Cell Cycle and Senescence

PTP1B Contributes to Calreticulin-Induced Metastatic Phenotypes in Esophageal Squamous Cell Carcinoma

Xiao-Min Wang, Li Shang, Yu Zhang, Jia-Jie Hao, Feng Shi, Wei Luo, Tong-Tong Zhang, Bo-Shi Wang, Yang Yang, Zhi-Hua Liu, Qi-Min Zhan, and Ming-Rong Wang

Abstract

Calreticulin (CRT) is a Ca$^{2+}$-binding chaperone protein that alters cellular Ca$^{2+}$-homeostasis in the endoplasmic reticulum (ER). Previously it was shown that CRT was overexpressed in esophageal squamous cell carcinoma (ESCC), and elevated CRT expression promoted the migration and invasion of ESCC cells. In the present study, the mechanisms underlying the role of CRT in esophageal carcinoma progression were investigated. Critically, depletion of CRT or protein-tyrosine phosphatase 1B (PTP1B) reduced ESCC cell migration and metastasis to the lung, whereas restoration of PTP1B protein levels rescued cell migration in CRT-silenced cells. Knockdown of CRT decreased PTP1B protein expression by reducing phosphorylation at the Y694 site of STAT5A, whereas knockdown of PTP1B reduced ERK1/2 phosphorylation at T204. Immunohistochemical analysis of CRT and PTP1B expression in ESCC patient tissues was strongly correlated. Importantly, PTP1B expression was associated with poor survival in patients with CRT overexpression. Overall, these data indicate a novel signaling pathway connecting CRT, STAT5A, PTP1B, and ERK1/2 in the regulation of ESCC cell migration.

Implications: These findings suggest that PTP1B is a downstream effector of CRT signaling, promotes tumor progression, and can potentially be used as a new drug target for ESCC. Mol Cancer Res; 11(9); 986–94. ©2013 AACR.

Introduction

Squamous cell carcinoma is the predominant esophageal cancer occurring in the Chinese population and is correlated with a poor prognosis and a 5-year survival rate of 15% to 25% (1). One reason for the poor prognosis is that esophageal squamous cell carcinoma (ESCC) already exhibits extensive local invasion and distant metastasis at the time of initial diagnosis (2). Therefore, having a better understanding of the molecular pathogenesis of ESCC is important for early diagnosis and effective treatment of the disease.

The metastatic process consists of a number of distinct steps. Tumor invasion and migration are parts of these sequential steps. A multitude of molecular changes in tumor cells could enable these cells to acquire highly motile properties that overcome cell–cell contacts and cell–extracellular matrix barriers. In a previous study, we showed that one of such molecular alterations is the overexpression of calreticulin (CRT) in ESCCs (3).

PTP1B is a unique endoplasmic reticulum luminal protein that participates in many cellular functions, including lectin-like chaperoning, Ca$^{2+}$ storage and signaling, regulation of gene expression, cell adhesion, wound healing, and autoimmunity (4). Many studies have shown that CRT plays an important role in regulating cell motility. For example, mouse embryonic fibroblasts K42 cells with CRT deficiency migrate poorly on fibronectin and laminin when compared with wild-type (WT) cells (5). The interaction of CRT with thrombospondin-1 increases cell migration (6). Kidney epithelial cells with CRT overexpression show enhanced migration and a reduction in E-cadherin expression (7). CRT has also been shown to promote the malignant phenotype and is associated with a poor prognosis in various cancers. For example, in gastric cancer, overexpression of CRT enhances cell proliferation and migration and upregulates the expression and secretion of placenta growth factor and VEGF (8). In bladder cancer, mice injected with CRT-silenced J82 cells have fewer metastatic sites in the lung and liver when compared with the control mice (9). We previously reported that a reduction in CRT expression could inhibit the invasion and migration of esophageal carcinoma cells, and the overexpression of CRT in ESCC is associated with a poor prognosis of the patients (10). However, the underlying mechanisms of CRT promoting cell motility and cancer metastasis remains unclear.

