

# Role of GDF15 in Radiosensitivity of Breast Cancer Cells

## Research Article

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**Abstract:** The Growth Differentiation Factor-15 gene (GDF15) is a member of TGF- $\beta$  superfamily and this cytokine family is considered to be a promising target for cancer therapy. The purpose of this study was to investigate the effect of tumor derived GDF15 on proliferation and radiosensitivity of breast cancer cells *in vitro* and *in vivo*. A mouse breast cancer LM2 cell line with stable transfection of full-length mouse GDF15 cDNA was established. Cell growth and proliferation was observed using WST assay and impedance-based method. Radiation induced GDF15 and TGF- $\beta$ 1 expression was determined by qRT-PCR. Radiosensitivity was measured by a colony formation assay *in vitro* and by a tumor growth delay assay *in vivo*. Cells with more than a 10-fold increase in GDF15 expression had a higher growth rate than parental control cells *in vitro* and *in vivo*. The radiation induced elevation of the expression of TGF $\beta$ 1 was reduced in GDF15 overexpressing cells. GDF15 may play a role in the radiation response of breast cancer cells by effecting cell survival, inhibiting radiation-induced cell death, and inhibiting the TGF- $\beta$ 1 related cytotoxic action.

**Keywords:** Radiosensitivity • GDF15 • Biomarker • Breast cancer

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## 1. Introduction

The development of biomarkers as indicators for radiation exposure, predictors of biological early and late effects, and individual radiation susceptibility are likely to gain recognition and priority in radiation research.

Growth differentiation factor-15 (GDF15) is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, and GDF15 overexpression has been found in several cancers. GDF15 is published as a prognostic biomarker in ovarian cancer [1], a marker for tumor recurrence in colorectal cancer [2], and a novel serum marker for metastasis in uveal melanoma [3]. GDF15 expression is rapidly induced by a variety of cellular stresses in a p53-dependent

and –independent manner, and it mediates cell cycle arrest and apoptosis in response to DNA damage, toxins, anoxia, liver injury, and other cellular stresses in different models [4–6]. Overexpression of GDF15 by transfection in prostate cancer cells induces aggressive cell growth, whereas knocking down GDF15 in LNCaP-derived subclones using antisense oligonucleotides inhibits cell growth and proliferation [7]. A contradictory observation was made in a study of gastric cancer where the overexpression of GDF15 in gastric cancer cell lines reduced the growth of xenograft tumors [8], although the high level of serum GDF15 was positively correlated with advanced tumor stage and metastasis [9]. Despite ambiguous observations in various tumor cell types, data obtained from clinical studies has established

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that serum GDF15 is the most promising diagnostic marker of bone metastasis in prostate cancer [10]. Inflammatory cytokines such as GDF15 could contribute to the tumor promotion environment, and the resulting tumorigenic function could be modified by educating the macrophages. Another possibility is that this tumor derived GDF15 is secreted together with other cytokines to promote vascular development, mediated by serine/threonine-protein kinases signaling as reported in malignant melanoma [11]. Our earlier studies showed that GDF15 is a radioresponse gene, and that the sensitivity to ionizing radiation of cells is strongly associated with the radiation induced expression of GDF15 [12]. The importance of members of TGF-family in driving tumor progression and metastasis is now widely recognized; however, the response of each tumor type will be different since the signaling pathway can have tumor suppressing and tumor progressing activity, and these cytokines can affect both tumor cells as well as all cells of the tumor microenvironment. Radiation-induced vascular injury and endothelial dysfunction are mediated in part by TGF- $\beta$ 1 [13], a pluripotent growth factor that is part of many normal tissue radiation responses [14,15]. Previous studies have shown upregulation of TGF- $\beta$ 1 in rat models with radiation induced damage after localized heart irradiation with 20 Gy or 5 fractions of 9 Gy [16]. Cardiac radiation fibrosis is more severe in animals that have been administered the TGF- $\beta$ 1 inducing compound during the 6-month follow-up time after irradiation, and pre-clinical studies involving TGF-receptor inhibition are being undertaken [17]. GDF15 belongs to the TGF- superfamily, therefore the protein-coding gene is a promising target for radiation research. An integrated molecular and biological approach is warranted because, although the biochemistry of GDF15 signal transduction has been well characterized *in vitro*, much remains unresolved concerning functional interactions that occur between this signaling pathway and other biological pathways *in vivo*. Our proposal is to determine whether overexpression of GDF15 signaling prior to irradiation sensitizes mouse adenocarcinoma cells *in vitro* and *in vivo*.

## 2. Experimental procedures

### 2.1 Cell culture

Mouse mammary adenocarcinoma cells LM2 were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, US), supplemented with 10% fetal calf serum, plus penicillin (100 U mL<sup>-1</sup>) and streptomycin (100  $\mu$ g mL<sup>-1</sup>) (Gibco, Grand Island, NY). The cells were incubated at 37°C in a humidified atmosphere of 95%

air and 5% CO<sub>2</sub>. Cells were sub-cultured every 2-4 days according to the previous subculture ratio.

### 2.2 Establish stable GDF15 overexpressing cell line

The cells were transfected with True ORFGDF15-GFP tagged/pCMV6 plasmid or control empty vector (OriGene, Rockville, MD, US) using TurboFect (Fermentas, Amherst, NY, US) according to the manufacturer's instructions. Briefly, 5  $\mu$ g plasmid was incubated overnight with cells at about 50%–60% confluence. Then the transfected cells were incubated in the medium containing G418 (0.5 mg mL<sup>-1</sup>). The G418-resistant colonies were subcloned approximately 3 weeks after transfection for further experiments. The mRNA expression of GDF15 was validated by qRT-PCR, and transfected cells were also analyzed for GFP fluorescence (FACS Calibur Becton-Dickinson, San Jose, CA, US). The stable clone was named as LM2-GDF15, and the parental empty vector transfected LM2 was the control for comparison. For *in vitro* experiments, transfected cells were cultured for indicated G418.

