The role of jak2a in zebrafish hematopoiesis

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Abstract

Janus kinase 2 (Jak2) transduces signals from hematopoietic cytokines and a gain-of-function mutation (Jak2^{V617F}) is associated with myeloproliferative diseases particularly polycythemia vera. In this study, we examined the role of jak2a in zebrafish embryos in knock-down and over-expression studies using morpholinos (MOs) targeting the 5’untranslated region (UTR) (jak2a^{UTR}-MO) and splice-site junction (jak2a^{SS}-MO) of jak2a, a Jak inhibitor AG490 and a constitutive-active form of jak2a (jak2a^{ca}). At 18 and 24 hours-post-fertilization (hpf), jak2a is expressed predominantly in the intermediate cell mass (ICM, site of primitive hematopoiesis) of wild-type and chordin morphant embryos (characterized by expansion of ICM). Both jak2a MOs and AG490 reduced gata1^{+} (erythroid) cells in Tg(gata1:GFP) embryos, stat5 phosphorylation and gene expression associated with early progenitors (scl and lmo2), erythroid (gata1, ahel and βhe1) and myeloid [spi1 (early) and mpo (late)] lineages. The chordin morphant is associated with increased stat5 phosphorylation and both jak2a MOs and treatment with AG490 significantly ameliorated ICM expansion and hematopoietic gene up-regulation in these embryos. Injection of plasmid encoding jak2a^{ca} significantly increased erythropoiesis and expression of gata1, ahel and βhe1, spi1, mpo and l-plastin. In conclusion, zebrafish jak2a is involved in primitive hematopoiesis under normal and deregulated conditions.
Introduction

The Janus kinase (Jak)/signal transducer and activation of transcription (Stat) cascade is a ubiquitous intracellular pathway that transduces signals from extracellular ligands. In mammals, the Jak family of protein tyrosine kinases consists of four members (Jak1-3, Tyk2) of which Jak2 transduces signals of hematopoietic cytokines to enhance erythroid cell proliferation.¹ The recent identification of a gain-of-function mutation of Jak2 in human polycythemia vera (PV) has revised the diagnosis and perhaps future treatment of this disorder.² In PV, substitution of valine by phenylalanine at amino acid 617 in the auto-regulatory JH2 domain (V617F) results in constitutive activation of Jak2 (Jak2⁶¹⁷F), conferring proliferative and survival advantage to erythroid progenitors.³ As a result, patients suffer from erythrocytosis and hence thrombosis and bleeding. A similar gain-of-function mutation in the Jak JH2 domain has also been described in the single Drosophila Jak, which is encoded by the hopscotch (hop) locus. A glutamic acid to lysine substitution at residue 695 (E695K) in the JH2 domain of the Hop protein resulted in hyperphosphorylation of the Drosophila Stat (D-Stat) and over-production of primitive blood cells.⁴

The zebrafish has emerged as a model organism of the study of hematopoiesis during embryonic development and neoplastic transformation.⁵ In this organism, primitive
and definitive hematopoiesis arise successively in the intermediate cell mass (ICM) and the ventral wall of the dorsal aorta in the developing embryos.\textsuperscript{6} In zebrafish, the gene encoding for Jak2 has undergone duplication and subsequent specialization: \textit{jak2a} is expressed predominantly in the ICM and \textit{jak2b} in the developing eyes and pronephric ducts.\textsuperscript{7} However, functional studies of \textit{jak2a} in zebrafish are lacking. We have previously demonstrated using microarray analysis that \textit{jak2a} expression was significantly up-regulated in the ICM of the zebrafish \textit{chordin} morphant that is characterized by expansion of primitive hematopoiesis.\textsuperscript{8} This suggested that \textit{jak2a} may be involved in the hematopoietic expansion of this morphant.

In this study, we performed both qualitative and quantitative analyses to specifically examine the role of \textit{jak2a} in zebrafish hematopoiesis using morpholino knock-down. Furthermore, we investigated if \textit{jak2a} activation is involved in the hematopoietic expansion of the \textit{chordin} morphant and evaluated the effects of constitutive activation of \textit{jak2a} on hematopoiesis.

