Rac1/2 activation promotes FGFR1 driven leukemogenesis in stem cell leukemia/lymphoma syndrome

The Rac proteins are a subfamily of the Rho-GTPases which cycle between activated GTP-bound and inactivate GDP-bound forms to coordinate various cell responses with signals from extracellular stimuli.[1] In particular, the Rac proteins play an essential role in normal hematopoiesis as well as leukemogenesis, where they act as intermediates for activation of cancer driving proteins such as RAS, PI3 kinase, ERK, p38 and AKT.[2] In normal hematopoiesis, Rac1 and Rac2 have been shown to have important influences on engraftment into the stem cell niche, cell cycle progression and survival of stem cells as well as retention in the microenvironment.[3] Rac activation has also been shown to be critical for progression of leukemogenesis driven by the *BCR-ABL1* oncogene,[4-5] which has some similarities to the stem cell leukemia/lymphoma syndrome (SCLL) driven by ligand-independent, constitutively activated FGFR1 kinase (referred to by the World Health Organization (WHO) as myeloid and lymphoid malignancies with abnormalities of FGFR1). FGFR1 activation is facilitated through dimerization motifs contributed by various partner proteins that are juxtaposed to the kinase domain as a result of chromosome translocations.[6-7] FGFR1 normally promotes activation of cell proliferation pathways involving p38, ERK and AKT, which is likely facilitated by Rac activation.[8] Here we have used a combination of studies involving murine models of SCLL and cell lines derived from them, and show that Rac1 and Rac2 are important for the progression of SCLL leukemogenesis *in vivo* and further, that pharmacological inhibition of Rac leads to suppression of leukemogenesis due to increased apoptosis.

Using affinity-binding assays, high levels of activated Rac were detected in three murine SCLL cell lines carrying different FGFR1 chimeric proteins (Figure 1A). Similarly, Rac was highly activated in the human KG1 SCLL cell line. When BBC2 cells, expressing BCR-FGFR1, and KG1 cells, expressing FGFR1OP2-FGFR1, were treated with the BGJ398 FGFR1 inhibitor,[9] levels of activated Rac were suppressed (Figure 1B) demonstrating that FGFR1 activation is associated with increased Rac activation. No change in Rac activation was seen following BGJ398 treatment of human leukemia cell lines MOLT-4 and HL-60, which do not overexpress FGFR1 (Figure 1B). Increased activation of the p38, ERK and AKT, downstream effectors (Figure 1C) of Rac, was seen in the murine cell lines, suggesting functional consequences of

