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Zebrafish Embryonic Stromal Trunk (ZEST) cells support hematopoietic stem and progenitor cell (HSPC) proliferation, survival, and differentiation

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Abstract

Forward genetic screens in zebrafish have been utilized to identify genes essential for the generation of primitive blood and the emergence of hematopoietic stem cells (HSCs), but have not elucidated genes essential for hematopoietic stem and progenitor cell (HSPC) proliferation and differentiation due to a lack of methodologies to functionally assess these processes. We previously described techniques to test the developmental potential of HSPCs by culturing them on zebrafish kidney stromal (ZKS) cells, derived from the main site of hematopoiesis in the adult teleost. Here we describe an additional primary stromal cell line we refer to as zebrafish embryonic stromal trunk (ZEST) cells, derived from tissue surrounding the embryonic dorsal aorta, the site of HSC emergence in developing fish. ZEST cells encouraged HSPC differentiation towards the myeloid, lymphoid, and erythroid pathways when assessed by morphological and qRT-PCR analyses. Additionally, ZEST cells significantly expanded the number of cultured HSPCs *in vitro*, indicating that these stromal cells are supportive of both HSPC proliferation and multilineage differentiation. Examination of ZEST cells indicates that they express numerous cytokines and Notch ligands, and possess endothelial characteristics. Further characterization of ZEST cells should prove to be invaluable in understanding the complex signaling cascades instigated by the embryonic hematopoietic niche required to expand and differentiate HSPCs. Elucidating these processes and identifying possibilities for the modulation of these molecular pathways should allow the *in vitro* expansion of HSPCs for a multitude of therapeutic uses.

Graphical Abstract

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Keywords

hematopoietic stem cells; hematopoietic stem and progenitor cells; in vitro hematopoietic culture; zebrafish embryonic stromal trunk cells; developmental hematopoiesis

Introduction

Hematopoiesis, the production of blood, is an essential cellular process that occurs constantly over an organism's lifespan and is a paradigm for stem cell biology¹. This process starts with the hematopoietic stem cell (HSC), a tissue-specific stem cell that has the ability to both self renew and differentiate into more developmentally restricted progenitor cells that subsequently mature into the full repertoire of blood and immune cells. Importantly, this process is tightly regulated; perturbations in the proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) can lead to a host of hematopoietic disorders, including anemia, thrombocytopenia, leukopenia, and leukemia.

Zebrafish provide an excellent vertebrate model system to study hematopoiesis²⁻⁴. Their external development, high fecundity, and optical transparency have made them instrumental in the visualization of *de novo* HSC production^{5,6} and leukocyte behavior⁷⁻¹⁰. Zebrafish possess the full repertoire of mammalian blood cells including an innate¹¹⁻¹³ and adaptive immune system^{14,15}, and the genetic control of hematopoiesis is well conserved among fish and mammals. Importantly, zebrafish are useful in permitting large-scale forward genetic mutagenesis¹⁶⁻¹⁸ and drug screens¹⁹⁻²³. Their utility as a screening platform has resulted in identifying genes required for primitive hematopoiesis^{18,24} and drugs now in clinical trials to treat hematologic disorders²⁵.

Because the zebrafish is a relatively new model system, functional means of identifying *bona fide* HSPCs have been lacking. Clonal lines of zebrafish have only recently been developed²⁶⁻²⁸, making transplantation of HSPCs into immune-matched hosts problematic. While advances have been made in HSPC transplantation²⁹, these experiments are still technically difficult. To approach this problem in another way, we developed the first *in*

vitro assays to test HSPC function. Our original approach was to create zebrafish kidney stroma (ZKS) cells³⁰, a primary cell line derived from the main site of hematopoiesis in the adult zebrafish. The development of this line allowed us to identify cytokines produced by ZKS cells, permitting the development of clonal methylcellulose assays to test HSPC development³¹. As mammalian cytokines show little cross reactivity with paralogous zebrafish receptors³², the identification and validation of zebrafish cytokines has proven invaluable for understanding signaling molecules involved in teleost hematopoiesis.

To identify more cytokines responsible for zebrafish HSPC proliferation and differentiation, we isolated tissue near the embryonic dorsal aorta, the first site of definitive hematopoiesis and HSC formation in the zebrafish, culturing these cells *in vitro*. We have termed these primary cells zebrafish embryonic stromal trunk (ZEST) cells. While ZEST cells do not express hematopoietic markers, they do encourage the proliferation and differentiation of HSPCs in culture. We also show that ZEST cells can easily be utilized for quantification of multilineage HSPCs. Like their adult hematopoietic stromal counterparts, ZEST cells produce hematopoietic-supportive cytokines; further characterization of their transcriptome should elucidate molecules important for the generation, expansion, and differentiation of vertebrate HSCs and HSPCs.

Materials and Methods

Zebrafish stocks and embryos

Zebrafish were mated, staged, raised, and maintained in accordance with University of California, San Diego and California State University, Chico IACUC guidelines. AB* wild-type (wt) fish and the transgenic *tg(-6.0itga2b:eGFP)*³³ (referred to throughout the manuscript as *cd41:GFP*) and *tg(-3.5ubi:EGFP)*³⁴ (referred to throughout the manuscript as *ubi:GFP*) were utilized.

Generation of ZEST cells

ZEST cells were isolated by surgically removing the dorsal aorta and surrounding tissue from the trunk of 48 hour post fertilization (hpf) AB* wt fish. At 48hpf, approximately 200 embryos were rinsed three times in sterile embryo medium in 10cm² plates. Using an Olympus SZ51 dissecting microscope, the tissue posterior to the yolk tube extension was removed and discarded. Then, the tissue anterior to the yolk tube extension (including the large yolk ball) was removed with a sterile scalpel and discarded (see Figure 1A; hatched area denotes the region that was isolated). The remaining trunk of the embryo was finely minced with a surgical scalpel and grown in zebrafish tissue culture medium³⁰ in a 12.5cm² tissue culture flask. The mincing of the tissue destroyed most of the ventral yolk tube extension, but any that remained in the culture media did not attach to the surface of the flasks. The cells that attached to the surface of the flask were grown at 32°C in 5% CO₂ until cells achieved 80% confluence. Cells were trypsinized for 5 minutes and expanded onto 75-cm² tissue culture flasks.

Morphological characterization of ZEST cells

ZEST cells were grown on glass coverslips in culture media in 24-well tissue culture plates. When cells reached 100% confluence, they were fixed and stained with May-Grünwald Giemsa and visualized by microscopy³⁵.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of ZEST cells

RNA was isolated from ZEST cells using a QIAGEN RNeasy kit, and cDNA was generated using the BioRad iScript cDNA synthesis kit. Primers, product sizes, and annealing temperatures used for the RT-PCR characterization of ZEST cells are listed in Table 1.

