Mechanistic Insights Into Small-molecule Inhibitor MS-444 Arresting Embryonic Development Revealed by Low-input RNA-seq and STORM

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Research Article

Keywords: Embryonic development, MS-444, HuR dimerization, Fertility maintenance, Nanometer, STORM

Posted Date: January 25th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1260089/v1>

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Abstract

Background

With the improvement of the survival rate of cancer patients, fertility maintenance has become a major concern of cancer treatment for women of reproductive age. Thus, it is important to examine the impact on fertility of anticancer drugs that have been clinically used or are undergoing trials. The HuR small-molecule inhibitor MS-444 has been used in many cancer treatment studies, but its reproductive toxicity in females is unknown.

Results

Combining analysis of low-input RNA-seq for MS-444-treated 2-cell embryos and mapping binding sites of RNA binding protein, Agbl2 was predicted to be the target gene of MS-444. For further confirmation, RNAi experiment in wild-type zygotes showed that Agbl2 knockdown reduced the proportion of embryos successfully develop to the blastocyst stage: from 71% in controls to 23%. Furthermore, RNA-FISH and luciferase reporter analysis showed that MS-444 blocked the nucleocytoplasmic transport of Agbl2 mRNA and reduced its stability by inhibiting HuR dimerization. In addition, optimized stochastic optical reconstruction microscopy (STORM) imaging showed that MS-444 significantly reduced the HuR dimerization, and HuR mainly existed in the form of clusters in 2-cell stage embryos.

Conclusion

Those results showed that MS-444 blocked the nucleocytoplasmic transport of Agbl2 mRNA by inhibiting HuR dimerization, resulting in the developmental arrest of 2-cell stage embryos in mouse. This study provides clinical guidance for maintaining fertility during the treatment of cancer with MS-444 in women of reproductive age. And also, our research provides guidance for the application of STORM in the nanometer scale study of embryonic cells.

Introduction

More than 10% of new cancer diagnoses occurs in women of reproductive age, impacting 870 women per million annually [1]. With the emergence of new chemotherapeutic drugs, and the improvements of chemotherapy and radiotherapy regimens and the early cancer diagnosis rate, the survival rate of young female patients has greatly improved [2–4]. The significant improvement in the survival rate makes it possible for most female cancer patients at reproductive age to have children [5, 6]. It is important to comprehensively understand and evaluate anticancer drugs that are clinically used and undergoing trials for their effects on the fertility of female cancer patients of reproductive age.

Fertility is the natural ability to conceive and give birth to live babies, which involves oogenesis and embryonic development. Oogenesis is a complex molecular and cellular process, which is regulated by a large number of factors inside and outside the ovary [7–13]. Primary oocytes are arrested in prophase I of

meiosis, also known as the germinal vesicle (GV) stage. With the surge of hormones, oocytes resume meiosis I (MI) division, and are then arrested again in metaphase II (MII) and await fertilization [14–16]. During the development of mouse preimplantation embryos, the first important developmental stage is the 2-cell stage, which contains major zygotic genome activation (ZGA) and the first cell fate differentiation of embryos [17–19]. Abnormality of ZGA can arrest embryonic development, usually in the 2-cell stage in mouse and the 8-cell stage in human [20–22]. The drugs that are currently commonly used for treating cancer often cause irreversible fertility decline, serious damage to fertility, or infertility [23–25]. Moreover, many new and upcoming anticancer drugs such as MS-444 have an increasingly clear anticancer effects, but whether they affect fertility has not yet been studied [26].

MS-444 has become the most studied small-molecule inhibitor of HuR (encoded by the *Elavl1* gene) [26–31]. Studies have found that almost every feature of cancer can be regulated and promoted by HuR [32]. High cytoplasmic HuR accumulation, which can be inhibited by MS-444, was found in many cancers, such as colorectal cancer, ovarian cancer, breast cancer, and oral cancer, which are associated with worse clinical outcomes, worse histological grade, and increased incidence of lymphatic spread and distant metastasis [26, 33–37]. However, the conventional microscopy cannot directly resolve the cytoplasmic HuR accumulation due to diffraction limit on optical resolution [38, 39].

Stochastic optical reconstruction microscopy (STORM) surpasses the resolution limit by optical diffraction based on single molecule imaging and centroid localization of switchable fluorescent probes enabling nanoscopic imaging of biological specimen [40, 41]. Thus, STORM provides an ideal tool for directly visualizing the cytoplasmic HuR accumulation within subcellular regions at nanometer resolution. However, the study of STORM in embryonic development has not been reported so far because of the heavily scatters light caused by the large cell diameter and complex cytoplasmic components of embryonic cells.

In the present study, we found that MS-444 blocked the nucleocytoplasmic transport of Agbl2 mRNA by inhibiting HuR dimerization, resulting in embryonic development arrest at the 2-cell phase. STORM with our optimized protocol showed that HuR tended to form elliptical and dense clusters in 2-cell stage embryos, but MS-444 disrupted the intracellular distribution of HuR. In addition, MS-444 blocked the nucleocytoplasmic transport of Agbl2 mRNA and reduced its stability by inhibiting HuR dimerization. This study provides a theoretical basis for understanding the biological characteristics of the small-molecule inhibitor MS-444 and provides guidance for maintaining fertility during the clinical treatment of cancer patients with MS-444.

Results

MS-444 resulted in embryonic development arrest at the 2-cell stage

We investigated the effects of the HuR inhibitor MS-444 on oogenesis and early embryo development by its intraperitoneal injection and culture of oocytes and embryos *in vitro* (Figure 1A). We initially examined the roles of MS-444 in follicular development and oocyte maturation *in vivo*. Female mice

intraperitoneally injected with MS-444 had normal folliculogenesis and corpora lutea, which did not differ significantly from those of the control (Figure 1B). Meanwhile, the serum hormone levels of mice in the injection group and control group were determined. There were no significant differences ($p=0.71$ for estrogen, $p=0.71$ for progesterone) in serum estrogen (E2) and progesterone (prog) levels between MS-444-injected mice and the control group (Figure 1C, D).

