Original Paper

Up-Regulation of Glioma-Associated Oncogene Homolog 1 Expression by Serum Starvation Promotes Cell Survival in ER-Positive Breast Cancer Cells

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Key Words

Gli1 • Breast cancer • Cell survival • cIAP2 • NF-κB

Abstract

Background/Aims: Cancer cells are resistant to ischemia and starvation. Glioma-associated oncogene homolog 1 (Gli1) is a positive transcriptional activator of Hedgehog (Hh) pathway and plays an essential role in the development of cancers, including breast cancer. However, how Gli1 promotes cell survival remains elusive. The main purpose of this study is to investigate the pro-survival effect of Gli1 under serum starvation and its molecular mechanism in ER-positive breast cancer cells. **Methods:** Gene expression was determined by quantitative real-time PCR (QRT-PCR) and Western blot. The survival of Gli1 stably transfected ER-positive breast cancer cell lines (Gli1-MCF-7 and Gli1-T47D cells) and their untransfected control cells was estimated by WST-8 assay. Microarray analysis was performed to screen downstream Hh/Gli1 target genes in Gli1-overexpressed MCF-7 cells. Transcriptional activities of NF-κappaB were measured by luciferase assays. ChIP analysis was performed to explore whether cIAP2 was a direct target gene of Gli1. **Results:** Serum starvation significantly up-regulated the expression of Gli1 gene through activating PI3K/AKT pathway. Over-expression of Gli1 markedly promoted cell survival under serum starvation. Microarray analysis revealed that 338 genes were differentially expressed in Gli1-MCF-7 cells compared with those in the control cells. Among these genes, cellular inhibitor of apoptosis 2 (cIAP2), coding an anti-apoptosis and pro-survival protein, was significantly up-regulated not only by Hh/Gli1 pathway, but also by serum starvation. However, ChIP assay revealed no binding of Gli1 to cIAP2 promoter at the region of -1792 to -1568bp. Moreover, over-expression of Gli1 resulted in enhanced trans-activation of transcriptional factor NF-κB. Suppression of NF-κB signaling with NF-κB inhibitor

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Bay11-7082, significantly reduced the expression of cIAP2 and the cell survival under serum starvation. **Conclusion:** Serum starvation significantly up-regulated the expression of Gli1, which in turn increased its key target cIAP2 expression and enhanced NF- κ B/cIAP2 pathway, resulting in promoting cell survival under serum starvation. These findings may provide new insights into the pro-survival mechanisms of Gli1 in breast cancer.

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Introduction

Glioma-associated oncogene homolog (Gli) family includes Gli1, Gli2 and Gli3, which are zinc finger transcription factors and act as nuclear mediators of the Hedgehog (Hh) signaling pathway. Gli1 functions as a strong positive transcriptional activator of the downstream of Hh pathway, whereas Gli2 and Gli3 function as either transcriptional activators or repressors. They coordinately regulate the expression of genes [1]. The activation of the canonical Hh pathway is initiated by the secreted Sonic Hedgehog (Shh) ligand that binds to its receptor PTCH and then relieves the repression of the Smoothened (Smo). Activated Smo releases Glis from cytoplasmic sequestration mediated by a protein complex and allows the translocation of Glis to the nucleus [2, 3], where Gli1 binds to a consensus Gli1-binding element within target genes and results in their activation [4]. Notably, Gli1 itself is a transcriptional target of Hh signaling [5]. Gli1 expression and activity are modulated not only by the Hh pathway, but also by other signaling pathways, such as transforming growth factor beta (TGF β), Ras/ERK, phosphatidylinositol-3-kinase (PI3K)/AKT, etc [6-8].

The Hh/Gli1 pathway plays an essential role in vertebrate organogenesis as well as the development of some cancers by regulating proliferation, differentiation, epithelial-mesenchymal transition and survival [9-12]. The aberrant activation of Hh pathway has been described in a growing number of cancers, including breast cancer cells [13-17]. Studies have shown that the Hh ligand or Gli1 was abnormally expressed in breast cancer tissues and cells, which was associated with tamoxifen-resistance of breast cancer cells [7], increased metastasis risk and poor survival of breast cancer patients [7, 13, 14, 18-20]. Moreover, advanced mammary tumors developed in transgenic mice, in which Gli1 was conditionally expressed under the control of a doxycyclin-inducible MMTV promoter in the mammary gland epithelium [21]. Our previous study found that the stable expression of Gli1 in ER-positive breast cancer cell lines MCF-7 and T47D induced estrogen-independent proliferation and promoted G1/S phase transition [22]. These findings reveal that the activation of Hh/Gli1 pathway facilitates growth and progression of breast cancer. However, the precise mechanism, by which Gli1 contributes to breast cancer development, remains unclear.