**Methods and Materials**

The study has been approved by the Committee of the Use of Laboratory and Research Animals (CULATR) in the University of Hong Kong.
**Zebrafish and modulation of jak2a**

Danio rerio (wild-type) were obtained from local aquarium and were maintained and raised under standard conditions at 28°C. Wild-type embryos were obtained from natural spawning and were staged according to Kimmel et al.9 Transgenic zebrafish lines Tg(gata1:GFP)10 and Tg(fli1:GFP)11 were obtained from Tsinghua University (gift from Dr. Anming Meng) and ZFIN. Anti-sense morpholinos (MOs) (Gene-Tools, LLC, OR, USA) were designed to target at the 5’untranslated region (UTR) of the respective genes. MOs were also designed at the intron-exon junction (splicing site, SS) to induce defective splicing (Table S1). Where appropriate, a random sequence MO was used as a control,12,13 which has no effect on the morphology and erythropoiesis of the developing embryos (unpublished). Solutions of these were prepared and injected into embryos at the 1-4 cell stage, which were maintained at 28°C until analyzed. In some experiments, jak2a activity was suppressed by incubating 1-cell stage embryos with a soluble Jak2 inhibitor, AG490 (Calbiochem, EMD Bioscience, San Diego,CA, USA)14 until analysis. Protocols for whole-mount in-situ hybridization and O-dianisidine staining have been described previously.8,12,13

A wild-type zebrafish jak2a clone in the mammalian expression vector pCS2+ has been described previously.7 Mutagenesis to generate a hyperactive E629K mutant
equivalent to the Drosphila hopscotch T42 mutation⁴ was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), following the manufacturer’s recommendations. Sequence analysis confirmed that the change had been correctly introduced in the absence of extraneous mutations. For rescue experiments, RNA encoding wild-type jak2a was generated by in-vitro transcription and injected as described above.

Western Blotting

Total embryo protein extract was prepared by homogenizing 50 embryos at 18 hpf in 50 μl protein extraction buffer [Tris.HCl (63 mmol/L), pH 6.8, glycerol (10%, v/v), β-mercaptoethanol (5%, v/v), SDS (3.5%, w/v)] and boiling for 1 minute. Protein extracts (100 μg) were fractionated on a 10% (w/v) SDS polyacrylamide gel and electro-transferred to nitrocellulose membrane (Protran®, PerkinElmer Life Science, Boston, MA, USA). The membrane was then blocked in blocking buffer (5% non-fat milk in Tris-buffered Saline (TBS)) for 1 hr at room temperature, followed by overnight incubation at 4°C with primary antibody, either 1:250 rabbit anti-Stat5 antibody (Cell Signaling Technology, Beverly, MA, USA) or 1:250 rabbit anti-phospho-Stat5 antibody (Zymed laboratories, South San Francisco, CA, USA). Afterwards, the membrane was washed in TBS + 0.05% (v/v) Tween-20 (TBST)
followed by incubation with HRP-linked ECL donkey anti-rabbit IgG antibody (1:1000) at 4°C (Amersham Biosciences, Buckinghamshire, UK). The membrane was then washed with TBST followed by TBS before detection with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and Hyperfilm™ ECL (Amersham Biosciences, Buckinghamshire, UK). To re-probe the same membrane with another primary antibody, the membrane was incubated in stripping buffer [Tris.HCl (62.4 mmol/L at pH 6.7), β-mercaptoethanol (100 mmol/L), SDS (2%, w/v)] at 50°C for 15 min, followed by repeated washing in TBST.

Flow cytometry

Tg(gata1:GFP) embryos at 18 hpf were dechorionated and digested with 0.05% Trypsin/EDTA solution (Invitrogen, Carlsbad, CA, USA) for 15 min at 28°C, then completely dissociated to single-cell suspension by pipetting. Trypsin digestion was terminated by CaCl₂ (2 mmol/L) and the whole suspension was filtered through a 40 µm cell strainer (BD Falcon, BD Biosciences Discovery Labware, Bedford, MA, USA). Cells were washed and harvested in PBS (phosphate-buffered saline) with 2% (v/v) FBS and the percentage of GFP⁺-cells enumerated by flow cytometry.

Real-time quantitative RT-PCR (Q-RT-PCR) assay
Q-RT-PCR was performed to examine the relative expression of genes specific for early progenitor (*sel, lmo-2, gata2*), erythroid (*gata1, alpha and beta embryonic Hb 1, ahe1 and bhe1*), early myeloid (*spi1*), heterophilic granulocyte (*myeloperoxidase, mpo*) and macrophage (*l-plastin*) lineages using the ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA, USA). Embryos at 12 and 18 hpf were dechorionated and RNA was extracted using Trizol reagent as per manufacturer’s protocol (Life Technologies, OK, USA). Thirty embryos were included in each experiment. RNA was reverse transcribed and PCR was performed using the SYBR Green Method. Primer sequences used for Q-RT-PCR were shown in Table S1.

