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Silencing STAT3 may inhibit cell growth through regulating signaling pathway, telomerase, cell cycle, apoptosis and angiogenesis in hepatocellular carcinoma: potential uses for gene therapy

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The genesis and development of hepatocellular carcinoma (HCC) is related to the abnormity of signaling pathway, telomerase, cell cycle, apoptosis, angiogenesis, and others, in which STAT3 signaling pathway plays a key role. The HCC cell line $HepG_2$ was transfected with small interfering RNA (siRNA) directed against STAT3. After 72 h, cell growth and cycle were analysed by MTT and Flow cytometry. Then, the protein was extracted and the protein expression of STAT3, Smad3, p44/42, TERT, caspase-3, XIAP, Grp-78, HSP-27, MMP-2, MMP-9, VEGF-A, cyclin A, and cyclin E was detected by Western blot. After the transfection, HCC cell growth was inhibited during the 24–72 h time period and the cell cycle was arrested in G0/G1. STAT3 protein expression was inhibited at 72 h after the transfection. Interestingly, Smad3, p-caspase-3, p-p44/42, Grp78, cyclin A, and cyclin E protein expression was increased at 72 h, while TERT, caspase-3, XIAP, MMP-2, MMP-9, and VEGF-A protein expression decreased at 72 h. However, P44/42, and HSP27 protein expression showed no change following transfection. The results demonstrated that STAT3 signaling pathway may participate in HCC genesis and development through regulating the protein expression of other signaling pathway, telomerase, apoptosis, cell cycle and angiogenesis; thereby, blockade of the Stat3 pathway represents a potential strategy for future treatment.

Key words: STAT3, signaling pathway, telomerase, cell cycle, apoptosis, angiogenesis

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. The prognoses of the patients are very poor due to the late diagnosis and lack of effective treatment. To date, it has been proved that the genesis and development of HCC are caused by the abnormity of multiple factors, which in common form a complicated network. These factors include signaling pathway, telomerase, cell cycle, apoptosis, angiogenesis, and others. Studies on pathogenetic network of HCC will provide us further understandings on the genesis and development of HCC and contribute to the prevention and treatment of HCC.

STAT3 has been classified as an oncogene, because constitutively active STAT3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice. Since STAT3 appears to play an important role in oncogensis, it is of interest to investigate STAT3-regulated genes and elucidate STAT3-mediated oncogenesis. STAT3-regulated genes are related to these factors including signaling pathway, telomerase, cell cycle, apoptosis, angiogenesis, and others.

Up to date, people have studied some STAT3-regulated genes. For example, some studies suggest that STAT pathway plays an important role in cell-cycle progression and resistance to apoptosis through the regulation of cyclin A [1], E [1, 2], D1 [1, 3], XIAP [1, 3], Survivin [1, 4], and caspase [1, 5]. In prostate cancer cells, the activation of STAT3 may affect the telomerase activity [6]. In breast cancer cells and epithelial cells, STAT3 may modulates MEK5 and HSP27 protein expression [7, 8]. In addition, STAT3 signaling may regulate the expression of MMP [4, 9-12], VEGF [4, 11, 12], and Smad [13, 14]. However, STAT3-regulated genes are still unclear in HCC, so in this study, we investigated the STAT3-regulated genes in HCC cells.

Considering the above observations, we silenced STAT3 gene using RNA interference (RNAi) technology and observed cell growth and the expression of other signaling pathways (p44/42 and Smad3), telomerase (TERT), apoptosis (caspase-3, XIAP, Grp-78, and HSP-27), angiogenesis (MMP-2, MMP-9, and VEGF-A), and cell-cycle regulators (cyclin A and cyclin

E) in the HCC cell line $HepG_2$, so as to further elucidate their relationship, and better understanding the role of STAT3 signaling pathway during HCC genesis and development, and look for the efective treatment.

