MECHANISMS OF DEVELOPMENT 125 (2008) 906–917

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/modo

Promotion of spermatogonial proliferation by neuregulin 1 in newt (*Cynops pyrrhogaster*) testis ☆

Ozlem Oral[a], *Ichiro Uchida*[a], *Ko Eto*[a], *Yuki Nakayama*[a], *Osamu Nishimura*[b], *Yukako Hirao*[b], *Junko Ueda*[b], *Hiroshi Tarui*[b], *Kiyokazu Agata*[c], *Shin-Ichi Abé*[a,*]

[a]*Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan*
[b]*RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[c]*Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan*

ARTICLE INFO

Article history:
Received 19 April 2008
Received in revised form
20 May 2008
Accepted 2 June 2008
Available online 11 June 2008

Keywords:
Newt
Microarray
FSH
Spermatogonia
Neuregulin 1
Ig-type
CRD-type
Culture

ABSTRACT

We have previously shown that mammalian follicle-stimulating hormone (FSH) promotes the proliferation of spermatogonia and their differentiation into primary spermatocytes in organ culture of newt testis. In the current study, we performed microarray analysis to isolate local factors secreted from somatic cells upon FSH treatment and acting on the germ cells. We identified neuregulin 1 (NRG1) as a novel FSH-upregulated clone homologous to mouse NRG1 known to control cell proliferation, differentiation and survival in various tissues. We further isolated cDNAs encoding two different clones. Amino acid sequences of the two clones were 75% and 94% identical to *Xenopus leavis* immunoglobulin (Ig)-type and cysteine-rich domain (CRD)-type NRG1, respectively, which had distinct sequences in their N-terminal region but identical in their epidermal growth factor (EGF)-like domain. Semi-quantitative and quantitative PCR analyses indicated that both clones were highly expressed at spermatogonial stage than at spermatocyte stage. *In vitro* FSH treatment increased newt Ig-NRG1 (nIg-NRG1) mRNA expression markedly in somatic cells, whereas newt CRD-NRG1 (nCRD-NRG1) mRNA was only slightly increased by FSH. To elucidate the function of newt NRG1 (nNRG1) in spermatogenesis, recombinant EGF domain of nNRG1 (nNRG1-EGF) was added to organ and reaggregated cultures with or without somatic cells: it promoted spermatogonial proliferation in all cases. Treatment of the cultures with the antibody against nNRG1-EGF caused remarkable suppression of spermatogonial proliferation activated by FSH. These results indicated that nNRG1 plays a pivotal role in promoting spermatogonial proliferation by both direct effect on spermatogonia and indirect effect via somatic cells in newt testes.

1. Introduction

Spermatogenesis is an essential process for sexual reproduction in development. It is triggered by the sequential mitotic divisions of spermatogonia, followed by their differentiation into spermatocytes. After two meiotic divisions, spermatocytes develop into spermatids, which possess half the normal complement of genetic material, and then

☆ This work was supported by Grants-in-Aid for Scientific Research (No. 18370025) from the Ministry of Education, Science, Sports and Culture of Japan.

* *Corresponding author. Tel./fax: +81 96 342 3437.*
E-mail address: abeshin@gpo.kumamoto-u.ac.jp (S.-I. Abé).

0925-4773/$ - see front matter
doi:10.1016/j.mod.2008.06.004

into spermatozoa, mature male gametes in many sexually reproducing organisms. This complex process is controlled by cooperation with several hormones and testicular somatic cells. Follicle-stimulating hormone (FSH) is one of the most important hormones required for early gonadal development, maturity and function (Sairam and Krishnamurthy, 2001). It is secreted by the pituitary gland and act on testicular somatic cells (Means et al., 1980; Ritzen et al., 1981; Hodgson et al., 1983; Griswold et al., 1988), mainly Sertoli cells, through the specific receptor. Sertoli cells have essential roles in the regulation of spermatogenesis. They not only represent the only cellular component of the blood–testis barrier but also produce and secrete local factors to germ cells (Dym et al., 1997).

In mammalian testis, Sertoli cells have been shown to synthesize stem cell factor (SCF) (Motro et al., 1991; Tajima et al., 1991), which plays a pivotal role in survival (Packer et al., 1995; Yan et al., 2000), proliferation and differentiation (Yoshinaga et al., 1991; Sette et al., 2000) of germ cells expressing the receptor c-kit (Manova et al., 1990; Sorrentino et al., 1991). Insulin-like growth factor-I (IGF-I) is another factor shown to act specifically on spermatogonia and primary spermatocytes of rainbow trout (Loir and Le Gac, 1994). The mRNAs for IGF-I and its receptors are present to a greater extent in Sertoli cell-enriched populations and in those containing spermatogonia and primary spermatocytes (Le Gac et al., 1996). These findings may define SCF and IGF-I as paracrine factors derived from Sertoli cells in the regulation of spermatogenesis.

