Modeling the human 8p11-myeloproliferative syndrome in immunodeficient mice

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The 8p11 myeloproliferative syndrome (EMS), also referred to as stem cell leukemia/lymphoma, is a chronic myeloproliferative disorder that rapidly progresses into acute leukemia. Molecularly, EMS is characterized by fusion of various partner genes to the *FGFR1* gene, resulting in constitutive activation of the tyrosine kinases in FGFR1. To date, no previous study has addressed the functional consequences of ectopic *FGFR1* expression in the potentially most relevant cellular context, that of normal primary human hematopoietic cells. Herein, we report that expression of *ZMYM2/FGFR1* (previously known as *ZNF198/FGFR1*) or *BCR/FGFR1* in normal human CD34$^+$ cells from umbilical-cord blood leads to increased cellular proliferation and differentiation toward the erythroid lineage in vitro. In immunodeficient mice, expression of *ZMYM2/FGFR1* or *BCR/FGFR1* in human cells induces several features of human EMS, including expansion of several myeloid cell lineages and accumulation of blasts in bone marrow. Moreover, bone marrow fibrosis together with increased extramedullary hematopoiesis is observed. This study suggests that *FGFR1* fusion oncogenes, by themselves, are capable of initiating an EMS-like disorder, and provides the first humanized model of a myeloproliferative disorder transforming into acute leukemia in mice. The established in vivo EMS model should provide a valuable tool for future studies of this disorder. (*Blood*. 2010;116(12):2103-2111)

Introduction

The 8p11 myeloproliferative syndrome (EMS), also known as stem cell leukemia/lymphoma, is a myeloproliferative disorder (MPD) that WHO recently classified as belonging to the group of myeloid neoplasms associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*.[1] Clinically, EMS is characterized by leukocytosis, eosinophilia, splenomegaly, and a short chronic phase that rapidly progresses into aggressive acute myeloid (AML) or lymphoblastic (ALL) leukemia. A prominent feature of the disease is an increased incidence of T-cell lymphomas, seen in 30% of the cases, suggesting that the target cell of transformation is a multipotent progenitor cell.[2] Although several FGFR1 inhibitors have been tested with promising effects in vitro,[3-5] the only cure for this disease at present is allogeneic bone marrow (BM) transplantation.

At the molecular level EMS is characterized by various translocations fusing at least 8 different 5′ partner genes to the 3′ part of the *FGFR1* gene that encodes the tyrosine kinase domain.[6] In most of these fusion proteins, the amino terminal regions contain structural properties that allow them to dimerize or oligomerize, resulting in constitutive activation of FGFR1.[7]

A limited number of studies in murine cells have addressed how *FGFR1* fusion oncogenes elicit their transforming activities, mainly focusing on the 2 most common fusion gene variants *ZMYM2/FGFR1* (previously known as *ZNF198/FGFR1*) and *BCR/FGFR1*. Expression of *ZMYM2/FGFR1* in IL-3 dependent murine Ba/F3 cells resulted in growth-factor independent growth in which signal transducer and activator of transcription (STAT) 1, 2, 3, 4, 5, and 6 become constitutively tyrosine phoshorylated.[8,9] Mice receiving transplants with *ZMYM2/FGFR1*-transduced mouse BM cells develop a condition that resemble EMS with MPD and T-cell lymphoma.[10] Moreover, mice that receive *BCR/FGFR1*-transduced BM cell transplants develop a rapid fatal chronic myeloid leukemia (CML)–like disease.[10] Although these studies in murine models have provided valuable pathogenetic insights into the different *FGFR1* fusion genes, the functional consequences of their expression in primary human cells have not been addressed. In fact, the introduction of leukemia-associated fusion genes in primary human cells, followed by transplantation into immunodeficient mice has, so far, been successful in reproducing the corresponding human disease only for the 3 fusion genes *MLL/AF9(MLLT3), MLL/ENL(MLLT1)*, and *TEL(ETV6)/JAK2*.[11-14]

Herein, we demonstrate that retroviral expression of *ZMYM2/FGFR1* or *BCR/FGFR1* in human CD34$^+$ hematopoietic cells induces an increased cellular proliferation and erythropoietin (EPO)–independent differentiation toward the erythroid lineage in vitro. In immunodeficient mice, both fusion oncogenes induce an MPD-like disorder, accompanied by bone marrow fibrosis and blast accumulation, consistent with features observed in EMS patients.[15,16]

Methods

Isolation, transduction, and sorting of cord blood CD34$^+$ cells

The collection of cord blood (CB) was approved by the Lund University ethics committee and performed after informed consent in accordance with

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the Declaration of Helsinki. Samples from different donors were pooled, mononuclear cells were isolated by centrifugation over Lymphoprep (Axis-Shield PoC A/S), and the $CD34^+$ cells were enriched by the use of MACS separation columns and isolation kit (Miltenyi Biotec). The purity of the $CD34^+$ cells was routinely higher than 95% as assessed by flow cytometry.

The retroviral vectors MSCV-IRES-*GFP* (MIG), MIG-*BCR/FGFR1*, MIG *ZMYM2/FGFR1*, MIG-*BCR/ABL1*, MIG-*BCR/FGFR1* Y653/654F, MIG-*ZMYM2/FGFR1* Y653/654F, and the lentiviral vectors short-hairpin RNA (shRNA) scramble and anti-STAT5 were used in this study. For origins, modifications, and production of these vectors, see supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). $CD34^+$ cells were thawed and prestimulated for 48 hours in Dulbeccos Modified Eagle Media with GlutaMAX (Invitrogen Corporation), containing 10% fetal calf serum (FCS; Invitrogen Corporation), 100 units/mL penicillin, and 100 μg/mL streptomycin. The medium was supplemented with the following cytokines (Peprotech Inc): 50 ng/mL thrombopoietin (TPO), 100 ng/mL stem cell factor (SCF), and 100 ng/mL Flt-3-ligand (FL). Viral vectors were preloaded using Retronectin (Takara Bio Inc). In the anti-STAT5 shRNA experiments, the lentiviral vectors, with previously well documented effects,[17] were used together with the retroviral vectors. The prestimulated $CD34^+$ cells were resuspended in prestimulation medium with the addition of 4 μg/mL protamine sulfate, and added to the wells at a density of 1×10^5 to 1.5×10^5 cells/mL. After 48 hours in culture, the GFP^+ or GFP^+/RFP^+ cells were sorted using a FACSAria cellsorter (BD).

Cell proliferation analysis and colony forming assay

For the cell proliferation analysis, 1×10^4 GFP^+ or GFP^+/RFP^+ cells were sorted into wells of a 96-well plate containing 200 μL serum-free Stemspan SFEM medium (StemCell Technologies Inc), 100 units/mL penicillin, and 100 μg/mL streptomycin. The medium was supplemented with 50 ng/mL TPO, 100 ng/mL SCF, 100 ng/mL FL, 25 ng/mL IL-3, and 10 ng/mL IL-6. Cultured cells were given fresh medium regularly. Cell numbers were counted at days 7 and 14.