Tumor microenvironment is a characteristic of ischemia and starvation of nutrient. Cancer cells are capable of enhancing their resistance to unfavorable microenvironment to promote cell survival, which is very important for development of cancer. In present study, we investigated the expression and pro-survival effect of Gli1 under serum starvation and the possible mechanisms of pro-survival of the gene by identifying its target genes and signaling pathway in ER-positive breast cancer cells. This study facilitates understanding the pro-survival mechanisms of Gli1 and promotes the development of novel therapeutic strategies.

Materials and Methods

Reagents and plasmids

Wortmanin and Bay11-7082 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Recombinant human SHH N-terminus (rhSHH-N, R&D system, Minneapolis, MN,

USA) was dissolved in PBS containing 0.1% bovine serum albumin (BSA) according to the manufacturer's recommendation. Human Gli1 expression vector pcDNA3.1-Gli1 and the vehicle vector pcDNA3.1 were provided by Dr. Hiroshi Sasaki (RIKEN Kobe, Japan). The pGL3-NF- κ B-luc containing two-copies of wild-type NF- κ B-luc-responsive elements was kindly provided by Dr. H.M. Xu [23]. The pRL-SV40-luc vector was obtained from Promega (Madison, WI, USA).

Cell culture and drug treatment

The human breast cancer cell lines MCF-7 and T47D are estrogen receptor (ER) positive cells that express ERs and grow in response to the estrogen. These cells were incubated at 37°C and in 5% CO₂ humidified atmosphere with the RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (PAA, Pasching, Austria), 100U/ml penicillin and 100µg/ml streptomycin (Huashun Technology, Inc., Shanghai, China). For Wortmanin treatment, cells were 60%-70% confluent and then treated with 100nM Wortmanin or an equimolar amount of vehicle DMSO in phenol-red free RPMI-1640 containing 2.5% or 0.5% charcoal-dextran stripped FBS (CD-FBS) to avoid possible interference with serum endogenous steroids for 24h.

Establishment of stable cell lines

The cells were seeded at 1×10^6 per 60 mm culture dishes for 24h and then were transiently transfected with 8.0µg pcDNA3.1-Gli1 or pcDNA3.1 plasmids using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) per manufacturer's instructions. After 48h, the cells were plated at a low density in medium containing 500µg/ml geneticin (G418) for MCF-7 cells or 600µg/ml G418 for T47D cells. Once colonies were formed, individual colonies were isolated and expanded. The cells were continuously maintained in medium containing 250µg/ml G418 for MCF-7 or 300µg/ml G418 for T47D.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) and 2µg total RNA was reversely transcribed using Reverse Transcription Reagents (MBI Fermentas, Vilnius, Lithuania) following manufacturer's protocol. Quantitative real-time PCR (QRT-PCR) was performed in triplicate using SYBR Green PCR Master Mix (Toyobo, Japan) on a Mastercycler ep realplex (Eppendorf, German). The mRNA levels were normalized to GAPDH (internal control) and relatively quantified by the DDCT method. Primer sequences were listed in Table1.

Western blotting analysis

Total cell lysates were prepared with 1×SDS lysis buffer with 100mM Dithiothreitol and 2µg/ml protease inhibitors containing 0.1mM leupeptin, aprotinin, and pepstatin. After electrophoresis, proteins were transferred to nitrocellulose membrane, blocked with 5% nonfat milk, and probed overnight with primary antibodies against Gli1 (1:200; Abcam, MA, USA), β -actin (1:10000, Sigma-Aldrich Chemicals, St. Louis, MO, USA), cIAP2 (1:200, Biolegend, CA, USA), p-Akt1/2/3 (Ser 473) (sc-7985, 1:1000, Santa Cruz, Texas, USA), Akt1/2/3 (sc-8312, 1:1000, Santa Cruz, Texas, USA). The membranes were washed three times and incubated with HRP-conjugated secondary antibodies (1:5000, Rockland Immunochemicals, PA, USA) for 2h. Finally blots were detected by ECL chemiluminescence (Pierce, Rockford, IL, USA). Protein bands were quantified with ImageJ software (NIH) using β -actin as an internal control.