We also studied the roles of the HuR inhibitor MS-444 in oocyte maturation and early embryo development *in vitro*. The maturation rate of oocytes treated with different concentrations of MS-444 did not differ significantly from that in the control group (Figure 1E). These results suggested that MS-444 did not affect the maturation of mouse oocytes. Moreover, MS-444 treatment did not affect early 1-cell to 2-cell embryonic development (Figure 1F). By adding MS-444 to KSOM medium, late 1-cell stage embryos were found to be arrested at the 2-cell stage *in vitro*, and the degree of arrest was proportional to the inhibitor concentration (Figure 1G). All embryos were arrested at the 2-cell stage, when cultured with 75 μ M MS-444. Furthermore, MS-444 did not cause an abnormal morphology of the embryos (Figure 1H, Supplementary Figure S1A and B). These results showed that MS-444 did not affect oocyte maturation, but blocked embryonic development at the 2-cell stage. Moreover, 5-ethynyl-2'-deoxyuridine (EdU) incorporation studies showed that embryos were blocked at G₂ phase because MS-444-treated embryos incorporated EdU at S phase and nuclei were clearly present (Supplementary Figure S2A).

To explore the mechanism by which MS-444 arrests embryonic development, its effects on DNA replication and transcriptional activity were examined in the arrest of embryonic development at the 2-cell stage. The results of EdU incorporation showed that MS-444 treatment did not cause changes in DNA replication in 2-cell embryos ($p=0.28$) (Supplementary Figure S2A and B). Compared with the control embryos, the 5-ethynyl uridine (EU) signal intensity of 2-cell stage embryos in the MS-444 treated group was significantly reduced ($p<0.001$, Supplementary Figure S2C and D), which revealed the deficiency of transcriptional activity.

MS-444 blocked 2-cell stage embryonic development by inhibiting HuR dimerization

To further study the mechanism by which MS-444 arrests embryonic development, we conducted western blotting analysis to reveal bands of both monomeric and dimeric HuR. Compared with the levels in the control group, the level of HuR monomer increased significantly in MS-444-treated embryos ($p<0.0001$), while HuR dimer decreased significantly ($p<0.0001$, Figure 2A).

Since MS-444 blocks embryonic development by inhibiting the function of HuR, the expression and localization of HuR in oocytes and embryos were determined. The mRNA of mouse HuR in oocytes and early embryos was determined by reverse-transcription polymerase chain reaction (RT-PCR). The results showed that HuR transcripts were ubiquitously expressed in mouse oocytes and early embryos (Figure 2B). Immunofluorescence staining showed that HuR was evenly distributed throughout the MII oocytes, but it was mainly distributed in the nucleus from the 2-cell stage to blastocyst (Figure 2C).

To confirm that MS-444 blocks the development of 2-cell stage embryos *via* HuR dimerization, HuR mRNA was injected into zygotes (1-cell stage embryos) treated with MS-444. After the overexpression of HuR, the proportion of embryos that developed to the 4-cell stage increased from 0–49%, indicating that some embryos arrested at the 2-cell stage were rescued (Figure 2D and E). Taken together, these findings showed that MS-444 blocks 2-cell stage embryonic development by inhibiting HuR dimerization.

MS-444 regulated the expression of a large number of genes including Agbl2 in 2-cell stage embryos

To explore the potential genes involved in MS-444 arresting the development of 2-cell stage embryos, we performed low-input RNA-seq analysis of late 2-cell stage embryos cultured from the late 1-cell stage with DMSO or MS-444. A total of 1640 differentially expressed genes were yielded by principal component analysis (PCA) and differential gene analysis, of which 915 were upregulated and 725 were downregulated (Figure 3A, B and Supplementary Figure S3A). GO terms for the MS-444-treated 2-cell stage embryos and their controls showed particular associations with RNA binding, poly(A) RNA binding, and protein binding activity (Figure 3C). Combined with the result that MS-444 blocked HuR dimerization in 2-cell stage embryos, we speculated that MS-444 regulated gene expression by inhibiting the ability of HuR to bind RNA. Collectively, these results suggest that the HuR inhibitor MS-444 causes extensive changes of gene transcription in 2-cell stage embryos.

To identify genes that may be regulated by MS-444, we compared the genes downregulated in the RNA-seq with the genes of the control [21]. From this comparison, 20 overlapping genes were identified (Figure 3D). Using the mapping binding sites of RNA binding protein (RBPmap) online tool [42] to predict the binding site of these overlapping genes, such as Agbl2 (Supplementary Figure S4A), it was found that 90% of these genes have a large number of HuR binding sites in the 3'UTR. This result further confirmed that the downregulation of transcription during 2-cell embryonic development was due to the inhibition of HuR dimerization. The mRNA levels of these overlapping genes in the 2-cell stage embryos were evaluated using qRT-PCR, and the 15 genes with the highest fold changes were largely consistent with the RNA-seq data (Figure 3E). Meanwhile, qRT-PCR was used to determine the expression levels at the 2-cell stage relative to the 1-cell stage for the 10 genes with the highest fold changes (Figure 3F). Among these overlapping genes, the difference between Agbl2 and Bcat2 was the most significant ($p < 0.001$). We then focused on Agbl2 because Bcat2 has no HuR binding site. In addition, the expression of Agbl2 at the 2-cell stage was 4 folds higher than that in 1-cell stage embryos, so Agbl2 may be a target gene by which MS-444 blocks embryonic development.

