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·Original Article·

Gene functional research using polyethylenimine-mediated *in vivo* gene transfection into mouse spermatogenic cells

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Abstract

Aim: To study polyethylenimine (PEI)-mediated *in vivo* gene transfection into testis cells and preliminary functional research of spermatogenic cell-specific gene *NYD-SP12* using this method. **Methods:** PEI/DNA complexes were introduced into the seminiferous tubules of mouse testes using intratesticular injection. Transfection efficiency and speciality were analyzed on the third day of transfection with fluorescent microscopy and hematoxylin staining. The long-lasting expression of the GFP-NYD-SP12 fusion protein and its subcelluar localization in spermatogenic cells at different stages were analyzed with fluorescent microscopy and propidium iodide staining. **Results:** With the mediation of PEI, the *GFP-NYD-SP12* fusion gene was efficiently transferred and expressed in the germ cells (especially in primary spermatocytes). Transfection into Sertoli cells was not observed. The subcellular localization of the GFP-NYD-SP2 fusion protein showed dynamic shifts in spermatogenic cells at different stages during spermatogenesis. **Conclusion:** PEI can efficiently mediate gene transfer into spermatocytes. Thus, it might be useful for the functional research of spermatogenic-cell specific genes such as the *NYD-SP12* gene. In our study, the NYD-SP12 protein was visualized and was involved in the formation of acrosome during spermatogenesis. Our research will continue into the detailed function of *NYD-SP12* in spermatocytes. *(Asian J Androl 2006 Jan; 8: 53-59)*

Keywords: gene transfer techniques; polyethylenimine; *NYD-SP12* gene; spermatogenic cells; spermatogenesis

1 Introduction

Spermatogenesis, the fundamental function of testis, occurs in successive mitotic, meiotic and postmeiotic phases of germ cells. The germ cells move from the periphery to the lumen of the seminiferous tubule during this process. Spermatogenesis is basically controlled by the programmed expression of a number of stage-specific genes, some of which have so far been identified as spermatogenic cell-specific genes that perform intrinsic regulation on spermatogenesis [1, 2]. Functional research of these genes would shed light on the understanding of the biological mechanisms of spermatogenesis and facilitate the treatment of male infertility. Our laboratory has identified a number of spermatogenic cell-specific genes by microchip screening [1, 2]. One of these, NYD-SP12, was thought to be a Golgi apparatus-associated protein [1]. In order to know the potential role of these genes in spermatogenesis, we are seeking a convenient and efficient method that could circumvent gene target-

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ing and spermatogenic cell culture.

Although transgenic mice offer ideal models for research into gene function, the technique of producing transgenic mice is laborious and time-consuming. Moreover, lethality before adolescence in transgenic mice precludes detecting the role of a candidate gene in spermatogenesis. Even when survival to later stages occurs, extrinsic defects might confound the interpretation of spermatogenic phenotypes. Under such circumstan-ces, several *in vivo* gene transfer methods were developed during the past decade, such as microparticle bombardment [3], electroporation [3, 4], adenoviral mediation [5] and lentiviral mediation [6]. However, these techniques were found to preferentially transfect Sertoli cells or both Sertoli and germ cells, but not spermatogenic cells alone, which disappointed us in conducting functional research of spermatogenic cell-specific genes. The recently developed technique of germ cell transplantation into the seminiferous tubules [7–9] offered an excellent *in vivo* model for intrinsic regulation research of spermatogenesis. Nevertheless, this technique was greatly limited by the insufficient availability of spermatogenic stem cells in the testis. Thus, a simple and effective germ cell-oriented *in vivo* gene transfer method was urgently needed for the functional research of spermatogenic cell-specific genes.

Recently, the linear 22 kDa form of polyethylenimine (PEI) has shown to function as an effective nonviral vector for both *in vitro* and *in vivo* gene transfer [10, 11]. Because its receptor proteoglycan is a common component of cell membranes [12], PEI was supposed to be used to mediate gene transfection into all kinds of cells. However, it has not been tested in testis. In this study, we tested whether PEI could mediate gene transfer into spermatogenic cells using intratesticular injection. Then, using the same technique, we further investigated the functional role of the *NYD-SP12* gene by its subcellular localization in spermatogenic cells at different stages. The *NYD-SP12* gene is a spermatogenic cell-specific gene and its protein has been proved to be associated with Golgi apparatus [1]. Golgi is an important organelle in sperm acrosome formation, therefore the functional role of the *NYD-SP12* gene was predicted to be involved in this process. In this study, long-term observational results revealed that *NYD-SP12* was involved in the formation of acrosome, which implicated its potential role in spermatogenesis and sperm-egg fusion.

