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Cloning, Expression and Investigation of Biological Function of Growth Hormone Releasing Hormone (GHRH) in MDA-MB-231 Breast Cancer Cells

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Abstract

Aim: Our aim is to clone the GHRH cDNA and demonstrate the biological function on cell growth, proliferation *via* generating GHRH overexpressing MDA-MB-231 cells.

Methods: GHRH cDNA were amplified from LNCaP prostate cancer cells, that have highest transcriptional and translational GHRH expression. EcoRI and HindIII restriction enzymes were used to insert GHRH cDNA in pcDNA 3.1 vector and generated plasmid transformed to *E. coli* HB101 strain, positive clones were selected by colony PCR. Forced GHRH expressing MDA-MB-231 breast cancer cells were generated by lyposomal transfection of GHRH cDNA-PC3.1 plasmid and neomycin selection. Highest GHRH expressing MDA-MB-231 breast cancer cells were selected by qRT-PCR, and immunoblotting.

Results: Increased GHRH expression leads cell growth, proliferation, colony formation in time dependent-manner in MDA-MB-231 breast cancer cells. Moreover, significant difference on EMT pathway key molecules expression profile has been observed when compared to parental MDA-MB-231 breast cancer cells. Autocrine GHRH expression induced invasion-metastasis through upregulation of E-cadherin, Vimentin, c-jun, MMP-2 and MMP-9 expressions in MDA-MB-231 cells within 48 h.

Conclusion: we cloned GHRH variant 1 cDNA in pcDNA3.1 vector and stable cell line generated by GHRH cDNA inserted plasmid triggered invasion-metastatic profile in MDA-MB-231 breast cancer cells *via* EMT pathway.

Keywords: Growth hormone releasing hormone; Cloning; Epithelial Mesenchymal Transition; Invasion; Metastasis.

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Introduction

Growth Hormone (GH), an anterior pituitary derived hormone, is essential for postnatal growth through activating Insulin like Growth Factor-I (IGF-I) signaling in liver. GH translational expression and secretion is under control hypothalamic factor; growth hormone-releasing hormone (GHRH) [1]. Following expression of GHRH in the arcuate nucleus of the hypothalamus, it reaches to pituitary somatotrophs *via* portal vasculature. Binding of GHRH ligand to its receptor (GHRHR) on the surface of the pituitary somatotrophs activates the GHRH signaling that leads GH expression and secretion [2]. Following binding of GHRH to GHRHR stimulates G protein to activate adenylyl cyclase to produce cAMP and accelerated intracellular cAMP levels activate the protein kinase A (PKA). Activated PKA and nuclear localization induced Ca^{2+} influx to stimulate GH exocytosis [3]. Concomitantly, transcriptional activation of GHRHR expression through stimulated PKA and cAMP accumulation triggered cAMP response element-binding protein (CREB) [2]. Alternatively, GH production and secretion are performed by inositol phosphate–diacylglycerol–protein kinase C (PKC), L-type calcium channels, and arachidonic acid–eicosanoic pathways [4].

Although GHRHR majorly expressed on the pituitary gland, recently it has been demonstrated in various body parts, such as myocardium, lymphocytes, testes, ovaries, skin, and pancreas. Similarly, the expression of GHRH has been detected in peripheral tissue such as breast, brain, kidney, heart and retina [5-7]. Various hypothalamic- and pituitary-derived peptide hormones and their receptor's expressions in peripheral tissue implicated association between cancer progression and active autocrine/paracrine signaling mechanism. Recently, active GHRH signaling has been demonstrated in prostate, colon, breast cancer tumors [8]. Concomitantly, exogenous GHRH expression can trigger cell proliferation, survival, invasion-metastasis and prevent drug mediated apoptotic cell death in colon, prostate and breast cancer [5].

Autocrine/Paracrine GHRH signaling triggered peripheral cell proliferation and growth through PI3K-Akt, MAPK activation and ERK phosphorylation [9]. Because of biologically active GHRH signaling effect on cellular growth and proliferation leads to develop agonists and antagonists against GHRH ligand. Synthetic peptide agonist against GHRH (1-44) acted as cardioprotective and pulmonary edema preventive agent because of its wound healing feature [8]. On the other hand, antagonists against GHRH 1-44 and 1-29 amino acid residues were developed and investigated for their efficiency on growth inhibition. By this point of view, the JV-1-36 and JV-1-38 GHRH antagonists prevents invasion-metastasis and induce caspase dependent apoptotic cell death in various cancer cells such as prostate, colon, breast cancer *in vitro* and *in vivo* studies [10]. Thus, cloning of GHRH cDNA is essential for generation of stable GHRH expressing cell line models *in vitro* and *in vivo* and also evaluate the role of GHRH overexpression in Epithelial Mesenchymal Transition (EMT) pathway mediated invasion-metastasis in breast cancer cells.

Methods

Strains, plasmids, and reagents: *Escherichia coli* HB101 (Invitrogen, Beijing, China) was used for plasmid amplification. The plasmids pcDNA3.1 and pJET (Invitrogen, Beijing, China) were used for the production of GHRH cDNA. The *E. coli* HB101 strain was grown in LB medium at 37°C. The AMV reverse transcriptase, T4 DNA ligase, Taq DNA polymerase, restriction enzymes (NcoI, XhoI, EcoRI, Hind III), 1 kb DNA marker, pre-stained protein marker were purchased from Fermentas (Thermo Fisher Scientific, Waltham, USA) and Pierce (Thermo Fisher Scientific, Waltham, USA), respectively. Plasmid Mini-prep Kit, Gel Extraction Kits were purchased from Zymogene (Irvine, USA).

Cell lines and antibodies: Anti-Slug, anti-N-cadherin, anti-FAK, anti-Vimentin, anti-FAK, anti-Talin-1, anti-c-Jun, anti-Ras, anti-β-catenin, anti-p42/44, anti-E-cadherin, anti-β-actin, anti-PI3K (each 1:1000, CST, Danvers, MA, USA) antibodies and HRP-conjugated secondary anti-rabbit and anti-mouse antibodies (1:5000) were purchased from CST. GHRH and GHRHR primary antibodies were obtained from Origene (Rockville, USA). Human breast cancer and prostate cancer cell lines LNCaP (CRL-1740), MCF-7 (HTB-22), T-47D (HTB-133), BT-20 (HTB-19), BT-474 (HTB-20), MDA-MB-468 (HTB-132), MDA-MB-231 (HTB-26), MDA-MB-453 (HTB-131), SK-BR-3 (HTB-30) and MCF10A (CRL-10317) cells were purchased from American Type Culture Collection. Cells were grown in DMEM and RPMI medium (PAN Biotech, Aidenbach, Germany), supplemented with 10% fetal bovine serum (PAN Biotech) and 10000 U penicillin/ml, 10 mg streptomycin/ml (PAN Biotech). Cells were cultured at 37°C in a humidified 5% CO_2 incubator (HERAcell 150; Thermo Electron Corporation, Waltham, MA, USA).

