Transcribing the missing hemangioblast: Single embryo gene expression profiling in zebrafish *cloche* embryos at the 5 somite stage

Gerhard J. Weber and Leonard I. Zon
Children's Hospital Stem Cell Program and Department of Hematology/Oncology, Howard Hughes Medical Institute
Children's Hospital Boston, Harvard Medical School
Boston MA 02115

The differentiation of hematopoietic stem cells (HSCs) into distinct lineages is orchestrated by tight regulation of critical genes during blood development. Forward genetic screens in zebrafish have identified mutants with defects that disrupt specific stages of hematopoiesis and vasculogenesis, including *cloche* (*clo*). $clo^{-/-}$ embryos are characterized by an almost complete absence of hematopoietic and vascular cells. The still elusive genetic defect is considered to occur on the level of the hemangioblast, and *in situ* studies in $clo^{-/-}$ have confirmed the absence of hemangioblast markers, such as *scl* or *lmo2*, as early as the 5 somite stage[2]. We have previously shown that transcriptional profiling of whole zebrafish blood mutants is capable of probing the genetic program of HSC differentiation[3]. These studies, however, relied on morphological identification of mutants and analyzed developmental stages (14 somites and 36 hours post fertilization) that occur after hemangioblast formation. In the study presented herein, we demonstrate that individual *clo* mutants can be distinguished at the 5 somite stage, before the visual appearance of the blood phenotype, based on their differential expression of *scl*. We further show that such separation of single mutant and wild type (wt) embryos, combined with RNA amplification, allows transcriptional profiling of zebrafish embryos during the earliest stages of blood development.

After individual homogenization of embryos at the 5 somite stage, total RNA was extracted using 500μl of Trizol (Invitrogen) / embryo, according to the manufacturer's recommendations, and resuspended in 15μl of RNAse-free H_2O. Purity and integrity of the isolated total RNA was confirmed by analysis of 1μl of total RNA on a Bioanalyzer 2100 (Agilent), yielding on average 468 ± 51ng (n=20) of total RNA / embryo (Figure 1). 60ng of total RNA from each embryo were then reverse-transcribed (SuperScript III, Invitrogen) and resulting cDNAs were tested for differential expression of *scl* by PCR, using the following primer sets: scl_1s (5'-TTAACAGTGGTTTTGCTGGAGATGCG-3'), scl_1as (5'-CGTTCACATTCTGCTGTCGCCA-3'), scl_2s (5'-AGTCCGCCCGCTTTCCCTCTC-3'), scl_2as (5'-CGTTCACATTCTGCTGTCGCCA-3'), scl_3s (5'-CGCAGACCTGCACCTTATGAG-3'), scl_3as (5'-CTCGTTCTTGCTGAGTTTCTTGTC-3'). Applied control primers were mapkapk2_s (5'-GCTGGGACTGGGCATCA-3'), mapkapk2_as (5'-GGCACTTCCTGTTTTGGTAGAG-3'), Dr.12322.1.A1_s (5'-ATCGGGGCAGAATGGTGTG-3'), Dr.12322.1.A1_as (5'-TTCTCTGGCTGTTCTTCTCCTGAC-3'). PCR parameters were 94°C for 20 seconds; 63°C or 59°C for *scl* or *mapkapk2 /Dr.12322.1.A1* primers, respectively, for 30 seconds; 68°C for 10 seconds, 32 cycles, 10 minute elongation at 68°C.

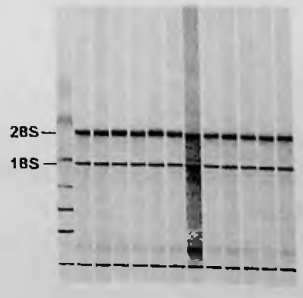

Fig. 1. Computed gel image of total RNA, confirming integrity of the isolated RNA from single embryos. (Lane 1: RNA ladder; lanes 2-13: total RNA of 12 individual embryos at the 5 somite stage).