To ensure that the amplification efficiency for all marker genes in the Q-RT-PCR was equal, RNA was extracted before the project from a group of 40 embryos at 24 hpf followed by reverse transcription. Serial dilutions of samples ranging from 0.0025 to 125 ng of input cDNA were frozen at -20°C in small aliquots (standards). Once thawed, each aliquot was used only once. Relative quantification of each gene expression was performed by the comparative $C_T$ methods.

Statistical analysis
Data were expressed as mean ± S.E.M. (standard error of mean, which is calculated by dividing the standard deviation (S.D.) by the square root of the number of replicate experiments) Comparisons between numerical data were evaluated by paired Students’ t-tests. A p-value of < 0.05 was considered statistically significant.
Results

jak2a is expressed in the ICM of wild-type embryos and chordin morphants

We first examined the expression of jak2a during zebrafish embryonic development using whole-mount in-situ hybridization. In agreement with another study, jak2a was expressed predominantly in the ICM of zebrafish embryos at 18 and 24 hpf (Figure 1A,B,E). Expression of jak2a was significantly up-regulated within the expanded ICM of the chordin morphant at both time points (Figure 1C,D,F), confirming our previous observations based on microarray analysis.

Knockdown of jak2a affects hematopoiesis

To investigate the role of jak2a in primitive hematopoiesis, we injected the jak2a^{UTR}-MO into zebrafish embryos at the 1-4 cells stage. Initial experiments showed that most embryos tolerated 7.5 ng and developed normally at 24 and 48 hpf (Figure 1G-J). However, at higher doses this MO caused excessive toxicity and lethality. Therefore, in all subsequent experiments the embryos were injected with a dose of 7.5 ng (referred hereafter as jak2a^{UTR} embryos). Erythropoiesis was reduced in these embryos at 48 hpf as shown by O-dianisidine staining (Figure 1K,L). To confirm the specificity of this result, a jak2a splice-site MO targeting an exon-intron junction (jak2a^{SS}-MO, 2 ng) was injected. These jak2a^{SS} embryos showed a similarly
reduced erythropoiesis at 48 hpf (Figure 1M). Moreover, the efficacy of the 

jak2a\textsuperscript{SS}-MO could be demonstrated by RT-PCR showing significantly reduced levels of \textit{jak2a} transcript (Figure 1O-P). To further correlate tyrosine kinase activity with primitive hematopoiesis, we incubated freshly spawned embryos with AG490 (50 \textmu mol/L), a known soluble inhibitor of Jak2 (jak2a\textsuperscript{AG} embryos). At 48 hpf, O-dianisidine staining was also markedly reduced in these embryos, corroborating with the proposition that jak2a regulates primitive hematopoiesis during zebrafish embryonic development (Figure 1N).

We performed quantitative analysis on the effects of jak2a knock-down on gata1\textsuperscript{+} population using transgenic Tg(gata1:GFP) embryos at 18 hpf, before the onset of functional circulation. In these embryos, GFP\textsuperscript{+} cells represent cells committed to the erythroid lineage. In uninjected embryos, the GFP\textsuperscript{+} population constitutes 4.47±0.18\% of the dissociated embryos. A significant reduction in the GFP\textsuperscript{+} population was observed in jak2a\textsuperscript{UTR} (3.56±0.58\%, \textit{p}=0.017), jak2a\textsuperscript{SS} (2.68±0.40\%, \textit{p}=0.01) and jak2a\textsuperscript{AG} (3.31±0.18\%, \textit{p}=0.003) embryos (Figure 2A). Furthermore, co-injection of embryos with both jak2a\textsuperscript{UTR-MO} (7.5 ng) and jak2a\textsuperscript{SS-MO} (2 ng) resulted in a further reduction of erythropoiesis (2.15±0.10\%, \textit{p}<0.001) compared with either of these MOs alone, demonstrating synergism and hence specificity of
these MOs on jak2a gene function. Specificity of jak2a\textsuperscript{UTR}-MO was further demonstrated by a reversal of its inhibitory effect on erythropoiesis after rescue by wild-type jak2a mRNA (75 pg) co-injection (4.25±0.03%, p=0.86). Collectively, these observations confirmed the findings with O-dianisidine staining that jak2a knock-down resulted in a decrease in erythropoiesis.

