Dominant-negative Stat5 inhibits growth and induces apoptosis in T47D-derived tumors in nude mice

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Signal transducer and activator of transcription 5 (Stat5) regulates growth, differentiation, and survival of mammary and hematopoietic cells. The role of Stat5 in breast cancer has not been established, although Stat5 is critical for some hematopoietic malignancies. In this study, we have analyzed the role of Stat5 in progression of the estrogen receptor-positive T47D human breast cancer cell line, in which Stat5b is constitutively activated. Expression of Stat5-regulated genes, such as *cyclin D1* and *bcl-xL*, was strongly suppressed in T47D cells infected with the dominant-negative Stat5 adenovirus, AdStat5aΔ740. We also determined the phenotypic effects of introduction of dominant-negative Stat5 on T47D-derived tumors in nude mice. Tumors injected with AdStat5aΔ740 showed a 60% reduction in size, which was associated with the induction of apoptosis. Our results indicate the possibility of using dominant-negative Stat5 to induce apoptosis in certain Stat5-activated breast cancers. (Cancer Sci 2004; 95: 662–665)

Signal transducer and activator of transcription 5 (Stat5) is well established as a key factor in mammary epithelial cell growth and differentiation. Prolactin receptor-mediated signal transduction through the Jak2-Stat5 pathway has been considered to be essential for proliferation and differentiation of normal mammary epithelial cells.[1, 2] Stat5 homologues, namely Stat5a and Stat5b, are stimulated by a number of cytokines and growth factors, including erythropoietin, thrombopoietin, growth hormone, epidermal growth factor, platelet-derived growth factor, and insulin, as well as many of the interleukins and colony-stimulating factors.[3] Stat5 is considered a survival factor for hematopoietic cells and has been implicated in the progression of leukemias. Constitutively activated Stats, in particular Stat3 and Stat5, have been demonstrated to contribute directly to oncogenesis by stimulating cell proliferation and preventing apoptosis in various cancers. Moreover, the process of activated Stat signaling in human tumors may offer promising molecular targets for therapeutic intervention.[4, 5] We have previously indicated that Stat5b is constitutively activated in human breast cancer cells, and have analyzed the role of Stat5 in progression of estrogen receptor-positive breast cancer using a dominant-negative variant of Stat5, Stat5aΔ740.[6, 7] Our previous study showed that Stat5aΔ740 completely blocked transcriptional activity of endogenous estrogen receptors and that introduction of dominant-negative Stat5 by an adenovirus-based system induced apoptosis via the caspase-3 mediated pathway in T47D cells. Based on this encouraging prior *in vitro* data, we have extended our studies to examine the effect of dominant-negative Stat5 on T47D-derived tumor growth *in vivo*. The present study demonstrates that overexpression of dominant-negative Stat5 inhibits growth of T47D-derived tumors in nude mice via the induction of apoptosis.

Materials and Methods

Cell lines and recombinant adenoviruses. T47D cells (ATCC, USA) were grown in RPMI-1640 containing 10% FCS, 2 m*M* L-glutamine and penicillin-streptomycin (50 IU/ml and 50 mg/ml, respectively), at 37°C with 5% CO_2. The replication-defective recombinant adenovirus carrying Stat5aΔ740, AdStat5aΔ740, was generated as previously described.[7] Control adenovirus (Adcontrol), an adenovirus vector d1312 containing no insert[8] and AdenoLacZ[7] were also used. Infection with recombinant adenovirus *in vitro* was accomplished by exposing cells to virus in serum-free RPMI-1640 medium for 1.5 h followed by addition of medium with 10% FCS.

Semi-quantitative RT-PCR. Total RNA was extracted using an Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and RT-PCR was performed as described.[7] Primers for PCR were as follows: cyclin D1, 5′ sense GGATGCTGGAGGTCTGCGA and 3′ antisense AGAGGCCACGAACATGCAAG (146 bp); bcl-xL, 5′ sense GAACAATGCAGCAGCCGAG and 3′ antisense GTAGAGTGGATGGTCAGTGT (140 bp); p21, 5′ sense CTGGAGACTCTCAGGGTCGAA and 3′ antisense CAGGACTGCAGGCTTCCTGT (122 bp). PCR conditions were determined from the cycle curve. Each product was run on a 2% agarose gel, and the bands were visualized by means of ethidium bromide staining. As an internal control, a fragment of human GAPDH was amplified from parallel samples by PCR using the following primers: 5′ sense AAATCAAGTGGGGCGATGCTG and 3′ antisense GCAGAGATGATGACCCTTTTG (118 bp). The intensity of each band was quantified with NIH Image.