For the colony-forming assay, 50 GFP^+ or GFP^+/RFP^+ cells were sorted into wells of a 48-well plate containing 200 μL methylcellulose medium Methocult (StemCell Technologies Inc) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 5U/mL EPO, 25 ng/mL SCF, 50 ng/mL granulocyte-macrophage colony-stimulating factor, and 25 ng/mL IL-3. After 2 weeks in culture, colonies were counted and replated at a dilution of 1:100. Two weeks later the secondary colonies were counted.

Cytospin preparations, flow cytometric analysis, and Western blot analysis

Cells grown in suspension culture were collected at days 7 and 14. For morphologic examination, cytospin slides were prepared by applying 1×10^4 cells onto glass slides followed by May-Grünwald and Giemsa staining. Cells harvested at the same time points were stained with allophycocyanin (APC)– or phycoerythrin (PE)–conjugated antibodies against CD34, CD13, CD71, or CD235a (glycophorin A, GPA; BD Immunocytometry Systems) and analyzed by flow cytometry using a FACSCalibur or FACSCanto (BD). Western blot was performed on cells harvested after 6 days in suspension culture. Details regarding the Western blot analysis are given in the supplemental Methods.

Gene-expression microarray analysis

2 days after transduction, GFP^+ cells were collected in 3 biologic replicates and RNA was isolated using the RNeasy isolation kit (QIAGEN). RNA quality was assessed using a Bioanalyzer (Agilent Technologies Inc). Samples were labeled, fragmented, and hybridized to Affymetrix U133 plus 2.0 microarrays (Affymetrix Inc) containing 54 000 probe sets representing approximately 38 500 genes according to the manufacturer's instructions. The data pre-processing is described in more detail in the supplemental Methods. Primary data from the gene-expression analysis is available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; accession number GSE15811).

Immunodeficient mouse xeno-transplantation assay

Human $CD34^+$ CB cells were prestimulated and 2 to 4×10^5 cells/mouse were transduced with *BCR/FGFR1*, *ZMYM2/FGFR1*, or the MIG control vector as described in "Isolation, transduction, and sorting of cord blood $CD34^+$ cells." One day after transduction, unsorted transduced cells were injected via the tail vein or into the femur of sublethally (300-350 Rad) irradiated NOD/SCID, NOD/SCID $B2m^{-/-}$, or NOD/SCID IL2–receptor gamma deficient (NSG) mice.[18,19] After irradiation, the mice were given antibiotics (ciprofloxacin) and powder food. At 6 and 9 or 12 weeks after injection of human cells, BM was collected from femur and cells were double stained with APC-conjugated anti-CD45 and PE-conjugated anti-CD33/CD15, -CD34, or -CD235a (BD) antibodies, followed by flow cytometric analyses as described.[20] The mice were killed at 9 to 12 weeks after injection of human cells, or earlier if found moribund. Spleen and femur (from leg not subjected to BM injection or aspiration) were collected in 4% formalin, embedded in paraffin, and sectioned. Slides were stained for reticulin, with hematoxylin and eosin (HE), or immunostained with antibodies against CD3, CD4, CD15, CD20, CD34, CD45, CD68, CD79a, CD117, CD235a, and myeloperoxidase (MPO; Dako). Antibodies used for immunostaining were controlled for cross-reactions with murine cells on BM sections from nontransplanted NOD/SCID control mice (supplemental Figure 5). Slides were analyzed using a Nikon Eclipse 50i microscope (Nikon Instruments Inc) with Plan Flour objective lenses (magnification/aperture 10×/0.30 and 40×/0.75). Micrographs were collected using a Pixera Pro 150 ES camera (Pixera Corp) and Viewfinder software (Pixera Corp). Subsequent adjustments in size and color balance of the pictures were made using Photoshop software (Adobe Systems Inc). BM sections were also examined using Fluorescence in situ hybridization (FISH) analysis as described in the supplemental Methods.

Results

Expression of *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* in $CD34^+$ CB cells leads to increased cellular proliferation and EPO-independent erythroid differentiation

To study the direct consequences of *FGFR1* fusion genes on proliferation and differentiation in primitive cells, $CD34^+$ CB cells were transduced with *BCR/FGFR1* or *ZMYM2/FGFR1*. The P210 *BCR/ABL1* fusion gene, associated with CML, was used as reference fusion oncogene. At 2 days after transduction, on average 39% of the *BCR/FGFR1*-transduced cells expressed GFP, 10% of the *ZMYM2/FGFR1*, 19% of the *BCR/ABL1*, and 36% of the MIG ($n = 7$; supplemental Figure 1A). Successful expression of fusion proteins was confirmed by Western blot analyses (supplemental Figure 1B).

$CD34^+$ CB cells expressing *ZMYM2/FGFR1, BCR/FGFR1* or *BCR/ABL1* increased in number 35 times more than MIG control cells during 2 weeks of suspension culture (Figure 1A). In contrast to the MIG cells that differentiated toward the myeloid lineage, mainly with a CD13-positive immunophenotype, *ZMYM2/FGFR1*-, *BCR/FGFR1*-, and *BCR/ABL1*-expressing cells differentiated into an erythroid phenotype ($CD235a^+$, $CD71^+$) in an EPO-independent manner (Figure 1B). Morphologic examination of cells from the suspension culture revealed that cells expressing either of the 3 fusion genes were at the pronormoblast stage of erythroid differentiation, consistent with our own and other investigators previous findings for *BCR/ABL1* (Figure 1C).[17,21,22] To investigate if the FGFR1 tyrosine kinase activity was required for the increased proliferation and erythroid differentiation, we constructed variants previously shown to abolish kinase activity.[10,23] $CD34^+$ CB cells

