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Cysteine and glycine-rich protein 1 is involved in spinal cord regeneration in adult zebrafish

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Abstract

In contrast to mammals, adult zebrafish have the ability to regrow descending axons and gain locomotor recovery after spinal cord injury (SCI). In zebrafish, a decisive factor for successful spinal cord regeneration is the inherent ability of some neurons to regrow their axons via (re)expressing growth associated genes during the regeneration period. The nucleus of the medial longitudinal fascicle (NMLF) is one of the nuclei capable of regenerative response after SCI. Using microarray analysis with laser capture microdissected NMLF, we show that cysteine and glycine-rich protein 1 (CRP1, encoded by the *csrpl1* gene in zebrafish), whose function is largely unknown in the nervous system, was upregulated after SCI. *In situ* hybridization confirmed the upregulation of *csrpl1* in neurons during the axon growth phase after SCI, not only in the NMLF, but also in other nuclei capable of regeneration, such as the intermediate reticular formation (IMRF) and the superior reticular formation (SRF). The upregulation of *csrpl1* in regenerating nuclei started at 3 days after SCI and continued to 21 days post-injury, the longest time point studied. *In vivo* knockdown of CRP1 using two different anti-sense morpholino oligonucleotides impaired axon regeneration and locomotor recovery when compared to a control morpholino, demonstrating that CRP1 upregulation is an important part of the innate regeneration capability in injured neurons of adult zebrafish. Together, this study is the first to demonstrate the requirement of CRP1 for zebrafish spinal cord regeneration.

Keywords

CRP1; spinal cord regeneration; zebrafish; NMLF; microarray

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Introduction

In adult mammals, the failure of injured axons to regenerate after spinal cord injury often results in permanent disabilities. Inhibitory molecules in the spinal cord are known to be major contributors to regeneration failure (David & Aguayo, 1981; Schwab & Bartholdi, 1996; Silver & Miller, 2004). However, removing inhibitory activities only induces limited axon regeneration which is insufficient for successful functional recovery (Zheng *et al.*, 2003; Case & Tessier-Lavigne, 2005; Yiu & He, 2006; Cafferty *et al.*, 2008), pointing to the importance of understanding the mechanisms for the intrinsic regenerative competence of neurons. Unlike mammals, adult zebrafish have the innate ability to regenerate descending axons after spinal cord injury (SCI), leading to locomotor recovery. In zebrafish, a crucial contributor for successful axon regeneration is the inherent ability of neurons to (re)express growth associated genes and regrow their axons (Becker *et al.*, 1997; Bernhardt, 1999; Becker *et al.*, 2004; Becker & Becker, 2008). *In vivo* depletion of these growth associated genes dramatically impairs axon regeneration and locomotor recovery (Becker *et al.*, 2004; Yu *et al.*, 2011a; Yu *et al.*, 2011b). The ability to upregulate growth associated genes during the regeneration period makes the adult zebrafish a valuable model for identifying molecules involved in successful spinal cord regeneration.

The nucleus of medial longitudinal fascicle (NMLF), an anatomically well defined nucleus, is one of several nuclei exhibiting a robust regenerative response after SCI as evidenced by the upregulation of growth associated genes and axonal regrowth (Becker *et al.*, 1997; Becker *et al.*, 1998; Becker *et al.*, 2004; Yu *et al.*, 2011a; Yu *et al.*, 2011b). To investigate the molecular mechanisms underlying this successful regeneration response, we performed microarray analysis with laser capture microdissected NMLF. The list of upregulated genes in the growth phase included known growth associated genes such as growth associated protein 43 (GAP-43), as well as novel genes whose role in axon regeneration are uncharacterized, such as the cysteine and glycine-rich protein 1 (CRP1).

CRP1 is a member of the LIM-domain protein CRP family including CRP1 (encoded by *csrp1a* gene in zebrafish), CRP2, and CRP3/MLP (Louis *et al.*, 1997). All CRP family members are highly expressed in muscle cells (Jain *et al.*, 1998) and CRP1 is regarded as marker for smooth muscle (Henderson *et al.*, 1999). CRP1 is involved in many different cellular functions, acting as a transcriptional cofactor (Chang *et al.*, 2003), suppressing cell proliferation, protecting cells from stress-induced death (Latonen *et al.*, 2008), regulating cell movement during zebrafish development (Miyasaka *et al.*, 2007), and promoting neointima formation (Lilly *et al.*, 2010). CRP1 localizes to the nucleus and actin cytoskeleton (Tran *et al.*, 2005; Jang & Greenwood, 2009), and interacts with α -actinin (Pomies *et al.*, 1997) and zyxin (Sadler *et al.*, 1992; Schmeichel & Beckerle, 1998). CRP1 is the only CRP family member that has detectable expression in the mammalian central nervous system (CNS) (Jain *et al.*, 1998). However, little is known about functions of CRP1 in the CNS. In the present study, we report that *csrp1a* is upregulated after SCI and its expression *in vivo* is essential for successful axonal regeneration and locomotor recovery. Our results identify CRP1 as a key component of functional spinal cord regeneration in adult zebrafish.

Materials and Methods

Spinal cord injury in adult zebrafish

Male adult zebrafish (*Danio rerio*, age > 6 months) were purchased from Aquatica Tropicals Inc. (Plant City, FL, USA). The fish were kept on a 14-hour light and 10-hour dark cycle at 28°C.

Spinal cord injury (SCI) was performed as described (Becker *et al.*, 1997; Becker *et al.*, 2004; Guo *et al.*, 2011; Yu *et al.*, 2011a; Yu *et al.*, 2011b). Briefly, fish were immersed in 0.033% aminobenzoic acid ethylmethylester (MS222; Sigma, St. Louis, MO, USA) for 5 min. After a longitudinal incision was made to expose the vertebral column, a complete transection of the spinal cord was performed between two vertebrae, about 1 mm (for microarray analysis and *in situ* hybridization) or 3.5 mm (for morpholino (MO) treatment) caudal to the brainstem-spinal cord junction, corresponding to the second and eighth vertebra respectively. The number of animals used for each experiment can be found in figure legends. The sham-lesioned control had identical surgical procedures except that the spinal cord was not cut (referred to as control or CON). The wound was sealed with Histoacryl (B. Braun, Melsungen, Germany).

All animal experiments were carried out according to a protocol approved by the Rutgers University Institutional Animal Use and Care Committee, which conformed to NIH guidelines.