### 2.3 Irradiation of cells

Cells in the logarithmic growth phase were subjected to X-ray irradiation with a THX-250 with 1 mm Cu filter and a dose-rate of 1.07 Gy min<sup>-1</sup>. After irradiation at a different dose, the cells were incubated for different time intervals in cultured medium, at 37°C, under 5% of CO<sub>2</sub>. A mock control was performed with cells under the same conditions but sham -irradiated (0 Gy) in all experiments.

### 2.4 Clonogenic assay

The cell cultures were maintained in a 75 mm<sup>2</sup> cell culture flask, in Dulbecco's modified Eagle Medium (Sigma-Aldrich St Louis, MO, US) supplemented with 10% fetal calf serum and appropriate antibiotics. The experiment was completed as previously described [18]. Six hours before radiation treatment, the cells were seeded in 100 mm<sup>2</sup> culture cell flasks, 500 and 1000 cell/dose/flasks. The cells were exposed to various doses of ionizing radiation (IR) (0, 2 and 4 Gy) at a rate of 1.07 Gy min<sup>-1</sup>. After the radiation, the cells were incubated 7-9 days at 37°C. After incubation time the colonies were stained and counted for colony assay, and the survival fractions and plating efficiency were calculated [19].

### 2.5 Animals and irradiation procedures

A total of 30 female BALB/c mice aged 10-12 $\pm$ 1 weeks that were purchased from Charles River Laboratories (Charles River Laboratories, Research Models and Services, Germany GmbH) were used in all experiments.

Exponentially growing cells cultured in DMEM medium supplemented with 10% fetal bovine serum were washed in PBS buffer and inoculated subcutaneously into the right flank of mice ( $1 \times 10^6$  cells/10 ml). The growth delay assay was performed when the LM2 tumor reached 2 mm in average diameter (6 days after inoculation). When the tumors were palpable, the mice were immobilized (without anesthesia) in specially designed jigs and the tumor was locally irradiated with 2 Gy using a THX-250 device operated at 200 kV, and a tube current of 20 mA and a beam filter of 0.6 cm Cu, resulting in a dose rate of  $1.07 \text{ Gy min}^{-1}$ . A two-dimensional measurement was performed three times per week, and the tumor volume was expressed as a relative tumor volume in  $\text{mm}^3$ ; data are mean  $\pm$  SE. Aged-matched sham-irradiated (0 Gy) animals were included and compared with irradiated groups at each time point ( $n = 5$  mice per cell type and time point). For tumor tissue sampling, the animals were euthanized with ether and the tumor tissues were isolated and kept in  $-70^\circ\text{C}$  until RNA processing.

## 2.6 WST-1-based cytotoxicity assay

LM2 cells were harvested using commercial 4% trypsin-EDTA solution, and viable cell concentrations were determined by Trypan blue staining. To allow cells to attach to 96-well tissue culture plate wells,  $1 \times 10^3$  viable cells were added to each well one day before radiation treatment and incubated overnight at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  in a humidified incubator. Cells were incubated for 6 days and the cell growth rate was measured by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) assay. 10  $\mu\text{L}$  of cell proliferation reagent WST-1 (Roche, Basel, Switzerland) was added to each well and incubated at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  in a humidified incubator for 1 h. The absorbance was measured in a microplate reader (Biotek, Synergy HT Winooski, US) at 450/630 nm. The WST-1 reagent incubation time of 1 h was found to be optimal since the color intensity did not change after 1 h (data not shown), and this condition was used in all subsequent assays.

## 2.7 Real time cell impedance measurements

Real time cell electronic sensing is an impedance-based, label-free measurement technique for dynamic monitoring of living cells. The RTCA-SP instrument (ACEA Biosciences, US) measures impedance continuously and is automatized to non-invasively quantify adherent cell proliferation and viability in real-time. This method has been effectively utilized previously to quantify and measure the number, adherence, growth, and viability of cells [20–24]. 50  $\mu\text{L}$  of cell culture medium was added to each well of the microtiter E-plates for impedance background measurements, and then 50  $\mu\text{L}$

cell suspensions were dispensed at the density of  $10^3$  cells/well. Mouse mammary gland tumor cell line LM2 was cultured with standard cell-culture medium and also with 50% normal – 50% conditioned medium (CM) of LM2-GDF15 containing 40 ng  $\text{mL}^{-1}$  GDF-15 parallel to the previous one. Cells were put into the incubator at  $37^\circ\text{C}$  and cultured for 7 days to monitor cell growth dynamics. Impedance was measured every 5 minutes. At each time point, the cell index was calculated by the instrument as  $(R_n - R_b)/15$ , where  $R_n$  is the cell-electrode impedance of each well containing cells, and  $R_b$  is the background impedance of the well with the medium alone.

