Critical Role of STAT5 Activation in Transformation Mediated by ZNF198-FGFR1*

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The 8p11 myeloproliferative syndrome is an aggressive disorder caused by FGFR1 fusion proteins resulting from a subset of acquired translocations that target chromosome band 8p11. These chimeric proteins have constitutive FGFR1 tyrosine kinase activity and are believed to deregulate hemopoietic development in a manner analogous to BCR-ABL in chronic myeloid leukemia. Here we have studied the role of STAT proteins in transformation mediated by the most common of these fusions, ZNF198-FGFR1. We found that STATs 1, 3, and 5 were activated constitutively in ZNF198-FGFR1-transformed Ba/F3 cells and that STATs 2, 4, and 6 were also tyrosine-phosphorylated. Induction of dominant negative STAT mutants showed that activation of STAT5, but not STATs 1 or 3, was essential for the anti-apoptotic effect of ZNF198-FGFR1 and that STAT5 activation is essential for the elevated levels of Bcixl in transformed cells. STAT5 activation was also shown to be required for continued cell cycle progression of Ba/F3/ZNF198-FGFR1 cells in conditions of cytokine deprivation and for up-regulation of the DNA repair protein Rad51. These findings suggest a critical role of STAT5 activation in transformation mediated by ZNF198-FGFR1.

Chromosomal translocations that disrupt FGFR1 are associated with the disease known as the 8p11 myeloproliferative syndrome (EMS),† a stem cell disorder associated with eosinophilia, lymphadenopathy, and rapid progression to acute leukemia (1, 2). The most common translocation in EMS is the t(8, 13), which results in the fusion of the N-terminal half of ZNF198 to the entire catalytic domain of FGFR1 (3–6). ZNF198-FGFR1 is a constitutively active tyrosine kinase that transforms growth-factor dependent cell lines to factor independence (7–9). Several other translocations have been identified that fuse different partner genes to FGFR1 (1); notable among these is BCR-FGFR1, which is associated with a disease that more closely resembles BCR-ABL-positive chronic myeloid leukemia than EMS (8, 10).

Definition of signal transduction pathways that are required for transformation by fusion tyrosine kinases is critical to the development of novel targeted therapies. Fusion tyrosine kinases share the ability to activate members of the STAT family of latent cytoplasmic transcription factors, and it has been suggested that STAT activation is a critical event in transformation mediated by oncogenic proteins such as BCR-ABL, TEL-JAK2, and TEL-PDGFβR (11–17). Using murine models, STAT5 has been shown to be essential for hematological disease induced by TEL-JAK (18) but not BCR-ABL (19), indicating that different fusion tyrosine kinases have distinct modes of action. STAT5 is activated by a wide variety of cytokines (20, 21) and has, for example, been shown to be essential for fetal red blood cell production by rescuing committed progenitors from apoptosis, at least in part by direct induction of Bcixl (22).

Constitutive activation of STAT3 also occurs in many cancers (23, 24). STAT3 is normally activated by diverse ligands, including epidermal growth factor, platelet-derived growth factor, IL-6, oncostatin M, and leukemia inhibitory factor; a role for STAT3 activation in suppression of apoptosis has also been suggested (25–27). In contrast, activation of STAT1 appears to be associated with negative regulation of cell growth, e.g. STAT1-mediated down-regulation of chondrocyte proliferation in forms of dwarfism that result from activating mutations in FGFR3 (28).

In this study we have investigated the importance of STAT activation in transformation mediated by the ZNF198-FGFR1. We have also characterized the relative transforming properties of ZNF198-FGFR1, BCR-ABL, and BCR-FGFR1.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine IL-3-dependent pro-lymphoid cell line Ba/F3 was maintained in RPMI 1640 supplemented with glutamine, penicillin, streptomycin, 10% fetal calf serum, and 10% WEHI-3B-conditioned media as a source of IL-3.

Transfections—Exponentially growing Ba/F3 cells were washed and resuspended in phosphate-buffered saline. For each transfection, 4 × 10⁶ cells were mixed with 16 μg of plasmid DNA and subjected to electroporation using a Bio-Rad gene pulser set at 250 V and 960 μF. After transfection, cells were cultured for 48 h before selection with either G418 1 mg/ml or hygromycin at 0.5 mg/ml. Individual clones were obtained by selection in limiting dilution in 96-well plates as described (29).