2 Materials and methods

2.1 Mice

Adult (8-week-old) ICR male mice (weight 30-40 g) were obtained from the Animal Center of Nanjing Medical University (Nanjing, China). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University.

2.2 Plasmids

Plasmid pEGFP, encoding green fluorescent protein (GFP) driven by the cytomegalovirus (CMV) promoter, was purchased from Clontech (Mountain View, CA, USA). Plasmid *pEGFP-NYD-SP12* was constructed by inserting the *NYD-SP12* open reading frame (nucleotides 131–1840) into plasmid *pEGFP*, as described in detail by Xu *et al*. [1]. The plasmids were amplified in DH5a host bacteria, then extracted and purified with the Qiagen Plasmid Mini Kit (Valencia, CA, USA) according to the manufacturer's instructions. The extracted plasmids were dissolved in 5 % sterilized glucose and stored at –20 ºC. For *in vivo* gene transfections, plasmid and PEI (ExGen 500, Fermentas, Burlington, Ontario, Canada) were mixed at a PEI nitrogen: DNA phosphate ratio of 6, according to the manufacturer's suggestion. The total volume of 10 μL (containing 3 μg plasmid) was injected into each testis.

2.3 Intratesticular injection

Mice were anesthetized with urethane and testes were exteriorized through a midline abdominal incision of approximately 5 mm. Glass microneedles were produced from capillary tubing (G-100; Narishige Instruments, Setagaya-ku, Tokyo, Japan) using a vertical pipette puller (model PB-7; Narishige Instruments, Setagaya-ku, Tokyo, Japan) and the tips were finely beveled with a pipette grinder (model EG-4; Narishige Instruments, Setagaya-ku, Tokyo, Japan) to a final diameter of 30 μm–50 μm. The intratesticular injection procedure was previously described [13], through which 70 % – 85 % of the seminiferous tubules could be introduced with transfection materials [6, 13]. After injection, the testes were put back into the abdomen and the incisions were sutured. The mice were recovered and raised until analysis.

Initially, 0.2 % trypan blue was used as an indicator to make sure that the microinjection was successful. However, as the dye was found to form adverse deposits with PEI, no trypan blue was used throughout the

gene transfection procedures.

2.4 PEI-mediated gene transfection analysis

The mice were divided into three groups: PEI (PEI alone); *pEGFP-NYD-SP12* (*pEGFP-NYD-SP12* alone); and PEI+*pEGFP-NYD-SP12* (PEI-mediated gene transfection of *pEGFP-NYD-SP12*). The former two groups served as controls. Each group contained three mice.

Three days after gene transfection, the mice were killed by cervical dislocation and the testes were removed, embedded in OCT (Frozen tissue matrix) and frozen-sectioned at a thickness of 5 μm. The slides were then analyzed by fluorescent microscope (Zeiss Axioskop 2; Carl Zeiss, Oberkochen, Germany), to determine the transfection efficiency of PEI and subsequently visualized by light microscopy after 4 g/L hematoxylin (Sigma, Louis, MO, USA) staining. The corresponding fluorescent and light images were then contraposed for the identification of positively transfected cell types in the seminiferous tubules.

2.5 Identification of NYD-SP12 functional role by subcellular localization in different stages of spermatogenic cells

PEI plus *pEGFP-NYD-SP12* was injected into mice seminiferous tubules as described previously [13]. The mice were killed on days 3, 6, 10, 15, 20, 30 and 60 after gene transfection. PEI-mediated pEGFP gene transfection was used as a control. Mice in the PEI+pEGFP group were killed at day 10 after gene transfection. Three mice were killed at each time point. The testes were removed, embedded in OCT (Frozen tissue matrix) and frozen-sectioned at a thickness of 5 μm. After 4 % paraformaldehyde (PFA) fixation for 20 s, the sections were stained for 5 s in 0.5 % propidium iodide (PI) for the visualization of nuclei. The slides were then rinsed with distilled water, alcohol dehydrated, and visualized under fluorescent microscope for the detection of *GFP-NYD-SP12* gene expression. The images were then superimposed to determine the subcellular localization of *GFP-NYD-SP12* gene expression.

