Heat Shock 70-kDa Protein 5 (Hspa5) Is Essential for Pronephros Formation by Mediating Retinoic Acid Signaling*

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Weili Shi1,2, Gang Xu31, Chengdong Wang34, Steven M. Sperber3, Yonglong Chen**, Qin Zhou44, Yi Deng53, and Hui Zhao444

From the 1Key Laboratory for Regenerative Medicine, Ministry of Education, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong Special Administrative Region (SAR), China, 2School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Hong Kong SAR, China, 3Department of Biology, South University of Science and Technology of China, Shenzhen 518055, China, 4Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen 518057, China, 5Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, New York 10029-6574, 6Key Laboratory of Regenerative Biology, Guangzhou Institutes of Biomedical and Health, Chinese Academy of Sciences, Guangzhou 510530, China, and 7Division of Molecular Nephrology and Creative Training Center for Undergraduates, Ministry of Education Key Laboratory of Laboratory Medicine Diagnostics, College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China

Background: The function of Hspa5 in early embryonic development is not well understood.

Results: Hspa5 is involved in mediating retinoic acid signaling and is required for pronephros.

Conclusion: Hspa5 is essential for pronephros formation by mediating retinoic acid signaling.

Significance: This is the first report on the cross-talk between physiological ER stress and transduction of retinoic acid signaling.

Heat shock 70-kDa protein 5 (Hspa5), also known as binding immunoglobulin protein (Bip) or glucose-regulated protein 78 (Grp78), belongs to the heat shock protein 70 kDa family. As a multifunctional protein, it participates in protein folding and calcium homeostasis and serves as an essential regulator of the endoplasmic reticulum (ER) stress response. It has also been implicated in signal transduction by acting as a receptor or co-receptor residing at the plasma membrane. Its function during embryonic development, however, remains elusive. In this study, we used morpholino antisense oligonucleotides (MOs) to knock down Hspa5 activity in Xenopus embryos. In Hspa5 morphants, pronephros formation was strongly inhibited with the reduction of pronephric marker genes Lim homeobox protein 1 (Lhx1), pax2, and B1 subunit of Na/K-ATPase (atp1b1). Pronephros tissue was induced in vitro by treating animal caps with all-trans-retinoic acid and activin. Depletion of Hspa5 in animal caps, however, blocked the induction of pronephros as well as reduced the expression of retinoic acid (RA)-responsive genes, suggesting that knockdown of Hspa5 attenuated RA signaling. Knockdown of Hspa5 in animal caps resulted in decreased expression of lhx1, a transcription factor directly regulated by RA signaling and essential for pronephros specification. Co-injection of Hspa5MO with lhx1 mRNA partially rescued the phenotype induced by Hspa5MO. These results suggest that the RA-Lhx1 signaling cascade is involved in Hspa5MO-induced pronephros malformation. This study shows that Hspa5, a key regulator of the unfolded protein response, plays an essential role in pronephros formation, which is mediated in part through RA signaling during early embryonic development.

The kidney is an essential organ that maintains homeostasis of the internal environment by filtering and excreting waste products and maintaining water and salt balance in the body. In vertebrates, the renal system develops from three successive developmental forms, the pronephros, mesonephros, and metanephros, originating from somatic and splanchnic intermediate mesoderm during embryonic development (1, 2). Although there are profound differences in anatomical structure between these three forms, the nephron is the basic structural and functional unit. The nephron contains three components: the glomerulus, tubule, and duct (3–5). From the pronephros to the metanephros, the successive forms become more complicated in the number and organization of nephrons. In higher vertebrates, the metanephros functions as the adult kidney, whereas in amphibians such as Xenopus, the mesonephros serves as the adult kidney, and the pronephros functions at embryonic stages. Although the developmental end point may differ in the vertebrate classes, the molecular mechanisms involved in kidney development are evolutionarily conserved in zebrafish, frog, mouse, rat, and humans (6).

Kidney development is a complex multistep process. It is initiated from the intermediate mesoderm at neurula stages. Many signaling pathways including bone morphogenetic protein (7), Wnt (8), FGF (9), Notch (10), and retinoic acid (RA)5

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1 Both authors contributed equally to this work.
2 Supported by a graduate studentship from The Chinese University of Hong Kong.
3 To whom correspondence may be addressed. E-mail: deng.y@sustc.edu.cn.
4 To whom correspondence may be addressed. E-mail: zhaohui@cuhk.edu.hk.

5 The abbreviations used are: RA, retinoic acid; Hspa5, heat shock 70-kDa protein 5; ER, endoplasmic reticulum; MO, morpholino antisense oligonucleotide; atRA, all-trans-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, RA response element; Lhx1, Lim homeobox protein 1; Red-Gal, 6-chloro-3-indolyl β-D-galactopyranoside; atp1b1, β1 subunit of Na/K-ATPase.
signaling have been implicated in pronephros development. These coordinated signaling cascades trigger the expression of a series of transcription factors that include Lim homeobox protein 1 (Lhx1), Wt1, and Pax8, which orchestrate the induction of the pronephros primordium.