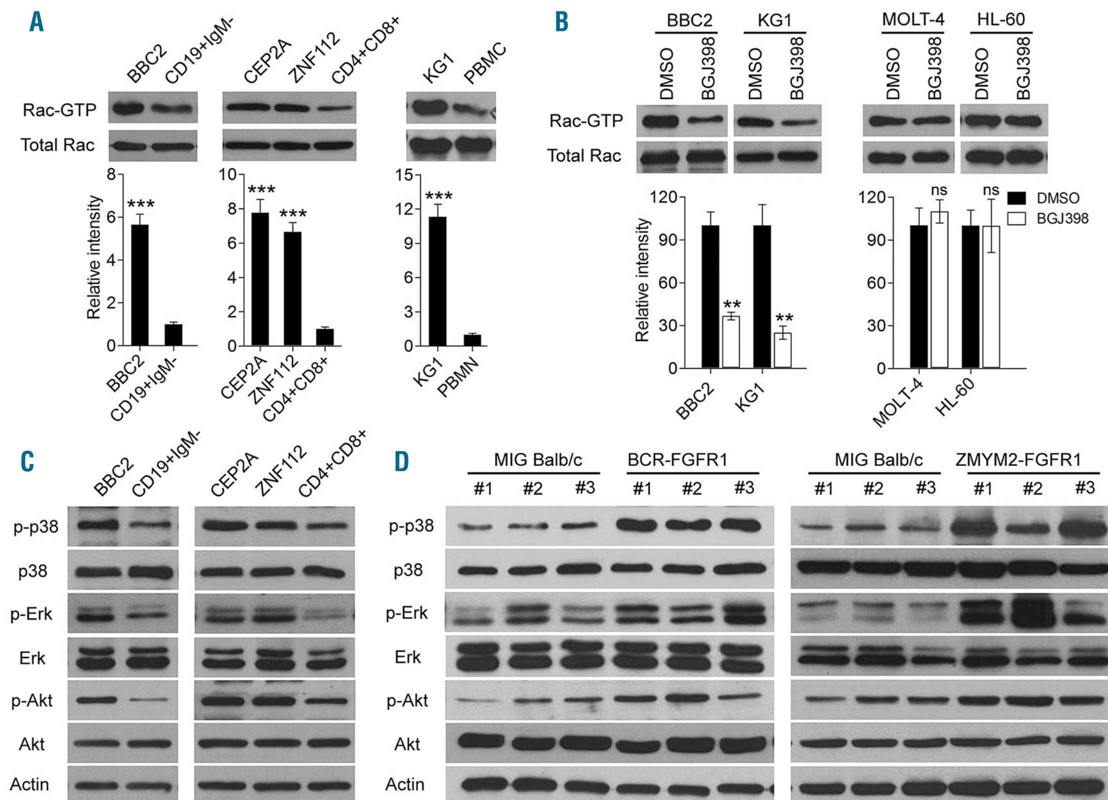

Figure 1. Affinity precipitation assays (Cytoskeleton, inc) were used to assess Rac activation levels in a series of cell lines expressing chimeric FGFR1 kinases. BBC2 (B-lymphoma) expresses BCR-FGFR1, CEP2A (T-lymphoma) expresses (CNTRL-FGFR1), ZNF112 (T-lymphoma) expresses ZMYM2-FGFR1. KG1 (AML), the only human cell line in the series, expresses FGFR1OP2-FGFR1. In the three murine cell lines (A), compared with flow sorted murine $CD19^{+}IgM^{-}$ B-cells or $CD4^{+}CD8^{+}$ T-cells, Rac-GTP levels were significantly increased (N=3). Similarly, KG1 cells, compared with mononuclear cells, show significantly increased levels of activated Rac (A). Analysis of Rac activation levels in BBC2 and KG1 cells after 72 hours treatment with the BGJ398 FGFR1 inhibitor, demonstrate significantly reduced levels of activated Rac (B), whereas the human MOLT-4 and HL60 leukemic cells do not. Analysis of downstream effectors of Rac (C) demonstrated increased levels of activated p38, Erk and Akt in the murine cell lines. Comparison between spleen cells derived from mice following transplantation of primary bone marrow cells transduced with the empty MIG vector or either BCR-FGFR1 (D, left) or ZMYM2-FGFR1 (D, right) supports the observation of increased activation of p38, Erk and Akt *in vivo*. Using Student's *t* test; ***P*=< 0.01, ****P*=< 0.001. Error bars represent standard deviation. Identity of SCLL cells is constantly verified by the presence of the uniquely sized chimeric kinases definitive for each cell line.

its activation. When primary hematopoietic stem cells are transformed *ex vivo* with chimeric FGFR1 kinases, and transplanted into lethally irradiated mice, SCLL develops consistently with different immunophenotypes depending on the particular oncokinase used. Analysis of these primary leukemic cells from *in vivo* models of the BCR-FGFR1[6] and ZMYM-FGFR1[7] oncokinases also demonstrated increased activation levels of p38, ERK and AKT (Figure 1D).