## 2.8 Quantitative RT-PCR

Total RNA was isolated with Qiagen RNeasy kit (Qiagen GmbH, Hilden, Germany). A 1-mg quantity of total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, US) according to the manufacturer's instructions. The effect of X-ray on the expression of GDF15 and TGF- $\beta$ 1 also was measured. We irradiated exponentially growing cells with doses 2, 4 and 6 Gy, and expression of genes were quantified at time point 24–42 hours. The specific primers for PCR for mouse GDF15 were Forward: 5'-AACACGCATGCGCAGATCAAAGC-3' and Reverse: 5'-AAGTCTGCAGTGACACACCACTGT-3', and for b-actin were Forward: 5'-GGCTGTAATCCCCTCCATCGT-3' and Reverse: 5'-CGTACATGGCTGGGGTGTGTA-3'. For measurement of TGF- $\beta$ 1 expression changes the following primer pair was used: Forward: 5'-CCCTATATTGGAGCCTGGA-3' Reverse: 5'-GTTGGTTGTAGAGGGCAAGG-3'. Gene-specific primers were designed with the aid of Applied Biosystems' Primer Express software. The qPCR reactions were performed with the Rotor-Gene 3000A Real Time PCR System using Universal PCR Master Mix (Maxima Probe qPCR Master Mixes, Thermo Scientific, Waltham, MA, US), following the manufacturer's instructions. All samples were run in duplicate. Relative -fold inductions were calculated by the DDCT method, as previously used [19], with averaged relative levels of ACTB, PolG2 and HGPRT used for normalization. PolG2 Forward primer: 5'-TGTGGTCACTCAGGTCAACAAGGT-3' and Reverse: 5'-TGTCTACACGCGTGCCTACAATCT-3' for HGPRT gene Forward primer: 5'-CCTGCTGGATTACATTAAAGCACTG-3' and Reverse: 5'-GTCAAGGGCATATCCAACAACAAA-3'.

## 2.9 ELISA

$5 \times 10^5$  cells/flask were seeded in T-25 flasks, and one day later the cells were irradiated with 2 Gy. The cell culture supernatants were collected after 48 h of treatment

and the amount of TGF- $\beta$ 1 determined by sandwich ELISA using a Mouse/Rat/Porcine/Canine TGF- $\beta$ 1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, US), according to the manufacturer's protocol. Recombinant mouse TGF- $\beta$ 1 (R&D Systems) diluted in medium ( $31.2\text{--}1000\text{ pg mL}^{-1}$ ) was used as a standard to determine the total amount of TGF- $\beta$ 1 secreted by the cells. The numbers of cells per flasks were determined by direct counting. Minimum sensitivity of this assay is  $< 4\text{ pg mL}^{-1}$  and measurement is performed 450 nm using a Microplate Reader (Biotek, Synergy HT Winooski, US).

## 2.10 Statistical Analysis

The graphical data are presented as the mean $\pm$ SEM. Statistical significance among groups and between groups was determined using one-way ANOVA following application of Bonferroni multiple comparison posttest, and Student t-test, respectively. Significance was assumed for  $P < 0.05$  (\*)  $P < 0.01$  (\*\*). Statistical analysis was performed using GraphPad Prism 6.0 statistical package, v.6.04 (GraphPad Software, La Jolla California USA).

## 3. Results

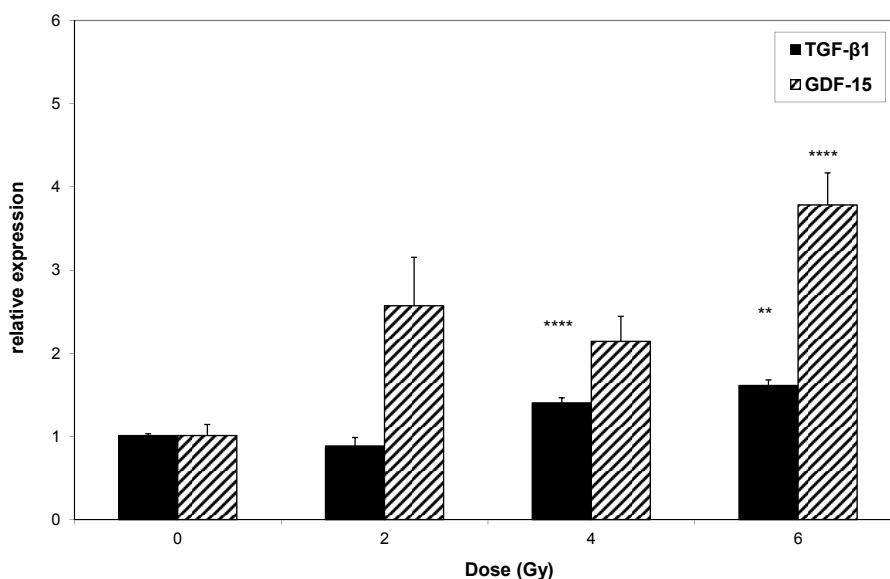
### 3.1 GDF15 and TGF- $\beta$ 1 mRNA expression in X-ray irradiated LM2 cells

To assess the utility of GDF-15 as a radiation response marker, the mouse mammary carcinoma cell line LM2,