In this study, we investigated the molecular mechanisms responsible for CRT-dependent ESCC progression. We showed that reduced expression of CRT represses PTPNI gene expression through the inhibition of Stat5a transcriptional activity. We found that protein-tyrosine phosphatase...
1B (PTP1B) is a potent downstream effector of CRT in ESCC. cell motility and lung metastasis, and PTP1B expression is correlated with a poor prognosis in patients with CRT-positive ESCC. These findings establish an important role for PTP1B in CRT-dependent ESCC progression and support the idea that PTP1B is a potential therapeutic target for the treatment of ESCC.

Materials and Methods

Cell culture and tissue specimens

The human ESCC cell lines, KYSE150 and KYSE510, were generously provided by Dr. Y. Shimada (Kyoto University, Kyoto, Japan; ref. 11). Cells were cultured in RPMI-1640 medium containing 10% FBS (Invitrogen), penicillin (100 U/mL), and streptomycin (100 mg/mL).

Fresh ESCC tissues were procured from surgical resection specimens collected by the Department of Pathology in the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. All of the samples used in this study were residual specimens collected after sampling for pathologic diagnosis. None of the patients received treatment before surgery, and all of the patients signed informed consent forms from the Cancer Hospital, CAMS/PUMC for sample collection. The study was approved by the Ethics Committee/Institutional Review Board of Cancer Hospital/Institute, PUMC/CAMS (No. 12-097631).

Western blot analysis

Immunoblotting was conducted with primary antibodies against CRT (Abcam), PTP1B (Sigma), Stat5a, phosphorylated Stat5 (p-Stat5; yr694; Cell Signaling Technology), extracellular signal-regulated kinase (Erk)-1/2, phosphorylated Erk1/2 (p-Erk; Tyr204), and c-MYC (Santa Cruz Biotechnology). The signal was visualized using the super enhanced chemiluminescence detection reagent (Applygen).

Real-time PCR

Total RNA was isolated from differentially treated cells using TRIZOL reagent (Invitrogen), according to the manufacturer’s instructions and was used as a template for the reverse transcription reaction (Invitrogen). The PCR reactions were conducted using a SYBR Green PCR Master Mix (Applied Biosystems). PCR amplification and detection were conducted using a 7900 Real-Time System (Applied Biosystems). The relative mRNA expression levels of CRT, PTP1B, and Stat5a were calculated on the basis of the mean glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level.

Immunohistochemistry

ESCC samples were prepared for immunohistochemical analysis as described previously (10). The samples were incubated with anti-CRT (Abcam) or anti-PTP1B antibody (Sigma) at 4°C overnight followed by incubation with biotinylated secondary antibody (Santa) and 3,3’-diaminobenzidine (Sigma). The staining intensity was graded as followed: negative expression (−); weak expression (+); moderate expression (++); and strong expression (+++). Tumors with weak to strong immunostaining were classified as positive expression (+ to +++), whereas tumors with absent immunostaining were classified as negative expression (−).

Luciferase assay

The luciferase reporter assays were conducted according to the manufacturer’s instructions (Promega). Each sample was carried out in duplicate, and the experiment was repeated at least 3 times. The transfection efficiency was measured by cotransfection with a Renilla luciferase expression plasmid pRL-SV40 (Promega). The data were presented as the ratio of firefly luciferase activity to Renilla luciferase activity. The results were presented as the mean ± SE.

Chromatin immunoprecipitation

A chromatin immunoprecipitation assay (ChIP) was conducted using the EZ-ChIP Kit, according to the manufacturer’s instructions (Upstate Biotechnology). Chromatin samples were immune precipitated with an anti-Stat5a antibody (Cell Signaling Technology) and anti-rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology) as a negative control. Precipitated DNA was amplified by PCR using 5′-CGCCTGGGCTCCTATGAT-3′ as the forward primer and 5′-CAGGATCCGCAGCTTCCCT-3′ as the reverse primer.
and 5' -AAGCCCACTGCTCTCTAAGG-3' as the reverse primer. Non-immunoprecipitated chromatin fragments were used as an input control.

Xenograft assays in NOD/SCID mice

The research protocols involving animal studies were approved by Beijing Medical Experimental Animal Care Commission. Age-matched female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were injected with 1 × 10⁶ cells that were transduced with pGLV-shRNA CRT or pGLV-shRNA PTP1B lentivirus via the tail vein. The mice were sacrificed 8 weeks after injection and examined for lung metastases. The tissues were fixed in Bouin’s solution, embedded in paraffin, and sectioned and stained with hematoxylin and eosin (H&E).