MS-444 blocked embryonic development by disrupting the interaction between HuR dimerization and Agbl2 mRNA

To investigate whether HuR can interact with Agbl2 mRNA, the 3'UTR of Agbl2 was cloned downstream of hRluc in the psiCHECK2 plasmid. The activity of the contained luciferase reporter was monitored (Figure 4A). Compared with the control, the 3'UTR of Agbl2 significantly increased the expression of luciferase ($p < 0.0001$). The luciferase activity in the group co-transfected with HuR overexpression plasmid was significantly ($p < 0.01$) higher than that in the group with single transfection of Agbl2-3'UTR reporter

plasmid. Moreover, in luciferase reporter assay, MS-444 induced a strong response to Agbl2-3'UTR in a dose-dependent manner (Figure 4B). These findings demonstrated a molecular interaction between HuR and Agbl2 mRNA. Since HuR can regulate the post-transcriptional modification of genes, we next examined the effect of HuR on the stability of Agbl2 mRNA. The psicheck2-Agbl2-3'UTR plasmid and HuR overexpression plasmid were co-transfected into 3T3 cells. Twenty-four hours later, MS-444 was added to inhibit the HuR dimer, and DMSO was used as a control. After 24 h of MS-444 or DMSO treatment, 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB) was added to stop the transcription and harvested a time course. Then, the RNA decay rate of hRluc was detected by qRT-PCR. The half-life of hRluc mRNA was decreased in the group with MS-444 treatment (HuR $T_{1/2}$ = 4.4 h) compared with that in the control (Control $T_{1/2}$ = 7 h) (Figure 4C), while no difference was observed for Luc that was independently transcribed in the plasmid (Figure 4D). Therefore, MS-444 reduces the stability of Agbl2 3'UTR by inhibiting HuR dimerization.

To verify the role of Agbl2 in embryonic development, an Agbl2 knockdown experiment was performed in wild-type zygotes (Figure 4E). As shown by qRT-PCR analysis, a significant decrease ($p < 0.0001$) in the mRNA level of Agbl2 occurred in Agbl2 knockdown embryos (Figure 4F). The embryos in which Agbl2 was knocked down had a lower embryonic development rate than the control group (Figure 4G, H). Following Agbl2 knockdown at the zygote stage, approximately 52% of the embryos with Agbl2 knockdown cleaved to the 4C stage and approximately 23% developed into blastocysts (Figure 4H). Meanwhile, upon the injection of Agbl2 mRNA into zygotes cultured with MS-444, this Agbl2 overexpression saved some 2-cell stage embryos from the developmental arrest caused by MS-444, and 17% of 2-cell embryos entered the blastocyst stage (Supplementary Figure S5A). These results indicated that Agbl2 is a target gene for HuR and Agbl2 is essential for regulating embryonic development. Together, these findings suggest that the interactions between HuR dimerization and Agbl2 is necessary for early embryonic development, and that MS-444 destroys this process.

MS-444 blocked the subcellular transport of Agbl2 mRNA by inhibiting HuR dimerization

To explore the effect of MS-444 on the subcellular localization of Agbl2 mRNA, RNA fluorescence *in situ* hybridization (FISH) analysis of Agbl2 in 2-cell stage embryos treated with MS-444 or DMSO was carried out. RNA-FISH showed that Agbl2 mRNA was mainly distributed around the nuclear membrane in the early 2-cell stage (Figure 5A). The results suggested that the transcripts of Agbl2 accumulated at the nuclear membrane of early 2-cell embryos. With the development of 2-cell stage embryos, Agbl2 mRNA was transported to and randomly distributed in the cytoplasm (Figure 5B). The results indicated that Agbl2 mRNA was no longer retained in the nucleus and was transported to the cytoplasm when needed for embryonic development. Compared with the control group (Figure 5B), a large amount of mRNA was retained around the nuclear membrane in the MS-444 group (Figure 5C). The results demonstrated that MS-444 treatment disrupted the nucleocytoplasmic transport of Agbl2 mRNA. The statistical results showed that the nuclear/cytoplasmic ratio of the Agbl2 mRNA fluorescence signal decreased significantly ($p < 0.05$) with the development of 2-cell stage embryos (Figure 5D). Compared with that in the control group (DMSO-20h), MS-444 treatment significantly increased ($p < 0.05$) the

nuclear/cytoplasmic ratio of Agbl2 mRNA fluorescence signal (Figure 5D). These results indicated that MS-444 blocked the nucleocytoplasmic transport of Agbl2 mRNA by inhibiting HuR dimerization in 2-cell stage embryos.

MS-444 inhibited HuR dimerization and destroyed its mRNA transport function visualized by STORM imaging

To visualize the effect of HuR dimerization on the nucleocytoplasmic translocation of targeted mRNA, the super-resolution optical microscopy, i.e., STORM, was employed to perform imaging of the embryo at nanometer resolution with our optimized sample preparation protocols. We first developed a method to retain the embryonic cells on a petri dish with a coverslip at the bottom for STORM imaging. By exposure to the external flame of an alcohol lamp for about 5 seconds, the cells can strongly adhesive on the petri dish. In order to reduce the background signal caused by excitation light scattering inside embryonic cell which contains high amount of lipid droplets. Inspired by previous reports on reducing light scattering and increase tissue transparency using lipase-based digestion of lipid droplets, we also treated our embryonic cells before STORM imaging with an optimized lipase concentration and time [43, 44]. The wide-field images showed that HuR was dispersed in the cells and part of them formed fluorescent spots, but they showed little difference between the MS-444-treated group and the control group (Figure 6A-i and B-i). However, the resolution of reconstructed STORM images was significantly improved (Figure 6A-ii and B-ii). From these images, we can see that the HuR fluorescence signal in the cytoplasm decreased significantly in the MS-444-treated group (Figure 6A-ii and B-ii). Moreover, the magnified super-resolution images reveal distinct structural features: some HuR proteins are clustered, while others are scattered. Interestingly, there seemed to be smaller and fewer clusters in the MS-444-treated group than in the control (Figure 6A-iii and B-iii).