Isolation and enumeration of WKM

WKM was isolated as described previously³⁵ and enumerated by trypan blue exclusion and counting with a hemocytometer. WKM cells were added on top of ZEST cells when the stroma was 80% confluent; ZEST cells divide slowly and do not need to be mitotically arrested with mitomycin C or irradiation. To enumerate WKM cells after being cultured on ZEST cells, the stroma was gently rinsed to remove the WKM from the cell monolayers. Cells were concentrated by centrifugation at 300 g, cytopun onto slides, stained with May-Grünwald Giemsa and visualized by microscopy.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of cultured hematopoietic cells

RNA was isolated from cultured ZEST cells using a QIAGEN RNeasy kit, and cDNA was generated using a BioRad iScript cDNA synthesis kit. qRT-PCR was performed on an Eppendorf Mastercycler with BioRad SsoAdvanced Universal SYBR Green Supermix. Primers have been previously described^{36,37}. Fold expression was determined by the Ct method, using *ef1α* as a reference gene, and kidney as a reference tissue.

Fluorescence-activated cell sorting (FACS)

WKM from *ubi:GFP* fish was isolated and resuspended in PBS with 0.9% fetal bovine serum. Lymphoid and precursor fractions were sorted and analyzed on a FACS Aria II (BD Biosciences) by utilizing their unique forward and side scatter characteristics³⁸. Sytox red (Life technologies) was used as a cell viability stain.

Proliferation of HSPCs on ZEST cells by flow cytometry

Lymphoid and precursor fractions from WKM were isolated and stained with the membrane dye PKH-26 (Sigma), and plated onto monolayers of ZEST cells. PKH-26 fluorescence was analyzed before the cells were plated and after five days in culture on an LSR II flow cytometer (BD Biosciences). Proliferation calculations were performed with FloJo software version 9 (Treestar), specifically utilizing the Proliferation Platform, which was used to determine the percent of WKM cells that divided, as well as determine the culture's Division Index. The Division Index is defined as the average number of cell divisions that a WKM cell underwent over the course of the experiment.

Differentiation analysis of HSPCs on ZEST

Single *cd41:GFP*⁺ cells from the lymphoid fraction were plated into individual wells of 96 well plates on top of ZEST monolayers. Iron supplement (1:1000, Sigma) and carp serum (1:1000) was added to each well to encourage erythroid development³⁰. After five days of *cd41:GFP*⁺ cell growth and differentiation, RNA was collected from the wells and processed for qRT-PCR. qRT-PCR was performed with primers specific for erythroid, myeloid, and lymphoid differentiation. As a control, plates with ZEST cells only were also processed and analyzed for the same transcripts. Individual cell sorting into plates was verified by microscopic analysis of fluorescent cells.

Sequence alignment and read counts

All sequenced libraries were processed using the following procedures. First, copy-duplicates were removed from sequenced reads. Two sequenced reads are considered to be copy-duplicates, the product of PCR, if they were exact copies of each other. Only one copy of exact copy-duplicates was kept. Quality control was carried out using FastQC tool (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Since base sequence content (count of A, C, G, T along the reads' lengths) in the first ten base pairs of the reads showed abnormal distribution, the first ten bases were trimmed. Another measurement, namely, *Kmer Content* of FastQC showed high k-mer enrichment at the end of reads. A k-mer is a short k-bases-long substring of a read. Various adapter trimming tools were used to reduce adapter contamination, and, hence, k-mer enrichment; however, none of the tools used significantly affected the results of k-mer enrichment. Therefore, to reduce k-mer enrichment, we used our own method to trim unaligned ends of the reads (potential adapter contamination). First, the sequenced reads were aligned using *bowtie-2* tool³⁹ to cDNA sequences of zebrafish (zv9, Ensembl), using *local* mode of alignment and loose parameters that allowed mapping with low alignment score, with the following options: --score-min L,-2,0.3, -L 18, --ma 3, --mp 1,1, --np 1, --rdg 50,50, --rfg 50,50, --ignore-quals, -k 1. Then our own script processed the results of the alignments and extracted only high-quality aligned consecutive stretches of the reads, requiring the minimum length of this stretch to be at least 32bp. In other words, loosely mapped ends of the sequenced reads were trimmed. Next, trimmed reads were mapped to cDNA sequences using *bowtie-2* with default parameters and option -k 23 (this option finds at most 23 alignments for each read). Since there are at most 22 different transcripts for each gene in zebrafish, this option ensured the finding of alignments to different genes. Based on the mapping results, only uniquely mapped reads were kept for further analysis. A read was considered *unique* if it was mapped to the transcripts of the same gene only; in other words, if a read was mapped to transcripts of two different genes with the same best alignment score, then the read was considered to be ambiguous and was discarded from further analysis. Our own script was used together with the Ensembl GTF file with the gene information of zebrafish to count the total number of unique reads mapped to each gene. These read counts were used to identify differentially expressed genes in each sample compared to the negative control, ZF4 cells.

Normalization and differential expression

To identify differentially expressed genes in ZEST/ZKS samples compared to the ZF4 cells, the Microsoft online tool FDR Calculator based on the Fisher's Exact Test was used: <http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/falsediscoveryrate/default.aspx>. The tool calculated the p-value and q-value for each gene from the read counts. A gene was considered to be differentially expressed in a sample ZEST/ZKS compared to ZF4 if its p-value was less than or equal to 0.05, and its q-value was less than or equal to 0.1, and the maximum reads count in two samples was at least 10. To identify over-expressed genes in a sample ZEST/ZKS compared to ZF4, we used RPKM (reads per kilo base per million mapped reads), calculated as the read count multiplied by 10^9 and divided by the gene's length and total read count per sample. The gene's length was calculated as the sum of lengths of all its exons. If the RPKM value for a differentially expressed gene in a sample ZEST/ZKS was greater than the RPKM value for the same gene in sample ZF4, then the gene was considered over-expressed.

To compare over-expressed genes in ZEST and ZKS samples, only over-expressed genes with $2\times$ fold change in a ZEST/ZKS sample compared to ZF4 were considered. The Venn diagrams were built using over-expressed genes in ZEST/ZKS whose RPKM value in a ZEST/ZKS sample was at least twice its RPKM value in the ZF4 sample.

To build the heat-map clustergram, we considered the top 100 over-expressed genes with $2\times$ fold change in a ZEST/ZKS sample compared to the ZF4 sample. Out of these genes, the genes with the highest average RPKM values were used to build the heat-map clustergram.

Results

ZEST cells are derived from embryonic trunk stroma

Due to the fact that the site of HSC emergence in zebrafish is endothelium located in the floor of the dorsal aorta^{5,6}, we hypothesized that stromal cells isolated from this embryonic area would support hematopoiesis. To create ZEST cells, we dissociated the trunk tissue of 48hpf wt embryos by removing tissue anterior and posterior to the yolk tube extension (Figure 1A). The tissue dorsal to the yolk tube extension was then finely minced with a scalpel and grown in zebrafish tissue culture medium³⁰ in tissue culture flasks until cells were confluent. Upon confluence, cells were trypsinized and passaged for further culture. While cells were not transformed in any way, they have continued in culture for over 90 passages with no signs of senescence (data not shown).

Cultured ZEST cells displayed a fibroblastic morphology when grown on glass coverslips and stained with May-Grünwald Giemsa (Figure 1B). To confirm that these ZEST cells were of stromal origin and did not contain hematopoietic cell lineages, RT-PCR was performed for both the pan-leukocytic marker *cd45* and the red blood cell-specific *gatal* (Figure 1Ci). Neither of these transcripts were present, confirming that ZEST cultures were not hematopoietic or contaminated with mature hematopoietic cells.