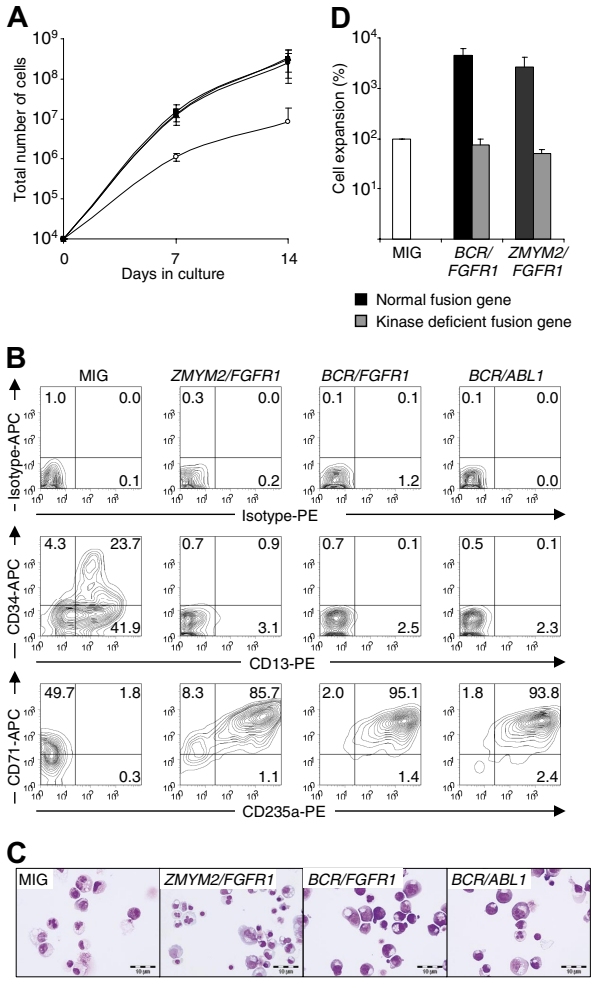

Figure 1. Expression of *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* in human CD34⁺ CB cells leads to increased cellular proliferation and erythroid differentiation. (A) Cells transduced with *ZMYM2/FGFR1* (●), *BCR/FGFR1* (■), or *BCR/ABL1* (▲) increase in numbers approximately 35-fold compared with MIG control cells (○) during 14 days of suspension culture. Cells were counted at days 7 and 14 after sorting and the mean values of 3 separate experiments are shown. (B) The immunophenotype of cultured cells was assessed after 14 days of culturing. At this stage, cells expressing *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* had become erythroid as shown by high levels of CD235a- and CD71-antigen expression. (C) Morphologic examination by May-Grünwald and Giemsa staining of cells on cytospin slides after 14 days of suspension culture shows that cells expressing either of the 3 fusion genes were at the pronormoblast stage of erythroid differentiation. (D) The proliferation rate of CD34⁺ CB cells expressing the kinase dead mutants *BCR/FGFR1* 653/654Y or *ZMYM2/FGFR1* 653/654Y (gray) was significantly reduced compared with cells expressing normal *BCR/FGFR1* and *ZMYM2/FGFR1* (black) after 2 weeks of suspension culture. The result shown is the mean value from 3 separate experiments with MIG control cell expansion (white) set to 100%. Error bars show SD.

that expressed *BCR/FGFR1* Y653/654F or *ZMYM2/FGFR1* Y653/654F did not display an increased proliferation or differentiation toward erythroid cells, suggesting that the kinase activity is crucial for the FGFR1 fusion oncogenic effects of the 2 fusion genes (Figure 1D and supplemental Figure 2).

To investigate whether the erythroid differentiation of the *ZMYM2/FGFR1*-, *BCR/FGFR1*-, and *BCR/ABL1*-expressing cells was related to specific properties of CB cells to commit to the erythroid lineage, the suspension culture experiment was repeated using normal human CD34⁺ BM cells, yielding a similar pattern of differentiation toward the erythroid lineage (data not shown). To study the effect on colony forming capacity, cells were cultured in methylcellulose medium. CD34⁺ CB cells expressing *ZMYM2/FGFR1*, *BCR/FGFR1* or *BCR/ABL1* primarily gave rise to burst-forming units erythroid (BFU-E) colonies, whereas MIG control cells formed equal numbers of BFU-E and colony-forming units granulocyte-macrophage (CFU-GM) colonies (Figure 2A). After replating, cells expressing any of the 3 fusion genes almost exclusively gave rise to massive BFU-E colonies, in contrast to the few MIG control colonies formed, demonstrating that *ZMYM2/FGFR1*, *BCR/FGFR1*, and *BCR/ABL1* increased the BFU-E colony forming capacity (Figure 2B).

We have previously demonstrated that *BCR/ABL1*-induced increase of erythroid cells is STAT5-dependent in CD34⁺ CB cells.[17] Because *ZMYM2/FGFR1* and *BCR/FGFR1* expression in murine Ba/F3 cells results in STAT5 activation,[9] we analyzed the phosphorylation pattern of STATs 3, 5, and 6 in cells after 6 days in suspension culture. Similar levels of STAT5 phosphorylation were detected for BCR/FGFR1 and BCR/ABL1, whereas ZMYM2/FGFR1 induced a much lower, but still detectable, level of phosphorylation of STAT5 (Figure 2C).

ZMYM2/FGFR1-, *BCR/FGFR1*-, and *BCR/ABL1*-expressing cells display similar but distinct gene-expression profiles

To study the deregulation of transcriptional programs induced by *FGFR1* fusion genes, global gene-expression analysis on cells expressing *ZMYM2/FGFR1*, *BCR/FGFR1*, *BCR/ABL1*, or the MIG control vector was performed. Unsupervised hierarchical clustering revealed a clear separation into 2 main clusters, with one comprising the biologic replicates of MIG and the other the 3 fusion oncogenes, thus demonstrating a fundamental difference in their gene expression profiles (supplemental Figure 3A). Notably, in a supervised multiclass SAM analysis, comparing the 3 biologic replicates of MIG, *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1*, cells expressing the 3 different fusion genes also formed separate subclusters indicating a smaller, but significant, difference in their expression profiles (supplemental Figure 3B).

Two-class unpaired SAM analysis, comparing MIG with each of the 3 fusion gene-expressing cells separately, identified 77 commonly up-regulated genes at a FDR of $< 1\%$ (Figure 2D; supplemental Table 1). Gene ontology analysis of these 77 genes revealed that signal transducer activity was the main top-ranked category (25 genes, Ease score $3.08 \times 10\text{-}^{4}$). Notably, as many as 11 of the 77 genes (14%) were involved in the JAK-STAT signaling pathway, including *CISH, LEPR*, *LIF*, *OSM, PIM1*, *SOCS1-3, SOS1,* and the interleukin receptors *IL2RB* and *IL7R*. In parallel, 74 genes were found to be commonly down-regulated by *ZMYM2/FGFR1*, *BCR/FGFR1*, and *BCR/ABL1* compared with MIG (Figure 2E; supplemental Table 2). Although many genes were commonly regulated in the fusion gene-expressing cells, the individual fusion genes still displayed distinct gene-expression profiles with *ZMYM2/FGFR1* and *BCR/FGFR1* being more similar than *BCR/ABL1* using multiclass SAM analysis (supplemental Figure 3B).

