Knockdown of STAT3 Expression by RNA Interference Inhibits the Induction of Breast Tumors in Immunocompetent Mice

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Abstract
Constitutively activated STAT3 is involved in the formation of multiple types of tumors including breast cancer. We examined the effects of Stat3 protein knockdown by RNA interference using a dicistronic lentivirus small hairpin (shRNA) delivery system on the growth of mammary tumors in BALB/c mice induced by the 4T1 cell line. A single exposure of 4T1 cells to shRNA/STAT3 lentivirus transduced 75% of the cells with green fluorescent protein (GFP) within 96 hours. In cells selected for GFP expression, neither Stat3 protein nor phosphoryrosine Stat3 was detected. Tumor formation induced by injecting 4T1 cells into the mammary fat pad was blocked by expression of the shRNA for STAT3 whereas all mice injected with 4T1 cells expressing only GFP efficiently formed tumors. c-Myc expression was reduced 75% in cells expressing greatly reduced levels of Stat3 compared with the GFP control. Of interest, the level of activated Src, which is known to activate Stat3, was virtually eliminated but the level of the Src protein itself remained the same. Importantly, expression of Twist protein, a metastatic regulator, was eliminated in STAT3 knockdown cells. Invasion activity of STAT3 knockdown cells was strongly inhibited. However, the proliferation rate of cells in Stat3 knockdown cells was similar to that of the GFP control; the cell cycle was also not affected. We conclude from these studies that activated Stat3 protein plays a critical role in the induction of breast tumors induced by 4T1 cells by enhancing the expression of several important genes including c-Myc and the metastatic regulator Twist. These studies suggest that stable expression of small interfering RNA for STAT3 has potential as a therapeutic strategy for breast cancer. (Cancer Res 2005; 65(7): 2532-6)

Introduction
There is abundant evidence to show that constitutive activated signal transducer and activator of transcription 3 (Stat3) is frequently found in breast cancer samples (1). Molecular mechanism studies suggested that c-Myc is up-regulated by activated STAT3, which plays a role in the transformation of the mammary gland cells (2). Most interestingly, there is evidence that activation of Stat3 is associated with abnormal differentiation of dendritic cells in cancer as well as regulation of innate and adaptive immune responses (3, 4). These effects of activated Stat3 on immune function may allow cancer cells to evade the immune surveillance of the host. Recently, there was a report that STAT3 knockdown by RNA interference (RNAi) in human neuron tumor cells induces apoptosis (5). Although Stat3 is constitutively activated in breast cancer, it is not clear what kind of biological effects will result from the inhibition of Stat3 protein expression.

The 4T1 cell line was originally derived from a spontaneous mouse mammary carcinoma from the BALB/c strain (6). It has been reported that 4T1 cells mimic the effects of human mammary carcinoma in that morbidity is due to outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early during primary tumor growth (7). Therefore, the injection of the 4T1 cell line in BALB/c mice is an appropriate model to mimic human breast cancer with regard to tumor growth and tumor metastasis in an in vivo immunocompetent mouse model. Thus, the 4T1/BALB/c mouse system provides a useful experimental model to explore the role of STAT3 activation and its biological function in mammary tumorigenesis.

In this study, we used lentivirus infection to deliver a specially designed small interfering RNA (siRNA) for mouse Stat3 into a mouse breast cancer cell line, 4T1, to inhibit Stat3 protein expression. Therefore, we were able to analyze the effects on upstream and downstream components of the STAT3 pathway and the ability of treated cells to form breast tumors in immunocompetent BALB/c mice following injection of STAT3 knockdown 4T1 cells.

Materials and Methods

Cell line and antibodies. The 4T1 cell line was a gift from Dr. Mien-Chie Hung (M.D. Anderson Cancer Center). The cells were maintained in DMEM plus 10% fetal bovine serum. Anti-p-Tyr-STAT3 [phospho-Tyr705 (pTyr705)], anti-STAT3, anti-p-S5, anti-p-Src (pY416), anti-Src, and anti-p-Akt (pSer473) were obtained from Cell Signaling (Beverly, MA). Anti-c-Myc and anti-Twist were obtained from Santa Cruz (San Diego, CA); anti-β-actin was from Sigma Life Science (St. Louis, MO). Cell Invasion Kit was from Chemicon (Temecula, CA).

