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Sensitising Breast Cancer Cells to Chemotherapy by Down Regulation of Lifeguard

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Abstract

Introduction: Lifeguard (LFG) is an anti-apoptotic protein that inhibits programmed cell death mediated by Fas in tumour cells. The exact mechanism of action and the molecular function from LFG in the carcinogenesis of human breast cells is not clear. But the expression of LFG mRNA correlates with LEF-1 transcription factor activity.

Methods: In the present study, chemotherapeutic-induced apoptotic effects were studied using MCF-7 cells as an *in vitro* test model. Molecular (Western blot and RT-PCR) techniques were used to investigate LFG expression. To investigate the breast cancer cell proliferation in presence of siRNA-LFG we performed fluorescent cell viability assays.

Results: The results indicated that, a decrease of LFG expression using siRNA correlates with an increased sensitivity to Trastzumab and Erlotinib. Moreover, cell cycle analysis of LEF-1 siRNA-transfected human breast cancer cells revealed a significant arrest in G_2 phase.

Conclusion: Taken together, our results indicate a pivotal role of LFG in the regulation of apoptosis in MCF-7 breast cancer cells.

Introduction

Breast cancer as one of the most frequent diagnosed cancers at all, is the leading cause of cancer death among women worldwide and hence demands new effective therapeutic prospects [1]. Numerous studies characterized the role of the PI3-K/Akt signalling pathway as a pivotal one in cancer progression since various up regulated pathway components have been observed in cancer cells and most notably since inhibition of the Akt signal cascade has been described as resulting in apoptosis, suppression of cell survival and thus inhibition of tumour genesis [2-9].

Human epidermal growth factor receptor (HER2/neu), a member of the epidermal growth factor receptor family (EGFR) and responsible for increased levels of active Akt, is a potent target for anticancer management [10-13]. Pharmacological applications of anti HER2 monoclonal antibodies like Trastzumab (Herceptin) or EGFR inhibitors like Erlotinib (Tarceva) have been in clinical use already and afford proved inhibition of cell proliferation as well as downregulation of PI3K/Akt pathway activity [10,14,15].

While recent studies presented a large number of patients who no longer respond to chemotherapy like Erlotinib and revealed a correlation to the deregulated PI3K-PTEN-Akt signal cascade [15,31], research on new medical options to overcome these resistance seems to be essential.

The anti-apoptotic protein Lifeguard (LFG), responsible for inhibition of FasL-mediated programmed cell death [16,17], was previously analysed as structurally and functionally associated to the BI-1 and Bcl-2 family [18,19], but recently to the so called LFG gene family [20]. Several studies showed that a decreased sensitivity to FasL-induced apoptosis is based on a high LFG mRNA and protein expression rate, which was notably discovered in breast cancer cell lines. LFG downregulation therefore implicates an attractive therapeutic chance for anticancer therapy via antisense oligonucleotides or siRNA

[21-23]. While the exact molecular mechanism of LFG regulation has not yet been fully understood, it is hypothesized as mediated by the PI3-K/Akt/LEF-1 signalling pathway because PI3-K/Akt inhibition in MCF-7 and MDA-MB-231 breast cancer cells leads to increased rates of apoptosis and reduced amounts of LFG mRNA and Protein [23,24]. The transcription factor LEF-1 plays a crucial part in the regulation of cellular development and differentiation [25,26] in breast cancer [27,28].

Using MCF-7 breast cancer cells, we investigated the impact of LFG and LEF-1 suppression on cell proliferation, viability and apoptosis.

Materials and Methods

Cell lines

Human breast carcinoma cell lines MCF-7 (ATCC, Manassas, USA) were used in this study and grown in Dulbecco`s modified Eagle`s medium (DMEM, PAA, Cölbe, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany) and 50 mg/ml penicillin-streptomycin. Cultures were maintained at 37°C with 5% carbon dioxide in a humidified atmosphere. The medium was changed every 2 to 3 days, and cells were subcultured by treatment with 0.25% Trypsin/0.53 mM ethylenediaminetetraaceticacid (EDTA) (Biochrom, Berlin, Germany) solution.

