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Aspp2 negatively regulates body growth but not developmental timing by modulating IRS signaling in zebrafish embryos

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ABSTRACT

The growth and developmental rate of developing embryos and fetus are tightly controlled and coordinated to maintain proper body shape and size. The insulin receptor substrate (IRS) proteins, key intracellular transducers of insulin and insulin-like growth factor signaling, play essential roles in the regulation of growth and development. A short isoform of apoptosis-stimulating protein of p53 2 (ASPP2) was recently identified as a binding partner of IRS-1 and IRS-2 in mammalian cells *in vitro*. However, it is unclear whether ASPP2 plays any role in vertebrate embryonic growth and development. Here, we show that zebrafish Aspp2a and Aspp2b negatively regulate embryonic growth without affecting developmental rate. Human ASPP2 had similar effects on body growth in zebrafish embryos. Aspp2a and 2b inhibit Akt signaling. This inhibition was reversed by coinjection of *myr*-Akt1, a constitutively active form of Akt1. Zebrafish Aspp2a and Aspp2b physically bound with Irs-1, and the growth inhibitory effects of ASPP2/Aspp2 depend on the presence of their ankyrin repeats and SH3 domains. These findings uncover a novel role of Aspp2 in regulating vertebrate embryonic growth.

1. Introduction

The growth of vertebrate embryos and fetus is tightly controlled and strictly coordinated with developmental rate to maintain proper growth size and shape. For example, changes in biparietal diameter, head circumference, and abdominal circumference are critical criteria used to assess growth and development rate during growth of the fetus (Hobbins, 1997). Similarly, during embryogenesis of teleosts, the body length and somite numbers are strictly correlated and coordinated (Kimmel et al., 1995). Spatial and temporal growth and development are highly consistent features of early embryogenesis throughout the animal kingdom.

Insulin receptor substrate (IRS) proteins are key signaling intermediates that transduce signals emanating from insulin and insulin-like growth factors (IGFs) through the insulin receptor (IR) and IGF1 receptor (IGF1R). Insulin and IGFs are evolutionarily conserved peptides that play pivotal roles in regulating glucose metabolism as well as growth and development in vertebrates (Wood et al., 2005). Insulin and IGF bind to the extracellular domains of IR and IGF1R and induce their intrinsic protein tyrosine kinase activities, which then phosphorylate IRS proteins on specific tyrosine residues (Gronborg et al., 1993; Long et al., 2011; Sadagurski and White, 2013). Tyrosine phosphorylation of IRSs activates several downstream signaling cascades, including the PI3K–AKT–TOR and RAS–MEK–ERK pathways that are well conserved in mammals and fish (Duan et al., 2010; Engert et al., 1996). Activation of P13K–AKT–TOR induces a phosphorylation cascade that increases protein synthesis and inhibits protein degradation (Beckert et al., 2005). Activation of the RAS–MEK–ERK pathway causes ERK to translocate to the nucleus where it phosphorylates transcription factors, which are important in promoting growth and preventing apoptosis of multiple cell types (Steelman et al., 2011). For example, IRS-1 knockout mice were significantly smaller than wild-type mice (Gazdag et al., 1999). The combined deficiency of IRS-1 and IRS-2 in skeletal and cardiac muscle severely reduced skeletal muscle growth and myofiber cross-sectional area caused by reducing Akt–mTOR activity (Steelman et al., 2011).

Apoptosis-stimulating protein of p53 2 (ASPP2) was first identified in complexes with p53 family members, such as p53,

Abbreviations: aa, amino acids; Akt/PKB, protein kinase B; Ank, ankyrin repeats; AO, Acridine orange; BrdU, 5-bromo-2′-deoxyuridine; EGFP, enhanced GFP; Erk, extracellular signal-regulated kinases; HEK, human embryonic kidney; hpf, hour post fertilization; IGF-IR, IGF-I receptor; IGFs, insulin-like growth factors; IRS, insulin receptor substrate; SH3, Src homology 3.

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p63 and p73. ASPP2 enhances the ability of p53 to stimulate specifically the expression of pro-apoptotic target genes but not genes involved in cell-cycle arrest (Bergamaschi et al., 2004; Samuels-Lev et al., 2001). Furthermore, ASPP2 regulates epithelial cell polarity in cooperation with PAR-3 to form an active PAR complex (Cong et al., 2010; Sottocornola et al., 2010). ASPP2 facilitates the interaction between TAZ and PP1 to promote TAZ function, which is a downstream component of the Hippo pathway that regulates organ size by controlling cell proliferation and apoptosis (Liu et al., 2011). 53BP2S (also known as BBP), an alternatively spliced ASPP2 isoform lacking the N terminus has been reported that interacted with IRS-1 and IRS-2 (Hakuno et al., 2007). However, the role of ASPP2 in early embryonic growth and development is unclear.

The accessible, transparent zebrafish embryo, and the evolutionarily conserved signaling transduction system in zebrafish make them suitable models for investigating the function of IRSs and related proteins in early embryonic growth and development (Duan et al., 2003; Kamei et al., 2011). In the present study, using zebrafish as an experimental model organism, we investigated the *in vivo* function of Aspp2 in early embryonic growth and development. We showed that Aspp2 reduces embryonic growth but not developmental timing through its interaction with IRSs. This action requires the presence of its ankyrin repeats and SH3 domains.

