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Mycotoxin ochratoxin A disrupts renal development via miR-731/prolactin receptor axis in zebrafish

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Abstract

Mycotoxin ochratoxin A (OTA) frequently contaminates various food and feed products, including cereals, coffee and wine. While the nephrotoxicity and teratogenicity of OTA have been extensively documented, the molecular mechanisms associated with OTA toxicity remained poorly understood in a developing organism. We showed that zebrafish embryos exposed to OTA demonstrated incorrect heart looping and small heart chambers. OTA also impaired the renal morphology and reduced the glomerular filtration rate of embryonic zebrafish. The treatment of embryos with OTA attenuated the expression of the prolactin receptor a gene (PRLRa) that has a key role in organogenesis and osmoregulation in vertebrates. OTA not only inhibited the phosphorylation of STAT5 and AKT, but also down-regulated the level of serpina1 mRNA in a dose-dependent manner. On the other hand, the microRNA profiling based on RNA sequencing revealed the up-regulation of microRNA-731 (miR-731) in OTA-treated embryos. Further *in silico* analysis predicted that PRLRa was a target gene of miR-731. AntagomiR-731 restored PRLRa levels that had been reduced by OTA and also recovered the pronephros morphology that was damaged by OTA. These observations suggest that exposure to OTA adversely affected the organogenesis of zebrafish, and the modulation of miR-731 and PRLR signaling cascade contributed to the abnormal renal development mediated by OTA.

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Introduction

Ochratoxin A (OTA, Fig. S1) is a secondary metabolite that is produced by certain *Aspergillus* and *Penicillium* species. It is frequently found in various foodstuffs of plant and animal origin, such as cereal products, coffee, grapes, wine, and pork/poultry.[1] Epidemiological studies have revealed that OTA is a possible cause of Balkan endemic nephropathy and interstitial nephritis in humans.[2] The derivatives of OTA have also been detected in human blood and urine samples, and even in the breast milk of nursing women in some European countries.[3]

OTA is a nephrotoxin and renal carcinogen to various mammalian species.[4] It has a major role in the etiology of porcine nephropathy in Europe[5]; symptoms such as interstitial fibrosis and the atrophy of the proximal/distal tubules are observed in both pigs and chickens.[6, 7] Additionally, OTA is a teratogen to rodents, chick embryos, and rabbits.[8, 9] The treatment of maternal rabbits with OTA leads to histopathological changes in the kidneys and hearts of rabbit fetuses.[10] Administration of a single dose of OTA to pregnant rats causes enlarged renal pelvis and myocardial hemorrhages in developing rat fetuses.[11]

Several transcriptomic and microRNA profiling studies focused on OTA have been performed in cultured cell lines and adult rats.[12-16]. The gene expression profiles from the kidney and liver of male rats that were given dietary OTA for 7 days to 12 months indicate that the prolactin receptor (PRLR) is one of the genes down-regulated by OTA.[17] PRLR, with prolactin (PRL) and growth hormone as ligands, interacts strongly with the Janus kinases (JAKs)/signal transducers and activators of transcription (STAT) pathway to promote cell growth and differentiation during development.[18, 19] PRL/PRLR signaling plays an important role in the physiological

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control of water and electrolyte balance in rats, birds, and teleost fish.[20-22] Disruption of osmoregulation may interfere the development and functions of kidneys and skin in fish and humans.[23]

MicroRNAs are a class of small noncoding RNA molecules that consist of ~22 nucleotides and negatively regulate gene expression. Diverse miRNAs are known to participate in such processes as embryonic development, cell proliferation, and organogenesis in various models.[24, 25] MicroRNA-731 (miR-731), identified only in teleost fish, is found to be strongly up-regulated during the virus-fish interaction, and the induction is mediated by the type I interferon.[26] An ortholog of miR-731, hsa-miR-425, is recently identified in humans and the hsa-miR-425 cluster is functionally associated with cell cycle regulation.[27]

Although many studies have investigated the nephrotoxicity and teratogenicity associated with OTA, its mode of action in developing vertebrates is still poorly defined. Zebrafish embryos provide an ideal vertebrate model for studying developmental toxicology because of their external post-fertilization and quick maturation.[28] Furthermore, the patterning of pronephric nephrons in zebrafish is in a similar fashion as the metanephric nephrons of mammalian species.[29] Therefore, we applied embryonic zebrafish in the present study and found that OTA down-regulated the PRLR/STAT5 signaling in developing fish. The miR-731, induced by OTA, was involved in PRLR regulation and the development of renal morphology.

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Materials and Methods

Test species and husbandry

Wild-type (WT) AB strain zebrafish (Danio rerio) were provided by Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS). The pronephron specific transgenic line Tg(wt1b:GFP) was kindly provided by Dr. Christoph Englert at Leibniz-Institute for Age Research Fritz-Lipmann-Institute in Germany. All fishes were raised at 28^{o}C with a 14-h/10-h light/dark cycle. Anesthesia and euthanasia of zebrafish was conducted according to Matthews and Varga.[30] All procedures regarding zebrafish were performed in compliance with the relevant laws and institutional guidelines, and the Institutional Animal Care and Use Committee(s) have approved the experiments (IACUC Approval Number : 20140416).

OTA exposure

OTA purchased from Sigma-Aldrich Co (St. Louis, MO) was first dissolved in ethanol at a concentration of 10 mM, and then further diluted with 0.01M phosphate buffered saline (PBS) to 1 mM for storage at -20℃. To evaluate the survival rate and LD50 of embryos, healthy and normally developing WT embryos at 6 hour post-fertilization (hpf) were collected and kept in 96-well plates (one embryo/ well) in 200 μl egg water (60 mg/l ocean salts in distilled water) for further toxin treatment. For other experiments, normal WT or Tg (wt1b:GFP) embryos at 6 hpf were placed in 24-well plates (10 embryos/1ml egg water/well) and then exposed to vehicle or various concentrations of OTA according to the experimental design.

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Whole-mount immunostaining

The heart ventricle and atrium were marked with monoclonal antibodies MF20 and S46 (Developmental Studies Hybridoma Bank, University of Iowa), respectively. WT embryos were treated with vehicle or OTA (0.25, 0.5 and 1 μM) from 6 to 72 hpf and then the following procedure was conducted according to Wu et al.[31]

Dextran clearance assay

Embryos of WT strain were treated with vehicle or 0.1 to 0.5 μM OTA from 6 to 72 hpf, and the clearance assay was conducted according to Hentschel et al.[32] After anesthesia by 0.4% Tricaine solution, surviving embryos at 72 hpf were injected with 2.3 nanoliter of 10 kDa lysine-fixable rhodamine dextran (1% in PBS solution) into the cardiac venous sinus using Nanoject II injection device (Broomall, PA). Each injected embryo was transferred into a 96-well plate and imaged at 0 and 7 h post-injection (hpi) under Zeiss fluorescence microscope (Axiovert 200M) equipped with rhodamine filter. All images were captured at constant gain and exposure time. The fluorescence intensity of individual cardiac area was determined by using the ImageGauge program Ver. 3.46 (Fuji Photo Film, Tokyo).

RNA sequencing (RNA-seq) for microRNA profiling

WT embryos at 6 hpf were treated with vehicle (n=20) or 0.5 μM OTA (n=20) and collected at 48 hpf for total RNA extraction with TRIzol-reagent (invitrogen, Carlsbad, CA).[33] Three independent replicates were conducted and extracted microRNA samples were pooled together for the following experiment. The quality of purified RNA was confirmed by using a Bioanalyzer 2100 (Agilent Technology, USA) with

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RNA 6000 labchip kit (Agilent Technologies, USA).The small RNA library construction and deep sequencing was carried out at Welgene Biotech Co. (Taipei, Taiwan). Libraries were sequenced on an Illumina GAIIx instrument (50 cycle single read) and sequencing data was processed with the Illumina software. Qualified reads after filtering low-quality data were analyzed before aligning reads to the Zebrafish genome (version Zv9). Only reads that mapped perfectly to the genome five or less times were used for miRNA detection. MiRDeep2 software was used to estimate expression levels of known miRNAs and also identifies novel miRNAs.[34] The RNA-seq data has been deposited in NCBI-GEO under accession numbers GSE71346.