Table 1. Primers for amplification of genes in this study. F: forward; R: reward

Gene	Primer sequence
GAPDH	F: 5'-CATGAGAAGTATGACAACAGCCT-3' R: 5'-AGTCCTTCCACGATACCAAAGT-3'
GLI1	F: 5'-GGAAGTCATACTCAGCCCTCGA-3' R: 5'-CATTGCTGAAGGCTTTACTGCA-3'
cIAP2	F: 5'-AGCTGAAGCTGTGTTATATGAGC-3' R: 5'-ACTGTACCCTTGATTGTACTCCT-3'
UBD	F: 5'-CCGTTCCGAGGAATGGGATTT-3' R: 5'-GCCATAAGATGAGAGGCTTCTCC-3'
SLFN5	F: 5'-GAGTGTGTTGTAGATGCAGGAA-3' R: 5'-ACTGCTCGCAGGATGATTTCA-3'
CSAG1	F: 5'-GATCCAGCCAGGGCAGAG-3' R: 5'-TCTTGGAACCTCTTTGGTGT-3'
SLPI	F: 5'-GAGATGTTGTCTGACACTGTG-3' R: 5'-GCTTCCTCCTTGTGGGTTTG-3'
NDRG1	F: 5'-CTCCTGCAAGAGTTTGATGTCC-3' R: 5'-AGAGCCATGTAAAGTCTCGATGT-3'
ROCK1	F: 5'-AACATGCTGCTGGATAAATCTGG-3' R: 5'-TGTATCACATCGTACCATGCCT-3'

Table 2. List of differentially expressed genes by cDNA microarray analysis

Function	Accession	Gene	Ratio
Metabolism	NM_001353	AKR1C1	6.1395
	NM_001354	AKR1C2	7.04
	NM_003739	AKR1C3	4.1276
	NM_001818	AKR1C4	7.8424
Apoptosis	NM_182962	cIAP2	6.5846
	NM_004874	BAG4	0.4672
	NM_006290	TNFAIP3	2.5447
	NM_012385	NUPR1	3.0479
Cytoskeleton	NM_019114	EPB41L4B	6.6875
	NM_007124	UTRN	4.8194
	NM_003088	FSCN1	2.3869
	NM_002298	LCP1	2.1482
	NM_006207	CFL2	2.8904
	NM_016357	LIMA1	0.4886
	NM_005219	DIAPH1	0.4351
	XM_086186	SYDE2	0.3112
	NM_014547	TMOD3	0.3955
	NM_006096	NDRG1	0.3062
Regulation of inflammatory response	NM_006197	PCM1	0.4887
	NM_002982	CCL2	22.5694
	NM_002985	CCL5	3.1066
	NM_005408	CCL13	2.6249
Innate immunity	NM_030754	SAA2	1691.37
	NM_005514	HLA-B	15.1126
	NM_002117	HLA-C	6.7695
	NM_005516	HLA-E	3.2261
	NM_018950	HLA-F	5.8991
	NM_002127	HLA-H/HLA-G	14.7074
	NM_002125	HLA-DRB5	3.3043
	NM_033554	NP_061984.2	69.5012
Proliferation	NM_022555	HLA-DRB3	6.2563
	NM_001287188	ODC1	2.5858
	NM_001204502	FLT3LG	3.2152
	NM_003088	FSCN1	2.3869
	NM_005862	STAG1	0.2242
	NM_003157	NEK4	0.3464
	NM_015384	NIPBL	0.355
	NM_006472	TXNIP	0.3877
	XM_088331	ESCO2	0.3945
	NM_020382	SETD8	0.4844
	NM_057749	CCNE2	0.288

WST-8 assay for cell viability

Following the standard procedures provided by manufacturer, cell viability was evaluated by WST-8 assay modified from a method using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Japan). 1×10^3 cells were plated in 96-well plates in triplicate, and then allowed to adhere overnight. Next day (0 day), the medium was removed and the cells were incubated in phenol-red free RPMI-1640 containing 0.5% CD-FBS for indicated times. The cells were refreshed every other day and cell viability was measured on set days. Then, the optical density (O.D.) was measured at a wavelength of 450nm using a Labsystem multiskan microplate reader (Merck Eurolab, Dietikon, Switzerland).

Microarray analysis

Following the manufacturer's instructions, the stable subclone Gli1-MCF-7 and mock-transfected cells were picked. Oligonucleotide array analysis was performed by the CapitalBio Corp. (Beijing, P.R. China) using a 35K Human Genome Array containing 35,035 70-mer probes representing 25,100 human gene transcripts. Data processing and normalization were performed according to standard procedures using a LOWESS program. The raw microarray data were released into the GEO-database (accession number GSE53729) and

linked to <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=opwrmowetziijrmb&acc=GSE53729>. The genes shown in Table 2 were at least two-fold up-regulated or down-regulated in stable Gli1-MCF-7 cells.