PCR amplification revealed abundant transcripts of *scl* in 14/20 embryos, and complete absence of *scl* expression in 6/20 embryos. Transcripts of the control genes *Dr.12322.1.A1* and *mapkapk2* were consistently detected in all mutants. Embryos were separated into wt or *clo* mutants, respectively, based on presence / absence of *scl* expression by PCR, which followed approximately the Mendelian ratio of 3:1 expected for the *clo* phenotype. A representative gel image is shown in Figure 2.

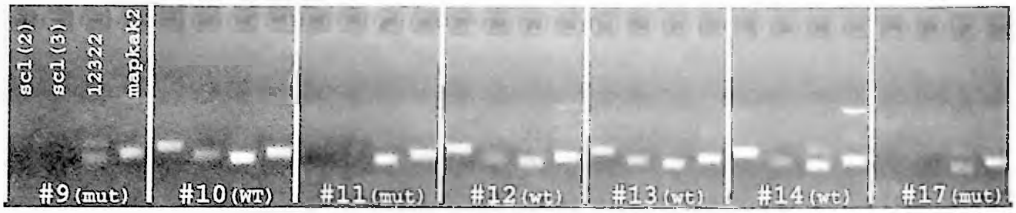

Fig. 2. RT-PCR for *scl*, *Dr.12322.1.A1*, and *mapkapk2* in 5 somite stage embryos. Absence of transcripts for *scl* identifies *clo* mutants.

The remaining total RNA of five putative wt and five putative *clo*$^{-/-}$ embryos were then individually subjected to two rounds of amplification, according to standard protocols (http://www.ambion.com/techlib/prot/fm_1750.pdf)[3]. After amplification, biotin-labeled cRNA was hybridized to zebrafish GeneChips (Affymetrix, Santa Clara, CA). Normalization and statistical data analysis were performed using publicly available software[1-3].

The generated gene expression profiles of the single embryos confirmed the genotypes of the putative wt and *clo* mutants. For all embryos that lacked *scl* expression by RT-PCR, microarray analysis revealed similar low expression of other early hematopoietic markers, such as *lmo2*, *hhex*, or *gata1*. Additional representative genes and their regulation are listed in Table 1.

Gene Title	Gene Symbol	log2(fold change)	q-value
T-cell acute lymphocytic leukemia 1	tal1 (scl)	-3.7	0.13
LIM domain only 2	lmo2	-1.7	0.13
GATA-binding protein 1	gata1	-1.6	0.39
Retinoic acid receptor gamma	rarg	-1.4	0.43
Homeobox protein pbx4	pbx4	-1.0	0.28
Homeo box A10b	hoxa10b	2.1	0.34
RGM repulsive guidance molecule	rgma	3.6	0.40

Table 1. Representative genes regulated in *clo*[s5]. Fold changes are shown as log2[mean(signal mutant / signal wt)].

In summary, our data demonstrate that gene expression profiling of single zebrafish embryos enables analysis of mutant transcriptomes before morphological phenotypes become apparent. We detect gene regulation in *clo*$^{-/-}$ that occurs in response to failing formation of hemangioblasts. In conjunction with the high conservation of vertebrate hematopoiesis, our approach represents a powerful tool to unravel the genetic program during the early stages of blood and blood vessel development.

We would like to thank Nishad Jayasundara for excellent technical assistance. The research was supported by a New Investigators Award to L.I.Z. L.I.Z. is an investigator of the Howard Hughes Medical Institute.

1. **Choe SE, Boutros M, Michelson AM, Church GM, and Halfon MS.** Preferred analysis methods for Affymetrix GeneChips revealed by a wholly defined control dataset. *Genome Biol* 6: R16, 2005.
2. **Liao EC, Paw BH, Oates AC, Pratt SJ, Postlethwait JH, and Zon LI.** SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev* 12: 621-626, 1998.
3. **Weber GJ, Choe SE, Dooley KA, Paffett-Lugassy NN, Zhou Y, and Zon LI.** Mutant-specific gene programs in the zebrafish. *Blood* 106: 521-530, 2005.