Silencing of STAT5 with shRNA results in decreased proliferation of cells expressing *BCR/FGFR1*, *ZMYM2/FGFR1*, or *BCR/ABL1*

We have previously demonstrated that *BCR/ABL1*-induced cell proliferation and erythroid differentiation in human CD34⁺ CB cells is partially STAT5 dependent.[17] In this study, gene expression profiling revealed that *BCR/FGFR1*, *ZMYM2/FGFR1*, and *BCR/ABL1* all up-regulated several genes involved in JAK/STAT signaling. This finding agreed well with our Western blot data demonstrating a clear STAT5 phosphorylation for BCR/FGFR1

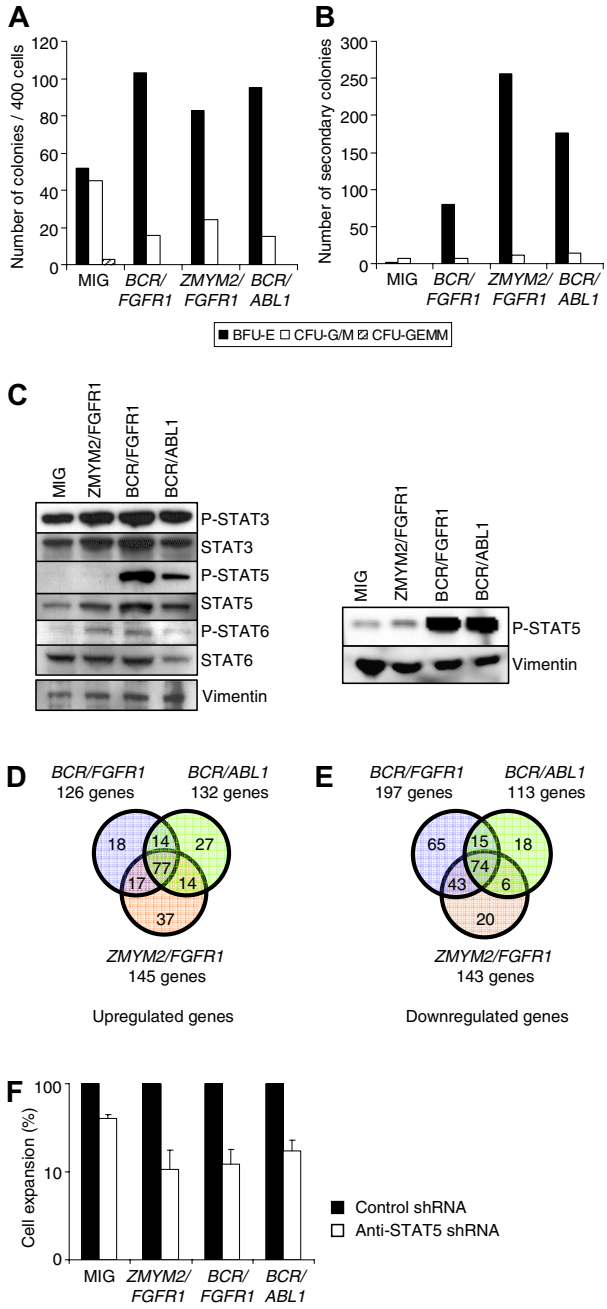

Figure 2. Expression of *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* in human CD34$^+$ CB cells leads to expansion of erythroid colonies and induces similar but distinct gene-expression profiles. (A) Cells expressing *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* mainly gave rise to BFU-E colonies after 2 weeks of culture in methylcellulose medium. (B) After replating of colonies, cells expressing a fusion gene almost exclusively formed BFU-E colonies. One representative experiment of 4 is shown. (C) Western blot analysis of STAT phosphorylation after 6 days suspension culture showing that ZMYM2/FGFR1 induced a much lower level of STAT5 phosphorylation than BCR/FGFR1 or BCR/ABL1 (left panel). The right panel shows an independent Western blot analysis, using longer exposure time, in which a weak but distinct band of phosphorylated STAT5 in ZMYM2/FGFR1-expressing cells is observed. Vimentin was used as an endogenous control for equal loading. (D-E) Venn diagrams showing the number of genes that are differentially expressed and commonly regulated in cells expressing *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* compared with MIG control cells. (F) Cells co-expressing *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* and an anti-STAT5 shRNA showed a significant reduction in cell proliferation after 7 days in suspension culture. The data are displayed as the mean cellular expansion for the anti-STAT5 shRNA-expressing cells in percentage of the corresponding control scramble shRNA-expressing cells from 3 separate experiments, with error bars representing SD.

and BCR/ABL1, while ZMYM2/FGFR1 unexpectedly showed a markedly lower level of STAT5 phosphorylation. To study mechanistically if STAT5 activation was equally important for the 3 different fusion oncogenes in terms of their effects on cellular proliferation, we cotransduced CD34$^+$ CB cells with anti-STAT5 shRNA and the different fusion genes. After sorting of GFP$^+$/RFP$^+$ cells as previously described,[17] a significant decrease in cell proliferation was observed for all 3 fusion oncogenes (Figure 2F). Moreover, negative effects in erythroid differentiation and colony formation were observed after STAT5 silencing, but as similar effects on MIG control cells were observed (data not shown), it is currently unclear to what extent these effects are related to the fusion genes. Nevertheless, the strong dependence on STAT5 for inducing cell proliferation by cells expressing all 3 fusion genes, suggests that STAT5 activation is an important mechanism whereby FGFR1 fusion oncogenes elicit their transforming effects.

BCR/FGFR1 and *ZMYM2/FGFR1* induce a human EMS-like disorder in mice

To investigate how *FGFR1* fusion oncogenes affects primary human cells in vivo, CD34$^+$ CB cells expressing *BCR/FGFR1, ZMYM2/FGFR1*, or the MIG control vector were transplanted into immunodeficient NOD/SCID or NOD/SCID *B2m*$^{-/-}$ mice, hereafter referred to as *BCR/FGFR1* mice, *ZMYM2/FGFR1* mice, or MIG mice. In a second set of experiments, we also used the more severely immunocompromised NSG mouse strain. 6 weeks after transplantation into NOD/SCID or NOD/SCID *B2m*$^{-/-}$ mice, the engraftment of human cells (GFP$^+$ and/or CD45$^+$) in the mouse BM was on average 46% for *BCR/FGFR1*, 41% for *ZMYM2/FGFR1*, and 22% for MIG control (Figure 3A). No difference in engraftment of human cells related to mouse strain, or if tail vein or intrafemoral injections were used, was noted (supplemental Table 3 and data not shown). *BCR/FGFR1* expression directed the human cells toward an increased myeloid and erythroid cell fate as shown by higher levels of CD33/CD15 and CD235a, whereas *ZMYM2/FGFR1* at this stage did not differ significantly from the MIG control (Figure 3B-D). At 9 to 12 weeks after transplantation, the *ZMYM2/FGFR1*-induced effects were similar to that observed for *BCR/FGFR1*, including increased levels of human myeloid and erythroid cells (supplemental Figure 4).