Additionally, sperms were collected from the epididymis of transfected mice and washed three times with phosphate-buffered saline. After fixation in 4 % PFA for 30 min, sperms were spread on slides and viewed by fluorescence microscopy. Sperms from three mice were examined separately.

3 Results

3.1 Intratesticular injection

Successful intratesticular injection was achieved, as traced by trypan blue, shown in Figure 1.

3.2 PEI-mediated gene transfection efficiency

PEI-mediated *pEGFP-NYD-SP12* gene transfection efficiency was much higher than that of *pEGFP-NYD-SP12* alone (Figure 2). *pEGFP-NYD-SP12* was hardly transferred into the seminiferous tubules when PEI was not applied (Figure 2).

3.3 PEI-mediated gene transfer was spermatocyte-preferential in seminiferous tubules

When we contraposed fluorescent and light microscopic images, we found that PEI-mediated transfection of *pEGFP-NYD-SP12* was achieved mostly in spermatocytes (Figure 3A). Most *GFP-NYD-SP12* positive spermatocytes (white arrow, Figure 3A) also had a strongly stained particle within its nucleus (dark arrow, Figure 3B), which represented the condensed PEI [14]. However, approximately 10 % of PEI positive spermatocytes were *GFP-NYD-SP12* negative (Figure 3, yellow circles). Sertoli cells seemed to be neither GFP-*NYD-SP12* nor PEI positive (Figure 3, red arrows).

3.4 Identification of NYD-SP12 functional role by subcellular localization in different stages of spermatogenic cells

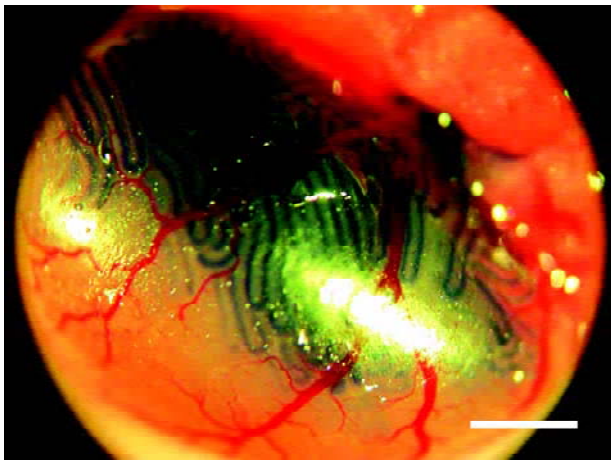

Figure 1. Intratesticular injection through the efferent duct, traced by 0.2 % trypan blue. Stereomicroscopic image, Bar = 1 mm.