Growth studies of T47D-derived solid tumors in nude mice. T47D cells (1×10^7 cells) in 0.1 ml of 50% Matrigel (BD Biosciences, Bedford, MA) plus 50% unsupplemented RPMI-1640 were injected subcutaneously into 8-week-old, female, athymic nude mice (BALB/cAnNCrj-nu/nu, Charles River Japan). Ten days after injection, tumors had developed and the mice were divided into 3 groups (8 tumors per group), each of which was injected with mock preparation, Adcontrol, or AdStat5aΔ740. Doses of 2.5×10^6 plaque-forming units (pfu) were injected into the tumors on 3 successive days. The sizes of the tumors in three dimensions were measured 7 days after injection, followed by excision of the tumors. Tumor size (mm^3) was calculated using the formula: (3.14×length×width×depth)/6, with statistical significance being evaluated by using Student's paired *t* test.

TUNEL assay and immunohistochemistry. Four-micrometer sections of T47D-derived tumors, previously embedded in paraffin blocks, were first stained with hematoxylin and eosin (H&E), followed by processing of serial sections for terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) for detection of apoptosis and immunohistochemistry for cleaved caspase-3, Ki67, cyclin D1, bcl-x, and p21. Internucleosomal DNA cleavage characteristic of apoptotic cells was detected by TUNEL (In Situ Cell Death Detection kit, Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. For immunohistochemical staining, primary antibodies used included a polyclonal rabbit anti-human

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caspase-3 antibody that detects only activated caspase-3 (Cleaved Caspase-3, Asp175; Cell Signaling, Beverly, MA) at 1:50 dilution, a polyclonal rabbit anti-human Ki67 antibody (Ab-3; Neo Markers, Fremont, CA) at 1:50 dilution, a polyclonal rabbit anti-human cyclin D1 antibody (Ab-4; Neo Markers) at 1:100 dilution, a polyclonal rabbit anti-human bcl-x antibody (DAKO, Glostrup, Denmark) at 1:100 dilution, and a polyclonal rabbit anti-human p21 antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution. The DAKO Envision system (DAKO EnVision+, Peroxidase, Rabbit) was used for detection of immune complexes.

Results

Expression of Stat5-regulated genes in AdStat5aΔ740-infected T47D cells *in vitro*. Expression of *cyclin D1*, *bcl-xL*, and *p21*, which are reported to be Stat5 target genes, was analyzed in AdStat5aΔ740-infected T47D cells. In our previous study, we demonstrated that Stat5b was constitutively activated in T47D cells, whereas Stat5a was expressed but not detectably phosphorylated on tyrosine, and that human Stat5aΔ741 and human Stat5bΔ746, which are naturally occurring dominant-negative variants, were expressed at low levels in these cells. Moreover, activated caspase-3 was observed 24 h after AdStat5aΔ740 treatment at multiplicity of infection (MOI) 10 in T47D cells and was markedly enhanced 30 h after infection. In addition, TUNEL staining of fragmented DNA verified an increased number of apoptotic cells 96 h after infection with AdStat5aΔ740.[7] We, therefore, examined whether expression of Stat5-regulated genes was suppressed by overexpression of dominant-negative Stat5. T47D cells were either mock-infected or infected with Adcontrol or AdStat5aΔ740 at MOI 10 and cultured in medium containing 10% FCS. Total RNA was extracted 24 h, 48 h, and 72 h after infection and RT-PCR was performed. Expression levels of cyclin D1 and bcl-xL were gradually diminished in AdStat5aΔ740-infected cells, whereas infection with either mock or Adcontrol preparations had little effect on the expression levels of these genes (Fig. 1, top and second panels, lanes 7–9 vs. lanes 1–6). On the other hand, overexpression of Stat5aΔ740 led to a significant increase in the expression levels of p21 (Fig. 1, third panel, lanes 7–9 vs. lanes 1–6). These results demonstrated that adenovirus-based introduction of Stat5aΔ740 affected expression of cyclin D1, bcl-xL, and p21, as well as inducing apoptosis, in T47D cells.

