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Chicken Embryo Fibroblasts are Effective Cells to Study the Function of Chicken Neurexophilin 1 Gene

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Authors' Contribution
GQL and XPJ designed the experiments and wrote the manuscript. DS, MC and YL performed the experiments. HIA performed the analysis with constructive discussions.

Key words
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ABSTRACT

Neurexophilin has been proved to be a differentially expressed gene in chicken sperm storage tubules. In this work, Immunocytochemistry, immunofluorescence and Western-blot were performed to detect the expression of neurexophilin 1 in epithelial cells of chicken utero-vaginal junction, chicken embryo fibroblasts (DF-1 cells) and Hela cells. The immunocytochemistry results showed that neurexophilin 1 was expressed in both the epithelial cells of chicken genital tract and DF-1 cells. Immunofluorescence revealed that neurexophilin 1 was expressed in cytoplasm as well as cell nucleus of chicken fibroblasts and Hela cells. Western-blot analysis displayed neurexophilin 1 in the cell and cell culture fluid of DF-1 cells and Hela cells, and higher expression of neurexophilin 1 in cell culture fluid of DF-1 cells than that of Hela cells. These findings suggested that DF-1 cells are effective and a better choice than Hela cells to do researches on the function of neurexophilin 1 gene.

INTRODUCTION

Artificial insemination is practiced extensively with commercial turkeys and egg type breeder hens. Chicken semen begins to lose fertilizing ability when stored >0.5 hr at room temperature (Tang *et al*., 2012), therefore insemination with fresh semen in chicken production is executed within half an hour in China. In marked contrast to the short storage period of fowl sperm in vitro, sperm of all avian species are stored in the female oviduct at body temperature of 41°C for days to weeks, depending on species (García-Herreros, 2016; Matsuzaki and Sasanami, 2017). The anatomical structures associated with prolonged sperm storage are the infundibulum and the uterovaginal junction (UVJ), and the latter is the primary sperm storage tubules (SSTs) (Bakst, 2011). Since the discovery of the SST in the hen oviduct, many researches focused on the SST-oriented mechanisms underlying the prolonged fowl sperm survival. However, little is known concerning the mechanism of sperm storage in birds. Specific gene expression within the SSTs may involve in the prolonged sperm storage. So differentially expressed genes were searched in SST in recent researches. Neurexophilin 1 is one of the differentially expressed genes, whose expression was elevated in the UVJ of high fertility hens (Liu *et al*., 2009). NXPH1 expression level is prominent in high fertility hens, compared with low fertility hens. NXPH1 binds tightly to the extracellular domain of neurexin Ia (Craig and Kang, 2007) that binds to a-latrotoxin only in the presence of Ca2+ that are important factors for sperm motility (Wang and Li, 2017). Further study will focus on the function of this gene with overexpression, interference, or knock-out of gene. These methods of studying the function of genes will use suitable cells. This study will detect the expression of neurexophilin 1 gene in the epithelial cells of the UVJ, DF-1 cells and Hela cells, aims to look for convenient cells when studying the function of neurexophilin 1 gene in chicken genital tract.

MATERIALS AND METHODS

Cell culture

The primary cells of chicken genital tract were cultured as follows. Briefly, mature Hyline female chickens aged 30-40 weeks were reared individually in cages, fed standard diets adlibitum, and kept under a 14L: 10D photoperiod. Hens were killed and the utero-vaginal junction of the oviduct were excised, soaked in PBS buffer

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and cleared without fat, connective tissue and adventitia. The remained tissue was cut into pieces of 1mm³. Then the pieces were moved into culture bottle and incubated with DMEM (Lot. 8117105, Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (Gibco,Thermo Fisher Scientific) and 5% CO2 at 37°C for 2-4 h until the plots were adhered to each other, then the cells were cultured until the bottle was filled with genital tract epithelium cells during which the nutrient solution was changed every three days. The DF-1 cells and Hela cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 incubator.