Animals. Six- to 8-week-old female BALB/c mice were purchased from Jackson Lake laboratory (Bar Harbor, ME) and maintained in our institute’s conventional animal facility.

Small hairpin RNA of mouse STAT3 lentivirus gene transfer vector construct. The STAT3 hairpin oligo (aggcaagccgatctaggcagatgccaccctgactgcagatgctgtttttttt ouraaggt) was designed by selecting appropriate sequences from the mouse STAT3 complete mRNA (Medline: 22388257); the cdNA of the small hairpin (shRNA) was inserted into our lentivirus gene transfer vector. The double stranded shRNA oligo was cloned into pLVTH lentivirus vector (a very generous gift from Dr. D. Trono) using the ClaI restriction enzyme site. The construct (Fig. 1) was verified by sequencing. Because the green fluorescent protein (GFP) sequence is encoded in the lentivirus transduction vector under the control of a separate promoter, GFP is expressed in lentivirus-infected cells as the marker for cell sorting and to indicate that the cells express the shRNA for STAT3 (Fig. 2A and 8). A control shRNA unrelated to STAT3 sequences was used as a negative control.

Preparation of lentivirus STAT3 shRNA. Lentiviruses were prepared by transfecting three plasmids into 293T cells as described (8, 9). The plasmids are pCMVΔR2 (GAG-POL DNA), the vesicular stomatitis virus (VSV-G) envelope plasmid pMD.G and gene transfer plasmid pLVTH containing the self-inactivating LTR (8).
Lentivirus siRNA gene transduction. Cells were infected by the spin inoculation method as described (10). At 72 hours after infection, the culture was monitored/sorted for GFP fluorescence by means of flow cytometry. The lentivirus encodes a dicistronic RNA (shRNA for STAT3 and GFP).

Selection of STAT3 siRNA-positive 4T1 cells. Because cells that express shRNA of STAT3 will also express GFP marker, as indicated the gene transfer vector diagram in Fig. 1. shRNA STAT3-positive 4T1 cells were selected by fluorescence-activated cell sorting using GFP as the marker. GFP-positive cells were expanded after the cell sorting.

Western blotting. Western blotting was done as described (11).

Cell proliferation assay. Both 4T1/GFP and 4T1/STAT3 shRNA/GFP cells were seeded in to 6-well plate with a concentration of 1 × 10⁵ cells per well in triplicate. Total viable cell number in each well was determined by a Vi-cell analyzer (Beckman Coulter, Miami, FL).

Cell cycle analysis. Propidium iodide staining was used to analyze DNA content using fluorescence-activated analysis.

Matrix invasion assay. The assay was done in matrix chambers as described (12).

Mouse tumor formation and metastasis assay. Various doses of cells were injected to establish levels needed for tumor formation. 7 × 10⁴ 4T1/GFP cells or 4T1/STAT3 shRNA/GFP cells were injected into mammary fat pad of 8-week-old female BALB/c mouse. Tumor formation at the site of injection and at distant tissue sites (metastasis) was monitored. Tumor metastasis was assayed as described (13).

Results

Stat3 is constitutively activated in mouse breast cancer cell line 4T1. We first examined STAT3 expression by Western blotting with anti-Tyr705/Stat3 antibody, and we found that the Stat3 protein is constitutively activated in 4T1 cells (Fig. 2C).

Knockdown of Stat3 protein by infection with replication-defective lentivirus encoding a STAT3 shRNA. After a single exposure of 4T1 cells to the lentivirus encoding shRNA of mouse STAT3 and GFP, a high percentage of the cells expressed GFP 96 hours after the infection. Cell sorting was carried out by selecting cells expressing the GFP marker (Fig. 2A and B). As shown in Fig. 2C, Stat3 protein expression was virtually eliminated from 4T1 cells after STAT3 shRNA transduction, indicating that our shRNA for mouse STAT3 can target STAT3 mRNA efficiently. As Stat3 was virtually eliminated from 4T1 cells, pTyr705-STAT3 was no longer detectable (Fig. 2C). In contrast, STAT3 expression was not affected by an unrelated shRNA lentivirus (Fig. 2D).

