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Global analysis of hematopoietic and vascular endothelial gene expression by tissue specific microarray profiling in zebrafish

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Abstract

In this study, we utilize fluorescent activated cell sorting (FACS) of cells from transgenic zebrafish coupled with microarray analysis to globally analyze expression of cell type specific genes. We find that it is possible to isolate cell populations from *Tg(fli1:egfp)*^{y1} zebrafish embryos that are enriched in vascular, hematopoietic and pharyngeal arch cell types. Microarray analysis of GFP$^+$ versus GFP$^-$ cells isolated from *Tg(fli1:egfp)*^{y1} embryos identifies genes expressed in hematopoietic, vascular and pharyngeal arch tissue, consistent with the expression of the *fli1:egfp* transgene in these cell types. Comparison of expression profiles from GFP$^+$ cells isolated from embryos at two different time points reveals that genes expressed in different *fli1*$^+$ cell types display distinct temporal expression profiles. We also demonstrate the utility of this approach for gene discovery by identifying numerous previously uncharacterized genes that we find are expressed in *fli1:egfp*-positive cells, including new markers of blood, endothelial and pharyngeal arch cell types. In parallel, we have developed a database to allow easy access to both our microarray and in situ results. Our results demonstrate that this is a robust approach for identification of cell type specific genes as well as for global analysis of cell type specific gene expression in zebrafish embryos.

Keywords: Zebrafish; Hematopoietic; Vascular; Endothelial; Microarray

Introduction

The zebrafish has become an ideal model organism for the study of vertebrate embryogenesis. The transparency and accessibility of zebrafish embryos allow for a variety of detailed manipulations and phenotypic analyses, while its small size, fecundity and ease of maintenance have helped to establish it as a powerful genetic system. Numerous mutants have been described in the course of several large- and small-scale forward genetic screens (Driever et al., 1996; Haffter et al., 1996; Patton and Zon, 2001). Increasingly dense genetic (Gates et al., 1999; Kelly et al., 2000; Knapik et al., 1998; Postlethwait et al., 1994) and physical maps (Geisler et al., 1999; Hukriede et al., 1999) have greatly aided the subsequent positional or candidate cloning of the genes responsible for these mutant phenotypes, and large-scale screens utilizing retroviral mutagenesis now allow rapid cloning of affected genes (Amsterdam, 2003). In addition to classical forward genetic approaches, the advent of modified antisense oligonucleotides that block translation or splicing in a gene specific manner (referred to as Morpholinos) has allowed reverse genetic approaches as well (Nasevicius and Ekker, 2000). The current availability of genomic sequence has greatly benefited both forward and reverse genetic approaches by facilitating the identification of candidate genes of interest.

The transparency of the zebrafish embryo and the ability to collect hundreds of embryos at specific time points has also allowed detailed temporal and spatial analysis of gene expression through whole mount in situ hybridization. Several

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groups have initiated large-scale in situ screens in order to provide tissue specific expression data in parallel to current expressed sequence tag (EST) projects. In some cases, these screens are unbiased and involve random selection of clones from normalized libraries derived from different embryonic stages (Kudoh et al., 2001; Thisse et al., 2004). Thus far, several thousand expression patterns have been generated from these efforts and curated by the Zebrafish Information Network (ZFIN). In other cases, researchers have focused their expression screens on genes containing particular motifs (Crosier et al., 2001) or involved in specific developmental processes (Yoda et al., 2003). In some cases, these approaches have proven to be useful at identifying new components of known signaling pathways since genes with common expression patterns often have functional relationships that are important for development. For example, both the *sef* gene and *dusp6* were identified in an in situ screen based on the similarity of their expression patterns to genes known to be involved in fibroblast growth factor (FGF) signaling pathway, such as *fgf8* (Furthauer et al., 2002; Tsang et al., 2002, 2004). Subsequent functional analysis of both *sef* and *dusp6* revealed their role as negative regulators of FGF signaling.

In addition to whole mount in situ hybridization, researchers have begun to utilize microarrays for global analysis of gene expression in zebrafish embryos. Several studies have used microarray analysis of zebrafish mutants in order to characterize changes in gene expression associated with defects in hematopoietic and vascular development (Qian et al., 2005; Sumanas et al., 2005; Weber et al., 2005). Thus far, this work has proven useful at identifying a number of new hematopoietic and vascular markers and new genes required for development of these cell types (Sumanas and Lin, 2006). Additionally, microarray analysis has allowed the identification of pathway-specific molecular signatures through the combinatorial comparison of multiple related mutant lines (Weber et al., 2005). In most of the microarray studies to date, researchers relied on whole embryos as a source for RNA for microarray analysis. However, there are drawbacks to the use of whole embryo array profiling. First, the primary cell type affected in a particular mutant may only represent a small proportion of the embryo. This is certainly the case for many of the mutants that affect development of endothelial or hematopoietic cell types. Second, given the complexity of transcripts obtained from a whole embryo, it is likely that moderate and low abundant transcripts in a cell type of interest will be poorly detected in this context. Finally, transcript levels for many genes expressed in multiple tissues may change in only the affected cell types in a particular mutant. These same genes would still be expressed normally in other tissue type and would likely appear to be unchanged by microarray analysis using whole embryos.