Laser capture microdissection, RNA isolation and amplification

A photoablation and laser microdissection system (PALM, Carl Zeiss, Thornwood, NY, USA) was used as previously described (Liss *et al.*, 2005). At 4 hours, 12 hours or 11 days after SCI, the brain was removed, embedded in Tissue-Tek OTC, and snap frozen in isopentane (cooled on dry ice). Utilizing the atlas of zebrafish brain (Wullmann, 1996), coronal cryosections (20 µm) of zebrafish brainstem containing NMLF (approximately 30 sections) were cut and mounted on RNase-free, ultraviolet light-treated, and poly-L-lysine coated membrane slides (1 mm polyethylene-naphthalate membrane, PALM). Sections were fixed with ethanol (four washes with 70%, 95%, 100% ethanol, respectively) and stained with 1% cresyl violet in 100% ethanol, and dried. The NMLF area was cut and captured directly into an adhesive cap (PALM). The buffer RLT (RNeasy Micro Kit; Qiagen, Hilden, Germany) supplemented with β-mercaptoethanol (10 µl/ml) was added into the tube which was then incubated upside-down for 30 min at room temperature. Each condition (control or SCI) consisted of three microarrays. The RNA sample for each microarray was prepared from NMLF tissues pooled from 3 animals.

Total RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's directions with the addition of linear acrylamide (Ambion, Austin, TX, USA) as RNA carrier to the lysate. The RNA was amplified using the RampUP RNA amplification kit (Genisphere, Hatfield, PA, USA) following the manufacturer's protocol.

Microarray analysis and quantitative real-time PCR

Affymetrix GeneChip® Zebrafish Genome Arrays (Affymetrix, Santa Clara, CA, USA) containing 15,617 probes for ~14,900 transcripts were used for gene expression analysis, according to the manufacturer's instructions. Complementary DNA was reverse transcribed from the amplified RNA and hybridized to microarrays. Following hybridization, the arrays were washed and scanned following standard Affymetrix protocols. Data were extracted from CEL files using the RMA method (Irizarry *et al.*, 2003) as implemented in GeneSpring GX (Agilent, Santa Clara CA, USA). The exploratory list (Table 1 and Supplementary Table 1) was generated in GeneSpring by selecting genes with a *t*-test *P* value of 0.05 or less and a fold-change from control of 1.5 or more, both at 11 days after injury. The table lists average fold-change expression at each time point (calculated by taking the antilog of the log normalization values for each replicate array in GeneSpring) and the standard error of the mean (SEM). Data files have been deposited in the NIH GEO repository with a study accession number GSE28470.

Quantitative real-time polymerase chain reaction (qPCR) was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) as previously described (Goff *et al.*, 2004). The comparative cycle threshold C_t method (C_t method) was used for data analysis. Results were expressed relative to control, sham-injured fish. The same cDNA used for the microarrays was used to test the expression of GAP-43 and CRP1 in the NMLF after SCI. To study the expression of CRP1 in spinal cord caudal to the lesion site, total RNA was prepared from 4 millimeter pieces of whole spinal cord directly caudal to the lesion site. The primers used are as follows: zebrafish *csrp1a* (forward: 5'-TGCTTCCTGTGCATGGTTTG-3' and reverse: 5'-GGCCACCGTGGTACTGTCA-3'); zebrafish *GAP-43* (forward: 5'-TCAGGAGATCAAGCAGGATGG-3' and reverse: 5'-GCCTTGTGAGCGTTTTTCCTC-3'); zebrafish ribosomal protein *P0* (forward: 5'-TCGGCTACCCAACCTCTTGCT-3' and reverse: 5'-TGTTTCGACAGTGACAGCCAG-3').

In situ hybridization

Digoxigenin (DIG) labeled RNA sense and anti-sense probes for zebrafish *csrp1a* (NM_205567, 125bp – 554bp of coding sequence) was generated using the Megascript™ system (Ambion) according to the manufacturer's protocol and *in situ* hybridization was performed with some modifications as previously described (Becker *et al.*, 1998; Lieberoth *et al.*, 2003). No significant similarity was found with other zebrafish genes demonstrating its specificity for *csrp1a*. Briefly, 20 µm-thick coronal sections containing NMLF, the intermediate reticular formation (IMRF) and the superior reticular formation (SRF), were incubated with 0.1 N HCl for 10 minutes, followed by three washes in phosphate buffered saline (PBS), pH 7.4, and then digested for 10 minutes with 10 µg/ml proteinase-K (Roche, Indianapolis, IN, USA) at room temperature, followed by fixation in 4% paraformaldehyde after 2 washes with glycine (2 mg/ml) in PBS, pH 7.4. Next, the sections were acetylated, dehydrated, air-dried, prehybridized, and subsequently hybridized with DIG-labeled probe at 55°C overnight. The hybridized probes were detected using alkaline phosphatase-coupled anti-DIG antibody (Roche) and color developed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche). The same development time was used for sections from control and SCI animals. *In situ* hybridization was performed in parallel on sections from

injured animals and no significant signal was observed. The neurons in NMLF can be easily identified by their anatomical location according to the atlas of zebrafish brain (Wullmann, 1996) and their large size (13 – 23 μ m in diameter) (Becker *et al.*, 1997), which is distinguished from the small size of glial cells (Becker *et al.*, 1998). For profile counting, positively stained neurons in the NMLF, or IMRF, or SRF from each animal were counted with the experimenter blinded to the treatment of each fish.

Double staining for in situ hybridization and immunohistochemistry

The immunostaining of NeuN was performed after *csrp1a* signal development by *in situ* hybridization. Slides were rinsed in PBS for 3 times (5 min per wash) and antigen retrieval was performed by incubation of the slides with 10 mM citrate buffer (pH 6.0) at 95 °C for 15 min as described previously (Ma *et al.*, 2006). The slides were allowed to cool to room temperature naturally. After three washes with PBS, the slides were blocked with 1% bovine serum albumin and 3% goat serum in PBS, and incubated with mouse anti-NeuN antibody (1:150, A-60; Millipore, Billerica, MA, USA) at 4 °C overnight. Then, the slides were washed three times in PBS and incubated with secondary antibody Alexa Fluor 555 (1:600, Invitrogen, Carlsbad, CA, USA).

