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SLC7 family transporters control the establishment of left-right asymmetry during organogenesis in medaka by activating mTOR signaling

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ABSTRACT

The precise government of the left-right (LR) specification of an organ is an essential aspect of its morphogenesis. Multiple signaling cascades have been implicated in the establishment of vertebrate LR asymmetry. Recently, mTOR signaling was found to critically regulate the development of LR asymmetry in zebrafish. However, the upstream factor(s) that activate mTOR signaling in the context of LR specification are as yet unknown. In this study, we identify the SLC7 amino acid transporters Slc7a7 and Slc7a8 as novel regulators of LR asymmetry development in the small fish medaka. Knockdown of Slc7a7 and/or Slc7a8 in medaka embryos disrupted LR organ asymmetries. Depletion of Slc7a7 hindered left-sided expression of the *southpaw* (*spaw*) gene, which is responsible for LR axis determination. Work at the cellular level revealed that Slc7a7 coordinates ciliogenesis in the epithelium of Kupffer's vesicle and thereby the generation of the nodal fluid flow required for LR asymmetry. Interestingly, knockdown of Slc7a7 depressed mTOR signaling activity in medaka embryos. Treatment with rapamycin, an inhibitor of mTOR signaling, together with Slc7a7 knockdown synergistically perturbed *spaw* expression, indicating an interaction between Slc7a7 and mTOR signaling affecting gene expression required for LR specification. Taken together, our results demonstrate that Slc7a7 governs the regulation of LR asymmetry development via the activation of mTOR signaling.

1. Introduction

The establishment of left-right (LR) asymmetry in the body is a fundamental embryonic process, and many internal organs, including the liver and heart, exhibit obvious LR asymmetric morphology in vertebrate species [1]. Defects of LR asymmetry induce disorders such as abnormal arrangement of viscera, congenital malformations, and asplenia [2,3]. An evolutionarily conserved mechanism has been identified as responsible for the establishment of vertebrate LR asymmetry [1,4]. In its first step, a ciliated organ, called the node in mice or Kupffer's vesicle (KV) in small fish, makes directional counter-clockwise nodal flow. Subsequently, this nodal flow conveys cues that induce the left-sided expression of early laterality genes, such as *nodal* in mice and *southpaw* (*spaw*) in small fish, as well as *pitx2* in the lateral plate mesoderm (LPM). The expression of these genes then guides asymmetric organ morphogenesis.

A number of signaling cascades, including the TGF-β and Bmp pathways, are reportedly important for LR asymmetry [5]. Recently, mTOR signaling has also emerged as an essential regulator of LR specification during early zebrafish development [6,7]. mTOR is a serine/threonine protein kinase that senses signals emanating from diverse environmental stimuli and triggers a pathway leading to protein synthesis. First, mTOR complex1 (mTORC1) mediates the phosphorylation of ribosomal protein S6 kinase 1 (S6K1), a key translational regulator. Phospho-S6K1 induces the up-regulation of ribosome biosynthesis, which facilitates an increase in protein translation [8]. Intriguingly, knockdown of Tsc1a, a negative regulator of mTORC1, induces the elongation of nodal cilia and

Abbreviations: spaw, southpaw; KV, Kupffer's vesicle; LPM, lateral plate mesoderm; mTORC1, mTOR complex1; S6K1, S6 kinase 1; HAT, heteromeric amino acid transporter.

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randomization of *spaw* expression in zebrafish [6]. In addition, Grk5l-mediated inhibition of mTORC1 activity influences symmetry breaking during zebrafish embryogenesis [9]. However, little is known about the upstream factor(s) that positively regulate mTOR signaling in the context of LR axis determination.

Amino acids, especially leucine, are well known to be important upstream activators of mTOR signaling [10,11], and are taken up by the SLC7 family of amino acid transporters, which are crucial transducers of stimulation mediated by extracellular amino acids to the mTOR signaling pathway [12–14]. For example, leucine activates mTOR signaling in *Xenopus* oocytes expressing SLC7 family transporters [14]. Based on these findings, we hypothesized that SLC7 transporters might contribute to LR asymmetry development during early embryogenesis by activating mTOR signaling. To investigate this theory, we used an *in vivo* knockdown system in medaka (*Oryzias latipes*). Medaka embryos develop *ex utero*, making it easy to observe them and manipulate their tissues and cells [15]. Here, we provide evidence that Slc7a7 plays a critical role in LR axis determination during early medaka embryogenesis by positively regulating mTOR signaling.

2. Materials and methods

2.1. Medaka maintenance

The Cab wild type (WT) strain was raised and maintained under standard laboratory conditions at ~27 °C. Embryos were produced by natural matings, raised at 30 °C, and staged by standard morphological criteria as previously described [16].

