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Microarray analysis of prothrombin knockdown in zebrafish

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ABSTRACT

The serine protease thrombin is generated from its precursor, prothrombin, in the coagulation cascade and plays a central role in fibrin deposition and platelet activation mediated through the protease activated receptors. Knockdown of prothrombin in the zebrafish was previously shown to recapitulate the phenotype observed in prothrombin knockout mice, such as an absence of blood pericardial edema, and hemorrhage. However, the role of thrombin during embryogenesis is not fully understood. To find genes affected by potential thrombin signaling in embryogenesis before blood circulation, microarray analysis was performed using total RNA prepared from antisense-injected, knockdown embryos versus mismatch-injected at 20 h post fertilization. A total of 63 upregulated and downregulated genes were identified with duplicate microarrays using dye reversal and a two-fold difference limitation. Real time RT-PCR for 10 selected genes identified by the microarray confirmed the expression changes in these genes. One particular gene, *phlda3*, was at least eleven fold upregulated, and in situ hybridization revealed expansion of phlda3 expression in the central nervous system, branchial arches, and head endoderm in knockdown embryos. The identification of these genes regulated by thrombin according to microarray analysis should provide a greater understanding of the effects of thrombin activity in the early vertebrate embryo.

Introduction

Thrombin is the central serine protease of the blood coagulation cascade that converts fibrinogen to fibrin and is generated from its zymogen precursor prothrombin by the action of Xa. Thrombin also mediates a cellular response primarily through cleavage and activation of the G-protein-coupled, protease activated receptor-1 (PAR-1) that has been extensively characterized in platelet activation [1]. In addition to being a potent platelet agonist, thrombin also exerts activity in fibroblasts, smooth muscle, neurons, endothelium and other cell types [1–5].

Targeted gene inactivation of prothrombin in mice leads to embryonic lethality at approximately midgestation [6–8]. A prothrombin-deficient phenotype exhibited many developmental defects related to yolk sac vascular integrity such as enlarged and dilated capillary structure, vessels devoid of blood, and flattening of visceral yolk sac endoderm. In the zebrafish, knockdown of prothrombin produced an early phenotype in which greater than one third of all embryos have abnormalities in their overall growth with defects in the anterior and posterior regions [9]. When grown to 48 h post fertilization (hpf), these embryos recapitulated the prothrombin-deficient phenotype observed in the mouse embryo. They exhibited an absence or reduced number of blood cells, reduced blood flow, pericardial edema, and blood clots in the trunk region. These results showed a conserved role for thrombin in vertebrate embryonic development.

To elucidate changes in gene expression affected by prothrombin knockdown, hybridizations on microarrays consisting of oligonucleotides representing 14,000 genes were performed using RNAs made from 20 hpf antisense morpholino-injected (ASMO) embryos displaying the previously characterized abnormalities versus mismatch control-injected (MMMO) embryos. Hybridization results revealed a total of 63 upregulated and downregulated genes using a two-fold expression change limit. Real time quantitative RT-PCR (QRT-PCR) was used to confirm microarray results for 10 genes identified. A gene that was 11-fold upregulated, called *phlda3*, encodes a small protein containing a pleckstrin homology domain that could be involved in regulating IP_3 release, recruitment of proteins to the intracellular membrane surface, and membrane shape changes. Phlda3 was shown to be upregulated in the CNS with possible expansion of expression in the branchial arches and anterior endoderm. Additionally, the sry-related HMG box transcription factor, sox21, was shown to be downregulated greater than two-fold. Sox21 is duplicated in the zebrafish genome and is termed as sox21a or sox21b [10]. Sox21a is expressed throughout the forebrain, midbrain and hindbrain, but shows the greatest expression in the midbrain–hindbrain boundary (MHB) [11]. Reduction of sox21a MHB expression in knockdown embryos further verified microarray results. All of these results taken together indicate a potential role for thrombin signaling in the embryonic brain. The identification of *sox21a*, *phlda3*, and the other

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genes regulated by thrombin according to microarray analysis will provide a greater understanding of the effects of thrombin activity in the early vertebrate embryo.

Materials and methods

Morpholino oligonucleotides and microinjections of zebrafish embryos

A prothrombin antisense morpholino oligonucleotide, 5′GTTT-GGCTCCCATCCTTGAGAGTGA-3′ (ASMO) against the 5′-UTR to target the translational start site of the zebrafish prothrombin mRNA and a control oligonucleotide 5′GTTTCGCTCGCATGCTTCAGACTGA-3′ (MMMO) with 5 base mismatches (mismatches indicated by underlines) were purchased from Gene-Tools LLC, Philomath, OR. Embryos were microinjected with 4.5 nl ASMO (1 mg/ml) or MMMO diluted in Danieau buffer into the yolk of one to four cell stage embryos [12]. Injected embryos were maintained in embryo medium at 28 °C until 20 hpf. Embryos that exhibited early phenotype (depicted in Fig. 1) as previously reported were collected [9].

Oligonucleotide microarray experiments

Total RNA was isolated from 20 hpf embryos collected from 4 independent microinjection experiments. The first batch of RNA was made from 16 embryos (each embryo was injected with 4.5 ng ASMO) displaying the early phenotype, and 16 embryos injected with 4.5 ng MMMO that were normal in appearance. The same selection procedure was used for 3 more experiments using 12 embryos each for ASMO and MMMO groups. Total RNA was isolated from embryos, and independent RNA preparations were pooled, run through columns provided in the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA), and final RNA integrity, purity, and concentration were again estimated by UV spectrophotometry and gel electrophoresis.