Application of morpholinos and biocytin

Two different anti-sense morpholino (MO) oligonucleotides for zebrafish CRP1 (NM_205567) (CRP1 MO1: 5'-GTTTCCACCCCAAGAGGCATCCTG-3'; CRP1 MO2: 5'-CTGCTAGGTGTGTGGATATGAAGAG -3') were designed and synthesized by Gene Tools, LLC (Philomath, OR, USA). Both CRP1 MOs work by blocking translation. CRP1 MO1 was tagged with carboxyfluorescein at the 3' end. The specificity of CRP1 MO2 has been previously demonstrated that its effects on zebrafish embryos can be rescued by coinjection of CRP1 mRNA (Miyasaka *et al.*, 2007). CRP1 MO2 could not be coupled with carboxyfluorescein due to its high G content, but was included to compare with previous studies. Sequence for the standard control MO, which was also tagged with carboxyfluorescein at the 3' end, was 5'-CCTCTTACCTCAGTTACAATTTATA-3'. The MOs were dissolved in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) and applied as previously described (Becker *et al.*, 2004; Guo *et al.*, 2011; Yu *et al.*, 2011a; Yu *et al.*, 2011b).

Fish were treated with 600 ng MO (approximately 0.27 μ l, absorbed into Gelfoam, Upjohn, Kalamazoo, MI, USA) at the lesion site immediately after transection. The MO is taken up by lesioned axons and retrogradely transported to the cells of origin in the brain, where protein synthesis for the target gene is blocked. The MO-treated fish were allowed to survive for 6 weeks. The dosing of the MOs (600 ng/fish) was chosen based our previous publication showing that 400 ng L1.1 MO is effective for knocking down protein expression and locomotor recovery in the identical zebrafish injury model (Becker *et al.*, 2004). Also, the effectiveness of this dosing has been confirmed by several publications from our group (Guo *et al.*, 2011; Yu *et al.*, 2011a; Yu *et al.*, 2011b).

Six weeks after the MO application, the neuronal tracer biocytin (Sigma) saturated solution, approximately 0.27 μ l, absorbed in Gelfoam) was applied to a secondary lesion site, which

was 8 mm caudal to the brainstem-spinal cord junction, i.e. 4 mm caudal to the first spinal lesion site for MO application. Twenty-four hours later, the brains were dissected, embedded in OCT medium, and transversely frozen-sectioned. The biocytin was detected with the Vectastain ABC-DAB kit (Vector Laboratories, Burlingame, CA, USA) as previously described (Becker *et al.*, 1997; Yu *et al.*, 2011a). For profile counting, all positively stained neurons in the NMLF, IMRF, and SRF of each animal were counted with the experimenter blinded to the treatment of each fish.

Locomotor analysis

Six weeks after SCI and MO application (before the application of biocytin), the total distance moved by the MO-treated fish was measured to examine locomotor recovery. Tracking of freely moving fish was performed as previously described (Becker *et al.*, 2004; Guo *et al.*, 2011; Yu *et al.*, 2011a; Yu *et al.*, 2011b). Each fish was placed in a glass tank (50 × 30 cm) and allowed to move freely for 5 minutes while a video recording was captured using a camera mounted above the tank. Swim paths were tracked and calculated with ANY-maze video tracking system (Stoelting Co, Wood Dale, IL, USA).

Western blot analysis

At 11 days after SCI and MO treatments as described above, 2 mm tissue from spinal cords, centered on the transection site, was dissected and lysed in RIPA lysis buffer (Sigma) supplemented with protease inhibitors (Roche). After homogenization, the lysate was pelleted and the protein concentration was determined with the BCA protein assay kit (Promega, Madison, WI, USA). Proteins were resolved on a 4–12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to PVDF membrane. The blot was probed by a polyclonal CRP1 antibody produced in rabbit against the C-terminal of rat CRP1 (PKGFGFGQGAGALVHSE) at 1.12 µg/ml. The antibody was a kind gift from Dr. Greenwood (Oregon University, USA). α -tubulin (1:1000, T9026, Sigma) served as a loading control on the same membrane after stripping. HRP-conjugated goat anti-rabbit (Invitrogen, 1:5000) or HRP-conjugated goat anti-mouse (Millipore, 1:5000) were used as secondary antibodies. The ECL detection system (Pierce, Rockford, IL, USA) was used for signal detection. Quantitative analysis was performed using Kodak molecular imaging software version 4.0 (Carestream Molecular Imaging, New Haven, CT, USA).

Statistical analysis

Images were captured using a digital CCD camera attached to a microscope driven by AxioVision software (Zeiss). Two-tailed Student's *t*-test was used for microarray and q-PCR results of *GAP-43* and *csrp1a* both in NMLF and spinal cord, results of *csrp1a*-positive neuronal profiles in different regenerative nuclei at 11 days after SCI, and results of fluorescein-positive cell profiles of control MO and CRP1 MO1 at 6 weeks after MO treatment. One-way ANOVA followed by Tukey *post-hoc* test when appropriate was used for results of densitometric analysis of CRP1 expression after MO treatment, results of locomotor recovery of fish after MO treatment and results of neuronal profiles retrogradely labeled after MO treatments in different regenerative nuclei. Two-way ANOVA followed by Tukey *post-hoc* test when appropriate was used for results of *csrp1a*-positive neuronal

profiles in different regenerative nuclei at different time points. The level of significant was set at $P < 0.05$ for all analysis. Data are shown as mean values $\pm$ SEM. Statistical analysis were performed using R 2.12.2 (a free statistical software from www.r-project.org).

Results

Gene expression profiling of regenerating NMLF neurons

In adult zebrafish, there are 20 distinct brain nuclei projecting to the spinal cord. Neurons in most brain nuclei grow axons beyond the transection site at 3.5 mm caudal to the brainstem/spinal cord transition zone (Becker *et al.*, 1997). It has been reported that different nuclei with the capacity to regenerate their axons have similar expression patterns for growth associated genes, suggesting a shared molecular mechanism (Becker *et al.*, 1998). The NMLF neurons mount a robust regeneration response after SCI (Becker *et al.*, 1997; Becker *et al.*, 1998; Becker *et al.*, 2004; Yu *et al.*, 2011a; Yu *et al.*, 2011b). In addition, the NMLF is anatomically well defined, making it an excellent candidate to perform a screening assay such as microarray analysis. Spinal cord transection was performed one millimeter caudal to the brainstem-spinal cord junction (Fig. 1). As the distance between spinal cord and the brainstem-spinal cord junction increases, the number of neurons in brain projecting to spinal cord decreases (Becker *et al.*, 1997). Thus, transection at the relatively rostral one millimeter level affects most neurons of the NMLF. Brain samples were collected at different time points to distinguish early (4 and 12 hr) regulation in response to axotomy from regulation associated with the growth phase of descending axons (11 d). This time is when neurons increase expression of growth associated genes and exhibit active axon growth after SCI (Becker *et al.*, 1998; Becker *et al.*, 2004). To concentrate the effect of injury response in relatively few cells within the region, laser capture microdissection was used to harvest NMLF from brain sections of sham-lesioned control or spinal cord lesioned animals (Fig. 1). Because of the limited number of neurons in the NMLF and the small quantity of RNA recovered from captured NMLF, RNA amplification was performed and the resulting cDNA was used for microarray analysis (Fig. 1). Each condition (control or SCI) consisted of three microarrays, with the total RNA sample for each microarray prepared from laser-dissected NMLF tissues pooled from 3 animals.