Statistical analysis

Experimental results were analyzed using an ANOVA test. Significance for all of the tests was set at P < 0.05. The correlation between CRT and PTP1B expression levels in primary ESCC tissues was analyzed by using the Pearson correlation test. * P < 0.05.

Results

CRT regulates PTP1B mRNA and protein expression in ESCC cells

In our previous study, we showed that reduced CRT expression decreases ESCC cell motility (3). To identify the underlying mechanisms, we conducted a cDNA array analysis in CRT-siRNA KYSE150 cells using nontargeting siRNA cells as a control. Genes that have at least a 2-fold difference in their expression levels were selected for further analysis. Of these, 9 upregulated and 11 downregulated genes associated with cell motility were chosen for real-time PCR and Western blot analysis to measure their mRNA and protein expression in CRT-silenced and control cells (Supplementary Table S1). We found that the mRNA and protein levels of PTP1B were significantly lower in CRT-siRNA–transfected KYSE150 cells and CRT-siRNA–transfected KYSE510 cells (Fig. 1A and B and Supplementary Fig. S1A and S1B). Interestingly, MMP-9, a CRT target implicated in CRT-mediated signaling, also showed lower expression. This is consistent with the current data (12). To determine whether a reduction in CRT expression directly leads to PTP1B downregulation, we transfected CRT-silenced KYSE150 cells with pcDNA3.1-CRT* (CRT mutant with myc tag, active form) to evaluate the ability of CRT expression to rescue the knockdown phenotype. We found that the expression of the exogenous fusion protein CRT-myc was restored to 50% of the WT CRT levels. In addition, PTP1B mRNA expression and protein levels were recovered (Fig. 1C and D). However, knocking down PTP1B had no effect on cellular CRT expression in KYSE150 cells (Supplementary Fig. S1C). These results indicate that CRT regulates PTP1B expression in ESCC cells.

![Figure 1](image_url)

Figure 1. Downregulation of CRT expression reduces PTP1B mRNA and protein expression in ESCC cells. A, KYSE150 cells were transfected with CRT-specific siRNA (siRNA CRT) or a nontargeting siRNA control (siRNA cont) for 48 hours. Total RNA or protein from transfected cells or nontreated cells (Cont) was isolated for analysis by real-time PCR and Western blotting. Real-time PCR analysis showed that PTP1B mRNA levels were reduced significantly in cells transfected with CRT siRNA when compared with the control (P < 0.01). B, Western blot analysis showed that PTP1B protein levels were also decreased. Representative photos are shown. C, KYSE150 cells were transfected with CRT-specific lentil-shRNA or nontargeting lentil-shRNA as a control. At 48 hours after the transduction, CRT-silenced cells were transfected with pcDNA3.1-CRT-mutant (CRT*) or pcDNA3.1 control. After an additional 48 hours, real-time PCR and Western blotting were used to determine PTP1B mRNA and protein levels. Real-time PCR showed that PTP1B mRNA is increased in CRT-rescued cells when compared with CRT-silenced cells. D, Western blot analysis showed that when the CRT-myc fusion protein was overexpressed, PTP1B protein levels were restored in CRT knockdown cells (P < 0.01).
Reduction of PTP1B expression inhibits ESCC cell motility

To examine the effect of PTP1B on ESCC cell motility, KYSE150 and KYSE510 cells were infected with PTP1B siRNA for 24 hours and then seeded on a Transwell plate for an additional 24 hours. The results showed that PTP1B-silenced cells had greatly reduced migration and invasion capabilities when compared with nontargeting siRNA control cells or nontreated cells in both KYSE150 and KYSE510 cells (Fig. 2A–C and Supplementary Fig. S2A and S2B). To investigate whether the inhibition of motility in PTP1B-silenced cells was due to a decrease in cell growth, a CCK-8 assay was conducted to evaluate the rate of cell proliferation. The number of PTP1B-silenced cells that transversed the Transwell chamber was found to be reduced (Fig. 2D and Supplementary Fig. S2C). These results indicate that CRT regulates cell motility primarily through PTP1B in ESCC cells.