2.2. Rapamycin treatment

Embryos were treated with 1 or 2.5 μM rapamycin (Calbiochem) in 1% DMSO starting at stage 8. At stage 22 (9 somite stage), treated embryos were washed clean of treatment and collected. Control embryos were treated with 1% DMSO.

2.3. Antibodies

Antibodies (Abs) recognizing the following proteins were used in this study: S6K1 (611260) (BD Biosciences); phosphorylated S6K1 (108D2) (Cell Signaling Technology); and GAPDH (MAB374) (Millipore). Anti-mouse acetylated tubulin Ab has been described elsewhere [17].

2.4. Western blotting

Extracts of dechorionated embryos were prepared as previously described [18], fractionated by SDS-PAGE, and transferred electrophoretically to PVDF membranes. Membranes were incubated in blocking solution [2% nonfat skim milk in Tris-buffered saline (TBS)] for 1 h at room temperature (RT). Blocked membranes were incubated with rabbit anti-phospho-S6K1 (1:500), mouse anti-S6K1 (1:1000) or mouse anti-GAPDH (1:500) Ab in 5% BSA/TBS overnight at 4 °C. Blots were washed three times in 0.2% Tween 20 in TBS (TBST), and incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG Ab (1:3000) in 2% nonfat skim milk in TBS for 1hr followed by three washes in TBST. Proteins were visualized using the SuperSignal West Femto Kit (Pierce) and a ChemiDoc XRS system (Bio-Rad), as described elsewhere [19].

2.5. Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as previously described using an antisense digoxigenin (DIG)-labeled riboprobe generated from a partial medaka *spaw* cDNA [17].

2.6. Total RNA extraction and RT-PCR analysis

Total RNA was isolated from medaka embryos using TRIzol reagent according to the manufacturer's protocol (Invitrogen). First-strand cDNA was synthesized from 1 μg total RNA using SuperscriptIII reverse transcriptase (Invitrogen) and oligo-dT primer.

2.7. Antisense morpholinos against slc7a7 and slc7a8

Morpholino antisense oligos (MOs) were synthesized by GeneTools, LLC. Sequences of MOs used were: Slc7a7 spMO (splice-blocking), 5′-ACAGG AAGTC AGGCA CTCAC CACCG-3′; Slc7a7 augMO (translation-blocking), 5′-TGTTC CTGCT TAATC AGAAC AGTTT-3′; Slc7a8 spMO (splice-blocking), 5′-CTGAA TGCAT GTTAA CTCAC CCCGC-3′.

2.8. Synthesis of capped mRNA for microinjection

Capped sense strand mRNA was synthesized using T3 RNA polymerase and the mMESSAGE mMACHINE system (Ambion) according to the manufacturer's protocol. RNA injections were performed as described previously [20].

2.9. Analysis of KV fluid flow

For imaging of nodal flow dynamics in KV, medaka embryos around stage 21–22 (6–9 somite stage) were mounted in 1.5% agarose. Fluorescent beads (FluoSpheres carboxylate-modified microspheres, 0.5 μm; Thermo Fisher) were injected into KVs and imaged using a Biorevo All-in-One fluorescence microscope (Keyence). The quantitative analysis of bead trajectories was performed using ImageJ software.

2.10. Immunohistochemistry

To measure cilia length in KV, medaka embryos around stage 21–22 were fixed in 4% PFA in phosphate-buffered saline (PBS) overnight at RT. Embryo chorions were removed and the embryos washed twice with PBS containing 0.1% Tween 20 (PBST) prior to storage in methanol at −20 °C. Whole mount Ab staining was performed as described previously [17].

3. Results

3.1. mTOR signaling in left-right body patterning and expression of SLC7 transporters

To determine whether mTOR signaling is required for the establishment of LR asymmetry during medaka development, we treated WT medaka embryos with the mTOR inhibitor rapamycin and analyzed the expression pattern of *spaw*, a nodal-related gene expressed in the left LPM. Western blot analysis of embryos at stage 22 (9 somite stage) treated with 2.5 μM rapamycin revealed decreased levels of phospho-S6K1, confirming the efficacy of rapamycin treatment (Fig. 1A). Whereas ~80% of control embryos showed normal left-sided *spaw* expression, the rapamycin-treated embryos displayed random *spaw* expression, with 44% exhibiting left-sided *spaw* expression in the LPM but the remainder showing right-sided (37%) or bilateral (11%) *spaw* expression (Fig. 1B). This result indicates that, as is true in zebrafish, mTOR signaling is crucial for LR axis determination in medaka.

We next examined whether the SLC7 family of amino acid transporters was involved in the establishment of LR asymmetry

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