The Zebrafish GeneChip microarray from Affymetrix was used for microarray analysis. Since changes in gene expression at 11 days after SCI associate with the growth phase of descending axons, we initially tested for mRNA regulation due to axotomy injury at 11 days. When using a two-way ANOVA, no genes were found to be significantly affected by injury, probably due to the small fold changes and the limited number of arrays. However, to extract an exploratory list of potentially regulated genes, we selected 143 genes based solely on Student's *t*-test at $P = 0.05$ and a minimum 1.5-fold change (Supplementary Table 1). The gene symbols and descriptions were obtained with DAVID (<http://david.abcc.ncifcrf.gov/>) (Huang *et al.*, 2009a; b). The fold changes for gene expression in our microarray data were not as high as another study which used zebrafish retinal ganglion cells (RGCs) for microarray analysis (Veldman *et al.*, 2007), which is probably due to the limited number of NMLF neurons (about 60) in each brain, and the presence of non-neuronal cells in the laser-dissected NMLF nucleus and/or other neurons which are not

capable to regenerate after SCI, like cells in nucleus ruber (Becker *et al.*, 1997). Table 1 lists a subset of upregulated genes from the exploratory list, selected based on published results linking their function to SCI or based on association with mechanisms thought to be prominent in SCI. As shown in Table 1, few of the genes upregulated at 11 days post-injury had changes in expression level in the early phase after axotomy, consistent with a specific role for those genes during the axon regeneration phase. This list contains several genes whose roles in axon regeneration (outgrowth) have been reported, including matrix metalloproteinase 9 (MMP9) (Vaillant *et al.*, 2003), GAP-43 (Skene, 1989; Leu *et al.*, 2010), activating transcription factor 3 (ATF3) (Seijffers *et al.*, 2006) and decorin (Davies *et al.*, 2004; Minor *et al.*, 2008). We used antisense MOs to study the functional roles of several of these genes proposed to be important for spinal cord regeneration, such as ependymin, Wnt4b, jagged 1a, and CRP1. Since only CRP1 MO reduced recovery of locomotor function, we chose to focus on this molecule.

Csrp1a is upregulated in the NMLF after SCI

If CRP1 is a growth-associated protein it should be regulated in parallel with other genes known to participate in regenerative mechanisms. GAP-43 is a well-known marker of axonal growth (Skene, 1989) and is upregulated in different regenerating models (Bormann *et al.*, 1998; Costigan *et al.*, 2002; Veldman *et al.*, 2007). It is also upregulated in neurons of the NMLF and other nuclei capable of regeneration after SCI in adult zebrafish (Becker *et al.*, 1998). Here, GAP-43 is used as a positive control for genes that are upregulated after SCI. The microarray data showed that the normalized fold induction for *GAP-43* at 11 days after SCI was 2.3 (2.304 ± 0.156 , $n = 3$ experiments, two-tailed t -test, $P = 0.003$) compared to the sham-lesioned control (CON), while the fold induction for *csrpl1a* was 1.71 (1.714 ± 0.158 , $n = 3$ experiments, two-tailed t -test, $P = 0.03$) (Fig. 2A). The upregulation of *GAP-43* and *csrpl1a* in the microarray data was verified by quantitative real-time PCR (qPCR), which showed the fold induction values for *GAP-43* and *csrpl1a* were 3.45 (3.45 ± 0.7 , $n = 3$ experiments, two-tailed t -test, $P = 0.03$) and 1.44 (1.44 ± 0.1 , $n = 3$ experiments, two-tailed t -test, $P = 0.01$), respectively (Fig. 2B). The discrepancy in the fold inductions between microarray and qPCR could be due to the difference in the amplification methods and detection techniques used in those assays, however, the reproduction of the upregulation by qPCR agrees with the predicted change observed on microarrays. *Csrpl1a* expression is increased in NMLF after spinal axotomy, at a time when regenerative growth is observed.

In addition to neurons regenerating axons after SCI in the brain, the spinal cord region caudal to the lesion site, into which the regenerating axons must regrow, also contributes to regenerative capacity (Becker *et al.*, 1998; Becker *et al.*, 2004; Guo *et al.*, 2011). For example, adhesion molecule L1.1 is upregulated in the caudal spinal cord after SCI and likely promotes spinal cord regeneration by acting not only in regrowing axons, but also in the caudal part of the injured spinal cord affecting, for instance, neuronal survival, axonal growth of interneurons and/or synaptic plasticity (Becker *et al.*, 2004). The expression of *csrpl1a* in the spinal cord region caudal to the lesion site was therefore examined using qPCR to determine if it also functions in the regenerative milieu. Unlike the response found in the NMLF, the expression of *csrpl1a* caudal to the lesion site was slightly decreased at 11 days after SCI (0.79 ± 0.02 , $n = 3$ experiments, two-tailed t -test, $P = 0.001$) (Fig. 2C). With *in situ*

hybridization, the expression of *csrp1a* was observed in NeuN-positive neurons in spinal cord and no significant change of *csrp1a* expression was detected (data not shown). This suggests that increased *csrp1a* expression is mainly associated with the intrinsic regenerative response of neurons, and may not be involved in the extrinsic response that creates a permissive environment for axonal regrowth, since a molecule involved in creating a supportive environment ought to be upregulated in the caudal part of spinal cord, as L1.1 (Becker *et al.*, 2004).

