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NSrp70 is significant for embryonic growth and development, being a crucial factor for gastrulation and mesoderm induction

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ABSTRACT

NSrp70 (nuclear speckle-related protein 70), a recently discovered protein and it belongs to the serine/arginine (SR) rich related protein family. NSrp70 is recognized as an important splicing factor comprising RNA recognition motif (RRM) and arginine/serine (RS)-like regions at the N- and C-terminus respectively, along with two coiled coil domains at each terminus. However, other functions of NSrp70 remain unelucidated. In this study, we investigated the role of NSrp70 in *Xenopus* embryogenesis and found that its maternal expression plays a critical role in embryonic development. Knockdown of NSrp70 resulted in dramatic reduction in the length of developing tadpoles and mild to severe malformation in *Xenopus* embryos. In addition, knockdown of NSrp70 resulted in an extremely short axis by blocking gastrulation and convergent extension. Further, animal cap assays along with activin A treatment revealed that NSrp70 is an essential factor for dorsal mesoderm induction as knockdown of NSrp70 caused a dramatic down-regulation of dorsal mesoderm specific genes and its loss significantly shortened the elongation region of animal caps. In conclusion, NSrp70 is crucial for early embryonic development, influencing gastrulation and mesoderm induction.

1. Introduction

Alternative splicing is an important process that regulates gene expression and leads to protein diversity and complexity [1,2]. The regulation of this process is essential for correct mRNA expression [3]. This process of alternative splicing is controlled at various stages by multiple cis-acting elements and trans-acting factors [1–4]. There are two major classes of trans-acting protein factors: serine/arginine (SR) rich proteins and heterogeneous nuclear proteins (hnRNPs) [1]. SR proteins usually tend to stimulate the inclusion of exons whereas opposite effect is observed for hnRNP [5,6].

The human SR protein family is phylogenetically conserved, comprising of 12 proteins. SR proteins are composed of one or more RNA recognition motifs (RRMs) and an arginine/serine rich (RS) domain at the C-terminus. Mostly members of SR protein family are mainly localized in nucleus, whereas its six members, including SRSF1, can shuttle between the nucleus and cytoplasm [7]. SRSF1 was the first SR protein discovered that not only regulates splicing but has other functions related to genome stability and chromatin association [8–10].

Nuclear speckle-related protein 70 (NSrp70) is a recently discovered, SR-related protein that differs from SR proteins in having no or different RRMs [11]. Fluorescence microscopic analyses have shown nuclear speckles as irregular sub-nuclear structures that vary greatly in their shape and size [12]. Nuclear speckles have been recognized as a storage site for pre-mRNA splicing components and thus, could play a significant role in alternative splicing [13].

As the name indicates, NSrp70 is localized in the nuclear speckles and is comprised of N-terminus RRMs, a C-terminus RS like domain and two coiled coil domains each at the N and C-terminus [11]. NSrp70 is recognized as an important factor in the splicing machinery, playing a significant role in RNA splicing [11,14].

NSrp70 controls RNA splicing by selecting the splice site for a number of pre-mRNAs, including CD44 exon v5 [9,14]. In this

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process, NSrp70 works in collaboration with SRSF1 and SRSF2 within nuclear speckles, acting through its RS-like domain. The RS-like domain of NSrp70 is subdivided into three regions: RS-1, RS-2, and RS-3. All these regions are essential for alternative splice-site selection [11,14]. Moreover, NSrp70 has a coiled coil domain at the N-terminus that is also significant for splicing regulation. Deletion of this coiled coil domain leads to improper configuration of nuclear speckles and hence, hinders the process of splicing [9,11,14].

NSrp70, initially identified as CCD55 (coiled coil domain 55), governs the process of splicing by counter-acting the function of two SR-proteins: SRSF1 and SRSF2. NSrp70 works antagonistically to SRSF1 and SRSF2 in regulating splicing of the CD44 exon v5 minigene [7,11,14]. Although NSrp70 has been recognized as a significant splicing factor, its physiological functions are still not known.

In this study, we investigated *Xenopus* embryos microinjected with NSrp70 morpholino oligonucleotides (MOs) that block translation of NSrp70. Our results showed that NSrp70 is expressed maternally in embryos from stage 0 to tadpoles and is enriched in dorso anterior structures of developing embryos. Down-regulation of NSrp70 resulted in phenotypic defects, acting in an NSrp70-MO dose-dependent manner. Low doses of NSrp70 MO induced short-form tadpoles, whereas high doses of NSrp70 caused failure of blastopore closure. Moreover, NSrp70 knockdown also hindered the gastrulation movements and affected the dorsal mesoderm induction. Collectively, these results indicate that NSrp70 is essential for *Xenopus* embryonic development.