Adenoviral infectivity of T47D-derived tumors *in vivo*. To establish the adenoviral infectivity of our direct intratumoral approach in T47D-derived tumors, 2.5×10^6 pfu of AdenoLacZ was injected into the tumors on 3 successive days. Expression of β-galactosidase protein was detected 2 days later by X-gal staining of frozen tissue sections of the removed tumors. Approximately 20% of the T47D cells within the tumors were positively stained (data not shown). Thus, adenoviral gene delivery into T47D-derived tumors is feasible.

Dominant-negative Stat5 adenoviral treatment inhibits growth of T47D-derived tumors in nude mice. Based on the findings that dominant-negative Stat5 inhibits cell growth and induces apoptosis *in vitro*, we extended our study to analyze the effect of dominant-negative Stat5 on T47D-derived tumors in nude mice. Tumors were treated by injections of 2.5×10^6 pfu of either mock, Adcontrol, or AdStat5aΔ740 on 3 successive days. Injection of AdStat5aΔ740 induced a 60% reduction in tumor size 7 days after treatment (P=0.0009), whereas injection of either mock or Adcontrol did not suppress growth of T47D-derived tumors (Fig. 2). We concluded from this analysis that overexpression of dominant-negative Stat5 inhibited growth of T47D-derived tumors *in vivo*.

Dominant-negative Stat5 induces apoptosis in T47D-derived tumors *in vivo*. To determine whether infection with dominant-negative Stat5 adenovirus induced apoptosis *in vivo*, apoptotic cells in tumors were detected by TUNEL assay, as well as immunohistochemistry for cleaved caspase-3. H&E staining of tissues revealed a marked decrease in cell number and tumor size in the AdStat5aΔ740-injected tumors (Fig. 3, top panels). TUNEL-based DNA fragmentation analysis showed an increased number of apoptotic cells in tumors injected with AdStat5aΔ740, while few cells were positively stained by TUNEL in either mock- or Adcontrol-injected tumors (Fig. 3, second panels). Cleaved caspase-3 was strongly expressed in 2–5% of T47D cells within AdStat5aΔ740-injected tumors, but was not observed in either mock- or Adcontrol-injected tumors (Fig. 3, third panels). Immunohistochemical staining for Ki67 verified the decreased number of positive cells in tumors injected with AdStat5aΔ740 compared with those in tumors injected with either mock or Adcontrol preparations (Fig. 3, fourth panels). Expression of cyclin D1 and bcl-x was diminished in AdStat5aΔ740-injected tumors. Most cell nuclei were strongly stained for cyclin D1 expression in mock- or Adcontrol-injected tumors, whereas only slight expression of cyclin D1 was observed in AdStat5aΔ740-injected tumors (Fig. 3, fifth panels). Similarly, bcl-x expression in the cytoplasm was markedly decreased in AdStat5aΔ740-injected tumors (Fig. 3, sixth panels). The anti-bcl-x antibody used in this study recognizes both bcl-xL and bcl-xS proteins. Since little expression of bcl-xS is observed in T47D cells, the positive staining detected

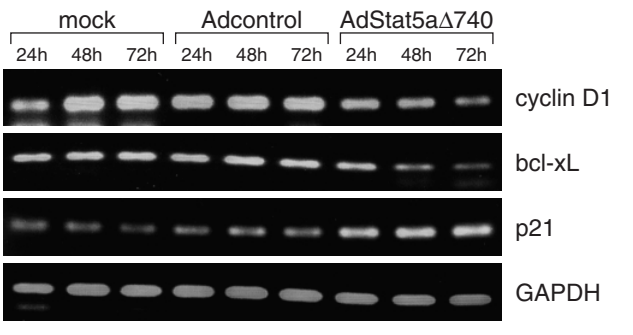

Fig. 1. Expression of Stat5-regulated genes in AdStat5aΔ740-infected T47D cells. T47D cells were plated on 6-well plates, uninfected (mock) or infected with Adcontrol or AdStat5aΔ740 at MOI 10, and incubated in RPMI-1640 containing 10% FCS for 1–3 days. Total RNA was extracted and semi-quantitative RT-PCR was performed for detection of cyclin D1, bcl-xL, p21, and GAPDH mRNA transcripts. Representative results from one of three independent experiments are shown.