RA signaling plays distinct roles during pronephros development. In early Xenopus gastrulae, treatment with all-trans-retinoic acid (atRA) and activin induces pluripotent ectodermal cells to differentiate into pronephros tissue (11–14). Inhibition of RA signaling impairs pronephros formation (15, 16). During early embryonic development, RA signaling is initiated mainly by atRA, a metabolite of vitamin A. atRA binds to a family of nuclear receptors, RARs (α/β/γ), which in turn form heterodimers with retinoid X receptors (RXRs) (α/β/γ) to activate expression of RA response element (RARE)-containing target genes (16, 17). lhx1 is one of the earliest genes that is expressed in the nphric mesenchyme and has been implicated in kidney development (18–22). The promoter region of lhx1 contains RAREs, and its expression is directly regulated by the RA signaling pathway (15). In Xenopus embryos, overexpression of lhx1 leads to the expansion of the pronephric field, whereas depletion of lhx1 severely reduces the pronephric field during organogenesis (15, 21). In Xenopus, it has been reported previously that RA can induce lhx1 expression in animal caps (23).

Heat shock 70-kDa protein 5 (Hspa5), also known as binding immunoglobulin protein (Bip) or glucose-regulated protein 78 (Grp78), belongs to the heat shock protein 70 kDa family of molecular chaperones (24). It functions in endoplasmic reticulum (ER) homeostasis and is a key regulator of the unfolded protein stress response. ER stress refers to any environmental condition such as thermal stress or heavy metal insults that interfere with the proper tertiary folding of proteins. In response to ER stress, cells activate the unfolded protein response (see the diagram in Fig. 1). Under normal conditions, Ire1α is constrained in an inactive form by binding to Hspa5. In response to ER stress, Hspa5 releases Ire1α, allowing free Ire1α to access full-length Xbp1 mRNA, generating the active form of Xbp1. Xbp1 in turn functions in regulation of biogenesis to recover homeostasis in the ER. It is notable that a number of key components including Xbp1 and Ire1β are highly expressed during embryonic development; however, their functional roles during embryogenesis are not well defined (25–27). In mammalian cells, Hspa5 is predominantly localized to the ER lumen and considered a chaperone, but recent reports indicate that it is also distributed at the cell membrane and participates in signal transduction (28–30).

Here, we report for the first time that Hspa5 plays an essential role in pronephros development in Xenopus embryos. We show that Xenopus hspa5 is strongly expressed in pronephros throughout tail bud stages and knockdown of Hspa5 function results in the inhibition of pronephros formation. We further show evidence that RA signaling and Lhx1 are involved in Hspa5MO-induced pronephros malformation.

Supporting these results, overexpression of lhx1 partially rescued the morphant phenotype induced by Hspa5MO. Collectively, our studies demonstrate the importance of Hspa5 in kidney development.

**EXPERIMENTAL PROCEDURES**

Embryo Manipulations—Female frog ovulation was stimulated by injection with 500 IU of human chorion gonadotropin (Sigma-Aldrich) into the dorsal lymph sac. In vitro fertilization was performed by mixing male testis homogenates and eggs. After fertilization, embryos were dejellied with 2% cysteine solution, pH 8 and cultured in 0.2× MMR (20 mM NaCl, 0.2 mM KCl, 0.2 mM MgSO4, 0.4 mM CaCl2, 0.02 mM EDTA, 1 mM HEPES, pH 7.8). Embryos were staged according to Nieuwkoop and Faber (31).

Whole Mount in Situ Hybridization, β-Galactosidase (LacZ) Staining, and Vibratome Sectioning—Whole mount in situ hybridizations were performed according to the standard protocol (32, 33). LacZ staining was carried out as published previously (34). Briefly, 100 pg of LacZ mRNA with either the indicated morpholino (Hspa5MO1 or Hspa5MO2) or mRNA was injected into one blastomere of Xenopus embryos at the indicated stages for lineage tracing. After fixation with HEMFA (0.1 mM HEPES, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), embryos were stained in X-Gal or Red-Gal staining solution (1 mg/ml X-Gal or Red-Gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2 in 1× PBS) at room temperature. After fixation, embryos were dehydrated and stored in absolute ethanol at −20 °C until whole mount in situ hybridization. Vibratome sectioning was performed as described elsewhere (35, 36). After whole mount in situ hybridization, embryos were embedded in gelatin-albumin solution (5 g/liter gelatin, 380 g/liter chick egg albumin, 200 g/liter sucrose in 0.1 M phosphate buffer, pH 7.4) mixed with 1/10 volume 25% (v/v) glutaraldehyde and sectioned with vibratome at a thickness of 20 μm.

Microinjection Experiments—Hspa5MOs and standard control MO were purchased from Gene Tools (Philomath, OR). For overexpression, mRNAs were synthesized in vitro with the mMessage mMachine SP6 kit (AM 1340, Ambion). Microinjection was performed as published previously (25).

In Vitro Induction of Pronephros—Animal caps were dissected from stage 9 embryos in 1× MMR and then cultured in animal cap culture solution (67% L-15 medium, 7.5 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) until sibling embryos reached the desired stages. About 20 embryo animal caps were collected to extract RNA (33). For pronephros induction, the dissected animal caps were treated with activin at 10 mg/ml and atRA at 10−8 M for 3 h and then transferred into animal cap culture medium until the sibling embryos reached stage 15 or 32.