To investigate the fusion oncogene-induced histopathologic changes in NOD/SCID and NOD/SCID *B2m*$^{-/-}$ mice in more detail, femurs and spleens from 15 *BCR/FGFR1* mice, 6 *ZMYM2/FGFR1* mice, and 12 MIG mice were harvested at 6 to 12 weeks after transplantation. In agreement with the flow cytometric data, HE and MPO staining revealed expansion of the human myeloid cell compartment in *BCR/FGFR1* mice together with an increase in erythroid (CD235a$^+$) cells (Figure 4A and supplemental Figure 5A). Similar, but less pronounced effects were seen in *ZMYM2/FGFR1* mice. In 14 of the 15 *BCR/FGFR1* mice, and in 5 of 6 *ZMYM2/FGFR1* mice, we found clusters (foci) of histiocytes/macrophages as defined by CD68 staining and morphology (Figure 4B and supplemental Figure 5B). These clusters, which typically were more pronounced in *BCR/FGFR1* mice, also contained human mast cells as determined by staining for CD68 and CD117 (c-kit), and morphology (Figure 4B and supplemental Figure 5B). The human origin of the cells in these foci was confirmed by FISH using probes specific for human or mouse centromeres (supplemental Figure 5B). The expansion of the different myeloid lineages varied, with some mice displaying a pronounced accumulation of macrophages and mast cells, whereas

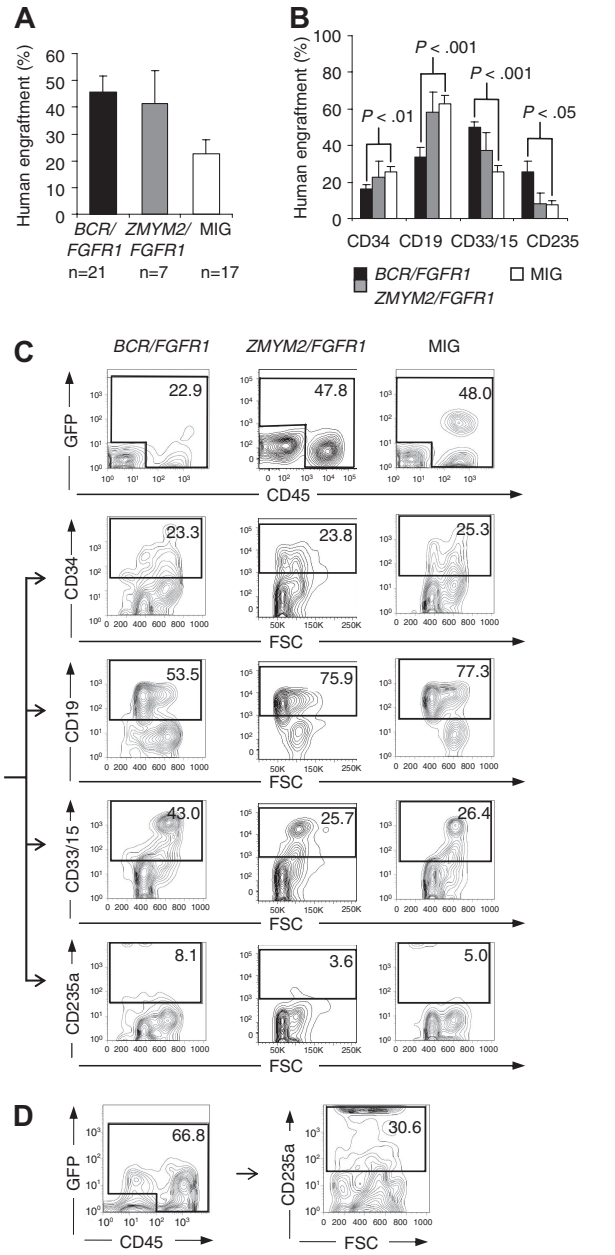

Figure 3. Flow cytometric analysis of human cells in NOD/SCID mouse BM around 6 weeks after transplantation reveals that *BCR/FGFR1* expression directs the differentiation to myeloid and erythroid cell fates. (A) Engraftment at 6 to 7 weeks after transplantation of human cells transduced with *BCR/FGFR1*, *ZMYM2/FGFR1*, or the MIG control vector. (B) Phenotype of human cells in the BM of *BCR/FGFR1* (black), *ZMYM2/FGFR1* (gray), or MIG (white) mice at 6 to 7 weeks after transplantation, revealing that *BCR/FGFR1* significantly directs grafted cells toward the myeloid and erythroid cell lineages. Data are presented as the mean value of all analyzed mice with error bars representing SEM (for data at 9-12 weeks after transplantation, see supplemental Figure 4B). (C) FACS plots showing the gating and differentiation pattern of human cells (GFP^+ and/or $CD45^+$; top panel) in BM from representative MIG, *ZMYM2/FGFR1*, or *BCR/FGFR1* mice, as detected by cell surface expression of CD34, CD19, CD33/15, and CD235a. (D) FACS plot showing increased erythroid differentiation of human cells in BM of an additional *BCR/FGFR1* mouse.

others showed a more prominent expansion of granulocytic cells, possibly being a result of either the type of cells being targeted by the 2 fusion genes or by variability in CB donors.

Human lymphoid cells in both *BCR/FGFR1* mice and *ZMYM2/FGFR1* mice were frequently found to be B-lymphocytic, as shown by staining for CD20 (Figure 4C). T cells positive for CD3 and CD4 were more rarely seen (Figure 4C). In 10 of the 15 *BCR/FGFR1* mice, human granulocytopoiesis displayed a left-shifted maturation pattern with marked expansion of myeloid cells at all stages of maturation, including eosinophilia, which is in agreement with findings described in the chronic phase of human EMS[7] (Figure 4D). Interestingly, 3 of the *BCR/FGFR1* animals displayed an accumulation of human blasts in the BM (Figure 4D). In 2 of the 3 animals, the blasts stained strongly positive for CD235a, suggesting the emergence of an acute erythroleukemia in these mice (Figure 4D). In 1 of these animals, the $CD235a^+$ blasts coexpressed CD117 indicating a more immature