AntagomiRs microinjection

The mirVana^{TM} antagomiR-731 or mirVana miRNA inhibitor negative control#1 (antagomiR-NC) (Life Technologies) at a level of 1600 pg was injected into yolk-stream of 1-2 cell stage WT or Tg(wt1b:GFP) embryos with Nanoject II nanoliter injector (Drummond Scientific). The normally developing embryos at 6 hpf were collected under stero-microscope (Nikon SMZ 800) and subsequently for vehicle and OTA treatment (at least 25 embryos/dose).

Total RNA isolation

WT embryos at 6 hpf were treated with OTA. Total RNAs, including mRNA and microRNA, were isolated at the designated time by TRIzol-reagent (Invitrogen) according to the protocols of manufacture and Mraz *et al.*[33]

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Determination of mRNA levels

The cDNA was obtained from 2 μg of total RNA using Super Script Ⅲ (Invitrogen, Carlsbad, CA), and PCR was followed using designed primers, including PRLRa (NM_001128677.1), serpina1 (serpin peptidase inhibitor, clade A, member1, NM_001077758.1), c-myc (NM_131412.1, official symbol in zebrafish: myca) and EF (eukaryotic translation elongation factor 1 α, AM422110.2) (Table S1). For amplification of EF and serpina1, the reactions were heated at 94°C for 5 min and cycled 25 times through the following procedures: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, with a final extension step at 72°C for 7 min. For amplification of c-myc and PRLRa, PCR was performed (94°C, 30 s; 54.3°C, 30 s; 72°C, 30 s) for 23 and 40 cycles, respectively.

qRT- PCR for microRNA

For miR-731 (MIMAT0003761) quantification, specific stem-loop primers designed based on Varkonyi-Gasic et al. (2007) were applied for cDNA production.[35] The quantitative PCR was performed in a reaction containing specific primers (Table S1), universal probeLibrary probe #21 (Roche), and FastStart Universal probe Master (Roche); U6 small nuclear RNA and miR-26a were used as the reference genes. Data were obtained from ABI 7000 thermocycler and calculated according to the manufacturer's description.

Western blot

Protein extraction was performed using the method reported by Link et al. with a slight modification.[36] Dechrionated and deyolked WT embryos at 72 hpf (n=20-25) were dissolved in 2 × SDS buffer (2 μl/embryos) and incubated at 95°C for 5 mins.

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Equal amounts of proteins (10 embryos/lane) were separated on an 8% SDS-PAGE, and then electrophoretically transferred to a nitrocellulose membrane. The membranes were blocked with PBS containing 10% skimmed milk for 1 h at room temperature, and soaked overnight at 4^{o}C with anti-p-STAT5 (Life Technologies #71-6900, MA), anti-p-AKT (EMD Millipore #124003, Germany), anti-p-ERK (Cell Signaling Technology #9101, MA), anti-c-myc (R &D systems #MAB3696, MN) and anti-tubulin antibodies (Upstate Biotechnology #05829, NY). Signals were visualized with anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase and revealed with chemiluminescence detection system (Millipore, France). The intensities of bands on blots were quantitated using the ImageGauge program Ver. 3.46 (Fuji Photo Film, Tokyo).

Statistical analysis

Data are presented as means±SEM. The unpaired two-tailed Student's *t*-test was used between two groups. One-way ANOVA plus Dunnett's multiple comparisons test was used between more than two groups. The statistical analyses were performed using GraphPad Prism (version 4.0, GraphPad Software Inc., San Diego, CA). The significance level was set smaller than 0.05.

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Results

Effects of OTA on viability and morphology of embryonic zebrafish

Zebrafish embryos at the 6 and 24 hpf stages were exposed to various concentrations of OTA. Methods I to VI in Fig. 1A were used to determine their viability and LC50 values were calculated for each method. As shown in Fig. 1A, the LC50 values obtained from Methods I (6 to 72 hpf) and V (24 to 96 hpf) were 1.00 and 0.97 μM, respectively, revealing that exposing the embryos to OTA at different time points leads to a slight difference in the embryo's viability.

The morphology of the zebrafish at 72 hpf was examined under a microscope after exposure to OTA from 6 hpf (Method I). Both the heart and the yolk of zebrafish are easily accessible organs for observing toxin-induced damage. As shown in Fig. 1B, embryos that were treated with more than 0.5 μM OTA exhibited pericardial edema, dark yolk sac and yolk sac edema.

The defects in the heart chambers that were caused by OTA were observed closely using whole-mount immunostaining with MF20 and S46 antibodies, which stain the myosin heavy chains in the ventricle and atrium, respectively.[37] In Fig. 2A, correct heart looping and chamber overlapping were observed in the vehicle-treated embryos (control) at 72 hpf, but, following exposure to 0.25 μM OTA toxin, the ventricle and atrium in the embryos were separated and stretched out. Additionally, in the 1 μM OTA-treated group, the distance between the sinus venosus and the bulbus arteriosus (SV-BA), an indicator of cardiac looping, had significantly increased to 1.5-fold of the control (Fig. 2B).

OTA altered structure and function of developing kidneys

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To study the effects of OTA on the morphology of embryonic kidneys, embryos of the transgenic zebrafish line (wt1b:GFP) expressing a green fluorescent protein in their pronephros were exposed to 0.1 to 0.5 μM OTA from 6 to 72 hpf. As shown in Fig 3A, the pronephros in the 0.5 μM OTA-treated groups had shrunk and arranged in a disorganized manner. The angle between the neck and pronephric tubule differed from that in the control group.

To investigate the effect of OTA on renal function, the dextran clearance method was applied to evaluate the glomerular filtration rate of the embryos.[38] Approximately 54.8% (17 out of 31) of the vehicle-treated control efficiently cleared the dextran with red fluorescence at 7 hours post-injection (hpi) (Fig. 3B). However, the dextran clearance rates in the embryos that were exposed to 0.25 and 0.5 μM OTA were 31.2 % (10 of 32) and 11.5 % (3 of 26), respectively, suggesting that OTA reduced the glomerular filtration rate in the embryonic fish. Furthermore, the clearance rates from three individual experiments were calculated and shown in Fig. 3C. The 0.5 μM OTA treatment caused only 15.5±7.8% of embryos not displaying fluorescence, but 50.4±12.4% of control embryos were in clearance status without fluorescence signal.

OTA modulated PRLR/STAT5 signaling in zebrafish embryos

OTA is reported to decrease the PRLR mRNA levels in rats, based on microarray data.[17] The treatment of zebrafish embryos with various concentrations of OTA inhibited the gene expression of PRLRa in a dose-dependent manner; as little as 0.25 μM OTA significantly reduced the PRLRa mRNA level to 47.3 ± 0.2% of that in the control group (Fig. 4A). The strong reduction of PRLRa expression by OTA was detected as early as 24 hpf (Fig. 4B).

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The major downstream pathway of PRLR in mammalian cells is the STAT5 signaling cascade;[19] AKT and ERK1/2 signaling in some human cells are also regulated under PRLR.[39, 40] Treating embryonic fish with OTA reduced the phosphorylated levels of STAT5 in a concentration-dependent manner (Fig. 5A). OTA at concentrations of 0.25 and 0.5 μM significantly reduced the strength of p-STAT5 signals to 52.6 ± 10.2% and 37.6 ± 6.0% of that in the control group, respectively. Administering OTA also suppressed the phosphorylation of the AKT protein (Fig. 5B). Nevertheless, OTA did not modulate the phosphorylated ERK1/2 signal, even at a concentration of 0.5 μM (Fig. 5C).

Previous studies have shown that serpina1 and c-myc genes are possible downstream targets of p-STAT5 in human cells after the introduction of tilapia and seabream PRLR into human HEK293 cultures.[41, 42] Serpina1 gene encodes an acute phase protein with anti-protease and immunoregulatory activities.[43] In zebrafish, OTA at 0.25 and 0.5 μM reduced the levels of serpina1 transcripts to 30.9 ± 13.3% and 17.1 ± 5.2% of that in the control group, respectively (Fig. 6A); it suggests that OTA modulates the PRLRa/STAT5/serpina1 pathway in embryonic zebrafish. However, exposure to OTA did not have a similar effect on the levels of c-myc transcripts and proteins (Fig. 6B and Fig. S2).