CRT regulates ESCC cells’ motility through PTP1B

Because our results indicate that CRT regulates PTP1B expression and a reduction in PTP1B expression inhibits ESCC cells motility, we hypothesized that CRT modulates cell metastasis through PTP1B. To test this, we transduced CRT-silenced KYSE150 cells with lentiviral PTP1B and examined their migration and invasion capabilities. The results showed that restoring PTP1B expression rescued CRT-silenced cell motility (Fig. 3A–C). Similarly, the cell proliferation rate was not altered in PTP1B-overexpressed cells 24 hours after seeding (Fig. 3D and Supplementary Fig. S3B). Furthermore, knockdown of PTP1B expression in CRT-overexpressed KYSE150 cells inhibited CRT-dependent cell motility in a cell growth-independent manner (Fig. 3E–H and Supplementary Fig. S3C). These results indicate that CRT regulates cell motility primarily through PTP1B in ESCC cells.

Reduction of CRT or PTP1B expression in ESCC cells inhibits metastasis to the lung in NOD/SCID mice

Because silencing CRT or PTP1B can inhibit ESCC cell migration and invasion in vitro, we tested the effect of reducing CRT and PTP1B expression in ESCC cells on lung metastasis in vivo. KYSE150 cells were first transduced with pGLV-CRT shRNA or pGLV-PTP1B shRNA lentivirus. After 24 hours, CRT-silenced cells were then transduced with pLVX-PTP1B to restore PTP1B expression. The expression levels of CRT and PTP1B were determined by Western blotting (Fig. 4A). Cells receiving various treatments were injected via the tail vein into 7 NOD/SCID mice per group for 8 weeks, and the animals were then sacrificed and examined for visible lung metastasis. The average number of lung metastases in mice injected with CRT shRNA or PTP1B shRNA lentivirus-infected cells was significantly lower than the number of metastases in mice injected with PTP1B-rescued cells and negative control cells, indicating that knockdown of PTP1B or CRT expression reduced ESCC cell metastasis to the lung. Restoring PTP1B expression in CRT-silenced cells rescued the ability of KYSE150 cells to metastasize to the lung (Fig. 4B and C). In addition, we found that the tumor tissues stained positively for Ki67 but normal lung tissue was negative for Ki67 expression, indicating that the cancer cells were proliferating (Supplementary Fig. S4, top). However, no F4/80 staining was detected in both tumor and normal lung tissues, indicating that the cancer inflammatory response was minimal in this mouse model.
and did not contribute to metastasis (Supplementary Fig. S4, bottom). These results suggested that a reduction in CRT or PTP1B expression can inhibit metastasis of ESCC cells to the lung in vivo.

**CRT regulates PTP1B transcription through Stat5a in ESCC cells**

To determine the mechanism by which PTP1B is regulated in cells at the transcription level, we searched...
Next, we asked whether CRT regulates Stat5a. We first confirmed that p-Stat5a (Tyr694) was decreased in CRT-silenced KYSE150 cells (Fig. 5C). We then transiently transfected KYSE150 cells with p-Stat5a-Luc (a vector encoding a luciferase reporter gene under the control of 6 × Stat5a response elements or a control vector) and treated cells with CRT-siRNA, Stat5a siRNA, or nontargeting siRNA. We observed that luciferase activity was decreased in CRT-silenced or Stat5a-silenced cells after 48 hours compared with negative siRNA control cells (Supplementary Fig. S5D). To further test whether the CRT-mediated transcriptional activity of Stat5a regulates PTP1B expression, we transfected an active form of the Stat5a-myc construct into CRT-silenced cells and analyzed the cells for CRT, Stat5a, and PTP1B expression. The results showed that expression of active Stat5a (detected using a myc antibody and a Stat5a antibody) in CRT-silenced cells restored PTP1B protein levels (Fig. 5E). We next examined whether CRT regulates Stat5a through PTP1B. A, KYSE150 cells were stably transduced with pGLV-shRNA PTP1B or CRT-shRNA (pGLV-shRNA CRT), CRT-silenced cells were infected with lenti-PTP1B or lenti-EGFP. Western blot analysis showing the protein levels of CRT and PTP1B in PTP1B-silenced, CRT-silenced, and overexpressing PTP1B CRT-silenced cells. B, following various treatments, 1 × 10⁶ cells were injected via the tail vein into NOD/SCID mice. Visible lung metastases were counted and graphed. C, the lungs were photographed after Bouin’s fixation (top) and stained with H&E. Immunohistochemistry staining of PTP1B and CRT in the lung tissues are also shown. Original magnification, ×200.
binding was significantly reduced in CRT-silenced KYSE150 cells (Fig. 5F). Taken together, these results indicate that CRT regulates PTP1B expression by modulating Stat5a transcriptional activity.