RNA Extraction, RT-PCR, and Real Time PCR—Total RNA was extracted from Xenopus embryos or animal caps using TRIzol (Invitrogen) and precipitated by isopropanol. The RNA extract was purified with RNeasy (Qiagen) after DNase I treatment. cDNA was synthesized by using Superscript III (Invitrogen) according to the manufacturer’s instruction. Semiquantitative PCR was carried out as we described previously (36, 37). Real time PCR was performed with the ABI 7900HT Fast Real-Time PCR system (Applied Biosystem) using SYBR Green PCR.
**TABLE 1**

| Name             | Sequence (5’–3’)                              | Refs. |
|------------------|-----------------------------------------------|-------|
| cdx4 Fw          | ATGTCGCGCTGTCCTTATACC                            | 63    |
| cdx4 Re          | ACCACCTTTGCTGTTATAG                              |       |
| hoxd1 Fw         | CAGGCGACTAGCTTTG                                  |       |
| hoxd1 Re         | CCGGGAGAGTTTGG                                   | 64    |
| gbx2 Fw          | CCCCCAAAACCTACACCTTTTAA                           |       |
| gbx2 Re          | TGGCTGCTCCTTACGACATT                             |       |
| pax2 Fw          | CTTGCTCTCCACGTAGCTTT                             | This study |
| pax2 Re          | TACGAGAATGTCTTTCTAC                              |       |
| smp30 Fw         | TTGACTGCTCTCTAGCACAC                             |       |
| smp30 Re         | CTGAGATGCTCTTACGATC                              |       |
| nephrin Fw       | GCCGACCTACATGACATG                                 |       |
| nephrin Re       | CCAACGAGCATCACATG                                 |       |
| pax8 Fw          | CAAGGAGCATCCGCTGATA                                |       |
| pax8 Re          | CCAAGGAGCATCCGCTGATA                               |       |
| hspa5 Fw         | TACGACCTTACACCTTGC                               |       |
| hspa5 Re         | CTTCACGGCCACAACATG                                |       |
| lhx1 Fw          | AGGAGGACATGACACATC                                | 23    |
| lhx1 Re          | CTTCCTTGGATCGCTGCTTGGACACAC                      |       |
| odc Fw           | CTAGACCTTGCTTGCTG                                 | 65    |
| odc Re           | CAAATGGAACCTTACACAC                               |       |
| Human HSPA5 Fw   | GGCGCAAGATGACTGAAAG                               | 66    |
| Human HSPA5 Re   | CTGTGCGCGCTCCTACATGAC                             |       |
| Human β-actin Fw | GGCTGCTACACATGACATG                                | 67    |
| Human β-actin Re | GGGTGGTACACCACTAAGAC                              |       |
| Human RARα Fw    | CTGCCACCTGACCCTGAC                                | 68    |
| Human RARα Re    | GGCAGCTGCTGACCTGAC                                |       |
| Human RXRα Fw    | CGTACACATGACCTGAC                                 | 69    |
| Human RXRα Re    | GGGGACACCTGACCTGAC                                |       |
| Human RXRβ Fw    | CGTACACATGACCTGAC                                 | 70    |
| Human RXRβ Re    | TTGCAGCGCTGACACATG                                |       |
| Human CYP26α Fw  | GGCGGCTGACGAGGACTG                                | 71    |
| Human CYP26α Re  | GGCGGCTGACGAGGACTG                                |       |
| Human RALDH1 Fw  | CTGTCGCGACAGAATG                                  | 72    |
| Human RALDH1 Re  | GTCACGACACAGAATG                                  |       |
| Human RALDH2 Fw  | AGAAGGAGGTACGACGAC                                | 73    |
| Human RALDH2 Re  | AGAAGGAGGTACGACGAC                                |       |
| Human RALDH3 Fw  | ACCGTGAGGAGCCTAACAG                                | 38    |
| Human RALDH3 Re  | ACCGTGAGGAGCCTAACAG                                |       |

Master Mix (TaKaRa) and specific primers. *Xenopus odc* and human β-actin were used as internal controls to normalize the target gene expression in *Xenopus* and HEK293T samples. All quantitative PCR experiments were performed in triplicate. The primers used in this study were either designed by Primer 3 or by following published studies. The primer sequences are listed in Table 1.

**Western Blot**—Proteins were extracted from *Xenopus* embryos and HEK293T cells with lysis buffer (137 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100), and separated by SDS-PAGE. Antibodies for detecting protein were diluted at 1:1000 for anti-HSPA5 antibody (Cell Signaling Technology) and 1:5000 for anti-tubulin antibody (Cell Signaling Technology). The HRP-labeled secondary antibodies were diluted at 1:10,000 and detected with enhanced chemiluminescence (Super Signal West Dura Extended Duration Substrate, Thermo Fisher Scientific).