Materials and methods

Materials. The HCC cell line $HepG_2$ was purchased from the cell bank of the Chinese Academy of Sciences. Lipofectamine™ 2000 was purchased from Invitrogen and cell cycle kit was purchased from BD Corporation. Small interfering RNA (siRNA) directed against STAT3 was synthesized by the Shanghai Genepharma Company. The siRNA sequences directed against STAT3 were: sense, 5'- CAUCUGCCUAGAUCGGCUAdTdT -3'; and anti-sense, 5'- UAGCCGAUCUAGGCAGAUGdTdT -3' (15). Dulbecco's minimum essential medium (DMEM) with high glucose was purchased from the Hecoly Corporation and fresh fetal calf serum (FCS) was purchased from TBD Biotechnology Corporation (Tianjin). The primary antibodies used were mouse anti-β-actin, rabbit anti-STAT3, rabbit anti-Smad3, rabbit anti-TERT, goat anti-Grp-78, mouse anti-HSP-27, rabbit anti-MMP-2, rabbit anti-MMP-9, mouse anti-VEGF-A, rabbit anti-cyclin A, rabbit anti-cyclin E(above from Santa Cruz Biotechnology), rabbit anti-p44/42, rabbit anti-p-p44/42, rabbit anti-caspase-3, rabbit anti-p-caspase-3, and goat anti-XIAP (above from cell Signaling Technology). The secondary antibodies of horseradish peroxidase (HRP)-conjugated goat anti-rat, goat anti-rabbit, and rabbit anti-goat were also from Santa Cruz.

Cell culture. $HepG_2$ cells were plated in culture flasks and cultured in DMEM supplemented with 10% vol/vol fresh FCS at 37°C in a humidified, 5% carbon dioxide atmosphere. Cells were replated following a 1 min 0.25% trypsin digestion when they reached confluency in order to maintain the cell line.

Transient transfection. A total of 3×105 cells were plated in six-well plates in triplicate and grown to approximately 30-50% confluency. For transfection, 500 µl of siRNA in Lipofectamine™ 2000 (Invitrogen) was applied to each well containing cells and 2 ml of DMEM media without FCS, and the plates were gently rocked back and forth. At 6 h after the transfection, the media was replaced with DMEM media containing 10% vol/vol FCS. The cells were harvested at 72 and 96 h, and mRNA and protein were isolated for later analyses. Control groups without the addition of transfection reagents were also analyzed. All experiments were performed in triplicate and representative results were reported.

Detection of HCC cell growth by methyl thiazolyl tetrazolium (MTT) assay. The obtained specimens were subdivided into the following groups: (1) control group without siRNA-Lipofectamine™ 2000 complexes; (2) Lipofectamine™2000 transfection group without siRNA; and (3) the siRNA transfection group.

A total of 1.5×104 cells were plated in 96-well plates in triplicate, which were grown to about 30-50% confluence at the time of transfection. A total of 50 µl of siRNA-Lipofectamine™ 2000 complexes were applied to each well containing cells and 100µl of DMEM medium without FCS, followed by gentle rocking. Medium was replaced by DMEM medium containing 10% vol/vol FCS at 6 h after the trasfection. A total of 20 µl (5 mg/ml) of MTT diluted in PBS was added to medium at 24, 48, and 72 h after the transfection. After subsequent 4 h incubation, the medium was removed and the sediments were left at the bottom of wells. Dimethyl sulphoxide (DMSO, 200 µl) was applied to each well and the sediments were redissolved by rocking the plate. Finally, the absorbance was measured using a microplate reader (Model MK3, Thermo Labsystems Co., USA) at 540 nm to determine the number of viable cells. All experiments were performed in triplicate. The data were normalized to their respective controls and presented as a bar graph.

Flow cytometry. At 72 h after transfection, the media were removed and the cells were washed in PBS, trypsinized, collected, and washed again in PBS, and then placed in 2 ml of ice cold 70% ethanol and preserved at 4°C. The cells were washed three times and the RNases and proteins were removed by using a cell cycle kit (BD Corporation, USA). After the cells were incubated in 10 g/ml propidium iodide (PI) at 4°C for 10 minutes in the dark, cell cycle was analyzed by a flow cytometer (Partec Co., Germany) in 2 hours.