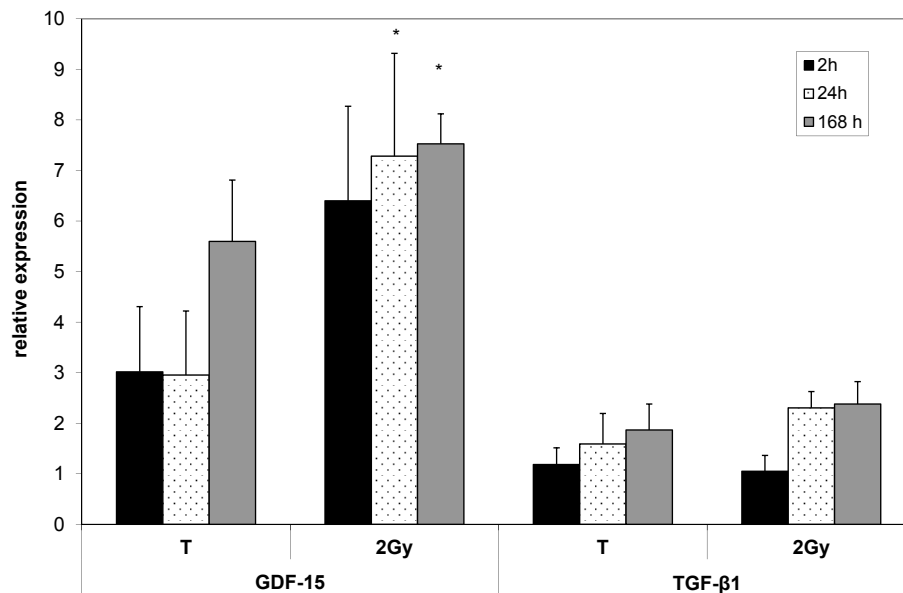
treated with X-ray, was chosen. Realtime PCR was performed to test the dose dependent changes in GDF-15 and TGF- $\beta$ 1 mRNA expression. As it was demonstrated in Figure 1, GDF-15 up-regulation is clearly observed in LM2 cells at 24 hours after irradiation at 6 Gy exposure ( $2.01\pm 0.54$ ,  $2.14\pm 0.299$ ,  $3.78\pm 0.384$ , respectively). 2 Gy induced elevation of TGF- $\beta$ 1 and GDF15 mRNA expression in these cells was not observed. Since a significant GDF15 upregulation of 3.78-fold change was detected in LM2 cells at a dose of 6 Gy, our results suggest that in this cancer cell line higher doses than 2 Gy would have been necessary to induce GDF15 and TGF- $\beta$ 1 expression (Figure 1). Expression of TGF- $\beta$ 1 in LM2 cells is induced significantly by radiation only at high doses as 4 Gy and 6 Gy ( $1.40\pm 0.06$ ,  $1.61\pm 0.066$ ).

### 3.2 Expression of GDF15 and TGF- $\beta$ 1 in locally irradiated tumor

We next performed qRT-PCR in order to detect alterations in GDF15 and TGF- $\beta$ 1 known to be involved in the radiation response in tumor models. The mRNA levels of the TGF- $\beta$ 1 gene was determined in irradiated and control tumor mass, removed 2, 24, and 168 h after treatment. Using a moderate dose (2 Gy) to irradiate a local tumor, approximately 2-fold GDF15 upregulation was observed ( $6.40\pm 1.869$ ,  $7.28\pm 2.034$ ,  $7.52\pm 0.594$ ) compared to the untreated tumor ( $3.01\pm 1.287$ ,  $2.95\pm 1.268$ ,  $5.59\pm 1.21$ ), with an increase of 2 hours after X-ray irradiation (Figure 2). Notably, at 168 hours, GDF15 levels elevated in the sham-treated tumor, with



**Figure 1.** X-ray induces the expression of GDF15 and TGF- $\beta$ 1 *in vitro*. Dose dependent GDF15 and TGF- $\beta$ 1 expression by qRT-PCR in LM2 cell lines, assessed 24 hours after 2, 4, 6 Gy X-ray irradiation events. Relative expression of both mRNA was calculated by  $\Delta\Delta CT$  method and the acquired -fold change values are relative to their respective non-treated mock control. Values are presented as mean normalized target gene expression  $\pm$ SEM. Pairwise comparisons within ANOVA were assessed by Bonferroni post hoc multiple comparisons test. Throughout, P values of  $< 0.05$  were considered significant. \* $P < 0.05$ , \*\* $< 0.01$  \*\*\*\* $< 0.0001$  compared to sham-irradiated control cells.



**Figure 2.** IR induces the expression of GDF15 and TGF- $\beta$ 1 in tumor tissue *in vivo*. GDF15 and TGF- $\beta$ 1 expression by qRT-PCR in LM2 tumor mass, assessed 2, 24 and 168 hours after 2 Gy X-ray irradiation events. Results are expressed as the ratio of induction in irradiated to untreated tumor at 0 hour. Means were calculated from the results of 2 independent experiment  $n=4$ . Relative expression of mRNAs were calculated by  $\Delta\Delta$ CT method. Values are presented as mean normalized target gene expression  $\pm$  SEM. Pairwise comparisons within ANOVA were assessed by Dunnett's multiple comparisons test, P values of  $<0.05$  were considered significant compared to sham treated tumor at 0 hour.

an approximately 2.5-fold change compared to the non-treated control at 0 time points, suggesting that the LM2 cells produce an abundant amount of GDF15. We also investigated the TGF- $\beta$ 1 expression levels in the tumor mass, and its expression was not altered in the growing tumor. We tested whether TGF- $\beta$ 1 was also upregulated in LM2 cells in response to moderate doses of X-ray irradiation. As demonstrated in Figure 2, TGF- $\beta$ 1 upregulation was not observed after irradiation. Our results suggest that higher doses would have been necessary to induce TGF- $\beta$ 1 expression *in vivo* in this cancer cell line.

### 3.3 Effect of GDF15 on radiation response *in vitro*

To determine whether GDF15 actually modulated the sensitivity of LM2 cell lines to radiation-induced cell death, LM2 cells were transfected with full-length GDF15 cDNA and an empty vector. As expected, a significant increase of GDF15 mRNA was detected in the GDF15-transfected cells, designated as LM2-GDF15, while no significant alteration of GDF15 mRNA expression was observed in the cells transfected with an "empty" pcDNA3 vector (as a negative control), designated as parental LM2 (Figure 3). These results indicate that LM2 cell line with overexpression of the GDF15 transgene was successfully established.