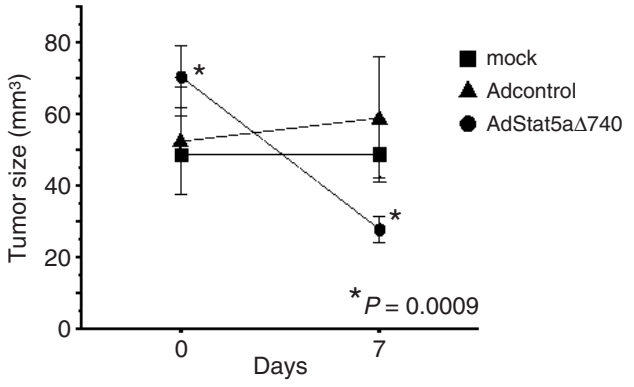

Fig. 2. Dominant-negative Stat5 adenoviral treatment inhibits growth of T47D-derived tumors in nude mice. Adenoviral vectors were injected into tumors on 3 successive days. The sizes of the tumors were measured 7 days after the first injection, and the tumor volume was calculated (bars, SE; n=8).

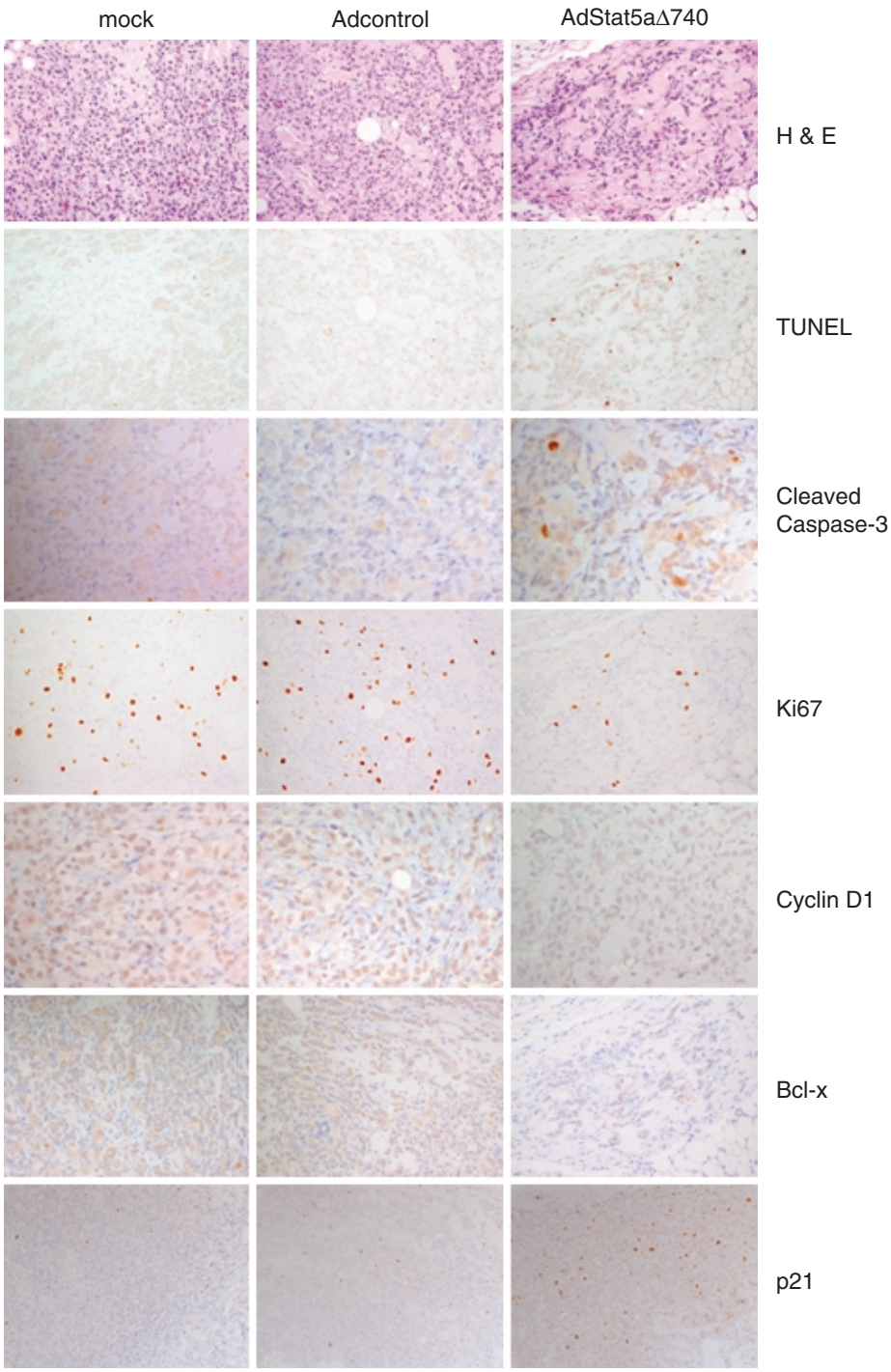

Fig. 3. Apoptotic effect of adenovirus-based introduction of dominant-negative Stat5 into T47D-derived tumor xenografts. T47D-derived tumors were removed 7 days after injection of adenoviral vectors. H&E staining, TUNEL, and immunohistochemistry for cleaved caspase-3, Ki67, cyclin D1, bcl-x, and p21 were performed. Representative results from one of 8 removed tumors are shown (magnification, 200×).

by immunohistochemistry for bcl-x is considered to indicate the expression of bcl-xL. In contrast, expression of p21 in the nuclei was markedly increased in AdStat5aΔ740-injected tumors, while only a few cells were stained for p21 in mock- or Adcontrol-injected tumors (Fig. 3, bottom panels). We concluded from these analyses that dominant-negative Stat5 induced apoptosis in T47D-derived tumors in nude mice via a caspase-3 mediated pathway and that expression of cyclin D1 and bcl-xL was suppressed and p21 expression was increased by overexpression of dominant-negative Stat5.

Discussion

The present study demonstrates that overexpression of dominant-negative Stat5 induces apoptosis in T47D-derived tumors in nude mice via a caspase-3 mediated pathway. Expression of cyclin D1 and bcl-xL was markedly reduced and p21 expression was increased in the tumor cells *in vitro* and *in vivo* when

