Biochemical and Biophysical Research Communications 436 (2013) 300–305

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

NGF induces adult stem Leydig cells to proliferate and differentiate during Leydig cell regeneration

Lei Zhang [a,1], Huaxi Wang [b,1], Yan Yang [c], Hui Liu [a], Qihao Zhang [a,d], Qi Xiang [a,d], Renshan Ge [e], Zhijian Su [a,*], Yadong Huang [a,d,*]

[a] Department of Cell Biology, College of Life Science and Technology, Jinan University, 510632 Guangzhou, PR China
[b] Southern Medical University, 510515 Guangzhou, PR China
[c] College of Pharmacy, Jinan University, 510632 Guangzhou, PR China
[d] National Engineering Research Center of Genetic Medicine, 510632 Guangzhou, PR China
[e] Population Council, Rockefeller University, 10065 New York, USA

ARTICLE INFO

Article history:
Received 17 May 2013
Available online 4 June 2013

Keywords:
Nerve growth factor (NGF)
Stem Leydig cells
Seminiferous tubules
Luteinizing hormone (LH)
Progenitor Leydig cells
Immature Leydig cells

ABSTRACT

Nerve growth factor (NGF) has been reported to be involved in male reproductive physiology. However, few reports have described the activity of NGF during Leydig cell development. The objective of the present study was to examine the role of NGF during stem-Leydig-cell (SLC) regeneration. We investigated the effects of NGF on Leydig-cell (LC) regeneration by measuring mRNA levels in the adult rat testis after ethane dimethanesulfonate (EDS) treatment. Furthermore, we used the established organ culture model of rat seminiferous tubules to examine the regulation of NGF during SLC proliferation and differentiation using EdU staining, real-time PCR and western blotting. Progenitor Leydig cells (PLCs) and immature Leydig cells (ILCs) were also used to investigate the effects of NGF on LCs at different developmental stages. NGF mRNA levels changed significantly during Leydig-cell regeneration *in vivo*. In vitro, NGF significantly promoted the proliferation of stem Leydig cells and also induced steroidogenic enzyme gene expression and 3β-HSD protein expression. The data from PLCs and ILCs showed that NGF could increase *Cyclin D1* and *Hsd 17b3* mRNA levels in PLCs and *Cyclin D1* mRNA levels in ILCs. These results indicate that NGF may play an important role during LC regeneration by regulating the proliferation and differentiation of LCs at different developmental stages, from SLCs to PLCs and from PLCs to ILCs. The discovery of this effect of NGF on Leydig cells will provide useful information for developing new potential therapies for PADAM (Partial Androgen Deficiency in the Aging Male).

1. Introduction

Adult Leydig cells are the testosterone-producing cells of the adult testis. These cells arise from stem Leydig cells (SLCs) and undergo four stages of differentiation [1]. Populations of cells at each of the following developmental stages are present in the testis: SLCs, progenitor Leydig cells (PLCs), immature Leydig cells (ILCs) and adult Leydig cells (ALCs). Previous studies in postnatal day 7 male rats showed strong evidence that SLCs may localize on the outer surface of the seminiferous tubules [2,3]. LC maturation involves a complex process of proliferation and differentiation under the control of endocrine and paracrine signals [4,5]. In adult rats, when a critical mass of mature LCs is achieved, the LC population becomes less mitotically active such that fully differentiated ALCs no longer divide. However, when adult male rats were treated with ethylene dimethanesulfonate (EDS), an alkylating toxicant that is selectively cytotoxic for differentiated LCs, ALCs were depleted, and the process of LC development began again, leading to the formation of a new generation of LCs [6,7].

Nerve growth factor (NGF), the first-identified neurotrophic protein, regulates the survival, growth and differentiation of neurons [8]. However, considerable evidence has accumulated to indicate additional activities in certain non-neuronal tissues and cells [9,10]. NGF mediates its cellular effects through interactions with two distinct receptors in Leydig cells, designated TrkA and p75NTR [11,12]. NGF binds preferentially to TrkA, and p75NTR can bind to NGF and also acts as a co-receptor for TrkA. p75NTR can increase the affinity and specificity of TrkA for NGF [13,14]. Peritubular myoid cells, Sertoli cells and germ cells have been identified as potential testicular NGF sources [15,16]. Many studies have focused on the NGF receptors; however, to date, the role of NGF during Leydig-cell development has not been determined. A