In addition to obtaining high-resolution HuR images, it is also important to use appropriate methods to quantitatively analyze the intracellular distribution of proteins. Here, the SR-Tesseler method, which can accurately segment the localizations and extract cluster information, was used to analyze the STORM data [45]. The nucleus was depicted as a region of interest (ROI) and polygons were created (Figure 6C-i). When a polygon met the condition that its localization density δ_i was higher than twice the average localization density of the cell δ , it was considered as an object (Figure 6C-ii). Those objects whose localization density was higher than the average localization density of all objects was extracted as clusters (Figure 6C-iii).

The data obtained by Image J were analyzed by the SR-Tesseler method as mentioned above. For the cluster properties, although HuR formed clusters in both groups of embryos, there were also significant differences in this regard. Data indicated that the localization density of HuR in the cytoplasm of MS-444-treated embryos was much lower than that of control embryos (Figure 6D). The results showed that MS-444 blocked the cytoplasmic translocation of HuR and destroyed its mRNA transport function. To distinguish these small clusters (one protein) from large clusters composed of several proteins or more, we first analyzed the “small clusters” formed by a single Alexa 647 binding protein by Image J

(Supplementary Figure S6A). The average diameter of these small clusters was 41.11 ± 4.25 nm (Supplementary Figure S6B), which was identified as the diameter of a single Alexa 647-conjugated HuR protein. The number of molecules in each cluster in the nucleus showed that the percentage of HuR single-molecule clusters in the MS-444-treated group (55.93%) increased significantly ($p < 0.0001$) compared with that in the control group (29.19%) (Figure 6E). In contrast, the percentage of HuR bimolecules in the MS-444-treated group (5.57%) was significantly ($p < 0.0001$) lower than that in the control group (11.68%). The results showed that MS-444 reduced the level of dimers of HuR, increased the monomers of HuR, and made the distribution of HuR more disperse in the nucleus.

Moreover, compared with the control 2-cell stage embryos, MS-444-treated embryos showed a significant decrease ($p < 0.05$) in the relative cluster area (Figure 6F). The results indicated that HuR tended to form smaller clusters in MS-444-treated embryos compared with the findings in the control. Then, the number of clusters per area of the nucleus under different treatment conditions was analyzed. We found that the number of clusters per μm^2 of MS-444-treated embryos was 1.6 times that of embryos in the control group (Figure 6G). The results indicated that MS-444 treatment reduced the number of HuR clusters in 2-cell stage embryos. These results showed that HuR mostly existed in the form of clusters in normal 2-cell stage embryos, while MS-444 treatment resulted in fewer and smaller clusters. In conclusion, MS-444 disrupted the normal distribution of HuR in 2-cell stage embryos and destroyed the mRNA transport function of HuR by inhibiting HuR dimerization.

Discussion

The small molecule inhibitor MS-444, as a blocker of HuR dimerization and nuclear/cytoplasmic shuttle, is increasingly used in research on tumor therapy[27, 46–48]. HuR is among the most well-known translation and turnover regulating RBPs, which is involved in many post-transcriptional gene regulation events, including mRNA stabilization, and translation[49–52]. HuR is highly expressed in the cytoplasm of many cancer cells and plays a regulatory and promoting role in almost every feature of cancer[26, 32]. Will MS-444 damage women's fertility when used in cancer treatment?

Our study found that MS-444 did not damage oocyte maturation and secretion of hormones (E2 and Prog), but caused 2-cell stage embryo arrest in a dose-dependent manner. Further experiments confirmed that MS-444 inhibited the formation of HuR dimer in 2-cell stage embryos. Using low input RNA-seq, we found that MS-444 treatment caused extensive downregulation of the transcription of 2-cell stage embryos. We speculate that HuR dimerization is important for ZGA, and MS-444 can inhibit this process. Previous studies of mouse brain showed that normal brain development and function required an appropriate subcellular localization of HuR, and HuR localization gradually shifted from the cytoplasm to the nucleus from postnatal day 7 to postnatal day 21 [53]. This study found that the location of HuR after fertilization was dynamic, and gradually shifted from the cytoplasm to the nucleus. This indicated that the cytoplasmic to nuclear translocation of HuR is developmentally regulated during embryonic development. Furthermore, Agbl2 was found to be a direct target gene of HuR required for early embryonic development, and its expression increased in 2-cell stage embryos. Previous studies showed

that, in the case of mRNA nuclear retention, mRNA had higher levels in the nucleus than in the cytoplasm, and it was randomly distributed in the nucleus [54]. Nuclear retention of mRNA is beneficial to reduce gene expression noise caused by burst transcription [54]. In this study, we found that Agbl2 mRNA mostly remained in the nucleus and gathered around the nuclear membrane at early 2-cell stage embryos. MS-444 blocked the subsequent nucleocytoplasmic transport of Agbl2 mRNA, which may disrupt the progress of ZGA. At the same time, this study found that MS-444 could reduce the stability of Agbl2 mRNA, which may be caused by the blocked transport of Agbl2 mRNA, and finally led to it being degraded by RNA exosomes. These results suggest that MS-444 should be avoided in preconception and early pregnant women when it is used for cancer treatment.

