Alternative *TEL-JAK2* fusions associated with T-cell acute lymphoblastic leukemia and atypical chronic myelogenous leukemia dissected in zebrafish

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ABSTRACT

Background
Chromosomal translocations resulting in alternative fusions of the human *TEL* (*ETV6*) and *JAK2* genes have been observed in cases of acute lymphoblastic leukemia and chronic myelogenous leukemia, but a full understanding of their role in disease etiology has remained elusive. In this study potential differences between these alternative *TEL-JAK2* fusions, including their lineage specificity, were investigated.

Design and Methods
TEL-JAK2 fusion types derived from both T-cell acute lymphoblastic leukemia and atypical chronic myelogenous leukemia were generated using the corresponding zebrafish *tel* and *jak2a* genes and placed under the control of either the white blood cell-specific *spi1* promoter or the ubiquitously-expressed cytomegalovirus promoter. These constructs were injected into zebrafish embryos and their effects on hematopoiesis examined using a range of molecular approaches. In addition, the functional properties of the alternative fusions were investigated *in vitro*.

Results
Injection of the T-cell acute lymphoblastic leukemia-derived *tel-jak2a* significantly perturbed lymphopoiesis with a lesser effect on myelopoiesis in zebrafish embryos. In contrast, injection of the atypical chronic myelogenous leukemia-derived *tel-jak2a* resulted in significant perturbation of the myeloid compartment. These phenotypes were observed regardless of whether expressed in a white blood cell-specific or ubiquitous manner, with no overt cellular proliferation outside of the hematopoietic cells. Functional studies revealed subtle differences between the alternative forms, with the acute lymphoblastic leukemia variant showing higher activity, but reduced downstream signal transducer and activator of transcription activation and decreased sensitivity to JAK2 inhibition. JAK2 activity was required to mediate the effects of both variants on zebrafish hematopoiesis.

Conclusions
This study indicates that the molecular structure of alternative *TEL-JAK2* fusions likely contributes to the etiology of disease. The data further suggest that this class of oncogene exerts its effects in a cell lineage-specific manner, which may be due to differences in downstream signaling.

Key words: oncogenesis, TEL-JAK2, leukemia, zebrafish.

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The online version of this article has a Supplementary Appendix.

Introduction

The Janus kinase - signal transducer and activator of transcription (JAK-STAT) pathway lies downstream of cytokine receptors where it plays an important role in regulating proliferation, differentiation, and function of several cell types, particularly those of the immune and hematopoietic systems.[1] Not surprisingly, therefore, deregulation of this pathway has been associated with various hematologic malignancies.[2] For example, activating JAK1 mutations have been identified in T-cell acute lymphoblastic leukemia (ALL),[3] where they are associated with poor prognosis,[4] and acute myeloid leukemia,[5] while activating mutations of JAK3 have been observed in acute megakaryoblastic leukemia.[6,7] Activating JAK2 mutations have been observed in a high percentage of patients with classical myeloproliferative disorders,[8-10] and at a lower frequency in those with acute myeloid leukemia and chronic neutrophilic leukemia.[11,12] JAK and STAT proteins are also constitutively activated in hematopoietic cell lines transformed with different oncogenic kinases and in cells from patients with acute myeloid leukemia, ALL, chronic myelogenous leukemia (CML), and chronic lymphoblastic leukemia.[13-15] In addition, *JAK2* has been identified as a fusion partner in several leukemic oncogenes.[16,17]

TEL-JAK2 fusions are generated as a result of translocation of the *JAK2* locus on 9p24 to the *TEL/ETV6* locus on 12p13. In cases of ALL this is mediated by either of two t(9;12)(p24;p13) translocations: that seen in the pre-B-cell form of the disease creates a transcriptional fusion between exon 4 of *TEL* and exon 17 of *JAK2*, whereas that seen in the T-cell form fuses exon 5 of *TEL* and exon 19 of *JAK2*. In addition, a case of atypical CML has been reported which is the result of a unique complex t(9;15;12)(p24;q15;p13) translocation that creates a fusion between exon 5 of *TEL* and exon 12 of *JAK2*, with no involvement of *BCR-ABL* or other known oncogenes.[16,18] Each of these TEL-JAK2 variants identified has in common the *pointed* (PNT) dimerization domain of TEL fused to the JH1 tyrosine kinase domain of JAK2, resulting in constitutive activation of the kinase as well as downstream signaling pathways.[18-21] However, the CML version also contains the complete JH2 pseudokinase domain, which is known to be important in regulating kinase activity[22,23] and recently identified as possessing dual-specificity kinase activity.[24] The transformation properties of isolated *TEL-JAK2* fusions have been demonstrated *in vitro* by their ability to enable cytokine-independent growth of murine hematopoietic Ba/F3 cells.[18,19,21,25] Mice reconstituted with bone marrow cells transduced to express the T-cell ALL-associated *TEL-JAK2* oncogene variant developed a fatal mixed myelo- and T-cell-lymphoproliferative disorder.[19] Moreover, transgenic mice expressing *TEL-JAK2* in the lymphoid lineage developed a rapid onset and fatal T-cell leukemia[20] and B-cell lymphoma/leukemia.[26] STAT5 is strongly activated by each of the *TEL-JAK2* fusions and its activation is at least partially responsible for their transforming properties.[20,27] Conversely, SOCS1, a negative regulator of the JAK-STAT pathway, is induced by TEL-JAK2 and can inhibit TEL-JAK2-mediated transformation.[28-31] However, possible differences between the various TEL-JAK2 forms have yet to be established.