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Real-time polymerase chain reaction

Total RNA was isolated from cultured cells using the NucleoSpin RNAII Kit (MN Macherey-Nagel, Duren, Germany) according to the manufacturer`s protocol. RNA concentration was measured by photometry at NanoDrop (PeqLab, Erlangen, Germany). The quality of total RNA was verified by the integrity of 18S/28S ribosomal RNA in 1% ethidium bromide-stained agarose gels. Reverse transcription (RT) was performed with 1 µg total RNA using iScript™ cDNA Kit (Bio-Rad Laboratories, Hercules, CA). Real-time polymerase chain reaction (Q-PCR) reaction was carried out in 20 µl samples with 5ng cDNA and 10 pmol of each forward and reverse primer and 2x SYBR green Sensi-Mix DNA Kit (Quantace, London, U.K.). Relative gene expression was determined by the fluorescence intensity ratio of the target gene to ß2-Microglobulin. The primers used in the real-time PCR reaction were designed based on information from the human genomic data base. The following primer sequences were used:

human LFG: forward: 5`-gactcatcctggccatcctcctac-3`

reverse: 5`-ggcgtcggttacccatcagc-3`;

human. LEF-1: forward: 5`agagaaaggagcag-3`,

reverse: 5`-attgtctcttgcag-3`,

ß2-Microglobulin: forward: 5`-atgagtatgcctgccgtgtga-3`

reverse:5`-ggcatcttcaaacctccatg-3`.

The initial denaturation step at 94°C for 4 min. was followed by 40 cycles of denaturation for 30 s, annealing at 65°C for 30 s, extension at 72°C for 1 min., and final extension step at 72°C for 10 min. All experiments were carried out in triplicate and repeated at least at three independent times. The specificity of the Q-PCR products was proven by the appropriate melting curves (specific melting temperature).

Western blot analysis

For Western blot analysis, cells were lysed in RIPA buffer containing 0.3M NaCl, 1% sodium desoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton-X-100, 20 mM Tris-HCL (pH 8), 1 mM EDTA, and 1 mM phenylmethyl sulfonyl fluoride. 25 µg of protein were fractionated by 15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Bedford, USA), then blocked in Odyssey buffer for 1 hr. Protein expression levels were determined by immunoblotting with the following antibodies: anti-hLFG (1:200 dilution) (IMGENEX, San Diego, CA), monoclonal anti-LEF-1 (at 1:500) purchased from Abcam, Cambrige, UK, and anti-goat-ß-Actin (at 1:1000) (Abcam, Cambrige, UK), at 4°C overnight. For quantification of protein expression levels, Odyssey 680/800 nm secondary conjugates were used and PVDF membranes were analysed using the Odyssey Infra-Red Imaging System and software (Li-Cor BioSciences, Lincoln, Nebraska, USA).

Small interfering RNA

In this study, we transfected MCF-7 cells with siRNA LFG-5`gggcaaagaaacattctatgt-3`, control siRNA LFG-5`ggaatctcattcgatgcatac-3` (designed by SuperArray Bioscience Corporation, USA) and with siRNA LEF-1-5`guugcugaguguacucuaa-3`, control siRNA LEF-1-5`uuagaguacacucagcaa-3` (designed by Ambion, USA). The cells were seeded at 1×10^4 cells and incubated at 37°C, 5% CO$_2$ in humidified atmosphere for 48 hrs before being analysed.

Caspase assay

Activation of caspase-3/7 was determined using the Apo-One Homogeneous Caspase-3/7 Assay (Promega, Madison, WI) following the protocol provided by the manufacturer. Briefly, 1×10^4 MCF-7 breast cancer cells were seeded per well of a 96 well plate and transfected with siRNA LFG and control siRNA, for 48 hrs. After 48 hrs cells were incubated with 0.25 mg/ml, 0.5mg/ml or 1mg/ml of Trastzumab (Hercetin®, Roche) or Erlotinib (Tarceva®, Roche) for 2 hrs and 4hrs, respectively. After treatment, cells were mixed with the same volume of Apo-One Homogeneous Caspase-3/7 reagent and incubated at room temperature for 2 hrs. Caspase-3/7 activation was estimated from sample fluorescence at the excitation wavelength of 492 nm and the emission wavelength of 521 nm using a fluorescence plate reader Tecan GENios (TECAN, Männedorf, Switzerland).