Cloning of the GHRH cDNA and construction of the expression plasmid: The transcriptional expression profile of GHRH gene was demonstrated by using primers against GHRH cDNA. In order to determine the annealing temperature of primers for GHRH gene, PCR amplification was performed by using genomic DNA isolated from LNCaP prostate cancer cells. The forward primer was (5'-ATGCAGATGCCATCTTCACCAA-3'), and the reverse primer was (5'-TGCTGTCTACCTGACGACCAA-3'). PCR conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s 50-65°C for 30 s and 72°C for 2 min and finally 72°C for 10 minutes final extension. Total RNA was isolated from the LNCaP prostate cancer cells (ATCC, CRL-1740) using TRIzol reagent (Invitrogen, Beijing, China). The cDNA was generated by RT-PCR using the iScript cDNA synthesis kit. The forward primer was (5'-GATCGGTACCCAGTATGCCCCACAAACC-3'), and the reverse primer was (5'-TTTGTTCTAGACTTAATTTGGATTCAGCATG-3'). Following amplified GHRH cDNA fragment was purified using the Gel Extraction Kit, it was ligated into the pJET vector, and transformed into the competent *E. coli* HB101 strain by calcium chloride activation. The positive colonies were identified by colony PCR by using cloning primers. Selected positive clone was used as a template to subclone GHRH cDNA from pJET vector to pcDNA 3.1 vector by using EcoRI and Hind III restriction enzyme recognition sites. The forward primer was (5'-AAGAATTCTCATCCCTGGGAGTTCCTGTGC TT-3'), and the reverse primer was (5'-TCTAAGCTTATGCCACTCTG GGTGTTCTTCTT-3'). The PCR product was purified, digested with EcoRI and HindIII, and ligated into the expression vector PCDNA3.1. The pcDNA3.1-GHRH cDNA plasmid was transformed into *E. coli* HB101, and positive transformants were selected by using ampicillin (25 μg/mL) resistance, colony PCR and restriction map-

ping (Invitrogen, USA).

Determination of recombinant GHRH expression in *E. coli* HB101 cells: The positive transformant strain (GHRH cDNA inserted pcDNA3.1 plasmid) and strains having mock vector (pcDNA 3.1) were inoculated to LB medium with 50 µg/ml of ampicillin and was shaken at 37°C, 180 rpm until its absorption at OD600 nm reached 0.4. The culture cells were isolated from LB medium by centrifugation and bacterial pellet washed once with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4), and cells were resuspended in the binding buffer (pH 8.0, 50 mM phosphate buffer with 300 mM NaCl and 10 mM imidazole). By ultrasonication, total protein extracts were disrupted, and the cell debris was separated by centrifugation. Total protein content of positive transformant and mock vector were determined by Bradford Assay (Thermo Scientific, Waltham, USA) according to manufactures instructions.

Transfection and overexpression of GHRH cDNA in MDA-MB-231 breast cancer cells: The recombinant plasmid PCDNA3.1-GHRH cDNA was transfected into MDA-MB-231 cells by FuGENE HD lyposomal agent and positive clones selected by neomycin-included media for 10-12 days. Single colonies of the transfectants were selected for overexpression of GHRH by using RT-PCR method (Invitrogen, Beijing, China). Each PCR reaction solution contained 12.5 µl of 2X PCR Master Mix (Thermo Scientific, Waltham, MA, USA), 2 µl of cDNA and 0.4 µl of each qPCR primer (10 µM), and 6.8 µl of ddH_2O. The program was run as follows: an initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, 57°C for 30 s, then a final extension at 72°C for 30 s and PCR products were electrophoresed with 2.5% agarose gel with EtBr (10 mg/ml). 18S rRNA gene was used as housekeeping for RT-PCR. The translational expression of GHRH was demonstrated by immunoblotting and immunofluorescence staining.

Cell viability assay: Cells were seeded at a 1 x 10^4 density per well in 96-well plates and incubated for 24, 48 and 72 h. 10 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye] (5 mg/ml) (Sigma-Aldrich, Taufkirchen, Germany) were added to each well and cells were kept at 37°C for 4 h. The resulting formazan crystals were solubilized in 200 µl dimethyl sulfoxide (DMSO). The density of the solubilized formazan was read at dual wavelength 570 and 655 nm spectrophotometrically (Bio-Rad, Hercules, CA, USA).

Trypan blue dye exclusion assay: In order to evaluate the time dependent effect of GHRH expression on MDA-MB-231 breast cancer cell growth, trypan blue assay was performed. MDA-MB-231 wt and GHRH+ cells were seeded at a 6-well plate at a seeding density of 50.000 cells and cells were incubated overnight to attach. Every 24 h, cells were trypsinized and stained with trypan blue, viable and death cells were counted under light microscopy. Obtained data were placed on a graph for the number of cells (y-axis) and time (x-axis).

Soft agar assay for colony formation: For the soft agar assay, the base agar was prepared creating a mixture with equal amounts of 2X DMEM medium (20% FBS and 2% penicillin/streptomycin) and 0.5% agarose in PBS and the mixture was dispersed as 1 ml into each well in 6-well plates. After solidification of gel, 2.5x10^5 cells/ml in a 1:1 mix of 2X DMEM medium and 0.3% agarose was added on top of the base gel. After solidification, 500 µl media was added on the gel and is incubated 37°C for 15 days. The cells were stained with 0.05% crystal violet for 30 min.

Fluorescence microscopy

Mitotracker/ DAPI staining: The cells were seeded in 12-well plates at a density of 1x10^5 cells per well. After 24, 48 and 72 h incubation period, cells were washed once with 1X PBS. The cells were stained with 1 µl/ml MitoTracker™ Red CMXRos (Thermo Fisher Scientific, Waltham, USA) and 1 µl/ml 4',6-diamidino-2-phenylindole (DAPI) (1 mg/ml stock concentration in 1X PBS) fluorescent probes and were incubated for 30 min and 10 min in the dark, respectively. Time dependent GHRH expression-induced cell proliferation/viability was visualized using fluorescent microscopy (Olympus, Japan).

$DiOC_6$ staining: MDA-MB-231 wt and GHRH+ breast cancer cells (1x10^5) were seeded into 12-well plates. Following incubation of cells for 24-72 h, they were washed once with 1X PBS, and then were stained with a 4 nM 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Calbiochem, La Jolla, CA, USA; 40 nM stock concentration in DMSO) fluorescent probe. $\Delta\psi m$ disruption was visualized by fluorescence microscopy (Olympus, Japan).

Scratch assay: MDA-MB-231 wt and GHRH+ cells (3 x 10^5) were seeded on 6-well plates. After 24 h incubation, when the cells reached 70-80% confluency, the cell monolayer was scratched gently with the tip of sterile 100 µl pipette across the center of the well and detached cells were removed by changing the medium. Then the cells were incubated for 24-96 h and radius of the resulting gap was measured every 24 h.

RNA isolation, cDNA synthesis and RT-PCR: RNA isolation from MDA-MB-231 wt and GHRH+ cells were performed by TriZol Reagent according to manufacturer's suggestions. In brief, cells were cultured in 60 mm culture dishes. After 24 h incubation, media were discarded and TriZol Reagent was added directly on the cells. Cell lysate was pipetted up and down several times, incubated for 5 minutes and chloroform was added to lysate. After incubation for 2-3 minutes, samples were centrifuged for 15 minutes at 12000 x g at 4°C. Upper aqueous phase was transferred to a new tube, isopropanol was added, incubated for 10 minutes and centrifuged for 10 minutes as above. Supernatant was discarded, pellet was resuspended in 75% ethanol and centrifuged for 5 minutes at 7500 x g at 4°C. Supernatant was discarded, pellet was air-dried and resuspended in RNase-free water. cDNA synthesis from isolated RNA was performed by iScript cDNA Synthesis Kit. 4 µl 5X iScript Reaction Mix, 1 µl iScript Reverse Transcriptase, 1000 ng RNA sample dH_2O (total volume 20 µl) were mixed in PCR tubes and incubated in thermal cycler as following protocol: 5 minutes at 25°C, 20 minutes at 46°C, 1 minute at 95°C. cDNA obtained in this reaction was used as template for RT-PCR.