**CRT and PTP1B modulate Erk1/2 activity**

Erk1/2 has been reported to be modulated by PTP1B, and Erk1/2 activation is associated with tumor metastasis. To determine whether CRT and/or PTP1B regulate metastasis via Erk1/2 in ESCC cells, Erk1/2 expression and activity were examined in PTP1B-silenced or CRT-silenced KYSE150 cells. Although the expression levels of endogenous Erk1/2 in PTP1B-silenced cells and siRNA control cells remained similar, the phosphorylated form of Erk1/2 (p-Erk) was decreased in PTP1B-silenced cells compared with control cells (Fig. 5G). When CRT was knocked down in KYSE150 cells, both p-Erk and PTP1B were also reduced (Fig. 5H). Furthermore, restoring PTP1B expression in CRT-silenced cells rescued Erk1/2 phosphorylation (Fig. 5I). These findings suggest that CRT regulates Erk1/2 activity through PTP1B and promotes ESCC cell metastasis.
CRT and PTP1B expression are positively correlated in primary ESCC tissues

To seek clinical relevance of our findings, we analyzed the relationship between CRT and PTP1B expression in 176 ESCC tissues using immunohistochemistry. The negative to positive staining patterns were determined by their staining intensity and marked as −− to +++. The percentage of tissues staining positive for CRT and PTP1B by immunostaining was 90% and 62%, respectively. Double-positive staining of CRT and PTP1B was observed in 58%, and double-negative staining only accounted for 6% of the tumors. Representative photos are shown (Supplementary Fig. S6). Further statistical analysis with Pearson correlation test showed that there was a positive correlation between CRT and PTP1B expression in the ESCC tissues tested (P = 0.013; Table 1).

PTP1B expression was associated with a poor prognosis in CRT-positive patients

Of the 159 ESCC tissues that were CRT positive, 133 patients had complete records of prognosis. We then divided these patients into 2 groups for analysis: those positive for PTP1B expression (CRT+/PTP1B+) and those negative for PTP1B expression (CRT+/PTP1B−) with the same criteria as described above. The representative immunostaining images are shown in Supplementary Fig. S7. Of the 133 tissues positive for CRT expression, 83 (62%) were positive for PTP1B expression and 50 (38%) were negative for PTP1B expression. The expression of PTP1B in CRT-positive ESCC tissues was analyzed for their association with overall survival using Kaplan–Meier analysis and log-rank test for significance estimates. It showed that higher PTP1B expression was associated with poor overall survival (log-rank test; P = 0.042; Fig. 6).

Discussion

In this study, we investigated the underlying mechanisms of CRT-dependent ESCC progression. We showed that PTP1B, an important member of PTP family, is involved downstream of the CRT-dependent ESCC cell motility pathway. We found that CRT regulates Stat5a activity at its transcriptional level, whereas Stat5a regulates PTP1B. We showed that Erk1/2 activity is balanced by CRT and/or PTP1B and affects cancer cell invasiveness and metastasis. Thus, we identified a new signaling pathway of CRT-dependent cell motility (Supplementary Fig. S8). The cooperative function of CRT and PTP1B in cancer cell motility identified in our studies is exemplified by the finding that CRT and PTP1B coexpressed in primary ESCC tissues.

PTP1B is a non-transmembrane tyrosine phosphatase that functions as a negative regulator of insulin and leptin signaling. PTP1B protein levels have been observed to be upregulated in various human cancers such as breast and ovarian cancers (13, 14). Ablation or inhibition of PTP1B activity can abrogate the malignant properties of prostate cancer cells, whereas PTP1B overexpression promoted the growth of gastric and colon tumor cells (15–17). In our studies, we identified PTP1B as a downstream effector of the CRT pathway. We showed that CRT regulated PTP1B expression and promoted ESCC cell invasion and metastasis. More importantly, we found that the expression of CRT is positively correlated with PTP1B expression in ESCC tissues. CRT expression in ESCC tissues has been established as an indicator for ESCC progression (10). Here, we showed that in ESCC tissues expressing CRT, PTP1B expression is an indicator of a poor prognosis. Thus, PTP1B plays an important role in CRT-dependent ESCC progression and may be a potential therapeutic target for the treatment of patients with ESCC.