To further characterize these cells at the transcriptional level, RT-PCR was performed to analyze the expression of genes known to play essential roles in hematopoietic maintenance,

development, and proliferation. Transcriptional analysis showed that ZEST cells expressed many hematopoietic progenitor supportive factors including *kitla*, *kitlb*, *bmp1l*, *fgf1a*, *fgf21*, *il-11a*, and *il-11b*, as well as cytokines and growth factors involved in lineage-specific signaling, maintenance, and differentiation such as *epo*⁴⁰, *gcsfa*⁴¹, and *gcsfb*⁴² (Figure 1Cii) and inflammatory signaling (Figure 1Ciii). Additionally, ZEST cells expressed Notch ligands involved in niche signaling, lymphocyte development, and maturation (Figure 1Civ). Finally, ZEST cells expressed transcripts indicative of skeletal (Figure 1Cv) and smooth muscle (Figure 1Cvi). Together, these data indicate that ZEST cells are stromal and produce a multitude of ligands important for the survival and proliferation of hematopoietic cells.

ZEST cells support hematopoiesis and promote hematopoietic proliferation

The main site of adult hematopoiesis in teleosts is the kidney; all mature blood cell types can be isolated from the whole kidney marrow (WKM)³⁸, as well as hematopoietic stem and progenitor cells (HSPCs)^{31,38,42,43}. In order to determine if ZEST cells were capable of supporting hematopoiesis, WKM from adult wt fish was plated on an 80% confluent layer of ZEST cells. WKM cells plated on ZEST cells or without stromal support were quantified at days 3, 6, 9, and 14. Compared with cells cultured without supportive stroma, WKM cells proliferated in the presence of ZEST cells, increasing nearly 5× over the course of two weeks (Figure 2A). To confirm that these expanding cells were hematopoietic, we examined cytocentrifuge preparations at each time point, analyzing their cellular morphologies (Figure 2B). While mature red blood cells persisted for only 6 days in culture, myeloid, lymphoid, and precursor cells were observed at all time points, indicating that ZEST cells are capable of maintaining these hematopoietic cell lineages in culture. Importantly, the support of hematopoiesis required cell-cell interaction between WKM cells and ZEST stroma; conditioned media from these stromal cells was incapable of expanding WKM (data not shown).

To investigate the ability of ZEST cells to encourage proliferation of HSPCs, we isolated purified lymphoid and precursor populations from fish by FACS. We utilized *ubi:GFP*³⁴ fish for these experiments because their blood is uniformly GFP⁺, allowing us to ensure that the our results were from the plated hematopoietic cells, and not the ZEST cells themselves. The small, agranular lymphoid fraction contains B and T lymphocytes, as well as HSPCs^{30,31,38,43}. The precursor fraction is slightly larger and more granular, and contains myeloid, lymphoid, and erythroid progenitors^{30,31,38,43}. These FACS-isolated cell populations were cultured on 80% confluent ZEST monolayers and quantified at day 3, 6, 9, and 13 (Figure 3A). Expansion of cells from the lymphoid and precursor cell fractions were observed at all time points, indicating that ZEST cells facilitate HSPC proliferation. To further corroborate these data, purified *ubi:GFP*⁺ lymphoid and precursor cell fractions were stained with the fluorescent red membrane dye PKH-26 and either plated on a monolayer of confluent ZEST cells or plated without supportive stroma. After 5 days, these cells were examined by flow cytometry for relative fluorescence intensity. Cells stained with PKH-26 maintain their red fluorescence until they divide, which decreases their fluorescence intensity by 50%. After 5 days in culture, over 60% of cells from the precursor fraction and 10% of the cells from the lymphoid fraction exhibited at least one cell division event when cultured on a ZEST monolayer (Figure 3B). Additionally, the lymphoid and precursor cells

plated on ZEST underwent more cell divisions than their counterparts plated with no stroma, indicated by the reduction in PKH-26 fluorescence (Figure 3C). While few cells plated with no stroma progressed past one (lymphoid fraction) or two divisions (precursor fraction), cells plated on ZEST had over five divisions in the lymphoid and precursor fraction over five days. These data can be quantitated into a “division index,” which is the average number of cell divisions that a cell in the original population underwent over the course of five days. Again, the division index of all cells plated on ZEST cells is at least 2× higher than when no stroma is present. Together, these data demonstrate that ZEST cells facilitate HSPC proliferation.

ZEST cells support HSPC differentiation

In order to determine if ZEST cells also encouraged the differentiation of hematopoietic cells, we purified *ubi:GFP⁺* lymphoid and precursors populations by FACS and co-cultured them for 12 days on ZEST monolayers, adding iron supplement and carp serum to improve erythroid differentiation³⁰ (Figure 4). In addition to identifying lymphoid and precursor fractions of zebrafish WKM by flow cytometry, it is also possible to identify mature myeloid and erythroid cells in this manner³⁸. Purified lymphoid and precursor populations started to differentiate by day 3 in culture when analyzed by flow cytometry (Figure 4A, top). The change in granularity of the lymphoid cells into erythroid, precursor, and myeloid fractions indicated that HSPCs present in this fraction had differentiated. Likewise, precursor cells changed their scatter characteristics to be more erythroid, lymphoid, and myeloid (Figure 4A, bottom). This differentiation continued over the course of 12 days in culture. To quantitate the actual number of cells that were generated in these cultures, the percentages of *ubi:GFP⁺* WKM cells in particular cellular fractions (from Figure 4A) were multiplied by the total number of WKM cells in the culture. Both lymphoid and precursor populations showed expansion of erythroid cells at every time point throughout the duration of the time course (Figure 4B, left panel). The precursor population showed an increased number of lymphoid cells at day 3 and day 6, eventually leveling out by day 9 and remaining relatively unchanged by day 12. On the other hand, the lymphoid population showed an increased number of lymphoid cells at day 3, followed by a gradual decline that continued until day 12 (Figure 4B, second panel), likely because the HSCs present in this population are rare; the majority of cells are post-mitotic lymphocytes and do not proliferate over the course of 12 days. The plated lymphoid population showed an increase in precursor cells up to day 6, at which point they remained relatively constant for the remainder of the time course, while the plated precursor population showed a decrease in precursor cells over time, likely due to these HSPCs differentiating into mature hematopoietic lineages (Figure 4B, third panel). Finally, both cells from lymphoid and precursor populations showed an expansion of mature myeloid cells over the course of the experiment (Figure 4B, right panel). To confirm that ZEST stroma encouraged the differentiation of HSPCs, RNA was extracted from these experiments and qRT-PCR was performed for mature hematopoietic markers. The erythroid-specific transcript *hbaa* was detected in both the lymphoid and precursor cultures at every time point during the duration of the assay, as were the myeloid-specific transcripts *mpx* and *csf3r*, and the lymphoid-specific transcripts *pax5* and *lck* (Figure 4C). Overall, the change in scatter profile, numbers, and gene expression indicate

that HSPCs within the lymphoid and precursor fraction differentiated into mature myeloid, lymphoid, and erythroid cells when plated on ZEST cells.

