1Ec isoform in MG-63 cells as assessed by trypan blue exclusion assays (mean±SEM of three independent experiments). Insulin, IGF1 and E peptide stimulated MG-63 cell proliferation. However, it can be observed that although MG-63 cells silenced for the IGF1R (a) and for INSR (b) were not stimulated by IGF1 (50 ng/ml) and insulin (30 ng/ml), respectively, they were stimulated by the synthetic E peptide (50 ng/ml) (c). *,p<0.05, significantly different from control (negative control stealth siRNA-treated cells).*

Discussion

Osteosarcoma is the most common non-hematologic primary malignant tumor of bone in childhood and adolescence; it usually occurs in the metaphyseal region of long bones, which are the anatomic sites that respond to increasing serum levels of GH/IGF1 (*i.e.* the distal femur and the proximal tibia). The survival of patients with osteosarcoma is poor, despite the use of combination therapy with surgery and chemotherapy (cisplatin, doxorubicin, ifosfamine, methotrexate) (25). Since the peak incidence of osteosarcoma coincides with the pubertal growth spurt, which in turn corresponds to the peak concentrations of circulating IGF1 levels, IGF1R has become an obvious research target in the pathophysiology of osteosarcoma (4-7, 25-28).

Anti-IGF1R therapies, such as R1507, a fully humanized monoclonal anti-IGF1R antibody, effectively inhibited the growth of a subset of human osteosarcoma tumors engrafted into mice (29). However, while some of these xenograft tumors respond to therapy, others show little to no growth inhibition (30-34). According to this, one can hypothesize that other mitogens beyond IGF1 may be involved in osteosarcoma biology. Herein, we documented that the synthetic E peptide, which is produced by the translation of the E domain of the *IGF1Ec* transcript, exerted mitogenic activity on MG-63 cells *via* an IGF1R- and INSR-independent mechanism. This may represent an explanation as to how the specific anti-IGF1R antibody fails to block the growth of osteosacoma cells *in vitro* (29). Nevertheless, we cannot exclude the possibility that other potent growth factors sustain such IGF1R-independent growth effects on osteosarcoma cells. In addition, DHT enhanced the expression of *IGF1Ea* and *IGF1Ec*, and induced the expression of *IGF1Eb* transcript, which was undetectable in control MG-63 cells. These data indicate that the expression of *IGF1* transcripts is hormonally regulated in MG-63

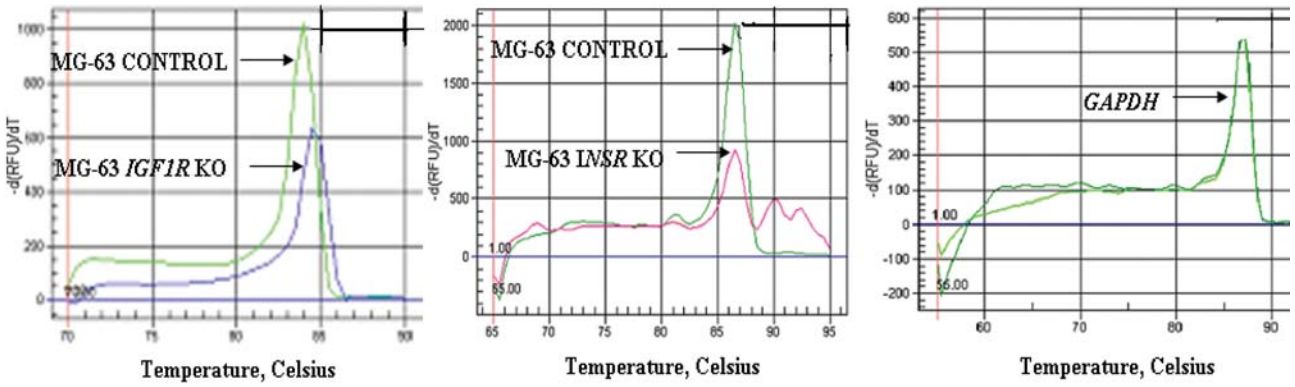

Figure 4. *Quantitative RT-PCR analysis for IGF1R and INSR mRNAs revealed a significant decrease in IGF1R and INSR mRNA levels 48 h after MG-63 cells were exposed to siRNA targeting for IGF1R and INSR, respectively. Left panel: Melting curve of IGF1R siRNA as compared with that of the control cells. The control MG-63 cells were treated with the universal negative control siRNA. Middle panel: Melting curve of INSR siRNA as compared with that of control MG-63 cells treated with the universal negative control siRNA. Right panel: Normalization in all cases was carried out using GAPDH.*

osteosarcoma cells, and could explain the fact that osteosarcoma affects more males (2.27-fold) than females during the pubertal spurt (35).

Several questions remained to be answered regarding the role of the IGF1 system in the biology of osteosarcoma, such as why different transcripts are employed for the production of the same bioactive molecule, namely IGF1. To investigate this, we examined the effects of the E peptide (a specific translation product of the E domain of *IGF1Ec*) in MG-63 cells. Our data suggest that the synthetic E peptide stimulated the cellular proliferation of MG-63 cells in a similar fashion to IGF1 and to insulin, suggesting that the *IGF1* transcripts may produce bioactive products other than IGF1.

The biological activity of IGF1 is mainly exerted through IGF1R, to a lesser extent through INSR and possibly *via* the IGF1R/INSR hybrid receptors. Consequently, we proceeded to undertake silencing experiments targeting *IGF1R* and *INSR* expression in MG-63 cells (*IGF1R* KO and *INSR* KO MG-63 cells). In this way, we also blocked the formation of hybrid IGF1R/INSR. In our experimental setting, we achieved the suppression of *IGF1R* and *INSR* expression by up to 80% in MG-63 cells, as assessed by qRT-PCR. The functional significance of such suppression was confirmed by the lack of detecting IGF1-mediated and insulin-mediated stimulation of growth of the *IGF1R* KO and *INSR* KO MG-63 cells, respectively. Interestingly, the exogenous administration of the synthetic E peptide stimulated the growth of *IGF1R* KO and *INSR* KO MG-63 cells, suggesting that the mitogenic activity of this E peptide is possibly mediated *via* an IGF1R-independent, INSR-independent and IGF1R/INSR-independent mechanism in these cells. Hence, our data further suggest that *IGF1* gene transcripts possibly generate bioactive products other than IGF1. However, the experimental design and the data of the present study do not allow us to corroborate our speculation that the E domain of IGF1Ec isoform is stable and is produced in sufficient amounts within these cells to elicit strong mitogenic effects on them; whether the E peptide is more stable within the full length IGF1Ec form and is processed to act immediately on its target remains to be elucidated. Thus, further studies are needed to illuminate the factors that regulate the processing and secretion of the E domain peptide of IGF1Ec isoform and the mechanism(s) of its action in osteosarcoma cells.

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