Zebrafish is now established as a useful model for the study of leukemic oncogenes.[32-34] In previous studies, we used relevant zebrafish orthologs to recapitulate both a myeloproliferative disease-derived *JAK2* mutant[35] and the atypical CML-derived *TEL-JAK2* fusion,[34] and demonstrated their ability to perturb hematopoiesis in zebrafish embryos. Here we use this model to further investigate the alternative *TEL-JAK2* fusions seen in ALL and CML, comparing their effects when expressed either in a white blood cell-specific or ubiquitous manner. This has delineated clear functional differences between the alternative *TEL-JAK2* forms, as well as demonstrating lineage specificity for this class of oncogene.

Design and Methods

Cloning of Flag-tagged tel-jak2a

Standard polymerase chain reaction (PCR) cloning techniques were used to splice together sequences encoding a double Flag tag, followed by residues 1-330 of zebrafish tel (equivalent to 1-337 of human TEL), followed by residues 776-1095 of zebrafish jak2a[36] (equivalent to 812-1132 of human JAK2) to recapitulate the T-cell ALL-derived TEL-JAK2 fusion.[18] After sequence verification, the fragment encoding Flag-tel-jak2a ALL was subcloned under the control of the zebrafish white blood cell-specific *spi1* promoter in pA308, as described for the equivalent CML-derived fusion.[34] Both were also cloned into pCS2+, to allow expression from the ubiquitous cytomegalovirus (CMV) promoter.[37]

Transient expression and analysis in human 293T cells

Human 293T cells were grown to 50-80% confluency before transfection with an empty vector (pCS2+) or constructs expressing either ALL- or CML-derived Flag-tel-jak2a using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). After incubation at 37°C in 10% (v/v) CO_2 for 2 days, a total cell lysate was prepared and subjected to western blot analysis with murine anti-Flag (Upstate Biotechnology Inc., Lake Placid, NY, USA), or immunoprecipitated with anti-Flag followed by western blotting with anti-phosphotyrosine 4G10 (Upstate), as previously described.[34] Alternatively, cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.5% (v/v) Triton X-100 in PBS, and washed three times with PBS, before blocking with 1% (w/v) bovine serum albumin in PBS. Cells were then incubated with 20 μg/mL anti-Flag antibody (Sigma) at 4°C overnight followed by incubation with a 1/2000 dilution of Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) for 1 h. Excess antibodies were washed away by PBS. Finally, cells were subjected to nuclear staining with 1 μg/mL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain (Sigma) and were visualized with an Olympus FluoView FV10i self-contained confocal laser scanning microscope system. In other experiments STAT3(m67)-luciferase, STAT5(β-cas)-luciferase, CMV-Renilla, CMV-stat1 and CMV-stat3 constructs were co-transfected and luciferase activity determined. JAK2 inhibitors (AG490 - 30 μM; WP1066 - 4 μM) were added as required.

Zebrafish husbandry and manipulation

Wild-type stocks of zebrafish were obtained from St Kilda aquarium (St Kilda, Victoria, Australia) and MAS imports (Coburg, Victoria, Australia), and maintained according to standard husbandry practices, as described elsewhere.[34] Microinjection was performed using Narishige micromanipulators mounted on a Leica stereomicroscope. Embryos at the one-cell stage were injected using a finely drawn capillary with approximately 1 nL of DNA solution diluted to 100 ng/μL in 0.25 M KCl. As controls, empty vector DNA and diluent only were injected in parallel. Embryos were subsequently allowed to develop in egg water [2.5% (w/v)

Na_2HPO_4; pH 6.0-6.3] on a warming tray set at 28°C. In some experiments, embryos were incubated in the presence of a pharmacological JAK2 inhibitor, or a diluent control (dimethylsulfoxide; DMSO), as performed previously.[38] Institutional and national guidelines for the care and use of laboratory animals were followed in all studies.

Molecular analyses

Techniques for whole mount *in situ* hybridization, immunohistochemistry with anti-Flag and anti-BrdU antibodies, diaminofluorene (DAF) and *O*-dianisidine staining of hemoglobin, May-Grünwald staining of blood smears and reverse transcriptase PCR analysis have been described previously.[34,38,39] For quantitation of *tel-jak2a* expression, the primers 5'-TTCATGGACTACAAAGAC and 5'-GAATGTGCTGCAGGAGCT were used, with RNA treated extensively with DNaseI prior to reverse transcriptase PCR to destroy contaminating plasmid DNA.

Results

Construction and expression of tel-jak2a fusions

For this study, a Flag-tagged fusion gene corresponding to the TEL-JAK2 form seen in T-cell ALL[18] was constructed using zebrafish *tel* and *jak2a* genes, guided by the high level of conservation at the fusion juncture (*Online Supplementary Figure S1A*). This was cloned downstream of the *spi1* promoter, for white blood cell-specific expression,[40] as previously described for the atypical CML-derived *tel-jak2a*.[34] Both fusions were also cloned downstream of the CMV promoter for ubiquitous expression[37] (*Online Supplementary Figure S1A*). The latter constructs were introduced into human 293T cells to verify the expression of the expected fusion proteins. Western blot analysis of total cell lysates using anti-Flag identified single bands with the expected molecular weight for each fusion which were absent in cells transfected with empty vector with GAPDH used as a loading control (*Online Supplementary Figure S1B*).