\textit{jak2a knock-down induces changes in stat5 phosphorylation}

To confirm that the reduced erythropoiesis in zebrafish embryos after down-modulation of jak2a was related to reduction in Jak/Stat signaling, we examined the phosphorylation status of endogenous stat5 protein in these embryos. Although the amount of stat5 protein was unchanged in jak2a\textsuperscript{UTR}, jak2a\textsuperscript{SS} and jak2a\textsuperscript{AG} embryos, stat5 phosphorylation was significantly reduced as compared with uninjected embryos (Figure 2B). The results supported the notion that stat5 mediates the downstream effects of jak2a.

\textit{jak2a knock-down has no effect on angiogenesis}

The effects of jak2a knock-down on hematopoiesis led us to examine its effects on angiogenesis as both processes arise from a common precursor, known as the hemangioblasts.\textsuperscript{15} Moreover, in the zebrafish \textit{cloche} mutant whose blood and
vascular formation are defective, *jak2a* expression was significantly down-regulated.\(^7\)

We injected *jak2a\(^{UTR}\)-MO into transgenic Tg(fli1:GFP) embryos at 1-4 cell stage. At 48 hpf, both vasculogenesis and angiogenesis was not affected as shown by the intact axial and inter-segmental vessels (Figure 2C,D). A patent circulation in these vessels was further confirmed by microangiography (data not shown).

*Gene expression analysis*

In order to understand the regulatory role of *jak2a* in the hematopoietic hierarchy, we examined the expression of a panel of hematopoietic genes in the *jak2a\(^{UTR}\)* embryos by Q-RT-PCR (Table 1). At 12 hpf, expression of *scl, lmo2, gata1, ahel and bhel* were significantly reduced in *jak2a\(^{UTR}\)* embryos, while expression of *mpo* was unaffected. At 18 hpf, expression of genes encoding for all these hematopoietic genes were reduced. At both time points, *spi1* appeared to be down-regulated although the difference from uninjected embryos did not reach statistical significance.

Expression of *fli1* was not affected in the *jak2a\(^{UTR}\)* embryos. Intriguingly, expression of *l-plastin* was significantly up-regulated in *jak2a\(^{UTR}\)* embryos at both time points.

*Hematopoietic expansion in the chordin morphant is mediated by jak2a*

The up-regulation of *jak2a* expression in the *chordin* morphant in which there is
expansion of primitive hematopoiesis led us to investigate whether increased jak2a activity may mediate the hematopoietic expansion in these embryos. Wild-type embryos injected with chordin MO (0.75 ng) induced expansion of ICM in nearly 90% of embryos (Figure 3A-B). However, co-injection of chordin and jak2a<sub>UTR</sub>MOs resulted in significant reduction in ICM expansion both in the average size of the expansion as well as the number of embryos showing significant expansion (defined arbitrarily by ICM ≥ 5 somites) (Figure 3C). Similar changes were recapitulated by co-injection of chordin and jak2a<sub>ss</sub>-MO or by incubating chordin MO injected embryos with AG490 (Figure 3D). Using flow cytometry on Tg(gata1:GFP) embryos at 18 hpf, jak2a<sub>UTR</sub>-MO, jak2a<sub>ss</sub>-MO as well as AG490 were able to reduce the increase in GFP<sup>+</sup> population in the chordin morphant (Figure 3E). Q-RT-PCR showed that the expression of scl, lmo2, gata1, ahl and βhe1, spil, mpo and l-plastin were up-regulated in the chordin morphant at 12 and 18 hpf (Table 1). With the exception of l-plastin, the increases in gene expression were ameliorated by co-injection with Jak2<sub>UTR</sub>-MO.

**Constitutive activation of jak2a stimulates hematopoiesis**

The results from the chordin morphant suggested that activation of jak2a in zebrafish embryos may increase hematopoiesis. To test this hypothesis, we injected the
plasmid containing the constitutive active form of jak2a (jak2a\textsuperscript{ca}, 150 pg) (Figure 4 A) into 1-cell stage wild-type or Tg(gata1:GFP) embryos and examined its effects on hematopoiesis, stat5 phosphorylation and gene expression. The jak2a\textsuperscript{ca} embryos had normal morphology (Figure 4 B-E). When jak2a\textsuperscript{ca} was injected into Tg(gata1:GFP), there was a significant increase in gata1\textsuperscript{+} cells (Uninjected embryos: 4.26±0.06% vs jak2a\textsuperscript{ca} embryos: 5.26±0.14%, p=0.019, n = 4 separate paired experiments including a total of 120 embryos) (Figure 4 F,G). Furthermore, stat5 phosphorylation was significantly increased in the jak2a\textsuperscript{ca} embryos. Similar increase was observed in the chordin morphant embryos (Figure 4H). In addition, expression of gata1, ahe1, bhe1, spil, mpo and l-plastin was significantly increased. On the other hand, those encoding for scl, lmo2 and fli1 did not show any changes in the jak2a\textsuperscript{ca} embryos (Table 2).
Discussions