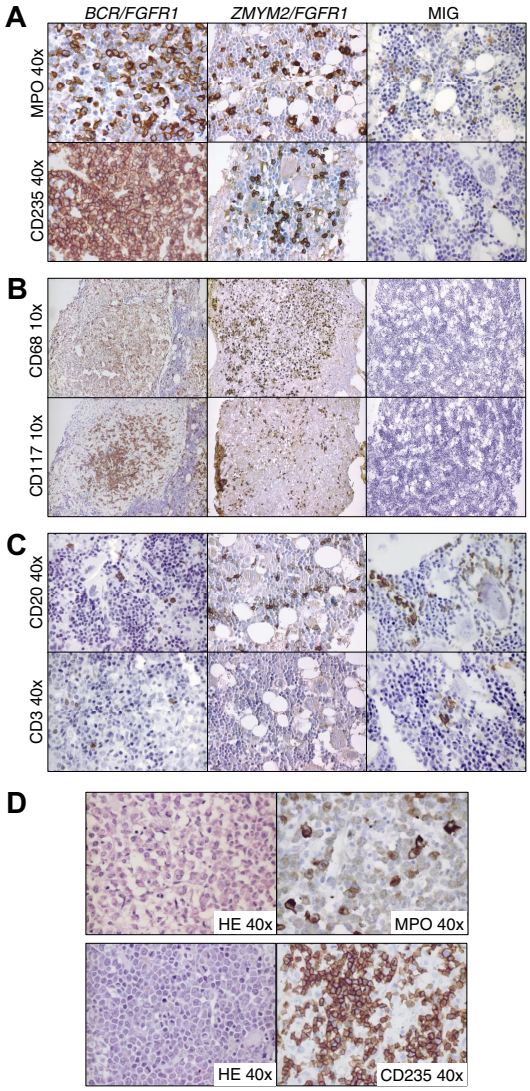

Figure 4. Immunostained BM sections from *BCR/FGFR1*, *ZMYM2/FGFR1*, and MIG NOD/SCID mice. (A) *BCR/FGFR1* and *ZMYM2/FGFR1* mice display increased levels of human granulocytic and erythroid cells in BM as shown by immunostaining for MPO and CD235a. BM sections from a representative MIG mouse reveal single granulocytic and erythroid human cells staining positive for MPO and CD235a. (B) Human cells in *BCR/FGFR1* and *ZMYM2/FGFR1* mice BM also differentiated into macrophages and mast cells as shown by morphology and positive staining for CD68 and CD117 in BM sections from representative mice. Typically, macrophages and mast cells formed round clusters of cells. In MIG mice, only few cells stained positive for CD68 or CD117. (C) In BM sections from *BCR/FGFR1* and *ZMYM2/FGFR1* mice, as well as from MIG mice, dispersed B-lymphoid cells, staining positive for CD20 were found, whereas T-lymphoid cells positive for CD3 were more rarely detected. (D) In *BCR/FGFR1* mice, human granulocytopoiesis frequently displayed a left-shifted maturation pattern with eosinophilia, as shown by morphology in HE stained sections and immunostaining for MPO (top panel). Furthermore, accumulation of erythroid blasts as shown in HE stained sections and by CD235a staining was observed (bottom panel).

phenotype (data not shown). In the third mouse, the blast refrained staining with all antibodies used (CD3, CD4, CD15, CD20, CD34, CD45, CD68, CD79a, CD117, CD235a, and MPO) suggesting an immature and primitive origin of these blasts (data not shown). All examined MIG mice (n = 12) displayed a relatively normal BM hematopoiesis with dispersed lymphoid and single granulocytic and erythroid human cells. (Figure 4A-C).

Eleven of the 15 *BCR/FGFR1* mice and 1 of the 6 *ZMYM2/FGFR1* mice suffered from mild to severe BM fibrosis as shown by reticulin and HE staining (Figure 5A and data not shown). The numbers of megakaryocytes in the BM were most often normal in count, but sometimes displayed abnormal nuclei in *BCR/FGFR1* mice. In accordance with what has been shown for *TEL/JAK2* in a similar experimental model,[12] the megakaryocytes were of murine origin as determined by FISH (Figure 5B).

Similar to patients with MPD, splenomegaly, consistent with an increased extramedullary hematopoiesis, was detected in several *BCR/FGFR1* mice, but not in *ZMYM2/FGFR1* mice (supplemental Figure 6A). In most cases the same abnormalities seen in the BM were also observed in the spleen, for example, expansion of human granulocytopoietic and erythropoietic cells and foci containing macrophages and mast cells (supplemental Figure 6B). Of the 21 *BCR/FGFR1* mice, 7 died due to disease during the study. Histopathologic and immunohistochemical analysis was performed on 5 of these animals, all displaying the characteristic features of disease, that is, left-shifted granulocytopoiesis with eosinophilia, BM fibrosis, and splenomegaly. Three of the dead animals showed accumulation of blasts in the BM. Peripheral blood examination during the disease course did not, however, reveal elevated white blood cell counts in any of the examined animals (data not shown).

None of the abnormalities observed in the BM or spleen of the *BCR/FGFR1* or *ZMYM2/FGFR1* mice were found in any of the MIG mice, and no MIG control mice died due to disease during the study.

Because the disease phenotype in *ZMYM2/FGFR1* NOD/SCID mice was relatively weak compared with *BCR/FGFR1*, we also transplanted human CD34$^+$ cells transduced with *BCR/FGFR1*, *ZMYM2/FGFR1*, or the MIG control into NSG mice. Whereas the *BCR/FGFR1* mice all died of disease at days 23 to 32 after transplantation, most likely because of BM insufficiency caused by human myeloid cell expansion and/or fibrosis, the *ZMYM2/FGFR1* mice survived until day 38 or longer (Figure 6A). The NSG mice displayed on average approximately 70% human cells, irrespectively if the mice were transplanted with cells expressing *BCR/FGFR1*, *ZMYM2/FGFR1*, or the MIG control (Figure 6B and supplemental Table 3). The increase in human myeloid and erythroid cells observed in *BCR/FGFR1* and *ZMYM2/FGFR1* NSG mice was typically more pronounced than seen in NOD/SCID mice (Figure 6C). Immunostainings of BM sections were similar to NOD/SCID mice (supplemental Figure 7). Both fusion oncogenes induced an MPD-like disorder with left-shifted granulocytopoiesis, sometimes accompanied by eosinophilia and/or dysplastic megakaryocytes (data not shown). *BCR/FGFR1* and *ZMYM2/FGFR1* NSG mice generally suffered from mild to severe BM fibrosis and displayed foci of human histiocytes and mast cells (supplemental Figure 7). Two *BCR/FGFR1* and 1 *ZMYM2/FGFR1* mouse showed blast expansion in bone marrow and/or spleen (supplemental Figure 8). These blasts stained positive for CD235a and CD117 (supplemental Figure 8 and data not shown). None of the NSG MIG control mice died due to disease during the study. There were no signs of lymphoid disease in any of the mice, irrespectively of the fusion gene used.

Given the close similarities in BM morphology between the *BCR/FGFR1* and *ZMYM2/FGFR1* mice and patients with EMS described in the literature,[24] we reexamined a BM biopsy taken from an EMS patient in which we originally isolated the *BCR/FGFR1* fusion gene (Figure 7).[16,25] At the stage when the EMS had

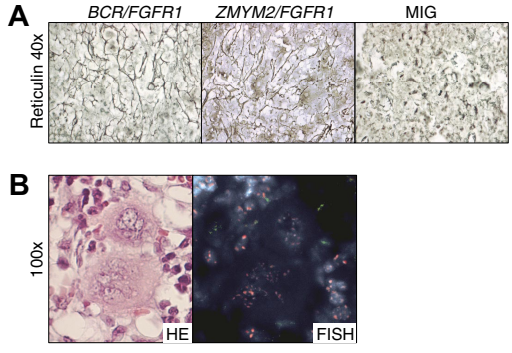

Figure 5. NOD/SCID mice transplanted with *BCR/FGFR1*- and *ZMYM2/FGFR1*-expressing cells display BM fibrosis. (A) Reticulin-stained BM sections from a representative *BCR/FGFR1* mouse and a ZMYM2/FGFR1 mouse reveal pronounced fibrosis, whereas all MIG mice displayed normal reticulin. (B) Two representative megakaryocytes from *BCR/FGFR1* mouse BM shown to be of murine origin as determined by FISH analysis (red signals, murine cells; green signals, human cells).