For the *in vivo* expression experiments, zebrafish embryos were microinjected with each of the four expression constructs at the one-cell stage. Quantitative reverse transcriptase PCR analysis on pooled embryos at 24 h post-fertilization (hpf) revealed higher expression from the *CMV* promoter compared to the *spi1* promoter but that the ALL and CML versions were expressed at similar levels in each case (*Online Supplementary Figure S1C*). Staining of embryos with an anti-Flag antibody showed that those injected with *spi1.tel-jak2a ALL* or *spi1.tel-jak2a CML* showed discrete Flag-positive cells in the posterior intermediate cell mass and across the yolk (*Online Supplementary Figure S1E,F*), a pattern consistent with endogenous *spi1*-expression.[40-42] In contrast, embryos injected with *CMV.tel-jak2a ALL* or *CMV.tel-jak2a CML* showed ubiquitous staining throughout the majority of the embryo (*Online Supplementary Figure S1G,H*). Only slight background staining was observed in uninjected embryos (*Online Supplementary Figure S1D*). This confirmed that all fusion constructs were expressed, and in appropriate cell populations.

Characterization of injected embryos

Injected embryos were examined for phenotypic perturbation from 24 hpf to 5 days post-fertilization (dpf) and compared to uninjected embryos or embryos injected with the relevant empty vector controls for the *spi1* and *CMV* promoters (pA308 and pCS2+, respectively), with particular attention to any phenotypes related to perturbation of the hematopoietic compartment. Each construct was injected on at least five independent occasions to ensure reproducibility of the results, with more than 300 embryos injected with each oncogenic construct for an accurate representation of the effects observed. Injection of all *tel-jak2a* constructs produced phenotypes consistent with severe perturbation of the hematopoietic compartment during embryonic development. Ubiquitous expression of both *tel-jak2a* constructs also caused a number of other phenotypes, including head defects as well as occasional disruption of circulation, pigmentation and curvature of the tail (*Online Supplementary Figures S2 and S3*), although no cellular hyperproliferation was observed outside the hematopoietic compartment.

At 24 hpf, all constructs produced an expansion of the posterior intermediate cell mass (Figure 1B-E,P), a site of zebrafish hematopoiesis at this time,[43] which was not observed in uninjected embryos (Figure 1A) or those injected with empty vectors (pA308 or pCS2+) (*data not shown*). This was unrelated to the infrequent circulation defects (*data not shown*). By 48 hpf, increased numbers of large, non-hemoglobinized cells were observed in the circulation in injected embryos (Figure 1G-J), compared to those not injected (Figure 1F). To examine the nature of the large non-hemoglobinized cells in more detail, blood smears were prepared and examined histologically. Samples taken from embryos injected with any of the *tel-jak2a* oncogenes contained significant numbers of white blood cells (Figure 1L-O), whereas blood from uninjected embryos contained almost exclusively red blood cells (Figure 1K). BrdU incorporation experiments confirmed that the posterior intermediate cell mass expansion observed in tel-jak2a-expressing embryos was due to increased cell proliferation (Figure 1Q-U).

Molecular analysis of hematologic compartments

To further investigate the hematologic perturbation apparent in these embryos, *in situ* hybridization was performed using specific molecular markers and stains. At 24 hpf, there was an increase in cells expressing *spi1*, an early white blood cell marker,[41,42] in embryos injected with *spi1.tel-jak2a CML* (Figure 2C,N) and *CMV.tel-jak2a CML* (Figure 2E,N), compared to uninjected embryos (Figure 2A,N). However, this was restricted to the rostral (anterior) population, with the caudal (posterior) population significantly depleted with *spi1.tel-jak2a CML*. The *spi1.tel-jak2a CML* and *CMV.tel-jak2a CML* embryos also showed a decrease in cells expressing *lysozyme* (*lyz*), a marker for mature myeloid cells derived from the rostral compartment[44] (Figure 2H,J,O), although cells expressing *mmp9*[45] were increased with *spi1.tel-jak2a CML* (Figure 2M,P), compared to uninjected embryos (Figure 2F and Figure 2K, respectively). In contrast, expression of the ALL-derived fusion did not alter the number of *lyz*+ (Figure 2G,I) or *mmp9*+ (Figure 2L,P) cells, and only *CMV.tel-jak2a ALL* led to an expansion of *spi1*+ cell numbers in the posterior intermediate cell mass (Figure 2D,N). This suggests that the effects on myelopoiesis were largely specific for the CML-derived *tel-jak2a* fusion gene. In addition, ubiquitous expression of ALL or CML-derived *tel-jak2a* fusions resulted in ectopic expression of *spi1* in a range of anterior tissues (Figure 2D,E) at 24 hpf.

At 24 hpf, there was a slight increase in the expression of *gata1*, an early erythroid marker,[46,47] in embryos injected

with *spi1.tel-jak2a CML* (Figure 3C), *CMV.tel-jak2a ALL* (Figure 3D), and *CMV.tel-jak2a CML* (Figure 3E) compared to uninjected controls (Figure 3A). In contrast, there was a slight but consistent decrease in embryos injected with *spi1.tel-jak2a ALL* (Figure 3B). Consistent with a visible anemia observed by light microscopy from 48 hpf, a significant decrease in DAF staining was seen in embryos injected with *spi1.tel-jak2a ALL* (Figure 3G) or *spi1.tel-jak2a CML* (Figure 3H) compared to uninjected controls at 52 hpf (Figure 3F). This was confirmed by O-dianisidine staining of hemoglobin (Figure 3K-M), which correlated with levels of β-embryonic globin expression (Figure 3N-O, and *data not shown*). Ubiquitous expression of these two *tel-jak2a* variants, on the other hand, caused no major differences in the number of hemoglobinized cells, although there was some disruption of circulation through the tail (Figure 3I,J).