ChIP assay

Chromatin immunoprecipitation assay were performed according to the instructions of the EZ ChIP™ Kit (Millipore) with minor modifications. Briefly, pcDNA3.1-MCF7 cells and pGli1-MCF7 cells were cross-linked and sonicated. Equal amounts of lysates were used for immunoprecipitation of chromatin with specific antibodies: Gli1 (R&D system), acetyl-histone H3 and normal mouse IgG (Millipore). The purified chromatin DNA was subjected to quantitative real-time PCR with primers for promoter of human *cIAP2* gene, with acetyl-histone H3 as a positive control and immunoglobulin G as a negative control. The primers were 5'-CCAAGTAGCTGGGACTACAGGC-3' (sense) and 5'-CTGGGATCATAAGGATCTAAGTGTTA-3' (antisense). The data were analyzed using the formula of $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (Ct[IP] - Ct[input])_{SA} - (Ct[IP] - Ct[input])_{NS}$. SA=Specific antibody, NS= Non-specific antibody. Three independent ChIPs were performed.

Luciferase assay

The cells were plated in triplicate into a 24-well plate at a density of 5×10^4 cells/well for 24h, and then were transiently co-transfected with a DNA mixture containing pGL3-NF- κ B-luc (200ng/well), pcDNA3.1-Gli1 (500ng/well) or pcDNA3.1 vector (500ng/well) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) for 24h. 1.0ng pRL-SV40-luc per well was co-transfected to normalize the transfection efficiency. The luciferase activity was determined by the dual luciferase assay system. Results were normalized against the internal renilla control and presented as fold induction over control.

Statistical analysis

Data were expressed as Mean $\pm$ SD of at least three determinations. Statistical significance between experimental groups was analyzed by ANOVA and the significant level was set at $P < 0.05$.

Results

Serum starvation induces the expression of Gli1 through activation of PI3K/AKT pathway in breast cancer cell lines

Tumor microenvironment is a characteristic of ischemia and starvation of nutrients, and the enhanced resistance of cancer cells to ischemia- and starvation-induced cell death plays a very pivotal role in cancer progression. First we examined the expression of Gli1 in MCF-7 and T47D cells cultured in medium containing decreasing serum concentration (5%, 2.5% and 0.5%) using QRT-PCR and Western blotting. We found that the expression of Gli1 was increased with decreasing the serum concentration. The mRNA levels of Gli1 in MCF-7 and T47D cells cultured in 0.5% CD-FBS medium increased by 2.67 fold ($P < 0.05$) and 2.23 fold ($P < 0.05$) compared with that cultured in 10% CD-FBS medium, respectively (Fig. 1A). Similarly, increased Gli1 proteins were also observed by Western blotting analysis in both cell lines (Fig. 1B). These results indicate that serum starvation up-regulates the expression of Gli1 in breast cancer cells.

AKT is a serine/threonine kinase downstream of PI3K. The PI3K/AKT pathway plays a critical role in cell survival, proliferation, metabolism and resistance to apoptosis [24, 25]. It has been reported that the expression and activity of Gli1 are modulated by PI3K/AKT pathway [7, 26, 27]. We therefore examined whether up-regulation of Gli1 by serum starvation was through the activation of PI3K/AKT pathway. Results showed that the level of phosphorylated AKT at S473 was significantly increased in MCF-7 and T47D cells cultured in 0.5% CD-FBS medium for 24h compared with that in cells cultured in medium with 10% CD-FBS (Fig. 1C). Treatment with 100nM of Wortmanin, a specific inhibitor of PI3K, significantly decreased the levels of Gli1 protein and mRNA in MCF-7 cells and T47D cells under a serum-starved condition (Fig. 1C and D). These results indicate that serum starvation up-regulates the expression of Gli1 at least in part through the activation of PI3K/AKT pathway.

Fig. 1. Effect of serum starvation on Gli1 expression in breast cancer cells. Breast cancer cells (MCF-7 and T47D) were cultured for 24h in complete medium and then in medium containing indicated concentrations of serum for another 24h. Gli1 mRNA (A), Gli1 protein (B) were detected by QRT-PCR and Western blotting, respectively. The cells grew for 24h in complete medium and then were treated with 100nM Wortmanin, or an equimolar amount of DMSO in phenol-red free RPMI-1640 containing indicated concentrations of CD-FBS for another 24h. Indicated proteins (C) and Gli1 mRNA (D) were evaluated in MCF-7 and T47D cells by Western blot and QRT-PCR, respectively. GAPDH was used as a normalization control for QRT-PCR

and β -actin as a loading control for Western blotting. The results were expressed as fold over control cells in complete medium. Data were representative of three independent experiments. Each bar represents mean $\pm$ S.D. * P <0.05 versus the 10% group. # P <0.05 versus the cells treated with the vehicle.