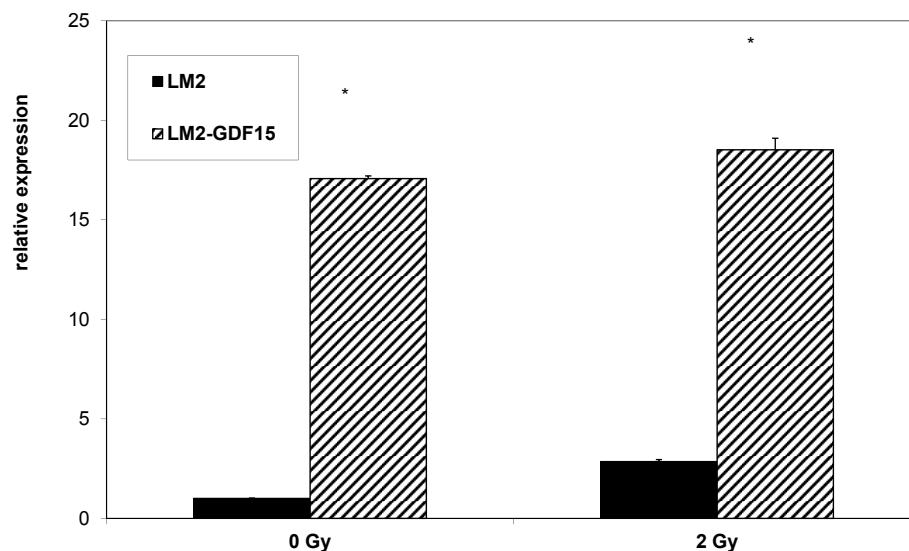
The clonogenic assay's results showed that the survival fraction of GDF15 overexpressing cells were

significantly higher than that of control groups at 2 Gy (Figure 4), showing a correlation between GDF15 expression and radiosensitivity of LM2 cells to X-rays. These results suggested that overexpression of GDF-15 decreased cell radiosensitivity *in vitro*.

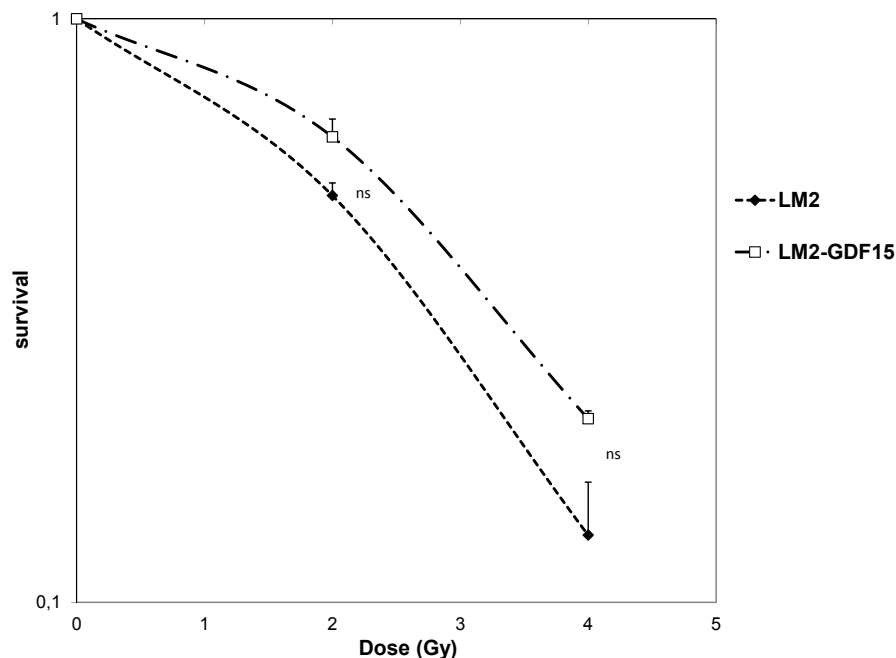
The growth of parental and LM2-GDF15 cells was evaluated and compared by WST assay. As shown in Figure 5A, enhanced endogenous expression of GDF15 slightly increased the growth rate in LM2 cells. The advanced proliferation was observed only from the fourth day of cultivation. Thus, the endogenous overexpression of GDF-15 was conferred, resulting in advanced proliferation in this mouse mammary adenocarcinoma cell line.

Next we sought to determine whether the GDF15 enriched conditioned medium affected the growth of parental LM2 cells. The growth of the two groups (LM2, LM2+CM) was monitored by the real time impedance sensing instrument. Cell growth was studied for 7 days with an initial cell number of  $10^3$  cells/well. The LM2 cells showed a good proliferation rate which was enhanced by the conditioned medium containing GDF-15. LM2 and LM2+CM cells reached a cell index over 2 after 3 days in culture, and the system showed an impedance drop at day 5 (Figure 5B).

To identify the effect of GDF15 overexpression on the basal expression of TGF- $\beta$ 1 and IR induced expression of TGF- $\beta$ 1, we performed quantitative