In this study, we examined the role of jak2a during embryonic hematopoiesis in zebrafish. Injection of jak2a^{UTR}-MO significantly reduced primitive hematopoiesis, as shown qualitatively by O-dianisidine staining and quantitatively by flow cytometry of dissociated Tg(gata1:GFP) embryos and by real-time quantitative PCR. Enumeration of gata1^{+} cells at 18 hpf is specific for erythropoiesis, as thrombopoiesis, which is also under gata1 regulation, occurs at a later time point during embryonic development. Specificity of the MO was shown by the synergism between this and the jak2a^{ss}-MO, which induced defective splicing of jak2a pre-mRNA, and by use of a pharmacologic inhibitor to reproduce the observed phenotypes. Moreover, the hematopoietic defects of the jak2a^{UTR} embryos could be rescued by wild-type jak2a mRNA. The two MOs were different in their efficacies of perturbing hematopoiesis under basal and stimulated conditions. Whereas the efficacy of jak2a^{ss}-MO could be tested by RT-PCR, that of jak2a^{UTR}-MO were not testable due to the lack of specific antibody against zebrafish jak2a. Notwithstanding this limitation, the overall results were consistent with earlier observations showing reduced jak2a expression in the cloche mutant and corroborated with the proposition that jak2a mediates primitive hematopoiesis in zebrafish. In mice, the function of Jak2 in embryonic hematopoiesis could not be examined mechanistically because homozygous Jak2^{null}
mice are embryonic lethal at day 12.0-13.0 post-coitus.\textsuperscript{18,19} Therefore, the zebrafish system has provided us with a unique model for the examination of this gene with hitherto uncertain function during embryonic development and we have made a number of observations that may shed light to our understandings of jak2a during normal and deregulated hematopoiesis.

First, we demonstrated that jak2a plays a non-redundant role in the initiation of primitive hematopoiesis. In particular, genes associated with early hematopoietic specification (\textit{scl} and \textit{lmo2}) as well as erythroid (\textit{gata1}, \textit{ahe1} and \textit{bhe1}) and myeloid [\textit{spi1} (early) and \textit{mpo} (late)] differentiation were down-regulated in the jak2a\textsuperscript{UTR} embryos as early as 12 hpf. Therefore, jak2a might be required at the level of early hematopoietic progenitor/stem cells before they differentiate into distinct erythroid and myeloid lineages. We have recently shown that zebrafish \textit{stat5.1} is crucial for multi-lineage hematopoietic development (RS Lewis, C Liongue, AC Ward, manuscript submitted). Given the tight correlation between jak2a activation and stat5 phosphorylation observed in this study, it is likely that the jak2a-stat5.1 signaling module is critically involved in the cell-fate decision of early hematopoietic progenitor/stem cells. Intriguingly, \textit{l-plastin}, which is associated with primitive macrophage development\textsuperscript{20}, was significantly upregulated in jak2a\textsuperscript{UTR} embryos at
both 12 and 18 hpf. This lineage appears to be derived from an anterior hematopoietic precursor population distinct from the posterior ICM population.\textsuperscript{21,22}

Whether the paradoxical up-regulation of \textit{l-plastin} represented skewing of hematopoietic progenitor cell-fate from erythro-myeloid towards a macrophage lineage, or from a posterior to an anterior precursor population, warrants further examination.