Plasmids—The ZNF198-FGFR1 and BCR-FGFR1 constructs in pCDNA3.1 have been described previously (8, 29). The coding sequence for human FGFR1 was similarly subcloned into pCDNA3.1. The plasmids containing pCMV-LacI, HA-tagged dominant negative (dn) STAT1 (Y701F), HA-dnSTAT3 (Y705F), and HA-dnSTAT5 (Y694F) in the vector POPRSV1 (30) were a kind gift from Drs. Y. Kanakura (Osaka University Medical School, Osaka, Japan) and H. Wakao (Helix Research Institute, Chiba, Japan) (30).

Measurement of Factor-independent Survival and Proliferation—Factor-independent growth was analyzed immediately after establishment of growth.
ment of G418-resistant clones in 96-well plates. Clones were replated into 24-well plates in the absence of IL-3 to determine the percentage of clones that survived without this growth factor. Surviving clones were replated into 12-well plates to determine the percentage of clones that proliferated without IL-3. FGFR1-expressing clones were maintained in media without IL-3 with the addition of acidic human fibroblast growth factor (First Link, West Midlands, UK) at 10 ng/ml and heparin (Sigma) at 2 mg/ml.

**Induction of Dominant Negative STATs**—To express inducible dn STATs, the Lac Switch II inducible expression system was used (Stratagene, La Jolla, CA). Ba/F3 cells and IL-3-independent ZNF198-FGFR1/BaF3 clones were each co-transfected with the Lac repressor pCMV-LacI and either HA-dnSTAT1, HA-dnSTAT3, or HA-dnSTAT5. Clones were selected by culture with hygromycin at limiting dilution. The POPRSV1 expression vector contains the Rous sarcoma virus promoter linked to the Escherichia coli lactose operon, and expression of target DNA is suppressed by the lactose repressor. Addition of isopropl-β-D-thiogalactoside at 0.5 mM to the culture media causes the release of Lac-R from the lactose operon and transcription of target DNA (30).

**Apoptosis and Cell Cycle Assays**—The percentage of dead cells was measured by Trypan blue exclusion, and the percentage of apoptotic cells was measured by analysis of the DNA content by flow cytometry. Apoptotic cells were defined as those with less than 2n DNA content. Cell cycle distribution was analyzed by the same procedure. After specific culture conditions (growth in the absence of exogenous IL-3 and/or in 0.5% fetal calf serum), 2 x 10⁶ cells were fixed in 70% ethanol for 30 min and then incubated in 1 ml of phosphate-buffered saline containing 1 mg of DNase-free RNase (Sigma) and propidium iodide (Sigma) at 50 μg/ml for 30 min. DNA content was then analyzed by flow cytometry (FACscan; BD Biosciences).

**Western Analysis**—Cells were solubilized in lysis buffer (20 mM Tris, pH 8, 1% Triton, 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, and protease inhibitors 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, plus phosphatase inhibitors 10 mM β-glycerophosphate and 1 mM sodium orthovanadate). Lysates were clarified by centrifugation and an aliquot removed for protein quantitation by the DC protein assay (Bio-Rad). 70 μg of total protein was boiled in 2× loading buffer (62 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 10 mM dithiothreitol, 0.01% bromphenol blue) and fractionated on SDS-PAGE gels before transfer to polyvinylidene difluoride membrane (Immobilon; Sigma). Membranes were probed with the following antibodies: STATs 1, 2, 3, 4, 5, and 6 (Santa Cruz Biotechnology), antiphosphotyrosine 4G10 (Upstate Biotechnology), Bcl2, and BclXL (BD Biosciences), Mcl-1 (Santa Cruz Biotechnology), Rad51 (Upstate Biotechnology), Mek (Santa Cruz Biotechnology), HA high affinity antibody 1–867-423 (Roche Applied Science). The bond antibody was detected using enhanced chemiluminescence (Amersham Biosciences).

For immunoprecipitations, total cell lysates from 2 x 10⁶ cells were mixed with 2 μl of RIPA buffer (10 mM Tris, pH 8, 150 mM NaCl, 1% Triton, 0.1% SDS) plus 2 μg of antibody at 4 °C overnight, followed by binding to agarose AG beads (Santa Cruz Biotechnology) at 4 °C for 2 h. Immunoprecipitates were washed three times in RIPA buffer plus protease and phosphatase inhibitors and then boiled in 2× loading buffer.