We demonstrated the potential of STORM-based location super-resolution microscopy, which can directly visualize the nanometer scale distribution of HuR in single embryo cells under different conditions. For large-diameter cells ($>40\text{ }\mu\text{m}$), no reports have been published of single-molecule imaging at the nano level using STORM microscopy. In this study, STORM was applied to the study of mammalian embryonic development for the first time. The super-resolution images of HuR marks revealed that HuR mostly existed in the form of clusters in the nucleus of normal 2-cell stage embryos. An increasing number of studies have shown that some proteins exist in cells in the form of clusters [55, 56]. The clustered distribution can shorten the distance between molecules and enable more rapid response to external signals[39]. Therefore, HuR clusters might be used as a functional platform to enhance their own interaction. The area and density of HuR clusters were smaller and lower in 2-cell stage embryos in which MS-444 caused embryonic development arrest. We speculate that MS-444 destroys the functional platform of HuR by inhibiting HuR dimerization, and then causes embryonic development arrest. As a consequence, a distinct protein organization that is either less prevalent or absent in normal cells probably causes abnormal embryonic development. Super-resolution optical microscopy has been applied to study the molecular mechanism of disease, such as using STORM to study the distribution of carbohydrate clusters in cancer cells [57–59]. The optimized STORM imaging enables us to observe the distribution of proteins that are key for embryonic development in embryonic cells at the single-molecule level. Therefore, we can use optimized STORM imaging with excellent resolution to examine the alterations of various proteins in abnormal embryonic development cells, so as to better confirm the cause of abnormal embryonic development according to their different distribution patterns (especially cluster size and cluster coverage). This may provide a new diagnostic strategy for the research and clinical diagnosis of embryonic development.

Conclusion

In conclusion, we found MS-444 blocked the nucleocytoplasmic transport of Agbl2 mRNA by inhibiting HuR dimerization, resulting in the development arrest of 2-cell embryos. Moreover, STORM was applied to the nanoscale study of embryonic cells for the first time. Single-molecule imaging showed that MS-444 significantly reduced the level of HuR dimers, and HuR mainly existed in 2-cell embryos in the form of clusters. This study provides a theoretical basis and clinical guidance for maintaining fertility in the clinical treatment of cancer patients with MS-444.

Materials And Methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai, and were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals. Six- to eight-week-old female ICR mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd.

Hormone assay

Six-week-old female ICR mice were randomly assigned to two groups with five mice in each group as follows: (1) control group mice, which were injected with the diluent DMSO; and (2) treatment group mice, which were injected with 25 mg/kg MS-444. Mice were consecutively intraperitoneally injected with MS-444 or the same dose of DMSO for 10 days. Blood was collected from the tail vein of the mice to detect progesterone and estrogen. Serum progesterone and estrogen levels were measured by the Access immunoassay system (Beckman Coulter, Brea, CA), an automated random access analysis method based on chemiluminescence, in accordance with the manufacturer's specifications.

Histology

Mouse ovaries were fixed in PBS containing 4% paraformaldehyde (PFA) at 4°C overnight, embedded in paraffin wax, and sectioned at a thickness of 5 µm. Slides used for histological analysis were stained with hematoxylin. Images were obtained with a Leica DM2500 microscope and a Leica DFC 550 digital camera.

MS-444 treatment

GV stage oocytes, early 1-cell stage embryos, or late 1-cell stage embryos were cultured in culture medium with 0, 25, 50, or 75 µM MS-444 (MCE) dissolved in 0.1% DMSO. To block zygotic transcription in 2-cell stage embryos, late 1-cell stage embryos were cultured in KSOM containing 80 µM 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB; Sigma-Aldrich). Oocytes/embryos were covered with mineral oil (Sigma-Aldrich) and maintained in 5% CO₂ atmosphere at 37°C.

Western blot analysis

Proteins collected from 60–80 oocytes or embryos were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was incubated with HuR (diluted 1:1000) primary antibody, followed by horseradish peroxidase-conjugated secondary antibody. Protein bands were detected using the SuperSignal West Femto agent (Thermo Scientific).

EdU and EU incorporation assays

EdU and EU incorporation assays were performed with Cell-Light EdU Apollo567 *In Vitro* Kit and Cell-Light EU Apollo567 RNA Imaging Kit (RiboBio, Shanghai, China). Briefly, 2-cell stage embryos from the control

group and the group with inhibition of HuR dimerization were collected. More than 12 embryos were used for each group per replicate and incubated in KSOM supplemented with EdU or EU for 8 h. Fixation, penetration, and staining were performed in accordance with the kit instructions. DAPI (10 µg/ml) was used to stain the nucleic acid within the cells. Images were obtained with a Leica fluorescence microscope. Image J software was used to quantify the intensity of EdU and EU signals. The fluorescence signal was calculated as the average intensity after background subtraction.

Oocyte/Embryocollection and microinjection

Six- to eight-week-old ICR female mice were superstimulated with 10 IU pregnant mare serum gonadotropin (PMSG). Forty-eight hours later, oocytes at the GV stage were collected in M2 medium (Sigma-Aldrich) and cultured in small drops of M16 medium (Sigma-Aldrich). After 48 h of PMSG injection, females were injected with 10 IU human chorionic gonadotropin (HCG). Females were mated with adult ICR males post-HCG injection, and 1-cell stage embryos were collected from the oviducts at 18 h after HCG injection. One-cell stage embryos were cultured in small drops of KSOM medium (Sigma-Aldrich). Oocytes/embryos were covered with mineral oil (Sigma-Aldrich) and maintained in a 5% CO₂ atmosphere at 37°C.

For microinjection, 1-cell stage embryos were microinjected using an Olympus IX51 inverted microscope equipped with an Eppendorf micromanipulator system (InjectMan 4). To overexpress genes in embryos, embryos were microinjected with a synthetic mRNA (500 ng/ml) into the cytoplasm between 25 and 28 h after HCG injection. mMACHINE™ T7 transcription kit (Invitrogen) was used to transcribe mRNA *in vitro*. To deplete Agbl2, embryos at the 1-cell stage were injected with siRNAs at a concentration of 50 µM. siRNA (5'-CAAUAGAUGUUCUCUUUAAAG-3') was obtained from RiboBio.