Detection of the protein expression by Western blot. The siRNA was transferred into $HepG_2$ cells (same procedures as described above) and cells were lysed in RIPA buffer (Solarbio technology Co., Beijing, China) at 72 h post-transfection for protein isolation. Protein concentrations were determined using the BCA assay (Beyotime Biotechnology Co., Shanghai, China). Proteins samples were electrophoresed on 10% SDS-PAGE gels (STAT3, Smad3, p44/42, TERT, Grp-78, MMP-2, MMP-9, cyclin A, and cyclin E) or 15% SDS-PAGE gels (caspase-3, XIAP, HSP-27, and VEGF-A) and transferred onto PVDF membranes (Millipore Corporate, Billerica, MA, USA). The membranes were blocked by incubation in PBS containing 5% skim milk at 37°C for 1 h, and then were incubated with specific antibodies (1:200 or 1:1000 dilution) at 4 overnight. The next day, the membranes were washed 3 times with PBS, followed by incubation with HRP-conjugated secondary antibodies (1:5000 dilution) at 37°C for 1 h. After a second wash of 3 times with PBS, the target proteins were developed in 3, 3'diaminobenzidine (DAB). Once protein bands were visible on the membranes, color development was stopped and the membranes were rinsed in distilled water. Images were captured using a gel imaging system and were analyzed using Quantity One software. The quantitative results of gray-scale analysis were used for statistical analysis.

Statistical analysis. Data were compared using the Student's *t*-test and variance analysis. $p < 0.05$ or $F < 0.05$ was considered to be statistically significant.

Results

HCC cell growth by MTT assay. The results of the MTT assays are listed in Figure 1. Cell viability in the control

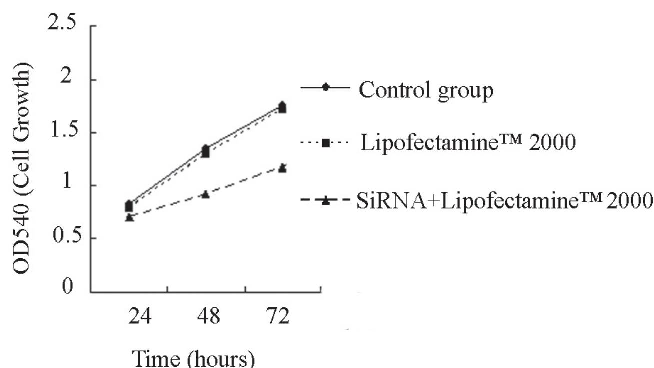


Figure 1. MTT assay. HCC HepG2 cells were grown in 96-well plates in triplicate and transiently transfected with random siRNA oligonucleotides or STAT3 siRNA for 72 h. A MTT assay was preformed to detect the change in cell viability. The percentage of cell growth was calculated using the formula: % of control = ODt/ODc × 100, where ODt and ODc are the optical densities for treated and control cells, respectively. 1. control group. 2. Lipofectamine™ 2000. 3. siRNA+Lipofectamine™ 2000.

group showed no significant difference relative to that of the Lipofectamine™ 2000 only group ($P > 0.05$); however, the siRNA transfection group showed a significant decrease in the number of viable cells ($P < 0.01$). Cell growth inhibition ratios induced by the siRNA were14.3 % at 24 h, 31.8 % at 48 h, and 33.4% at 72 h. (Fig. 1.) These results indicated that HCC cell growth was inhibited during the 24–72 h time period after siRNA transfection. In other words, the short-term inhibition of cell growth might be obtained by transient transfection of siRNA.