Immunoblotting Analysis: Cells were cultured in 60 mm Petri dishes in complete medium, then media were discarded and washed with ice-cold 1X PBS and lysed with ProteoJET Mammalian cell lysis buffer (Fermentas, St. Leon-Rot, Germany). Total protein levels were determined by Bradford method (Bio-Rad,

Hercules, CA, USA). Total cell lysates were separated by 12% SDS–PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche) subjected to electrophoresis. Membranes were washed in tris-buffered saline with Tween-20 (TBS-T) [10 mM Tris-HCl (pH 8.3), 0.05% Tween-20] (Tween 20, Sigma Ultra, St. Louis, MO, USA). Blocking was proceeded by 5% (w/v) skim milk containing TBS-T milk overnight at 4°C. PVDF membranes were incubated with primary antibody buffer containing 5% (w/v) skim milk solution with appropriate antibodies. Membranes were rinsed with TBS-Tween-20 and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, 1:5000 (v:v)) at 4°C overnight. Following addition of enhanced chemiluminescence reagent (Lumi-Light Western Blotting Substrate; (Roche, Mannheim, Germany) membranes were exposed to X-ray films (Roche, Mannheim, Germany).

Immunofluorescence Assay: For immunofluorescence assay, 2.5 x 10^4 cells were seeded on coverslips. After overnight incubation, cells were washed three times with 1X PBS and fixed with ice-cold methanol for 10 minutes. The fixed cells were treated with Triton-X-100 for 15 minutes and washed three times with 1X PBS and three times with PBB solution (1X PBS with 0.5% BSA w/v). Then the cells were incubated with blocking solution (1X PBS with 2% BSA w/v) for one hour at room temperature, washed with PBB solution and incubated overnight with primary antibody (1:50 v/v, in PBB). After incubation, cells were washed with PBB solution and treated with IgG (H+L), F(ab')2 Fragment Alexa Fluor® 488 Conjugate (1:250 v/v, in PBB solution, Cell Signaling Technology, Denvers, USA) for 1h +4°C. Cells were washed with PBB solution, incubated for 15 minutes with 1 µl/ml 4',6-diamidino-2-phenylindole (DAPI) and visualized by fluorescence microscopy with appropriate filters (Olympus, Japan).

ELISA: GHRH ELISA was performed as manufacturer's suggestions (Elabscience, Houston, USA). In brief, 100 µl reference standard or sample was added to GHRH antibody coated wells and incubated 90 min at 37°C. The liquid was removed, 100 µl Biotinylated Detection Antibody was added to each well and incubated 1 h at 37°C. Then the wells were washed with wash buffer, incubated with HRP conjugate for 30 min, washed with wash buffer and treated with Substrate reagent for 15 minutes. After incubation, stop solution was added to each well and absorbances were measured at 450 nm wavelength.

Statistical analysis: Quantification of all experiments was conducted using Graphpad Prism version 6.01 for statistical analyses. Data presented are the mean -/+S.D. from at least three independent experiments. In all experiments, significance of difference between two group was calculated by Bonferroni's test after two-way analysis of variance (ANOVA).

Results

Cloning of GHRH cDNA in pJET vector and obtaining positive transformants: In order to select the major GHRH cDNA expressing cell line, we first determine the annealing temperature for GHRH cDNA primers. Genomic DNA was isolated from 1x10^6 LNCaP cells and 2 µg genomic DNA was used as a template to amplify the GHRH gene by gradient PCR (50-65°C). According to gradient PCR, 59.3-64°C were shown an exact band profile of genomic length of GHRH gene. As after 55.9°C non-specific band profile was observed by using GHRH primers that corresponds as a 2200 bp PCR product length (Figure 1A). After determining the annealing temperature for GHRH cDNA primers, we isolated RNA from LNCaP (CRL-1740), MCF-7 (HTB-22), T-47D (HTB-133), BT-20 (HTB-19), BT-474 (HTB-20), MDA-MB-468 (HTB-132), MDA-MB-231 (HTB-26), MDA-MB-453 (HTB-131), SK-BR-3 (HTB-30) and MCF10a (CRL-10317) cell lines by Trizol method and total RNA's were converted to cDNA by iScript cDNA synthesis kit. All the cDNA's isolated from different cell lines in our laboratory, the most GHRH cDNA expression was determined in LNCaP cells (Figure 1B). Following selection of mammalian cell line, we amplified GHRH cDNA by cloning primers with NcoI and XhoI recognition sites by PCR. PCR product was extracted from 2% low melting agarose by using Gel Extraction Kit (E.Z.N.A., Omega Bio-tek, Norcross, USA) and purified PCR product was digested by NcoI and XhoI enzymes, double digestion product was visualized by 2% agarose gel electrophoresis (Figure 1C). The first cloning vector pJET was transformed to *E. coli* HB101 cells and plasmid isolated by SpinClean Plasmid DNA Miniprep Kit (Biomatik, Ontario, Canada) and plasmid was double digested with the same enzymes cutting GHRH cDNA and visualized in 0.8% agarose gel electrophoresis (Figure 1D). The cloned GHRH cDNA was transformed to *E. coli* HB101 cells by using heat shock method, selected by kanamycin and positive strains were selected by colony PCR (Figure 1E).

Subcloning of GHRH cDNA in pcDNA3.1 vector: As pJET vector has no neomycin selection marker, we subcloned the GHRH cDNA inserted pJET vector by amplifying GHRH cDNA primers with EcoRI and HindIII recognition sites. PCR product was digested with EcoRI and HindIII enzymes and extracted from 2% low melting agarose and visualized by 2% agarose gel electrophoresis (Figure 2A). pcDNA3.1 vector was digested with the same restriction enzymes and visualized in 0.8% agarose gel electrophoresis (Figure 2B). Double digested pcDNA3.1 and GHRH cDNA was ligated by T4 ligase and ligation products were transformed to competent *E. coli* HB101 cells *via* heat shock method and selected by ampicillin including LB agar plates. Selected potential 12 transformants used as a template for colony PCR (Figure 2C). Major GHRH cDNA expression was observed in clone 5, 10 and 12. In order to be sure for GHRH cDNA insertion in pcDNA3.1 vector, we cultured these three potential clones in LB medium with ampicillin overnight. Following incubation, plasmid isolation was performed, and plasmids were double digested with EcoRI and HindIII enzymes. Digested products were run on a 2% agarose gel and GHRH cDNA insertion was determined in clone 5, 10 and 12 (Figure 2D). The translational expression profile of GHRH cDNA in each potential positive clone were determined by SDS-PAGE electrophoresis of total protein lysates (Figure 2E).

Generation of GHRH cDNA overexpressing MDA-MB-231 breast cancer cells: Cloned GHRH cDNA was transfected to MDA-MB-231 breast cancer cell by Fugene HD lyposomal agent and following 48 h incubation period, neomycin G-418 was treated to transfected cells with increasing concentration (200-1000 ng/ml) for 10 days. Six potential positive clones were picked up from G-418 selected cells and RNA isolated from these colonies and cDNA synthesized and GHRH cDNA expression was determined by PCR amplification using GHRH primers. The mostly GHRH expressing clone 4 and 5 has been determined but, clone 4 was selected

for further studies (Figure 3A). After determining the transcriptional expression of GHRH in clone 4, we performed immunofluorescence staining by using GHRH antibody. According to IF assay, selected potential GHRH expressing clone of MDA-MB-231 cells demonstrated GHRH puncta frequently compared to un-transfected MDA-MB-231 wt breast cancer cells (Figure 3B). To support the immunofluorescence data, ELISA and immunoblotting assays were performed to determine the increase in GHRH expression in MDA-MB-231 GHRH+ cells both in media (Figure 3C) and cells (Figure 3D). According to ELISA assay, secreted GHRH concentration in media was calculated as 480,388 pg/ml and 1282,611 pg/ml for MDA-MB-231 wt and GHRH+ cells, respectively.