Second, we demonstrated that erythrocytosis in zebrafish embryos is mediated by jak2a activation, consistent with enforced expression of a tel-jak2a fusion.\textsuperscript{23} The proposition was first supported by the study of \textit{chordin} morphant whose hematopoietic expansion was significantly ameliorated by jak2a knock-down. Furthermore, constitutive activation of jak2a also resulted in a significant increase (24\%) in hematopoiesis, as defined quantitatively by flow cytometry in Tg\textit{(gata1:GFP)} embryos. Such an increase in erythropoiesis might not manifest morphologically in the developing embryos and non-quantitative whole-mount in-situ hybridization did not show any consistent increase in gata1 staining (unpublished). However, in consistent with the data from flow cytometry, we were able to demonstrate both an increase in erythroid (\textit{gata1, ahel} and \textit{βhel}) and myelomonocytic gene expression (\textit{spi1, mpo} and \textit{l-plastin}) using quantitative real-time PCR as well as an increase in
stat5 phosphorylation with Western blotting. Notably, constitutive activation of stat5.1 has also been shown to increase \( gata1^{+} \) and \( spi1^{+} \) populations in zebrafish embryos.\(^{21}\) The \( jak2a^{ca} \) used in the present study was generated based on the Drosophila Hop\(^{T42}\) mutant carrying a gain-of-function mutation (E695K) at the JH2 domain.\(^{4}\) In this mutant, proliferation of primitive hematopoietic cells was increased and the Drosophila Jak/Stat pathway was activated.\(^{24}\) Our observations therefore highlight a conserved phenomenon in which gain-of-function mutations of the JH2 domain in Jak2 induce hematopoietic proliferation in Drosophila, zebrafish and human via activation of Stat signaling. Intriguingly, constitutive \( jak2a \) activation had no effect on \( scl \) or \( lmo2 \) expression. Therefore, the effects of \( jak2a \) stimulation (presumably via stat5.1) appear to be restricted to the lineage-committed erythroid/myeloid precursors rather than the early hematopoietic progenitor cells.

Third, we demonstrated that neither knock-down or constitutive activation of \( jak2a \) had any effect on endothelial cell specification, as shown by Q-RT-PCR for \( fli-1 \) and the intact vasculature in Tg(fli1:GFP) embryos injected with \( jak2a^{UTR} \)-MO. This issue has not been addressed in murine model as Jak2 knock-out results in early embryonic lethality.\(^{18,19}\) Remarkably, the \( jak2a^{UTR} \) embryos showed specific reduction of gene expression associated with early hematopoiesis but not
vasculogenesis, and may be useful as a tool whereby these hitherto intertwined processes can be dissected. Our data also suggested that jak2a functions at a higher level in the hematopoietic hierarchy than phospholipase C gamma 1 (PLCγ1) during development as specific knock-down of PLCγ1 reduces gata1, ahel and bhe1 but not scl or lmo2 expression.13

In the present study, the use of flow cytometry in dissociated transgenic embryos and Q-RT-PCR has enabled us to examine zebrafish hematopoiesis objectively and quantitatively. In fact, the differential gene expression induced by jak2aUTR-MO could not be differentiated reliably by whole-mount in-situ hybridization (unpublished data), which is non-quantitative and is subject to biases due to variations in embryo staining. Therefore, quantitative analyses such as those described herein represent a useful supplement to non-quantitative tests in future study of zebrafish embryonic hematopoiesis.

The results of this study are of clinical relevance. Recent identification of Jak2V617F in PV23 suggested that specific targeting of Jak2 signaling may lead to better treatment for this disorder. The jak2aCa embryos may therefore shed light to our understanding of human PV and provide us with a robust model for the screening of
potential therapeutic agents used for this and other Jak2 related blood diseases. A zebrafish equivalent of jak2^{V617F} mutation has also been generated (unpublished data) and this model will enable us to dissect the pathogenesis of jak2^{V617F} induced neoplastic transformation. On the other hand, the upregulation of jak2a in chordin morphants and the amelioration of expanded hematopoiesis by jak2a knock-down have provided us with two possible leads for further studies. First, a possible link between BMP and jak2a signaling should be examined (Figure 5). In particular, whether the increase in jak2a signaling in the embryos was due to a cross-talk between smad and stat pathways as reported in neural stem cells^{25} or a simple increase in hematopoietic cell population in the embryos should be further studied. Second, previous studies have demonstrated the pivotal role of BMP4 in mesoderm induction and hematopoietic differentiation during embryonic development^{26}, whether and how deregulation of BMP/Smad signaling might link to human leukemia would have to be further evaluated.
Acknowledgements

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Authors’ Contribution

A.C.H. Ma conducted the study and wrote the manuscript. A.C. Ward conducted part of the experiments. R. Liang analyzed the data and wrote the manuscript. A.Y.H. Leung designed the research, analyzed the data and wrote the manuscript.
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Table 1. Differential gene expression in wild-type, jak2aUTR, chordin (chd) morphant and Chd+ jak2aUTR embryos