PRLRa is the potential target of miR-731

To further study the molecular mechanism of OTA toxicity, the microRNA profile of embryos after OTA exposure was obtained by RNA-seq. After embryos had been treated for 42 h (6 to 48 hpf) with 0.5 μM OTA, a total of twelve microRNAs were found to be differentially expressed (> 2.0 folds) relative to the control group. Seven

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microRNAs were up-regulated and five were down-regulated (Table 1). *In silica* analysis with TargetsScanFish and miRMAP software revealed that, among these twelve microRNAs, miR-731 was the only one that had the potential to recognize the core sequence on the 3'UTR of PRLRa (Fig. 7A).

To validate the miR-731 data from the RNA-seq, qRT-PCR was performed to elucidate the effects of OTA on miR-731 expression. As displayed in Fig. 7B, treating 6 hpf embryos with 0.5 μM OTA increased their miR-731 levels in a dose-dependent manner at 48 hpf (Fig. 7B). The differential expression of miR-731 was observed as early as 24 hpf, and the differences relative to the control embryos reached their peaks at 48 hpf (Fig. 7C).

To evaluate whether PRLRa mRNA is a target of miR-731, the antagomiR-NC (control) or antagomiR-731 was microinjected into embryos that were subsequently exposed to 0.5 μM OTA at 6 hpf. The knockdown efficiency of antagomiR-731 was evaluated by the down-regulation of mature miR-731 in OTA-exposed embryos (Fig. 8A). Moreover, a comparison with the antagomiR-NC group revealed that the presence of antagomiR-731 not only recovered the levels of PRLRa that were reduced by OTA (from 17.4 ± 5.8% to 73.4 ± 26.2%), but also concomitantly enhanced the PRLRa mRNA to 146.6 ± 15.3% in the absence of OTA (Fig. 8B), suggesting that PRLRa is a target gene for miR-731.

miR-731 effects on renal morphology that is impaired by OTA

After being injected with antagomiRs at 1-2 cell stage and exposed to vehicle at 6 hpf, the pronephro images of Tg(wt1b:GFP) embryos at 72 hpf were displayed in Fig. 9A (a and b); 97.7% of antagomiR-731 injected embryos retained normal pronephric

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structure (n=42/43). On the other hand, approximately 60.6 % of embryos (n=20/33), treated with antagomiR-NC and then 0.5 μM OTA, had shrunken and disorganized pronephros as shown in Fig. 9A (c). The presence of antagomiR-731 resulted in 73.2 % of peonephros (n= 30/41) showing a relative normal structure (Fig. 9A (d)). In Fig. 9B, 0.5 μM OTA, following antagomiR-NC injection, caused 58.5 ± 2.9% of the embryos to develop defective pronephros, as defined in Fig. 9A (c). However, the microinjection of antagomiR-731 before OTA administration reduced the defective rate to 19.4±3.3 %, suggesting the miR-731 is involved in OTA-induced renal defects.

Discussion

It has been reported that OTA is able to decrease the mRNA levels of PRLR in the kidney/liver of Fischer 344 rats based on data obtained from Affymetric RGU34A array.[17] We also applied Agilent Zebrafish V3 array to investigate the mRNA profiling of OTA-treated zebrafish embryos and found that the expression of PRLRa was dramatically down-regulated after OTA exposure (un published data, GEO accession numbers GSE71345). These information strongly suggest the effects of OTA on PRLR is common in both teleost fish and mammals. From the perspective of gene structure, the PRLR gene of teleost fish has two isoforms, PRLRa and PRLRb, and both isoforms have considerable structural similarity to the long-form PRLR in mammalian species.[42] However, PRLRa and PRLRb locate on different chromosomes of zebrafish and demonstrate only 30% of amino acid sequence similarity.[42] Moreover, these two isoforms in tilapia fish are functionally distinct from each other and mediate different signaling events.[41] Therefore, it is not out of our expectation that OTA

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exposure only decreased the levels of PRLRa (Fig. 4), but did not modulate the PRLRb expression (Fig. S3).

Injection of antagomiR-731 into embryos not only led to PRLR elevation, but also partially rescued the renal morphology impaired by OTA (Figs. 8B and 9). The PRL/PRLR interaction is known to play a critical role in water and electrolyte balance in fresh-water fish by decreasing water uptake and increasing ion retention.[44] PRL is secreted by anterior pituitary gland as a autocrine/paracrine factor and PRLR is widely found in all the osmoregulatory organs in fish, including kidney, gill, skin and intestine.[45] PRL in mice also regulates water and salt balance by reducing renal Na^+/K^+ ATPase activity and increasing intestine water.[23] Therefore, the loss of PRLR after OTA treatment is highly possible to disturb the function and structure of developing kidney, and also may contributes to the pericardial and yolk edema as shown in Fig. 1B.

In addition to miR-731, other microRNAs shown in Table 1 may also contribute to OTA's embryonic toxicity. Both miR-731 and miR-462 were up-regulated by OTA to a similar extent in either RNA-seq or qRT-PCR (Table 1 and unpublished data), because they located 124 base pairs apart as a gene cluster on the chromosome 8 of zebrafish. Bioinformatic analysis suggests that miR-731/462 are orthologues of human miR-425/191.[27] This human cluster is involved in cell cycle control and carcinogenesis,[46] which is consistent with our findings that the alteration of miR-731/462 levels is associated with developmental abnormalities in zebrafish. On the other hand, OTA treatment reduced the expression of miR-129 (Table 1). Microarray data reported by Dai et al. indicate the miR-129 expression is inhibited in the kidneys of rats fed with 210 μg/kg OTA for 13 weeks.[12] Cao et al (2012) showed that inhibition of miR-129 in zebrafish embryos suppressed the ciliation in the

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pronephros and Kupffer's vesicle.[47] It has been known that cilia-driven fluid flow in pronephros, brain, and Kupffer's vesicle is required for normal organogenesis of embryonic zebrafish.[48]

Our findings for the first time suggest that PRLRa has a biological function to activate STAT 5 and AKT signaling pathways in zebrafish (Fig. 5). STAT molecules are known to serve as transcription factors to promote differentiation and proliferation during zebrafish embryogenesis.[49] However, among the two potential downstream targets of STAT5, only sepina1 gene but not c-myc was modulated by OTA (Fig. 6). Fiol et al. (2009) has documented that when human HEK293 cultures were transfected with tilapia PRLR1 and then stimulated with two tilapia PRL variants, PRL177 and PRL188, the former variant activates both human spi 2.1 (named serpina1 in zebrafish) and c-myc and the latter one only affects spi 2.1. [41] Recently, a novel prolactin-like protein (PRL2) expressed in extra-pituitary tissues has been discovered in zebrafish.[50] Therefore, the complex interaction between PRL variants and PRLR isoforms may direct the signaling pathway to stimulate various downstream genes. Besides, it is reasonable that the transduction cascades observed in human cell line are not always consistent with those in developing zebrafish.

In conclusion, we have demonstrated that OTA impaired the morphology and biological functions of embryonic zebrafish. The possible molecular mechanism involved in OTA-triggered renal damage is summarized in Fig. 10. OTA exerted part of its adverse effects on renal development through activation of miR-731 expression, suppression of PRLRa, and subsequent attenuation of p-STAT5 and p-AKT signaling; all of these factors are known to be strongly associated with the proliferation and differentiation of embryos. The presence of OTA has been reported in the blood and milk samples of pregnant women, as well as in fetal cord blood samples.[3,51] Moreover,

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the transplacental transfer of OTA has been demonstrated in rats and horses.[52, 53] These findings raise concerns about the potential health hazards of OTA on developing organisms. Thus, an understanding of the toxic levels and mechanism of OTA in vertebrate embryos may provide a basis for evaluating OTA's health and safety impact on the public.

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Conflict of Interest

Authors declare that there are no conflicts of interest.