Inhibition of Stat3 protein expression by STAT3 shRNA down-regulates its upstream and downstream targets in 4T1 cells. We examined the upstream and downstream targets of Stat3 in 4T1 cells after STAT3 knockdown. We found that the downstream target of Stat3, c-Myc, is reduced (Fig. 2E). Quantification of c-Myc, calculated after normalization for protein loading, indicated that c-Myc protein was reduced 75%, as shown in Fig. 2F. The phosphorylation of Src, the upstream regulator of Stat3, was completely eliminated in 4T1 cells after STAT3 knockdown. However, Src protein levels remained the same, as shown in Fig. 2E. To evaluate the other possible effects of Stat3 protein reduction, we also examined the p53 level in 4T1/GFP cells and STAT3 knocked down 4T1 cells. As shown in Fig. 2E, we did not find any change in the p53 level as a result of Stat3 protein knockdown. In addition, after STAT3 knockdown in 4T1 cells, Akt phosphorylation at Ser473 was greatly reduced (Fig. 2E), indicating that activation of Akt is also interfered with by reducing the level of the functional Stat3 protein. Importantly, Twist, a regulator of metastasis (14), was not detectable in STAT3 knockdown 4T1 cells (Fig. 2E).

Proliferation of 4T1 cells is not affected by STAT3 knockdown. The effect of Stat3 protein reduction on 4T1 cell proliferation was examined. Cell proliferation was measured by counting total viable cell number after STAT3 knockdown in 4T1 cells. The results showed that there was no change in cell proliferation after the STAT3 knockdown in 4T1 cells, as shown in Fig. 2G. STAT3 knockdown also had no significant affect on the cell cycle (Fig. 2H).

The tumorigenic effects of 4T1 cells after STAT3 knockdown is blocked in an immunocompetent mouse model. We examined the ability of 4T1 cells to produce mammary tumors after STAT3 knockdown. For this purpose, we first established the mouse breast cancer model in immunocompetent mouse, BALB/c. As shown in Table 1, both 4T1 and 4T1/GFP cells can rapidly form mammary tumors after injection into the mammary fat pad in BALB/c mice without any notable rejection. No effect of GFP expression on tumor formation was observed (Table 1). We assessed tumor-forming ability of 4T1 cells in which Stat3 protein was severely reduced by STAT3 shRNA. As shown in Fig. 3A and Table 1, tumors were not observed in mice that were injected STAT3 knockdown 4T1 cells through day 73 whereas GFP controls had large tumors within 20 days. No signs of illness were noted in mice injected with 4T1 cells expressing STAT3 shRNA. In addition, GFP+ cells were not observed at the injection site in mice injected 4T1/STAT3 knockdown cells (data not shown).

STAT3 knockdown prevents metastasis. 4T1 cells produce metastasis in various tissues (6, 7). No evidence of metastatic was obtained in lung, liver, brain and peripheral blood, using the sensitive assay for 4T1 cells (13), in mice injected with 4T1 STAT3 knockdown cells (Fig. 3B and data not shown). These results are consistent with our finding that Twist protein expression was completely blocked in 4T1 STAT3 knockdown cells. In addition, 4T1 STAT3 knockdown cells were severely inhibited in their ability to invade matrix in vitro compared with GFP-transduced 4T1 cells (Fig. 3C).

Discussion

Our results indicate that transduction of shRNA cDNA of STAT3 into mouse 4T1 breast cancer cells by lentivirus infection virtually eliminated Stat3 protein expression, and completely inhibited the tyrosine phosphorylation of c-Src, the STAT3 upstream regulator. Importantly, STAT3 knockdown down-regulated the STAT3 effector molecule, c-Myc, by as much as 75% (Fig. 2E and F). We anticipated that STAT3 knockdown would down regulate c-Myc expression because there are a number of reports indicating that c-Myc is
downstream of STAT3 (2). In addition, knockdown of STAT3 also drastically inhibited Akt phosphorylation. As Akt is regulated by Src through phosphatidylinositol-3 kinase pathway (15–17), it is not unexpected that Akt activation would be down regulated in 4T1 cells after STAT3 knockdown since Src kinase activation was eliminated by STAT3 RNAi. However, we did not expect that STAT3 knockdown would have such strong negative feedback effect on its upstream regulator, Src. The mechanism for this decrease in the level of activated Src following STAT3 knockdown is unknown. As Src also regulates with Akt (15–17), the inhibition of Akt phosphorylation by STAT3 knockdown is likely the result of Src kinase inactivation.