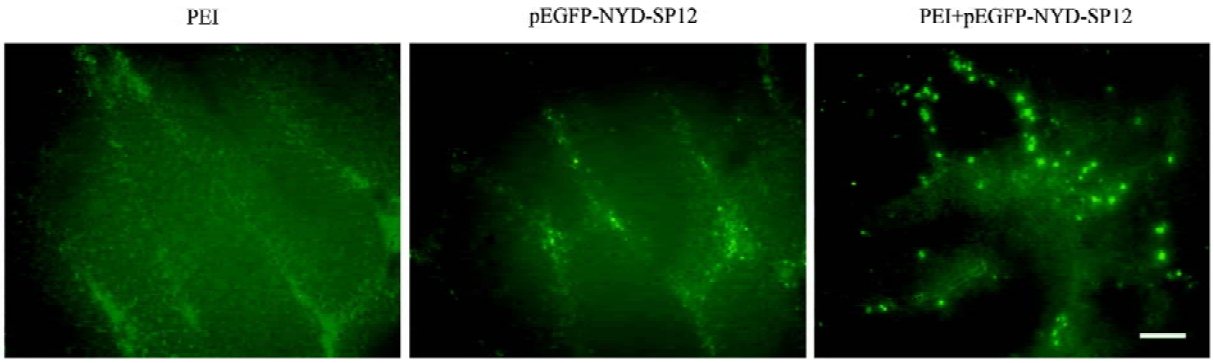

Figure 2. Polyethylenimine (PEI)-mediated gene transfection efficiency. The transgene was highly expressed in the seminiferous tubules in the PEI+ *pEGFP-NYD-SP12* group compared with the *pEGFP-NYD-SP12* group. PEI, PEI injection group; *pEGFP-NYD-SP12*, *pEGFP-NYD-SP12* injection group; PEI+*pEGFP-NYD-SP12*, PEI plus *pEGFP-NYD-SP12* injection group. Fluorescent microscope images (at 450 nm – 490 nm excitation wavelength). Bar = 50 μm.

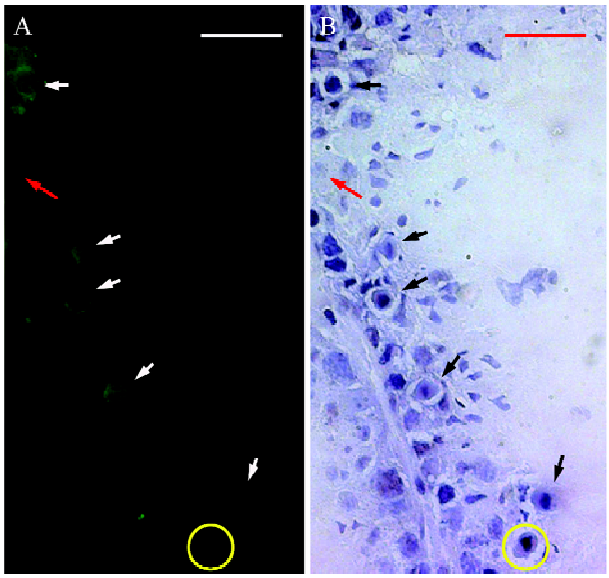

Figure 3. Polyethylenimine (PEI)-mediated *pEGFP-NYD-SP12* gene transfection was spermatogenic cell preferential. (A): Fluorescent microscope image (450 nm – 490 nm excitation), (B): Light microscope image (hematoxylin staining). Positively transfected cells were identified as green fluorescent protein (GFP) positive (white arrows in A) and PEI positive (deeply stained PEI particles in the nucleus, dark arrows in B). Most transfected cells (GFP and PEI positive) were spermatocytes. Approximately 10 % of PEI positive spermocytes were GFP negative (yellow circles). Sertoli cell (red arrow) was GFP and PEI negative. Bar = 10 μm.

Three days after gene transfection, *GFP-NYD-SP12* gene expression could be detected in some spermatogonia, mostly spermatocytes and a few round spermatids in the seminiferous tubules. However, transgene expression in elongated spermatids could not be detected until day 10. At this point, round and elongated spermatids made up the majority of the *GFP-NYD-SP12* positive cells. *GFP-NYD-SP12* gene expression in the seminiferous tubules could last for more than 1 month after transfection.

Furthermore, the subcellular localization of *GFP-NYD-SP12* revealed that the fusion protein appeared to shift from Golgi to acrosome (Figure 4A–G), whereas GFP did not exhibit such a translocation profile (Figure 4H, I). Twenty days after transfection, GFP-NYD-SP12 protein could be detected in the acrosomes of sperms in the epididymis (Figure 4G), however, the efficiency was rather low, approximately 1:10 000.

4 Discussion

Functional research of spermatogenic cell-specific genes is important for the understanding of spermatogenesis and would facilitate the treatment of male infertility. Previous studies using *in vivo* gene transfer methods [3–6] circumvented the gene targeting and spermatogenic cell *in vitro* culture. However, these newly-developed methods preferentially affected Sertoli cells but not germ cells. In this study, we showed that the PEI-mediated *in vivo* gene transfection method using intratesticular injection was efficient and convenient, and that this method was spermatogenic cell preferential. This offered a new method for the functional research of spermatogenic cell-specific genes. However, it needs to be further investigated whether the method was a selective transfer into sper-