Over-expressed Gli1 promotes survival of breast cancer cells cultured in serum-starved medium

Serum supports cell growth by providing a broad spectrum of nutrients, hormones (such as estrogen), growth factors and attachment factors. Other groups had also reported that serum starvation could reduce cell viability and induce cell apoptosis [28, 29]. Our previous studies also demonstrated that serum starvation (0.5% CD-FBS) obviously decreased cell proliferation and increased cell death (data not shown). Therefore, we then examined whether up-regulation of Gli1 expression could promote survival of breast cancer cell in a serum-starved condition using established stable Gli1-transfectants (Gli1-MCF-7 and Gli1-

Fig. 2. Effect of over-expressing Gli1 on breast cancer cell survival in serum-starved medium. The cells were transfected with pcDNA3.1-Gli1 or control plasmid, and single-cell-derived clones were obtained as described in “Materials and methods”. The mRNA (A) and protein (B) levels of Gli1 in control cells and Gli1-transfectants were analyzed by QRT-PCR and Western blotting, respectively. GAPDH was used as a normalization control for QRT-PCR and β -actin as a loading control for Western blotting. (C and D) Stably Gli1-over-expressing breast cancer cells (MCF-7 and T47D) and corresponding control ones (1,000 cells/ well) were plated in 96-well plates and allowed to adhere overnight. Next day (0 day), the medium was removed and the cells were incubated in medium containing 0.5% CD-FBS as indicated for different days. Cell viability was evaluated by WST-8 cell viability assay. Data were representative of three separate experiments and expressed as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ versus corresponding empty vector control.

cells (MCF-7 and T47D) and corresponding control ones (1,000 cells/ well) were plated in 96-well plates and allowed to adhere overnight. Next day (0 day), the medium was removed and the cells were incubated in medium containing 0.5% CD-FBS as indicated for different days. Cell viability was evaluated by WST-8 cell viability assay. Data were representative of three separate experiments and expressed as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ versus corresponding empty vector control.

T47D cells) and their control cells (pcDNA3.1-MCF-7 and pcDNA3.1-T47D). The increased expression of Gli1 mRNA and protein in Gli1-transfectants were confirmed by QRT-PCR (Fig. 2A) and Western blotting (Fig. 2B). The cell survival in a serum-starved condition was assayed by WST-8 cell viability analysis at indicated time points. As shown in Fig 2C, the viability of Gli1-MCF-7 cells was higher than control cells in medium with 0.5% CD-FBS at day 4 and 6 (1.4 and 2.7 fold of control, $P < 0.01$, respectively) (Fig. 2C). Similarly, the significantly increased viability was also observed in Gli1-T47D cells in medium with 0.5% CD-FBS at day 4 (1.53 fold of control, $P < 0.01$) (Fig. 2D).

Identifying Gli1-regulated genes in MCF-7 cells by microarray analysis

In order to screen for downstream Hh/Gli1 target genes that are likely to be involved in pro-survival effect of Gli1 in breast cancer cells, microarray analysis was performed on Gli1-MCF-7 cells over-expressing Gli1 and mock-transfected cells. Results of three independent microarray experiments showed that 179 genes were significantly up-regulated by at least two-fold (≥ 2 -fold), whereas 159 targets were down-regulated by at least 50% as compared with the control cells (Fig. 3A). All of the 338 Gli1-regulated genes could be further divided into six functional groups: metabolism, apoptosis, cytoskeleton, proliferation, inflammation and innate immunity (Table 2). Among Gli1-regulated genes, five up-regulated targets, including cIAP2 (cellular inhibitor of apoptosis 2), SLFN5 (a member of the Schlafen family), SLPI (secretory leucocyte protease inhibitor), UBD (Ubiquitin D/ Ubiquitin-like protein FAT 10), CSAG1 (chondrosarcoma associated gene 1) and two down-regulated targets, NDRG1 (a member of the N-myc down-regulated family) and ROCK1 (rho-associated, coiled-coil-containing protein kinase 1) were further confirmed by QRT-PCR in Gli1-MCF-7 cells (Fig. 3B). Except CSAG1 and ROCK1, similar up-regulation of cIAP2, SLFN5, UBD, SLPI genes and down-regulation of NDRG1 gene were also seen in Gli1-T47D cells (Fig. 3C).

Fig. 3. Validation and identification of Gli1-regulated genes in breast cancer cells. (A) Microarray analysis was performed as described in “Materials and methods”. Log-Log Scatter plot showed genes differentially expressed between Gli1-MCF-7 and pcDNA3.1-MCF-7 cells (high expression in red and low expression in green). Five up-regulated genes and two down-regulated genes were confirmed by QRT-PCR in the stably transfected MCF-7 (B) or T47D (C) cells. cIAP2 protein expressions were determined by Western blotting (D) in stably transfected MCF-7 and T47D cells. GAPDH was used as a normalization control for QRT-PCR and β -actin as a loading control for Western blotting. Data were representative of three independent experiments and expressed as mean $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the empty vector control.