Using the same transduction and transplantation approach, as described previously,[6] we investigated the role of Rac in transformation of primary bone marrow cells with the BCR-FGFR1 oncokinase using cells derived from three different strains of mice; (1) wild-type (2) Rac2 mutant null mice and (3) Rac2 mutant null mice carrying floxed Rac1 alleles allowing induced Cre-mediated deletion of the Rac1 gene.[10] Since Rac2 null mice survive to term and are viable, we transduced bone marrow cells from these mice with *BCR-FGFR1* and transplanted them into lethally irradiated C57Bl/6 mice. Deletion of Rac1, however, is embryonic lethal and so in order to be able to study the effects of combined Rac1/2 inactivation, we used bone marrow cells from the $Rac1^{flox/flox}/Rac2^{-/-}$ mouse strain, in which Rac1 deletion can be induced in hematopoietic cells by exposure to poly I/C.[10] Bone marrow cells from the $Rac1^{flox/flox}/Rac2^{-/-}$ strain of mice were transduced with *BCR-FGFR1* and engrafted *via* the tail vein into lethally irradiated mice.[7] These cells were allowed to engraft and establish for seven days before being treated with five intraperitoneal (i.p.) injections of 300 μg of poly I/C dissolved in PBS every other day as described previously.[10] Disease progression was monitored in these mice through weekly flow cytometry analysis of GFP^+ cells in peripheral blood obtained from the tail vein, and were ultimately sacrificed when morbidity was observed. As shown in Figure 2A, on autopsy, western blot analysis of bone marrow cells showed high-level depletion of Rac1 in cells from mice treated with poly I/C, confirming loss of Rac1. In these *in vivo* studies, mice receiving bone marrow cells from wild-type