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Legend of Figures

Figure 1. (A) Exposure methods and corresponding LC50 values in OTA-treated zebrafish. The mortality of wild-type (WT) AB line fish exposed to OTA was observed under stereo microscope and the LC50 values were estimated based on a fitted curve. Data were obtained from 4-5 independent experiments and 20-25 embryos were used in every treated group of individual experiment. (B) Lateral view images of embryos after exposure to vehicle, 0.25, 0.5 and 1 μM OTA from 6 to 72 hpf (Method I). Arrow and arrowhead indicate the heart and yolk areas, respectively.

Figure 2. Cardiac defects in OTA-treated embryos. The WT embryos were exposed to vehicle, 0.25, 0.5 and 1 μM OTA. (A) Whole-mount immunostaining of 72 hpf WT embryos with chamber-specific antibodies MF20 (ventricle, red) and S46 (atrium, green). Lateral view images were taken under Zeiss fluorescence microscope (magnification × 200). The measured SV-BA distance is indicated by double arrow; the quantitative results are displayed in (B) panel. Data are mean ± SEM from 4 independent experiments and each experiment consisted of 15 embryos/dose. **, *p*

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<0.01. V, ventricle; A, atrium.

Figure 3. Effects of OTA on pronephric morphology and renal function. (A) Embryos of Tg (wt1b:GFP) were exposed to vehicle and OTA from 6 to 72 hpf. Images of pronephric phenotype were taken from dorsal view under fluorescence microscope (magnification x 200). gl, glomerulus; N, neck; pt, pronephric tubule. (B) WT embryos at 6 hpf were first exposed to vehicle or OTA, and then 10-kDa rhodamine-labeled dextran were injected at 72 hpf according to Materials and Methods. Individual fish's fluorescence intensity was measured at baseline and 7 hour post-injection (hpi). The N value on the bar means the number of examined fish without fluorescence (clearance) or with fluorescence (non-clearance). (C) The clearance ratio was determined by dividing the number of embryos without fluorescence with total examined number. Data represents mean ± SEM from three independent replicates, and each replicate consisted of 8-10 embryos/dose. *$p < 0.05$, significantly different compared to the vehicle group.

Figure 4. OTA decreased PRLRa mRNA expression in zebrafish embryos. (A) The WT embryos at 6 hpf were exposed to vehicle or 0.1-0.5 μM OTA. The PRLRa mRNA levels of 48 hpf embryos were determined by RT-PCR and the relative PRLRa/EF intensity is shown in the lower panel. Data are mean±SEM from 4 replicates and each replicate consisted of 20 fish/dose. (B) WT embryos at 6 hpf were exposed to vehicle or 0.5 μM OTA, and PRLRa mRNAs were detected in the embryos of 24, 48, and 72 hpf by RT-PCR. EF, elongation factor, was used as an internal control.

Figure 5. OTA modulated the PRLRa downstream signals. WT embryos were treated with vehicle or OTA from 6 to 72 hpf. Total protein extracts of 72 hpf embryos were subjected to Western blotting using antibodies specific for p-STAT5 (A), p-AKT (B), p-ERK (C) and tubulin (internal control). The relative levels shown

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in the lower panel are the mean ± SEM from 3 independent replicates and each replicate consisted of 20-25 embryos/dose. *, $p < 0.05$; **, $p < 0.01$.

Figure 6. Effects of OTA on the gene expression of serpina1 and c-myc. WT embryos at 6 hpf were treated with vehicle or OTA. The mRNA levels of serpina1 (A) and c-myc (B) in 48 hpf embryos were determined by RT-PCR. The relative levels shown in the lower panel are normalized with EF (internal control). Data are mean ± SEM from three independent replicates and each replicate consisted of 20 embryos/dose.

Figure 7. OTA induced miR-731 expression in zebrafish embryos. (A) The schema of miR-731 binding site in corresponding 3'-UTR sequence of zebrafish PRLRa (seed sequence highlighted in red). (B) WT embryos at 6 hpf were exposed to vehicle or OTA (0.1 to 0.5 μM), and miR-731 levels were determined at 48 hpf by qRT-PCR. (C) WT embryos that had been exposed to vehicle or 0.5 μM OTA at 6 hpf were collected at 24, 48 and 72 hpf for qRT-PCR quantification of miR-731.Values were expressed as mean ± SEM form three independent replicates and each replicate consisted of 20 embryos/dose. *, $p < 0.05$.

Figure 8. PRLRa is the potential target of miR-731**.** WT embryos injected with antagomiRs at 1-2 cells stage were treated with vehicle or 0.5 μM OTA from 6-48 hpf. Total RNA samples containing both mRNA and microRNA were prepared according to Materials and Methods. (A) The miR-731 levels determined by qRT-PCR were presented as mean ± SEM from four replicates (20 embryos/dose/replicate). **$p < 0.01$. (B) PRLRa mRNA was detected by RT-PCR; the relative levels shown in the lower illustration are the mean ± SEM from four replicates (20 embryos/dose/ replicate). *, $p < 0.05$; **, $p < 0.01$.

Figure 9. The antagomiR-731 rescued the pronephric defect caused by OTA. Tg (wt1b:GFP) embryos at 1-2 cells stage were injected with 1600 pg of antagomiR-NC or antagomiR-731, and then treated with vehicle or 0.5 μM OTA at 6 hpf. (A) The

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representative pronephric phenotypes after antagomiR-NC and antogomiR-731 introduction and toxin exposure. All the images were taken at 72 hpf from dorsal view under fluorescence microscope (magnification x 200). (B) The percentage of pronephric defects was determined at 72 hpf by dividing the number of embryos with malformed kidney with the total treated number. Data represents mean ± SEM from three independent experiments, and at least 10 embryos per treatment were included in each experiment. **$p < 0.01$, significantly different between compared groups.

Figure 10. A putative model depicts the mechanism of OTA-induced renal defects in embryonic zebrafish. OTA pressure promotes the miR-731expression and then attenuates PRLRa levels. The phosphorylation of downstream STAT5 and AKT is further blocked and subsequently lead to abnormal renal morphology.

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Table 1. MicroRNAs altered in OTA-treated embryos

microRNA	Fold Change[a]
dre-miR-462	8.46
dre-miR-731	8.05
dre-miR-2185-5p	4.81
dre-miR-738	3.45
dre-miR-7145	2.95
dre-miR-2192	2.86
dre-miR-732	2.50
dre-let-7e	-2.09
dre-miR-129-3p	-2.15
dre-miR-190b	-2.39
dre-miR-219	-2.81
dre-miR-122	-3.45

a) RNA-seq analysis revealed the microRNAs with expression altered greater than 2-fold after 0.5 μM OTA treatment

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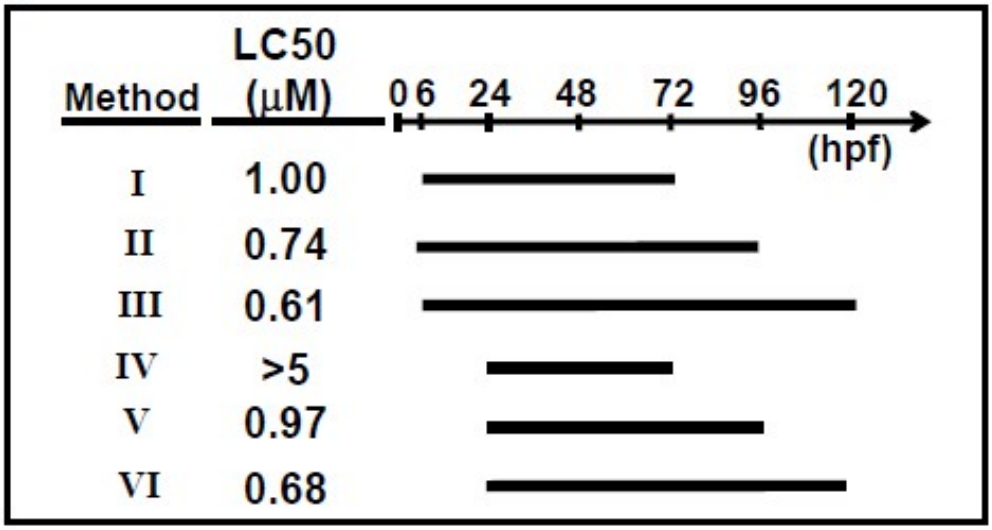
Figure 1