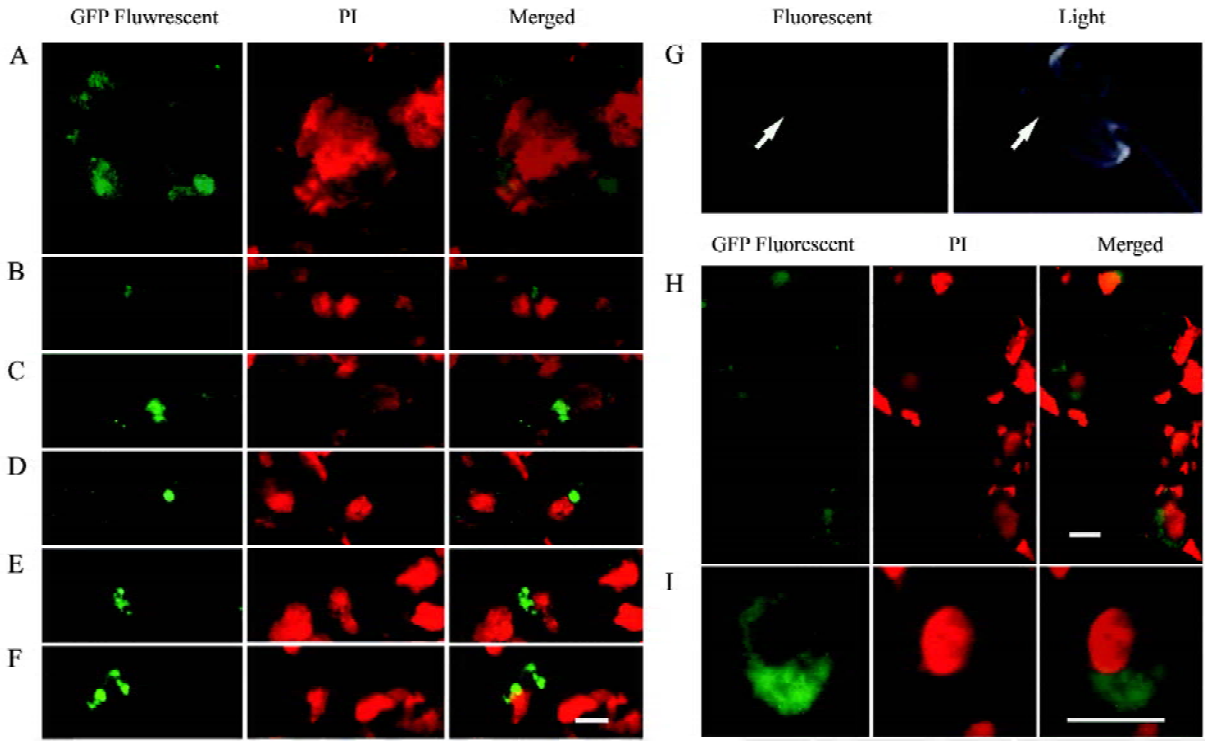

Figure 4. Subcellular localization of *NYD-SP12* in spermatogenic cells at different stages. (A – G): PEI+*pEGFP-NYD-SP12* transfection, (H, I): PEI+pEGFP transfection served as the control. (A): Primary spermatocyte with *GFP-NYD-SP12* dispersed in cytoplasm; (B): spermatocyte in the telophase of meiosis I or II. *GFP-NYD-SP12* localized at one pole and the middle zone between the two daughter cells; (C): round spermatid. *GFP-NYD-SP12* congregated at one pole; (D): round spermatid. *GFP-NYD-SP12* condensed in an acrosome vehicle-like structure at one pole; (E): elongating spermatid. *GFP-NYD-SP12* initiated elongation along one side of the nucleus; (F): elongated spermatid. *GFP-NYD-SP12* further elongated along one side of the nucleus; (G): spermatozoon. *GFP-NYD-SP12* located in acrosome of spermatozoon; (H): polyethylenimine-mediated pEGFP transfection in seminiferous tubules; (I): round spermatid. Green fluorescent protein (GFP) diffused in cytoplasm. PI, propidium iodide. Bar = 5 μm.

matogenic cells.