The biological effect of GHRH overexpression on MDA-MB-231 breast cancer cell proliferation, growth and colony formation: In order to evaluate the biological activity of cloned GHRH cDNA in MDA-MB-231 cells, we performed growth assay, MTT cell viability assay for 72 h and colony formation by soft agar. When we checked the effect of GHRH overexpression on MDA-MB-231 cell growth, we observed that clone 4 carrying GHRH cDNA inserted pcDNA3.1 vector showed an increase in cell growth (Figure 4A) and proliferation in time dependent manner as compared to wt MDA-MB-231 breast cancer cells (Figure 4B). In order to determine the effect of cloned GHRH expressing MDA-MB-231 cells on colony formation, we performed colony formation assay. Autocrine GHRH expressing MDA-MB-231 cells demonstrated an increase in colony formation compared to wt cells in time dependent manner by colony (Figure 4C) and soft agar assays, respectively (Figure 4D).

Overexpression of GHRH triggered invasion and metastasis through MAPK and EMT pathway: To evaluate the morphological differentiation and growth induction, we performed Mitotracker/DAPI and $DiOC_6$ staining (Figure 5A). Mitotracker/DAPI and $DioC_6$ staining results, overexpression of GHRH triggered cell proliferation and growth through mitochondrial membrane potential inclination in MDA-MB-231 breast cancer cells as compared to parental cells. Moreover, scratch assay was conducted to demonstrate the wound healing potential of increased GHRH signaling in MDA-MB-231 breast cancer cells (Figure 5B). Time-dependent migration of MDA-MB-231 breast cancer cells was inducted through autocrine GHRH expression. In order to observe the effect of GHRH overexpression on invasion-metastasis, we checked the expression of key molecules of Epithelial-Mesenchymal Transition (EMT) and MAPK pathways. Firstly, we performed immunofluorescence assay to detect the expression profile of FAK, Talin-1 and β-catenin proteins, which are mainly involved in focal adhesion of cells, and we observed the increased expression of these proteins in GHRH+ cells compared to MDA-MB-231 wt cells (Figure 5C). In the meantime, we checked the translational expression of EMT markers and MAPK signaling pathways key molecules expression profile in time dependent manner by immunoblotting (Figure 5D-E). Autocrine GHRH expression-mediated VEGF, MMP-9, MMP-2 mRNA upregulations were determined only after 24 h in MDA-MB-231 breast cancer cells as compared to parental cells (Figure 5D). Time-dependent forced GHRH expression triggered MAPK signaling key players; Ras, p44/42, c-jun and EMT markers; E-cadherin, N-cadherin, vimentin, slug, FAK, Vimentin and ß-catenin expressions upregulated as compared to parental MDA-MB-231 breast cancer cell. Moreover, sharp upregulation in vimentin, N-cadherin, Slug and ß-catenin has been determined after 48 h (Figure 5E).

Discussion

Postnatal human growth was under control of Growth Hormone (GH) secreted from pituitary gland *via* induction of somatotroph cell by Growth Hormone Releasing Hormone (GHRH) synthesized from hypothalamus [1]. Following binding of GHRH to its receptor GHRHR, GHRH/GHRHR heteroduplex complex formation triggers receptor-dependent cAMP activation through intracytoplasmic G-protein induction-mediated Ca^{+2} release. GHRH signal was finalized by GH synthesis and exocytosis from somatotrophs [3]. Beside its indirect effect on postnatal growth, GHRH has function on food uptake and sleep induction [11].

GHRH was isolated as a peptide with 40-44 amino acid residue from pancreatic tumor of acromegaly patient in 1982. Human GHRH peptide synthesized as a 108 amino acid residue preprohormone from GHRH gene localized in human chromosome 20 band at 20q11.23 locus. Biological activity of GHRH protein demonstrated that 29 amino acid residues at N' terminus had major function. Although receptor for GHRH is majorly expresses in pituitary gland, the transcriptional and translational expression of GHRH has been determined various peripheral tissue such as in ovary, placenta, testis, pancreas, gastrointestinal tract, prostate, immune system cells [12]. Biological activity of GHRH signaling has been embark on binding GHRH to its receptor GHRHR which is a member of G- protein coupled receptor family. GHRHR, a 423 amino acid length receptor, is transcribed from gene localized in human chromosome 7 at 7p14.3 [13]. The expression of GHRHR has been determined in placenta, kidney, gastric mucosa lung, liver and prostate cells [14]. Moreover, pituitary derived GHRHR splice variants (SVs),(SV1, 2, 3, 4), has been detected in various cancers such as lymphoma, glioblastoma, non-small cell lung cancer cell lines and lung cancer biopsy samples [12]. Both GHRH and GHRHR SVs has been determined in LNCaP, MDA-MB468, MDA-MB435S, T47D and NC-H838 cells. MCF-7III, MDA-MB468, T47D cells express GHRHR SV1 isoforms and MCF-7MIII, T47D cells express GHRHR SV2 [15]. In addition, translational expression of GHRH has been detected in various tumor biopsy samples and cancer cell lines such as prostate, breast, ovary, endometrium, adrenal, lung, gastric, colorectal, brain, pancreatic cancer, lymphoma, renal carcinoma [8]. Transcriptional and translational expression of GHRH has been detected in T47D, LNCaP, MDA-MB-231, MDA-MB-468 and PC3 cells by RT-PCR and immunoblotting [16,17]. Thus, we checked the transcriptional and translational expression of GHRH cDNA from various cancer cells such as LNCaP, MCF-7, T47D, BT-474, BT-20, MDA-MB-468, MDA-MB-231, MDA-MB-453, SK-BR-3, MCF-10A cells (Figure 1B). Similar to previous studies, the expression of GHRH was detected majorly in LNCaP prostate cancer and MDA-MB-231, T47D breast cancer cell lines (Figure 1B). In order to clone the human GHRH cDNA within the pcDNA3.1 plasmid, we selected LNCaP prostate cancer cells. Because of the basal GHRH expression was low in various cell lines, cloning directly to pcDNA3.1 has not been performed and we firstly clone the GHRH cDNA to pJET Blunt cloning plasmid (Figure 1C-1E) and then subcloned to pcDNA3.1 vector (Figure 2A-2B).

Cloning a target protein within an expression vector has

various steps and the essential point is to determine the positive clone expressing transformants. To demonstrate the positive clone expressing transformants, generally colony PCR, restriction enzyme analysis, immunoblotting, sequencing techniques are generally performed [18]. By this study, we first scanned the positive clones expressing GHRH cDNA by colony PCR (Figure 1E, Figure 2C) and then perform cloning restriction enzyme digestion for potential positive clones determined by colony PCR (Figure 2D). Recombinant GH expression has been demonstrated in *E.coli* BL-21 strains [19] and also HEK293 cells by SDS-PAGE electrophoresis and immunoblotting [20]. In order to determine the translational expression of cloned GHRH cDNA in bacterial system we performed SDS-PAGE electrophoresis and immunoblotting. In addition, secretion levels of GHRH protein from MDA-MB-231 breast cancer cells that are stably expressing GHRH protein has been measured by GHRH ELISA method.