Differentiation of clonal HSPCs on ZEST stroma

Due to the fact that ZEST cells support multi-lineage differentiation of HSPCs, we hypothesized that they had utility to identify HSPCs *in vitro*. To develop an assay to test HSPC differentiation (Figure 5A), we isolated cells from the WKM marked with the *cd41*:GFP transgene, previously identified as a marker for HSCs with *in vivo* experiments⁴⁴. As previous work identified the lymphoid fraction as the scatter fraction where HSCs were enriched³⁸, we isolated *cd41*:GFP⁺ lymphoid cells from these fish, and utilized FACS to deposit individual cells into individual wells of a 96 well plate. On this 96 well plate was a monolayer of ZEST cells with iron supplement and carp serum, and the *cd41*:GFP⁺ cells were allowed to proliferate and differentiate over the course of five days. After five days, RNA was isolated from individual wells, cDNA was generated, and qRT-PCR was performed for lineage-specific markers, including the myeloid-specific transcripts *cmpl*, *cd41*, *csf1r*, *mpx*, and *mpeg*; the erythroid-specific *band3* and *hbaa*; the B cell-specific *igm* and *pax5*; and the T cell-specific *lck*. To simplify the presentation of these data, we generated a color-coded schematic, whereby every column represents an individual well that received an individual *cd41*:GFP⁺ cell that was interrogated for lineage-specific transcripts (Figure 5). If there was any expression of that specific gene, a colored box is shown; a black box indicates no expression. All samples were normalized to *ef1a* as a housekeeping gene, so all columns have a yellow box in that row. All samples were also normalized to gene expression in the kidney, which is the far right column, also uniformly yellow. As a control, we performed qRT-PCR for the fold-change of these markers when no *cd41*:GFP⁺ cells were plated on ZEST cells; only very few wells indicated any levels of these markers (Figure 5B). Importantly, no samples had multilineage transcript expression. However, when *cd41*:GFP⁺ cells were plated on ZEST cells and allowed to differentiate for five days, there was a striking increase in numbers of wells that had multilineage transcript readouts. We considered a well (column) that had both myeloerythroid and lymphoid transcripts to be multilineage; 57% of wells had this characteristic (Figure 5C). Importantly, only one sample out of 49 failed to generate a hematopoietic readout, indicating that *cd41*:GFP⁺ cells survive and differentiate well on the ZEST monolayer. Overall, these data indicate that ZEST cells are an excellent *in vitro* model for analyzing the differentiation capability of HSPCs. These assays should allow the identification and testing of putative HSPCs and the validation of previously identified HSPCs.

ZEST and ZKS cells are different stromal cell lines

While ZEST and ZKS cells both support HSPC proliferation and multilineage differentiation, they are derived from different temporal and spatial locations in the zebrafish. As such, we hypothesized that they would have different properties. First, we compared the ability of ZEST cells and ZKS cells to expand WKM in culture, plating cells and enumerating them over the course of 14 days. While ZKS expanded WKM, ZEST monolayers encouraged approximately 4× more expansion over the course of the experiment (Figure 6A), indicating that they had different expansion ability. To more thoroughly investigate the differences and similarities between these cell lines, we performed RNA

Sequencing (RNASeq) to analyze differences in the transcriptome of ZEST and ZKS cells, comparing expressed transcripts in these cell lines to non-hematopoietic-supportive ZF4 stromal cells (data not shown). While ZKS and ZEST cells shared 970 transcripts over 2× upregulated when compared to ZF4 cells, our studies also indicated that ZEST cells expressed 1410 unique transcripts from ZKS cells (Figure 6B). Additionally, ZKS cells expressed 1408 unique transcripts when compared to ZEST cells (Figure 6B). To more carefully examine the expression differences between the shared upregulated transcripts, we took the top 100 genes overexpressed in both ZKS and ZEST cells (from the subset of 970 shared genes), and plotted their expression in a heat map (Figure 6C and Table 2). In this figure, green represents genes expressed at similar levels to ZF4 cells, while red denotes genes more highly upregulated compared to ZF4 cells. These studies indicated that there are subsets of genes similarly overexpressed in ZKS and ZEST cells; analysis of gene ontology (GO) terms over represented in these 100 transcripts indicated that the top two enriched GO terms were “translation” and “protein metabolic processes,” which fits with the fact that these stromal cells are producing hematopoietic-supportive proteins (Table 3). However, there are also subsets of genes expressed differentially in these cell types (Figure 6C). The gene names of these top 100 genes are listed in Table 2 in the same order presented in Figure 6C, along with their reads per kilobase of transcript per million reads mapped (RPKM) values. Overall, these data indicate that ZEST cells and ZKS cells, while they appear to function similarly in support of hematopoiesis *in vitro*, have very different transcript expression.

Discussion

Over the past 20 years, zebrafish have become a more popular model system for investigating hematopoietic development and dysregulation. The transparency and external development of zebrafish have been instrumental in observing early hematopoietic ontogeny, and the ability to perform large-scale mutagenesis and drug screens has been critical in the identification of genes and pathways involved in hematopoiesis. However, the zebrafish as a model system has traditionally lacked the functional assays needed to investigate HSPC proliferation and differentiation. Importantly, HSPCs are essential to understanding molecular pathways essential for hematopoiesis; these multi-, oligo-, and uni-potent cells are commonly dysregulated during hematologic disease^{45–47}.

HSPCs in mammals were discovered and characterized largely due to the development of *in vitro* assays to functionally assess their differentiation capabilities^{48–51}. As HSPCs were not well defined in zebrafish, yet are essential to understanding, preventing, and treating hematologic disorders, we designed assays to investigate them. Our first studies involved creating ZKS cells, a stromal cell line from adult zebrafish, which is the main site of hematopoiesis in teleosts. These cells allowed the rescue of erythroid genetic mutants, underlining their usefulness in investigating the differentiation of HSPCs³⁰. ZKS cells also expressed multiple cytokines such as *epo*, *gcsfa*, *gcsfb*, and *tpo*, allowing the development of clonal methylcellulose assays to further refine the isolation and testing of zebrafish HSPCs^{31,42,43}. While methylcellulose assays allowed the clonal investigation of myeloerythroid HSPCs, assays to assess lymphoid progenitors remain elusive; lympho-supportive cytokines have yet to be identified and recombinantly generated, and T cell

development requires cell-to-cell contact⁵². The benefit of ZKS and ZEST cells is that they do support lymphoid cell proliferation and differentiation, likely by their expression of lympho-supportive cytokines and Notch ligands. Further examination of the transcriptome of these two cell lines is likely to yield important advances in this area.

Importantly, stromal cells are useful for understanding not only normal hematopoiesis, but also the effect of the hematopoietic niche on hematologic disease development⁵³. Other groups created zebrafish hematopoietic supportive mesenchymal stem cell lines from *sdf1*:DsRed transgenic animals⁵⁴, allowing investigation of the hematopoietic niche, positing the zebrafish as a useful model system to study niche/HSPC interactions. Importantly, ZEST cells are easily transfected, and with the advent of CRISPR/Cas9 technology, it should be possible to quickly and effectively modulate putatively important stromal factors involved in hematopoiesis in this *in vitro* system.