Immunofluorescence

Oocytes and embryos were fixed in methanol for 30 min and permeabilized in PBS containing 0.4% Triton X-100 for 6 h. After being blocked with 1% bovine serum albumin (BSA) in PBS for 6 h, the oocytes and embryos were incubated with HuR antibodies (diluted 1:100) at 4°C overnight, washed three times, and incubated with Alexa Fluor 594 anti-rabbit IgG (diluted 1:200) at 37°C for 0.5 h and 10 µg/ml DAPI for 3 min.

RNA-seq and analysis

For RNA-sequencing, late 2-cell stage embryos were collected from MS-444-treated and control groups (16 embryos per group, 3 replicates). Each sample was collected in 7 µl of buffer (1000 µl of buffer composed of 1/500 dilution of Phusion HF buffer and RNase inhibitor, 400 µl of 2 µM unique barcode primer), frozen with dry ice, and stored at -80°C for cDNA synthesis using the simplified SCRB-seq method. The RNA sequencing protocol was performed as described previously [60]. Briefly, cells were lysed by freeze-thaw and reverse-transcription reactions were performed using Minus Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). All samples were pooled together and the cDNA was purified using a purification kit (Zymo Research, Irvine, CA, USA). Full-length cDNA was amplified using

the Terra PCR Direct Polymerase Mix buffer (Clontech, Mountain View, CA, USA). Sequencing libraries were constructed using TruePrep DNA Library Prep Kit (Vazyme, Nanjing, China), in accordance with the manufacturer's instructions. Finally, the libraries were sequenced on an Illumina HiSeq system (Illumina, San Diego, CA, USA).

All sequence reads of the libraries were mapped to the mouse genome (mm10; reference set: <http://genome.ucsc.edu/>). Read pairs with correct cell barcodes were aligned with mouse Ensembl genes for further analysis. The gene expression matrix was generated by calculating the number of unique UMIS associated with the gene in each sample and the Ensembl gene. In each case, genes with an adjusted p-value (p.adjust) below 0.05 and fold change over 1.5 were considered significant. GO analysis of gene enrichment was performed using the Database for Annotation, Visualization, and Integrated Discovery.

Quantitative reverse transcription PCR

Embryos were collected and frozen at -80°C . Cells were lysed by freeze-thaw and reverse-transcribed with a reverse transcription kit (Takara), in accordance with the manufacturer's recommendations. The cDNA from cells was subjected to targeted pre-amplification for 15 cycles. The pre-amplified template was then 10 $\times$ diluted and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) analysis with the Applied Biosystems® 7500 Real-Time PCR System. Each experiment was performed in triplicate using gene-specific primers. Primer sequences are shown in Supplementary Table S1 in the Supporting Information.

Luciferase assay

DMEM/high glucose (Gibco) supplemented with 10% fetal bovine serum was used for 3T3 cell growth, and cells were incubated with 5% CO $_2$ at 37°C . The 3'-UTR sequence of Agbl2 was amplified by PCR and cloned into the psiCHECK™-2 vector (Promega) between synthetic *Renilla* luciferase gene and synthetic poly(A). Reporter plasmid was co-transfected into cells with the HuR overexpression vector (HuR-pcDNA3.1) or control vector (pcDNA3.1) using Lipofectamine 3000 transfection reagent (Invitrogen). 3T3 cells were collected 48 h after transfection, and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega), in accordance with the manufacturer's instructions.

RNA decay analysis

The Agbl2-3'UTR luciferase reporter plasmid and HuR overexpression vector (HuR-pcDNA3.1) or control vector (pcDNA3.1) were co-transfected into 3T3 cells using the Lipofectamine 3000 transfection reagent (Invitrogen). After 24 h, 3T3 cells were treated with 80 μM DRB (Sigma-Aldrich) and RNA was harvested at different times as indicated in the figures. The same proportion of RNA was obtained at different time points for real-time PCR. Primer sequences are shown in Supplementary Table S1 in the Supporting Information.

The CT of each sample was used to calculate the remaining mRNA percentage of each point. These data were fit into a non-linear regression curve (one-phase decay) using GraphPad Prism or similar software to

derive the half-life of the mRNAs.

RNA-FISH

Cy3-labeled FISH probes were synthesized by GenePharma. Probe sequences (probe1+2+3) are provided in Supplementary Table S1. We performed RNA-FISH in accordance with the instructions of the Fluorescent *In Situ* Hybridization Kit (GenePharma). In short, 2-cell embryos were fixed in 4% paraformaldehyde for 1 h and blocked with Buffer C for 30 min at 37°C. Two-cell embryos were incubated with 2 µM Agbl2 FISH probe in hybridization buffer in the dark at 37°C overnight, washed with Buffer C, and incubated with 10 µg/ml DAPI for 3 min. Then, a confocal fluorescence microscope (iXplorespinsr; Olympus, Tokyo, Japan) was used for imaging analysis.

Preparation of samples for STORM

For super-resolution imaging of HuR in embryos, 2-cell stage embryos after removal of the zona pellucida with pronase (Sigma) were transferred to a 29 mm glass-bottomed Petri dish (*In Vitro* Science) and dried as soon as possible using an alcohol lamp. We optimized the method for the adhesion of embryonic cells and tested the adhesion effects under different treatment conditions: 80°C for 2 min, 80°C for 5 min, 80°C for 10 min, the external flame of an alcohol lamp for 2 s, the external flame of an alcohol lamp for 5 s, and the external flame of an alcohol lamp for 10 s. Embryos were fixed, permeabilized, and blocked as described above. Prior to incubation with primary antibodies, lipid droplets in embryos were digested with lipases from *Candida rugosa* (Sigma-Aldrich) in 50 mM Tris (pH 7.2), 400 mM NaCl, 5 mM CaCl₂, and EDTA-free Protease Inhibitor. Referring to the concentration of lipase of 4000 U/ml [43, 44], the cell processing time of lipase was optimized: room temperature for 20 min, room temperature for 40 min, and room temperature 60 for min. Incubation with primary antibody (HuR 1:100) was performed in 0.4% Triton X-100+BSA at 4°C overnight, followed by washing three times. Embryos were incubated with secondary antibody labeled with Alexa Fluor 647 (diluted 1:200) at 37°C for 0.5 h and counterstained with 10 µg/ml DAPI for 3 min.