As peripheral expression profile of both GHRH and GHRHR has been demonstrated, the essential role of GHRH signaling and cancer progression was realized. According to various studies, autocrine/paracrine GHRH expression triggers pathogenesis of neoplasm has been reported [12]. Moreover, GHRH signaling induced cell survival, proliferation through triggering Mitogen Activating Kinase (MAPK) has been demonstrated [8,9]. Functional GHRH peptide has been used for treating growth deficiency syndrome children with low/non-functional GH and sleep disorders. Industrial recombinant GHRH peptide was approved by Food and Drug Adminstration (FDA) and marketing with trademark as Tesamorelin (Egrifta, Thera Technologies) and used as a therapeutic agent for HIV patient's lipodystrophy symptoms in 2010 [12]. Similarly, GHRH hormone agonists treatment induces cardioprotective agent through its preventive effect on pnomolisin in pulmonary permeability edema [8]. It is reported that proliferative effect of GHRH hormone agonist on pancreatic islet cells *via* activating cell cycle mediated cell division [11]. Regarding all these study results, GHRH peptide assumed as a postnatal growth induction, irregular sleep regulator, wound healing and necrosis mediated myocardial infraction treatment agent. Thus, by this study we performed recombinant GHRH protein expressing *E. coli* HB101 bacterial clones for further studies in order to obtain large scale GHRH peptide and demonstrate its biological activity *in vitro* and *in vivo* studies.

Contrast to GHRH peptide agonists on cell proliferation, antagonists targeting GHRH has been demonstrated as an inhibitive agent on neoplasm. By this point of view, first GHRH antagonist (GHRHa) peptide has been designed by Robberecht and collegues in 1985 [21]. First designed GHRHa peptide has alanine to arginine alteration at 2. amino acid residue from N' terminal side of GHRH 1-29 peptide. 30-40 % suppressive effect of GH in acromegaly rat treated with this antagonist has been performed [22]. In addition, Zarandi and colleagues modified this antagonist through altering N' to hydrophobic feature and C' terminal with agmatine and also 4-chlorophenyalanin, 2-amino-butric acid and norleucin modifications termed as MZ- of GHRHa peptides[23]. Following MZ series, increased binding activity of GHRH to its receptor through acetylation of N' terminal fatty acids (monocarbocylic and dicarboxylic; 6-7 C atom) called as MZ-J series GHRH antagonists. Inhibitive effect of MZ-J GHRHa peptides on prostate and pancreatic cancer cell proliferation has been demonstrated [24]. Moreover, 3 μM GHRHa treatment inhibit cell proliferation and induced apoptotic cell death in time dependent manner in MDA-MB-231 breast cancer cells [25]. Molecular machinery of GHRHa MZ-J-7-118 has been demonstrated to prevent cell proliferation and induced apoptotic cell death in HCC1806, HCC1937 (ER-/PR-/HER2-) breast cancer cells through targeting MAPK signaling [26]. GHRHa JMR-132 increased the cytotoxic drugs (5-FU, irinotecan or cisplatin) mediated cell cycle arrest at S-phase and triggered apoptotic cell death *in vitro* and *in vivo* colon cancer [27]. In addition, JMR-132 prevent tumor growth through inhibition of GHRH expression in PC-3 prostate cancer cells *via* acting on Raf/MEK/ERK pathway and inactivates PI3K and Akt in xenograft mice models [28]. Similarly, dose-dependent JMR-132 treatment inhibits cell proliferation and induced caspase-3 dependent apoptotic cell death in SKOV3 and CaOV3 ovarian cancer cells *via* acting suppressive effect on EGFR/Akt pathway [29]. In order to develop more efficient therapeutic agent, GHRHa molecular machinery has to be investigated in various cancer cells such as prostate, colon, endometrium, breast and lung. GHRHa and doxorubicin combined treatment increased apoptotic cell death potential of doxorubicin agent in triple negative breast cancer cells *in vitro* and *in vivo* [30]. JMR-132 and docetaxel increased the cell viability loss in dose-dependent manner in MDA-MB-231 triple negative breast cancer cells and also combined treatment significantly increase the suppressive effect of alone agent treatment on tumor growth in xenograft mice models *in vivo* [31].

GHRH has been found to be present in many cancer types such as prostate, lung, breast and colon cancers [8]. Although it is produced in hypothalamus and induce the release of growth hormone in the anterior pituitary cells, it also affects cells other than pituitary and exerts direct activities such as survival, antiapoptotic effects and wound healing [9]. Among these activities, wound healing is an important process for gaining migration property of cells and triggering malignant progression. Therefore, we checked the wound healing capability of the cells for 72 hours and observed that while MDA-MB-231 wt cells migrate more slowly and heal the wound in 72 hours, GHRH overexpressing cells migrate more rapidly and heal the wound within 48 hours (Figure 5B). In the light of this data, we examined the translational levels of migration-related proteins. As focal Adhesion Kinase (FAK) is a protein tyrosine kinase that mediates the signaling between cells and extracellular matrix and has been linked to motility of cell [32], we first performed IF assay for FAK expression in each cell line. According to IF results, following 48 h forced GHRH expression triggered FAK localization within MDA-MB-231 cells. Concomitantly, ß-catenin, a transcription factor promoting cell growth, proliferation, has been increased intracellular translational profile in GHRH+ MDA-MB-231 cells. Because of its effects on cell-cell adhesion and migration induction [24], induction of colony formation and migration profile *via* accelerated GHRH signaling through ß-catenin upregulation. Moreover, Talin-1 is a cytoskeletal protein which plays a central role in regulation of integrin family of cell adhesion proteins and binds to many adhesion molecules as integrins and have major role in tumor formation, invasion-metastasis [33]. By this stud, we demonstrated that forced GHRH expression triggered Talin, ß-catenin and FAK cytoplasmic localization as compared to parental MDA-MB-231 cells by IF and IB assay results. Thus, Talin, ß-catenin and FAK might be downstream target for GHRH signaling that leads cell migration and metastasis. Similarly, FAK strongly influence the expression

of VEGF and MMPs. Vascular endothelial growth factor (VEGF) is an endothelial mitogen and proangiogenic factor which regulates survival, proliferation and migration when binds to its receptor. Matrix metalloproteinases are markers of a tumor's invasion potential. In particular, MMP-2 and MMP-9 expression is important for invasion. These enzymes degrade the components of extracellular matrix and allow cells to invade nearby tissues [34]. To go further, we performed RT-PCR to determine the transcriptional levels of these migration-associated genes and observed upregulation of VEGF in 24 h and MMP-2 and MMP-9 in 48 hours (Figure 5D). Epithelial cells should undergo epithelial-mesenchymal transition (EMT) and gain mesenchymal characteristics in order to migrate. Cadherins, that mediate the organization of cytoskeleton of the cells, play the major role in this process along with vimentin, snail, slug and twist. One of these cadherins, E-cadherin, is localized on the surface of epithelial cells and important in cell-cell adhesion. Upregulation of N-cadherin and suppression of E-cadherin leads to dysfunction of cell-cell adhesion and increase in invasiveness [35]. We demonstrated that accelerated GHRH signalling triggers proliferation, invasion-metastasis through acting on mesenchymal marker; N-cadherin, Vimentin and Slug in time-dependent manner in active GHRH signalling.

Conclusion

In conclusion, we demonstrated the GHRH transcriptional expression profile of various frequently laboratory using cancer cell lines such as prostate, breast cancer cell lines with different genomic content. In the meantime, cloning of GHRH cDNA within pcDNA3.1 plasmid and generate GHRH overexpressing MDA-MB-231 breast cancer cells. Although MDA-MB-231 breast cancer cells expresses GHRH, we perform a forced GHRH expressing MDA-MB-231 breast cancer cell line in order to understand the role of GHRH invasion-metastatic profile and molecular machinery underlying this process. Thus, overexpression of GHRH induced cell proliferation, invasion-metastasis and oncogenic induction through acting on MAPK signalling pathway and EMT key molecules in time-dependent manner. Finally, we generated hormone-dependent increased metastatic profile triple negative breast cancer cell line for further investigations on drug-mediated resistance *in vitro* and *in vivo* cancer models.