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<th>jak2aUTR</th>
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<th>Chd</th>
<th>Chd+ jak2aUTR</th>
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<th>Chd</th>
<th>Chd+ jak2aUTR</th>
<th>p-value$^\psi$</th>
</tr>
</thead>
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<tr>
<td>18 hpf</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scl</td>
<td>1.00</td>
<td>0.17±0.02</td>
<td>0.001</td>
<td>9.56±1.51</td>
<td>0.19±0.02</td>
<td>0.000</td>
</tr>
<tr>
<td>lmo-2</td>
<td>1.00</td>
<td>0.66±0.05</td>
<td>0.011</td>
<td>3.55±0.30</td>
<td>0.82±0.05</td>
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<tr>
<td>gata1</td>
<td>1.00</td>
<td>0.57±0.07</td>
<td>0.018</td>
<td>24.89±2.89</td>
<td>12.64±0.77</td>
<td>0.002</td>
</tr>
<tr>
<td>αhe1</td>
<td>1.00</td>
<td>0.48±0.09</td>
<td>0.036</td>
<td>9.24±0.85</td>
<td>0.52±0.13</td>
<td>0.006</td>
</tr>
<tr>
<td>βhe1</td>
<td>1.00</td>
<td>0.46±0.09</td>
<td>0.037</td>
<td>2.37±0.04</td>
<td>0.53±0.08</td>
<td>0.005</td>
</tr>
<tr>
<td>spi1</td>
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<td>0.51±0.15</td>
<td>0.107</td>
<td>12.48±2.61</td>
<td>0.61±0.17</td>
<td>0.017</td>
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<tr>
<td>mpo</td>
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<td>0.05±0.02</td>
<td>0.004</td>
<td>31.32±6.09</td>
<td>0.03±0.02</td>
<td>0.000</td>
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<tr>
<td>fli1</td>
<td>1.00</td>
<td>1.09±0.03</td>
<td>0.184</td>
<td>1.11±0.05</td>
<td>1.01±0.02</td>
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<tr>
<td>l-plastin</td>
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<td>3.23±0.22</td>
<td>0.002</td>
<td>9.10±0.83</td>
<td>9.87±1.34</td>
<td>0.195</td>
</tr>
</tbody>
</table>

Results are presented in mean±SEM of three to four separate experiments. $^+$: comparison between WT and jak2aUTR embryos using Students’ paired t-test. $^\psi$: comparison between Chd and Chd+ jak2aUTR embryos using Students’ paired t-test.
Table 2. Differential gene expression in wild-type and jak2a\textsuperscript{ca} embryos at 18 hpf.

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>jak2a\textsuperscript{ca}</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>scl</td>
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<td>1.06±0.05</td>
<td>0.386</td>
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<tr>
<td>lmo-2</td>
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<td>0.248</td>
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<tr>
<td>gata1</td>
<td>1.00</td>
<td>1.55±0.06</td>
<td>0.001</td>
</tr>
<tr>
<td>αhe1</td>
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<td>1.74±0.06</td>
<td>0.001</td>
</tr>
<tr>
<td>βhe1</td>
<td>1.00</td>
<td>1.88±0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>spi1</td>
<td>1.00</td>
<td>2.13±0.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mpo</td>
<td>1.00</td>
<td>1.30±0.03</td>
<td>0.002</td>
</tr>
<tr>
<td>fli1</td>
<td>1.00</td>
<td>1.05±0.03</td>
<td>0.116</td>
</tr>
<tr>
<td>l-plastin</td>
<td>1.00</td>
<td>1.16±0.02</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Results are presented as mean±SEM of four separate experiments. Comparison between wild-type (WT) and embryos injected with jak2a\textsuperscript{ca} were evaluated by Student’s paired t-tests.
Legends

**Figure 1. jak2a expression and effects of jak2a knock-down.** (A,B,E): Whole-mount ISH for jak2a mRNA showing specific expression in the ICM (black arrows) of 18 (A,B) and 24 hpf embryos (F). (C,D,F): Increased jak2a expression in the expanded ICM (white arrows) of the *chordin* morphant. (G-J): Knock-down of jak2a by jak2a<sup>UTR</sup>-MO had no effects on embryonic development at 24 (G,H) and 48 hpf (I,J). (K-N): O-dianisidine staining (black arrows) in 48 hpf embryos showing reduced hematopoiesis by morpholinos against jak2a (L,M) and a soluble jak2a inhibitor AG490 (N). (O,P): Morpholinos against an intron-exon junction in jak2a resulting in reduced levels of jak2a mRNA shown by Q-RT-PCR. 