The lymphoid compartment was also investigated by analysis of the expression of the lymphoid-specific genes, *rag1*[48] and *ikaros*.[49] In embryos injected with the *spi1.tel-jak2a ALL* oncogene, expression of *rag1* (Figure 4B) and *ikaros* (Figure 4G) was increased compared to that in uninjected embryos (Figure 4A,F). However, embryos injected with the three other *tel-jak2a* constructs exhibited normal *rag1* and *ikaros* expression patterns (Figure 4C-E,H-J). Further analysis of embryos injected with the *spi1.tel-jak2a ALL* oncogene at 7 dpf demonstrated strong *spi1* expression (Figure 4L) that was absent in controls (Figure 4K), indicating that the *spi1* promoter remains strongly active in the thymus of these embryos.

Biochemical analysis of TEL-JAK2 variants

We next sought to investigate potential functional differences between the different TEL-JAK2 variants to explain their lineage specificity. To compare their relative activities, both forms were immunoprecipitated from transfected HEK293T cell lysates with anti-Flag antibody with the resultant immune complexes analyzed by western blot with anti-phosphotyrosine antibody as a surrogate marker of activity (Figure 5A). This revealed that both fusion proteins were phosphorylated, probably through auto-phosphorylation as occurs in mammalian TEL-JAK fusion proteins.[21] Re-probing the blot with anti-Flag antibody allowed normalization of the phosphotyrosine band intensity, which revealed a greater activity of the ALL variant compared to the CML variant. To assess potential biochemical differences further, activation of the downstream STAT3 and STAT5 was analyzed using relevant luciferase reporters normalized relative to a CMV-Renilla control. This indicat-

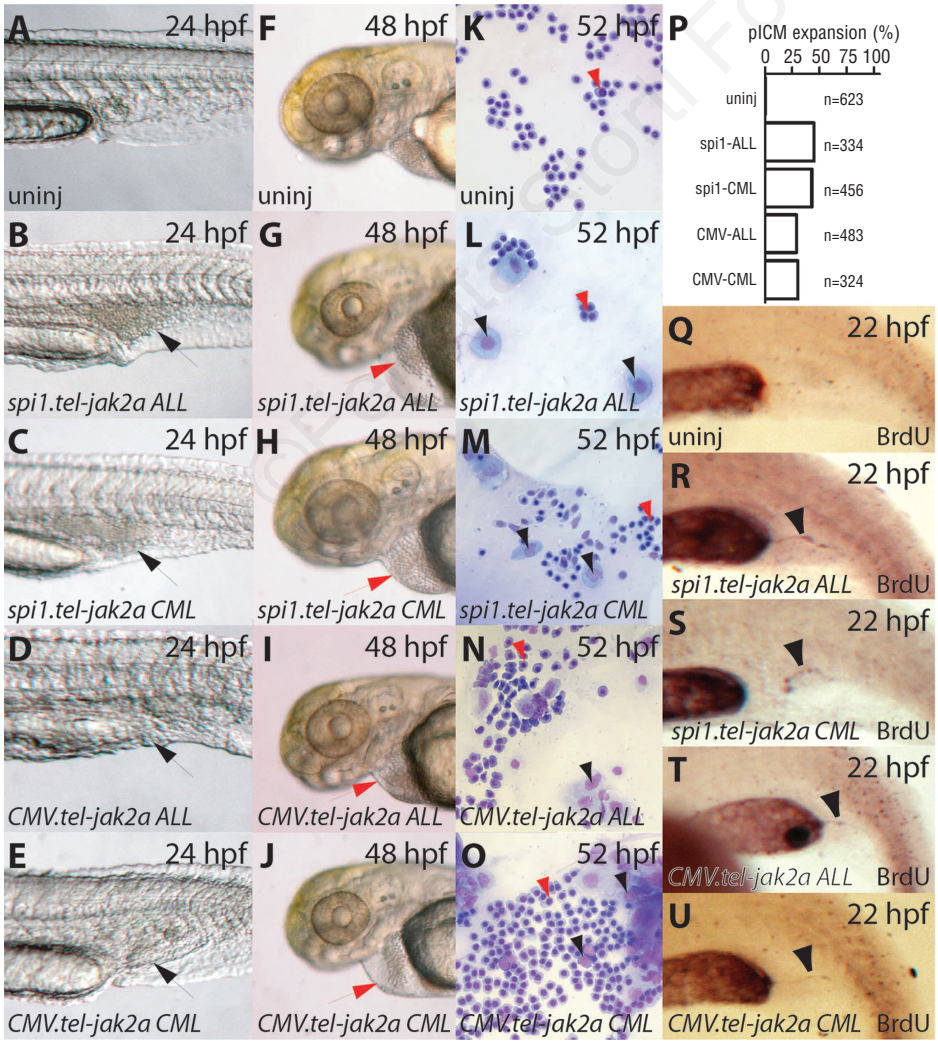

Figure 1. Hematopoietic defects caused by *tel-jak2a* fusions. (A-J) Light microscopy. Images of uninjected embryos (uninj: A, F) or embryos injected with *spi1.tel-jak2a ALL* (B, G), *spi1.tel-jak2a CML* (C, H), *CMV.tel-jak2a ALL* (D, I), or *CML.tel-jak2a CML* (E, J) at the times indicated. Expanded posterior intermediate cell mass regions in panels B-E are indicated by black arrowheads and enlarged pericardia in panels G-J indicated by red arrowheads. (K-O) Blood analysis. May-Grünwald stained blood smears from 52 hpf embryos, either uninjected (uninj: K), or injected with *spi1.tel-jak2a ALL* (L), *spi1.tel-jak2a CML* (M), *CMV.tel-jak2a ALL* (N), or *CMV.tel-jak2a CML* (O). Red arrowheads indicate red blood cells and black arrowheads indicate white blood cells. (P) Quantification of posterior intermediate cell mass expansion. (Q-U). Cell proliferation. BrdU incorporation in uninjected embryos (Q) or those injected with *spi1.tel-jak2a ALL* (R), *spi1.tel-jak2a CML* (S), *CMV.tel-jak2a ALL* (T) or *CMV.tel-jak2a CML* (U).