Importantly, we showed that knockdown of STAT3 completely blocked Twist protein expression. Twist has been shown to be

Figure 2. STAT3 knockdown in mouse breast cancer cell line 4T1. GFP and shRNA cDNA for mouse STAT3 were expressed in 4T1 cells by lentivirus infection. A, flow cytometry pattern of 4T1 cells following a single exposure to shRNA/GFP lentivirus infection; the level of GFP was estimated to be expressed in 75% of the cells. B, photographs of 4T1 cells taken after cell sorting; taken under fluorescent microscope (top); same view as the top but with a light microscope (bottom). As a control, 4T1 cells were transduced with a GFP lentivirus. C, Western blotting was conducted on cell lysates with antibodies to Stat3, pTyr Stat3, and actin as a loading control. Equal amount of protein was loaded on each gel lane. D, negative control shRNA lentivirus had no effect on STAT3 expression and Stat3 tyrosine phosphorylation. E, knockdown of STAT3 in 4T1 cells: effects on its upstream regulator and downstream targets. Western blotting was conducted for the indicated proteins in either 4T1 cells expressing GFP or 4T1 cells expressing both shRNA for STAT3 and GFP. Equal amounts of protein were loaded on each gel lane. F, knockdown of STAT3 from 4T1 cell by RNAi reduced c-Myc expression. A quantitative densitometry analyses after normalization for actin expression done on Western blots from STAT3 knockdown 4T1 cells and from control was cells that were transduced with GFP only. G, cell proliferation analysis after STAT3 knockdown in 4T1 cells. Cell proliferation was analyzed after STAT3 was knockdown from 4T1 cells compared with 4T1 cells expressing only GFP. In this analysis, we used the V-Cell computerized cell analyzer. Cells (n = 2,500) were analyzed for each sample in triplicate. H, cell cycle analysis after STAT3 knockdown.
involved in the regulation of metastasis (14). The inhibition of Twist expression by STAT3 knockdown is consistent with our findings that 4T1 cells having STAT3 knockdown do not induce metastasis in mice (Fig. 3B) and also interferes with cell invasion in vitro (Fig. 3C).

Most importantly, knockdown STAT3 in 4T1 mouse breast cancer cells also eliminated the ability of the cells to induce breast tumors in immunocompetent mice (Fig. 3A and B; Table 1). In addition, to our knowledge, this is the first report showing that knockdown of STAT3 in mouse breast cancer cell line by RNAi can down regulate c-Src and Akt activity and can prevent breast tumor formation in immunocompetent mouse model.

Wang et al. (4) showed that activated Stat3 triggers the expression of several factors in cancer cells that have a potent inhibitory effect on functional dendritic cell maturation. Thus, cancer cells might be able to escape from immune system surveillance. Therefore, activated STAT3 in cancer cells does not just function as a mediator for intracellular signaling but also affects cell-cell interaction. Obviously, our findings, which show that 4T1 cells lose their ability to form mammary tumors and metastatic tumors in immunocompetent mice after STAT3 knockdown, support the above hypothesis. It would be interesting to explore further the molecular basis of the block in primary mammary and metastatic tumor formation after STAT3 knockdown, especially with regard to the inhibition of the metastatic regulator Twist. In addition, we did not observe any significant increase in apoptosis in 4T1 cells after STAT3 knockdown (Fig. 2H), whereas others reported that apoptosis occurred after STAT3 knockdown in astrocytoma cells (5), and in human breast and ovarian cancer cells treated with the Jak 2 kinase inhibitor AG490 (18). Cell context effects may explain the different results with STAT3 knockdown in brain and breast cells. With regard to the Jak2 inhibitor studies (18), one or more of the Jak kinases is an upstream regulator of Stat3 (19). In our studies, this lack of apoptotic effects after STAT3 knockdown could be because STAT3 knockdown is quite specific compared with AG490 treatment. AG490 inhibits Stat3 activity through a Jak kinase pathway (2, 3). In that situation, apoptosis of the AG490-treated

<table>
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<td>18-20</td>
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NOTE: 4T1/GFP cells were injected as the indicated dose into the mammary fat pad of immunocompetent BALB/c mice; tumors formed at the indicated time for both types of 4T1 cells. Mice formed tumors faster when injected with a higher dose of 4T1 cells. No tumor formation by STAT3 RNAi in 4T1 cells in BALB/c mice was observed. The mice remained healthy after more than 70 days following mammary fat pad injection.