Despite the relatively low transfection efficiency as compared with viral vectors, cationic vectors retain a high attractiveness in gene transfer due to their theoretically excellent safety profile. As a new member of the cationic vector family, PEI performed more efficiently in mediating gene transfection and expression in mammalian cells as compared with other cationic vectors [15]. The transfer activity of PEI is related to its ability to condense DNA, interact with anionic proteoglycans of the cell membrane [10,16,17], protect DNA [18], and induce endosomal swelling and rupture prior to DNA degradation [19]. PEI or PEI/DNA complexes could be transported to the nucleus and exhibited as distinct structures [12], which could be visualized by light microscopy after hematoxylin staining. By contraposing the fluorescent and light images we found that the positive cells were mainly spermatocytes. Transfection into Sertoli cells was not observed. Because the CMV promoter is well known for its ubiquitous activity of initiating transcription in various eukaryotic systems, the possibility of a successful *GFP-NYD-SP12* gene transfection into Sertoli cells without transcription/expression under a CMV promoter is very slim. Furthermore, in order to make clear whether this was caused by the difference between a spermatogenic-specific gene (like *GFP-NYD-SP12*) and a non-spermatogenic-specific gene, a GFP vector (without the fusion with the *NYD-SP12* gene) was used as a control. The results were the same as that for *GFP-NYD-SP12* (data not shown). Thus, the most reasonable conclusion is that PEI-mediated gene transfection is spermatogenic cell selective. However, the detailed mechanisms of this pro-

cess need to be investigated further.

PEI-mediated gene transfection in the seminiferous tubules occurred in a restricted population of spermatogenic cells, mainly the spermatocytes. The efficiency was approximately five cells per tubule. Approximately 10 % of PEI positive cells did not show a GFP signal, which might have resulted from the sole transfection of PEI (excessive PEI might be applied for PEI/DNA interaction), or the expression silence of the unlinearized plasmid DNA. The lower transfection efficiency in spermatogonia was most likely due to their location in the basal compartment of the seminiferous tubules where the PEI/DNA complexes were less accessible. We also noticed that much more *GFP-NYD-SP12* positive round and elongated spermatids could be found on day 10 compared with day 3. We thought that GFP-NYD-SP12 protein in round and elongated spermatids might not be the result of direct transfection but that of inheritance from the positively transfected spermatocytes. GFP-NYD-SP12 protein detected in spermatogozoa on day 20 might be explained in the same way. This could also explain why there were fewer GFP positive sperms and weaker GFP signals in sperms as compared with spermatocytes. After meiosis, a diploid spermatocyte divides into four haploid spermatids, so the transgene (*GFP-NYD-SP12*) and the GFP signal are also distributed into four daughter cells. PEI-mediated gene transfection is also transient. Nevertheless, more efficient transfection could be achieved if this method was modified and developed.

The *NYD-SP12* gene was cloned from human testis through the microarray technique in our previous work [1]. It was specifically expressed in spermatogenic cells, and its product was proved to localize in Golgi apparatus [1]. As Golgi is an important organelle in the formation of sperm acrosome, the *NYD-SP12* gene was assumed to relate to this process. In the present study, this proposal was confirmed by the PEI-mediated *in vivo* gene transfection method. By the subcellular localization of exogenous *GFP-NYD-SP12* in different stages of spermatogenic cells, the functional role of the *NYD-SP12* gene in spermatogenesis was revealed to be involved in the process of acrosome formation. In brief, along with spermatogenesis, the GFP-NYD-SP12 fusion protein dispersed in the cytoplasm of spermatocytes at the beginning of transfection, and subsequently congregated at one pole in round spermatids where it further formed the acrosome vehicle-like structure. It then elongated along one side of the nucleus in elongated spermatids, and finally located in the acrosome of spermatozoon. All of this implicated that NYD-SP12 protein might play important roles in acrosome formation, spermatogenesis and, probably, in the later sperm-egg fusion.

PEI-mediated *in vivo* gene transfection offered a convenient and efficient method for the functional research of genes related to the intrinsic regulation of spermatogenesis. By this method, the functional role of the *NYD-SP12* gene related to Golgi apparatus/acrosome formation was visually identified, which provided important information for further research.

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