(A)



(B)

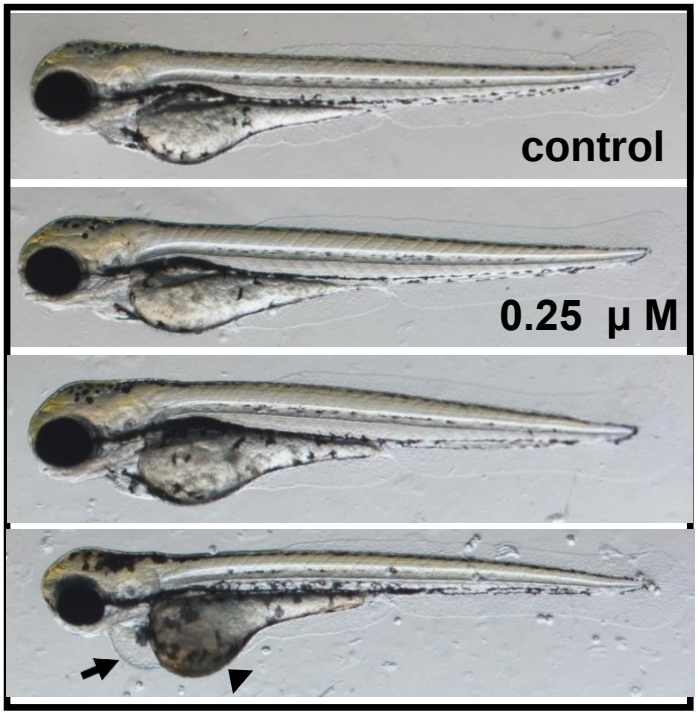


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Figure 2

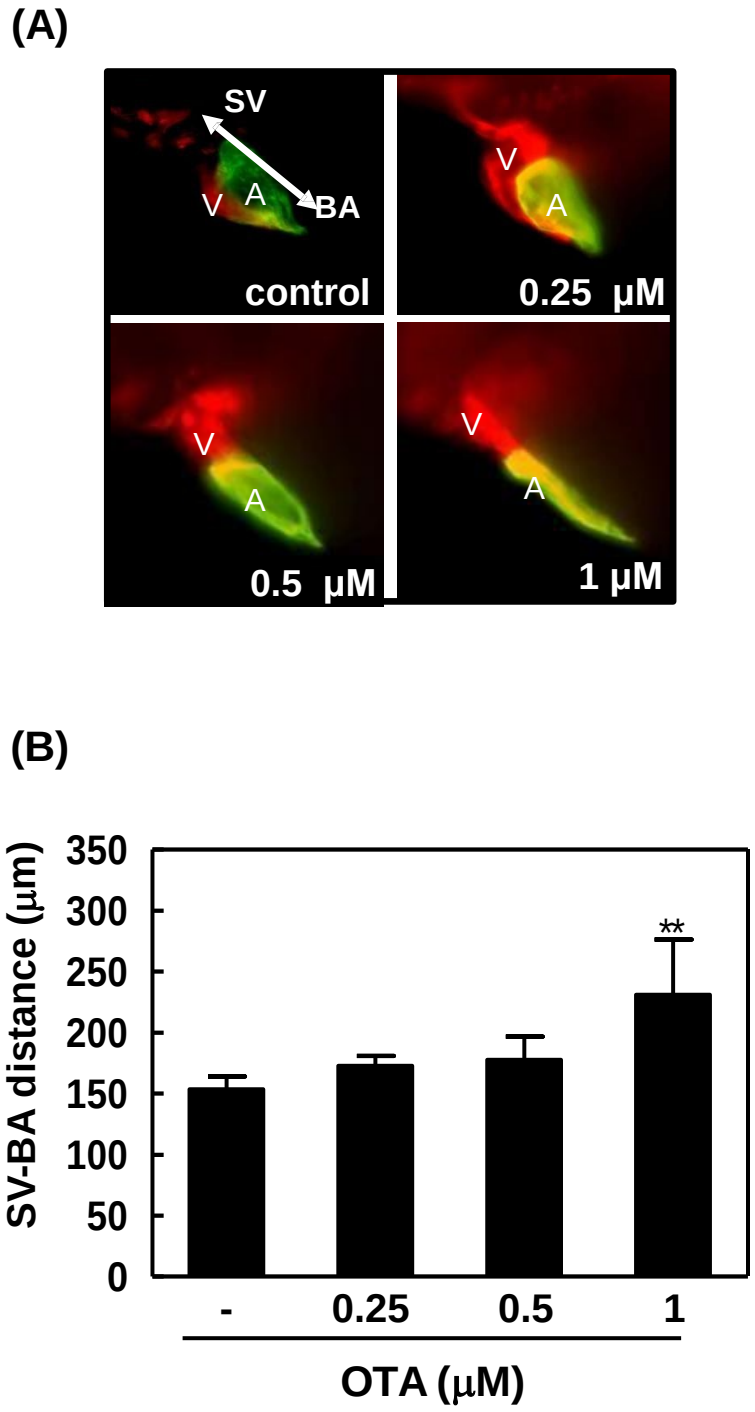

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Figure 3

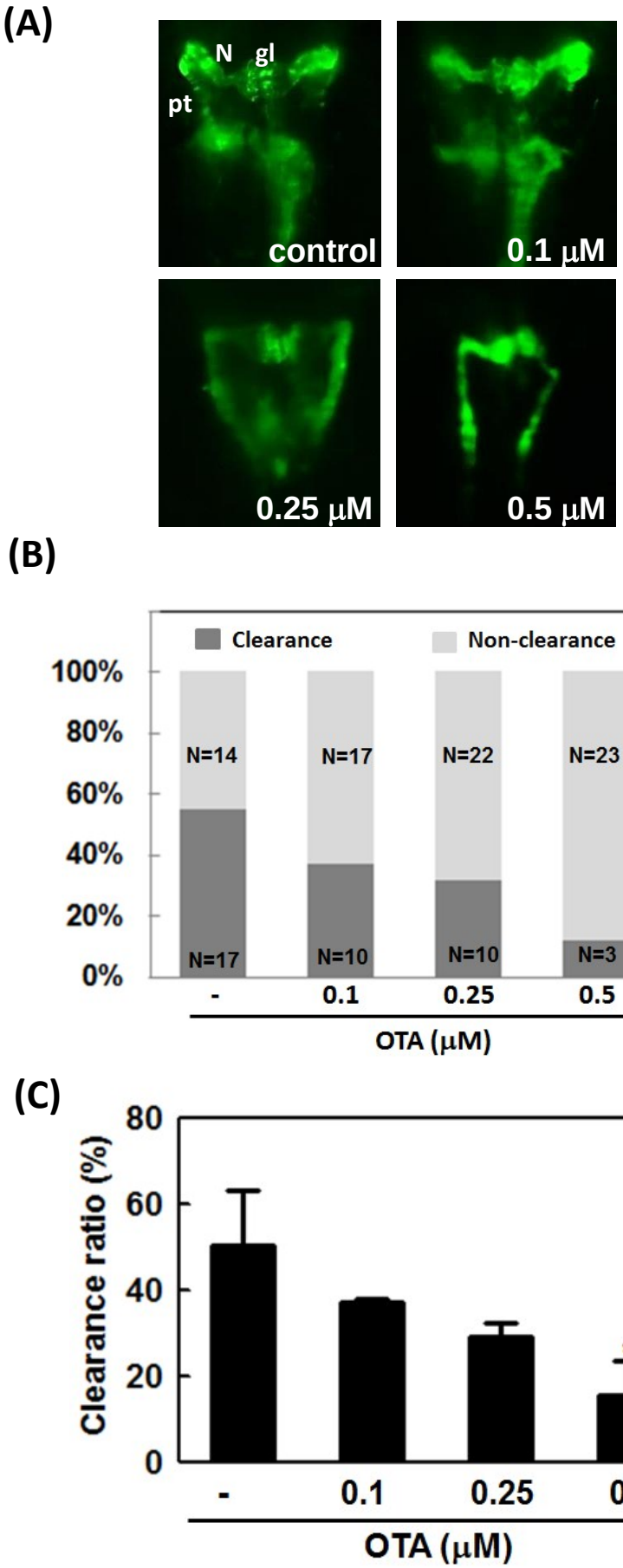