**Luciferase Assay**—DNA constructs of HSPA5 shRNA-pLKO.1 and a no-target control shRNA were purchased from Sigma. The HSPA5 shRNA sequence is CCAGCTTGTGGTGGCTGACTGCAGCTGACGACACAGTTT. shRNA-pLKO.1 was transfected into HEK293T cells along with pCMV-ΔR8.91 and vesicular stomatitis virus G. Forty-eight hours after transfection, lentiviral particles were collected and used to infect HEK293T cells. Twenty-four hours after infection, HEK293T cells were transfected with pGL3-RARE-luciferase and pRL-Revilla by Lipofectamine 2000 (Invitrogen). AtRA (2 × 10⁻⁸ M) was added into culture medium 6 h after transfection. A luciferase assay was performed with the Dual-Luciferase reporter assay system (Promega) 48 h after transfection. Measurements were carried out in triplicate and are expressed as mean ± S.D. The following small interfering RNAs (siRNAs) were also used for knockdown of HSPA5 in HEK293T cells: siRNA1: sense, 5’-GGAGCCGCAUUUGAUACUAGATT-3’; antisense, 5’-UCUAGUAUCUAGUGCCUCCTT-3’; siRNA2: sense, 5’-GGCCGUAAUUUGGAAAGATT-3’; antisense, 5’-UCCUUCCAAUUAGGCUCCTT-3’. Two siRNAs were mixed in a 1:1 ratio at a final concentration of 60 nM. The siRNAs as well as pGL3-RARE-luciferase and pRL-Revilla were transfected into HEK293T cells using Lipofectamine 2000. Six hours after transfection, atRA was added to the cell culture medium. The luciferase assay was performed with the Dual-Luciferase reporter assay system (Promega) 48 h after transfection.

**Immunostaining and Confocal Imaging**—For immunostaining with *Xenopus* embryos, the embryos were bleached with 10% H₂O₂ in methanol for 3 h after fixation with HEMFA. After washing with 1X PBS, embryos were then blocked (155 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10% PBS, 5% DMSO) for 1 h at room temperature. Embryos were incubated with the first antibody, 3G8 or 4A6 (European Xenopus Resource Centre), for 3 days at 4°C. Subsequently, embryos were washed five times with 1X PBS and then incubated with the secondary anti-mouse antibody conjugated with Alexa Fluor 568 (Invitrogen). Embryos were then photographed with a microscope (SZX16, Olympus).

For immunostaining with mammalian cells, SH-SY5Y cells were harvested 48 h after transfection with HSPA5 siRNA and fixed with 4% formaldehyde solution in 1X PBS. The endogenous HSPA5 and RARα were detected using rabbit anti-HSPA5 antibody (Cell Signaling Technology) and mouse anti-RARα (Abcam), respectively. Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 568-conjugated anti-mouse antibodies were then used to visualize the localization and density of HSPA5 and RARα, respectively. Slides were mounted using Fluoromount (Southern Biotechnology) and viewed using an Olympus FluoviewTM FV1000 confocal microscope. Sequential scanning mode was utilized to prevent possible bleed-through of fluorescence emission.

**Statistical Analysis**—Data are shown as an average with a S.D. of measurements derived from at least three trials. One-way analysis of variance analysis was used to determine whether the difference was statistically significant.

**RESULTS**

**Expression Pattern of hspa5 in Xenopus Embryos**—Whole mount in situ hybridization was carried out to examine the spatial expression pattern of *hspa5* in *Xenopus* embryos. *Hspa5* was expressed in the animal half of the embryo from the two-cell stage to blastula stage (Fig. 1, A and B). During gastrulation, *hspa5* expression was detected in the ventral ectoderm. In addition, a distinct expression domain was found in the dorsal blastopore lip (red arrow), whereas the prospective neural ectoderm lacked staining (Fig. 1, C–E). At neurula stage, staining was observed anterior to the neural plate (red arrow), corre-
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FIGURE 1. Expression pattern of hspa5 in developing Xenopus embryos. A–K, spatial expression pattern of hspa5 analyzed by whole mount in situ hybridization. Embryos are shown in different views (dorsal-lateral and vegetal). A and B, Hspa5 was expressed in the animal half prior to gastrulation stages. C–E, during gastrulation, hspa5 is strongly expressed in ventral ectoderm, dorsal blastopore lip, and the developing notochord but is absent from the prospective neural plate. In C and D, the red arrow indicates dorsal blastopore lip; in E, the white arrowhead indicates the notochord. F and G, at neurula stages, hspa5 expression is restricted to the notochord (white arrow) and the region anterior to the neural plate (red arrow) that corresponds to the future cement gland. H and I, at stage 22, strong expression is found in the cement and hatching glands. H, anterior view; I, lateral view. J and K, at the tail bud stage, hspa5 is expressed in the cement gland, pronephros (red arrow), ear vesicle, and epidermis. It is notable that the hspa5 signal can also be found in the liver primordium (K, black arrow). L and M, transverse sections of tail bud stages (J) showing hspa5 expression in pronephros (pn) and notochord (nc) tissue. N and O, transverse sections of a late tail bud stage embryo (K) showing hspa5 expression in the pronephros (pn) and liver (li). P–S, Xbp1 expression in Xenopus embryos at the stages indicated. T, RT-PCR shows the temporal expression pattern of hspa5. Numbers above the lanes indicate the corresponding embryonic stages. A negative control (RT–) without reverse transcriptase was included. odc expression was used as the internal standard control. U, schematic diagram illustrating the interaction of Xbp1 and Hspa5 in the unfolded protein response. V, real time PCR indicates that hspa5 is up-regulated by overexpression of xbp1. xbp1 mRNA at the indicated doses was injected into Xenopus embryos at the two-cell stage. Animal caps were dissected at stage 9 and cultured for 2 h. The expression of hspa5 was analyzed by real time PCR, and its expression was normalized to odc. The animal cap assay was performed twice. St, stage.