Cell viability assay

Metabolic activity was determined by Cell Titer Blue cell viability assays (Promega, Madison, USA). Briefly, 1×10^4 cells from MCF-7 breast cancer cells were seeded, 1×10^5 cells per well of a 96 well plate and transfected with siRNA LEF-1 and control siRNA, for 48 hrs (HiPerFect Transfection Reagent; Fa. Qiagen). Relative numbers of viable cells were measured in comparison to the untreated control and the solvent control according to the manufacturer's instructions at $560_{Ex}/590_{Em}$ nm in a fluorescence plate reader TecanGENios (TECAN, Männedorf, Switzerland).
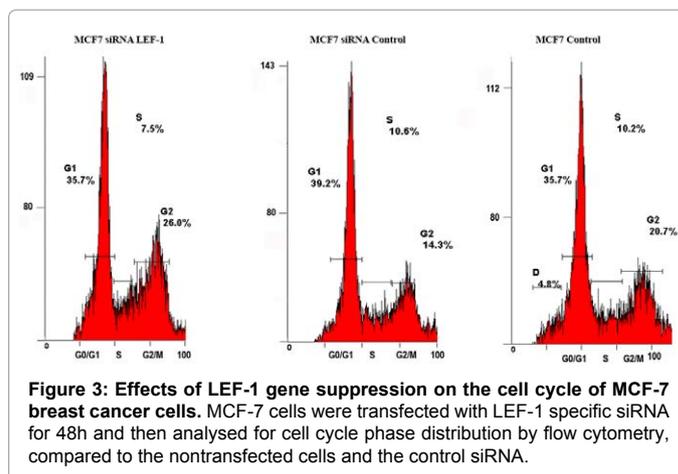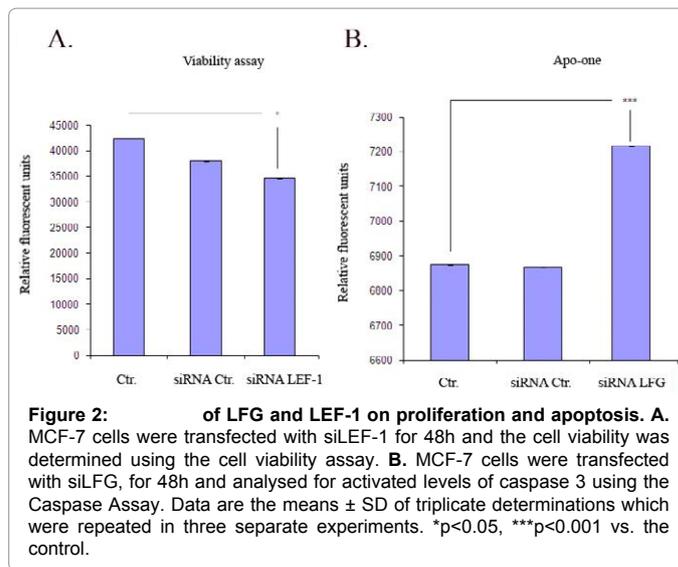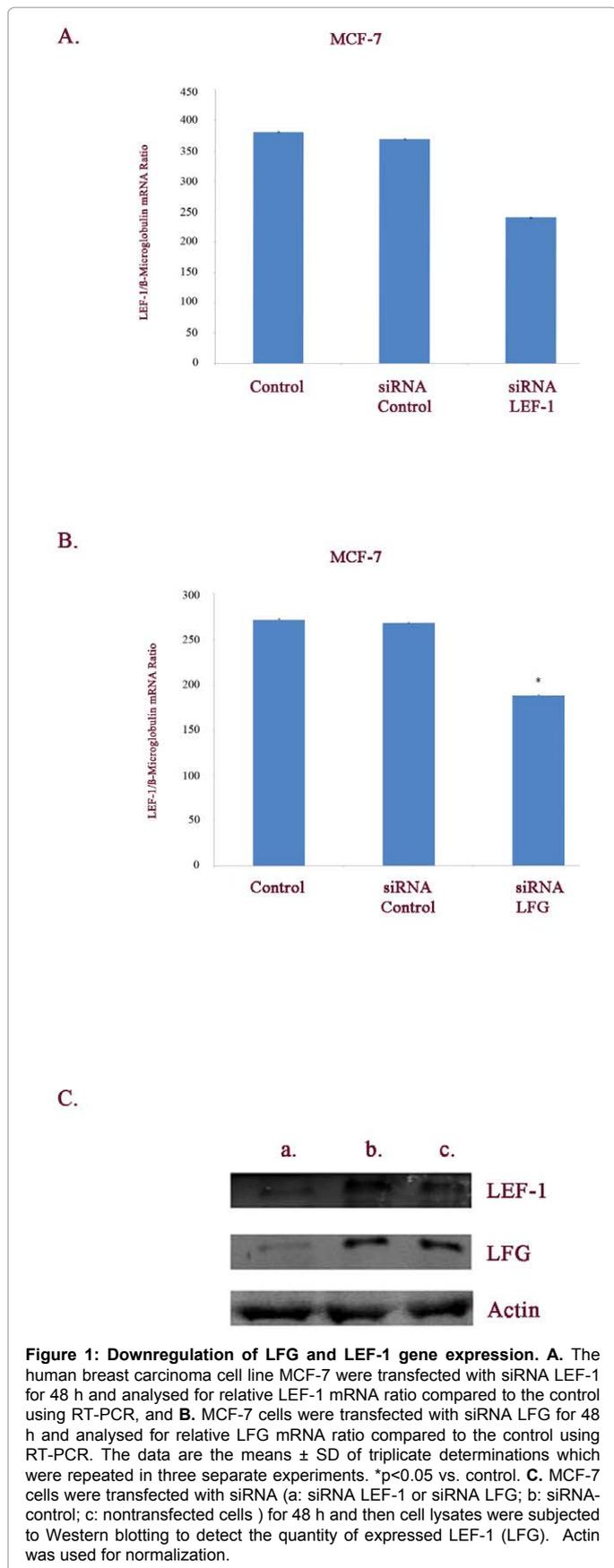
Cell cycle analysis by flow cytometry