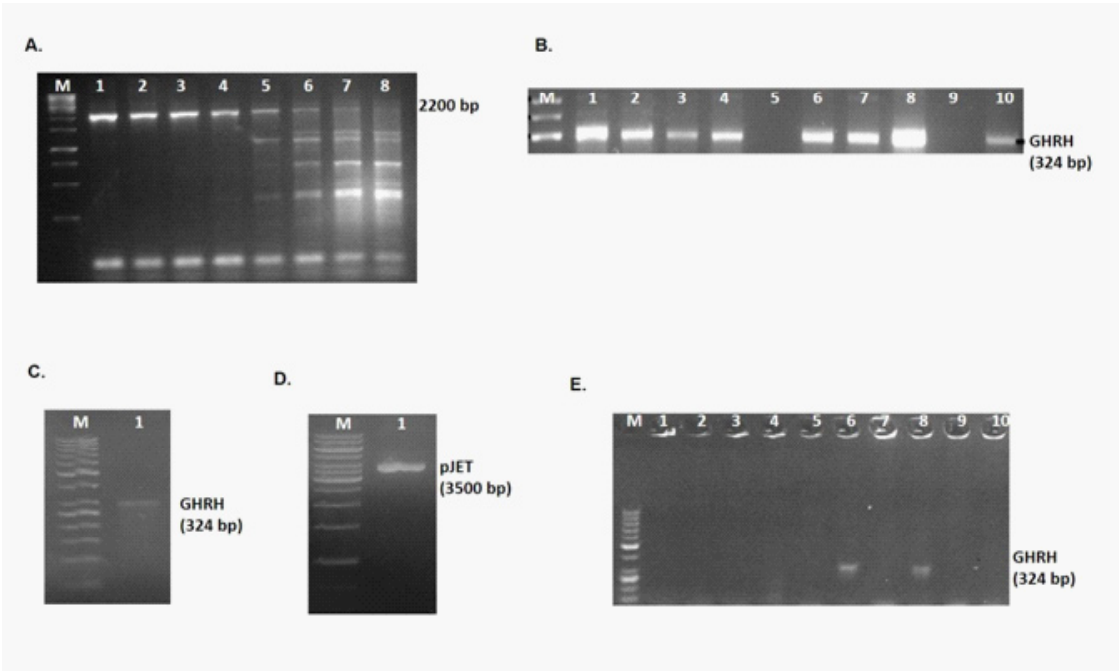

Figure 1: Amplification of GHRH cDNA and cloning in pJET vector.
A. Annealing temperature for GHRH cDNA primers was determined by gradient PCR using genomic DNA of LNCaP cells. PCR products were run on 2% agarose gel. M: 1 kb DNA ladder, 1: 65°C, 2: 64°C, 3: 62°C, 4: 59.3°C, 5: 55.9°C, 6: 53.2°C, 7: 54.8°C, 8: 52.5°C.
B. The transcriptional expression of GHRH cDNA was demonstrated in various cancer cells by using RT-PCR. M: 100 bp ladder, 1: LNCaP, 2: MCF-7, 3: T-47D, 4: BT-474, 5: BT-20, 6: MDA-MB-468, 7: MDA-MB-231, 8: MDA-MB-453, 9: SK-BR-3, 10: MCF-10A.
C. LNCaP GHRH cDNA and
D. pJET1.2/blunt cloning vector were cleaved with NcoI and XhoI restriction endonucleases, digestion products were visualized by 2% and 0.8% agarose gel electrophoresis, respectively.
E. Digested cDNA and pJET1.2/blunt cloning vector were ligated with T4 DNA Ligase, transformed to *E. coli* HB101 cells and colony PCR was performed to select the colonies expressing GHRH cDNA by using cloning primers, and PCR results were visualized by %2 agarose gel electrophoresis.