2. Materials and methods

2.1. Chemicals and reagents

Restriction endonucleases were purchased from New England BioLabs Inc. (Beverly, MA). RNA polymerase and RNase-free DNase were purchased from Promega (Madison, WI). KOD DNA polymerase was purchased from Toyobo (Osaka, Japan). Superscript II reverse transcriptase, MMLV reverse transcriptase, and oligonucleotide primers were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Acridine orange (AO), 5-bromo-2′-deoxyuridine (BrdU), and anti-BrdU antibody were purchased from Sigma–Aldrich (St. Louis, MO). Anti-GFP antibody was purchased from Torrey Pines Biolabs (Secaucus, NJ). Anti-Akt, anti-phosphorylated Akt (Ser473), anti-Erk, and anti-phosphorylated Erk (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Experimental animals

Wild-type zebrafish (*Danio rerio*) were kept at 28 °C under a light/dark cycle of 14 and 10 h and feed twice daily. Embryos were obtained by natural cross. Fertilized eggs were raised at 28.5 °C and staged according to Kimmel et al. (1995). A 0.003% (w/v) solution of 2-phenylthiourea was added to the embryo-rearing solution in some experiments to inhibit embryo pigment formation. All experiments were conducted in accordance with the guidelines approved by the Ethical Committee of Experimental Animal Care at Ocean University of China (OUC, China).

2.3. Plasmid construction

Using human the ASPP2 amino acid sequence as a query, the zebrafish genome database (http://www.ensembl.org/Danio_rerio/blastview), was searched using BLASTN. Two zebrafish ASPP2-like sequences were found (NM_214814 and XM_001923552). The primers were designed as follows based on sequences deposited in GenBank: *aspp2a* (forward, 5′-ATGATGCCTATGTTCCTGACGGTG-3′; reverse, 5′-TTAAGCCA-GACTCCGTTGGCGAAG-3′); *aspp2b* (forward, 5′-ATGTTCC-TAACGGTGTACCTG-3′; reverse, 5′-CTACGCCAGGCTCCTCTGT-3′). The primers used to generate the C-terminal truncated Aspp2s mutants that lack ankyrin repeats and SH3 domains are as follows: *aspp2a-2Δ* (forward, 5′-GACTGATTCGAAGCCATGATGCCTATGTTCCTGAC-3′; reverse, 5′-GACTGAACTAGTGAAGCGCACCCGCATGCTGTGATCG-3′); *aspp2b-2Δ* (forward, 5′-GGGCCCATCGATAGCCACCATGCCGATGT TCCTAACGGTGTACC-3′; reverse, 5′-TTTCCGGAATTCGAAAGCG-CACGCGCATCCCG-3′). Amplified DNAs were subcloned into the pCS2 + enhanced GFP (EGFP) expression vector (Li et al., 2005) and confirmed by sequencing (Shanghai Sangon Co. Ltd., China).

Zebrafish *irs-1* (GenBank: XM_682610) cDNA was cloned into the pcDNA™ 3.1 vector (Invitrogen Life Technologies, Carlsbad, CA). Sequences of primers were: forward, 5′-ATAAGAATGCGGCCGCGCCACCATGGCAAGCCCGACTACGGA-3′; reverse, 5′-GGGCCCAAGCTTGGG TTCTTCTCTGTGTGTGAGAT-3′.

2.4. Reverse transcriptase-polymerase chain reactions (RT-PCR) and whole-mount in situ hybridization

Total RNA was isolated from embryos or tissue samples using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA). The cDNAs were reverse-transcribed using oligo(dT) (Sangon, Shanghai, China) and SuperScript® II reverse transcriptase according to the manufacturer's instructions. RT-PCR was performed using TaqDNA polymerase with the primers as follows: *aspp2a* (forward, 5′-GAAGAGCGGACAGATGGATGCC-3′; reverse, 5′-GATGCCCTGCTGTTGCTGTGAA-3′); *aspp2b* (forward, 5′-GGCTACA-TACCTCGCAATCT-3′; reverse, 5′-TCTAAGTCTACTGGTCCAAAGC-3′).

Partial sequences of *aspp2a* and *aspp2b* were amplified for the whole mount *in situ* hybridization experiments. These sequences were subcloned into pBSSK plasmid (Stratagene, La Jolla, CA). Whole-mount *in situ* hybridization was performed as reported previously (Maures and Duan, 2002).

2.5. Microinjection

Capped mRNA synthesis was carried out using a commercial kit and linearized plasmid DNA as template (mMESSAGE mMACHINE Kit; Ambion, Inc.). Capped mRNA (800 pg/embryo) was microinjected into zebrafish embryos at the 1- to 2-cell stage. Green fluorescent protein (GFP) mRNA was used as a control. After injection, embryos were placed in embryo-rearing medium and kept at 28.5 °C.

2.6. Body size and somite number measurements

Body length and somite number were measured at 24 hpf as described previously (Zhou et al., 2008) with some modifications. Briefly, Head length was defined as the curvilinear distance from the top of the head to the middle of the otic vesicle. Body length was defined as the curvilinear distance from the middle of the otic vesicle to the end of tail through the trunk midline. Full length was defined as the sum of head length and body length. All images were measured using the Image J software (National Institutes of Health, Bethesda, Maryland).

2.7. Acridine orange (AO) staining and immunohistochemistry

For detection of apoptotic cells, embryos were stained with AO (Sigma A1121) (Tucker and Lardelli, 2007). Embryos were incubated for 30 min in 2 μg/ml AO at 28.5 °C, washed in distilled water several times, and anaesthetized with MS-222 for observation. For quantification of the effects, AO-labeled cells in the trunk were analyzed by manual counting of at least 10 embryos for each condition (Tucker and Lardelli, 2007).

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