**Electrophoretic Mobility Shift Assay (EMSA) Analysis**—Nuclear extracts were prepared by suspending cell pellets in 500 μl of hypotonic buffer (20 mM Hepes, pH 7.9, 0.2% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol) plus protease and phosphatase inhibitors as above. After a 10-min incubation on ice, nuclei were recovered by centrifugation at 1000 x g for 5 min. Nuclei were then extracted with gentle rocking on ice in 150 μl of high salt buffer (20 mM Hepes, pH 7.8, 0.2% Nonidet P-40, 0.4 mM NaCl, 13.3% glycerol, 1 mM dithiothreitol) plus protease and phosphatase inhibitors. Lysates were then clarified by centrifugation at 1300 x g for 5 min, and the supernatant was recovered as the nuclear extract. The extracts were rapidly frozen at ~70 °C. An aliquot was taken and used to determine the protein concentration.

Nuclear extracts (10 μg for each reaction) were incubated with 5 femtomoles of 32P end-labeled probe containing a specific STAT binding sequence in 4% Ficoll, 0.5 μg/ml bovine serum albumin, 20 mM Hepes, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM spermidine, 50 μg/ml poly(dI-dC) for 30 min on ice. For supershift analysis the cell extract in binding buffer was incubated with specific antibody on ice for 30 min before addition of labeled probe. For specific competition analysis, nuclear extracts were preincubated with 500 femtols of unlabeled probe for 30 min prior to incubation with labeled probe. Samples were separated on a non-denaturing 5% gel in 0.2× TBE (1× TBE is 50 mM Tris borate, 1 mM EDTA, 0.5 mM EDTA). Gels were dried and visualized by autoradiography.

For analysis of STAT5 binding activity, the ~105 probe from the β-casein promoter was used (sense sequence 5′-AGATTCTAGGAGATTTCCGGTAATGTTTAGCTAACCTTTGTTACCAT-3′) (31). For analysis of STAT1 and STAT3 binding the SIE m67 probe derived from the c-fos promoter was used (sense sequence 5′-CATTCCCGTAATGTTTAGCTAACCTTTGTTACCAT-3′) (32). For analysis of binding to the BCLXL promoter, a region derived from the mouse BCLXL gene was used (sense sequence 5′-TTTGGAGGGCAACATTTCCGAATAA-3′) (33). All oligos were annealed to their corresponding antisense sequences prior to analysis.

**RESULTS**

STATs 1, 2, 3, 4, 5, and 6 Are Activated by ZNF198-FGFR1—We have previously shown that STATs 1 and 5 are constitutively phosphorylated in cycling Ba/F3 cells transformed to IL-3 independence by the ZNF198-FGFR1 fusion (29). To further characterize STAT activation by ZNF198-FGFR1, we investigated the tyrosine phosphorylation of all six STAT proteins after 24 h of IL-3 withdrawal in three transformed clones. STAT activation was compared with parental Ba/F3 cells treated in the same way. Cell lysates were immunoprecipitated with a specific STAT antibody; half the sample was probed with the same STAT antibody to check for loading and expression levels, and the other half was probed with 4G10 (antiphosphotyrosine antibody). In cells transformed by the fusion, but not in control Ba/F3 cells, tyrosine phosphorylation of each STAT protein was detected (Fig. 1).

**Constitutive Binding of STATs 1, 3, and 5 to Cognate DNA Sequences Occurs in Ba/F3 Cells Transformed by the ZNF198-
FGFR1 Fusion—Activation of STATs 1, 3, and 5 has been associated with transformation induced by BCR-ABL (12, 13), and therefore we focused on the activity of these proteins. To determine whether STAT 1, 3, or 5 tyrosine phosphorylation induced functional activation of DNA binding, we carried out EMSAs. Specific activation of STAT1 and STAT3 was achieved by using the high affinity m67sie sequence derived from the c-fos promoter. Three different complexes, which correspond to STAT1 homodimers, STAT1/3 heterodimers, and STAT 3 homodimers, respectively (32), have been characterized to form with this probe. Nuclear extracts from parental Ba/F3 cells that had been grown continuously in the presence of IL-3 or deprived of IL-3 for 24 h were compared with ZNF198-FGFR1-transformed cells that had been treated in the same way. We found a very low level of STAT1 or STAT3 binding in extracts from parental Ba/F3 cells but strong binding in transformed cells. Competition with excess cold m67sie probe confirmed that the complexes observed are specific. The top two complexes were confirmed to contain STAT3 by supershifts induced by STAT3 antibody. Addition of STAT1 antibody induced a reduction in the complexes with STAT1 homo- and heterodimers. (Fig. 2A). Thus, tyrosine phosphorylation of STATs 1 and 3 in fusion-transformed cells confers DNA binding activity.