STORM imaging

To ensure accurate quantification of the density of HuR, all samples underwent exactly the same imaging conditions. The freshly prepared imaging buffer was dropped in a glass-bottomed Petri dish containing 10% w/v glucose (Sigma-Aldrich), 0.5 mg/ml glucose oxidase (Sigma-Aldrich), 53 µg/ml catalase (Sigma-Aldrich), and 10 mM MEA (Sigma-Aldrich) in TN buffer (50 mM Tris and 10 mM NaCl, pH 8.0). The N-STORM (Nikon, Japan) system equipped with a 100× objective lens (PlanApoTIRF, NA 1.49; Nikon) were used for acquiring the super-resolution images. A 640 nm semiconductor laser was used to excite Alexa-647 fluorophore (50 mW power), while a laser with a wavelength of 405 nm (0.5 mW power) was used to activate the fluorophores in dark state. All images were recorded onto a 256 × 256 pixel region of an electron-multiplying CCD camera (iXon 3; Andor). The exposure time was set to 16 ms, and the conversion gain and EM gain were set at 1× and 200×, respectively. During the image acquisition, the perfect focusing system (PFS) was used to keep the specimen in focus in real time.

STORM data analysis

We reconstructed the STORM raw image data using the Image J plugin ThunderSTORM [61]. Quantitative information for each localization, including x and y positions, background noise, standard deviation (σ), and localization precision, was automatically determined by this program. Then, the total localization number was divided by the cell area to calculate the localization density.

To determine the cluster from the STORM images, we used SR-Tesseler [45]. The STORM data processed by Image J were first imported into SR-Tesseler software in “csv” format and a reconstructed image was obtained. The nucleus was then circled as a region of interest (ROI) and polygons were created inside the ROI. The polygons whose density δ_i satisfied the condition of localization density $\delta_i > 2\delta$ were defined as objects. Here, δ is the average localization density of the nucleus. Next, the objects whose localization density in the nucleus was higher than the average density of all objects were extracted as clusters. Finally, the outlines of the clusters were shown in the image, and cluster information including the number, area, circularity, and the localization number in clusters was obtained directly.

Statistical analysis

Two-tailed t-tests or Mann–Whitney tests were used for comparisons between two groups. The results from multiple groups were analyzed by one-way ANOVA. The statistical software GraphPad Prism was used for all analyses.

Declarations

Acknowledgements

We appreciate the support from National Key Research and Development Program of China, National Nature Science Foundation of China and National Major Scientific Instruments and Equipment Development Project, National Nature Science Foundation of China.

Contributions

Yongqiang Nie designed and performed the experiments, analyzed the data, and wrote the draft of the manuscript. Wei Xu performed the experiments of RNA-seq. Geng G. Tian helped to analyze the data. Xiaowei Li, Yan Guo, Xuefeng Liu, Lin He, and Zhifeng Shao gave guidance to the experiment. Xiaoyong Li gave guidance to the experiment, analyzed the data, and revised the manuscript. Ji Wu conceptualized and supervised the entire project, analyzed the data, revised the manuscript, and funded the acquisition.

Funding

We are grateful for the financial support from the National Key Research and Development Program of China (2017YFA0504201), National Nature Science Foundation of China (81720108017, 32000806),

National Major Scientific Instruments and Equipment Development Project, National Nature Science Foundation of China (61827814).

Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Data Availability

Low-input RNA sequencing and processed files have been deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE192494.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai and performed in accordance with the National Research Council Guide for Care and Use of Laboratory Animals. The ethical approval number for our research is A2016084.

Consent for publication

All authors in the paper agree to be published.

Competing interests

The authors declare no competing financial interest.

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Figures

Figure 1

Effects of MS-444 on oogenesis and early embryonic development

A) Schematic diagram showing the analysis of the effects of MS-444 on follicular development, oocyte maturation, and early embryonic development. B) Ovarian histology of DMSO- or MS-444-treated females with hematoxylin staining. CL, corpus luteum. Scale bar, 100 μ m. C) E2 hormone levels in control or MS-

444-treated females. E2, estrogen. D) Progesterone hormone levels in control or MS-444-treated females. Prog, progesterone. E) Percentage of MII oocytes following culture with different concentrations of MS-444. The experiment was repeated three times. Scale bar, 50 μ m. F) Percentage of early zygotes that reached the 2-cell stage following culture with different concentrations of MS-444. The experiment was repeated three times. Scale bar, 50 μ m. G) Percentage of late zygotes that reached specified stages following culture with different concentrations of MS-444. 2C, 2-cell stage embryo; 4C, 4-cell stage embryo; Mor, morula; Blast, blastocyst. The experiment was repeated three times. * $p < 0.05$. n.s., no significance. H) Morphology of embryos at 20, 28, 64, and 78 h following culture from late zygotes treated with DMSO or 75 mM MS-444. Scale bar, 50 μ m.