* Corresponding authors. Address: College of Life Science and Technology, Jinan University, 510632 Guangzhou, PR China. Fax: +86 20 85221303-808 (Z. Su, Y. Huang).
E-mail addresses: tjnuszj@jnu.edu.cn (Z. Su), tydhuang@jnu.edu.cn (Y. Huang).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.05.098

study using rat embryonic tissues [17] reported the presence of p75NTR mRNA in the mesenchymal cells surrounding the epithelia of the developing seminiferous tubules. Immunohistochemical analyses showed that p75NTR-expressing cells were located in the intertubular compartment in the embryonic testis and that during postnatal development these cells become organized in a cellular layer that surrounds the myoid cells of the seminiferous tubules [18]. Consistent with these results, both TrkA and p75NTR gene products were detected in immature rat testes, with maximal expression in 10- and 20-day-old rats. However, the expression of TrkA and p75NTR was barely detectable in 90-day-old adult rats [19]. Furthermore, Leydig cells were positively stained for NGF, as well as for TrkA and p75NTR [20], and the embryonic testes of TrkA knockout mice were found to be developmentally delayed when compared with their wild-type counterparts [21].

The present study was designed to investigate the role of NGF in SLCs proliferation and differentiation during LC regeneration. We examined changes in the mRNA and protein levels of key steroidogenic components after NGF and LH treatment in isolated EDS-treated seminiferous tubules *in vitro*. Furthermore, we isolated PLCs and ILCs to further examine the effects of NGF at different developmental stages.

2. Materials and methods

2.1. Chemicals and kits

EDS was provided by Dr. Jiyan Pang (Sun Yat-Sen University, China). A radioimmunoassay kit for testosterone was purchased from Beijing North Institute of Biological Technology. A Click-iT EdU HCS Assays kit (C10350) was purchased from Life Technology Corporation.

2.2. Animals and treatment

Sprague–Dawley rats were purchased from the Experimental Animal Center of Guangdong Province. The rats received a single, i.p. injection of EDS (75 mg/kg body weight). On days 7, 28, 42 and 63, the testicles of a subset of the rats were removed under deep chloral hydrate anesthesia for RNA extraction, and blood samples were collected to examine serum testosterone. The animal protocol was approved by the Institutional Animal Care and Use Committee of Jinan University.

2.3. Isolation and culture of rat seminiferous tubules

The animals were treated 1 week prior to the beginning of experimentation with ethane dimethanesulfonate (EDS, 75 mg/kg body weight, by i.p. injection). The seminiferous tubules were isolated as previously described [22]. These tubules were transferred to 24-well plates and cultured in basic DMEM/F12 medium for 24 h.

2.4. SLC proliferation and differentiation assay in rat seminiferous tubules

The isolated tubules were cultured in 24-well plates with DMEM/F12 medium. The tubules were divided into three treatment groups: Insulin-Transferrin-Selenite (ITS) (treated with ITS and 0.1% BSA), LH (treated with ITS, 0.1% BSA and 1 ng/ml LH) and NGF (treated with ITS, 0.1% BSA, 10 ng/ml NGF, 100 ng/ml NGF and 200 ng/ml NGF). For the cell proliferation assays, the treated tissues were incubated for 24 h, and cell proliferation was measured using Click-iT EdU HCS assays. To measure cell differentiation, the ITS and LH groups were incubated with the medium described for 21 days. The NGF group was incubated for 3 days and then treated with DMEM/F12-ITS media containing ITS, 0.1% BSA and 1 ng/ml LH for up to 21 days.

2.5. Isolation of Leydig cells and treatment

Leydig cells were isolated as previously described [23]. Leydig cells were typically enriched more than 90% and showed intense staining. PLCs and ILCs were cultured for 24 h in DMEM/F12 medium with 1 mg/ml cholesterol-rich lipids (Sigma, C7305) alone, with a stimulating dose of LH (1 ng/ml), or with NGF (100 ng/ml) for 24 h.

2.6. RIA for testosterone

The culture media from the seminiferous tubules were collected on day 21 and stored at −20 °C prior to analysis. Testosterone concentrations in the media were measured using I^{125}-based RIA.