ZEST cells, like their ZKS counterparts, appear to resist senescence. ZEST cells have been grown for over 100 passages, never underwent a massive die off followed by a clonal recovery during their establishment, and show no growth slowing or functional deviation. We have created ten different clonal lines of ZEST cells, and all have the same functional properties; they all support the expansion and differentiation of hematopoietic cells (data not shown). Also, similar to ZKS cells, ZEST cells support the proliferation and differentiation of all blood cells; lymphoid cells and myeloid cell types were expanded and observed. While ZEST cells do not support the survival of post-mitotic red blood cells for more than six days in culture, they do encourage differentiation from erythroid precursors when iron supplement and carp serum is added to isolated HSPCs. While ZEST cells support adult HSPCs, future experiments are required to analyze embryonic HSPCs. However, as ZKS cells support embryonic HSPCs⁵⁵, we believe ZEST cells will also. Due to their embryonic origin, they may be more supportive.

The gold standard for identifying HSCs is to transplant putative HSCs into irradiated recipients and measure long-term hematopoietic reconstitution. In mice, studies indicate that only 0.01% of bone marrow (the mammalian location of HSCs; equivalent to fish WKM) is comprised of long- and short-term HSCs⁵⁷. In zebrafish, these studies have been problematic; few clonal fish strains exist, so immune-matching donors and recipients is challenging. Due to these issues, it has been difficult to quantitate zebrafish HSCs. Descriptive reports quantitating side-population (SP) HSCs indicated that $0.056 \pm 0.008\%$ of the WKM is comprised of SP HSCs⁵⁶, but this characterization was largely descriptive; there was no functional evidence that these cells were *bona fide* HSCs. Further refinement of this SP assay indicated that 20% of these SP cells in *cd41*:GFP transgenic animals expressed *cd41* at low levels⁴⁴. Transplanting these *cd41*:GFP^{lo} SP cells rescued irradiated hosts, indicating that about 0.018% of the WKM is comprised of HSCs⁴⁴. However, these studies did not isolate *cd41*:GFP^{lo} cells from the lymphoid scatter fraction, the fraction where HSCs reside³⁸. Additionally, these studies did not immune match donors and recipients, likely reducing the amount of successful engraftment and long-term reconstitution.

We took a different approach to quantitating zebrafish progenitors, developing a clonal *in vitro* assay to assess if they had the capability of multi-lineage differentiation. By plating

cd41:GFP^{lo} cells from the lymphoid scatter fraction on ZEST cells and analyzing a multi-lineage transcriptional readout five days later, we were able to enumerate HSPCs present in the zebrafish kidney. Our FACS isolation indicated that *cd41:GFP^{lo}* cells in the lymphoid fraction routinely comprised 0.3% of the total WKM. We detected both lymphoid and myeloerythroid differentiation from 57% of these cells, or 0.171% of the WKM. This percentage is higher than the estimated numbers of *cd41:GFP^{lo}* HSCs present in this population⁴⁴, indicating that this population is heterogeneous⁴⁴, and indicating that our assay detects other multipotent progenitors in this population. Further refinement of the assay, including sorting SP⁺ *cd41:GFP^{lo}* lymphoid cells may resolve this issue. While we can not assess if these *cd41:GFP^{lo}* lymphoid cells are *bona fide* HSCs with this assay, they represent a progenitor pool that expressed multiple mature hematopoietic lineage transcripts after culture on ZEST cells.

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Highlights

- ZEST cells are derived from the site of *de novo* HSC formation in the zebrafish
- ZEST cells support proliferation and differentiation of hematopoietic progenitors
- ZEST cells allow the clonal analysis of zebrafish HSPCs

Figure 1. ZEST cells are a primary stromal cell line derived from the zebrafish embryonic trunk tissue that expresses hematopoietic-supportive transcripts

(A) Schematic illustration of isolation and culture of ZEST cells from 48hpf zebrafish embryos.

(B) Morphologic characterization of ZEST cells with May-Grünwald/Giemsa staining indicates stromal morphology. Top image photographed at 400× (scale bar is 200µm); bottom image photographed at 1000× (scale bar is 50µm).

(C) Gene expression analysis of ZEST cells by RT-PCR for various transcripts. ZEST cells do not express the pan-leukocytic transcript *cd45* or the erythroid-specific transcription factor *gata1* (i). ZEST cells express certain transcripts involved in the proliferation and differentiation of progenitor cells (ii); hematopoietic lineage-specific signaling, maintenance, and differentiation (iii); Notch signaling and lymphoid development (iv);

muscle differentiation and development (v); and cardiac development (vi). Gene names are listed at left and whole kidney was used as a positive control. dH₂O controls were also universally negative, although not shown. RT-PCR primer sequences are listed in Table 1.

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Figure 2. ZEST cells encourage WKM proliferation and support hematopoiesis

(A) WKM cells plated on ZEST monolayers (black squares, solid line) or plated with no stroma (black triangles, dashed line) enumerated over 14 days.

(B) WKM cells from (A) were cytopspun and stained with May-Grünwald/Giemsa stain. Cell images were taken at 1000 $\times$ (scale bar is 25 μ m), and grouped into erythroid (top row), lymphoid (second row), precursor (third row), or myeloid (bottom row) morphologies.

Figure 3. ZEST cells encourage proliferation of lymphoid and precursor cell fractions

(A) Enumeration of WKM cells generated from isolated lymphoid (black squares, solid line) and precursor (black triangles, dashed line) cell populations plated on ZEST cells over 14 days.

(B) Cells stained with the cell membrane dye PKH-26 from isolated lymphoid (left) and precursor (right) cell fractions grown on ZEST cells (black squares, solid line) or with no stromal support (black triangles, dashed lines). After 5 days in culture, WKM cells derived from these cultures were analyzed by flow cytometry and FloJo's Proliferation Platform was used to calculate the percentage of cells in the cultures that divided.

(C) Cells stained with the cell membrane dye PKH-26 (PE-A, x-axis) from isolated lymphoid (left) and precursor (right) cell fractions grown with no stromal support (top) or on ZEST cells (bottom). After 5 days in culture, cells were analyzed by flow cytometry to

determine how many rounds of cell division occurred without (top) or with (bottom) ZEST support. Numbers (0–7) above the histograms (blue lines) represent the number of divisions that occurred over 5 days, with 0 corresponding to cells that did not undergo division. The blue histogram lines are calculated and drawn by the Proliferation Platform module within the FloJo software package. The numbers above each blue peak correspond to the number of divisions that each population underwent, and the height of the peak corresponds to the numbers of cells that divided. The program also calculates a Division Index of each culture, defined as the average number of cell divisions that a WKM cell underwent over 5 days.