Figure 2

MS-444 blocks embryonic development by inhibiting HuR dimerization

A) Western blot analysis of HuR monomer and dimer in 2-cell embryos treated with DMSO or MS-444. The experiment was repeated three times. **** $p < 0.0001$. B) RT-PCR showing HuR expression in oocytes and early embryos. MII, meiosis II oocytes; 1C, zygote; 2C, 2-cell stage embryo; 4C, 4-cell stage embryo;

Mor, morula; Blast, blastocyst. C) Immunofluorescence staining of HuR in mouse oocytes and preimplantation embryos. Scale bar, 10 μ m. MII, meiosis II oocytes; 1-cell, zygote; Mor, morula; Blast, blastocyst; (+), 3T3 cell. D) Percentage of zygotes that reached the specified stages following microinjection of HuR and EGFP mRNA in the late zygote with inhibited HuR dimerization. The experiment was repeated four times. * $p < 0.05$. n.s., no significance. E) Morphology of embryos at 2 and 4 days following HuR overexpression in late zygotes treated with MS-444. Scale bar, 50 μ m.

Figure 3

MS-444 regulates the expression of a large number of genes in 2-cell stage embryos

A) PCA of 2-cell stage embryonic transcriptome in control (DMSO) and MS-444-treated groups. B) Volcano plot showing the upregulated and downregulated genes in 2-cell stage embryos treated with MS-444. Different dots indicate the transcription change. The red dots indicate upregulated genes, the green dots indicate downregulated genes, and the black dots indicate no change in transcription. C) Gene Ontology analysis of the genes downregulated in MS-444-treated embryos compared with the levels in control embryos at the 2-cell stage. D) Comparison of gene expression differences of overlapping genes between MS-444-treated and control embryos. E) qRT-PCR analysis showing the expression of transcripts in control and MS-444-treated embryos at the 2-cell stage. The experiment was repeated three times. F) qRT-PCR analysis showing the expression of transcripts in 1-cell and 2-cell stage embryos. 1C, zygote. 2C, 2-cell stage embryo. The experiment was repeated three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4

MS-444 blocks embryonic development through Agbl2

A) Luciferase reporter activity in control, Agbl2-3'UTR, and Agbl2-3'UTR and HuR overexpression groups. The experiment was repeated three times. ** $p < 0.01$, **** $p < 0.0001$. B) The luciferase activity of Agbl2 in response to MS-444 with different concentrations. The experiment was repeated three times.

**** $p < 0.0001$. C, D) Agbl2 RNA half-life analysis in MS-444 and control groups over the time course of DRB treatment. The experiment was repeated three times. E) Schematic diagram showing the microinjection of mouse zygotes and subsequent embryo analysis at the molecular and developmental levels. F) qRT-PCR analysis showing the efficiency of Agbl2 knockdown. The experiment was repeated three times. **** $p < 0.0001$. G) Morphology of embryos at 2 and 4 days after injecting siRNAs targeting the Agbl2 gene at the late zygote stage. Scale bar, 50 μm . H) Percentage of zygotes that reached the specified stages following microinjection of control siRNA or siRNAs targeting Agbl2. The experiment was repeated three times. * $p < 0.05$, **** $p < 0.0001$.

Figure 5

HuR dimerization is essential for the nucleocytoplasmic transport of Agbl2 mRNA

A–C) RNA fluorescence *in situ* hybridization for Agbl2 mRNA. A) Localization of Agbl2 mRNA in 2-cell stage embryo after DMSO treatment for 12 h. DMSO-12h, DMSO treatment for 12 h. B) Localization of Agbl2 mRNA in 2-cell stage embryo after DMSO treatment for 20 h. DMSO-20h, DMSO treatment for 20 h.

C) Localization of Agbl2 mRNA in 2-cell stage embryo after MS-444 treatment for 20 h. MS-444-20h, MS-444 treatment for 20 h. Scale bar, 10 μm . D) Ratio of fluorescence intensity of Agbl2 mRNA in the nucleus and cytoplasm of embryos in each group. D-12h, DMSO-12h. D-20h, DMSO-20h. M-20h, MS-444-20h. * $p < 0.05$.

Figure 6

HuR distribution in MS-444-treated and normal 2-cell stage embryos revealed by STORM imaging

A-i) Wide-field images of HuR in normal 2-cell stage embryo. A-ii) The corresponding STORM images of the same cell as in (A-i). A-iii) Magnified view of STORM images boxed in green. B-i) Wide-field images of HuR in MS-444-treated 2-cell stage embryo. B-ii) The corresponding STORM images of the same cell as in (B-i). B-iii) Magnified view of STORM images boxed in green. C) Schematic diagram of SR-Tesseler analysis. White dotted line, edge of the nucleus in A-ii and B-ii. Green dotted line, cytoplasmic edge with the same circular area in A-ii and B-ii. Scale bars, 3.5 μm in (A-i and B-i), 4 μm in (A-ii and B-ii) and 400 nm in (A-iii and B-iii) C-i) Vorono diagram composed of many polygons. The edges of polygons are perpendicular bisectors from the two nearest localizations. C-ii) The objects (blue) extracted by thresholding the localization density of every polygon $\delta_i > 2\delta$. C-iii) The extracted clusters (green) have a higher density than the average density of objects. Scale bars, 300 nm in (C). D) The localization density of HuR in the cytoplasm of 2-cell stage embryos in the MS-444-treated and control groups. $**p < 0.01$. MS-444 vs. controls. E) Percentage of single HuR and bimolecular HuR in the nucleus of 2-cell embryos of the DMSO- and MS-444-treated groups. $****p < 0.0001$. MS-444 vs. corresponding controls. (F) The relative cluster area of HuR in the nucleus of the MS-444-treated and control groups. $*p < 0.05$. MS-444 vs. controls (G) The number of clusters per μm^2 in the nucleus of the MS-444-treated and control groups. All results were obtained from six embryo samples in three independent experiments. $****p < 0.0001$. MS-444 vs. controls.

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