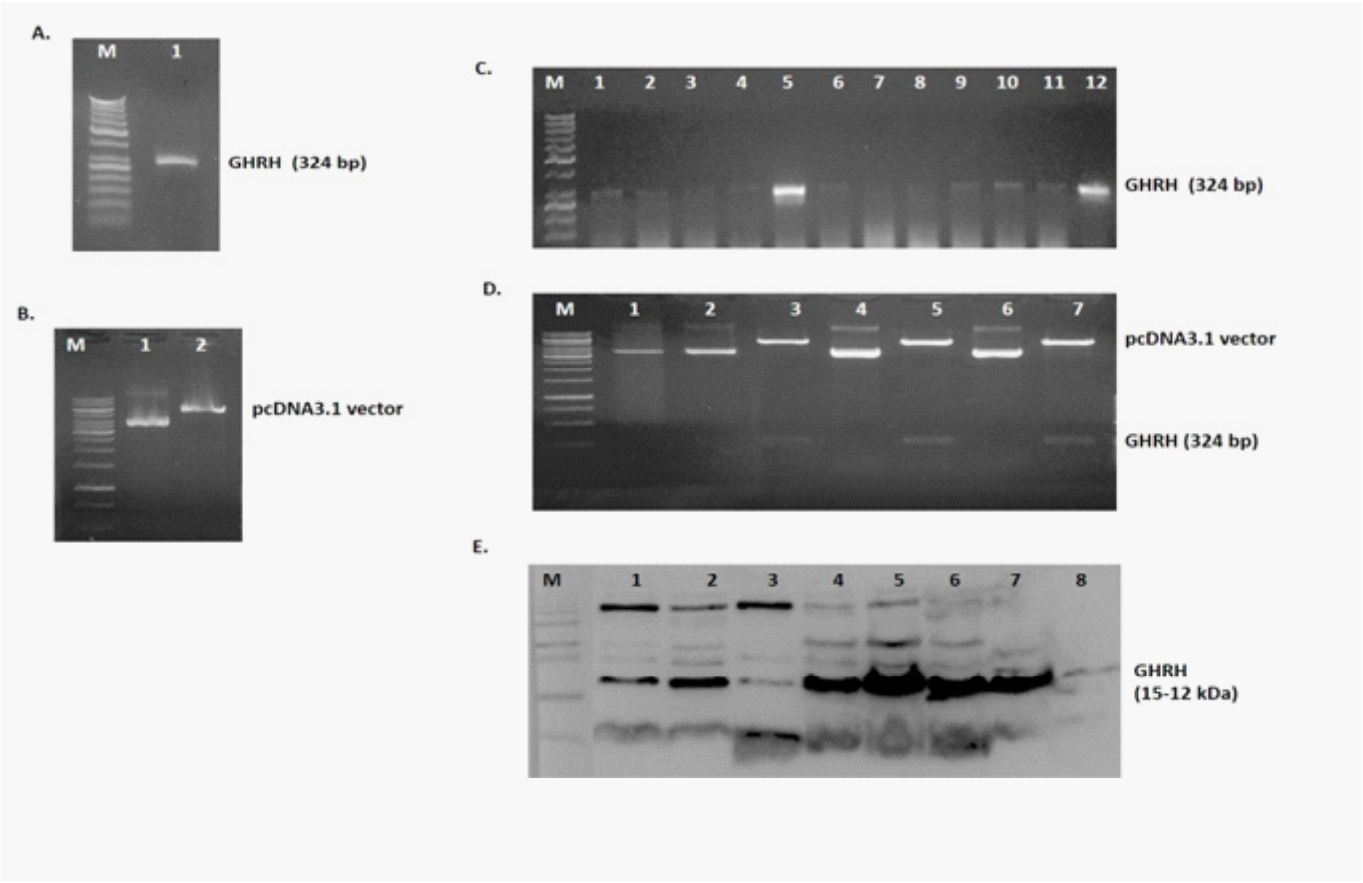

Figure 2: Subcloning of GHRH cDNA in pcDNA3.1 mammalian expression vector.
A. GHRH cDNA inserted pJET vector was amplified with primers having EcoRI and HindIII recognition sites, PCR product was digested with EcoRI and HindIII and purified from 2% low melting agarose gel M: 50 bp DNA ladder, 1. Purified PCR product.
B. pcDNA3.1 vector was double digested with EcoRI and HindIII and run on 0.8% agarose gel .M: 1 kb ladder, 1. uncut pcDNA3.1 vector, 2. double digested vector.
C. Double digested products were ligated and transformed to *E. coli* HB101 cells and GHRH cDNA expressing clones were selected by colony PCR.
D. Plasmids were isolated from selected positive clones and cut with EcoRI and HindIII restriction enzymes. M: 1 kb ladder; 1. Uncut control plasmid; 2-4-6. Uncut clones 5, 10 and 12, respectively; 3-5-7. Digested clones 5-10-12, respectively.
E. Selected positive clones inserted *E.coli* HB101 cells were incubated in 40 ml LB medium with kanamycin. Following incubation overnight, total protein was isolated and load on SDS-PAGE. The translational expression of GHRH in each clone were visualized by SDS-PAGE following Coomassie brilliant blue staining.

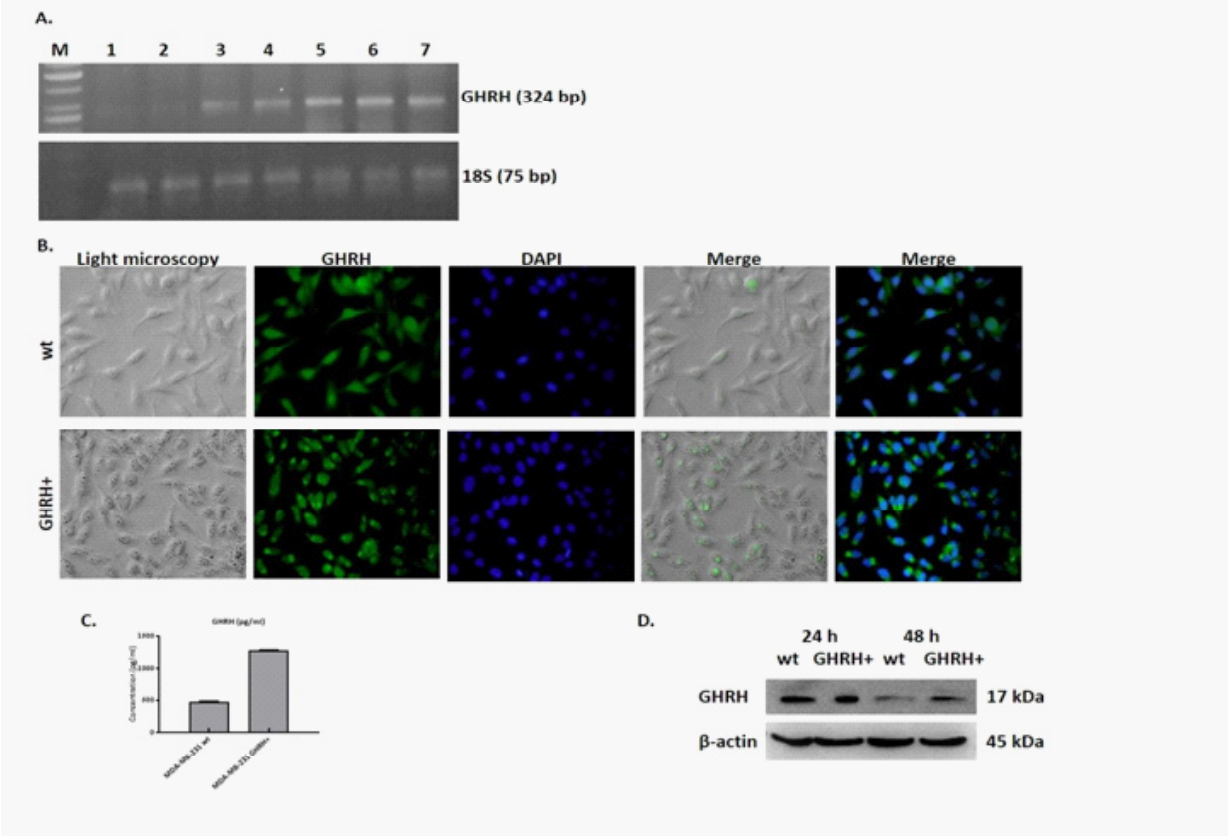

Figure 3: GHRH expression of MDA-MB-231 cells were enhanced by transfecting MDA-MB-231 wild type cells *via* transfection of GHRH cDNA inserted pcDNA3.1.

A. Plasmid were extracted from *E. coli* clone 3 and MDA-MB-231 wild type cells were transfected with this plasmid *via* liposomal agents. After 48 hours incubation, GHRH overexpressing MDA-MB-231 cells were selected with neomycin. RNA was isolated from selected clones and GHRH mRNA levels were checked by RT-PCR (above). 18S rRNA expression was also checked as loading control (below). M: 100 bp ladder, 1. MDA-MB-231 wt, 2. MDA-MB-231 GHRH clone-1, 3. MDA-MB-231 GHRH clone-2, 4. MDA-MB-231 GHRH clone-3, 5. MDA-MB-231 GHRH clone-4, 6. MDA-MB-231 GHRH clone-5, 7. MDA-MB-231 GHRH clone-6.

B. GHRH overexpression in MDA-MB-231 GHRH+ cells were demonstrated by immunofluorescence. MDA-MB-231 wt and GHRH+ cells were fixed and incubated with GHRH primary antibody. Cells were then subjected to Alexa Fluor-conjugated secondary antibody and observed by fluorescence microscopy.

C. ELISA assay was performed according to manufacturer's instructions. GHRH concentration in media was calculated according to reference standard.

D. Immunofluorescence results were confirmed by immunoblotting. MDA-MB-231 wt and GHRH+ cell lysates were obtained, proteins were separated on SDS-Polyacrylamide gel, transferred to PVDF membrane and incubated with antibody against GHRH.