Analysis of cell cycle by flow cytometry. The cell cycle was analyzed by flow cytometry at 72 h after the transfection. The distributions of the cell cycle for the control group were $G_1\%$ =20.408 , $G_2\%$ =47.542, S% =32.05; its distributions at 72 h after the transfection were $G_1\%$ =44.779, $G_2\%$ =15.207, S% =40.014. Apparently, the cell cycle was arrested in G_0/G_1 after the transfection ($p<0.05$) (Fig. 2).

Modulation of gene expression after knockdown of STATs expression . The protein expression was assessed by Western blot at 72 h after the transfection of siRNA against STAT3 into HCC $HepG_2$ cells. STAT3 protein expression was inhibited at 72 h after the transfection ($p<0.05$). Interestingly, Smad3, p-caspase-3, p-p44/42, Grp78, cyclin A, and cyclin E protein expression was increased at 72 h, while TERT, caspase-3, XIAP, MMP-2, MMP-9, and VEGF-A protein expression decreased at 72 h ($p<0.05$). However, P44/42, p38, and HSP-27 protein expression showed no change following transfection ($p>0.05$) (Figs. 3, 4 and 5).

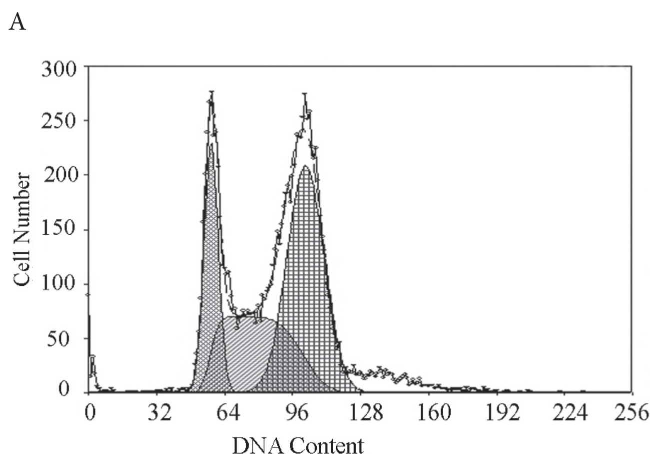
Discussion

The expression of STAT3 protein . RNAi, first reported by Fire et al. in 1998, is a gene silencing technique at the post-transcriptional level caused by introduction of a double-stranded RNA which induces the degradation of mRNA containing specific homologous sequences (16). To date, RNAi has been successfully applied in the study of gene functions and the interrelationships between the upstream and downstream factors in signaling pathways. RNAi may also potentially be applied in future tumor therapies. In our experiments, we found that STAT3 protein expression was inhibited at 72 h after the transfection of siRNA against STAT3 into HCC $HepG_2$ cells. This is just the result of RNAi technology in practical application.

The expression of other pathways (p44/42 and Smad3) and TERT protein . Previous work has demonstrated that many signaling pathways play key roles during the genesis and development of HCC. These pathways include Wnt/β-catenin, Ras/MAPK, PI3K/Akt, JNK/STAT, NF-κB, TGF-β/Smad, Hh, and p53 pathways, in which JNK/STAT, Ras/MAPK, and TGF-β/Smad pathways play particularly important and interrelated role.

At present, four MAPK pathways have been identified in mammalian cells, including the extracellular signal regulated protein kinase(ERK), c-Jun amino-terminal kinase(JNK)/

A



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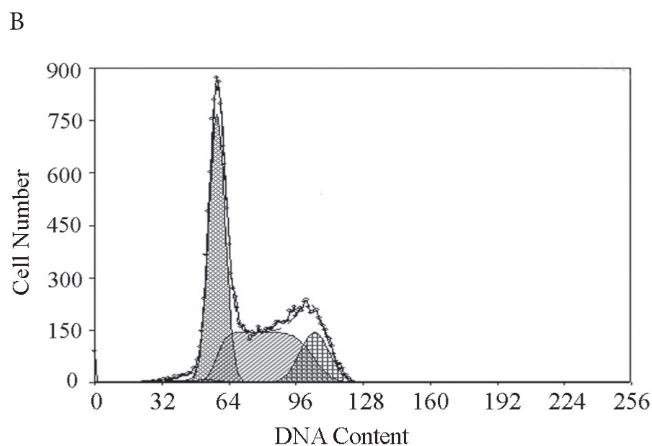

Figure 2. Flow cytometry assay. The cells were grown and transiently transfected with random siRNA oligonucleotides (A) or STAT3 siRNA for 72 h (B) and then subjected to a flow cytometry assay.