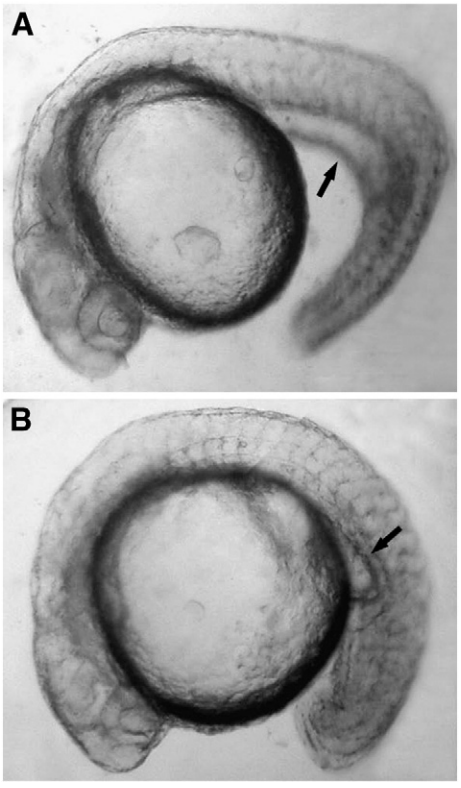

Fig. 1. Overall gross appearance of zebrafish embryos grown to 20 hpf after microinjection of morpholinos. Compared to the mismatch-injected control (A), there is aberrancy in the trunk and tail with a large reduction in the yolk sac extension (indicated by arrows) in the antisense-injected knockdown (B). All antisense embryos exhibiting this phenotype were selected for RNA preparation for microarray analysis.

The University of Texas Southwestern Medical Center at Dallas Microarray Core Facility performed the following steps using the high quality total RNA prepared. 5 μg of pooled ASMO and MMMO RNAs were used with the Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) for first strand cDNA synthesis. Second strand was synthesized using the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen). Reactions were incubated at 16 °C for 2 h, then 1 μl T4 polymerase was added, incubated for 10 min at 16 °C, and finally reactions were stopped by adding 10 μl of 0.5 M EDTA to inhibit all enzymes.

Complementary DNA was purified using the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) according to the protocol supplied by the manufacturer. Antisense RNA (aRNA) was amplified by T7 in vitro transcription using the MessageAmp aRNA Kit (Ambion, Austin, TX) with unlabeled NTPs, 16 μl cDNA, and incubated at 37 °C overnight according to protocol. The GeneChip Sample Cleanup Module (Affymetrix) was then used again for aRNA purification, and aRNA was quantified by spectrophotometry. Probes were labeled using the ASAP RNA Labeling Kit (Perkin Elmer, Boston, MA) in two separate reactions using 2 μg of each aRNA with ASAP Cyanine-3 Reagent or ASAP Cyanine-5 Reagent according to protocol. Each labeled probe was then combined, mixed into the same tube, and purified using a Microcon YM-30 filter column. RNA probe synthesis was also repeated by reverse labeling ASMO and MMMO RNA samples.

5 μl of probe were then mixed with preheated ASAP Hybridization buffer (Perkin Elmer) and placed on to two zebrafish 14K arrays (MWG Biotech Inc., High Point, NC) with each array being divided into two slides (array A and array B) each representing 14,067 total genes. Probes containing the MMMO-Cy3 and ASMO-Cy5 mixtures were used for two microarray A slides and the reverse label mixture (MMMO-Cy5 and ASMO-Cy3) was used for two microarray B slides for performing the hybridizations in duplicate. Probes were hybridized for 14–16 h at 62 °C. Slides were then washed, dried, and scanned for data acquisition using a GenePix® 4000B scanner (Axon Instruments, Inc., Union City, CA).

Oligonucleotide microarray data processing

Scanned GenePix data was imported into the online GeneTraffic™ DUO two-color microarray data analysis software version 2.8-9 (Iobion Informatics LLC, La Jolla, CA) for data analysis. LOWESS subgrid normalization was used for each slide, and background was automatically subtracted from a generated correction value. Two fold change criteria were used to select differentially expressed genes from the microarray to complete an annotated list of results with fold level changes greater than or equal to 2-fold or less than or equal to 0.5-fold. Data was sorted by selection of successful hybridizations. All genes were manually grouped according to function or subcellular localization.

Real time RT-PCR

Total RNA was made from early phenotype and MMMO-injected reference embryos at 20 hpf similar to RNAs prepared for the microarray as described below. Three independent RNA preparations were made from a pool of embryos for each ASMO and MMMO-injected group by homogenization in RNAzol™ B Isolation of RNA solution (Leedo Medical Laboratories, Houston, TX) using a Brinkman polytron homogenizer. RNA pellets were resuspended in nuclease-free water at 60 °C for 10 min. RNAs were treated with DNAse using the DNA-*free*™ DNase Treatment kit (Ambion, Austin, TX) at 37 °C for 30 min to eliminate any potentially contaminating genomic DNA, and 500 ng of treated total RNA were used for cDNA synthesis using