Corresponding to the future cement gland, as well as in ventral ectoderm. Consistent with early staining in the blastopore lip in the gastrula, distinct staining was also shown in the notochord (white arrow) (Fig. 1, F and G). At early tail bud stages (stage 22), hspa5 was expressed in the cement gland and hatching gland (Fig. 1, H and I). Embryos at late tail bud stages exhibited hspa5 expression in cement gland, pronephros (red arrow), liver (black arrow), ear vesicles, and epidermis (Fig. 1, J and K). Transverse sections confirmed hspa5 expression in the pronephros and notochord in early tail bud stages (Fig. 1, L and M) and in pronephros and the liver at late tail bud stages (Fig. 1, N and O). The spatial expression pattern of hspa5 was reminiscent of xbp1 (25), another key regulator of the unfolded protein response. A comparison of the expression pattern of xbp1 with that of hspa5 showed that xbp1 was co-expressed in the ventral ectoderm and dorsal blastopore lip (red arrow) during gastrulation (Fig. 1P), in the notochord and the region anterior to the neural plate (red arrow) at neurula stage (Fig. 1Q), and in the cement gland, pronephros (red arrow), and epidermis at tail bud stages (Fig. 1, R and S) (25). Our observations suggest that both hspa5 and xbp1 are as closely associated in embryonic development as they are in the ER stress response (39). Examination of the temporal expression pattern showed that hspa5 mRNA could be detected prior to gastrulation, and its expression was increased during gastrulation and maintained at higher levels onward (Fig. 1T). During the unfolded protein response, active Xbp1 can up-regulate Hspa5 expression (Fig. 1U). To demonstrate this interaction, we overexpressed Xenopus xbp1 in animal cap assays and found that hspa5 expression was indeed enhanced (Fig. 1V). Further confirmation of the up-regulation was observed in experiments repeated in Xenopus embryos (data not shown). Collectively, the complex expression of hspa5 in developing embryos suggests its importance during embryonic development.

Hspa5 Is Essential for Xenopus Embryonic Development—It has been reported that Hspa5 mutant mice are embryonic lethal (40), indicating an essential role for Hspa5 in early embryonic development. To further investigate the function of Hspa5 dur-
ing early embryonic development in Xenopus, we used two Hspa5 morpholino antisense oligonucleotides (MO1 and MO2) (Fig. 2A) to reduce endogenous Hspa5 protein expression through translational inhibition. We found that injection of the two Hspa5MOs into Xenopus embryos at the two-cell stage resulted in a dose-dependent reduction of endogenous Hspa5 protein translation in vivo. It is notable that injection of Hspa5MOs had no effect on the expression of β-tubulin. We examined the phenotypic effects of increasing doses of Hspa5MO1 injected into two blastomeres of two-cell stage embryos. Depletion of Hspa5 with 30 ng of Hspa5MO1 disrupted embryonic development. The injected embryos showed various degrees of developmental abnormalities including strong inhibition of head structures such as eyes as well as shortened anteroposterior axis. Lower injection doses (10 and 20 ng/embryo) induced a similar but less severely affected phenotype. We also examined the effects of Hspa5MO2 on embryonic development and observed the same phenotype. Based on the degree of severity, we categorized and quantified the phenotype of the injected embryos that developed to stage 32 into mild, moderate, and severe groups (Fig. 2F). The severity of the phenotype correlated with the doses of both Hspa5MO1 and Hspa5MO2 (Fig. 2G). Rescue experiments were performed using hspa5 mRNA containing six silent mutations (Fig. 2H) expected to be resistant to the binding of Hspa5MOs. The phenotype induced by 30 ng of Hspa5MO1 or Hspa5MO2/embryo was attenuated by co-injection with 200 pg of pCS2-hspa5 mRNA (Fig. 2I). In the Hspa5MO1 rescue experiments, the ratio of embryos exhibiting a severe phenotype was reduced from 58 to 13%, whereas the ratio of embryos in the mild and moderate categories increased from 41 to 87%. These observations indicate that the phenotype induced by Hspa5MO1 is specific. Consistent with Hspa5MO1, the phenotype induced by Hspa5MO2 was also partially rescued by pCS2-hspa5 mRNAs. In the Hspa5MO1 rescue experiments, the ratio
of embryos exhibiting a severe or moderate phenotype was reduced from 69 to 47%, whereas the ratio of embryos in the mild category increased from 31 to 53%. These results indicate that both Hspa5MOs are effective in knocking down Hspa5 expression, which is essential for embryonic development.