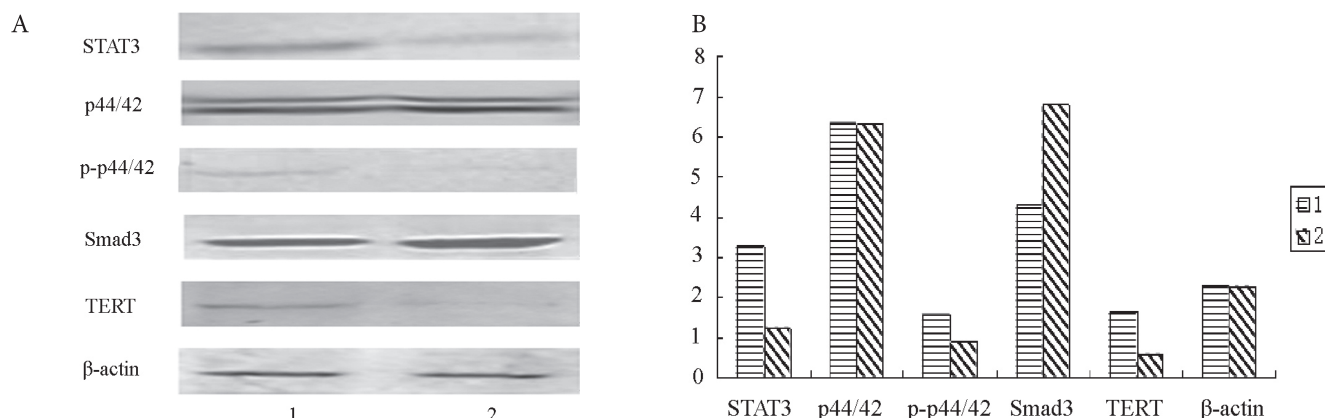

Figure 3. The protein expression of STAT3, p44/42, p-p44/42, Smad3, TERT, and β-actin after knockdown of STAT3 by siRNA in HCC HepG2 cells.
A: 1: The control group. 2: 72 h after the transfection.
B: (X axis)1: the control group. 2: 72 hours after the transfection.
Y axis is the quantitative results of gray-scale analysis.