Chd: Chordin morphant; jak2a<sup>UTR</sup> and jak2a<sup>ss</sup>: Embryos injected with jak2a<sup>UTR</sup> and jak2a<sup>ss</sup> morpholinos. CTL: Embryos injected with random sequence morpholino (see Methods and Materials). Images from A to N were representative pictures from at least three separate experiments containing 10 (A-J) and 20 (K-N) embryos per experiments. The columns in P represent mean values of three separate experiments using around 20 embryos per experiment. Comparison was made between uninjected embryos and those injected with jak2a<sup>UTR</sup> MO. (*, p<0.05)

**Figure 2.** Effects of jak2a knock-down on erythropoiesis, Stat signaling and
angiogenesis. (A): Flow cytometric analyses (x-axis: GFP, y-axis: side scatter) showing the effects of jak2a knock-down using jak2a\textsuperscript{UTR} and jak2a\textsuperscript{ss} morpholinos as well as a soluble jak2a inhibitor AG490 on GFP\textsuperscript{+} populations in Tg(gata1:GFP) embryos. Synergistic effect was seen when the embryos were co-injected with both morpholinos. Effects of jak2a\textsuperscript{UTR} morpholino could be rescued by wild-type jak2a mRNA. Each record was representative of three experiments using 20 embryos per experiment.  

(B): Western Blotting showing reduced phospho-stat5 upon knock-down of jak2a functions. About 30 embryos were used in each experiment and the result was representative of three experiments.  

(C,D): Fluorescent microscopy using Tg(fli1:GFP) showing knock-down of jak2a by jak2a\textsuperscript{UTR} morpholino had no effects on angiogenesis. AC: Axial circulation; ISV: Inter-segmental vessels. Results were representative of at least four separate experiments using more than five embryos each time.

**Figure 3.** jak2a mediates the expanded ICM phenotypes in the *chordin* morphants  

(A): WT 24 hpf embryos.  

(B): Chordin morphants at 24 hpf generated by injection of chordin morpholino at 1-4 cell stage. Bracket showed the extend of expanded ICM (arrowhead).  

(C): 24 hpf embryos co-injected with chordin and jak2a\textsuperscript{UTR} morpholinos showing reduced expansion of ICM. Each image
was representative pictures of 3-4 experiments using more than 20 embryos at each time. (D): Knock-down of jak2a by both UTR and splice-site morpholinos and AG490 reduced the width of ICM in 24 hpf chordin morphant embryos, expressed as the width of adjacent spanning somites (left panel) and % of embryos with ICM ≥ 5 somites (right panel). Each column represents mean value of 3-4 experiments using more than 20 embryos at each time. (E): Average results of flow cytometry showing mean ± S.E.M. of 3-4 experiments. P-values at the top of each column represent the results of statistical evaluation based on paired Students’ t-test when compared with chordin morphant.

Figure 4. Constitutive activation of jak2a. (A): Amino acid sequence alignment of zebrafish (ZF) jak2a, human (HM) and mouse (MO) jak2 and Drosophila (DP) Hopscotch at the JH2 domain. The conserved glutamic residue was marked with an asterisk. (B-E): Injection of jak2a<sup>ca</sup> had no effect on gross morphology at 24 (B,C) and 48 (D,E) hpf. Each image was representative of at least three experiments using more than 10 embryos each time (F-H): Quantitative analyses showed that jak2a<sup>ca</sup> resulted in increased erythropoiesis (F,G) and stat5 phosphorylation (H). The latter was also increased in chordin morphant embryos. Each graph is representative of three separate experiments using 20 (F-G) and 30 (H) embryos each time.
**Figure 5.** Diagrammatic representation of bone morphogenetic protein (BMP) and Jak/Stat signaling pathways. Chordin (Chd), together with other BMP antagonists such as twisted gastrulation, blocks the binding of BMP to its receptor. Therefore, knock-down of Chd with morpholino enhances BMP signaling. Potential crosstalk between BMP and Jak/Stat signaling pathways were shown by the dotted lines.
Figure 1
Figure 4

A

B 24 hpf

C 24 hpf

D 48 hpf Uninjected

E 48 hpf Jak2a<sup>Ca</sup>

F Side-Scatter

G Side-Scatter

H Phospho-

Stat-5

Wild-type

Chordin Morphant

Jak2α<sup>Ca</sup>

Stat-5
Figure 5

[Diagram showing the interaction of BMP receptors with ligand in the cytoplasm and nucleus, involving Smad proteins and JAK/STAT pathways.]
The role of jak2a in zebrafish hematopoiesis

Alvin C.H. Ma, Alister C. Ward, Raymond Liang and Anskar Y.H. Leung