Csrp1a is upregulated in neurons of regenerative nuclei in the axon regeneration phase after SCI

The upregulation of *csrp1a* in individual NMLF neurons after SCI was determined by *in situ* hybridization. With sense probe, no significant signal was observed when sections from injured animals were used under the same conditions. The NMLF neurons can be easily identified by their typical location and large size (13 – 23 μm in diameter) (Fig. 3A) (Wullimann, 1996; Becker *et al.*, 1998; Becker *et al.*, 2004), distinguishing them from glial cells, which are characterized by their small size (Becker *et al.*, 1998). Signal for *csrp1a* was observed in neurons in the NMLF (Fig. 3 and Fig. 4). The expression of *csrp1a* in neurons was further confirmed by showing the double labeling of *csrp1a* (*in situ* hybridization) and neuronal marker NeuN (immunohistochemistry) in cells of NMLF and the intermediate reticular formation (IMRF) (Fig. 4). The number of *csrp1a* positive neuronal profiles was strongly increased in the NMLF at 11 days after SCI (62.7 ± 5.7 , $n = 6$ fish, two-tailed *t*-test, $P = 0.001$) (Fig. 3B) when compared to sham-lesioned control (12.7 ± 1 , $n = 6$ fish). The expression of *csrp1a* was also studied in neurons from other nuclei capable of regeneration after SCI, such as IMRF and the superior reticular formation (SRF) (Becker *et al.*, 1997). At 11 days post-injury, the numbers of *csrp1a*-positive cell profiles were increased in both IMRF (119.3 ± 6.1 , $n = 3$ fish, two-tailed *t*-test, $P = 0.001$, Fig. 3) and SRF (59.3 ± 4.5 , $n = 3$ fish, two-tailed *t*-test, $P = 0.001$, Fig. 3) compared to sham-lesioned control (IMRF, 22.7 ± 4.3 , $n = 3$ fish; SRF, 16 ± 1.5 , $n = 3$ fish). In addition to the regenerative nuclei studied, the expression of *csrp1a* was also examined in neurons in other nuclei with no or limited regeneration after SCI, such as Mauthner cells (Becker *et al.*, 1997). Interestingly, no significant upregulation of *csrp1a* expression was observed in Mauthner cells at 11 days after SCI (Fig. 4), similar with that of L1.1 (Becker *et al.*, 1998), suggesting the upregulation of *csrp1a* is specifically associated with regeneration. The upregulation of *csrp1a* in different regenerative nuclei after SCI is consistent with the prediction that similar molecular mechanisms are shared by different nuclei for regeneration (Becker *et al.*, 1998).

Next, a study was performed to investigate the regulation of CRP1 expression over time following SCI. As shown in Table 1, the expression of CRP1 did not change from sham injury at 4 and 12 hours after axotomy, indicating that it is probably not involved in early response after SCI. The expression of *csrp1a* at an additional two time points, 3 and 21 days after SCI, was studied using *in situ* hybridization. The number of *csrp1a*-positive neuronal profiles in the NMLF was slightly increased at 3 days after SCI. The expression of *csrp1a* was highly upregulated at 11 days (Fig. 3 and Fig. 5). This upregulation continued to 21 days post-injury (Fig. 5A, SCI, 44 ± 3.7 , $n = 3$ fish, two-way ANOVA followed by Tukey *post-hoc* test, $P = 0.002$; CON, 12.3 ± 1.8 , $n = 3$ fish), the longest time point studied, albeit

to a lesser extent than that of 11 days post-injury. Other nuclei capable of regeneration showed similar regulation pattern of *csrp1a* expression after SCI (IMRF, Fig. 5B; SRF, Fig. 5C). The upregulation of *csrp1a* in the growth phase of descending axons suggest it may be a critical component of the neuron intrinsic regeneration response.

CRP1 is essential for spinal cord regeneration in zebrafish

Anti-sense morpholino (MO) oligonucleotides have been used effectively to investigate the role of specific genes in zebrafish spinal cord regeneration (Becker *et al.*, 2004; Guo *et al.*, 2011; Yu *et al.*, 2011a; Yu *et al.*, 2011b). Like conventional neuronal tracers, MO can be absorbed by the injured axons at the lesion site and retrogradely transported into neuronal somata where the protein translation of the target gene is blocked. In the zebrafish SCI model, this can be accomplished by placing MO-soaked Gelfoam into the spinal cord transection site. The inhibitory effect of MO can last for at least 6 weeks (the longest time point tested) (Becker *et al.*, 2004). Gelfoam alone or with the standard control morpholino did not show any effect on neuronal viability or spinal cord regeneration (Becker *et al.*, 2004).

The role of a gene in zebrafish spinal cord regeneration has been evaluated by two definitive parameters: locomotor recovery and axonal regrowth (Becker *et al.*, 2004; Yu *et al.*, 2011a; Yu *et al.*, 2011b). The total distance moved by fish in 5 minutes is used to measure locomotor activity. The locomotor activity is reduced to approximately 5% of that in unlesioned fish at one week after SCI and generally recovers to 60% at 6 weeks. No additional improvement is observed after this point (Becker *et al.*, 2004). The identification of neurons that have regenerated their axons beyond the lesion site is performed by applying the retrograde tracer biocytin 4 mm caudal to the transection site six weeks after the lesion. The degree of locomotor recovery and the number of retrogradely labeled neurons reflect the extent of spinal cord regeneration and these two parameters are usually correlated (Becker *et al.*, 2004).

The role of CRP1 in spinal cord regeneration was investigated using two different MOs specific for *csrp1a*. The effectiveness of the CRP1 MOs used was examined by Western blot analysis. The CRP1 antibody used here only detected one band of the correct size for CRP1, demonstrating its specificity (Fig. 6A). Signal for CRP1 was detected with samples from both brain and spinal cord, confirming the expression of *csrp1a* as shown by *in situ* hybridization. To test the effectiveness of CRP1 MOs, a two millimeter section spinal cord tissue, centered on the transection site, was collected at 11 days after MO treatment and was examined by Western blot analysis. Both CRP1 MO1 and CRP1 MO2 exhibited effective knock-down on CRP1 expression compared to control MO, with 30% (n = 3 experiments, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.01$) and 40% (n = 3 experiments, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.01$) knock-down effect respectively (Fig. 6B–C). Thus, both CRP1 MOs worked effectively in our system.