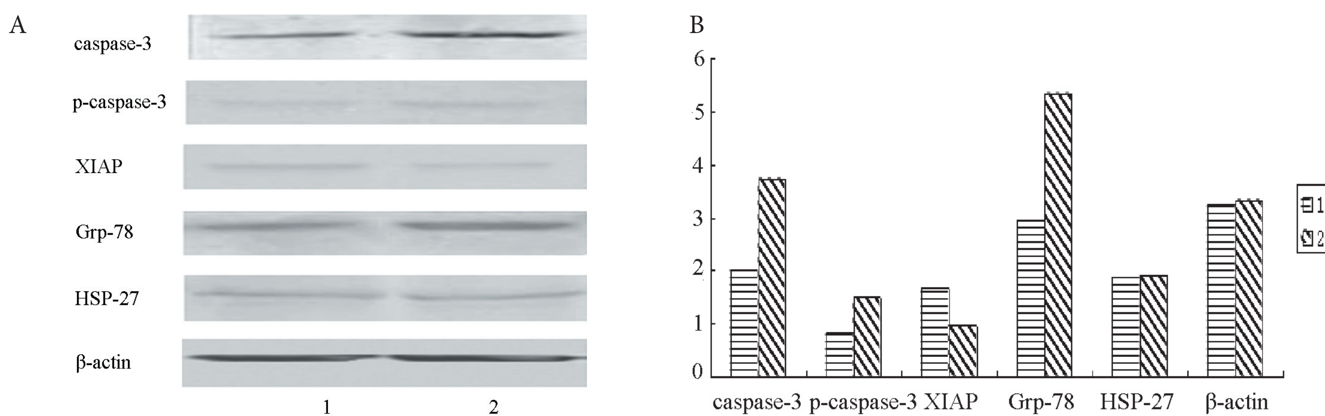

Figure 4. The protein expression of caspase-3, p-caspase-3, XIAP, Grp-78, HSP-27and β-actin after knockdown of STAT3 by siRNA in HCC HepG2 cells.
A: 1: The control group. 2: 72 h after the transfection.
B: (X axis)1: the control group. 2: 72 hours after the transfection.
Y axis is the quantitative results of gray-scale analysis.

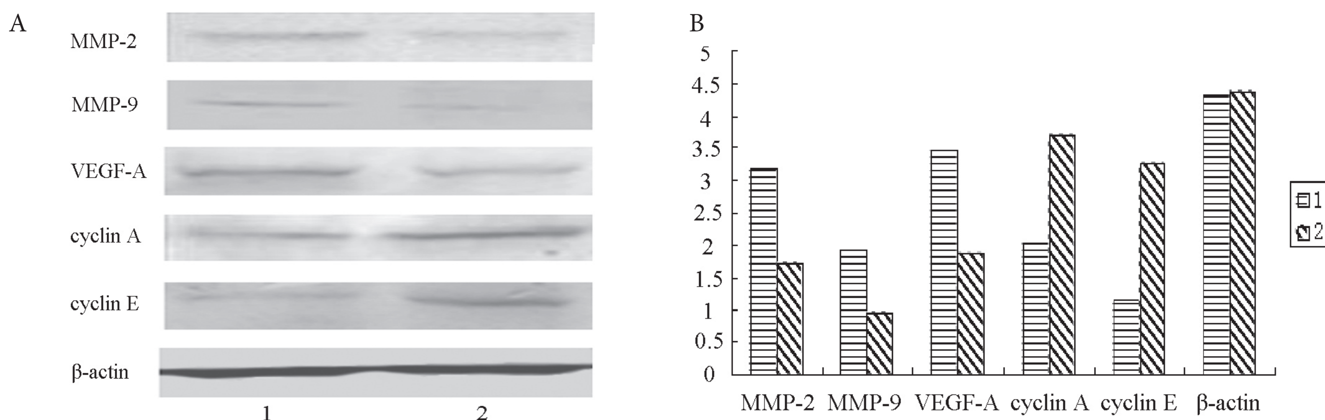

Figure 5. The protein expression of MMP-2, MMP-9, VEGF-A, cyclin A, cyclin E, and β-actin after knockdown of STAT3 by siRNA in HCC HepG2 cells.
A: 1: The control group. 2: 72 h after the transfection.
B: (X axis)1: the control group. 2: 72 hours after the transfection.
Y axis is the quantitative results of gray-scale analysis.

stress activated protein kinases(SAPK), p38MAPK, and big MAP kinase 1(BMK1)/ERK5 pathway. ERK1/2 (p44/42 MAPK) pathway is one of the most classic pathway. In our experiments, ERK1/2 (p42/p44 MAPK) protein expression after transfection showed no significant difference to levels measured before transfection; while phosphorylated (p) ERK1/2 (p-p42 MAPK) protein expression increased slightly at 72 h after transfection. The result indicated that silencing of the STAT3 gene might affect ERK1/2 pathway through the regulation of the phosphorylation. The result is different from Feng, et al (17), research where there was no significant correlation between p42/44(MAPK) and p-Stat3 in HCCs and their surrounding liver tissues, so we need to validate the result in other cell lines and animal studies.