Viability analysis of the cells was performed in a Vi-CELL Series Cell Viability Analyzer (Beckman Coulter GmbH, Krefeld, Germany). For distinct cell cycle phase distribution, about 10^6 breast cancer cells were analysed. Thus, the cells were harvested and fixed in 70% (v/v) ice-cold ethanol and kept at 4°C for 24 h. Thereafter, the fixed cells were stained with CyStain DNA 2 step kit (Partec GmbH, Münster, Germany) and filtered through a 50 µm filter. These samples were then analyzed in Galaxy flow cytometer (Dako, Hamburg, Germany) using FloMax analysis software (Partec) and the MultiCycle cell cycle software (Phoenix Flow Systems Inc., San Diego, CA, USA).

Results

Downregulation of LFG and LEF-1 expression by siRNA transfection

In order to demonstrate the impact of LFG and LEF-1 protein in breast cancer, MCF-7 cells were transfected as described in experimental procedures, with designed LFG specific siRNA and with designed LEF-1 specific siRNA, respectively. After 48 h, quantitative RT-PCR and Western blot analysis were performed to determine successful gene silencing. While the transfected cells exhibited decreased levels of LEF-1 and LFG mRNA respectively (Figures 1A and B), we also found, as expected, reduced amounts of LEF-1 and LFG protein (Figure 1C) compared to the si-Control and untreated Control cells.To examine the effect of LFG and LEF-1 downregulation on the apoptosis and viability in breast carcinoma cells, we transfected MCF-7 cells with siRNA LFG or siRNA LEF-1as before. Appropriate vitality measurements demonstrated reduced cell viability in LEF-1 siRNA transfectants as compared to control siRNA and untreated cells after 48 h (Figure 2A). Moreover, proliferative effects of LEF-1 down-modulation were assayed by cell cycle analysis. Following transfection of MCF-7 breast cancer cells with LEF-1 siRNA for 48 h, cell cycle phases were determined by flow cytometry analysis. Thus, LEF-1 siRNA transfection of MCF-7 demonstrated a significantly reduced amount of S phase cells and a marked accumulation of cells in G2 phase (Figure 3). Quantitative analysis of the cell cycle phases revealed a similar