Six weeks after SCI, fish treated with CRP1 MO1 showed a decrease in the total distance moved (278 ± 104 cm/5 min, n = 12 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.003$, Fig. 6D) compared with fish treated with control MO (1108 ± 365 cm/5 min, n = 9 fish). CRP1 MO2 treatment exhibited similar effect (CRP1 MO2, 261 ± 97 cm/5 min,

n = 5 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.008$, Fig. 6D). CRP1 MO2 has been used to knock down CRP1 expression and its specificity has been confirmed with a *csrp1a* rescue experiment (Miyasaka *et al.*, 2007). These data indicate that CRP1 MOs impaired locomotor recovery. After locomotor analysis, the same fish were used to retrogradely label neurons which had regrown their axons 4 mm beyond the lesion site. The number of neuronal profiles retrogradely labeled in the NMLF was reduced in animals treated with CRP1 MO1 (11 ± 2.3 , n = 12 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.001$) or CRP1 MO2 (13.2 ± 4.3 , n = 5 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.01$) compared to fish treated with control MO (27.7 ± 2.9 , n = 10 fish) (Fig. 7A,B). CRP1 MOs treatments also reduced the number of neuronal profiles retrogradely labeled in IMRF (Fig. 7A,B, CRP1 MO1, 15.3 ± 3.4 , n = 4 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.02$; CRP1 MO2, 13.8 ± 2.8 , n = 5 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.01$; CON MO, 36 ± 6.8 , n = 3 fish) and SRF (Fig. 7A,B, CRP1 MO1, 6.8 ± 1.8 , n = 4 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.02$; CRP1 MO2, 9.4 ± 2.7 , n = 5 fish, one-way ANOVA followed by Tukey *post-hoc* test, $P = 0.03$; CON MO, 25 ± 6.6 , n = 3 fish). These data suggest that knockdown of CRP1 inhibits locomotor recovery and axonal regrowth after SCI.

We then asked whether the reduced number of neuronal profiles retrogradely labeled is due to reduced cell viability after MO treatment. In this study, both the standard MO and CRP1 MO1 were tagged with fluorescein which does not affect cell viability and spinal cord regeneration (Becker *et al.*, 2004). No effect on cell viability by CRP1 MO2 has been reported (Miyasaka *et al.*, 2007). Since the inappropriateness of CRP1 MO2 to be coupled with fluorescein tag due to its high G content, it is not included in this experiment. In agreement with previous findings (Becker *et al.*, 2004), the fluorescein tag signal on the MOs was still detectable in the NMLF neurons 6 weeks after application. Since standard control MO does not show an effect on cell viability (Becker *et al.*, 2004), we compared the number of fluorescein-positive cell profiles from CRP1 MO1-treated fish with that from control MO-treated fish. No difference was found in the numbers of fluorescein-positive cell profiles in the NMLF between control and experimental animals (control MO, 25 ± 3.8 , n = 3 fish; CRP1 MO1, 24 ± 4.8 , n = 4 fish, two tailed *t*-test, $P = 0.85$) (Fig. 8), indicating that neurons survived the CRP1 MO1 and control MO applications equally well. Similar results were observed in IMRF and SRF (data not shown). In all, the combined observations show reduced locomotor recovery and reduced numbers of retrogradely labeled NMLF neurons in animals treated with CRP1 MO, indicating that CRP1 is required for successful spinal cord regeneration in zebrafish.

Discussion

Unlike mammals, adult zebrafish have the innate ability to (re)express growth associated genes and regenerate descending axons after spinal cord injury (SCI) leading to locomotor recovery (Becker *et al.*, 1997; Becker *et al.*, 1998; Bernhardt, 1999; Becker *et al.*, 2004). However, the molecular mechanisms for successful spinal cord regeneration have not been fully explored. In the current study, microarray analysis was used to obtain an exploratory list of potential growth-associated genes from regenerating NMLF. Among the potentially upregulated genes during the axon regeneration phase, several have been reported to be

induced during the regeneration period after nerve injury (Veldman *et al.*, 2007; McCurley & Callard, 2010; Saul *et al.*, 2010; Siebert *et al.*, 2010), such as matrix metalloproteinase 9, GAP-43, ATF3, tubulin beta 5 and SOCS3b, supporting the validity of this analysis. Furthermore, some of the identified genes appear to be specifically regulated in adult vertebrates competent for spinal cord regeneration. Decorin is a small, leucine-rich proteoglycan that has previously been shown to promote axon growth across adult rat spinal cord injuries (Davies *et al.*, 2004). Decorin also promotes robust axon growth on chondroitin sulfate proteoglycans (CSPGs) and myelin (Minor *et al.*, 2008), two major inhibitory factors for axon regeneration within the adult mammalian central nervous system (Morgenstern *et al.*, 2002; Yiu & He, 2006). Our microarray shows that decorin is likely upregulated during the axonal regeneration period after SCI in adult zebrafish (11 d). However, to the best of our knowledge, the upregulation of decorin has not been reported in other microarray analysis after nerve injury in adult mammals (Veldman *et al.*, 2007; McCurley & Callard, 2010; Saul *et al.*, 2010; Siebert *et al.*, 2010; Gibbs *et al.*, 2011), indicating it as one of the specific genes involved in spinal cord regeneration. On the other hand, L1.1, a well-known adhesion molecule for axon regeneration, was not found on our list. Although our microarray analysis showed a 1.82 fold change for L1.1 at 11 days after SCI, the result was not significant with 0.42 for the *p* value, likely due to a combination of measurement variability, low signal, and the limited number of replicates. Variability could be due to the limited number of the regenerative neurons in the tissue sample, the presence of other non-regenerative cells and the possible technical variation added by the two-step RNA amplification procedures involved in RNA preparation for microarray. Due to these limitations, our list may not include all of the upregulated mRNAs after SCI.

Quantitative PCR confirms predictions from the microarray, showing that both *GAP-43* and *csrp1a* are upregulated in the regenerating NMLF during the growth phase after SCI. Similarly, upregulation of CRP1 has also been reported in heart regeneration (Lien *et al.*, 2006), fin regeneration (Schebesta *et al.*, 2006) and optic nerve regeneration (McCurley & Callard, 2010), indicating CRP1 expression as a common molecular mechanism for regeneration. The upregulation of *csrp1a* in neurons in several regenerative nuclei after axotomy was confirmed by *in situ* hybridization. *In vivo* knockdown of CRP1 expression with specific MO impaired both locomotor recovery and axon regeneration, demonstrating that CRP1 upregulation is an essential part of the natural regeneration mechanism in spinal cord injured adult zebrafish. Successful spinal cord regeneration requires at least two critical factors: the ability of an injured neuron to regrow its axon and a supportive extracellular environment for axon regeneration (Bulsara *et al.*, 2002). In addition, rearrangements of intraspinal connections of interneurons involving pre- and post-synaptic structures need to be considered (Guo *et al.*, 2011). Interestingly, *csrp1a* levels are only upregulated in the cell bodies of neurons regenerating axons, but not in the caudal part of spinal cord, into which the severed axons regrow. This suggests that CRP1 is only associated with the neuron-intrinsic regeneration response and may not be involved in the extrinsic response that creates a permissive environment for axon regeneration. Thus, we propose that the effect of CRP1 MO on spinal cord regeneration is mainly due to the loss of CRP1 expression in those neurons that regrow their severed axons.