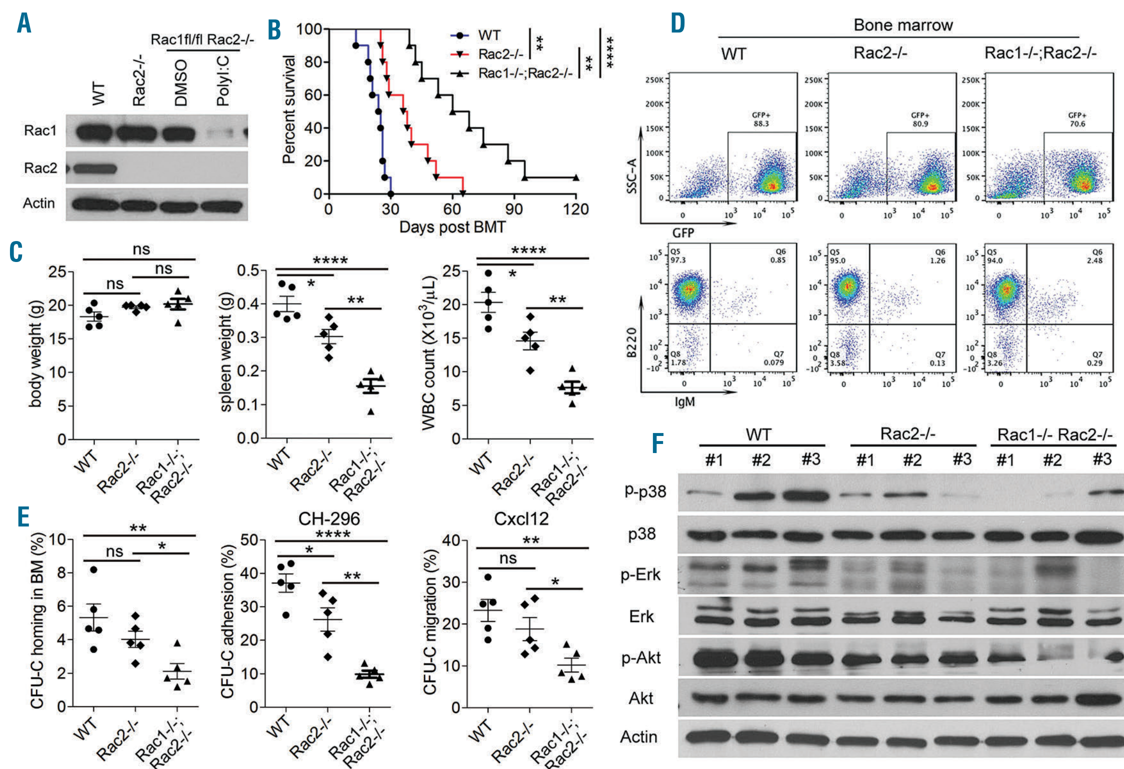

Figure 2. Western blot analysis of Rac expression in *BCR-FGFR1* transduced bone marrow stem cells derived from *Rac2* null mice shows absence of protein. In bone marrow cells from $Rac2^{null}/Rac1^{flox/flox}$ after poly I/C treatment demonstrates absence of the Rac1 and Rac2 proteins compared with DMSO treated mice (A). Kaplan-Meier analysis of cohorts of mice (n=10) following transduction and transplantation of primary bone marrow stem cells expressing the *BCR-FGFR1* kinase demonstrated the rapid onset of leukemia with survival times between 13-30 days (B). When *BCR-FGFR1* was transduced into bone marrow cells derived from *Rac2* null mice, although leukemia developed in all cases, survival times were increased to 25-65 days. An even more significant increase in survival was seen when bone marrow cells null for both *Rac1* and *Rac2* were transduced with *BCR-FGFR1*. On autopsy, the survival times were directly related to the size of the spleens and the white blood cell (WBC) count (C). Flow cytometry of bone marrow cells from these three cohorts shows a typical $B220^+IgM^-$, B-lymphoma phenotype (D). The consequences of Rac1/2 deletion on homing, adhesion and migration were assayed from hematopoietic stem cells from either wild-type $Rac2^{-/-}$ or *Rac1/2* null cells transformed with *BCR*-FGFR1 (E). Sixteen hours after injection of $2x10^6$ sorted GFP^+ cells into the tail vein of recipient mice, GFP^+ cells were recovered from the bone marrow of the transplanted animals (n=5) and colony forming unit cells (CFU-C) were compared in methylcellulose assay. While *Rac2* null cells showed only minor differences, the *Rac1/2* null cells showed a highly significant reduction in colony formation (E, left). Adhesion was assayed by plating $1x10^5$ GFP^+ cells onto fibronectin fragment CH-296 coated plates. Compared with input cells, there was a significant reduction in adhesion in the *Rac2* null cells but a highly significant reduction in adhesion for *Rac1/2* null cells (E, center). Transwell assays were used to measure migration, where the Cxcl12 chemokine (50 ng/ml) was placed in the lower chamber and $1x10^5$ GFP^+ cells introduced into the upper chamber. After four hours, cells in the lower chamber were recovered and the percentage of migrated hematopoietic progenitors determined using the CFU-C assay. There was no significant decrease for the *Rac2* null cells but the *Rac1/2* null cells showed a significant reduction in migration (E. right). Analysis of activated protein levels for p38, pERK1/2 and pAKT in the bone marrow cells from *Rac2* null and *Rac1/2* null mice (n=3) also shows reduced levels of p38, pERK and pAKT (F). Using the Student's *t* test; **P*=<0.01, ***P*=<0.001, ****P*=<0.0001, ****P*=0.00001. ns=not significant. Error bars represent standard deviation.

C57Bl/6 mice transduced with the *BCR-FGFR1* construct were used as controls, which showed a typically short survival time of approximately 25-30 days.[6] In contrast, in cohorts receiving transduced cells derived from *Rac2* null mice, survival was significantly extended and in mice transplanted with transduced cells that were depleted for Rac1 and Rac2, survival was extended significantly longer (Figure 2B). On autopsy, the spleen weights and white blood cell (WBC) counts from mice derived from these different strains reflected the aggressiveness of the disease in the individual cohorts (Figure 2C). We have demonstrated previously that *BCR-FGFR1* transformation of primary bone marrow cells results in pre-B-lymphomas with a B220$^+$IgM$^-$ immunophenotype.[6] The same phenotype was recorded in the lymphomas that arose in the mice in which either *Rac2* or *Rac1/2* was deleted in bone marrow cells expressing *BCR-FGFR1* (Figure 2D). Thus, loss of Rac expression affects tumor progression but the developmental (lineage) course of disease progression was unaffected. Rac also plays an important role in engraftment, cell migration and adhesion and when the same transduced cells were assayed for homing, adhesion and migration (Figure 2E), as described previously,[11] while there was only a modest reduction in these phenotypes in the *Rac2* null transformed cells, there was a highly significant suppression in the double null cells, suggesting a potential mechanism for suppressed leukemogenesis. While *Rac2* null cells showed spleen cells from the *Rac2* null engrafted cohort showed reduced pAKT levels but there was an almost complete absence of pAKT in the cohort receiving the *Rac1/2* double, mutant null cells (Figure 2F). Similarly, activation levels of p38 were suppressed in Rac deficient mice (Figure 2F). Thus, it appears that loss of *Rac2* can suppress FGFR1-driven leukemogenesis, while loss of both Rac family members has an even greater ability to suppress disease progression.