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Figure 4

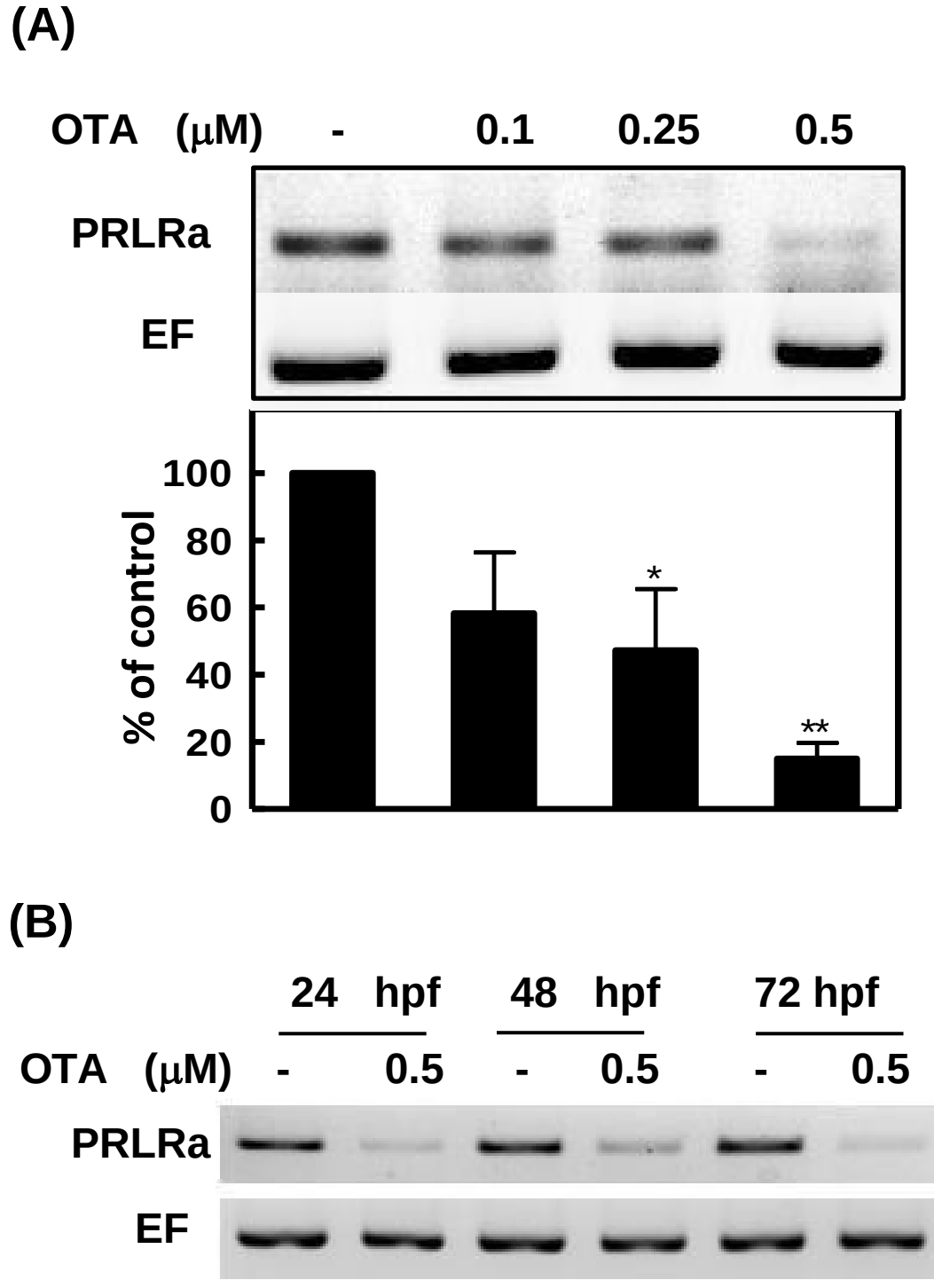

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Figure 5

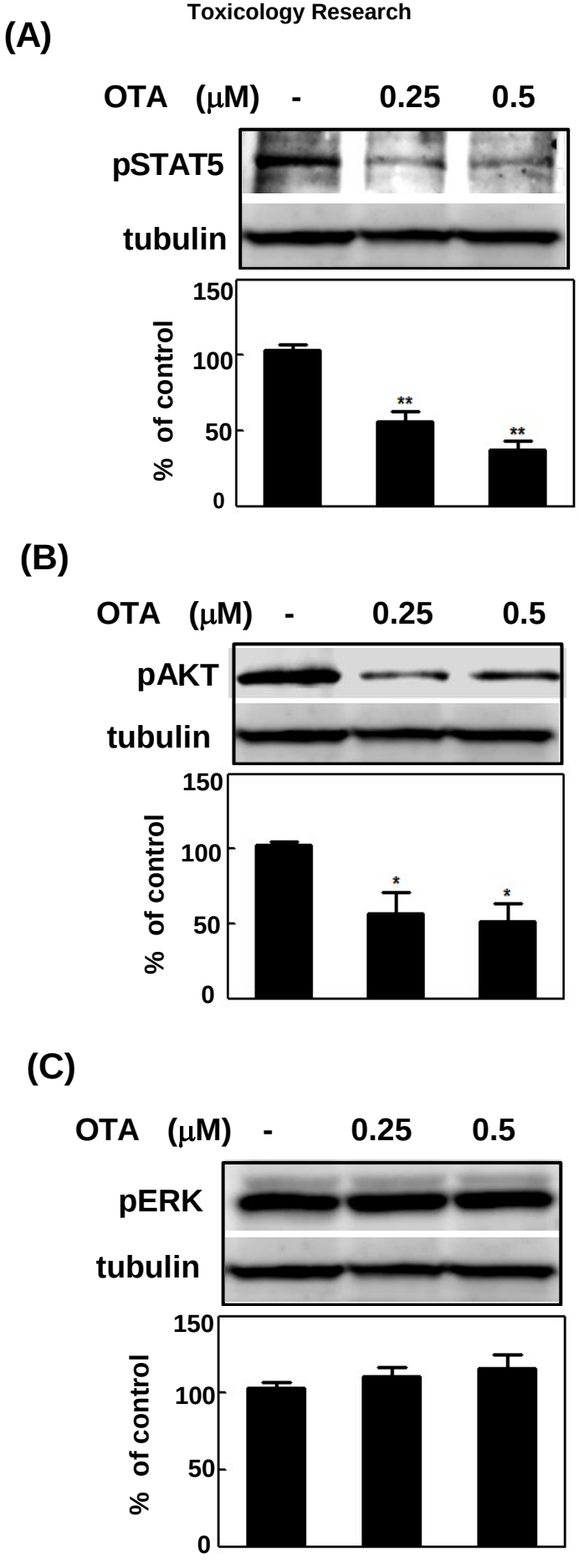

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Figure 6