Smad is a central factor in the TGF-β pathway. So far, Smad3 is the only proven substrate of the TGF-β receptor and is the key factor regulating TGF-β inhibitory action [18]. Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase and a major limiting factor of telomerase activation [19]. In our experiments, after the inhibition of STAT3 expression for 72 h, Smad3 protein expression increased at 72 h, while TERT protein expression was reduced at 72 h. This is similar to the previous study that the activation of STAT3 enhances telomerase activity in prostate cancer cells (6). And recent studies showed that Smad3 could successfully block telomerase activation, which is necessary for tumor cell proliferation, and that TGF-β negatively regulates telomerase activity via Smad3 interactions with c-Myc and the TERT gene promoter [20,21]. Though the relationship between STAT3 and Smad3 has been not reported, some studies demonstrated that mitogen-activated protein kinase kinase-1 (MEK1) is an important regulator of Smad3 expression [22]., Our results also indicated that STAT3 regulated the expression of MEK1, so STAT3 may affect Smad3 protein expression via the regulation of MEK1. The changes in TERT and Smad3 after transfection demonstrated that STAT3 signaling pathway might directly or indirectly affect their expression, though their expression is also regulated by many other factors.

The expression of apoptosis related factors (caspase-3, XIAP, Grp-78, and HSP-27). Apoptosis is regulated in part by caspase and inhibitor of apoptosis protein (IAP) family members. Caspase-3 plays a critical role in the process of apoptosis, and its abnormal expression has been demonstrated in many tumors. IAP family members are the only known endogenous caspase inhibitors, in which X linked inhibitor of apoptosis protein (XIAP) effect is the strongest. Abnormal expression of XIAP has been demonstrated in many tumors, including HCC (23,24) and ductal carcinomas [25]. In our experiments, after silencing STAT3, caspase-3 and XIAP protein expression was inhibited at 72 h, but p-caspase-3 protein expression increased at 72 h. XIAP is the strongest inhibitionor of caspase, so the reason for this result might be that the inhibition of XIAP expression made caspase-3 activated to p-caspase-3 at 72 h after transfection, thereby promoting cell apoptosis. Existing research indicates that Akt interacts with and phosphorylates XIAP at serine-87 in vitro and in vivo [26]. Furthermore, MAPK signaling can also increased XIAP expression [27]. Caspase 3 was activated by silencing Smad4 [28], ERK1/2 [29], and p38 MAPK [30]. These data indicated that XIAP and caspase-3 are the downstream targets of the STAT3 signal pathway and their expression is influenced by a variety of factors.

The Heat shock protein (HSP) family members are important molecular chaperones, and their functions are closely related to tumor genesis, development, and prognosis through regulation of cell proliferation, differentiation, and apoptosis. Glucose regulated protein-78 (Grp-78) is one of HSP-70 family members and HSP-27 is one of sHSP subfamily members. In our experiments, after silencing STAT3, Grp-78 protein expression increased at 72 h, while HSP-27 protein expression showed no difference following transfection. These observations indicate that the STAT3 signaling pathway regulates Grp-78 expression and does not affect the HSP-27 expression. As an ER molecular chaperone, Grp-78 protein is involved in conveying, controlling, and degrading ER-associated proteins; ER stress-adjustment; and ER calcium binding. Therefore, Grp-78 may play a variety of functions in the maintenance of cell homeostasis. And the presence of GRP-78 or a homologue in nearly every organism from bacteria to man, reflects the central roles it plays in cell survival [31]. Moreover, Grp-78 expression is affected by a number of factors. For example, glucose deprivation results in upregulation of Grp-78 in human cancer cells [32], overexpression of c-Myb can induce the endogenous Grp-78 gene [33], and Grp-78 is upregulated by endosulfan in A549 cells [34]. Thus, STAT3 signaling pathway might play a role during tumor genesis and development via direct or indirect regulation of Grp-78, but this relationship is still in need of further study. In addition, previous studies have demonstrated a specific interaction between β-catenin and HSP-27 in breast cancers [35], but our results indicated that β-catenin was not related to HSP-27. The reason may be associated with the different tumor types used in these studies.