Figure 1: Downregulation of LFG and LEF-1 gene expression. A. The human breast carcinoma cell line MCF-7 were transfected with siRNA LEF-1 for 48 h and analysed for relative LEF-1 mRNA ratio compared to the control using RT-PCR, and B. MCF-7 cells were transfected with siRNA LFG for 48 h and analysed for relative LFG mRNA ratio compared to the control using RT-PCR. The data are the means ± SD of triplicate determinations which were repeated in three separate experiments. *p<0.05 vs. control. C. MCF-7 cells were transfected with siRNA (a: siRNA LEF-1 or siRNA LFG; b: siRNA-control; c: nontransfected cells) for 48 h and then cell lysates were subjected to Western blotting to detect the quantity of expressed LEF-1 (LFG). Actin was used for normalization.

Figure 2: of LFG and LEF-1 on proliferation and apoptosis. A. MCF-7 cells were transfected with siLEF-1 for 48h and the cell viability was determined using the cell viability assay. B. MCF-7 cells were transfected with siLFG, for 48h and analysed for activated levels of caspase 3 using the Caspase Assay. Data are the means ± SD of triplicate determinations which were repeated in three separate experiments. *p<0.05, ***p<0.001 vs. the control.

Figure 3: Effects of LEF-1 gene suppression on the cell cycle of MCF-7 breast cancer cells. MCF-7 cells were transfected with LEF-1 specific siRNA for 48h and then analysed for cell cycle phase distribution by flow cytometry, compared to the nontransfected cells and the control siRNA.
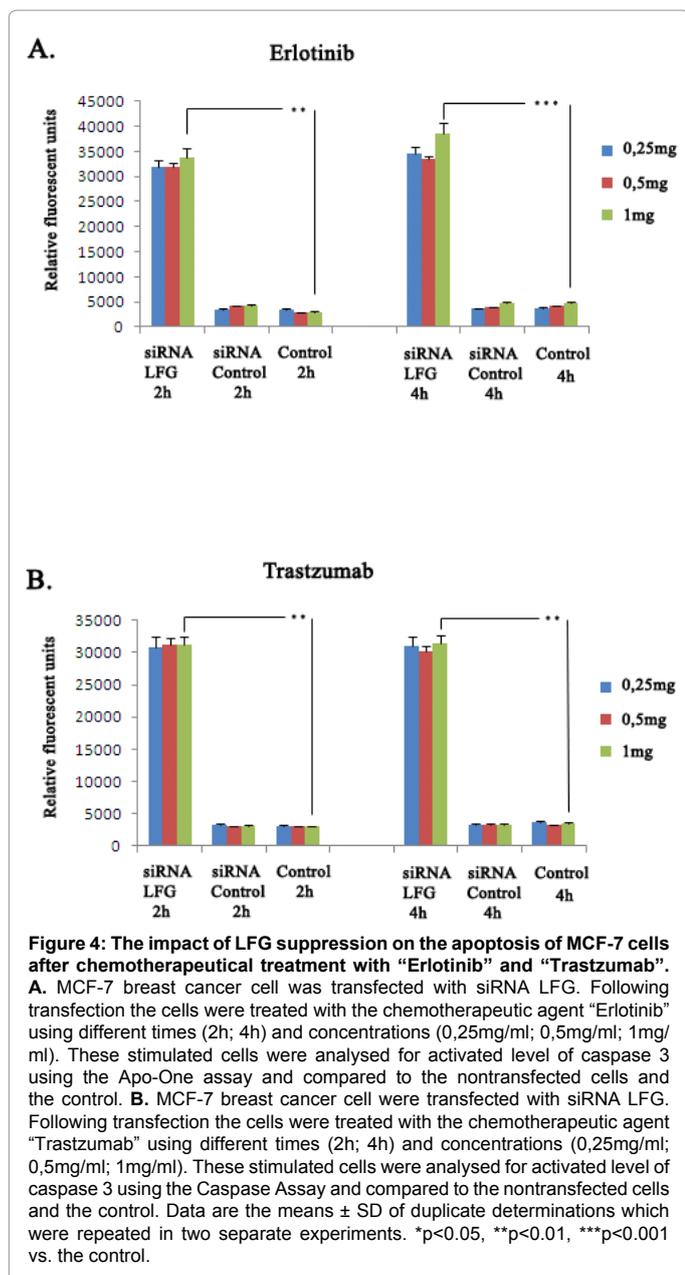
cell cycle distribution in MCF-7 control cells and the population's transfected with a control siRNA reaching about about 10.2% to 10.6% of cells in S phase and about 20.7% to 14.3% of cells in G2/M phase, respectively. In contrast, down-modulation of LEF-1 after 48 h of LEF-1 siRNA transduction was associated with a significant decline of the S phase to about 7.5% respectively (Figure 3). Conversely, a significant accumulation of cells in G2/M phase to about 26% detectable in 48 h LEF-1 siRNA transfectants (Figure 4B).