2. Materials & methods

2.1. Ethics statement

The present study was conducted according to the documented standards of the Animal Care and Use Committee, in agreement with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985). Moreover, we obtained a specific waiver from the Institutional Review Board of Kyungpook National University for the experimental use of amphibians or reptiles in Korea (#KNU-2013-222). Our research group attended educational and training courses on the appropriate care and usage of experimental animals. Adult *Xenopus laevis* were maintained in 12-h light/dark cycles at 18 °C in containers provided by the Institutional Review Board of Kyungpook National University and they are built according to the specifications for laboratory animal maintenance. There was no unexpected death of adult *Xenopus* during this study.

2.2. Plasmids and reagents

The partial NSrp70 gene obtained from ATCC (GenBank ID: BC_084673) was cloned using a pGEM®-T Easy Vector System (Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA) and subcloned into the pCS107 vector, using specifically designed primers that targeted *EcoR*I and *Xho*I restriction sites, as well as FLAG-tags. Protein BLAST was carried out to find the start codon of the *X. laevis* NSrp70 gene and NSrp70 MO (5′ AATTCCTCTCTCCTTGCTCGGACGC-3′), the latter of which was synthesized to block translation of this gene (Gene tools, Summerton Way, Philomath, USA).

2.3. In vitro fertilization

X. laevis were purchased from Nasco (Fort Atkinson, WI, USA) and maintained at 18 °C in plastic aquarium tanks containing dechlorinated water. *X. laevis* were maintained in a 12-h light/dark cycle and fed thrice a week. Ovulation was induced by injecting 750 IU of human chorionic gonadotropin (Sigma Aldrich, St. Louis, MO, USA) into the hind leg skin of female *X. laevis*. Eggs were collected the next day in a 60-mm petri dish containing 1X modified Barth's solution (MBS). MBS is composed of the following: 88 mM, NaCl, 5 mM Hepes, 2.5 mM $NaHCO_3$, 1 mM KCl, 1 mM $MgSO_4$, and 0.7 mM $CaCl_2$ (pH 7.8). *X. laevis* testes were obtained from males and stored in 4–5 mL of 1X MBS. After several washes with 0.1X MBS, eggs were fertilized with sperm suspension solution taken from euthanized males. After fertilization, the embryos were de-jellied using removing buffer (2% cysteine solution), and transferred into 0.5X MBS containing 2% Ficoll 400 (GE Healthcare, Sweden). Unfertilized eggs and dead embryos were removed and stored at 18 ± 2 °C.

2.4. mRNA synthesis and Xenopus embryo microinjection

Capped mRNAs were synthesized by *in vitro* transcription methods, using the SP6 mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA). The pCS107/NSrp70-flag construct was linearized using restriction enzyme *Apa*I and synthesized from the SP6 promoter. Morpholinos and mRNAs were injected together into two-cell staged embryos and incubated at 18–23 °C.

2.5. Western blot analysis

Protein lysates were prepared from extracts of embryos, using lysis buffer [137 mM NaCl, 20 mM Tris-Cl (pH 8.0), 1% NP-40 (Nonidet-P40), and 10% glycerol] supplemented with a mixture of 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium orthovanadate, and 1X protease inhibitor. Embryonic lysates were heated at 95 °C with loading buffer for 5 min and electrophoresed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Proteins were detected with anti-FLAG antibody conjugated to horseradish peroxidase (anti-FLAG HRP; Sigma Aldrich, St. Louis, MO, USA). Immunoreactive bands were detected using an ECL kit (Hyclo Kit, Denville Scientific, South Plainfields, NJ, USA).

3. RT-PCR

cDNA was prepared from total RNA extracted from *Xenopus* embryos at stages ranging from 0 to 40, using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Otsu, Shiga, Japan) according to the manufacturer's protocol. PCR reactions were carried out using specific primer pairs. The PCR products were run on a 1% agarose gel, and images captured using Wise Capture I-1000 software (see Table 1).

3.1. Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed in accordance with a previously described protocol [10,15]. Embryos were fixed in MEMFA [4% paraformaldehyde, 0.1 M MOPS (pH 7.4), 1 mM $MgSO_4$, and 2 mM EGTA] overnight at 4 °C and were hydrated before storing in 100% methanol at −20 °C. For the anti-sense digoxigenin (DIG)-labeling probe, SRSF1 DNA and Nkx-2.5 DNA templates were linearized using *Apa*I and *Nco*I, respectively. SRSF1 and Nkx-2.5 were generated using T7 RNA polymerase (Ambion, Austin, TX, USA). Probes were detected by alkaline phosphatase-labeled anti-DIG antibody and BM purple dye. Whole mount *in situ* hybridization was performed on the embryos from the oocyte to stage 32, using these RNA probes. The probes targeted NSrp70.

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