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Figure 4. ZEST cells encourage differentiation of lymphoid and precursor cell fractions
 (A) Isolated lymphoid (top left plot, black line) and precursor (bottom left plot, dashed line) cell fractions were plated on ZEST at Day 0, and differentiated over the course of 12 days. At each day, cells were removed and investigated on a flow cytometer to determine changes in the cells' scatter profiles.
 (B) Isolated lymphoid (black squares, solid line) and precursor (black triangles, dashed line) cell fractions were plated on ZEST at Day 0, and the number of differentiated erythroid (left), lymphoid (second from left), precursor (second from right), and myeloid (right) were enumerated over the course of 12 days. Flow cytometry plots in A were coupled with total cell counts at each time point to obtain these data. Each point represent the average of two biological replicates, and error bars represent SD.

(C) qRT-PCR results from cells isolated from differentiated isolated lymphoid (black bar) and precursor (striped bar) cell fractions. At each time point, cells were interrogated for the erythroid-specific marker *hbaa* (top), the myeloid markers *csf3r* and *mpx* (second and third row), the B cell marker *pax5* (fourth row), and T cell marker *lck* (bottom row). Bars represent the relative expression of 2 replicates averaged together, and error bars represent SD. Relative expression was determined by the Ct method, using *ef1α* as a reference gene.

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Figure 5. ZEST cells encourage multilineage differentiation of individual HSPCs

(A) Schematic of experiment.

(B) Visualization of qRT-PCR results from ZEST cells only. Each column is an individual sample (ZEST stroma only) grown for five days in culture. Far right column is kidney. Samples were interrogated for the genes listed on left side of the figure. Different color boxes shown at the bottom of the figure represent fold expression relative to kidney. Fold expression was determined by the Ct method, using *ef1a* as a reference gene and kidney as a reference tissue.

(C) Visualization of qRT-PCR results from ZEST cells only. Each column is an individual sample (*cd41:GFP^{low}* cells plated on ZEST stroma) grown for five days in culture. Far right column is kidney. Samples were interrogated for the genes listed on left side of the figure. Different color boxes shown at the bottom of the figure represent fold expression relative to kidney. Fold expression was determined by the Ct method, using *ef1a* as a reference gene and kidney as a reference tissue. If a column contained myelo-erythroid markers and a lymphoid marker, it was scored as multilineage (demarcated with *).

Figure 6. ZKS and ZEST cells both support hematopoiesis, but are different functionally and transcriptionally

(A) WKM cells plated on ZEST monolayers (blue squares, solid line) or ZKS monolayers (red circles, solid line) enumerated over 14 days.

(B) Venn diagram indicating the number of transcripts at least 2 $\times$ overexpressed in ZEST cells (blue; 2380), ZKS cells (red; 2378), and shared by both cell lines (intersection; 970) when compared to the non-hematopoietic supportive ZF4 cell line.

(C) Heat map representing the top 100 upregulated genes in ZEST or ZKS cells compared to ZF4 cells. Green represents genes underexpressed, and red denotes overexpression. Genes are listed in Table 2 in the same order (top-to-bottom), along with the RPKM values used to generate the heat map.

Table 1

Primer sets used for RT-PCR characterization of ZEST cells. Gene names are listed at far left, followed by the gene's description. Forward and reverse primer sequences are presented 5' to 3', followed by an optimized primer annealing temperature (°C). The generated product size (bp) is shown at far right.

Gene	Definition	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temp, °C	Product Size, bp
<i>acta1a</i>	<i>Danio rerio</i> actin, alpha 1a, skeletal muscle	GAAAAGAGCTACGAGCTTCC	GTAAGTGGTCTCGTGAATGC	50°	129
<i>acta2</i>	<i>Danio rerio</i> actin, alpha 2, smooth muscle	TGGATCTGGACTGTGTAAAG	ACTATCTTTCTGCCCCATTTC	50°	121
<i>actc1a</i>	<i>Danio rerio</i> actin, alpha, cardiac muscle 1a	TGCTGCTTTCCCTCTATTG	GAGTGAGGATACCCCTCTTG	50°	116
<i>bmp1l</i> ³⁰	<i>Danio rerio</i> bone morphogenic protein 1, like	GGATGGATAATTGGAGGAAAG	CTTTGTTCCGGTCTGTAAATCG	50°	230
<i>gcsfa</i> (<i>csf3</i>) ⁴²	<i>Danio rerio</i> colony stimulating factor 3a, granulocyte colony stimulating factor	AACTACATCTGAACCTCCTG	GACTGCTCTTCTGATGCTG	55°	165
<i>cxcl2a</i>	<i>Danio rerio</i> chemokine (C-X-C motif) 12a, stromal cell-derived factor 1a	CGCCATTTCATGCACCCGATTTC	GGTGGGCTGTCAGATTTCCTTGTC	50°	297
<i>cxcl2b</i>	<i>Danio rerio</i> chemokine (C-X-C motif) 12b, stromal cell-derived factor 1b	CGCCTTCTGGAGCCCCAGAGA	AGAGATTCTCCGCTGTCCTCC	50°	291
<i>dla</i> ³⁰	<i>Danio rerio</i> δA	ACGACGATTTGAGTATGACG	GGGATTGGCACCTTTATATCC	50°	186
<i>dlb</i> ³⁰	<i>Danio rerio</i> δB	TTCCGTGTTTAATGATTTGG	CACCTCCACAGAAACTCTTTGC	50°	158
<i>dlc</i> ³⁰	<i>Danio rerio</i> δC	TGGTGGACTACAATCTGAGC	ACCTCAGTAGCAAAACACACG	50°	169
<i>dld</i>	<i>Danio rerio</i> δD	AACCCAGACCGTCTGTATCAGT	CCGGGTTTGTGCGCAAAGGCCA	50°	308
<i>dll4</i> ³⁰	<i>Danio rerio</i> δ-like 4	CTCTTTCAGCACACCCAATTTC	TGAACATCCTGAGACCAATTC	50°	189
<i>efl1a</i> (<i>eefla111</i>) ⁵⁸	<i>Danio rerio</i> eukaryotic translation elongation factor 1 alpha 1, like 1	GAGAAAGTTCGAGAAGGAAGC	CGTAGTATTTGCTGGTCTCG	55°	123
<i>epo</i> ³⁰	<i>Danio rerio</i> erythropoietin	ACTTGTAAAGGACGATTGCAG	TATCTGTAATGAGCCGATGG	55°	156
<i>fgf1</i> ³⁰	<i>Danio rerio</i> fibroblast growth factor 1	ATACTGCGCATATAAAGCAAC	AGTGGTTTTCCTCCATCTTC	50°	154
<i>fig2l</i> ³⁰	<i>Danio rerio</i> fibroblast growth factor 21	CGGTGGTGTATGTATGTTCC	GTAGCTGCACCTCTGGATGAC	50°	203
<i>gata1a</i> ⁵⁸	<i>Danio rerio</i> GATA binding protein 1a	TGAAATGTGTGAAATTGTGGTG	ATTGCGTCTCCATAGTGTG	55°	650
<i>gcsfb</i> (<i>csf3b</i>) ⁴²	<i>Danio rerio</i> colony stimulating factor 3b, granulocyte colony stimulating factor b	GGAGCTCTGCGCACCCCAACA	GGCAGGGCTCCAGCAGCTTC	55°	184
<i>ifnγ</i>	<i>Danio rerio</i> interferon, gamma 1–2	TACATAAATGCACACCCCCATC	TCCTTTGTAGCTTTCATCCAC	55°	158
<i>il-1b</i>	<i>Danio rerio</i> interleukin 1, beta	TCCACATCTCGTACTCAAGG	CAGCTCGAAGTTAAATGATGC	50°	227
<i>il-10</i> ³⁰	<i>Danio rerio</i> interleukin 10	ATGAATCCCAACGATGACTTG	TCTTGCAATTCACCATATCC	50°	222
<i>il-11a</i> ³⁰	<i>Danio rerio</i> interleukin 11a	GACAAAGCTGAGCAATCAGAC	GGAGCTGAGAAAGAGTAGGC	50°	172