2.7. Real-time quantitative RT-PCR

Total RNA was extracted using RNeasy Plus Mini Kits (Qiagen, 74134). Total RNA was used as the template for cDNA synthesis primed with random hexamers (Bio-Rad, 170-8890). The reaction mixture was incubated at 42 °C for 30 min followed by 5 min at 85 °C. All PCRs were performed using Bio-Rad SsoAdvanced SYBR Green (172-5261). The Bio-Rad CFX connect Real-Time system (Bio-Rad Laboratories, California, USA) and Bio-Rad CFX Manager Software (version 2.0) were used to collect the PCR data. The primers were described previously [24,25] and were synthesized by Beijing Genomic Institue (BGI). The RNA levels of each gene were normalized to *Rps16*.

2.8. Western blot analysis

Western blot analysis was conducted as described previously [22]. Tissue proteins were isolated from the seminiferous tubules on day 21. The membranes were incubated with anti-StAR antibody (Santa Cruz, sc-25806, 1:1000), anti-Hsd3b1 antibody (abcam, ab150384) or anti-β-actin antibody (abcam, ab8227). The western blots were repeated three times using samples from three independent experiments. The protein levels were quantified using Image J software from BioRad and normalized to β-actin.

3. Results

3.1. Up-regulation of NGF mRNA during LC regeneration

Changes in serum testosterone levels indicated that ALCs were depleted, followed by LC regeneration. To investigate testicular NGF changes during this process, we measured NGF mRNA levels by real-time PCR. The results showed a sharp rise at day 7 (Fig. 1B). NGF mRNA expression returned to the baseline level on day 28 and remained at this level on days 42 and 63.

3.2. NGF induced SLCs to proliferate

We examined whether NGF induced SLCs to proliferate. In a culture model of seminiferous tubules, we examined the numbers of proliferating SLCs with EdU labeling. As shown in Fig. 2, using the Click-iT EdU cell proliferation assay, spindle-shaped, EdU-labeled cells were observed on the surfaces of the tubules during 24 h in culture. NGF induced marked cell proliferation, especially at 200 ng/ml. The NGF-stimulated groups showed significantly enhanced SLC proliferation, but the effect on cell proliferation varied significantly among the different NGF-stimulated groups. NGF

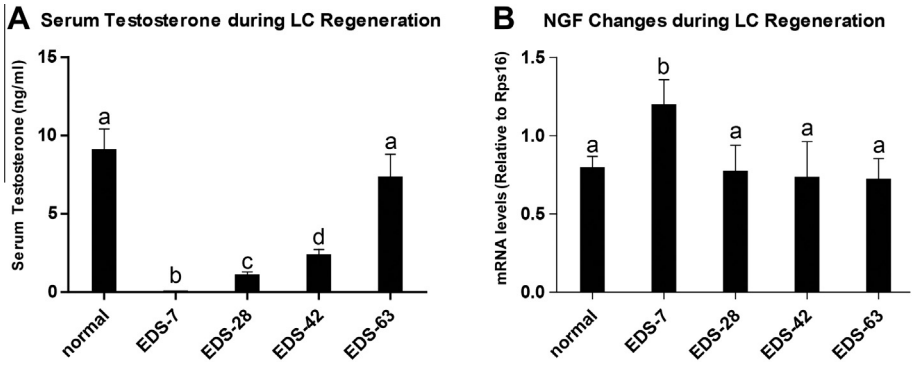

Fig. 1. The changes in testis NGF mRNA levels and serum testosterone during Leydig cell regeneration. (A) Serum testosterone concentrations on different days were measured using I^{125}-based RIA; (B) real-time PCR analysis of NGF mRNA expression on different days. The data are presented as the mean ± SEM ($n = 5$). Different letters(a, b, c, d) indicate significant differences ($P < 0.01$).

significantly promoted SLC proliferation (*vs.* ITS) with an obvious dose-response relationship.

3.3. NGF promoted testosterone production in seminiferous tubules in vitro

We used the established culture model of seminiferous tubules to assess the effects of NGF [3]. Fig. 3A shows the capacity of isolated seminiferous tubules pretreated with EDS to produce testosterone when stimulated with NGF for 3 days *in vitro*. After treatment with LH and NGF (100 ng/ml) for 3 days, the seminiferous tubules had a significantly increased ability to produce testosterone on day 21. However, after treatment with NGF (200 ng/ml and 10 ng/ml) for 3 days, the ability of these seminiferous tubules to produce testosterone failed to increase significantly. This result provides strong evidence that NGF can induce SLCs to differentiate into LCs.