Hspa5 Is Required for Pronephros Formation in Xenopus Embryos—Our results show that Xenopus hspa5 is highly expressed in developing pronephric tissue (Fig. 1). In a previous mouse study, heterozygous animals carrying an Hspa5 mutation developed glomerulosclerosis and tubulointerstitial nephritis disease (41). It has also been reported that dysfunction of Hspa5 accelerated kidney injury (42), and expression of Hspa5 was up-regulated in diabetic kidneys (43). We therefore wanted to investigate the functional role of Hspa5 in Xenopus pronephros development, which may provide valuable insights into explaining the reported observations. To avoid possible interference with initial mesoderm formation, a mixture of Hspa5MO and LacZ mRNA was injected into one of the ventral-vegetal blastomeres of Xenopus embryos at the eight-cell stage that largely contribute to the formation of the pronephros. The LacZ mRNA was used as a lineage tracer to identify the injected side at later stages. When the ventral-vegetal blastomeres were targeted with either 15 or 30 ng of Hspa5MO2, the morphology of the injected embryos was predominantly normal (Fig. 3, A–C). Whole mount in situ hybridization and immunostaining experiments were performed to examine the formation of the pronephros in embryos targeted on one side with 30 ng of Hspa5MO2/embryo using marker genes that include pax2 (44), lhx1 (21, 22), and β1 subunit of Na/K-ATPase (atp1b1) (45). The marker genes pax2 (60%; 18 of 30), lhx1 (67%; 24 of 36), and atp1b1 (85%; 29 of 34) were strongly suppressed on the injected side but not on the uninjected side (Fig. 3, D–F and D’–F’), which was further confirmed by section analysis (Fig. 3, D”–F”). We also examined the expression of genes involved in pronephros tubule and glomus development including smp30 (46) and nephrin (47). Expression of smp30 (tubule; 64%; 21 of 33) and nephrin (glomerulus; 55%; 17 of 31) was decreased on the Hspa5MO2-injected side when compared with the uninjected side (Fig. 3, G, H, G’, and H’). Inhibition was confirmed by analysis on serial sections (Fig. 3, G” and H”). The phenotypes were specific as the expression of pax2, lhx1, smp30, and nephrin remained normal when the same amount of standard control MO was injected into one vegetal-ventral blastomere of eight-cell embryos (Fig. 3, I–L’).

Similar to the whole mount in situ hybridization experiments, immunostaining with antibodies 3G8 and 4A6 that recognize pronephric tubules and ducts, respectively, also illustrated the inhibition of pronephros formation on the Hspa5MO-injected side (Fig. 4). The LacZ injection alone had little effect on pronephros formation (data not shown).
Depletion of Hspa5 Inhibits the Expression of Pronephros-specific Genes in Animal Cap Assays—Animal caps dissected from early gastrula embryos are pluripotent and can be induced to differentiate into pronephros tissue by treatment with atRA and activin (12–14). To further investigate the role that Hspa5 plays during pronephros development, we carried out an in vitro pronephros induction assay and examined two developmental time points, stages 15 and 32. In agreement with previous reports, treatment with activin and atRA induced animal caps to differentiate into pronephros tissue as revealed by up-regulation of lhx1, pax8, pax2, and wt1 when caps were collected at stage 15. Investigation of stage 32 treated caps showed an up-regulation of lhx1, pax8, pax2, smp30, and nephrin markers (Fig. 5, A–C). Conversely, knockdown of Hspa5 with Hspa5MO2 in animal caps resulted in decreased expression of the examined genes upon activin and atRA treatment at both stages (Fig. 5, B and C). These results were consistent with the inhibition of pronephros formation in Hspa5 morphants (Figs. 3 and 4). We also examined expression of lhx1 in Hspa5 morphants at stage 15. One dorsal blastomere of four-cell stage embryos was injected with either Hspa5MO1 (30 ng/embryo) or Hspa5MO2 (30 ng/embryo). The resulting lhx1 expression was reduced on the injected side (68% (13 of 19) in Hspa5MO1-injected embryos and 61% (14 of 23) in Hspa5MO2-injected embryos) when compared with the un.injected side (Fig. 5, D and E). These results support an important role for Hspa5 in pronephros formation both in vivo (embryos) and in vitro (animal cap assays).