ed that the levels of activation of both STAT3 and STAT5 were slightly greater in the CML variant, with the largest difference seen with STAT3 (Figure 5B). To investigate possible involvement of STAT1, zebrafish stat1 or stat3 expression constructs were co-transfected with the STAT3-luciferase reporter, which can also respond to STAT1. However, only transfection of stat3 led to increased luciferase activity (*data not shown*), implying that stat1 is not involved. The relative localization of the two TEL-JAK2 variants was also analyzed by immunohistochemistry with

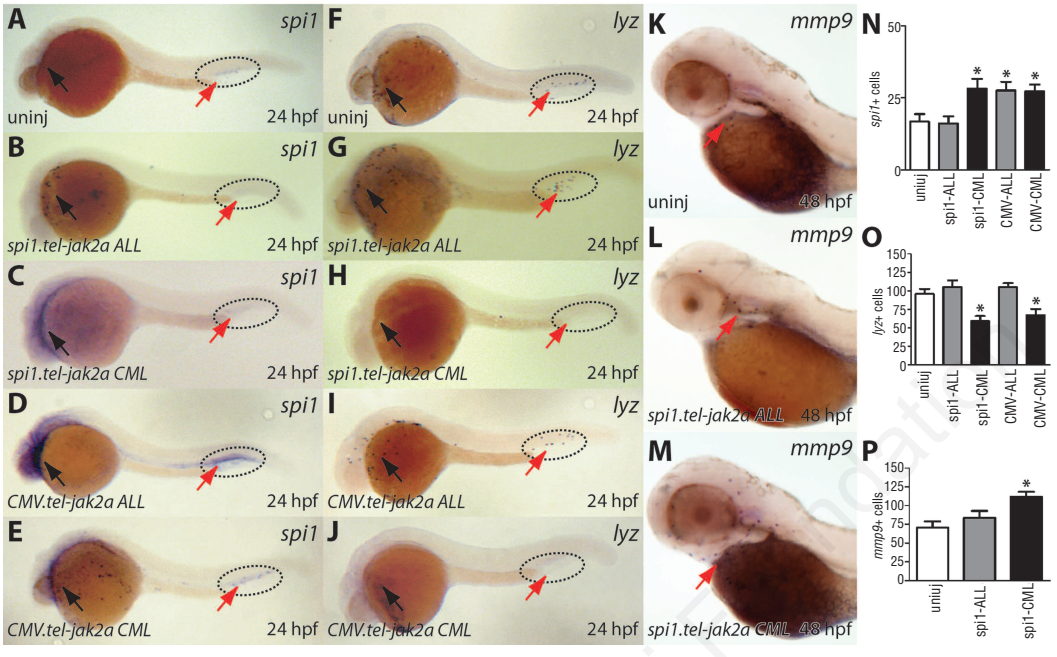

Figure 2. Characterization of myelopoiesis in embryos expressing *tel-jak2a* fusions. Uninjected embryos (uninj: A, F, K) or embryos injected with *spi1.tel-jak2a ALL* (B, G, L), *spi1.tel-jak2a CML* (C, H, M), *CMV.tel-jak2a ALL* (D, I) or *CMV.tel-jak2a CML* (E, J) were analyzed at 24 hpf for expression of the specific markers, *spi1* (A-E), *lysozyme (lyz)* (F-J) and *matrix metalloproteinase 9* (*mmp9*) (K-M), by *in situ* hybridization. The red arrows indicate the position of the yolk sac and the dotted line the area of the posterior intermediate cell mass. The numbers of *spi1* (N), *lyz* (O) and *mmp9* (P) cells were determined and are expressed as the mean ± SEM.