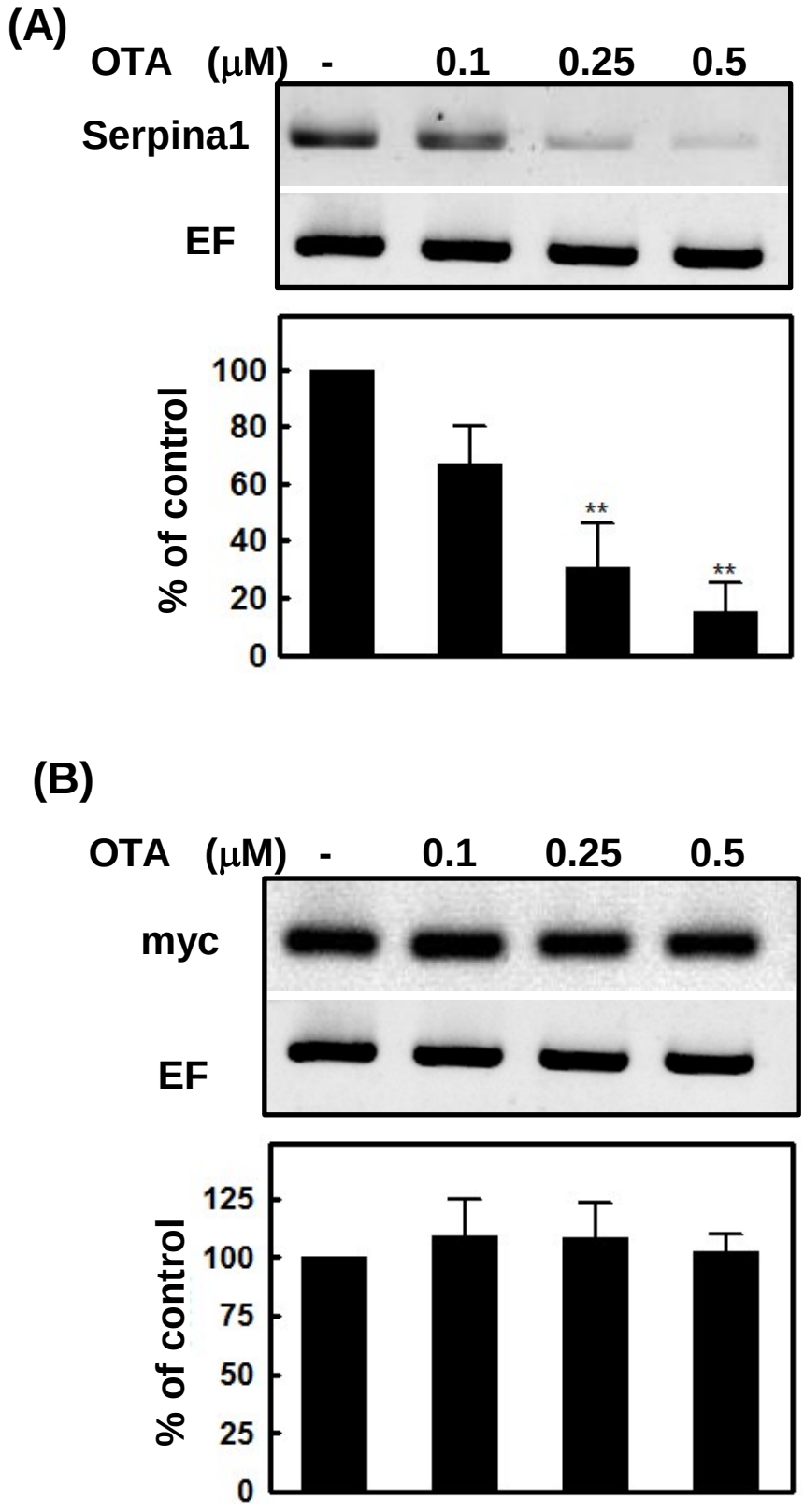

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Figure 7

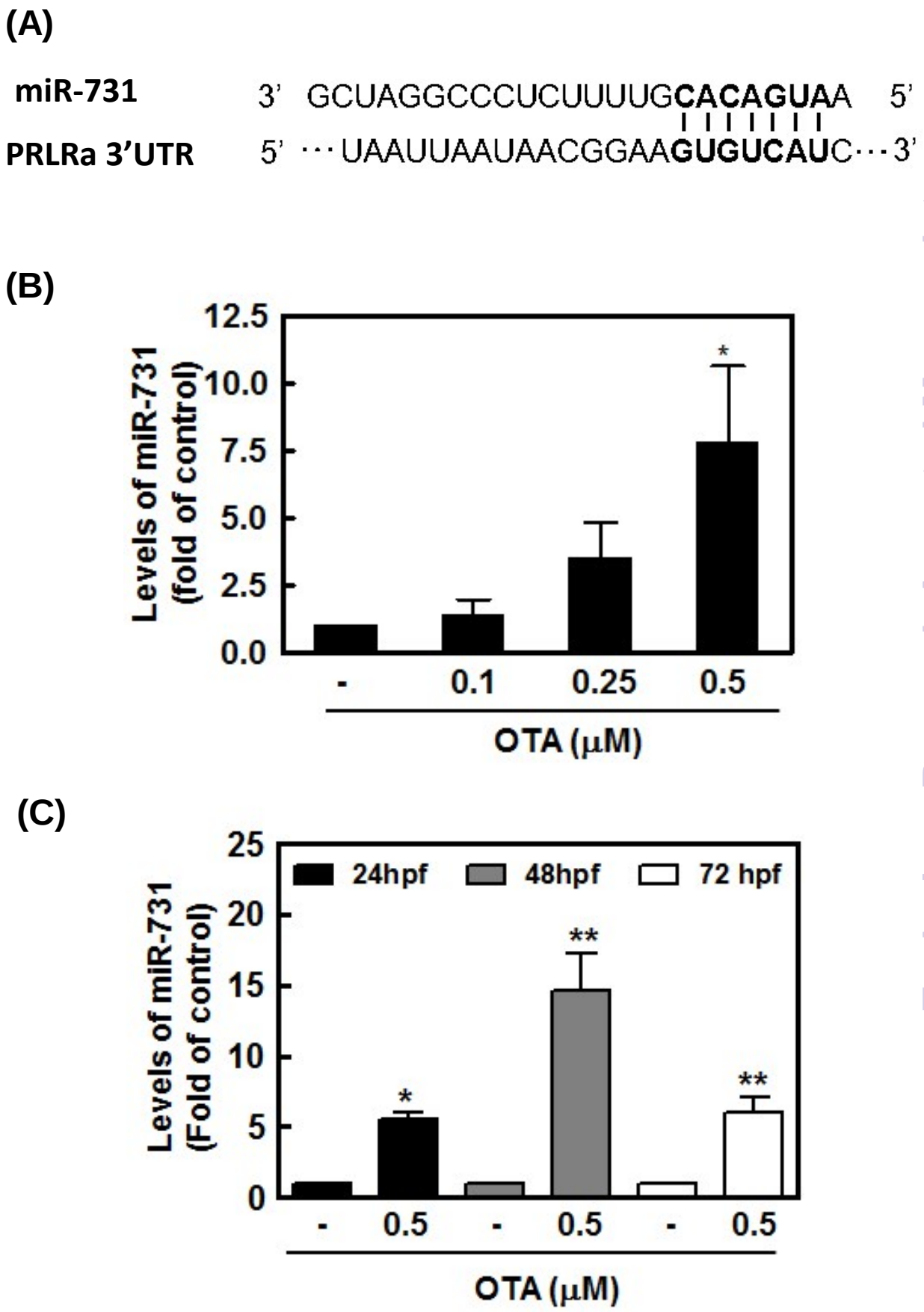

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Figure 8

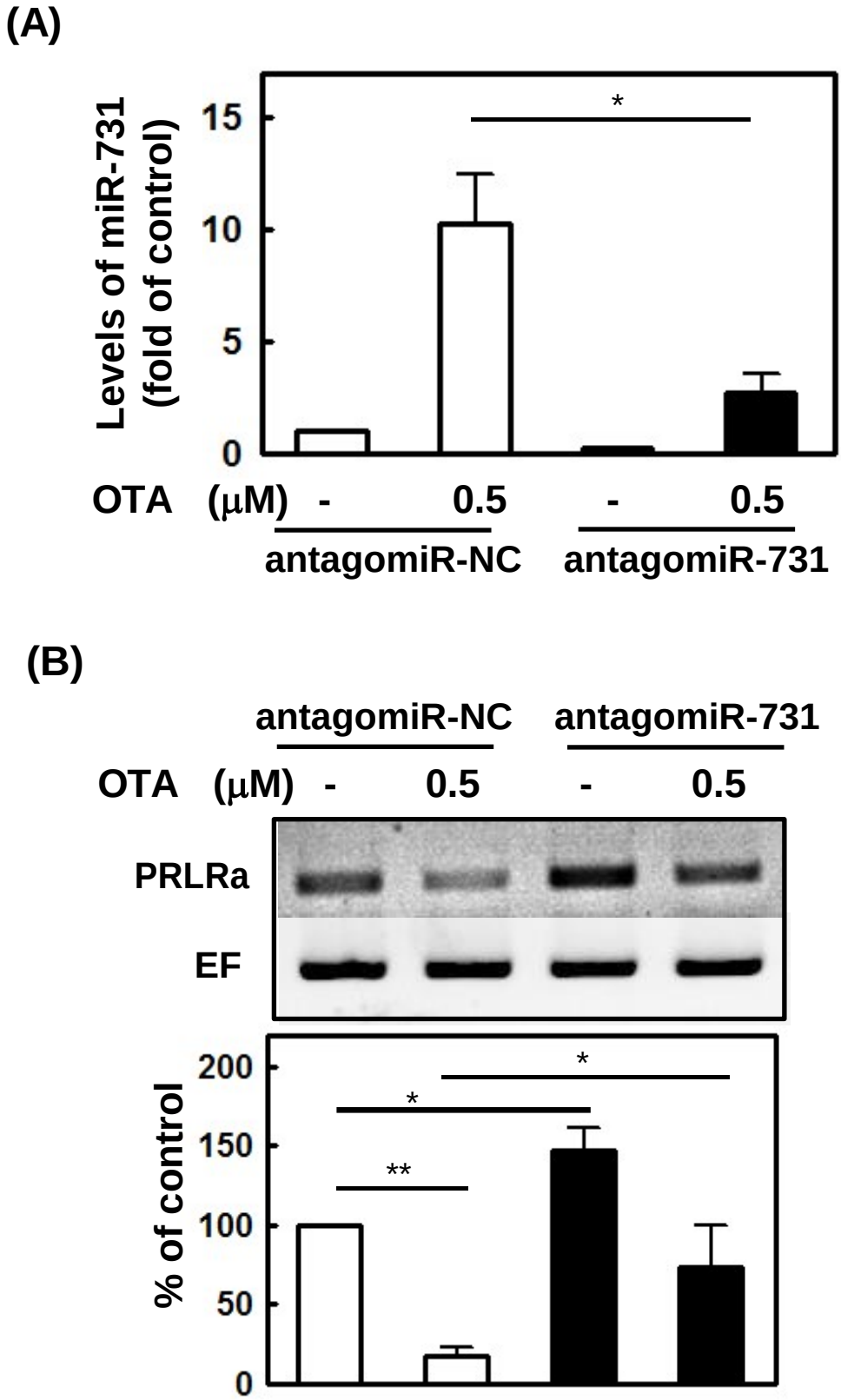

