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Role of Glial Cell Line–Derived Neurotrophic Factor in Germ-Line Stem Cell Fate

Laura Braydich-Stolle^a, Courtney Nolan^a, Martin Dym^b, and Marie-Claude Hofmann^a

^aDepartment of Biology, University of Dayton, Dayton, Ohio 45469, USA

^bDepartment of Cell Biology, Georgetown University Medical Center, Washington, DC 20057, USA

Abstract

The overall goal of this study is to unravel the role(s) played by glial cell line-derived neurotrophic factor (GDNF) in the fate of spermatogonial stem cells. There is great interest in the biology of spermatogonial stem cells, or A_{single} spermatogonia, because of their importance in the treatment of infertility, the development of contraceptives, and the understanding of the etiology of testicular cancer, particularly seminoma. In the mouse, spermatogonial stem cells express GFR α -1, the receptor for GDNF, and respond to this growth factor *in vivo* and *in vitro*. GDNF is produced by the adjacent Sertoli cells, which are part of the germ-line stem cell niche in vertebrates. We specifically isolated GFR α -1–positive spermatogonia using an immunomagnetic bead technique. We then stimulated the cells with 100 ng/mL of rGDNF for 10 hours; unstimulated cells served as negative controls. Microarray analysis, immunocytochemistry, and Western blotting revealed that Numb, a regulator of the Notch pathway, is upregulated by GDNF in spermatogonial stem cells. There are indications that in rats, mice, and humans, the Notch pathway promotes spermatogonial differentiation. We observed that an increase in Numb expression is concomitant with Notch degradation in these cells. Thus, through Numb, GDNF might inhibit differentiation and allows the maintenance of the stem cell pool in the mouse seminiferous epithelium.

Keywords

GDNF; Notch; Numb; spermatogonia; testis stem cells

INTRODUCTION

In mammals, spermatogenesis is a tightly controlled process that takes place within the seminiferous tubules of the testes and leads to sperm production. This process depends on the self-renewal and differentiation of spermatogonial stem cells (SSCs), or A_{single} spermatogonia, which are adjacent to the basement membrane of the seminiferous epithelium. Self-renewal and differentiation of the SSCs are regulated by soluble factors produced by the somatic Sertoli cells. One of these growth factors is glial cell line–derived neurotrophic factor (GDNF), which stimulates the proliferation of the A_{single} spermatogonia and their direct progeny, the A_{paired} spermatogonia, *in vivo* and *in vitro*.^{1,2} GDNF signals through a two-component receptor system comprising a GPI-anchored co-receptor called GFR α -1, and the Ret transmembrane receptor. Binding of GDNF to GFR α -1 and Ret activates Ret auto-phosphorylation and the intracellular relay of the signal.³

Signaling pathways leading to spermatogonial stem cell self-renewal and differentiation are poorly understood. This is due to the fact that this stem cell population is small (0.03% of the total number of germ cells) and difficult to specifically isolate. In order to elucidate which genes are differentially regulated by GDNF, we recently performed microarray analysis of pure GFR α -1-positive mouse spermatogonia isolated through the STAPUT method followed by magnetic activated cell sorting (MACS).² Some of the genes differentially regulated by GDNF appeared to belong to the Notch signaling pathway, as presented in TABLE 1. While GDNF does not influence the expression of Notch-1, -2, -3, or presenilin, it seems to upregulate the expression of the gene *numb*, a known repressor of Notch signaling.⁴ The Notch proteins (Notch 1–4) form a family of transmembrane receptors mediating juxtacrine signaling. These receptors and their ligands have been intensively studied in *Drosophila* and *C. elegans* and more recently in vertebrates.⁴ Notch signaling regulates stem cell fate choices in different tissues, generally by adjusting the cell's response to differentiation stimuli instead of specifying a cell's fate directly. Therefore, depending on the tissue and environmental cues, Notch signaling can promote stem cell self-renewal or differentiation. Once a Notch receptor is activated by its ligand, the receptor is cleaved proteolytically and an active form of the receptor is released. This active form is called the Notch intracellular domain (NICD). NICD can then enter the nucleus, where it acts as a transcription factor.⁵ One modulator of Notch is Numb, a cytoplasmic protein that binds to NICD and inhibits its action by promoting either its endocytosis or ubiquitination.^{6,7}

Notch receptors and their homologues are expressed in the germ line, and seem to promote germ cell proliferation in *C. elegans* and *Drosophila*.^{8,9} There are reports that the Notch signaling pathway promotes spermatogonial stem cell differentiation in humans.¹⁰ The exact function of Notch signaling in murine spermatogonial stem cells is not known, but it has been hypothesized that it could promote differentiation as well.^{11,12} In the mammalian testis, the ligands for the Notch receptors, Jagged-1 and Jagged-2, are expressed by Sertoli cells. The present study investigated the influence of GDNF on the protein expression of Numb and the Notch-1 intracellular domain (NICD) in spermatogonial stem cells. Our data indicate that GDNF might induce self-renewal through the production of Numb and the degradation of Notch-1.

MATERIAL AND METHODS

Isolation and Culture of GFR α -1-Positive Cells

Testes were isolated from 60 mouse pups at 6 dpp. The tunica was removed to expose the seminiferous tubules. The tubules were digested with a sequential enzymatic treatment to yield a single cell suspension. The cells were then separated using the STAPUT method followed by magnetic activated cell sorting (MACS) using an anti-mouse GFR α -1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).² The GFR α -1-positive cells were seeded in microtiter plates at a concentration of 5×10^4 cells per well, in minimal media consisting of D-MEM/F12 complemented with 10% NU synthetic serum (Fisher Scientific, Pittsburgh, PA).