As a LIM-domain protein, CRP1 plays a role in many different functions, such as gene transcription, cell movements, and cell proliferation (Chang *et al.*, 2003; Miyasaka *et al.*, 2007; Latonen *et al.*, 2008). The amino acid sequence of CRP1 is very similar among vertebrates with more than 80% identity between human, mouse, rat, chicken, quail and zebrafish, suggesting its conserved function over evolution (McLaughlin *et al.*, 1994). It has been reported that CRP1 binds actin filaments directly and cross-links them to generate actin bundles (Tran *et al.*, 2005; Jang & Greenwood, 2009). Actin bundles are the major component of filopodia at the leading edge of growth cones. Filopodia formation is critical for neurite outgrowth, neuritogenesis, neurite branching and spine formation in neurons (Mattila & Lappalainen, 2008). Expression of CRP1 in filopodia of growth cones and colocalization with actin filaments was observed by indirect immunofluorescence in cultured rat hippocampal neurons (Ma *et al.*, 2011), which is consistent with its actin-bundling ability reported (Tran *et al.*, 2005; Jang & Greenwood, 2009). Therefore, we propose that the role of CRP1 in spinal cord regeneration is likely due to its actin-bundling activity in filopodia formation. Since filopodia formation affects several different aspects of neurite modeling, such as axon outgrowth (Kwiatkowski *et al.*, 2007), neuritogenesis (Dent *et al.*, 2007) and neurite branching (Dailey & Smith, 1996), one or more aspects mentioned above may contribute to the requirement of CRP1 in spinal cord regeneration in zebrafish.

In addition to CRP1's interaction with the cytoskeleton, the involvement of CRP1 in other pathways has been reported. For example, it has been shown that CRP1 is involved in activation of the JNK pathway and interacts with Diversin and Dishevelled 2 (Miyasaka *et al.*, 2007). Moreover, it has been reported that CRP1 acts as a cofactor together with SRF to activate the transcription of target genes in smooth muscle (Chang *et al.*, 2003). Further investigations are needed to elucidate whether these mechanisms of CRP1 underlie successful spinal cord regeneration in zebrafish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CNS	central nervous system
CRP1	cysteine and glycine-rich protein 1, encoded by <i>csrplain</i> zebrafish
GAP-43	growth associated protein 43
IMRF	intermediate reticular formation
MMP9	matrix metalloproteinase 9

MO	morpholino
NMLF	nucleus of the medial longitudinal fascicle
qPCR	quantitative real-time PCR
RGC	retinal ganglion cells
SCI	spinal cord injury
SRF	superior reticular formation

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Figure 1. A cartoon diagramming the work flow for microarray analysis

Spinal cord injury (SCI) was performed with a complete transection at one millimeter caudal to the brainstem-spinal cord junction. Brains were removed at different time points after SCI and sections were collected. After staining with cresyl violet, laser capture microdissection was used to isolate the NMLF. Total RNA was extracted from the pooled NMLF tissue and was amplified. cDNA from amplified RNA was used for microarray analysis. Scale bar, 1 mm.

Figure 2. *Csrp1a* is upregulated in the NMLF at 11 days after SCI

(A) Graph of normalized fold changes for *GAP-43* and *csrp1a* from microarray analysis. (B) Quantitative real-time PCR (qPCR) shows that both *GAP-43* and *csrp1a* are upregulated in the NMLF at 11 days after SCI. (C) qPCR shows that *csrp1a* levels are slightly downregulated in the caudal part of the spinal cord at 11 days after SCI. n = 3 experiments.

* $P < 0.05$, t -test; Mean values $\pm$ SEM are shown.

Figure 3. *Csrp1a* is upregulated in the NMLF, IMRF and SRF at 11 days after SCI

(A) *In situ* hybridization was performed to study the expression of *csrp1a* in regenerative nuclei. Representative images depict *csrp1a* positive neurons in NMLF, IMRF and SRF at 11 days after SCI. Schematic drawings of the NMLF, IMRF and SRF are shown. Positive signal for *csrp1a* is observed in neurons, i.e. cells with a diameter more than 13 μ m. More positive neurons for *csrp1a* were observed in all three nuclei after SCI when compared with sham-lesioned control. With sense probe, no significant signal was observed. Arrows in the schematic drawings indicate the corresponding nucleus. Arrows in images indicate the brain

midline. **(B)** Quantification shows that *csrp1a* is upregulated in NMLF, IMRF and SRF at 11 days after SCI. NMLF (nucleus of the medial longitudinal fascicle), n = 6 fish; IMRF (intermediate reticular formation), n = 3 fish; SRF (superior reticular formation), n = 3 fish. * $P < 0.05$, t -test; Mean values $\pm$ SEM are shown. Scale bar, 50 μ m.

Figure 4. *Csrp1a* is upregulated in NeuN-positive neurons in regenerative nuclei after SCI
 Double staining of *csrp1a* (*in situ* hybridization) and NeuN (immunohistochemistry) was performed to examine whether *csrp1a*-positive cells are neurons or not. The signal for NeuN locates in nucleus and *csrp1a* locates in cytoplasm. Cells positive for *csrp1a* are also labeled by NeuN, demonstrating that *csrp1a* is expressed by neurons. More double labeling cells are observed at 11 days after SCI in regenerative nuclei, such as NMLF (upper two rows) and IMRF (middle two rows), when compared to control, while no significant upregulation of

cgrp1a expression is found after SCI in Mauthner cells (lower two rows) which are not capable to regenerate after SCI. n = 3 experiments. Scale bar, 50 μ m.

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Figure 5. *Csrp1a* is upregulated in the axon regeneration phase after SCI

In situ hybridization was performed to investigate the regulation pattern of *csrp1a* expression after SCI. Three different time points were included, 3 days, 11 days and 21 days after SCI. Quantification shows that *csrp1a* is slightly upregulated at 3 days. The expression of *csrp1a* is highly induced at 11 days post-injury and this upregulation continues to 21 days, the longest time point tested. Similar regulation pattern of *csrp1a* expression are observed in NMLF (A), IMRF (B) and SRF (C). $n = 3$ fish for each time points. * $P < 0.05$, two-way ANOVA with Tukey *post-hoc* test; Mean values $\pm$ SEM are shown.