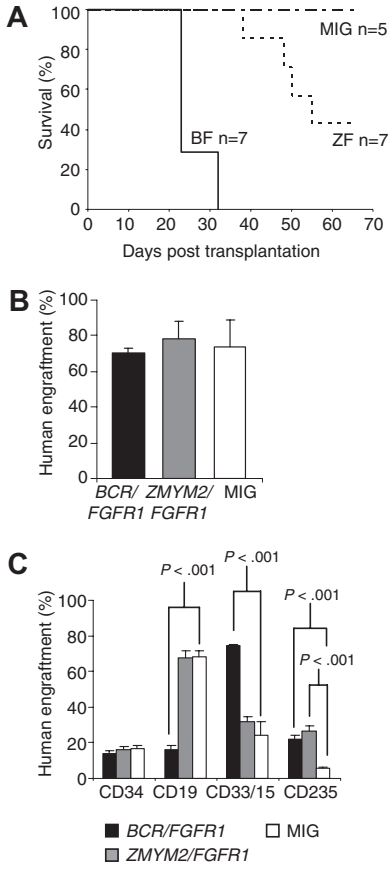

Figure 6. Results from NSG mice transplanted with human cells expressing *BCR/FGFR1* or *ZMYM2/FGFR1*. (A) *BCR/FGFR1* mice all died at day 32 or earlier, whereas *ZMYM2/FGFR1* mice survived longer. All MIG control mice, and 3 *ZMYM2/FGFR1* mice survived until end of study. (B) The engraftment of human cells was on average 70% in *BCR/FGFR1* mice, 74% in *ZMYM2/FGFR1* mice, and 78% in MIG control mice at end of life (23-65 days). Error bars represents SEM. (C) The percentage of human myeloid (CD33/15$^+$) cells was significantly higher in *BCR/FGFR1* mice (black), but not in *ZMYM2/FGFR1* mice (gray), compared with MIG control mice (white). The erythroid cell lineage (CD235a$^+$) was pronounced in both *BCR/FGFR1* mice and *ZMYM2/FGFR1* mice. Data are presented as the mean value of all analyzed mice with error bars representing SEM.

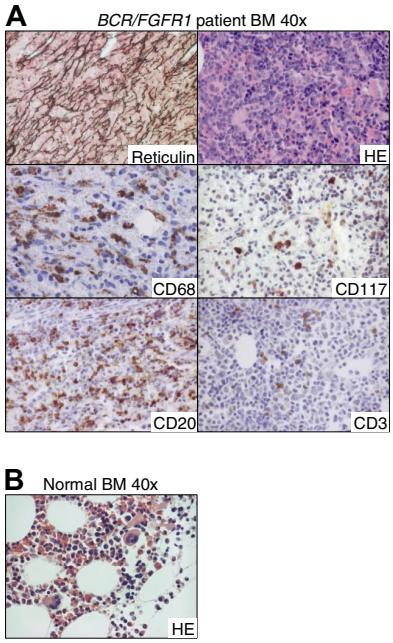

Figure 7. BM sections from the patient in which the *BCR/FGFR1* fusion gene originally was isolated show similarities with *BCR/FGFR1*-induced effects in the NOD/SCID and NSG mouse models. (A) Reticulin-stained section demonstrates a pronounced BM fibrosis and HE staining shows infiltration of blasts (top panel). Numerous macrophages staining positive for CD68 and occasionally mast cells, as defined by morphology and CD117 staining, are also found (middle panel). $CD20^+$ B cells are more frequent than $CD3^+$ T cells (bottom panel). Due to admixture of peripheral erythrocytes, a CD235 stained section could not be conclusively analyzed. (B) HE-stained section from a normal healthy BM.

progressed into acute leukemia, the reticulin stained section revealed a pronounced increase of reticulin. Furthermore, HE staining revealed eosinophilia and infiltration of blasts that on immunohistochemical staining were negative for all antibodies tested (CD3, CD4, CD8, CD15, CD20, CD34, CD45, CD68, CD79a, CD117, CD235, MPO, and TdT). This suggests an immature but otherwise unclassifiable phenotype of the blasts, similar to the finding in 1 of the NOD/SCID *BCR/FGFR1* mice with blast accumulation. Notably, numerous macrophages ($CD68^+$) and occasional mast cells ($CD68^+/CD117^+$) were found. In agreement with our finding in the immunodeficient mice, $CD20^+$ B cells were more frequent than $CD3^+$ T cells in the patient's BM.

Discussion

To study aberrant tyrosine kinases underlying human MPD, investigators have long been entirely dependent on leukemic cell lines and murine assays. Although these functional studies have contributed significantly to our knowledge regarding disease mechanisms in human MPD, there are noticeable limitations in both systems as cell lines typically acquire additional genetic aberrations for continuous growth in vitro, and tumorigenesis may differ substantially in human and mouse.[14,26,27]

In this study we assessed the consequences of ectopic *FGFR1* fusion gene expression in the cellular context of normal primitive human hematopoietic cells. $CD34^+$ CB cells expressing *ZMYM2/FGFR1* or *BCR/FGFR1* displayed EPO-independent erythroid differentiation and significant proliferation capacity in vitro. Importantly, we hereby establish the first human in vivo EMS model by transplanting *ZMYM2/FGFR1*- and *BCR/FGFR1*-expressing CB cells into immunodeficient mice. In this model, primitive human cells expressing the 2 fusion oncogenes induce an MPD-like disorder, accompanied by bone marrow fibrosis and accumulation of blasts, thereby closely resembling the disease features observed in EMS patients. An alternative approach to establish a human EMS disease model would be to transplant immunodeficient mice with primary cells obtained from EMS patients. Such studies have been performed using chronic phase CML cells, but in general, high-level engraftment and a faithful recapitulation of morphologic and clinical features observed in CML patients has been difficult to achieve.[28-30] Moreover, the relatively rare occurrence of human EMS, and the fact that EMS in many cases is diagnosed in the late AML or ALL stage of the disease, severely limit similar types of studies.

Upon retroviral expression of *ZMYM2/FGFR1* or *BCR/FGFR1* in normal human $CD34^+$ CB cells we found an *FGFR1* kinase dependent increased proliferation capacity and EPO-independent erythroid differentiation in vitro, which is similar to *BCR/ABL1*-induced effects and consistent with previously reported findings for *BCR/ABL1*.[17,21,22,31,32] This suggests that the *BCR/ABL1* and *FGFR1* fusion genes, at least partly, mediate their leukemogenic effects in a similar manner. In agreement with this, global gene-expression analysis confirmed that the 3 fusion oncogenes induced similar transcriptional responses with activation of genes involved in the JAK-STAT pathway. Moreover, all 3 fusion genes induced an increase of immature human erythroid cells. To test whether the erythroid bias of the fusion gene expressing cells was caused by a preferential retroviral targeting of early erythroid progenitor cells, we also included nontransduced control cells in the experiments. As the outcome of sorted GFP^+ MIG transduced cells did not differ significantly from the nontransduced cells, we conclude that the erythroid phenotype was not induced by a preferential targeting of erythroid committed cells during the transduction procedure (data not shown). Although the erythroid phenotype may seem unexpected, it is well-known that primary cells from CML patients form EPO-independent BFU-E colonies and that chronic phase CML patients display an increase of megakaryocyte and erythroid progenitor (MEP) cells.[32,33] Interestingly, similar in vitro studies of primary cells from patients with EMS have been shown to increase the number of BFU-E colonies in the absence of EPO.[3]