Cultures of Seminiferous Tubules

Testes were removed from immature and adult mice and the tunica was removed to expose the seminiferous tubules. The tubules were then digested with 1 mg/mL of collagenase and 1 μ g/mL DNase to remove the basement membrane and expose the GFR α -1-positive cells. The tubules were placed in minimal media in 24-well tissue culture plates overnight and used for the experiments the next day.

GDNF Treatment

GFR α -1–Positive Spermatogonia—After STAPUT/MACS isolation, the spermatogonia were cultured for 1 day in microtiter plates containing minimal media with or without 100 ng/mL of GDNF. The next day, the cultures were fixed *in situ* with ice-cold methanol, the protein Numb revealed by immunocytochemistry and its expression evaluated directly in the culture plates using an Olympus Nikon Eclipse TE 200 inverted microscope.

Organ Cultures—The digested seminiferous tubules were treated with 100 ng/mL GDNF (R&D Systems, Minneapolis, MN) or 100 ng/mL GDNF + 5 μ g/mL GDNF neutralizing antibody (R&D Systems). Untreated tubules were used as a control. Fresh treatments were applied daily for 3 days, and then proteins were isolated as described below.

Protein Isolation, Concentration, and Western Blotting

The seminiferous tubules were homogenized in a modified RIPA lysis buffer to extract the proteins. The samples were then concentrated using centrifugal concentrators from Millipore (cutoff = 30,000 Da)(Millipore, Bedford, MA). After the samples were concentrated, their protein content was determined using the Bio-Rad DC assay (BioRad Laboratories, Hercules, CA). Twenty-five μ g of proteins/lane were run on a 10% Tris-HCl gel and transferred to nitrocellulose membranes. The membranes were probed with antibodies for Numb and Notch-1 (Santa Cruz Biotechnology), and revealed using the immunoperoxidase technique and 4-chloronaphthol. Experiments were done in triplicate. The testis isoform of the Numb protein has a molecular weight of 72 kDa, and the Notch-1 intracellular domain has a molecular weight of 120 kDa.

RESULTS AND DISCUSSION

The study of spermatogonial stem cells and their niche is of paramount importance to understand the mechanisms of self-renewal and differentiation of unipotent stem cells, and to elucidate which disruptions of specific signaling pathways lead to male infertility or testicular neoplasias. In the present study, we showed that the treatment of isolated spermatogonial stem cells and seminiferous tubules with 100 ng/mL GDNF increases the expression of the protein Numb, which confirmed our results obtained by microarray analysis and semi-quantitative RT-PCR.² As shown in FIGURE 1, immunocytochemistry data revealed that after overnight treatment with GDNF *in vitro*, many A_{single} spermatogonia express Numb (73%), while the staining was not observed in absence of GDNF. Further, we specifically showed that only the testis isoform of Numb (MW = 72 kDa) is upregulated (FIG. 2, upper panel). Numb is a membrane-associated protein that functions as a determinant of cell fate during *Drosophila* and mouse development. Four specific isoforms of mammalian Numb exist, with molecular weights of 65, 66, 71, and 72 kDa that are generated by alternative splicing of the Numb mRNA. The expression of some of these isoforms is regulated in a cell type-specific manner.¹³ One function of Numb is the regulation of the Notch-1 signaling pathway by promoting the endocytosis or ubiquitination of the Notch intracellular domain (NICD).^{6–7} We next tested the samples for the expression of Notch-1 and the NICD protein in presence or absence of GDNF. As shown in FIGURE 2 (lower panel), the presence of GDNF lowers the amount of NICD available, in comparison to control samples without GDNF, or with GDNF treated with a neutralizing antibody. These data allow us to devise the following working model: the juxtracrine signaling initiated by the binding of Jagged-1 and Notch-1 induces differentiation, as suggested by others.^{10–12} GDNF, if present at a sufficient concentration, will promote the production of Numb, which will be able to degrade enough NICD to block the differentiation process and thus favor spermatogonial stem cell self-renewal. This model is in accord with the data of the group of H. Sariola, which showed that the dosage of GDNF is important. Indeed, in GDNF heterozygous knockout mice, spermatogonial differentiation prevails over self-

renewal, leading to spermatogonial depletion and Sertoli cell-only syndrome. On the other side, the overexpression of GDNF in transgenic mice induces aberrant proliferation of spermatogonial stem cells and eventually seminoma-like tumors.14•15

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FIGURE 1.

Expression of Numb in Asingle spermatogonia treated with GDNF. GFR α -1-positive spermatogonia were isolated using the STAPUT/MACS method, cultured overnight in presence or absence of 100 ng/mL GDNF and processed for Numb staining by immunocytochemistry. The percentage of positive/negative Asingle cells is shown.

FIGURE 2.

Expression of Numb and Notch-1 in seminiferous tubules treated with GDNF. Seminiferous tubules were cultured for 3 days in presence of 100 ng/mL GDNF and prepared for SDS-PAGE/ Western blotting. (A) No GDNF; (B) GDNF; (C) GDNF + 5 μg/mL neutralizing antibody.

Upper panel: Western blot for Numb, showing upregulation of the testis isoform of Numb by GDNF (arrowhead). *Lower panel:* Western blot for Notch-1, showing downregulation of Notch-1 and NICD by GDNF.

TABLE 1

Differential expression of genes influenced by GDNF and belonging to the Notch signaling pathway

Name	-Fold Expression (microarray analysis)	GenBank accession #
Notch-1	1.20	Z11886
Notch-2	1.10	D32210
Notch-3	1.10	X74760
Numb	4.44	U70674
Presenilin	-1.84	U57325