dominant-negative Stat5 was overexpressed. Our results indicate the feasibility of utilizing dominant-negative Stat5 gene therapy to induce apoptosis in certain Stat5-activated breast cancers.

Constitutively activated Stats have been demonstrated to contribute directly to oncogenesis by stimulating cell proliferation and preventing apoptosis in various cancers.[4, 5] Constitutive activation of Stat5 is found in a variety of hematopoietic malignancies, including chronic myelogenous leukemia, via the persistent activity of Bcr-Abl. Moreover, we have previously shown that Stat5b is constitutively activated in human breast cancer cell lines, such as T47D and MCF7.[7] Stat5 participates in oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators, including bcl-xL, cyclin D1, and p21.[5, 9]

The cyclin-dependent kinase inhibitor p21 was reported as a Stat5-regulated gene in the p53-deficient CMK human megakaryoblastic leukemia cell line. It was suggested that induction of p21 through Stat5 could be involved in thrombopoietin-induced megakaryocytic differentiation.[10] p21 is induced by both p53-dependent and p53-independent mechanisms following stress, and induction of p21 may cause cell cycle arrest.[11] T47D cells express mutant p53 protein as a result of a missense mutation of codon 194.[12] Our *in vitro* and *in vivo* data showed that overexpression of dominant-negative Stat5 resulted in marked expression of p21 in T47D cells, while p21 was weakly expressed in untreated cells. Further study is needed to identify the mechanism whereby dominant-negative Stat5 induces p21 levels.