We identified Stat5a as a regulator of PTP1B expression under the control of CRT. Stat5a is 1 of the 6 members of the STAT family (18). STAT protein activities are regulated by phosphorylation at their specific residues through the actions of tyrosine kinases and cytokines, which result in their nuclear translocation and binding to γ-IFN activation sites (GAS) of target genes (19). The phosphorylation of Tyr-694 is important for Stat5a transcriptional activity and has been used as a biochemical indicator of Stat5a activity (20). Our previous studies indicated that CRT regulates the transcriptional activity of Stat3, another member of the STAT family. Here, we found that decreasing CRT expression can reduce Stat5a phosphorylation (Tyr694) and silencing Stat5a but

Table 1. PTP1B expression positively correlates with CRT in 176 ESCCs

<table>
<thead>
<tr>
<th>CRT</th>
<th>PTP1B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>56</td>
<td>103</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>109</td>
</tr>
</tbody>
</table>

NOTE: P < 0.01, ANOVA test.

Figure 6. Correlation of PTP1B expression with overall survival in patients with CRT-expressing tumors. A Kaplan–Meier curve showing a correlation between patients with PTP1B-negative tumors and higher survival rates.
not Stat3 modulate PTP1B mRNA and protein expression. However, CRT may not directly regulate Stat5a phosphorylation. It is possible that CRT regulates Stat5a activity through CaMK II (a Ca2+/calmodulin-dependent kinase functioning in Ca2+ signaling) because previous studies have shown that the KN93 induced downregulation of CaMK II activation is associated with a marked reduction in the phosphorylation of Stat5a (Tyr694; ref. 21). Moreover, as a calcium-binding protein, CRT deficiency could reduce free Ca2+ concentrations (Ca2+), thus leading to the inhibition of CaMK II activity. Our current studies suggest that CRT regulates PTP1B signaling by controlling the phosphorylation status of Stat5a.

The Erk MAPks are a subfamily of mitogen-activated protein kinases (MAPK). Erk1/2 are important regulators of cell migration. They are also implicated in the PTP1B signaling pathway and promote cancer cell invasion and migration. We found that the activity of Erk1/2 was positively related to cellular CRT and/or PTP1B expression levels, suggesting that PTP1B supported lung metastasis of KYSE150 cells by activating the Erk1/2 signaling pathway and supporting cancer cell invasion and progression.

In summary, our studies reveal a novel mechanism by which CRT regulates ESCC invasion and metastasis through its downstream effectors of Stat5a, PTP1B, and Erk1/2 (Supplementary Fig. S8). The expression of CRT was relevant to that of PTP1B in ESCC patient tissues, and PTP1B expression was associated with a poor prognosis in patients with ESCC with CRT-positive tumors. Thus the CRT–Stat5a–PTP1B–Erk1/2 pathway contributes to the aggressive growth and metastasis of esophageal cancer, and PTP1B might be a potential drug target for cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: X. Wang, L. Shang, B.-S. Wang, M.-R. Wang
Development of methodology: F. Shi, W. Luo, T.-T. Zhang, Y. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wang, W. Luo, B.-S. Wang, Z. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, Y. Zhang, F. Shi, B.-S. Wang
Writing, review, and/or revision of the manuscript: X. Wang, M.-R. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhang, J.-J. Hao, F. Shi, T.-T. Zhang
Study supervision: Y. Zhang, Q. Zhan, M.-R. Wang

Acknowledgments
The authors thank Dr. Nakauchi (Institute of Medical Science, University of Tokyo, Tokyo, Japan) for providing us with the pCDNsnm-STAT5A 1’-IRES-EGFP (a constitutively active Stat5a mutant) plasmid.

Grant Support
This work was supported by Chinese Hi-Tech R&D Program Grant (2012AA020206, 2012AA02A503), National Science Fund (81021061), and Doctoral Program of Higher Education of China (201011001015).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 21, 2012; revised May 28, 2013; accepted May 29, 2013; published OnlineFirst June 27, 2013.