Among genes identified by microarray, the expression of three anti-apoptosis genes, including cIAP2, TNFAIP3 (TNF- α -induced protein 3) [30] and NUPR1 (nuclear protein 1) [31] were up-regulated, and the most highly up-regulated gene was *cIAP2* that has well-established functions as an anti-apoptotic and pro-survival protein [32–34]. The mRNA levels of cIAP2 tested by QRT-PCR increased by about 12 fold in Gli1-MCF-7 ($P < 0.01$) and 7 fold in Gli1-T47D cells ($P < 0.01$) compared with their control cells (Fig. 3B and C). The increased protein levels of cIAP2 in Gli1-transfectants were further confirmed by Western blotting analysis in breast cancer cell lines (Fig. 3D).

No direct binding of Gli1 to cIAP2 promoter region was found

In order to know whether cIAP2 is a direct target gene of Gli1, we searched putative Gli1 binding site in the promoter region of *cIAP2* gene through online transcription element search software analysis. Within -1681bp to -1741bp promoter region of *cIAP2* gene, we found a putative Gli1 binding site (5'-GATCCACCCA-3') that resembles a known consensus sequence of human Gli-binding site (5-GACCACCCA-3) [4, 35] (Fig. 4A). We further performed ChIP assay to figure out whether there is a direct binding of Gli1 to the promoter of *cIAP2* gene at the region of -1568 to -1792bp. Isotype control IgG and anti-acetyl-histone H3 were used as negative and positive controls. However, no direct binding of Gli1 to this region was found (Fig. 4B), suggesting that Gli1 might regulate the expression of cIAP2 in an indirect manner.

Fig. 4. No direct binding of Gli1 to the region of -1568 to -1792 bp in the *cIAP2* promoter. (A) Schematic representation of human *cIAP2* gene showing the relative location of DNA sequences that closely match Gli consensus binding site within about 3kb of the transcription initiation site. (B) ChIP assay was performed in pcDNA3.1-MCF7 and pGli1-MCF7 cells using indicated antibodies. Isotype control IgG and anti-acetyl-histone H3 were used as negative and positive controls, respectively. Data showed no recruitment of Gli1 to the promoter of *cIAP2* gene with primers listed in “Materials and methods”. All values were normalized by control IgG precipitations. Each bar represented the average result from three independent ChIP experiments. * $P < 0.05$ versus anti-control IgG.

cIAP2 expression is up-regulated by Hedgehog/Gli1 signaling pathway and serum starvation in breast cancer cells

Given that Gli1 is up-regulated by either Hh ligand-dependent or -independent mechanisms, we examined whether the expression of *cIAP2* is up-regulated by ligand of Hh/Gli1 pathway. MCF-7 and T47D cells were treated with 0.4 μ g/ml rhSHH-N, a ligand of hedgehog pathway, for different time (4h, 8h, 12h, and 24h), and then Gli1 and *cIAP2* mRNA were determined. The results showed that significant increases in Gli1 and *cIAP2* mRNA levels by rhSHH-N were only seen in MCF-7 cells for 4h (4.5 and 2.3 fold of controls, $P < 0.01$, respectively) or T47D cells for 12h (2.3 and 1.9 fold of controls, $P < 0.05$, respectively) (Fig. 5A). There was no significant change at other time points. This result indicated that Hh ligand could shortly up-regulate the expression of Gli1 and *cIAP2*.

It was showed that serum starvation up-regulated the expression of Gli1 and over-expressed Gli1 enhanced the survival of breast cancer cells. Therefore, we hypothesized that Gli1-induced the expression of *cIAP2* may account in part for the pro-survival effect of Gli1 under serum starvation. Being consistent with this hypothesis, *cIAP2* mRNA and protein levels in both MCF-7 cells and T47D cells cultured in medium with 0.5% serum concentration were significantly increased and the regulation patterns and extent of *cIAP2* were similar to Gli1 (Fig. 5B and C), strongly suggesting that *cIAP2* might be involved in the pro-survival effect of Gli1.