*No tumors were detected at 70 days post-challenge.

Figure 3. STAT3 knockdown in 4T1 cells eliminated their tumorigenic activity. After STAT3 was knockdown by RNAi in 4T1 cells, $7 \times 10^3$ cells were injected into BALB/c mice. A, no tumors were observed in any of the mice injected with STAT3 knockdown 4T1 cells (bottom), indicating knockdown of STAT3 blocked mammary tumor formation. In contrast, mouse injected 4T1/GFP formed tumor (top and middle). B, knockdown of STAT3 prevents formation of metastatic tumors. Lung and liver tissue from each of the five mice injected with STAT3 knockdown 4T1 cells were examined for the presence of 4T1 cells using the 6-thioguanine-selection assay (13) at 85 days after challenge with 4T1 STAT3 shRNA cells. C, knockdown of STAT3 inhibits in vitro invasion activity of 4T1 cells. STAT3 knockdown of 4T1 cells in mixtures of control GFP cells ranging from 4% to 90% of STAT3 activated cells were assayed for matrix invasion (12).
cells was observed. But it is likely that AG490 inhibits other kinases besides Jak2. Therefore, the knockdown of STAT3 may provide a more specific effect on signal transduction pathways than with AG490 treatment.

To investigate any possible non-specific side effects of shRNA expression after lentivirus infection, we tested a shRNA lentivirus encoding sequences unrelated to STAT3 (Fig. 2D). No effects of this negative control RNAi were observed on STAT3 expression. p53 levels in both STAT3 knockdown and vector control 4T1 cells were similar in both cell populations. This result was unexpected in view of the finding that STAT3 participates in the down-regulation of p53 mediated by oncostatin M (20). Nevertheless, our findings that p53 levels were not affected by STAT3 knockdown in the 4T1 breast cancer cell line suggests that STAT3 shRNA expression in 4T1 cells did not cause nonspecific affects, as originally observed with siRNA expression in some cells (21).

The results presented here are to our knowledge the first evidence that elimination of Stat3 protein by RNA interference in mouse breast cancer cells can block the tumorigenicity of these cells in immunocompetent mice. This interesting result has important clinical implications. First, STAT3 is an important target for breast cancer therapy, and second, siRNA for STAT3 can be an effective reagent for breast cancer therapy.

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References


**Structure of the shRNA for Human **STAT3** and the 3-Base Mismatch with Mouse **STAT3** Sequences**

In the article on how **STAT3** knockdown blocks breast tumor formation in the April 1, 2005 issue of Cancer Research (1) the human RNAi sequence instead of the mouse sequence was mistakenly used to cause the knockdown of mouse Stat3 protein. Several places in the text mentioned the use of mouse shRNA sequences but, in fact, human shRNA sequences, whose sequence is correctly listed in the Materials and Methods section but identified as mouse **STAT3** sequences, were used in the studies. The use of the human RNAi sequence in mouse cells was very effective in reducing the level of the Stat3 protein despite a three-base mismatch between the mouse and human RNAi sequences in the 3′ portion of the RNAi sequence (Fig. 1) (1). Based on studies by others (2, 3), the matching of the 5′ sequences is likely to be critical for the efficient knockdown that observed.

Also, in the Acknowledgments, the CA93792 was mistakenly used to identify the Hendricks Foundation, which is a private Foundation grant.


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**Figure 1.** The top half of the figure shows the essential sequences used to construct the shRNA to target human **STAT3** RNA. We emphasize that the human **STAT3** shRNA sequence has a 3-base mismatch when compared to the mouse mRNA. In the bottom half of the figure, we illustrate the targeted protein sequence (ICLDR) and its relative position in the three known isoforms of the Stat3 protein.
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