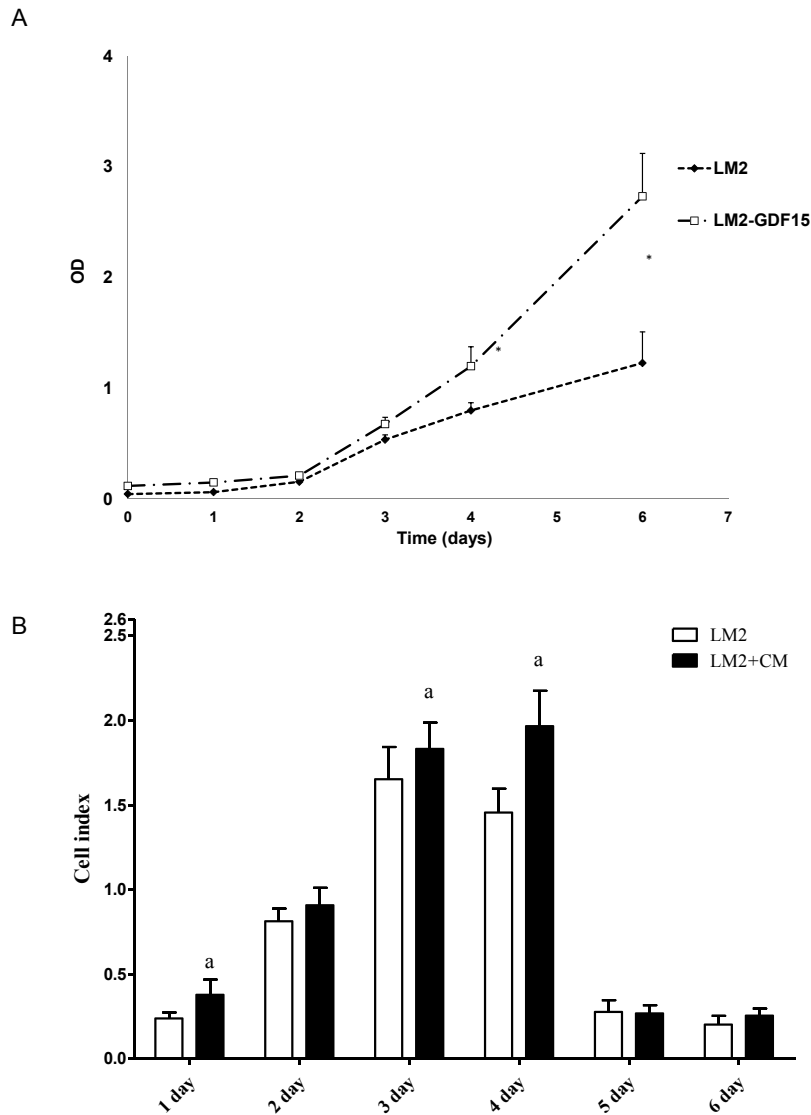
**Figure 3.** Overexpression of GDF15 is validated by qRT-PCR. The efficiency of transfection of full length GDF15-cDNA was determined by qRT-PCR. Basal and 2 Gy induced expression of GDF15 was compared in stable LM2-GDF15 cells and parental LM2 cells. Relative expression (-fold changes) was normalized to control unirradiated LM2 cells, and the data were calculated by  $\Delta\Delta\text{CT}$  method. Values are presented as mean normalized GDF15 expression  $\pm$  SEM. P values was compared between LM2 and LM2-GDF15 cells with ANOVA test as \* $P < 0.001$ .



**Figure 4.** Survival fraction is measured by clonogenic assay. Exponentially growing cells were exposed to indicated doses of X-ray and subjected to the clonogenic assay. Data shown are means  $\pm$  SEM of three independent experiments. Survival fraction was determined compared to untreated control cells; survival was significantly reduced by irradiation ( $P < 0.05$  versus control group), using one-way ANOVA with Bonferroni's multiple comparison test, but specifically, the survival was not statistically significant (ns) comparing irradiated LM2 with irradiated LM2-GDF15 cells.

analysis on mRNA and protein levels. Without irradiation in the basal condition, the expression of TGF- $\beta$ 1 was down-regulated in LM2-GDF15 cells compared to the LM2 ( $1 \pm 0.1$ ,  $0.6975$ ,  $0.69 \pm 0.0842$ ,  $P = 0.085$ ), while the 2 Gy induced expression of TGF- $\beta$ 1 was less inhibited

( $1.045 \pm 0.090$ ,  $0.072 \pm 0.0902$   $P = 0.0024$ ) in LM2-GDF15 cells at 24 hours postirradiation (Figure 6A). However, 48 hours after X-ray treatment, the expression of TGF- $\beta$ 1 was decreased in LM2-GDF15 cells as determined by protein levels measured by ELISA (Figure 6B).



**Figure 5.** Effect of GDF15 on cell growth. A. Cell growth was evaluated by WST assay. Data shown are means  $\pm$  SEM of three independent experiments. Significant difference was found from day 5 of postirradiation in comparison of LM2 to LM2-GDF15 cells (ANOVA test with Bonferroni's post hoc tests  $^* = P < 0.0001$ ). B. Growth column diagram representing the relationship between LM2 and LM2 kept in 50% conditioned medium every day. Cell index was expressed as an arbitrary unit and calculated from impedance measurements between cells and sensors. Data were presented as mean  $\pm$  SD,  $n = 20$ . Statistical analysis was performed with two-way ANOVA followed by Bonferroni test. Statistically significant differences ( $p < 0.05$ ) from LM2 growth (a) were indicated.