STAT5 binding activity was analyzed in a similar way using the β-casein probe. As shown on Fig. 2B, STAT5 binding was very weak in Ba/F3 cells but strong in transformed cells. Supershifting of the complex with STAT5 antibody confirmed that the complexes did indeed contain STAT5, and competition with excess unlabeled probe confirmed that the binding was specific. This experiment confirms activation of STAT5 by ZNF198-FGFR1.

ZNF198-FGFR1 Expression Blocks Apoptosis Following IL-3 or Serum Deprivation—To assess the ability of ZNF198-FGFR1 expression to block apoptosis induced by cytokine deprivation, we took three G418-resistant clones (C1-C3) and analyzed the percentage of apoptotic cells under different conditions. ZNF198-FGFR1-transformed cells were compared with parental Ba/F3 cells that were growing continuously in IL-3 or had been starved of IL-3 or IL-3 and serum for 24 or 48 h. The percentage of apoptotic cells was measured by FACS analysis (Fig. 3A), and the numbers of dead cells were counted by Trypan blue staining (not shown). As expected, we found a significant increase in apoptosis in parental Ba/F3 cells after 24 h of IL-3 withdrawal, which correlated with an increase in cell death. In contrast, cells transformed by the fusion had a low level of apoptosis and death after both 24 h (Fig. 3A) and 48 h (not shown) of IL-3 or serum withdrawal. Thus, expression of the ZNF198-FGFR1 fusion, which was expressed at similar levels in the three clones (Fig. 3B), protects transformed cells from apoptosis due to cytokine deprivation.

Effects of ZNF198-FGFR1 Expression on BclXL Protein Levels—To investigate further the signaling mechanisms involved in the resistance to apoptosis observed in ZNF198-FGFR1-transformed cells, we compared protein levels of the BCL-2 family members BclXL, Bcl2, and Mcl-1 by immunoblotting. Levels of these proteins were compared in parental Ba/F3 cells and transformed cells under the following conditions: cycling in the presence of IL-3, 24 and 48 h following IL-3 withdrawal or the same time points following serum and IL-3 withdrawal. We found that in parental Ba/F3 cells, down-regulation of BclXL protein levels occurred after 24 h of IL-3 withdrawal, with or without serum, but that the levels of other BCL2 family members were unaffected (Fig. 3C). In contrast, levels of BclXL protein in cells transformed by the fusion remained elevated after withdrawal of either IL-3 or serum and IL-3 at both 24 h (Fig. 3C) and 48 h (not shown).

Inducible Expression of Dominant Negative STATs in Cells Transformed by the ZNF198-FGFR1 Fusion—To investigate the function of specific STAT activation in transformation, proliferation, and resistance to apoptosis conferred by the fusion, we prepared several stable clones of ZNF198-FGFR1-transformed cells that inducibly expressed dominant negative forms of HA-tagged STAT1, STAT3, and STAT5, plus Ba/F3 cells that inducibly expressed dnSTAT5. Although stable transfectants expressing dnSTAT1 and dnSTAT3 were easily obtained in media without IL-3, we failed to obtain clones expressing dnSTAT5. We suspected a cytotoxic effect of low levels of dnSTAT 5 under these conditions as a consequence of leaky expression. Clones inducibly expressing dnSTAT 5 were, however, readily obtained in the presence of IL-3.