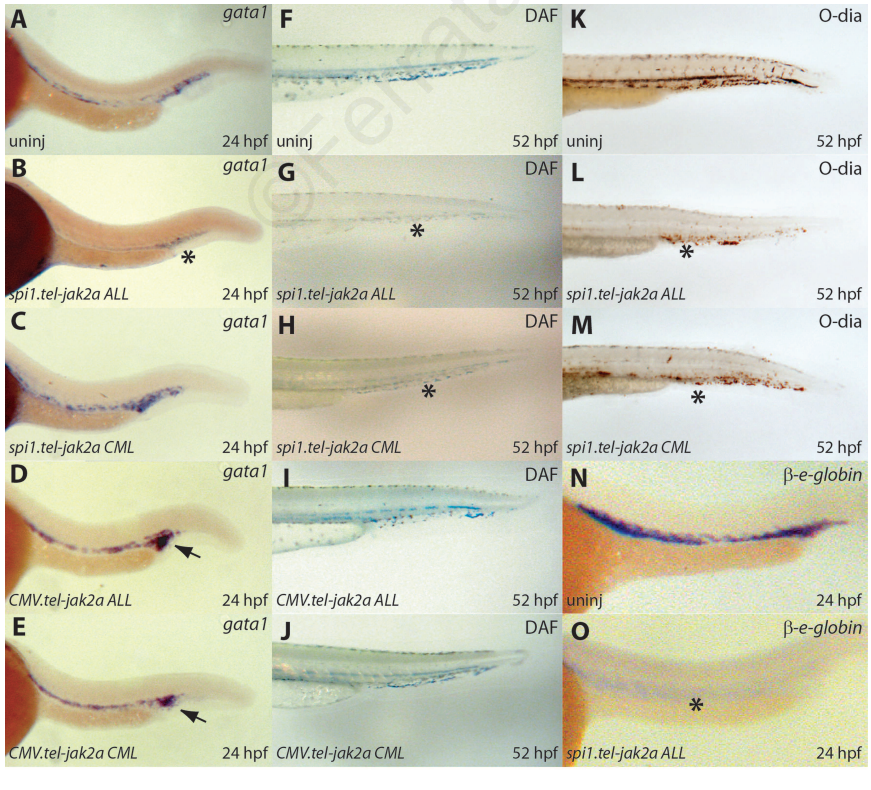

Figure 3. Characterization of erythropoiesis in embryos expressing *tel-jak2a* fusions. Uninjected embryos (uninj: A, F, K, N) or embryos injected with *spi1.tel-jak2a ALL* (B, G, L, O), *spi1.tel-jak2a CML* (C, H, M), *CMV.tel-jak2a ALL* (D, I), or *CMV.tel-jak2a CML* (E, J) were analyzed by whole-mount *in situ* hybridization (WISH) for expression of the erythroid specific markers *gata1* (A-E) and β-*embryonic globin* (β-*e-globin)* (N-O) at 24 hpf or stained with DAF (F-J) or *O*-dianisidine (K-M) at 52 hpf to detect hemoglobin (F-J). Reduced *gata1* expression in panel (B), β-*e-globin* expression in panel (O), and hemoglobin in panels (G-H) and (L-M) are indicated with black asterisks, while increased *gata1* expression is shown with a black arrow in panels (D) and (E).

anti-Flag antibody. This identified equivalent cytoplasmic expression patterns for both the ALL and CML variants (Figure 5C-E). Finally, the sensitivity of each variant to JAK2 inhibitors was investigated. By analysis of phosphotyrosine staining in transfected HEK 293T cells, both variants showed reduced activity with the inhibitor AG490, although the CML variant was more sensitive than the ALL variant (inhibition of 72% compared to 35%) (Figure 5F). A similar effect was also seen with the alternative inhibitor WP1066 (*data not shown*). Treatment of *tel-jak2a*-injected embryos with the JAK2 inhibitor AG490 largely blunted the expanded posterior intermediate cell mass mediated by both variants (Figure 5G).

Discussion

We and others have previously shown that zebrafish are susceptible to the pathological actions of a number of genes implicated in hematologic malignancies and diseases, including *Myc*, *RUNX1-CBF*, *JAK2* and *STAT5*,[32,33,35,38,50] as well as a zebrafish *tel-jak2a* fusion gene corresponding to the *TEL-JAK2* oncogene found in atypical CML[34] (Table 1). Collectively this work has demonstrated that zebrafish represents a suitable model organism for the study of leukemic oncogenes. Here we have taken advantage of this model to investigate two fundamental unanswered questions regarding *TEL-JAK2* fusions. Firstly, are there any intrinsic differences between the alternative forms of *TEL-JAK2* seen in lymphoid and myeloid leukemia that contribute to the type of malignancy induced? This was examined by directly comparing versions of *tel-jak2a* derived from T-cell ALL and atypical CML. Secondly, is *TEL-JAK2* a leukemia-specific oncogene, or is this association simply the result of being expressed in cells of this lineage via the *TEL* promoter? We investigated this question by comparing white blood cell-specific expression with ubiquitous expression of the alternative *tel-jak2a* fusions. Our results shed considerable light on both questions.

Expression of the T-cell ALL-derived zebrafish *tel-jak2a* gene under the control of the white blood cell-specific *spi1* promoter resulted in an expansion of the posterior intermediate cell mass, increased numbers of white blood cells in the circulation and anemia. Further analysis using *in situ* hybridization with lineage-specific markers revealed a later expansion of lymphoid cells. The late latency of the lymphoid phenotypes is consistent with the ontogeny of lymphoid development in zebrafish, which commences at around 3.5 dpf,[51] and is a consequence of the conserved ability of the *spi1* promoter to mediate expression in early lymphoid lineages.[52] The CML-derived *tel-jak2a* oncogene also resulted in elevated numbers of white blood cells and anemia. However, in this case there was a decrease in mature myeloid cells suggesting a block in myelopoiesis, as well as an increase in the number of immature erythroid cells, but no effects on cells of the lymphoid lineage. Collectively, this suggests that while both CML and ALL versions of *tel-jak2a* can exert a potent proliferative effect on early hematopoietic cells, different cell lineages are susceptible in each case. In contrast, the effects on the erythroid lineage were similar to those of the CML-derived fusion, with an increase in immature erythroid cells but with manifest anemia, suggesting a differentiation block in an erythroid progenitor population. This is in contrast to the effects of activating JAK2 mutants that lead to enhanced erythropoiesis.[53] There are two potential explanations for the difference. Firstly, the *tel-jak2a* fusions are expressed from the *spi1* promoter and so will not be expressed in erythroid cells, apart from the earliest precursors, in contrast to the JAK2 mutants that are expressed throughout erythropoiesis. Secondly, the activating JAK2 mutants act via receptor-associated pathways,[54] whereas the TEL-JAK2 forms lack the domains required for receptor association and are present throughout the cytoplasm, as we have demonstrated in this study.