NF- κ B/cIAP2 signaling partially contributes to the pro-survival role of Gli1 in ER-positive breast cancer cells

Activation of the transcription factor NF- κ B is a key pro-survival mechanism in cancer cells [36] and studies have shown that *cIAP2* is a target gene of NF- κ B [37, 38]. Therefore, we examined whether NF- κ B activation is involved in the pro-survival effect of Gli1 as well as up-regulation of *cIAP2* expression. The cells were transiently transfected with a DNA mixture containing pGL3-NF- κ B-luc, pRL-SV40-luc, pcDNA3.1-Gli1 or pcDNA3.1 vector for 24h, and then the luciferase activities were determined using the dual luciferase assay system. As shown in Fig. 6A, over-expression of Gli1 significantly increased the luciferase activities of NF- κ B (about 2.3 fold of controls, $P < 0.05$), indicating that Gli1 could enhance

Fig. 5. Effect of activation of Hedgehog/Gli1 signaling pathway and serum starvation on cIAP2 expression in breast cancer cells. (A) MCF-7 and T47D cells were cultured for 24h in complete medium and then treated with or without 0.4 μ g/ml rhSHH-N for indicated hours. Then Gli1 and cIAP2 mRNA expressions were determined by QRT-PCR. For serum starvation experiment, MCF-7 and T47D cells were incubated in medium containing indicated concentrations of serum for another 24h. Then cIAP2 mRNA (B) and protein (C) levels were tested. GAPDH was used as a normalization control for QRT-PCR and β -actin as a loading control for Western blotting. Data were representative of at least three independent experiments and expressed as mean $\pm$ S.D. * P <0.05, ** P <0.01 versus the 10% group or the cells treated with the vehicle.

transcriptional activity of NF- κ B in MCF-7 cells. Inhibition of NF- κ B trans-activation using Bay11-7082 (Bay), a specific inhibitor of NF- κ B, significantly decreased the Gli1-increased mRNA levels of cIAP2 in MCF-7 and T47D transfectants as compared with that treated by DMSO (from about 11 fold to 6 fold for Gli1-MCF-7, P <0.01, and from about 5 fold to 2.8 fold for Gli1-T47D, P <0.05, respectively)(Fig. 6B). Moreover, Bay not only attenuated basal cell viabilities, but also reduced Gli1-enhanced cell viabilities under serum-starved condition (Fig. 6C). These results demonstrate that pro-survival effect of Gli1 is partially mediated by NF- κ B activation and up-regulation of cIAP2 as a target gene of NF- κ B.

Discussion

Serum supplemented to cell culture media supports cell growth by providing a broad spectrum of nutrients, macromolecules, attachment factors, hormones and growth factors. Serum starvation could reduce cell viability and induce cellular apoptosis [28, 29]. Tumor

Fig. 6. Activation of NF-κB/cIAP2 signaling is a potential pro-survival mechanism of Gli1 in breast cancer cells. (A) MCF-7 cells were co-transfected with 200ng of pGL3-NF-κB-luc, 1.0ng of pRL-SV40-luc, 500ng of pcDNA3.1-Gli1 or pcDNA3.1 vector in 24-well plate for 24h. The luciferase activities were measured and expressed as fold over the empty vector control. (B) cIAP2 mRNA level was assayed by QRT-PCR in MCF-7 and T47D cells with or without 5μM Bay (NF-κB inhibitor) treatment for 12h. (C) MCF-7 or T47D cells were incubated in medium with 0.5% CD-FBS prior to treatment with 5μM Bay for 24h, and total incubation time was 4 days. The cell viability was determined at 450nm on a microplate reader using WST-8 assay. Data were representative of three independent experiments. Each bar represents mean ± S.D. * $P < 0.05$, ** $P < 0.01$ versus the empty vector control.

microenvironment is a characteristic of ischemia and starvation of nutrient, while the promotion of cell survival is a critical event in the development of cancer. In this study, we found that serum starvation significantly up-regulated the expression of Gli1 and over-expression of Gli1 promoted the survival of ER-positive breast cancer cells under a serum-starved condition. Furthermore, we found that serum starvation could activate AKT in MCF-7 cells and T47D cells. Inhibiting PI3K/AKT pathway with Wortmanin significantly reduced the serum starvation-induced the expression of Gli1. These results indicated that activated PI3K/AKT pathway contributed to induction of Gli1 expression and pro-survival effect of Gli1 in a less nourished condition. Besides PI3K/AKT pathway, TGFβ and Ras/ERK pathway also modulate Gli1 expression and activity [6, 7]. Although these two pathways play an important role in control of cell survival in cancer cells, whether they are also involved in promoting survival of breast cancer cells by up-regulation of Gli1 expression under serum starvation is needed to be clarified.