Western blot analysis of HA-immunoprecipitated proteins confirmed that the addition of 0.5 mM isopropyl-β-D-thiogalactoside to the culture media lead to the induction of dnSTAT1, dnSTAT3, and dnSTAT5 with maximum expression 24 h after induction. A slight variation in levels of expression of the dnSTATs was observed in different clones, and one dnSTAT1 clone was found to express protein without induction (Fig. 4). To evaluate the inhibitory effects of these dnSTATs on the constitutive binding to cognate DNA sequences observed with extracts from cells transformed by ZNF198-FGFR1, we carried out EMSA analysis. As before, nuclear extracts were isolated from parental Ba/F3 cells, Ba/F3 cells with induced dnSTAT5,
and from ZNF198-FGFR1-transformed cells with induction of either dnSTAT1, dnSTAT3, or dnSTAT5. Extracts were prepared from cells growing with IL-3, after IL-3 deprivation for 24 h, and with serum and IL-3 deprivation for 24 h. We found that induction of dnSTAT1 and dnSTAT3 greatly decreased the binding of these STATs to the m67sie probe observed in ZNF198-FGFR1-transformed cell lysates under all conditions (Fig. 5A). Similarly, induction of dnSTAT5 greatly decreased the complex formed with the β-casein probe but did not completely ablate binding (Fig. 5B). These experiments confirm that induction of the specific dnSTATs do indeed function to inhibit DNA binding of STATs activated by the ZNF198-FGFR1 fusion.

Dominant Negative STAT5 Induction Increases Cell Apoptosis on IL-3 and Serum Withdrawal of Ba/F3 Cells Transformed by ZNF198-FGFR1—To investigate the effects of inhibiting activation of specific STAT proteins on apoptosis of transformed Ba/F3 cells, we compared the proportion of cells that had a subdiploid DNA content or were dead in cells with or without induction of dnSTAT1, dnSTAT3, or dnSTAT5. We found that induction of either dnSTAT1 or dnSTAT3 had little or no discernable effect on the proportion of apoptotic cells in ZNF198-FGFR1-transformed cells after IL-3 or serum withdrawal (not shown). In contrast, induction of dnSTAT5 was found to greatly increase the percentage of apoptosis following both IL-3 or serum and IL-3 (Fig. 6A) in ZNF198-FGFR1-transformed clones that expressed comparable levels of the fusion protein. These results indicate that constitutive activation of STAT5 by ZNF198-FGFR1 plays an essential role in the observed resistance to apoptosis. Substantial levels of apoptotic cells were also seen in Ba/F3 cells expressing dnSTAT5, consistent with previous studies demonstrating that STAT5 plays an important role in IL-3-mediated signal transduction (31).

ZNF198-FGFR1-mediated STAT5 Activation Is Essential for the Elevated Levels of BclXL Protein in Transformed Cells—We used dnSTAT1, dnSTAT3, and dnSTAT5 induction to determine which STAT participates in the activation of BclXL protein expression observed in cells transformed by the ZNF198-FGFR1 fusion. We found that induction of either dnSTAT1 or dnSTAT3 had no effect on the elevated levels of BclXL protein found in cells expressing the fusion after serum or IL-3 deprivation. Protein levels of other BCL2 family members examined were also unaffected by dnSTAT1 or dnSTAT3 induction. In contrast, induction of dnSTAT5 caused a significant decrease in the level of BclXL protein expressed in fusion-transformed cells under all conditions (Fig. 6B). This is consistent with previous studies demonstrating that BCLXL is a direct transcriptional target of STAT5 (22). Protein levels of other BCL2
family members were not affected by dnSTAT5 expression (Fig. 6B), and no clear reduction in BclXL levels was seen on induction of STAT5 in Ba/F3 cells without the fusion (Fig. 6C). The decrease in BclXL protein levels due to dnSTAT5 induction correlates with the increase in apoptotic cell death observed. This suggests that activation of BclXL expression by the ZNF198-FGFR1 fusion via constitutive STAT5 expression is important in conferring the observed resistance to apoptosis.

