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A DN-*mda5* transgenic zebrafish model demonstrates that Mda5 plays an important role in snakehead rhabdovirus resistance

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ABSTRACT

Melanoma Differentiation-Associated protein 5 (MDA5) is a member of the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, which is a cytosolic pattern recognition receptor that detects viral nucleic acids. Here we show an Mda5-dependent response to rhabdovirus infection *in vivo* using a dominant-negative *mda5* transgenic zebrafish. Dominant-negative *mda5* zebrafish embryos displayed an impaired antiviral immune response compared to wild-type counterparts that can be rescued by recombinant full-length Mda5. To our knowledge, we have generated the first dominant-negative *mda5* transgenic zebrafish and demonstrated a critical role for Mda5 in the antiviral response to rhabdovirus.

1. Introduction

The innate immune system is the primary defense against infection and is pivotal in mediating an immediate response to pathogens and activating the adaptive immune response. Innate immunity involves germline-derived pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000). PRRs permit differentiation of self- and non-self, while retaining the capacity to respond immediately to infection. The retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are a family of PRRs that sense viral PAMPs and include RIG-1, MDA5 (also known as IFIH1), and laboratory of genetics and physiology 2 (LGP2). RLRs are expressed in the cytoplasm, and can detect a broad range of viruses (Loo and Gale, 2011). The three RLRs exhibit two conserved DExD/H box helicase domains that are connected to the C-terminal regulatory domain (CTD) (Luo et al., 2011). The CTD mediates the interaction of double-stranded RNA (dsRNA) with RIG-I and MDA5 and affords recognition of genomic viral RNA or replication intermediates of target viruses (Baum et al., 2010; Hornung et al., 2006; Peisley et al., 2011). RIG-I and MDA5 possess two N-terminal caspase activation and recruitment domains (CARDs) that are responsible for downstream signal transduction (Kowalinski et al., 2011; Wu et al., 2013). Activation of RIG-I or MDA5 leads to an interaction with the adapter molecule mitochondrial antiviral signaling protein (MAVS; also known as Cardif, IPS-1 or VISA) and culminates in the production of the antiviral protein type I interferon (IFN) (Kawai et al., 2005; Xu et al., 2005). Although RIG-I and MDA5 have conserved functional domains and bind dsRNA, there are differences in the structural basis for target recognition at the CTD that account for the varying modes of non-self RNA sensing. RIG-I engages in high affinity interactions with blunt-ended 5′-triphosphate containing RNA duplexes (Lu et al., 2010; Vela et al., 2012; Wang et al., 2010). Antigen recognition by MDA5 is dependent on the length of accessible RNA duplexes in a range of approximately 1–7 kilobases (Kato et al., 2008; Peisley et al., 2011). These structural differences in dsRNA sensing lead to MDA5 recognition restriction to picornoviruses and some flaviviridae while RIG-I has a broader range of viral species including paramyxoviruses, flaviviruses, influenza viruses and rhabdoviruses (Allen et al., 2007; Gitlin et al., 2006; Kato et al., 2006, 2008; Li et al., 2009; Loo et al., 2008; Takahasi et al., 2008).

The zebrafish (*Danio rerio*) model has provided novel insights into pathogenesis (Allen and Neely, 2010; Briolat et al., 2014; Gabor et al.,

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2014; Ludwig et al., 2011; Meijer and Spaink, 2011; Phelan et al., 2005b; Ramakrishnan, 2008; Sullivan and Kim, 2008; Tobin and Ramakrishnan, 2008; Yakoub et al., 2014; Zou et al., 2014b). Recently, a zebrafish MDA5 homolog and splice variant were described as transcriptionally induced upon virus infection and capable of conferring viral resistance when overexpressed *in vitro* (Zou et al., 2014b). The splice variant *mda5b* enhanced *Carassius auratus* interferon promoter activity when co-expressed with full-length *mda5* or *ips-1 in vitro* (Zou et al., 2014b). The study described herein utilizes zebrafish to further elucidate the antiviral properties of teleost Mda5 *in vivo* over the duration of virus infection. A dominant-negative *mda5* (DN-*mda5*) transgenic zebrafish was generated by removing the signaling CARD domains, while retaining viral RNA binding through the helicase domains. The DN-*mda5* transgene product appears to bind ligands, preventing endogenous Mda5 from mediating a response to snakehead rhabdovirus (SHRV). DN-*mda5* zebrafish were more susceptible to SHRV infection than wild-type, while overexpression of *mda5* conferred resistance to SHRV. The use of DN-*mda5* transgenic zebrafish provides the opportunity to further elucidate the role of RLR pathways in virus resistance.

2. Materials and methods

2.1. Ethics statement

Zebrafish used in this study were handled in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maine (Protocol Number: A2008-06-03). IACUC approved guidelines for zebrafish care were followed using standard procedures (www.zfin.org).

2.2. Constructs

Full-length *mda5* (Accession XM_689032) was isolated using 30 days post fertilization (dpf) zebrafish cDNA libraries and subsequently cloned into pGEM-T Easy (Promega). Constructs encoding full-length *mda5* and ΔCARD*mda5*, where the initial 200 codons were removed from the coding sequence, were subcloned into the expression vector Frm (Sullivan et al., 2009) using gene specific primers *mda5* KpnI fwd ACGACGGGTACCATGGATCCAAACATGAGCAG, ΔCARD*mda5* KpnI fwd ACTACTGGTACCATGCCGTGCGAGGGGGACGA and *mda5* SpeI rev ACGACGACTAGTTCAGTTAGTGTCCATATCTT.