Figure 6. Application of CRP1 MO inhibits locomotor recovery after SCI

(A) The CRP1 antibody used for Western blot analysis only detects one band with correct size for CRP1, demonstrating the specificity of the antibody. Signal for CRP1 was detected with both samples from zebrafish spinal cord and brain. SC: spinal cord. (B–C) Both CRP1 MO1 and CRP1 MO2 knock down CRP1 expression. Two millimeter spinal cord tissue, centered on the transection site, was collected at 11 days after SCI and MO treatment. α -tubulin serves as a loading control. CRP1 MO1 and CRP1 MO2 exhibited 30% and 40% knock-down effect on CRP1 expression compared to control MO, respectively, as

demonstrated by the Western blot analysis (**B**) and the densitometric analysis (**C**). (**D**) Total distance moved by animals treated with control (CON) MO, or CRP1 MO1, or CRP1 MO2 was measured during 5 minutes trial periods by video recording 6 weeks after MO application. CRP1 MO1 (n = 12 fish) or CRP1 MO2 (n = 5 fish) treatments reduce the total distance moved when compared to CON MO treatment (n = 9 fish). **A–C**, n = 3 experiments. * $P < 0.05$, one-way ANOVA with Tukey *post-hoc* test; Mean values $\pm$ SEM are shown.

Figure 7. CRP1 MO inhibits axon regeneration after SCI

(A) Representative images of retrogradely labeled neurons in NMLF, IMRF and SRF. The number of retrogradely labeled neurons in the NMLF, IMRF and SRF (biocytin label) is measured 6 weeks after SCI and MO application. Biocytin was applied 4 mm caudal to the lesion site and was detected 24 hours later. CRP1 MO1 and CRP1 MO2 treatments reduce biocytin-labeled neurons in all nuclei studied when compared to CON MO. Arrows in images indicate the brain midline. (B) Quantification shows a reduction in number of biocytin-labeled neurons in fish received CRP1 MO1 or CRP1 MO2 compared to fish

received CON MO. * $P < 0.05$, one-way ANOVA with Tukey *post-hoc* test; Mean values $\pm$ SEM are shown. Scale bar, 50 μm .

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Figure 8. CRP1 MO1 does not affect cell viability

(A) Representative images of neurons with fluorescein signal in the NMLF 6 weeks after MO application. The standard control MO and CRP1 MO1 were tagged with fluorescein and this signal is still detectable at 6 weeks after MO treatments. No difference was found between the numbers of cells positive for fluorescein in animals treated with CON MO or CRP1 MO1. Arrows in images indicate the brain midline. (B) Quantification shows no difference of fluorescein positive cells in the NMLF in animals treated with CON MO (n = 3 fish) or CRP1 MO1 (n = 4 fish). * $P < 0.05$, t -test; Mean values $\pm$ SEM are shown. Scale bar, 50 μ m.

Table 1

List of selected genes upregulated in their expression in NMILF at 11 days after SCI.

Probe Set ID	Gene Symbol	Description	4 hrs		12 hrs		11 d	
			Control	Injured	Control	Injured	Control	Injured
Dr.967.1.S1_at	Mmp9	matrix metalloproteinase 9	1.000 ± 0.084	1.493 ± 0.566	1.000 ± 0.162	1.186 ± 0.214	1.000 ± 0.157	10.438 ± 2.922
Dr.268.1.S1_at	epd	ependymin	1.000 ± 0.670	0.762 ± 0.350	1.000 ± 0.168	2.200 ± 0.640	1.000 ± 0.324	8.402 ± 0.919
Dr.20019.1.S1_at	THY1	Thy-1 cell surface antigen	1.000 ± 0.106	1.141 ± 0.065	1.000 ± 0.198	0.980 ± 0.126	1.000 ± 0.243	6.358 ± 2.135
Dr.8236.1.S1_at	wnt4b	wingless-type MMTV integration site family, member 4b	1.000 ± 0.258	0.951 ± 0.363	1.000 ± 0.121	1.903 ± 0.832	1.000 ± 0.316	5.311 ± 0.787
Dr.9617.1.A1_at	socs3b	suppressor of cytokine signaling 3b	1.000 ± 0.229	1.310 ± 0.072	1.000 ± 0.300	1.385 ± 0.419	1.000 ± 0.162	4.594 ± 0.512
Dr.14282.1.S1_at	atf3	activating transcription factor 3	1.000 ± 0.148	1.763 ± 0.335	1.000 ± 0.167	1.682 ± 0.134	1.000 ± 0.332	3.908 ± 1.115
Dr.4416.1.A1_at	Tubb5	tubulin, beta 5	1.000 ± 0.183	0.756 ± 0.139	1.000 ± 0.033	0.723 ± 0.122	1.000 ± 0.177	3.162 ± 0.306
Dr.10326.1.S1_at	junb	jun B proto-oncogene	1.000 ± 0.130	1.542 ± 0.252	1.000 ± 0.136	1.053 ± 0.074	1.000 ± 0.181	2.839 ± 0.460
Dr.8134.1.S1_at	PSME2	proteasome activator subunit 2	1.000 ± 0.134	1.404 ± 0.092	1.000 ± 0.043	1.121 ± 0.104	1.000 ± 0.084	2.753 ± 0.321
Dr.2644.1.A1_at	dhrrs3a	dehydrogenase/reductase (SDR family) member 3a	1.000 ± 0.137	1.642 ± 0.422	1.000 ± 0.154	1.602 ± 0.335	1.000 ± 0.228	2.728 ± 0.517
Dr.92.1.A1_at	gap43	growth associated protein 43	1.000 ± 0.188	0.907 ± 0.126	1.000 ± 0.020	0.884 ± 0.015	1.000 ± 0.111	2.304 ± 0.156
Dr.15857.1.A1_at	csrp1a	cysteine and glycine-rich protein 1	1.000 ± 0.137	1.200 ± 0.264	1.000 ± 0.026	1.008 ± 0.035	1.000 ± 0.148	1.714 ± 0.158
Dr.10428.1.S1_at	IRF7	interferon regulatory factor 7	1.000 ± 0.051	1.375 ± 0.095	1.000 ± 0.038	1.157 ± 0.121	1.000 ± 0.116	1.689 ± 0.149
Dr.16078.1.S1_at	DCN	decorin	1.000 ± 0.047	0.986 ± 0.119	1.000 ± 0.183	0.893 ± 0.130	1.000 ± 0.059	1.634 ± 0.108
Dr.12590.1.S1_at	jag1a	jagged 1a	1.000 ± 0.138	0.883 ± 0.032	1.000 ± 0.015	0.972 ± 0.061	1.000 ± 0.061	1.536 ± 0.127
Dr.8145.1.S1_at	igt2a	insulin-like growth factor 2a	1.000 ± 0.074	0.993 ± 0.040	1.000 ± 0.079	1.232 ± 0.033	1.000 ± 0.035	1.508 ± 0.095