**Activated STAT5 Binds to the BclXL Promoter in Cells Transformed by ZNF198-FGFR1, and dnSTAT5 Induction Inhibits This Binding**—We used EMSA to confirm that dnSTAT5 reduces BclXL levels by blocking the binding of STAT5 to the BCLXL promoter. As expected the 32P-labeled probe, which was derived from the murine BCLXL promoter, formed a complex in ZNF198-FGFR1-transformed cells, but this complex was markedly reduced in Ba/F3 cells cultured either in the presence or absence of IL-3 (Fig. 7). Preincubation of nuclear extracts from transformed cells with 100-fold molar excess of unlabeled probe prevented formation of this complex, confirming specificity. The complex was supershifted by anti-STAT5
8). Cells transformed by the fusion showed no G0/G1 arrest by serum deprivation for 24 h (lack of cells in S or G2/M phase; Fig. 7). We observed to progress to S or G2/M phase with dnSTAT5 inducive block in cell cycle progression. Hardly any cells were not shown). In contrast, induction of dnSTAT5 caused a drafed. We found that induction of neither dnSTAT1 nor dnSTAT5 in cells transformed by the fusion was then examined. We then investigated the effect of induction of either dnaSTAT1, dnaSTAT3, or dnaSTAT5 on formation of the complex with the BCLXL probe. Induction of dnaSTAT5 in cells transformed by the fusion caused a significant reduction in the bound complex under all conditions (Fig. 7). No effect on this complex formation was observed with induction of dnaSTAT1 or dnaSTAT3 (data not shown), and no or minimal effect was observed on induction of dnaSTAT5 in Ba/F3 cells (Fig. 7). These results confirm that ZNF198-FGFR1-mediated STAT5 activation causes transcriptional activation of BCLXL by direct binding of STAT5 to the BCLXL promoter.

**STAT5 Activation Is Essential for Continued Cell Cycle Progression in ZNF198-FGFR1-transformed Cells**—We sought to investigate whether STAT1, STAT3, or STAT5 activation affected cell cycle progression of Ba/F3 cells transformed by the fusion. Initially cell cycle progression of parental Ba/F3 cells, either growing with IL-3, starved of IL-3 for 24 or 48 h, or starved of serum and IL-3 for 24 or 48 h, was compared with that of similarly treated cells transformed by the ZNF198-FGFR1 fusion. Cell cycle status was examined by FACS analysis of cells after propidium iodide staining. We found that parental Ba/F3 cells arrested in G0/G1 following either IL-3 or cytokine withdrawal and continue to progress through the cell cycle under low serum or cytokine conditions. The resistance to apoptosis in fusion-transformed cells correlates with elevated levels of the anti-apoptotic protein BclXL. Identification of the critical signaling pathways downstream of FGFR1 fusion proteins that are responsible for these apoptotic and proliferative defects is important because it will aid the identification of specific inhibitory drugs for treatment of patients with EMS.

We and others have previously shown that several signaling pathways associated with the promotion of proliferation and the inhibition of apoptosis are activated by FGFR1 fusion proteins, including pathways that involve mitogen-activated protein kinase, phospholipase Cγ, phosphatidylinositol 3 kinase, and STATs (7–9, 18, 35). Here we have focused on the role of STAT proteins. We have shown that all six STATs are phosphorylated in Ba/F3/ZNF198-FGFR1 cells. This contrasts with a previous study that showed phosphorylation of STATs 1, 3,
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Fig. 8. Induction of dnSTAT5 causes a block in cell cycle progression of ZNF198-FGFR1-transformed cells. Cell cycle progression of Ba/F3 cells, ZNF198-FGFR1-transformed cells, and transformed cells with induction of dnSTAT5 growing with IL-3, deprived of IL-3 for 24 h, or deprived of both IL-3 and serum for 24 h. Cells were analyzed by propidium iodide staining and FACS analysis. The results are representative of four separate experiments.

Fig. 9. RAD 51 protein levels are reduced on dnSTAT5 induction. Rad51 protein levels were determined by Western analysis of Ba/F3 cells, ZNF198-FGFR1-transformed cells, transformed cells and Ba/F3 cells with induction of dnSTAT5 growing with IL-3 or deprived of IL-3 for 24 h. MEK protein levels were measured as a loading control.

Fig. 10. Relative transforming abilities of BCR-ABL, ZNF198-FGFR1, FGFR1, and BCR-FGFR1. Clones of transformed Ba/F3 cells were isolated by limited dilution and tested for survival and proliferation in the absence of IL-3. FGFR1-transformed clones were grown in the presence of acidic fibroblast growth factor plus heparin.

and 5, but not STATs 4 and 6, in ZNF198-FGFR1-transfected 293 cells (9). Lack of STAT4 and STAT6 phosphorylation was observed even in 293 cells that were engineered to express high levels of these proteins. One possible explanation for this difference could be that phosphorylation of STAT4 and STAT6 is not mediated directly by ZNF198-FGFR1 but rather through an intermediary that is expressed in Ba/F3 cells but not 293 cells.

We focused our analysis on STATs 1, 3, and 5 because these proteins have been shown to play an important role in hemo-
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