Gli1 functions as a strong positive transcriptional activator in the downstream of the Hh pathway. So we first identified putative target genes that mediated the role of Gli1 in breast cancer cells by microarray analysis. We found that 338 genes were differentially expressed in Gli1-MCF-7 cells as compared with their control cells, which could be further divided into six functional groups: metabolism, apoptosis, cytoskeleton, proliferation, inflammation and innate immunity. Furthermore, among Gli1-regulated genes, up-regulation of cIAP2, SLFN5, SLPI, UBD genes and down-regulation of NDRG1 gene were confirmed by QRT-PCR in both Gli1-MCF-7 cells and Gli1-T47D cells. SLFN5 is a member of the Schlafen (SLFN) family and is involved in important functions, such as the control of cell proliferation, induction of

immune responses, and the regulation of viral replication [39, 40]. SLPI (secretory leucocyte protease inhibitor) is a secreted protease inhibitor which is found in various secreted fluids including seminal plasma, cervical mucus, and bronchial secretions [41]. UBD (Ubiquitin D/Ubiquitin-like protein FAT 10) can be covalently attached to target protein and subsequently leads to their degradation by the 26S proteasome in a NUB1L-dependent manner [42]. NDRG1 is originally identified as a stress-responsive protein and belongs to a member of the N-myc down-regulated family [43]. These proteins have been reported not only to regulate cell proliferation and metastasis, such as SLFN5 [39, 40], but also to play important roles in induction of immune and inflammatory responses [41]. So it is interesting to investigate whether Gli1 also plays an important role in control of immune and inflammatory responses mediated by these genes.

Among genes identified by the microarray, we noticed that three anti-apoptosis genes including *cIAP2*, *TNFAIP3* and *NUPR1* were up-regulated in Gli1-MCF-7 cells. Another group also reported the up-regulation of *cIAP2* upon Gli1 over-expression in pancreatic cancer by microarray [44]. Since *cIAP2* has well-established functions as an anti-apoptotic and pro-survival protein [32-34], we further investigated whether *cIAP2* mediated the pro-survival effect of Gli1 as a potential target of Hh/Gli1 signaling. We found that expression of *cIAP2* was not only up-regulated by over-expression of Gli1 and rhSHH-N, a ligand of Hh/Gli1 pathway, but also significantly increased by serum starvation. The regulation pattern and extent of *cIAP2* were similar to the up-regulation of Gli1 by serum starvation, strongly suggesting that *cIAP2* might be involved in the pro-survival effect of Gli1 in ER-positive breast cancer cells. Moreover, we investigated whether *cIAP2* was a direct target gene of Gli1. Although a putative Gli1 binding site (5'-GATCCACCCA-3') within -1681bp to -1741bp promoter region of *cIAP2* gene has been identified by transcription element search software analysis, ChIP assay did not reveal the direct binding of Gli1 to *cIAP2* promoter at the region of -1568 to -1792 bp, suggesting that *cIAP2* is not a direct target gene of Gli1.

Transcription factor NF- κ B is a strong pro-survival mediator in breast cancer cells. Constitutive activation of NF- κ B in breast tumors is associated with highly aggressive ER-positive tumors [45, 46], development of the resistance to endocrine therapy [47, 48] and progression to estrogen-independent growth [49-51]. Ramirez E et al reported that activation of NF- κ B is associated with increased expression of Gli1 and Hh ligands since pharmacologic inhibition of NF- κ B pathway resulted in a decreased expression of Gli1 and Hh ligands in diffuse large B-cell lymphoma (DLBCL) [27]. On the contrary, we found that over-expression of Gli1 significantly enhanced transcriptional activity of NF- κ B in MCF-7 cells. Several anti-apoptotic and pro-survival genes such as *cIAP2*, *Bcl-2*, *Bcl-xL* and *Survivin* are regulated by NF- κ B [49, 51]. Inhibition of NF- κ B trans-activation using a specific inhibitor of NF- κ B, Bay11-7082 (Bay) not only significantly reduced the pro-survival effect of Gli1, but also markedly decreased the up-regulation of *cIAP2* mRNA expression in both Gli1-MCF-7 cells and Gli1-T47D cells under a serum-starved condition. These results revealed that enhancing NF- κ B signaling also contributed to the pro-survival effect of Gli1 by up-regulating its downstream anti-apoptotic and pro-survival genes, such as *cIAP2* in breast cancer cells.

In summary, our data revealed that the up-regulation of Gli1 and its target *cIAP2* by serum starvation promoted the survival of breast cancer cells. Gli1 also promoted cell survival by enhancing NF- κ B/*cIAP2* signaling in breast cancer cells. These findings provide new insights into the pro-survival mechanisms of Gli1 and evidence of cross-talk between Gli1 signaling and NF- κ B signaling in breast cancer. However, the mechanisms by which Gli1 enhances trans-activation of NF- κ B are still unclear and further study is needed.

Acknowledgements

The authors thank Dr. Hiroshi Sasaki and Dr. H.M. Xu for providing plasmids. This work was supported by a scientific research grant from the National Natural Science Foundation of China (grant no. 30971157).

Disclosure Statement

The authors declare that they have no conflict of interest.

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