Gene	Definition	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temp, °C	Product Size, bp
<i>il-11b</i> ³⁰	<i>Danio rerio</i> interleukin 11b	TTGAACATTGCTGCTATCATCC	GAGTAATCGTTCCCAATTC	50°	166
<i>il-12a</i> ³⁰	<i>Danio rerio</i> interleukin 12a	GTGAGTCTGCTGAAGGAGTG	AGTGACATCATTTCTGTGTC	50°	167
<i>il-15</i> ³⁰	<i>Danio rerio</i> interleukin 15, like	CCAAGTCCACAATTACATGC	TCTTTGTAGAGCTCGCAGAC	55°	166
<i>il-26</i> ³⁰	<i>Danio rerio</i> interleukin 26	TGAAAAAGATGTGGGATGAAC	ACTGATCCACAGCAAAACAC	55°	214
<i>jag1a</i> ³⁰	<i>Danio rerio</i> jagged 1a	TGATTGGTGGATACTTCTGTC	AATCCATTGAGTGTGTCCCTG	55°	238
<i>jag1b</i> ³⁰	<i>Danio rerio</i> jagged 1b	CTGTGAGCCATCTTCTTTCAG	AGCAAAAGAAACCAAGGTAGTC	55°	213
<i>jag2</i> ³⁰	<i>Danio rerio</i> jagged 2	AATGACTGTGTGAGCAATCC	GTCAATTGACCAGATCCACAC	50°	174
<i>kitlga</i> ³⁰	<i>Danio rerio</i> kit ligand a	GGATTCAATGCTTTGACTTTTG	TGTACTATGTTGCGCTGATG	50°	205
<i>kitlgb</i>	<i>Danio rerio</i> kit ligand b	GGCAACCAGTCCACCACAATAAG	CACCTTTCCCTTCTGTAGTGGC	50°	135
<i>m17</i>	<i>Danio rerio</i> il-6 subfamily cytokine M17	CTTGATTGCCGTTTCAGTTAG	TGACCGGAGATTGTAGACAC	50°	210
<i>myod1</i>	<i>Danio rerio</i> myogenic differentiation 1	ATGGCATGATGGATTTTATG	TTTATTATTCCGTGCGTCAG	50°	107
<i>cd45 (pprc)</i> ⁵⁸	<i>Danio rerio</i> protein tyrosine phosphatase, receptor type, C	AGTTCTCTGAAATGGAAAAAGC	GCACAGAAAAAGTCCAGTACG	55°	140
<i>vegfaa</i>	<i>Danio rerio</i> vascular endothelial growth factor Aa	GAAACGTCACATGGAGGGTG	TTCTTTGCTTTTGACTTCTGTC	50°	121
<i>vmhc</i>	<i>Danio rerio</i> ventricular myosin heavy chain	TTATTGACTTTTGGCATGGAC	AAAATGAGACTCTGGCTTCC	50°	203

Table 2
Top 100 transcripts overexpressed in hematopoietic supportive ZKS and ZEST cells compared to non-hematopoietic supportive ZF4 cells

Gene names shown based on Zv9 assembly, as well as reads per kilobase of transcript per million reads mapped (RPKM) of ZKS, ZEST, and ZF4 sample

Gene	RPKM (ZKS)	RPKM (ZEST)	RPKM (ZF4)
<i>mustn1a</i>	191.7311182	94.20002244	2.377441447
<i>myl9a</i>	179.6584981	96.56364658	2.276630133
si:busm1-57f23.1	182.0504465	89.25803193	13.64267158
<i>blmh</i>	184.0111916	86.10283629	29.39759063
<i>cmb1</i>	196.7707245	73.67679014	26.91899859
<i>scinla</i>	229.2763242	77.71766924	1.171371221
<i>pycard</i>	216.1110211	70.80059954	0.244784777
<i>wfdc2</i>	215.3192844	100.2819887	5.805860073
<i>atp6v1e1b</i>	211.5850639	104.0432062	50.77535662
<i>tnfaip6</i>	197.4394341	109.3081398	52.44479497
<i>arl6ip5b</i>	180.1080903	105.0459892	49.94297373
<i>ndufb11</i>	247.4927535	68.49972903	30.78048579
<i>psma6a</i>	227.9565636	80.43925949	36.57643139
<i>rpl13a</i>	262.095085	85.45651864	42.49472207
si:dkey-100n23.4	241.0388676	112.7794318	26.3597982
<i>rnd3b</i>	230.919067	106.1477521	20.10791448
<i>ada</i>	235.815493	146.7960562	31.49486791
<i>rpz5</i>	239.9595079	157.8233757	7.805466586
<i>vmhc</i>	277.2773505	124.2928784	3.342640338
<i>cahz</i>	355.7589188	56.55174506	1.48171963
<i>ost4</i>	293.697141	56.14183101	22.3880387
<i>txn</i>	428.0840844	170.734287	74.95409787
<i>fhl1a</i>	392.740221	209.8129055	51.64958797
si:dkey-12112.1	431.6353641	159.7228348	29.13243458
<i>ckba</i>	410.942751	156.7429014	16.93871335
<i>phex</i>	374.7902084	246.0473474	1.422658424
<i>CABZ01024686.1</i>	320.0454925	195.9867392	0.150924627
<i>rsl24d1</i>	303.7118765	226.5305973	110.1637925
<i>ap2m1a</i>	256.7847085	248.5305894	108.62445
<i>dlx3b</i>	285.6044597	300.5094173	33.95661813
<i>adamts17</i>	377.7311012	296.1991765	75.65003739
<i>anxa1a</i>	386.1286169	280.2930981	71.0710686
<i>cdk1</i>	157.1526365	92.45941086	41.08086644
ENSDARG00000087773 (novel gene; no gene name)	151.3186816	86.06460141	36.7259713