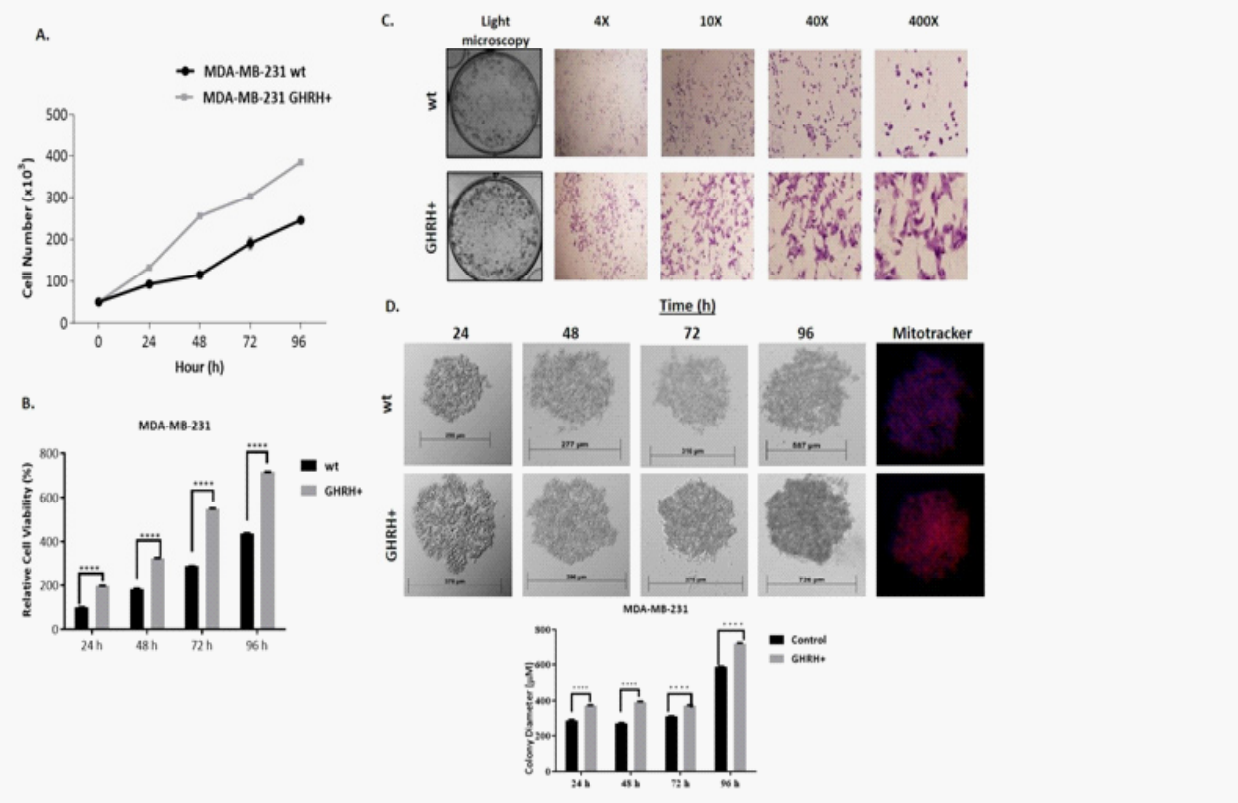

Figure 4: GHRH overexpression increases cell proliferation, colony formation in MDA-MB-231 breast cancer cells.
A. Cell growth enhancement effect of GHRH overexpression was determined by trypan blue dye exclusion assay.
B. Autocrine GHRH expression efficiency on cell proliferation was determined by MTT cell viability assay in time-dependent manner.
C. Inserted GHRH cDNA enhanced colony formation in GHRH+ cells with respect to parental MDA-MB-231 cells by soft agar method and indicated in bar graph.
D. MDA-MB-231 wt and GHRH+ cells were seeded on 6 well plate and incubated for 10 days. Following incubation period, colony formation was determined by light microscopy after fixed and crystal violate stained cells. Magnify: 40x, 100x, 400x. *p-value: <0.05, ** <0.01,***<0.001, **** <0.0001.

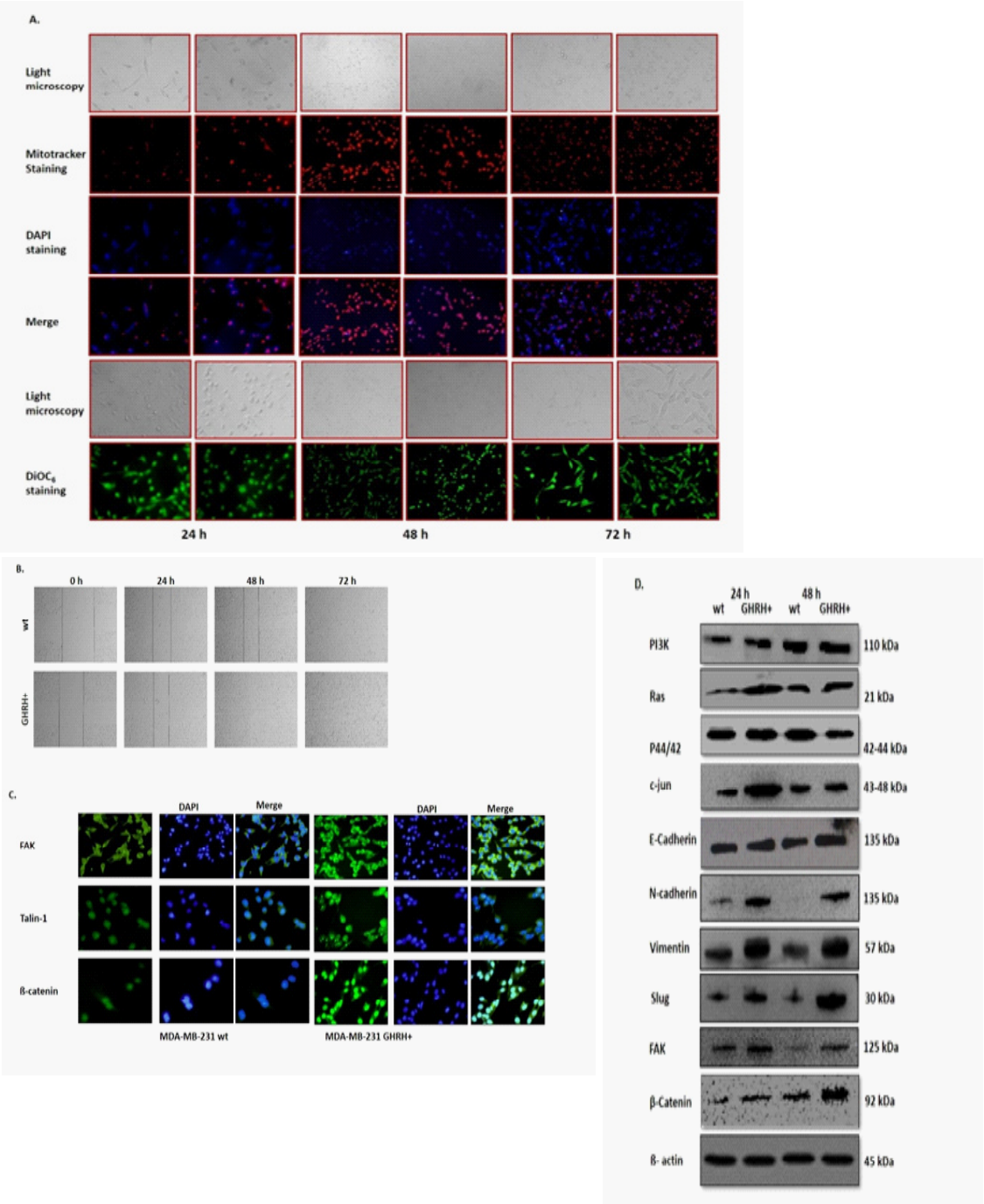

Figure 5: GHRH overexpression induces epithelial-mesenchymal transition.

A. MDA-MB-231 wt and GHRH + cells were seeded in 6 well plate with a cell density of $3x10^5$ cells/well. Following each 24 h time intervals, cells morphology and survival potential determined by Mitotracker/ DAPI and DioC6 staining by fluorescence microscopy. (Magnify: 400x).

B. For scratch assay, cells were seeded on 6-well plate ($3x10^5$ cells/well) and scratched with pipette tip. Radius of the resulting wound was measured every 24 h.

C. Immunofluorescence assay was performed to detect the protein expression of FAK, Talin-1 and ß-catenin proteins. Cells were fixed on coverslips and treated with stated antibodies (1:50 v/v in PBB solution). After incubation with Alexa-Fluor 488 conjugated seconder antibody (1:250 in PBB solution), protein expression was visualized by fluorescent microscopy. transcriptional expression profile of VEGF, MMP-2, -9 were determined by isolation of total RNA in MDA-MB-231 wt and GHRH+ cells in time dependent manner. 18S was used as a housekeeping in RT-PCR.

D. Protein expression of E-cadherin, Vimentin, ß-catenin, c-jun, Ras, p44/42 and PI3K were determined by immunoblotting. ß-actin was used as loading control and band intensities were determined by Image J program.

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