In this study, we describe an approach to globally analyze gene expression in specific cell types in the zebrafish embryo. We have taken advantage of the *Tg(fli1:egfp)* transgenic zebrafish line in which blood, endothelial and pharyngeal arch cells express enhanced green fluorescent protein. We demonstrate that it is possible to isolate these cells types from *Tg(fli1:egfp)*^{y1} embryos using fluorescence activated cell sorting (FACS). We show that comparison of expression profiles from GFP$^+$ and GFP$^-$ cells by microarray analysis allows identification of genes expressed in *fli1*$^+$ cell types. Furthermore, by isolating and comparing expression profiles in GFP$^+$ cells from embryos at two different developmental time points, we are able to globally observe dynamic changes in genes within these specific cell types. We also demonstrate the utility of this approach to identify new genes expressed in *fli1*$^+$ cell types. We have established a database of these microarray data, including in situ results, that is easily navigated with a web-browser interface and allows access to the general scientific community.

Methods

Zebrafish

Zebrafish and their embryos were handled according to standard protocols. *Tg(fli1:egfp)*^{y1}, *Tg(hsp70:gal4)*^{kca4}, *Tg(uas:notch1intra)*^{kca3} and *mib*^{ta52b} lines have been described elsewhere (Lawson et al., 2001) and are available through the Zebrafish International Resource Center (ZIRC). Heat shock treatment of *Tg(hsp70:gal4)*^{kca4}*;Tg(uas:notch1intra)*^{kca3} embryos was performed as described (Lawson et al., 2001). The *y18* allele of *phospholipase c gamma 1* is a splice mutation that truncates Plcg1 protein in the X catalytic domain (unpublished observations).

Embryo dissociation and fluorescence activated cell sorting (FACS)

Wild type *Tg(fli1:egfp)*^{y1} embryos were grown in egg water to indicated stage and were dechorionated by pronase treatment. Embryos were rinsed for 15 min in calcium free Ringer and passed several times through a 200 μL pipette tip to remove their yolk. Embryos were transferred into a 35 mm culture dish with 2 mL phosphate buffered saline (PBS, pH 8) containing 0.25% trypsin and 1 mM EDTA and incubated for 30 to 60 min at 28.5°C during which they were triturated with a 200 μL pipette tip every 10 min. The digest was stopped by adding $CaCl_2$ to a final concentration of 1 mM and fetal calf serum to 10%. Cells were centrifuged for 3 min at 3000 rpm, rinsed once with PBS and resuspended at 10^7 cells/mL with Leibovitz medium L15 without phenol red, 1% fetal calf serum, 0.8 mM $CaCl_2$, penicillin 50 U/mL and streptomycin 0.05 mg/mL. FACS of single cell suspensions was performed at room temperature under sterile conditions using a FACSVantage SE/DIVA (Becton Dickinson) with a Coherent Innova 70 laser at 488 nm and 200 mW power. GFP$^+$ and GFP$^-$ cells were separately collected in L15, 0.8 mM $CaCl_2$, 10% fetal calf serum, 10% zebrafish embryo extract, penicillin 50 U/mL and streptomycin 0.05 mg/mL. Following sorting, cell viability was greater than 90%. We were routinely able to obtain 7×10^5 GFP$^+$ from approximately 300 *Tg(fli1:egfp)*^{y1} embryos which was usually sufficient to obtain 1 μg of total RNA.

RNA isolation and quantitative RT-PCR

Equal numbers of GFP$^+$ and GFP$^-$ cells were centrifuged, resuspended in 250 μL of Trizol Reagent (Invitrogen) and stored at −80°C. Total RNA was isolated according to manufacturer's instructions. RNA pellets were resuspended in 20 μL nuclease-free water (Ambion). RNA integrity was confirmed by separation and visualization in ethidium stained formaldehyde/agarose gels according to standard protocols. For quantitative PCR, first strand cDNA was generated using 1 μg total RNA, 200 U Superscript III reverse transcriptase (Invitrogen) and 2.5 μM oligo dT primer in a 20 μL reaction. Both cDNA concentrations were adjusted to 200 ng/μL, and QPCR was performed using iTaq SYBR Green Supermix with ROX (Biorad) using 500 ng of cDNA and 0.6 μM gene specific primers in a 25 μL reaction and detected in ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCR primers were as follows: *gapdh*, 5′-TGCTGTAACCGAACTCATTGTC and 5′-CAAGCTTACTGGTATGGCCTTC; *ef1a*, 5′-ATACATCAAGAAGATCGGCTACAAC and 5′-AATACCTCCAATTTTGTACACAT; *bactin*, 5′-TGGCCCCTAGCACAATGAAG and 5′-GCCTCCGATCCAGACAGAGTAT; *fli1a*, 5′-CCGAGGT-