The expression of angiogenesis factors (MMP-2, MMP-9, and VEGF-A). In our experiments, after knockdown of STAT3 in HCC $HepG_2$ cells for 72 h, the protein expression of MMP-2, MMP-9, and VEGF-A all decreased. These findings indicate that STAT3 signaling pathway can regulate the expression of these proteins and they are downstream target proteins of this pathway. MMPs promote angiogenesis and contribute to tumor infiltration and metastasis not only through degradation of the extracellular matrix and vascular basilemma, but also through the active regulation of transforming growth factor β (TGF-β), bFGF, VEGF, and other important signaling molecules. The VEGF family regulates the formation of blood vessels and lymphatic vessels, vascular permeability, and endothelial cell survival. Angiogenesis and lymphangiogenesis may both promote the tumor metastasis. These factors play important roles in tumor angiogenesis, infiltration and metastasis [36].Thus STAT3 signaling pathway contributes to HCC angiogenesis, infiltration and metastasis through regulation of the expression of MMP-2, MMP-9, and VEGF-A.

In addition, MMP-2, MMP-9, and VEGF-A protein expression can be regulated by many other factors. For example, MAPK signaling pathway inhibited the expression of VEGF, bFGF, and STAT3 in fibrosarcoma [37], and p38 MAPK signaling pathway mediated VEGF expression in bone marrow mesenchymal stem cells [38]. In prostate cancer, MMP-2 and MMP-9 expression was regulated by the androgen receptor signaling pathway and was related to the tumor invasion [39]. They together take part in the regulation of MMP-2, MMP-9, and VEGF-A protein expression, thereby form the complex network of tumor pathogenesis.

Cell growth and cycle. In our experiments, the protein expression of cyclin A and cyclin E increased and the cell cycle was arrested in G_0/G_1 phase at 72 hour after knockdown of STAT3 by siRNA. The results demonstrate that the protein expression of cyclin A and cyclin E is related to STAT3 signaling pathway and the former is the downstream target protein of the latter. STAT3 signaling pathway possibly affects cell cycle through regulating the expression of cyclin A and cyclin E. This is similar to the previous study. For example, Akt was shown to affect the cell cycle and promote the cell proliferation through regulating the P21 phosphorylation and the combination of cyclin E/cdk2 and cyclin D/cdk4, and interleukin-6 (IL-6) was reported to decrease the expression of cyclin (A, D1, D3, and E) or cdk (cdk2, 4, and cdc2 p34) through a STAT3-dependent pathway, thereby the cell cycle was arrested in G_0/G_1 phase [40]. And small interfering RNA against Stat3 significantly reduced the MIA-MSLN cell cycle progression with a concomitant decrease in cyclin E expression(2).

In our experiments, HCC cell growth was inhibited during the 24–72 h time period after knockdown of STAT3 by siRNA. The reasons probably were that in signaling pathway, telomerase, cell cycle, apoptosis, angiogenesis, and others together played roles. In other words, β-catenin may regulate signaling pathway, telomerase, apoptosis, angiogenesis, and cell cyle, sequentially affect cell growth.

In short, STAT3 signaling pathway may regulate the protein expression of other signaling pathway (Smad3 and p44/42), telomerase (TERT), apoptosis (caspase-3, XIAP, HSP-27, and Grp-78), angiogenesis (MMP-2, MMP-9, and VEGF-A), thereby participating in HCC genesis and development. And this pathway is not related to HSP27 protein expression in HCC. These factors are inter-activated and form a complicated network. Also, the regulatory mechanism remains to be fully elucidated. These data will help us better understand HCC pathogenesis and the role of the STAT3 signaling pathway during the genesis and development of HCC. And blockade of the Stat3 pathway represents a potential strategy for future treatment. Further, by understanding these processes in detail, we may someday be able to treat tumors through silencing one or several key genes together.

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