Furthermore, we found significant increased levels of activated caspase-3/7 in MCF-7 cells with downregulated expression of LFG compared to control siRNA and untreated cells after 48 h (Figure 2B). Consequently we demonstrated that downregulation of LFG correlates with an increased sensitivity to chemotherapeutical treatment measured by increased rates of apoptosis.

Sensitisation of MCF-7 cells against chemotherapeutical treatment in consequence of LFG gene suppression

To corroborate our hypothesis, that downregulated expression of LFG has direct effects on the chemotherapeutic given of breast cancer cells, we transfected MCF-7 cells with LFG specific siRNA and treated them with 0.25 mg/ml; 0.5 mg/ml or 1 mg/ml of the cytostatic drugs "Erlotinib" or "Trastzumab" respectively. Following 2h and 4h of

Figure 4: The impact of LFG suppression on the apoptosis of MCF-7 cells after chemotherapeutical treatment with "Erlotinib" and "Trastzumab". **A.** MCF-7 breast cancer cell was transfected with siRNA LFG. Following transfection the cells were treated with the chemotherapeutic agent "Erlotinib" using different times (2h; 4h) and concentrations (0,25mg/ml; 0,5mg/ml; 1mg/ml). These stimulated cells were analysed for activated level of caspase 3 using the Apo-One assay and compared to the nontransfected cells and the control. **B.** MCF-7 breast cancer cell were transfected with siRNA LFG. Following transfection the cells were treated with the chemotherapeutic agent "Trastzumab" using different times (2h; 4h) and concentrations (0,25mg/ml; 0,5mg/ml; 1mg/ml). These stimulated cells were analysed for activated level of caspase 3 using the Caspase Assay and compared to the nontransfected cells and the control. Data are the means ± SD of duplicate determinations which were repeated in two separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. the control.

incubation we detected a significantly increased levels of caspase 3/7 in the LFG siRNA transfectants compared the si-Control and non-transfected cells (Figure 4). The activation of apoptosis demonstrated the potential of LFG gene silencing for sensitisation of MCF-7 breast cancer cells towards chemotherapeutical treatment.

Discussion

In addition to the fact that breast cancer is still the leading cause of cancer death in women worldwide [1], numerous studies have described the occurrence of resistances to conventional chemotherapy in cancer cells [14,29-33] and therefore underline the importance of understanding expression, regulation and function of signalling pathways. Dysfunction of this cell death system is contributed to several deregulations of antiapoptotic proteins like Bcl-2, Bcl-xl or BI-1 [19,39,40].