We have previously demonstrated that *BCR/ABL1*-induced erythroid differentiation and proliferation in human $CD34^+$ CB cells is STAT5-dependent,[17] and it could be speculated that the *FGFR1* fusion genes also mediate their effect through a similar mechanism. Unexpectedly, the level of STAT5 phosphorylation was markedly lower in *ZMYM2/FGFR1*-expressing cells compared with *BCR/FGFR1* and *BCR/ABL1*. Despite the differences in STAT5 phosphorylation, global gene-expression profiling revealed that all 3 fusion genes up-regulated several genes involved in the JAK-STAT signaling pathway as well as other sets of genes, indicating that these fusion proteins share similar molecular programs. Using lentiviral STAT5 shRNA constructs, we were able to demonstrate a significant effect on the proliferation rate of cells expressing either of the 3 oncogenes, suggesting that STAT5, in addition to its well-documented role in CML,[34] also plays an important role in FGFR1-mediated leukemogenesis.

Upon transplantation of $CD34^+$ cells into immunodeficient mice, *BCR/FGFR1* and *ZMYM2/FGFR1* expression rapidly lead to an increase of a variety of cell types including granulocytes, erythroid cells, macrophages, and mast cells. This suggests that

expression of *FGFR1* fusion oncogenes induce proliferation and differentiation of multiple myeloid cell lineages. Importantly, *BCR/FGFR1* and *ZMYM2/FGFR1* expression induced a marked increase of human granulocytopoietic cells at all stages of maturation with eosinophilia and dysplastic megakaryocytes also being observed. Furthermore, splenomegaly and increased extramedullary hematopoiesis was seen. Altogether, the *FGFR1* fusion oncogene-induced disease in mice in many ways closely resembles human EMS as these features all are key characteristics in this MPD subtype.[7] Moreover, blast accumulation in the BM or spleen of several mice suggest that the MPD was about to transform into AML. We failed to graft these blasts in secondary recipients through intravenous injections, suggesting that they were not fully immortalized blasts (data not shown), perhaps requiring secondary aberrations to become fully transformed. However, the short latency from transplantation to disease onset in this model indicates that *FGFR1* fusion oncogenes by themselves have the ability to induce, at least, the chronic phase of the disease. Albeit *ZMYM2/FGFR1* generally induced weaker phenotypic changes with longer survival of the mice compared with *BCR/FGFR1,* both fusion oncogenes induced similar disease phenotypes in immunodeficient mice. It is possible that the less pronounced disease pattern seen in *ZMYM2/FGFR1* mice could be explained by the markedly lower transduction efficiency achieved with the *ZMYM2/FGFR1* expressing retrovirus, thus our observations do not necessarily indicate that *BCR/FGFR1* is a more potent oncogene than *ZMYM2/FGFR1*.

Although the disease in mice much resembles human EMS, 3 distinct differences between the established model and human EMS are notable. First, patients with EMS often display a bilinear disease involving both the myeloid and the T-lymphoid lineage. We here failed to demonstrate a significant involvement of the human T-cell lineage in the immunodeficient mice. However, this may be explained by the inability of adult mice of both the NOD/SCID and the NSG strains to support T-lymphopoiesis after receiving transplants with human cells, possibly due to improper thymus development in these mice.[19,35] Moreover, while T-cell involvement has been primarily reported in patients expressing *ZMYM2/FGFR1*, to our knowledge no EMS patient harboring a *BCR/FGFR1* fusion gene has been reported to suffer from T-cell lymphoma/leukemia.[15,16,36-43] Secondly, the accumulation of macrophages and mast cells in the BM of both *BCR/FGFR1* and *ZMYM2/FGFR1* mice may seem unexpected. However, when reviewing published cases with EMS, presence of mast cells in the BM and systemic mastocytosis have been reported in 2 cases.[44,45] Upon reexamination of our previously described patient with EMS harboring a *BCR/FGFR1* fusion gene,[16,25] numerous mast cells and macrophages were found in the BM, although no prominent foci-like formations, as seen in the animal model, were observed. It is also interesting to note that mast cells often are enriched in eosinophilic myeloproliferative neoplasms caused by the closely related fusion genes involving *PDGFRA/B*.[46] Thirdly, in 5 of 6 animals with blast accumulation, the blasts displayed an erythroid phenotype. This is consistent with our in vitro studies that demonstrated a strong erythroid potential of *BCR/FGFR1*- and *ZMYM2/FGFR1*-expressing cells. So far, *FGFR1* fusion genes have not been associated with erythroleukemia, but we note with interest that 2 EMS patients were diagnosed with polycythemia vera, and 1 additional case developed erythroid BM hyperplasia.[47-49]In analogy with CML that may transform into an acute erythroleukemia,[50] we further speculate that kinase activating mutations of *FGFR1* may be found in a fraction of acute erythroleukemia, representing an acute phase of an undiagnosed EMS chronic phase.

Global gene-expression analysis strongly indicated that both BCR/FGFR1 and ZMYM2/FGFR1 activates the JAK-STAT pathway, and the myelofibrosis seen in *BCR/FGFR1* mice and *ZMYM2/FGFR1* mice is similar to a recently described study in which human primary hematopoietic cells expressing the *TEL/JAK2* fusion gene were transplanted into NOD/SCID mice.[12] Both our studies found atypical megakaryocytes of murine origin, suggesting that expression of a fusion oncogene such as *BCR/FGFR1* or *TEL/JAK2* in human cells can have cell nonautonomous effects on the mouse megakaryocytic lineage, perhaps contributing to fibrosis. In accordance with our in vivo findings, fibrosis in the BM has been reported in 3 described EMS cases verified to harbor the *BCR/FGFR1* fusion gene.[15,16]

In conclusion, we demonstrate that *FGFR1* fusion genes have the potential to support EPO-independent erythroid development in a similar manner as *BCR/ABL1*. More importantly, we have established a human in vivo model of EMS by introducing *BCR/FGFR1*- or *ZMYM2/FGFR1*-expressing human $CD34^+$ CB cells into immunodeficient mice. We show that *BCR/FGFR1* or *ZMYM2/FGFR1* expression alone induces rapid histopathologic changes that resemble several characteristic features observed in EMS patients, including increased granulocytopoiesis, eosinophilia, blast accumulation, and increased extramedullary hematopoiesis. Notably, this is the first humanized model of an MPD with features indicative of progression into an acute phase. As such it should constitute a valuable tool for obtaining further insights into *FGFR1* fusion gene mediated leukemogenesis and for the development and evaluation of new treatment strategies in EMS.

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Authorship

Contribution: H.Å., M.J., and T.F. designed the research and wrote the manuscript; and H.Å., M.J., A.A., P.J., N.H., C.L., M.R., D.G., T.O., J.R., X.F., M.E., and T.F. performed the research and analyzed the data.

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