**Hspa5 Regulates Pronephros Formation by Mediating RA Signaling**—Because knockdown of Hspa5 in animal caps impaired pronephros differentiation induced by activin and atRA, we sought to investigate how Hspa5 acts on transduction of RA or activin signaling. The animal caps dissected from stage 9 Hspa5MO2-injected embryos were treated with either atRA, activin, or a combination of both atRA and activin. In the control animal caps, atRA treatment led to an up-regulation of expression in RA-responsive genes including lhx1, cdx4, and gbx2 (Fig. 6A, lane 3) (37, 48). In contrast, RA-induced up-reg-
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**FIGURE 6. Hspa5 is required for transduction of RA signaling.** A, Hspa5MO2 down-regulates *lhx1*, *cdx4*, and *gbx2* gene expression in animal caps treated with atRA but not in animal caps treated with activin. Hspa5MO2 (MO2) (30 ng/embryo) was injected into both blastomeres of *Xenopus* embryos at the two-cell stage. Animal caps were dissected at stage 9, treated with either atRA or activin separately or a mixture of both for 3 h, and then cultured in normal animal cap culture medium for another 3 days. Expression of the indicated genes was examined by RT-PCR. Depletion of Hspa5 attenuates expression of *lhx1*, *cdx4*, and *gbx2* induced by atRA. Activin treatment up-regulates *lhx1*, *hoxd1*, *cdx4*, *gbx2*, and *chordin* expression. Knockdown of Hspa5 has little effect on the indicated genes except that *lhx1* expression is enhanced. Treatment of atRA and activin induces *lhx1*, *hoxd1*, and *gbx2* expression in control animal caps, whereas knockdown of Hspa5 causes a reduction of *lhx1* but not *hoxd1* or *gbx2*. AC, control animal cap; WE, sibling whole embryos. B, Hspa5MO decreases expression of atRA-responsive genes. Animal caps were treated with 0.1 or 1 μM atRA for 4 h at room temperature. RA-responsive genes *lhx1*, *gbx2*, *hoxd1*, and *cdx4* were induced by atRA treatment in a dose-dependent manner. Depletion of Hspa5 leads to decreased induction of these genes. C, real time PCR shows that HSPA5 shRNA effectively reduces HSPA5 mRNA levels in human HEK293T cells. Expression of HSPA5 in cells transfected with either control shRNA or HSPA5 shRNA was normalized to the control cells. D–G, endogenous HSPA5 expression is reduced in HSPA5 shRNA-infected (D and E) or HSPA5 siRNA-infected (F and G) HEK293T cells. The expression of HSPA5 was detected by Western blotting. β-Tubulin was used as a loading control (D and F). The quantified HSPA5 signal in either HSPA5 shRNA-infected cells or control shRNA-infected cells was normalized to the β-tubulin signal. The relative expression was normalized to that of control cells (E and G). The intensity of Western blot signals was quantified by a GS-800 calibrated imaging densitometer. H and I, depletion of HSPA5 by either Hspa5 shRNA (H) or Hspa5 siRNA (I) reduces the luciferase activity of RA luciferase reporter gene in HEK293T cells. AtRA treatment enhances the luciferase activity, whereas HSPA5 shRNA reduces the luciferase activity in a dose-dependent manner. BMS433, an RA signaling inhibitor, also inhibits the luciferase activity of RA luciferase reporter gene. DMSO treatment was used as a control. The asterisks indicate the statistically significant difference. con, control; RLU, relative luciferase units. Error bars represent S.D.

ulation was consistently reduced in the Hspa5MO2-injected caps (Fig. 6A, lane 4). Upon treatment with either 0.1 or 1 μM atRA, the RA-responsive genes including *lhx1*, *gbx2*, *hoxd1*, and *cdx4* were up-regulated in a dose-dependent manner. Therefore, attenuation of Hspa5 activity causes reduced expression of these genes (Fig. 6B). Similarly, activin treatment also resulted in induction of *lhx1*, *hoxd1*, *cdx4*, *gbx2*, and *chordin* in the uninjected animal caps (Fig. 6A, lane 5). Interestingly, knockdown of hspa5 under the same condition did not affect the expression of *hoxd1*, *cdx4*, *gbx2*, and *chordin* but did lead to an increase in *lhx1* expression (Fig. 6A, lane 6). These observations indicate that Hspa5 knockdown attenuates RA signaling but not activin signaling. When treated with both activin and atRA, the control animal caps showed increased expression of *hoxd1*, *lhx1* and *gbx2* but not chordin or *cdx4* (Fig. 6A, lane 7), whereas the Hspa5MO2-injected animals caps exhibited unchanged expression of *hoxd1*, *cdx4*, and *gbx2* except *lhx1*. Lhx1 is essential for pronephros formation, and its expression was reduced (Fig. 6A, lane 8). Collectively, these results provide evidence that RA signaling is attenuated when Hspa5 is depleted in the animal cap assay, suggesting that Hspa5 regulates pronephros formation at least in part through RA signaling.

We also examined the functional role of Hspa5 in RA signaling in cultured mammalian cells. Using shRNA and siRNA that specifically knock down HSPA5 in HEK293T cells, we carried out luciferase assays to quantitatively measure RA reporter activity in HSPA5-depleted cells. The knockdown efficiency was examined by real time PCR (Fig. 6C) and Western blotting.
which confirmed the reduction of HSPA5 expression at both the mRNA and protein levels. Luciferase assays were carried out in both control cells and the HSPA5-depleted cells to examine the activity of a RA reporter in the presence of atRA. As expected, atRA treatment significantly increased luciferase activity of the RA-responsive reporter gene in the control cells, whereas in HSPA5-depleted cells, the luciferase activity was lower compared with the control (Fig. 6, H and I). Moreover, the reduction was dose-dependent (Fig. 6H) and comparable with cells treated with BMS453, a known RA signaling inhibitor (49). These results indicate that knockdown of HSPA5 also attenuates RA signaling in HEK293T cells.

To further investigate the underlying mechanism as to how HSPA5 regulates RA signaling, we carried out real time PCR to examine the expression of genes involved in RA signaling transduction and atRA metabolism including RARα, RXRα, RXRβ, RALDH1, RALDH2, RALDH3, and CYP26A1 in HSPA5-depleted HEK293 cells. Knockdown of HSPA5 did not significantly alter the expression of the genes examined except that RALDH1 was moderately up-regulated (Fig. 7A). Thus it is likely that HSPA5 regulates expression of these genes at the
protein level or affects protein localization. We then investigated the effects of HSPA5 deletion, if any, on RARα subcellular localization and its expression level at the protein level in SH-SY5Y cells. Although RARα is a nuclear receptor, it is mainly located in the cytoplasm, and only a small amount of RARα could be found in the nucleus (Fig. 7, C2 and E2), which is in line with what has been reported previously (50). We also found that the expression of RARα in HSPA5-depleted SH-SH5Y cell was clearly reduced (Fig. 7, D2 and F2). The HSPA5 depletion was validated by immunostaining detecting endogenous HSPA5 expression in the same cells (Fig. 7, C1–F1). The reduction of HSPA5 was further confirmed by Western blotting with the protein extracts from the cells under the same experimental conditions (Fig. 7B). It is worth noting that strong nucleus-localized HSPA5 signals were detected in SH-SH5Y cells, consistent with a previous study (51). Collectively, we have provided evidence to support that HSPA5 is required to maintain the RARα expression at the protein level that may contribute to the inhibition of RA signaling.