Ubiquitous expression of either *tel-jak2a* variant caused some non-hematopoietic defects, including vasculature defects and general head and tail defects, which were also observed with activating forms of stat5 (*data not shown*), suggesting this is the likely downstream pathway. Importantly, however, while ubiquitous expression of either *tel-jak2a* variant resulted in expansion of white blood cells, no other cellular hyperproliferation was observed, indicating that the effects of this class of oncogenes are limited to cells of the hematopoietic lineage. Activating JAK2 mutations are known to promote cytokine receptor hypersensitivity[10,55] and altered regulation of downstream pathways.[56] Since the majority of cytokine receptors are expressed on immune and hematopoietic cells, this might provide some explanation for this observation.

Other studies have attempted to investigate the downstream pathways important for the effects of TEL-JAK2. These have identified STAT5,[27] TYK2,[57] SOCS1,[29] NF-κB,[58] PI-3K,[59] and various MAPK[60] as potentially playing a role. Several studies have investigated the efficacy of inhibitors

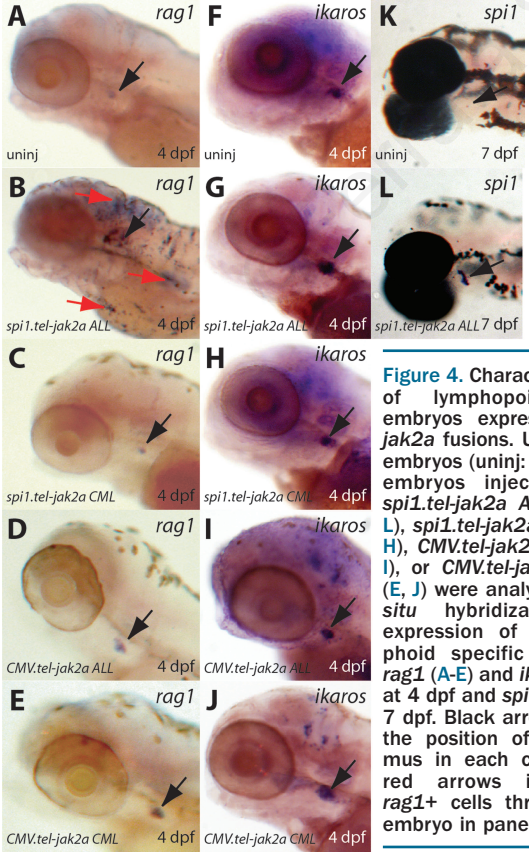

Figure 4. Characterization of lymphopoiesis in embryos expressing *tel-jak2a* fusions. Uninjected embryos (uninj: A, F, K) or embryos injected with *spi1.tel-jak2a ALL* (B, G, L), *spi1.tel-jak2a CML* (C, H), *CMV.tel-jak2a ALL* (D, I), or *CMV.tel-jak2a CML* (E, J) were analyzed by *in situ* hybridization for expression of the lymphoid specific markers *rag1* (A-E) and *ikaros* (F-J) at 4 dpf and *spi1* (K, L) at 7 dpf. Black arrows show the position of the thymus in each case, with red arrows indicating *rag1*+ cells through the embryo in panel B.