Immunocytochemistry of neurexophilin 1

Cells on Chamber petri dish were washed in PBS and fixed in 4% (v/v) paraformaldehyde for 20 min at room temperature. The immunohistochemistry detection of neurexophilin 1 was performed with the SABC kit (SA1023, Boster Biological technology Co., Ltd). Hydrogen peroxide was used to deactivate intrinsic peroxydase. Antigen retrieval was performed in a water bath using citrate buffer. Sections were incubated with diluted anti-neurexophilin 1 antibody (0.2 ng/ul; (Lot. CASE01, R&D, Bio-Techne China Co. Ltd.) Overnight at 4°C. Biotin labeled secondary antibody was added to the slides following horseradish peroxidase (HRP) labeled streptavidin. After staining with DAB and counterstaining with hematoxylin, the slides were imaged with software (Nikon, Tokyo, Japan).

Immunofluorescence assays

Cells were fixed with 4% paraformaldehyde for 20 min, rendered permeable with 0.5% Triton X-100 (Sigma-Aldrich) for 20 min and blocked with rabbit serum (Boster Bioscience) for 30 min at room temperature. Then the cells were incubated at 4°C overnight with primary antibodies against neurexophilin 1, and incubated at 37°C for 1 h with fluorescein isothiocyanate (FITC)-labelled secondary antibodies. Samples were mounted with 4′,6-diamidino-2-phenylindole (DAPI) and imaged with a laser-scanning, spectral, confocal microscope.

Western-blot

Cells were harvested and lysed with 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% NP40, and 1% PMSF (0754, GBCBIO Technologies Inc.). Lysates were centrifuged at 12,000 × g for 5 min at 4°C. The culture fluids were harvested and concentrated with TCA at 4°C overnight. After centrifuging at 12,000×g for 10 min, lower layer centrifugation were added with acetone at -20°C for 30 min and centrifuged again. The protein was dissolved with water and its content was measured with the BCA Protein Assay Kit (G3422, Solarbio Technologies). Equal amounts of protein was denatured by boiling and separated on 12% SDS-polyacrylamide gels. Proteins were then electroblotted to 0.45 μm PVDF membrane and blocked with 5% non-fat milk in TBS-Tween buffer for 2 h at room temperature. The membrane was then incubated with anti-neurexophilin 1 antibody (2 ng/ul; R&D) overnight in a 4°C refrigerator. The membrane was then washed with PBS-Tween-20 (PBST) three times and incubated with the HRP-conjugated rabbit anti-goat secondary antibody (1:5,000) in a 37°C shaker for 2 h. After washing three times with PBST, the membrane was visualized with an ECL system (Pierce Biotechnology, Inc.).

The densitometry analysis of western blot data was carried out with Image software. The relative band densities were normalized to appropriate β-actin bands used as reference protein.

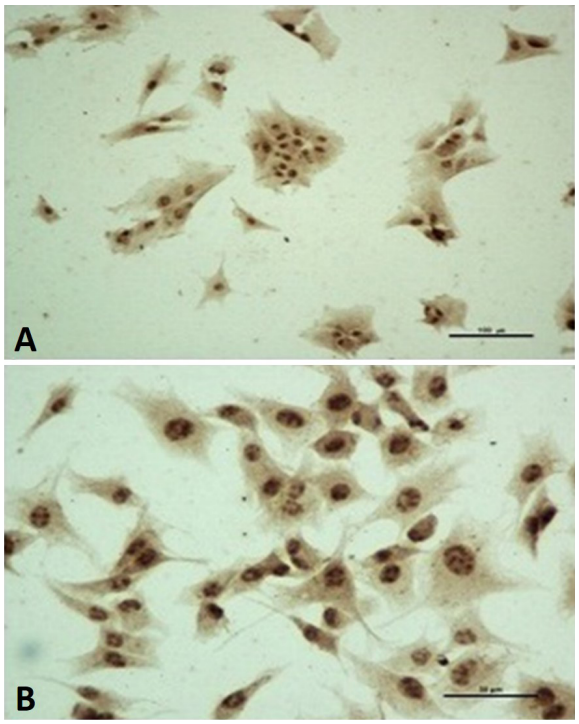

Fig. 1. Immuno-localization of NXPH1 in chicken genital tract epithelial cells and chicken fibroblasts (DF-1 cells). A, chicken genital tract epithelial cell; B, DF-1 cells.