2.3. Generation of Tg(*actb:*mda5,*myl7:EGFP) zebrafish line*

The Tol2 kit constructs p5E-*bactin2*, p3E-polyA, pDestTol2CG2 (Kwan et al., 2007), and pME-Δ*CARD-mda5* were used to assemble an expression vector by Gateway Recombination Cloning (Invitrogen). To create the middle entry clone pME-ΔCARD-*mda5*, primers GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCATGCCGTGCGAGGGGG ACGAGGGGAT and GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAG TTAGTGTCCATATCTTCAT were used to add attB1 and attB2 sites to the 5′- and 3′- ends of ΔCARD-*mda5*, respectively, followed by recombination into pDONR221 using Gateway BP Clonase (Invitrogen). Entry constructs were combined in a MultiSite Gateway recombination reaction using LR Clonase II Plus (Invitrogen) to create the expression vector pDestTol2CG2;actb:Δ*CARDmda5*-polyA,myl7:*EGFP*. Expression vector (25 ng/μl) combined with *Tol2* mRNA (50 pg/embryo) (Kwan et al., 2007) was injected into one-cell-stage *AB* zebrafish embryos. Individual adult F_0 founders were outcrossed with *AB* zebrafish and F_1 progeny were screened for EGFP. Positive F_1 adults were intercrossed and embryos positive for EGFP were used.

2.4. *Full-length* mda5 *RNA injection*

Full-length *mda5* was subcloned into pCS2$^+$ (Turner and Weintraub, 1994) using primers ACGACGCTCGAGCACCATGGATC CAAACATGAGCAG and ACGACGTCTAGATCAGTTAGTGTCCATATCTTCAT and synthesized *in vitro* using mMESSAGE mMACHINE SP6 transcription according to the manufacturer's recommendations (Life Technologies, Calsbad, CA). One-cell DN-*mda5* zebrafish were injected with 98.8 ng of full-length *mda5* mRNA or vehicle and subjected to downstream application.

2.5. Cell culture

EPC (*Epithelioma papulosum cyprini*) cells initially described as originating from common carp (*Cyprinus carpio*) epidermal herpes virus-induced hyperplastic lesions (Fijan, 1983) have subsequently been found to be contaminated with fathead minnow (*Pimephales promelas*) cells (Winton et al., 2010). EPC cells have a broad sensitivity for fish viruses and are commonly used for isolation, propagation, and diagnostic assays for fish viruses. EPC cells were maintained at 28 °C, 4% CO_2 in Minimum Essential Medium (MEM) (GIBCO–Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO–Invitrogen, Carlsbad, CA) and antibiotics. ZFL (zebrafish liver) cells were derived from normal adult zebrafish liver (Ghosh et al., 1994). ZFL cells display an epithelial morphology and it has been demonstrated that the cells exhibit properties in culture that are normally associated with liver cell function *in vivo* (Ghosh et al., 1994). ZFL cells were maintained at 28 °C, 0% CO_2 in LDF culture medium (50% Leibovitz's L-15 Medium, 35% Dulbecco's modified Eagle's Medium, and 15% F-12 Medium) supplemented with heat inactivated fetal bovine serum.

2.6. Virus propagation and infection

Snakehead rhabdovirus (SHRV) was propagated in EPC cells as previously described (Phelan et al., 2005b). Briefly, 70–80% confluent EPC cells were infected at a multiplicity of infection (MOI) of 0.1 in MEM without serum for 1 h at 28 °C, 4% CO_2 followed by addition of 4 volumes of MEM + 10% FBS. Twenty-four hours after infection, EPC cells were observed to exhibit 80–90% cytopathic effect (CPE). The supernatant was collected following centrifugation and filtered through a 0.22-μm filter to remove cellular debris and obtain virus at a titer of 3.16×10^7 50% tissue culture infectious doses ($TCID_{50}$)/ml. Wild-type, DN-*mda5* and DN-*mda5*-rescue zebrafish embryos were infected by static immersion 72 hours post fertilization (hpf) for 5 hours with 5×10^6 $TCID_{50}$/ml SHRV or maintained as uninfected controls. Twenty fish were collected 24–72 h post infection (hpi) for each treatment and homogenized in MEM with 50 μg/ml gentamycin. The homogenate was frozen at –80 °C before the $TCID_{50}$ assay.

2.7. Viral burden and plaque assays

The $TCID_{50}$ is an endpoint dilution assay that enables the determination of the quantity of a virus suspension that is needed to produce a pathological change (observed as cytopathic effects, or CPE) in 50% of inoculated cells in culture. CPE (i.e. infected cells) was manually observed and recorded for each virus dilution. For our experiments, 80% confluent EPC cells were infected with previously frozen supernatants that were diluted serially in MEM. After a seven day incubation at 28 °C, 4% CO_2, cells were monitored for cytopathic effects (CPE) and the $TCID_{50}$/ml of the virus was calculated according to the Reed–Muench formula (Reed and Muench, 1938).

Plaque assays were performed with 80–90% confluent ZFL cells in a 24-well plate 24 h following transfection of empty vector, *mda5*