We have established that Hspa5 is required for Xenopus pronephros formation and that depletion of Hspa5 inhibits the expression of pronephros-specific genes in animal cap assays. To further assess the underlying mechanisms as to how Hspa5 contributes to pronephros formation, we focused on lhx1. lhx1 is one of the earliest genes expressed in the pronephric anlagen, and its expression was reduced in Hspa5MO-injected animal caps and embryos (Figs. 3, E–E’; 5, D and E; and 6A). Both hspa5 and lhx1 are expressed in the dorsal blastopore lip at gastrula stages (52). Our data show that RA signaling is inhibited when Hspa5 is depleted both in animal caps and in HEK293T cells and that lhx1 is responsive to atRA treatment (Fig. 6, A and B). It has been reported that several putative RAREs have been identified in the lhx1 promoter region (15, 53). This observation prompted us to investigate whether Hspa5 regulated pronephros formation through RA-Lhx1 signaling in whole embryos.

Rescue experiments were performed to investigate whether Lhx1 activity could rescue the phenotypes induced by Hspa5MO. Hspa5MO1 alone or in combination with lhx1 mRNA was injected into one ventral-vegetal blastomere of Xenopus embryos at the eight-cell stage with LacZ mRNA. Pronephros formation was evaluated by examining the expression of markers including lhx1, pax2, nephrin, and smp30. Injection of Hspa5MO1 caused severe inhibition of pronephros at the injected side, whereas co-injection of lhx1 mRNA and Hspa5MO1 partially rescued the phenotype induced by Hspa5MO1 (Fig. 8). These results suggest that the role of Hspa5 in pronephros development is mediated by RA-Lhx1 activity.

**DISCUSSION**

Hspa5 is a multifunctional protein that has been implicated in many biological processes. There is increasing evidence indicating that Hspa5 is involved in biogenesis and signal transduction and that it plays important roles in carcinogenesis (54, 55). In this study, we investigated Hspa5 function during early embryonic development in *Xenopus* and showed for the first time that Hspa5 plays an essential role in pronephros formation that is mediated by RA-Lhx1 signaling.

A number of genes expressed in the pronephros have been implicated in regulating its formation (46, 56, 57). We found that hspa5 was highly expressed in the pronephros in *Xenopus* embryos. Injection of Hspa5MO into one of the ventral-vegetal blastomeres at the 8-cell stage, which contribute to the pronephros, resulted in a decrease of pronephric markers such as...
genes such as inhibitors such as BMS453, or overexpression of the RA-catab-

ual lines of evidence suggest that Hspa5 can interact with Cripto and promote Nodal signaling but attenuates activin A signaling (28, 58, 59). It is possible that knockdown of Hspa5 reduced (Fig. 5). Taken together, the data indicate that Hspa5 is required for pronephros formation.

RA signaling is crucial for pronephros formation. In Xenopus embryos, inhibition of RA signaling by either expression of a dominant-negative RA receptor, treatment with chemical inhibitors such as BMS453, or overexpression of the RA-catabolizing enzyme Cyp26 (15) results in the impairment of pronephros formation. Alternatively, the overactivation of RA signaling expands the size of the pronephros (15). In this study, we found that depletion of Hspa5 largely impaired pronephros formation. Moreover, the expression of retinoic acid-responsive genes such as hoxd1, gbx2, cdx4, and lhx1 were decreased in Hspa5-depleted animal caps treated with atRA (Fig. 6, A and B). Using confocal imaging, we also observed reduction of RARα in HSPA5-depleted SH-SY5Y cells that may contribute to the attenuation of RA signaling (Fig. 7). Interestingly, activin also induced the expression of hoxd1, gbx2, cdx4, and lhx1 in animal cap assays, but depletion of Hspa5 in activin-treated animal caps did not affect the expression of these genes (Fig. 6A).

Several lines of evidence suggest that Hspa5 can interact with Cripto and promote Nodal signaling but attenuates activin A signaling (28, 58, 59). It is possible that knockdown of Hspa5 enhances activin signaling to a certain extent, resulting in an increase of lhx1 expression. Taken together, our observations suggest that Hspa5 regulates pronephros formation through RA signaling rather than activin signaling in in vitro pronephros induction assays.

It has been suggested that RA, but not activin, induces the generation of Ca2+ transients, which are involved in the process of pronephric tubulogenesis (60). The inhibition of RA signaling by Hspa5 was also confirmed in HEK293T cells. We found that the RA luciferase reporter activity was drastically reduced by Hspa5 MO1 (Fig. 8).

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