RESULTS

Firstly, we conducted immunohistochemistry to examine the subcellular localizations of NXPH1 in chicken genital tract epithelial cells and DF-1 cells. The results showed that neurexophilin 1 existed in the cytoplasm and nucleus of the two types of cells (Fig. 1). This implied that

DF-1 cells can be used in studying the function of chicken neurexophilin 1.

Then immunofluorescence was used to detect the distribution of NXPH1 in DF-1 and Hela cells. It showed that green fluorescence of NXPH1 appeared in the cytoplasm and nuclei of chicken fibroblast cells and Hela cells, and the target protein were mainly localized in the cytoplasm (Fig. 2), which suggested that DF-1 and Hela cells can be used to study the function of chicken neurexophilin 1.

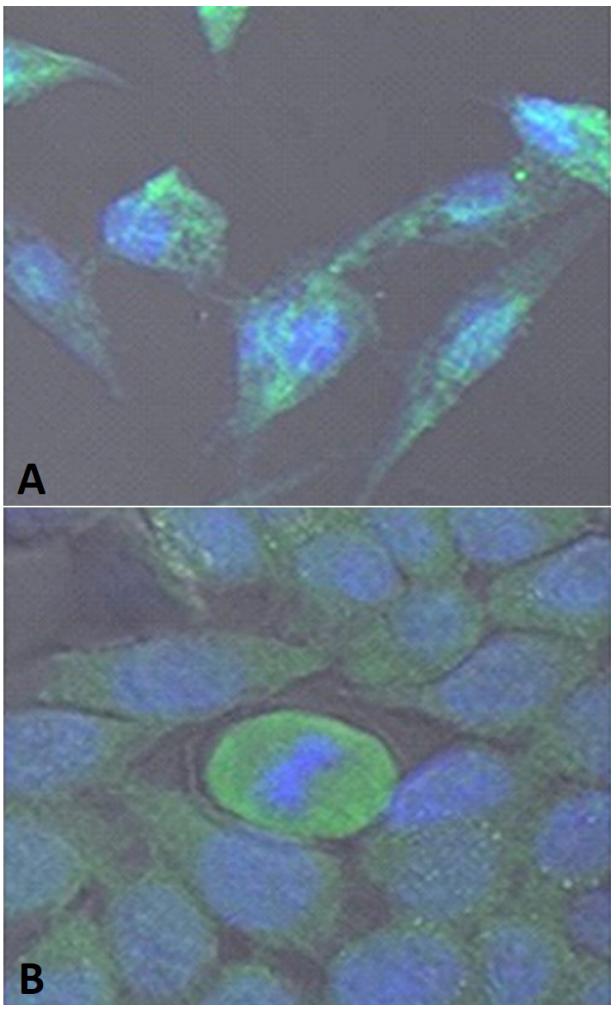

Fig. 2. Immunofluorescence against NXPH1 (Green, DAPI indicates nuclear staining) in DF-1 cells and Hela cells. A, DF-1 cell; B, Hela cell.

In order to detect whether NXPH1 is secreted from cells into culture medium, we used Western blot to determine the content of NXPH1 in the cells and culture medium. The results also showed that the target protein was found in chicken DF-1 cells and Hela cells, and the protein can be secreted into culture medium. The NXPH1 content of DF-1 cells was higher that of DF-1 culture fluid. The same trend was observed between Hela cells and its culture fluid. Moreover, NXPH1 content of DF-1 culture fluid was higher than that of Hela culture fluid (Fig. 3).