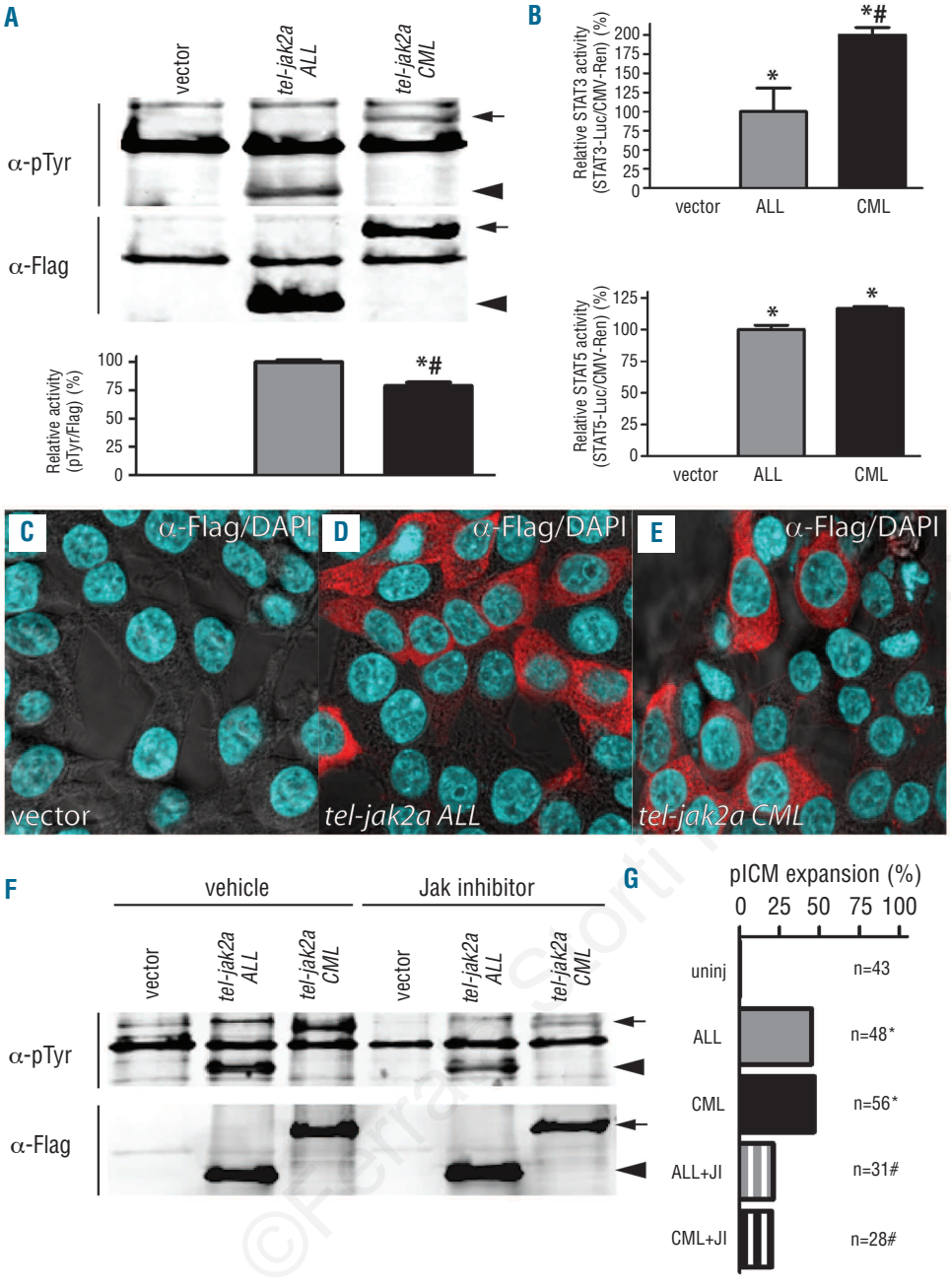

Figure 5. Functional properties of tel-jak2a fusions. (A) Activity of tel-jak2a fusions. Lysates from HEK293T cells transfected with the indicated constructs were immunoprecipitated with α-Flag and analyzed by western blot with α-phosphotyrosine (α-pTyr) and α-Flag (upper panels), with the relative levels of tyrosine phosphorylation (pTyr/Flag) determined by image analysis, with *tel-jak2a ALL* set at 100% (lower panel). The tel-jak2a CML protein is indicated with an arrow and the tel-jak2a ALL protein with an arrowhead. (B) Downstream STAT activation by tel-jak2a fusions. HEK293T cells were co-transfected with empty vector, *CMV.tel-jak2a ALL* and *CMV.tel-jak2a CML* as indicated, along with STAT-luciferase and CMV.Renilla constructs, with the relative activation of STAT3 (upper panel) or STAT5 (lower panel) reporter determined relative to *CMV.tel-jak2a ALL* at 100%. (C-E) Localization of tel-jak2a fusions. HEK293T cells were transfected with the indicated constructs and co-stained with α-Flag/Alexa fluor 568nm (red) and DAPI (blue). (F-G) Sensitivity of tel-jak2a fusions to JAK2 inhibitors. HEK293T cells were transfected and analyzed as in panel (A) in the absence or presence of 30 μM AG490 (F). Alternatively, zebrafish were injected with the indicated constructs with or without 30 μM AG490 and scored for posterior intermediate cell mass expansion (G).

Table 1. Summary of consequences of JAK2 perturbation in zebrafish.

Cell population	Marker/ assay	stat5.1[ca]	jak2a[ca]	tel-jak2a ALL	tel-jak2a CML
HSC/HPC	pICM	↑	±	↑	↑
early myeloid + lymphoid	*spi1*	↑	↑	±	↑
late myeloid	*lyz/ l-plastin*	↑	↑	±	↓
late myeloid	*mmp9/ mpo*	↑	↑	±	↑
early erythroid	*gata1*	↑	↑	↓	↓
late erythroid	e-globin/ Hb	↑	↑	↓	↓
lymphoid	*rag1/ ikaros*	↓	nt	↑	±

pICM: posterior intermediate cell mass; HSC: hematopoietic stem cells; HPC: hematopoietic progenitor cells.

that can suppress the effects of TEL-JAK2.[61,62] We showed that both forms of TEL-JAK2 were sensitive to JAK2 inhibitors, although the ALL form appeared slightly less sensitive. This information is clinically significant, and highlights the usefulness of zebrafish as a pre-clinical model to dissect these pathways.

The presence of a complete JH2 domain is the only difference between the two forms, and so this region of the fusion oncoprotein must be responsible. The mechanism by which this so-called "pseudo-kinase domain" facilitates the effect has remained unknown. This domain is known to act as a regulator of JAK kinase activity,[23,63] with mutation of this domain leading to myeloproliferative disorders in humans, mice, fish and flies.[22,35,38,64,65] Consistent with this, the ALL version that lacks the JH2 domain showed greater activity, which is in accordance with other studies on human TEL-JAK2 fusions.[66] Somewhat surprisingly, however, the CML version showed greater STAT activation, par-

ticularly of STAT3, which represents a novel downstream target for TEL-JAK2. Analysis of the conserved tyrosine residues within the JH2 domain which are unique to the CML-derived TEL-JAK2 provide a potential mechanism to explain this difference (*Online Supplementary Figure S4*). One of these, Y570, has the sequence DYG(E/Q)(V/L) which is in good agreement with consensus STAT5 docking sites.[67,68] Another, at Y637 has the sequence YLK(R/K), which fits the alternative STAT3 site of Y-hydrophobic-basic-large hydrophilic.[69] Recently, the JH2 domain was also found to possess both tyrosine and serine kinase activity,[24] which might also contribute to the differential levels of STAT activation – and potentially the phenotypic differences – observed. It is worth noting that activating JH2 domain mutants of JAK2 have also been identified in human lymphoid disorders.[70]

Authorship and Disclosures

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