In newt testis, we have demonstrated that FSH alone is sufficient to stimulate spermatogonial proliferation and differentiation into primary spermatocytes through the receptor expressed on Sertoli cells in both organ and reaggregate cultures (Abé and Ji, 1994; Abé, 2004), but not in the culture of spermatogonia alone (Ito and Abé, 1999). In addition, we reported that SCF and IGF-I, both of which are upregulated in response to FSH (unpublished data; Yamamoto et al., 2001), induce spermatogonial proliferation (Abé et al., 2002) and differentiation (Nakayama et al., 1999), respectively, in the organ culture. Recently, we have demonstrated that the blood-testis barrier formed by Sertoli cells show size selectivity, which allows small molecules (~500 Da) to penetrate into the cyst, but not larger ones (>1.9 kDa) in the organ culture (Jin et al., 2008). Therefore, SCF and IGF-I induce spermatogonial proliferation and differentiation indirectly in the organ culture. Though it is thus considered that the FSH effects are necessary to be mediated by local paracrine factors, which are secreted from Sertoli cells and act directly on spermatogonia within the testis, such factors have not been identified yet.

Because of the complex anatomical organization of the germinal compartment in mammalian testis (the Sertoli cells are normally associated with four or five germ cell generations), it is difficult to examine the stage-specific effects of the regulatory factors in the organ culture (Yamamoto et al., 2001). In contrast, clonally derived germ cells are enclosed in a cyst, the smallest unit of the testis, and surrounded by Sertoli cells within the lobules in newt testis (Callard, 1991; Abé, 2004). Therefore, newt testis is an appropriate model to isolate germ cells at a specific stage and study the effect of various factors on specific stage of spermatogenesis.

We aimed to identify FSH-upregulated local factors exerted directly in spermatogonial proliferation and differentiation. To this end, an EST (Expression Sequence Taq) library containing 5321 clones was prepared from newt testes and differentially screened by microarray analysis. We identified neuregulin 1, termed as newt neuregulin 1 (nNRG1), as one of the novel FSH-upregulated clones and isolated partial cDNAs encoding two different clones (newt immunoglobulin (nIg)-type and newt cysteine-rich domain (nCRD)-type). First, we examined their expressions in spermatogenic stages and testicular cell types by semi-quantitative and quantitative RT-PCR. Next, we tested the effects of nNRG1 on spermatogonial proliferation with recombinant epidermal growth factor (EGF)-like domain of nNRG1 (nNRG1-EGF) in organ culture and reaggregated culture with or without somatic cells. The data indicate that nNRG1 plays a pivotal role in promoting spermatogonial proliferation by acting both directly and indirectly via somatic cells on spermatogonia in newt testis.

2. Results

2.1. *cDNA cloning and structure of nNRG1 isoforms*

To screen out FSH-upregulated genes, we performed microarray analyses of a cDNA library including 5321 independent clones isolated from newt testis containing spermatogonial and early primary spermatocyte stages, and hybridized them with cDNA synthesized from total RNA extracted from testes 48 h after FSH or vehicle injection. NRG1 was identified as one of the 61 FSH-responsive cDNA clones. Nucleotide sequencing revealed that the clone contained an approximately 637 bp cDNA with 74% identity to mouse NRG1 belonging to members of the NRG family conserved among mammals (Falls, 2003) and amphibians (Yang et al., 1998, 1999). RT-PCR analysis confirmed that the mRNA expression of NRG1 gene was induced by FSH at the spermatogonial stages of newt testis (data not shown). Therefore we referred to the clone as newt NRG1 (nNRG1).

As the single NRG1 gene produces many isoforms (Burden and Yarden, 1997; Buonanno and Fischbach, 2001; Falls, 2003), we aimed to isolate the cDNAs encoding nNRG1 clones in the newt testis. PCR was performed with primers based on the sequences in the extracellular domain of *Xenopus laevis* Ig-type and CRD-type NRG1. Using newt testes cDNA as a template, two different clones of approximately 500 bp were obtained.

Cloning and sequence analysis of the clones revealed that the first clone was 483 bp in length, including several domains such as the Ig domain and EGF-like domain that was characterized as NRG1 Ig-type isoform of *Xenopus laevis* (Fig. 1A) (Yang et al., 1998). The domains in Ig-like molecules are grouped into four types: variable (V-type), constant-1 (C-1 type), constant-2 (C-2 type) and intermediate (I-type) (Smith and Xue, 1997). The spacing of the cysteine residues fits the consensus for an Ig-like domain of C2 type (Yang et al., 1998). The EGF-like domain is well defined with its six cysteine residues which form three disulfide bonds. Two types of EGF-like domains have been identified, α and β, which differ in the amino acid sequences between the 5th and 6th cysteine and in the region carboxyl-terminal to the 6th cysteine