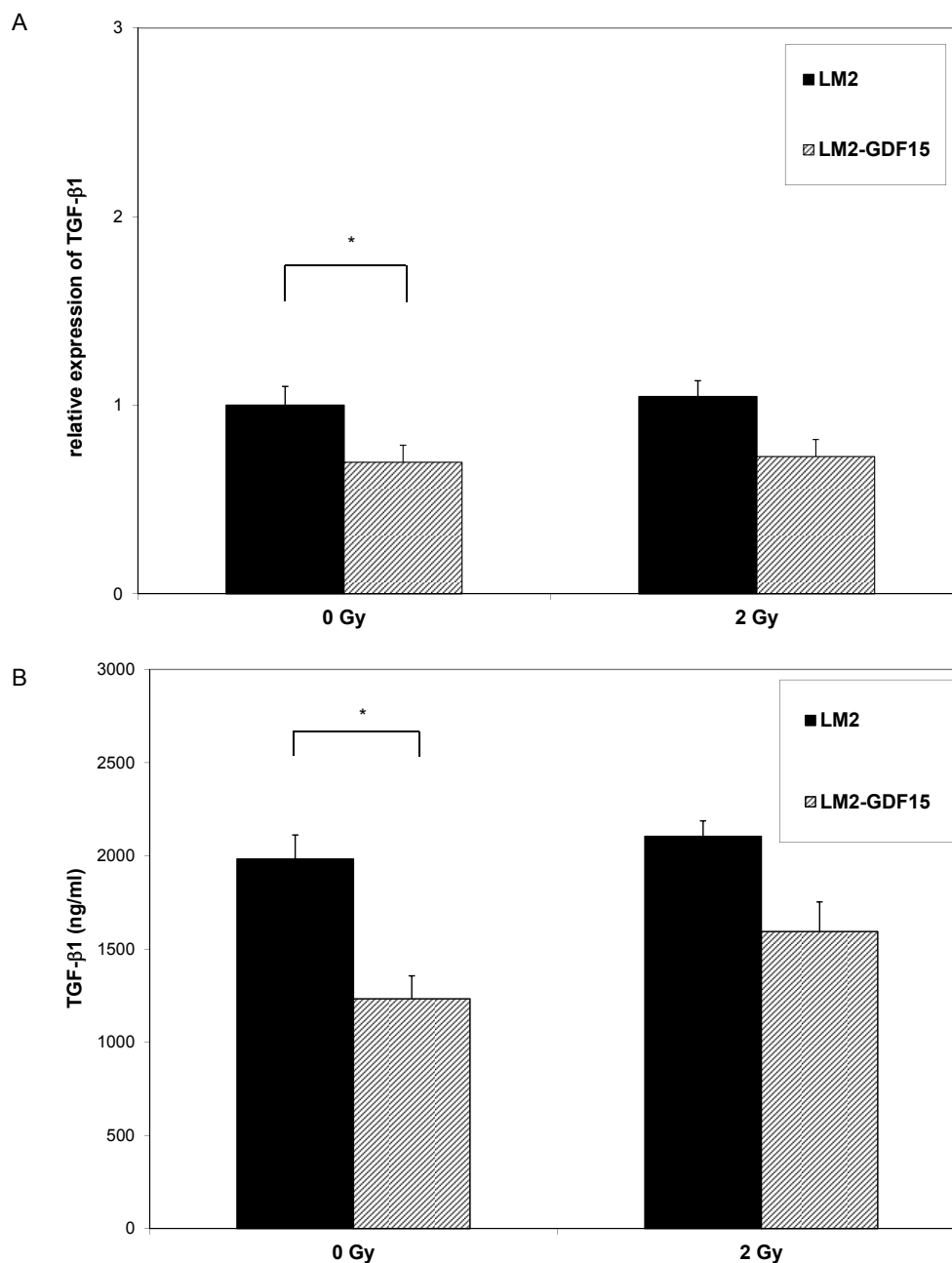
### 3.4 Overexpression of GDF15 expression enhances tumorigenicity

The effect of overproduction of GDF15 on the growth of tumors was evaluated in a mouse syngraft model. The LM2-GDF15 cells rapidly developed into visible tumors, while in contrast, LM2 breast carcinoma cells expressing normal levels of GDF15 showed minimal tumor formation over the observation period (Figure 7).

In order to confirm whether expression of GDF15 modified tumor growth after irradiation *in vivo*, we established a syngraft model with BALB/c mice using LM2 and LM2-GDF15 cells. After irradiation for a short

period of time, the tumor growth slowed, though rapid tumor recurrence was seen (Figure 7). After irradiation with 4 Gy, the growth rate of LM2 tumors was still significantly lower than the wild-type tumors and the LM2-GDF15 cell tumors. The size of LM2 tumors ( $3251.65 \pm 1523.91 \text{ mm}^3$ ,  $n=5$  mice) were smaller than the LM2-GDF15 tumors ( $13840.8 \pm 566.53$ ,  $n=6$  mice  $^* = P < 0.05$ ) at the end point, 25 days later of tumor cell inoculation. These results suggest that the tumor-derived elevated expression of GDF15 in BALB/c mice still enhanced the growth of breast cancer cells and increased radioresistance.





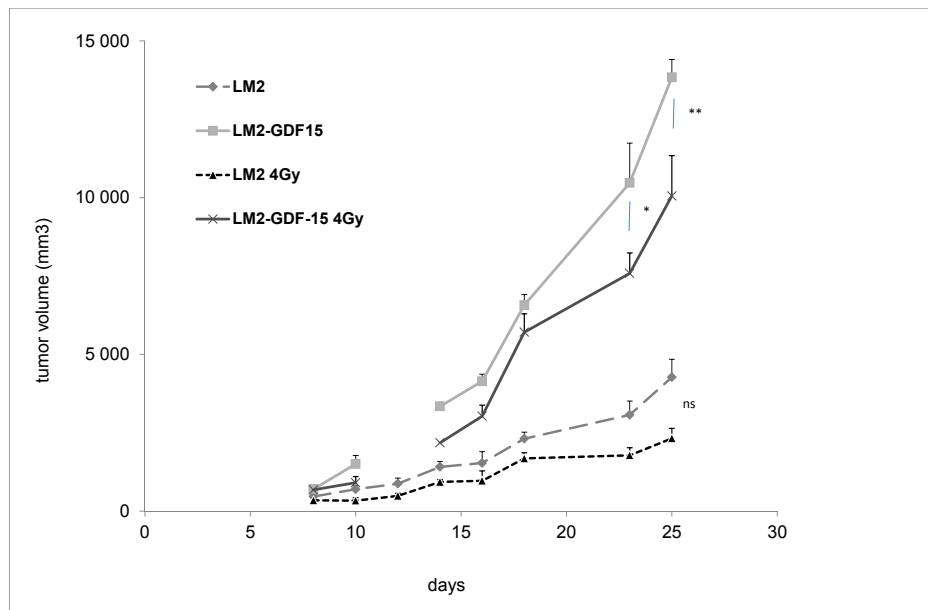
**Figure 6.** Effect of GDF15 on the expression of TGF- $\beta$ 1. Quantitative analysis of TGF- $\beta$ 1 expression in breast carcinoma cells from non-irradiated and irradiated cultures 24 hours post-irradiation was measured at mRNA and 48 hours postirradiation using secreted protein levels. A. Relative expression of TGF- $\beta$ 1 was evaluated by qRT-PCR. \* Significant difference was found when comparing expression of GDF15 in LM2 versus LM2-GDF15 cells ANOVA test  $*=P<0.05$ . B. Concentration of TGF- $\beta$ 1 was measured by ELISA. \* Significant difference was found between LM2 and LM2-GDF15 cells (ANOVA test  $*=P<0.05$ ).