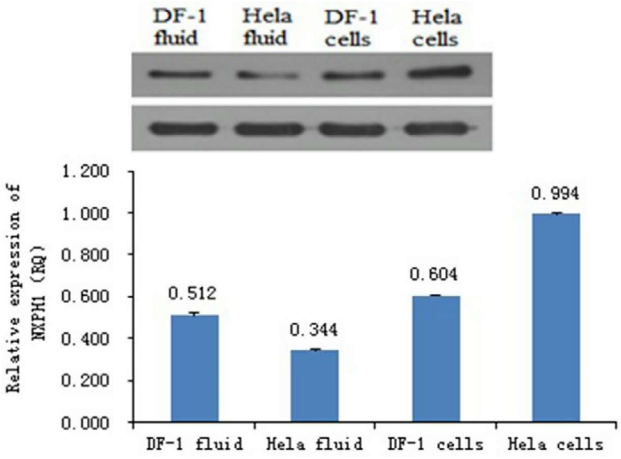

Fig. 3. Relative expression of neurexophilin 1 in cells and culture fluid.

DISCUSSION

Uterovaginal junction is the primary sperm storage tubules (SSTs) in poultry (Bakst, 2011). Neurons and small ganglia were identified in the UVJ of the turkey and chicken oviduct, and nerve fibres appeared to terminate on individual sperm storage tubules (Freedman *et al*., 2001), suggesting that neural factor may regulate sperm storage in and release of spermatozoa from the SST of hens. Neurexophilin, discovered as a 29 kDa protein that is purified with neurexin Ia on immobilized a-latrotoxin from brain (Petrenko *et al*., 1990), is synthesized in a subclass of neurons scattered throughout the nervous system (Petrenko *et al*., 1996). In rats and mice, neurexophilin 1 (Nxph1) bind tightly to the extracelluar domain of neurexin Iα (Craig and Kang, 2007) whose function involves calcium which are important factors for chicken sperm motility in vitro and in vivo storage (Thomson and Wishort, 1991; Holm *et al*., 2000). Expression of neurexophilin 1 gene in adult chicken UVJ was corresponded to fertility (Liu *et al*., 2009). Amino acid sequence analysis of NXPH1 among 18 species revealed that the second structure of NXPH1 in species with sperm storage tubules was different from that in species without SST (Liu *et al*., 2013). These data indicate that nervous system may be involved in sperm storage and/or release through Nxph1 gene in chicken.

To study the function of neurexophilin 1 in cells, it is necessary to know whether it expresses in cells. Das et

al. (2008) found that fowl spermatozoa were incubated with tissues from the infundibulum, magnum, isthmus, shell gland, uterovaginal junction and vagina can maintain motility for 12, 5, 4, 7, 11 and 4 days, respectively. The survival period was much longer for spermatozoa incubated with the tissues from the infundibulum and uterovaginal junction. The expression of neurexophilin 1 gene was elevated in the UVJ of high fertility hens (Liu *et al*., 2009). So the most suitable cells for studying the function of neurexophilin 1 are from the UVJ or infundibulum. However, these primary cells died after several generations. The established cell line, Hela cells, was found to have same effect on chicken spermatozoa motility and fertility as epithelial cells from fowl shell gland (Ashizawa *et al*., 1982). Chicken embryo fibroblast cell line (DF-1 cells) is widely used in chicken research. Here we detected that neurexophilin 1 expressed in the cytoplasm and nucleus of Hela cells, DF-1 cells and epithelial cells from chicken UVJ, suggesting that Hela and DF-1 cells can be used in studying the function of chicken neurexophilin 1. To study the effect of neuxophilin 1 on sperm motility, it is also necessary to know whether it is secreted from cells into culture medium. Neurexophilins are secreted glycoproteins and neurexophilin 1 is N-glycosylated immediately after synthesis (Craig and Kang, 2007). It's possible for NXPH1 being secreted into culture medium. We used western-blot and detected NXPH1 in Hela and DF-1 cells, and the culture liquid of these two cell lines, suggesting these two cell lines can be used to study the function of NXPH1. And the content of NXPH1 in the culture liquid of DF-1 cells were higher than that of Hela cells, indicating that it's better to use DF-1 cells in further researches than to use Hela cells.

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Compliance with ethical standards

All the essential experimental protocols included in this study were validated by the Law of Animal Husbandry in People's Republic of China (Dec 29, 2005). The entire protocols for sample collection were reviewed and legalized by the Biological Studies, Animal Care and Use Committee of National Animal Husbandry Services, Hubei, PR. China.

Statement of conflict of interest

Authors have declared no conflict of interest.

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