To further investigate the role of Rac in the development of SCLL, we used the pan-Rac, Ehop-016 pharmacological inhibitor[12] to evaluate the effect on viability in three different SCLL cell lines. As shown in Figure 3A, there is a dose-dependent suppression of viability following treatment in all cases. The ZMYM2-FGFR1 expressing cells were the most sensitive and KG1 cells were least

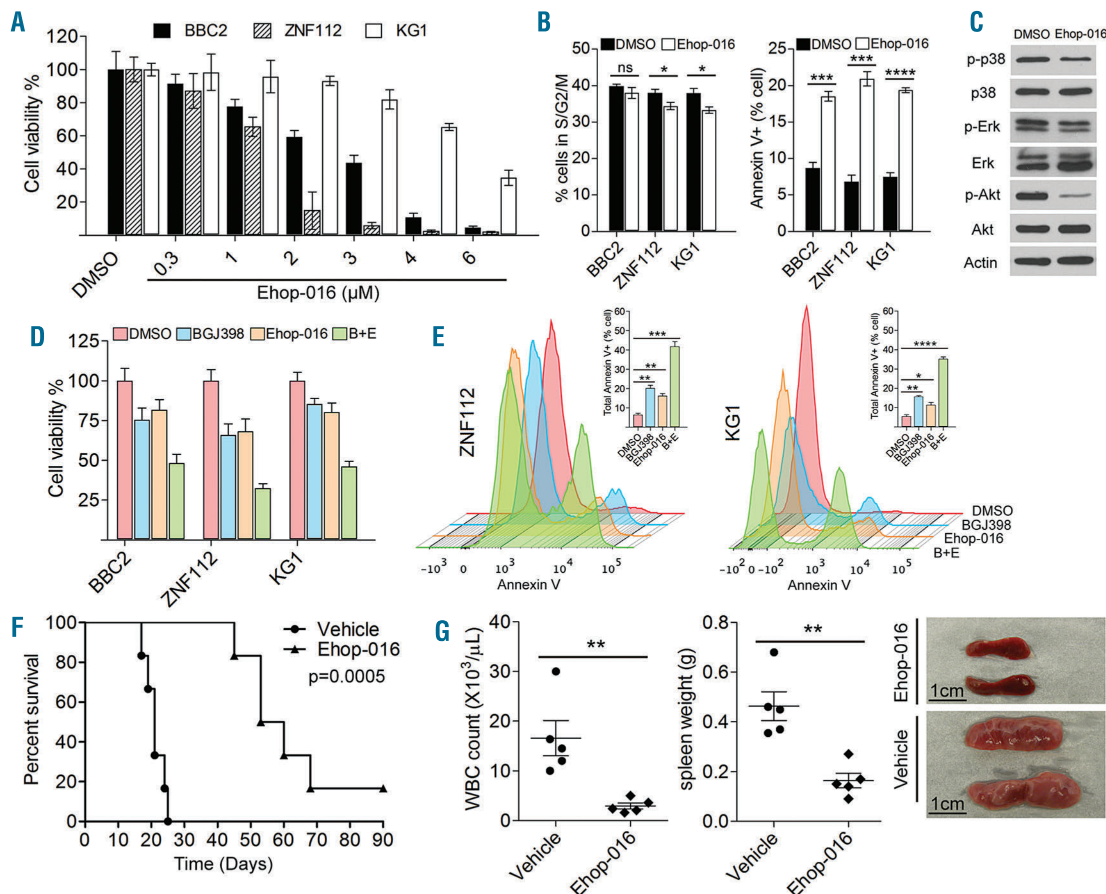

Figure 3. **When three different SCLL cell lines were treated with various concentrations of the Ehop-016 Rac inhibitor (A), there was a dose dependent reduction in cell viability in all (n=3) cases.** IC_{50} concentrations were 1.2 μM for ZNF112, 2.4 μM for BBC2 cells and 5.3 μM for KG1 cells. Inhibition of Rac in three cell lines following Ehop-016 treatment at the IC_{50} for 24 hours showed a mild but insignificant effect on progression through the cell cycle (B) but showed a significant increase in the levels of Annexin V expressing cells indicating increased apoptosis. As a result of Ehop-016 treatment, downregulation of activated p38, ERK and AKT was observed in ZNF112, for example (C). An additive effect on reduced cell viability was observed when the BGJ398 FGFR1 inhibitor (between 2 -20 nM depending on the cell line), was combined with Ehop-016 (D) which was associated with increased levels of Annexin V expression in the cells treated with the drug combination (E). *In vivo* studies showed that mice xenografted with primary bone marrow cells from the wild-type group showed a significant increase in survival when treated with Ehop-016 (25 mg/Kg i.p. 3x/week for two weeks) compared with vehicle-treated controls (F) and this was supported by decreased WBC counts in the peripheral blood and reduced spleen size in the Ehop-016 treated animals (G). Using the Student's *t* test; **P*=<0.01, ***P*=<0.001, ****P*=<0.0001, *****P*=<00001. Error bars represent standard deviation.

sensitive, with BBC2 showing an intermediate sensitivity (Figure 3A), which appears to reflect the endogenous levels of Rac activation in these cells (Figure 1A). Ehop-016 treatment only mildly affected cell cycle progression (Figure 3B, left) but dramatically increased apoptosis levels (Figure 3B, right), and in ZNF112 cells, for example, led to reduced activation of p38, Erk and Akt (C). These observations were confirmed in cell viability assays (Figure 3D), and the suppressive effect of Rac inhibition was further increased with the addition of an FGFR1 inhibitor (BGJ398), which showed an additive effect on apoptosis levels (Figure 3E). To evaluate the *in vivo* effects of Ehop-016 on leukemogenesis, we used the BCR-FGFR1 model where individual cohorts of mice were transplanted with 2×10^6 primary bone marrow cells transduced with *BCR-FGFR1* and, after one week, treated with i.p. injections of 25 mg/Kg Ehop-016 every other day for two weeks. Mice treated with Ehop-016 showed a highly significant increase in survival compared with those treated with the vehicle alone (Figure 3F). This observation was supported by the reduced white blood cells cell count in the peripheral blood and reduced spleen size on autopsy (Figure 3G).

SCLL is driven by constitutive activation of FGFR1 kinase, which in turn activates a variety of downstream signaling pathways. We have shown previously that STAT3 and STAT5 are activated in SCLL cells leading to increased MYC expression,[13] for example. Also through activation of the FRS2 domain in FGFR1, SRC is activated[14] and classic signaling cascades result from PLCG activation. We now show that part of the signaling process involves FGFR1-induced activation of Rac and that this activation leads to increased cell viability. Guanidine exchange factors (GEF) activate monomeric GTPases and GTPase activating proteins (GAP) mitigate Rac signaling.[1] The BCR protein contains a Rac GEF domain within a pleckstrin and tandem double homology domain and a GAP in the C-terminal domain,[15] which are retained in the BCR-FGFR1 chimeric kinase. The presence of both GEF and GAP domains in the same protein may suggest that the BCR-FGFR1 kinase can regulate both activation and deactivation of Rac signaling during the development of leukemia. Since Rac is also activated in the cells expressing three other FGFR1 chimeric kinases, however, which do not have homology to GEF or GAP domains, another mechanism of Rac activation in SCLL is suggested, possibly through either a PI3K⁻ or PLC-γ-dependent mechanism.[8] The implications that Rac is an essential part of the signaling cascade from FGFR1 activation also serves as a potential target in FGFR1-driven neoplasms as we have shown here, particularly in combination with FGFR1 inhibitors.

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