## 4. Discussion

Our study shows that functionally GDF15 is a survival and radioprotective factor for mouse breast cancer cells. This study is the first to demonstrate the role of GDF15 on LM2 cell survival *in vitro*. The elevation of GDF15 increased cell survival, confirming our previous observations [19]. Moreover, the tumor-derived GDF15

was able to significantly increase cell survival in LM2 cells treated with conditioned medium. In the real time measurement assay, it was revealed that the presence of GDF15 in conditioned medium helped the growth of cells. LM2 cells grew significantly faster with the help of conditioned medium compared to cells kept under standard culture conditions. Although these results contrast with those of GDF15 induced apoptosis of





**Figure 7.** Tumor promoting efficiency of GDF15 *in vivo*. BALB/c mice with established syngraft LM2 or LM2-GDF15 breast carcinomas (n=5-6 per group) received 4 Gy X-ray or sham-treatment. Tumor diameters were measured with digital calipers, and the tumor volume in mm<sup>3</sup> was calculated by the formula: Volume (mm<sup>3</sup>) = (width)<sup>2</sup> x length/2. ANOVA testing was performed to determine whether the change in volume during the time course was significant. \* P<0.05 and \*\*P<0.001 were considered statistically significant.

prostate cancer cells [25], they are notably consistent with the role of GDF15 supporting malignant glioma cell proliferation [26]. Additionally, GDF15 is known to promote apoptosis in colorectal tumor cells [27] and induces apoptosis in breast [28] and prostate cancer cell lines [25]. However, GDF15 has also been implicated in the progression and metastasis of malignant melanoma [29], consistent with our findings in the breast cancer model. Collectively, these studies indicate that the role and effect of GDF15 may be different depending on the cell type, stage, and extent of the tumor producing it and perhaps its secreted form, as has been found for other members of the TGF- $\beta$  superfamily. Alternatively, different tumors may also contain different mutations or signaling pathway alterations that could alter the effect of GDF15. A consistent interpretation of the function of GDF15 can be made for the breast cancer cells because the normal tumor cells and their transformed overexpressing counterparts have been compared *in vitro* and *in vivo*. Our research found that the growth and proliferation rate of the GDF15 stable cell line were significantly higher than the empty vector transfected control cells, suggesting that endogenous overexpression of GDF15 can promote breast cancer cell growth and proliferation. Moreover, we found that overexpression of GDF15 reduces LM2 cells' sensitivity to ionizing radiation. Further experiments indicated the modulation of radiation-caused cell growth inhibition may be connected with GDF15 mediated deregulation

of TGF- $\beta$ 1. Based upon our results, where the basal expression of TGF- $\beta$ 1 is upregulated in GDF15 overexpressing cells, we assume a possible new indirect molecular interaction between them.

Although GDF15 has been described in many solid tumors, a lot remains to be uncovered about its biology; in particular, the GDF15 receptor is still unknown today. There is some evidence for SMAD pathway activation, suggesting GDF15 acts through a TGF- $\beta$  receptor [30]. A recent study identified GDF15 as an acute-phase modifier of CCR2/ TGF- $\beta$ RII-dependent inflammatory responses to vascular injury [31]. Alternatively, Kim and colleagues elegantly showed that GDF15 induces the transactivation of ErbB2 tyrosine kinase in breast and gastric cancer cells [32]. We found evidence for crosstalk between GDF15 and TGF- $\beta$ 1 regulation: in our experimental system overexpression of GDF15 deregulated the secretion of TGF- $\beta$ 1; however the exact mechanism inside of this is still unknown. Ectopic overexpression of GDF15 may further lead to unphysiologically high cytokine levels in the tumor microenvironment that could affect radiation response [33]. Previous results demonstrated that in normal skin, TGF- $\beta$ 1 expression is activated by gamma-rays [34]. At the mRNA level, TGF- $\beta$ 1 was not increased at 24, 48 hours postirradiation. At the protein level, TGF- $\beta$ 1 was increased at 48 hours postirradiation, but the elevation was only 10%. Barcellos-Hoof *et al.* proposed that the elevation of TGF- $\beta$ 1 they found within 1 h in irradiated

mouse mammary glands was due to the activation of the latent pre-existing TGF- $\beta$ 1 proteins [13]. In our model, both IR induced transcriptional or posttranslational regulation processes can occur.

In this study, LM2-GDF15 cells exhibited more obvious tumor growth than control groups after X-ray irradiation. The results showed that overexpression of GDF15 still enhances the growth of breast cancer cells, thereby reducing the radiosensitivity *in vivo*.

In summary, we have shown here that GDF15 may play a crucial role in the survival of breast cancer cells. Current studies are aimed at understanding the processes by which this occurs in mouse breast cancer models. Elevation of tumor derived GDF15 supports

a proliferative advantage to the cells, thus contributing to the radiation resistance. Meanwhile, we propose that GDF15 may be suitable as a serum biomarker and may represent a target during the radiotherapy for the treatment of breast cancer.

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