Gene	RPKM (ZKS)	RPKM (ZEST)	RPKM (ZF4)
<i>lgals3l</i>	151.485882	94.79980323	28.5517658
<i>mcl1b</i>	138.0029954	100.3628761	34.55007944
<i>sumo3b</i>	142.0062334	85.88205698	40.71745045
<i>cx4oi2</i>	157.1675799	83.44086012	13.50501713
<i>capgb</i>	140.0313977	95.47507416	14.03742087
<i>fibina</i>	162.385075	111.5193532	8.63161174
<i>dcn</i>	138.738941	110.0299673	2.200388725
<i>fpr1</i>	129.1084434	117.6367545	0.801762529
<i>hspb9</i>	208.1065389	130.9142075	4.453978651
<i>urah</i>	181.7492844	153.0214309	2.606067373
<i>id3</i>	123.0298414	119.957024	32.53271352
<i>cd82b</i>	117.1867309	119.7501676	20.88039052
<i>dkk1b</i>	115.2674028	123.3601222	3.914853583
<i>cx43</i>	95.76255571	125.268914	20.60977394
<i>phlda3</i>	103.4830928	114.8205638	42.38678055
<i>pitpnaa</i>	102.4114719	110.3454892	46.22327706
<i>tstd1</i>	91.48324211	99.41705808	27.77823182
<i>timp2a</i>	110.5132145	134.7435353	45.08674135
<i>igflr1</i>	107.6665533	126.7691791	49.45986261
<i>ctsh</i>	116.9107597	129.9969604	52.32102958
<i>tspan4b</i>	124.4627775	134.439824	38.31343901
<i>kctd12.2</i>	70.17791498	131.2071169	10.41369721
<i>anxa5b</i>	223.4276827	190.0509067	82.02660608
<i>ccnd1</i>	204.0826607	198.000819	77.64559053
<i>cnm3a</i>	200.6466151	175.5868376	85.12488537
<i>lmo4b</i>	171.4754089	158.0390988	54.76838861
<i>iscu</i>	177.5601397	135.6039718	62.57678042
<i>serpine2</i>	155.1438879	185.8865435	41.16096343
<i>cxcl-c1c</i>	205.5342189	222.3706168	33.03549542
<i>fabp7a</i>	195.9918097	237.0929702	21.40297666
si:ch211-174h4.1	224.7857597	199.3655569	38.63959992
<i>ehd2</i>	161.7766861	245.0843826	56.47716098
<i>crabp2b</i>	105.0984741	221.8625863	49.8034191
si:ch211-222d3.3	95.00787666	229.3358097	21.15952131
<i>dup5</i>	126.478358	236.8883821	9.107537686
<i>itga10</i>	89.61611629	201.1502394	2.352461298
<i>tcn2l</i>	38.72650992	164.8295692	13.11416496
<i>cyp24a1</i>	18.72705544	177.0835255	2.519571099
<i>gdf10b</i>	33.67260166	219.1972506	0.695893287

Gene	RPKM (ZKS)	RPKM (ZEST)	RPKM (ZF4)
<i>hpdh</i>	81.65105717	369.7602972	1.681026612
<i>hbegfa</i>	237.5287195	432.953554	115.5311657
<i>timp2b</i>	201.697207	353.6519834	68.67404428
<i>thbs4b</i>	211.3951489	552.8064964	31.77418366
<i>phlda2</i>	661.6069058	447.6778629	148.3523463
<i>prdx1</i>	663.2337466	378.8825097	122.1185737
<i>gstp1</i>	585.6433683	324.3995674	107.5635844
<i>sncga</i>	506.5409161	491.6240584	33.91588988
<i>calm3a</i>	398.2576181	445.6855878	77.36093147
ENSDARG00000092903 (novel gene; gene name projected to be <i>C18H15orf48</i>)	570.4850827	238.5350614	41.45849625
<i>cd9b</i>	506.2882968	266.2312285	25.2919086
<i>snrpe</i>	514.8853227	160.8159508	78.98388808
<i>rpl31</i>	786.6591853	197.9255876	69.5006252
<i>ifitm5</i>	617.8582984	44.28435681	0.866051404
<i>soul5</i>	827.0453265	703.7188802	13.01478191
<i>rps16</i>	1058.353433	295.6625046	126.4458616
<i>rpl10a</i>	935.7242298	364.8865983	139.5790661
si:dkey-22i16.3	966.7386902	8.207114594	1.997906106
ENSDARG00000071626 (novel gene; no gene name)	858.6043295	26.27619774	1.428567107
<i>rps9</i>	1642.964348	524.3909043	228.2500494
<i>cox6b1</i>	1642.244168	266.1138185	132.3260874
si:ch211-12e1.3	1538.960816	697.6199432	27.15657434
<i>rpl22</i>	1350.323073	497.4139112	219.2842156
<i>clu</i>	1178.602248	1219.222027	348.8825399
<i>stc2a</i>	1171.390702	1231.42446	164.7996506
<i>rpl32</i>	3503.451624	1012.274554	501.3860264
<i>rpl6</i>	3084.841751	1276.133337	501.4586706

GO terms enriched in top 100 transcripts overexpressed in hematopoietic supportive ZKS and ZEST cells compared to non-hematopoietic supportive ZF4 cells

GO terms and description of transcripts in Table 2 analyzed by Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7^{59,60}. Gene names shown based on Zv9 assembly.

Term	Description	Count	%	P-Value	Gene	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GO:0006412	translation	9	10.46511628	1.76E-04	<i>rps16, rpl22, rpl10a, rs124d1, rpl32, rps9, rpl6, rpl13a, rpl31</i>	53	260	8389	5.479027576	0.06396177	0.06396177	0.24324761
GO:0016265	death	4	4.651162791	0.010687242	<i>phlda3, ckba, clu, pycard</i>	53	74	8389	8.555838858	0.98221296	0.866631938	13.79658808
GO:0008219	cell death	4	4.651162791	0.010687242	<i>phlda3, ckba, clu, pycard</i>	53	74	8389	8.555838858	0.98221296	0.866631938	13.79658808
GO:0007423	sensory organ development	5	5.813953488	0.040593182	<i>ccnd1, dlk1b, scinla, atp6v1e1b, dlx3b</i>	53	210	8389	3.768643306	0.999999822	0.994372052	43.5930556
GO:0019538	protein metabolic process	17	19.76744186	0.052330213	<i>dusp5, ctsh, rps16, rpl6, rpl13a, pycard, serpina2, psma6a, sumo3b, rpl22, blmh, rpl10a, rs124d1, rpl32, rps9, phex, rpl31</i>	53	1719	8389	1.565335265	0.999999998	0.993519671	52.41499751
GO:0001654	eye development	4	4.651162791	0.060506013	<i>ccnd1, scinla, atp6v1e1b, dlx3b</i>	53	145	8389	4.366428107	1	0.990730627	57.78407743
GO:0012501	programmed cell death	3	3.488372093	0.071435474	<i>phlda3, ckba, pycard</i>	53	71	8389	6.688014882	1	0.990266803	64.08636132
GO:0006915	apoptosis	3	3.488372093	0.071435474	<i>phlda3, ckba, pycard</i>	53	71	8389	6.688014882	1	0.990266803	64.08636132
GO:0042770	DNA damage response, signal transduction	2	2.325581395	0.077705368	<i>ccnd1, phlda3</i>	53	13	8389	24.35123367	1	0.986877438	67.29575354
GO:0009888	tissue development	5	5.813953488	0.096534022	<i>dlk1b, cx43, den, vmhc, dlx3b</i>	53	282	8389	2.806436505	1	0.991422401	75.40577817