Studies have shown that the antiapoptotic factor bcl-xL is a downstream target of Stat5.[13, 14] Cyclin D1 has been previously demonstrated to be also regulated by Stat5,[15] as well as by the estrogen receptor.[16] In addition, cyclin D1 is critical for lobuloalveolar proliferation in the mammary gland during pregnancy, and prolactin is suggested to activate the cyclin D1 promoter via the Jak2-Stat5 pathway in breast cancer cells, along with normal mammary epithelial cells.[17] This study has shown that adenovirus-based introduction of dominant-negative Stat5 markedly reduced the expression levels of bcl-xL and cyclin D1 both *in vitro* and *in vivo*, and inhibited growth of T47D cells. Moreover, our results provide evidence that dominant-negative Stat5 induces apoptosis in T47D-derived tumors *in vivo*. We previously reported that Stat5aΔ740 completely blocked transcriptional activity of estrogen receptors, as well as that of Stat5, in T47D cells, and that dominant-negative Stat5 induced apoptosis in T47D cells through an effect that might be due to combined inhibition of both Stat5 and estrogen receptor signaling pathways *in vitro*.[7] Our data on both *in vitro* and *in vivo* levels suggest that bcl-xL, cyclin D1, and p21 have important roles in the progression of breast cancer in which Stat5 is constitutively activated. Identifying the mechanisms of selective apoptosis induction by dominant-negative Stat5 is important in understanding the role of Stat5 in estrogen receptor-positive breast cancer.

In conclusion, we demonstrate here that overexpression of dominant-negative Stat5 inhibits growth of T47D-derived tumors in nude mice via the induction of apoptosis. Dominant-negative Stat5 gene therapy may be used to induce apoptosis in Stat5-activated, estrogen receptor-positive breast cancer.

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1. Hennighausen L, Robinson GW, Wagner KU, Liu W. Prolactin signaling in mammary gland development. *J Biol Chem* 1997; **272**: 7567–9.
2. Watson CJ. Stat transcription factors in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia* 2001; **6**: 115–27.
3. Grimley PM, Dong F, Rui H. Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. *Cytokine Growth Factor Rev* 1999; **10**: 131–57.
4. Bromberg J. Stat proteins and oncogenesis. *J Clin Invest* 2002; **109**: 1139–42.
5. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002; **8**: 945–54.
6. Yamashita H, Iwase H. The role of Stat5 in estrogen receptor-positive breast cancer. *Breast Cancer* 2002; **9**: 312–8.
7. Yamashita H, Iwase H, Toyama T, Fujii Y. Naturally occurring dominant-negative Stat5 suppresses transcriptional activity of estrogen receptors and induces apoptosis in T47D breast cancer cells. *Oncogene* 2003; **22**: 1638–52.
8. Jones N, Shenk T. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 1979; **17**: 683–9.
9. Battle TE, Frank DA. The role of STATs in apoptosis. *Curr Mol Med* 2002; **2**: 381–92.
10. Matsumura I, Ishikawa J, Nakajima K, Oritani K, Tomiyama Y, Miyagawa J, Kato T, Miyazaki H, Matsuzawa Y, Kanakura Y. Thrombopoietin-induced differentiation of a human megakaryoblastic leukemia cell line, CMK, involves transcriptional activation of p21(WAF1/Cip1) by STAT5. *Mol Cell Biol* 1997; **17**: 2933–43.
11. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther* 2002; **1**: 639–49.
12. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P *et al*. Mutations in the p53 gene occur in diverse human tumour types. *Nature* 1989; **342**: 705–8.
13. Gesbert F, Griffin JD. Bcr/Abl activates transcription of the Bcl-X gene through STAT5. *Blood* 2000; **96**: 2269–76.
14. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a$^{-/-}$5b$^{-/-}$mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 1999; **98**: 181–91.
15. Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, Downward J, Pestell RG, Kanakura Y. Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J* 1999; **18**: 1367–77.
16. Castro-Rivera E, Samudio I, Safe S. Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *J Biol Chem* 2001; **276**: 30853–61.
17. Brockman JL, Schroeder MD, Schuler LA. PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Mol Endocrinol* 2002; **16**: 774–84.