Cognition of the oncogenic potential from HER2 that is based on aberrant overexpression correlating with tumorigenesis and poor clinical outcome in breast cancer reveals the role of anti HER2 therapy [10-12]. Trastzumab is a humanized monoclonal antibody, which binds directly on the extracellular domain of the HER2 to initiate several mechanisms include the PI3K/Akt pathway and the MAPK pathway whereas Erlotinib, a small molecular EGFR inhibitor, targets the ATP-binding site of EGFR. Thereby, activity of the tyrosin kinase gets depressed and results in promoting inhibition of proliferation and induction of apoptosis in cancer cells [15,37,38]. Both drugs interfere with the crucial PI3K-PTEN-Akt signal cascade, which is moreover determined to be deregulated in breast cancer cell and hence responsible for drug resistance [10,14,29-31,39,40]. LFG an antiapoptotic protein with potential involvement in cancer progression and previously well described [16-21], is the target gene of the AKT/LEF-1 pathway are for this studies one potential regulator of apoptosis in tumour cells [23,24].

After treatment of MCF-7 breast cancer cells with LFG-specific siRNA, successful downregulation of LFG expression was proved by analysing the amount of mRNA and protein (Figures 1A and C), we demonstrated that this kind of gene silencing affects a significant raised level of activated caspase-3/7, which indicates an increased rate of apoptosis (Figure 2B).

Investigating clinical possibilities given by these findings, we detected here for the first time convincing evidence that LFG gene suppression sensitizes breast cancer cells to chemotherapeutical treatment (Figure 4). Following LFG gene silencing via specific siRNA transfection in MCF-7 breast cancer cells, our data clearly revealed significantly increased levels of activated caspase-3/7 after incubation with cytostatic drugs (e.g. Erlotinib or Trastzumab), compared to the si-Control and nontransfected cells. To contrast this results with the rate of apoptotis in LFG downregulated cells without chemotherapeutical treatment, we even showed a fivefold advance, which indicate the combined use of siRNA and chemotherapy as an enhanced effective strategy (Figure 2A and 4). Our findings are congruent with studies focused on other anti-apoptotic proteins like bcl-2, bcl-xl or p53 [32-36], and demonstrated as well favourable prospects using siRNA to overcome chemoresistance by inhibiting protein expression.

Our results displayed a slightly more potent treatment of Erlotinib to initiate apoptotic processes in LFG downregulated MCF-7 cells as compared with Trastzumab treated ones (Figure 4). This effect possibly originated from their different mechanism of inhibition, but still needs to be further elucidated. In both experimental parts the level of activated caspase-3/7 raised up on the sevenfold already 2h after treatment with lowest dose of 0.25 mg/ml, whereas an increasing concentrations on the double or fourfold no increased apoptosis proportional of the used chemotherapeutic concentration shows. This considerable suggest the high potential of sensitising.

LEF-1, member of the TCF/ LEF family and hence a regulatory participant in cell maturation and development [25,26], was identified as a LFG controlling transcription factor [24] and is therefore an important part of our studies. In the present study we demonstrated the critical role of LEF-1 on viability and proliferation in MCF-7 breast cancer cell. siRNA mediated downregulation of LEF-1 mRNA and protein (Figure 1A and C) led to an obvious reduction of cell viability in general (Figure 2A) and to an inhibition of proliferation caused by an increased accumulation of cells in the G2 phase of cell cycle (Figure 3). Based on previous reports, which have described the upregulation or aberrant activation of LEF-1 during cancer progression [28,41,42],

our findings indicated that LEF-1 may be suited as a new target in anticancer management.

In conclusion the present study corroborates the essential role of LFG and its regulatory transcription factor LEF-1 as well as its regulatory mechanisms. Moreover demonstrates here for the first time sensitisation of breast cancer cells to chemotherapeutics, caused by LFG gene suppression. In prospective, our results have to be tested in context with other resistant types of cancer plus different chemotherapeutics and finally proved in preclinical *in vivo* experiments.

The authors declare that they have no competing interests.

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