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Figure 9

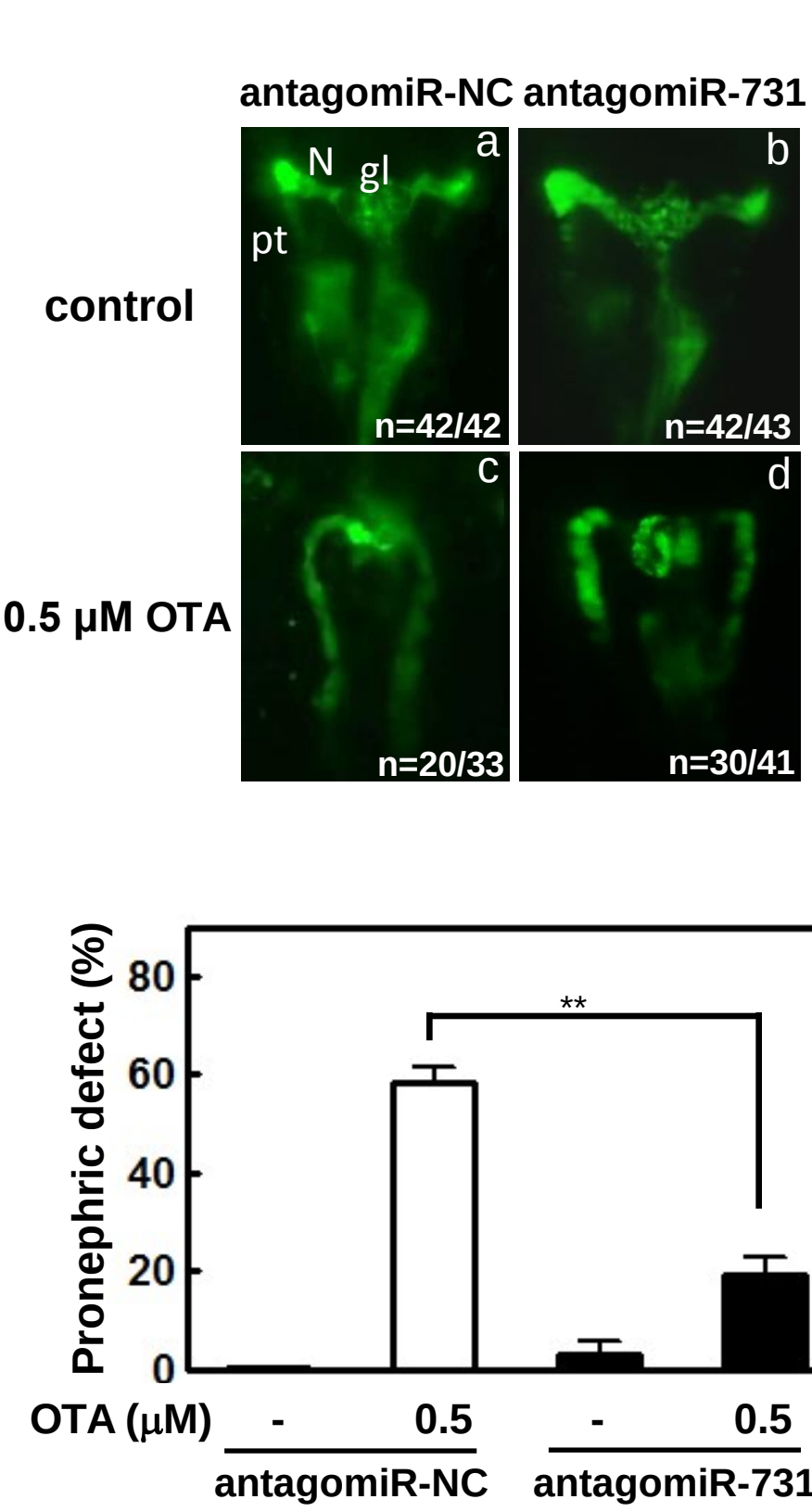

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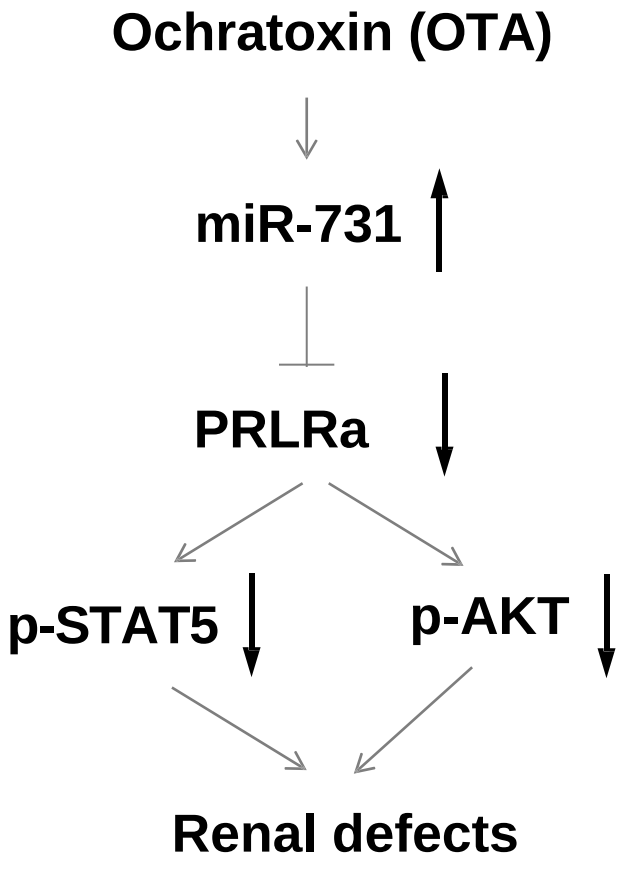
Figure 10



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