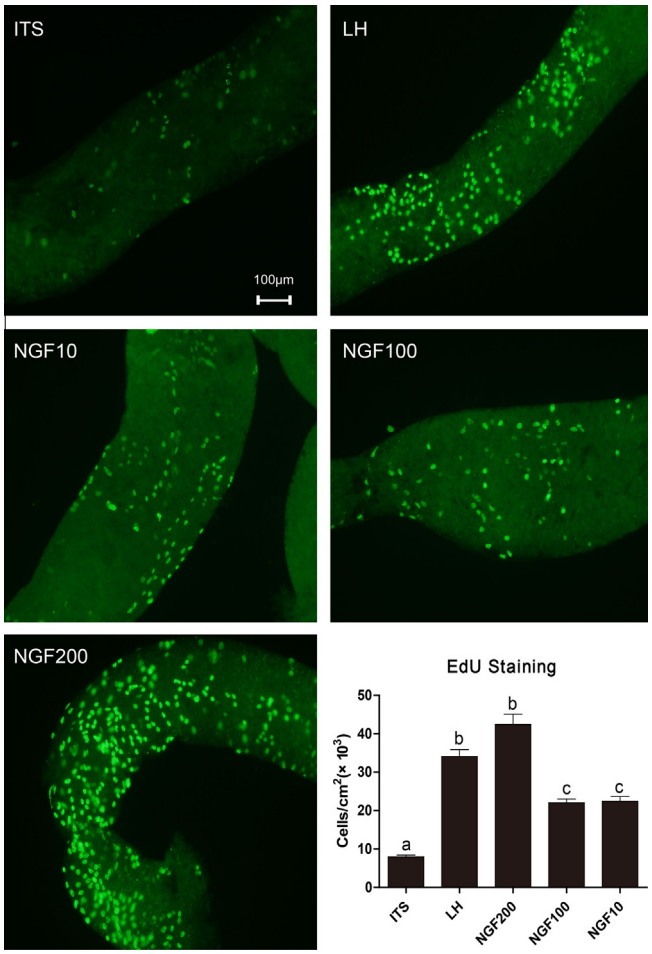

Fig. 2. The numbers of proliferating SLCs in seminiferous tubules after treatment with different concentrations of NGF for 24 h. Measurement of the numbers of SLCs was performed with Image-Pro Plus software. The numbers of SLCs are expressed as the mean ± SEM ($n = 5$). Different letters indicate significant differences ($P < 0.01$).

3.4. NGF up-regulated steroidogenic enzyme gene expression in seminiferous tubules

As shown in Fig. 3A, NGF induced high testosterone production. However, we know little about the effects of NGF on steroidogenesis. To investigate how NGF might induce steroidogenesis, we measured mRNA levels for *Star* and the steroidogenic enzyme genes *Cyp11a1*, *Hsd3b1*, *Hsd17b3* and *Srd5a1* by real-time PCR (Fig. 3B). The steroidogenic acute regulatory (StAR) protein is the rate-limiting enzyme in steroidogenesis. The mRNA level of *Star* showed a significant increase in all treatment groups compared with the control ($P < 0.01$), especially in the group treated with 100 ng/ml NGF. For *Cyp11a1* and *Hsd3b1*, the NGF-stimulated group (100 ng/ml) showed a significant increase in expression compared with the LH-stimulated group. The mRNA expression of *HSD17b3* in seminiferous tubules that had been exposed to NGF (10 ng/ml, 100 ng/ml and 200 ng/ml) was significantly increased compared with the control. Stimulation with 100 ng/ml NGF significantly increased *Srd5a1* expression relative to both the control and LH-stimulated groups. However, the mRNA levels of *Srd5a1* in the groups stimulated with other concentrations of NGF (200 ng/ml and 10 ng/ml) were similar to those in the LH-simulated group and the control.

3.5. NGF up-regulated 3β-HSD and StAR protein levels in seminiferous tubules

We measured protein levels for StAR and 3β-HSD in NGF-stimulated seminiferous tubules. These two key proteins in the steroidogenic pathway were significantly induced in response to NGF stimulation after 3 days in culture (Fig. 3C). The levels of StAR protein in the seminiferous tubules that had been treated with NGF were close to those of the LH-stimulated tubules. The 3β-HSD protein level in the seminiferous tubules that had been exposed to NGF (100 ng/ml) was 3-fold higher than in the control and LH-stimulated groups. 3β-HSD protein expression in the LH-stimulated group was similar to that of the control.

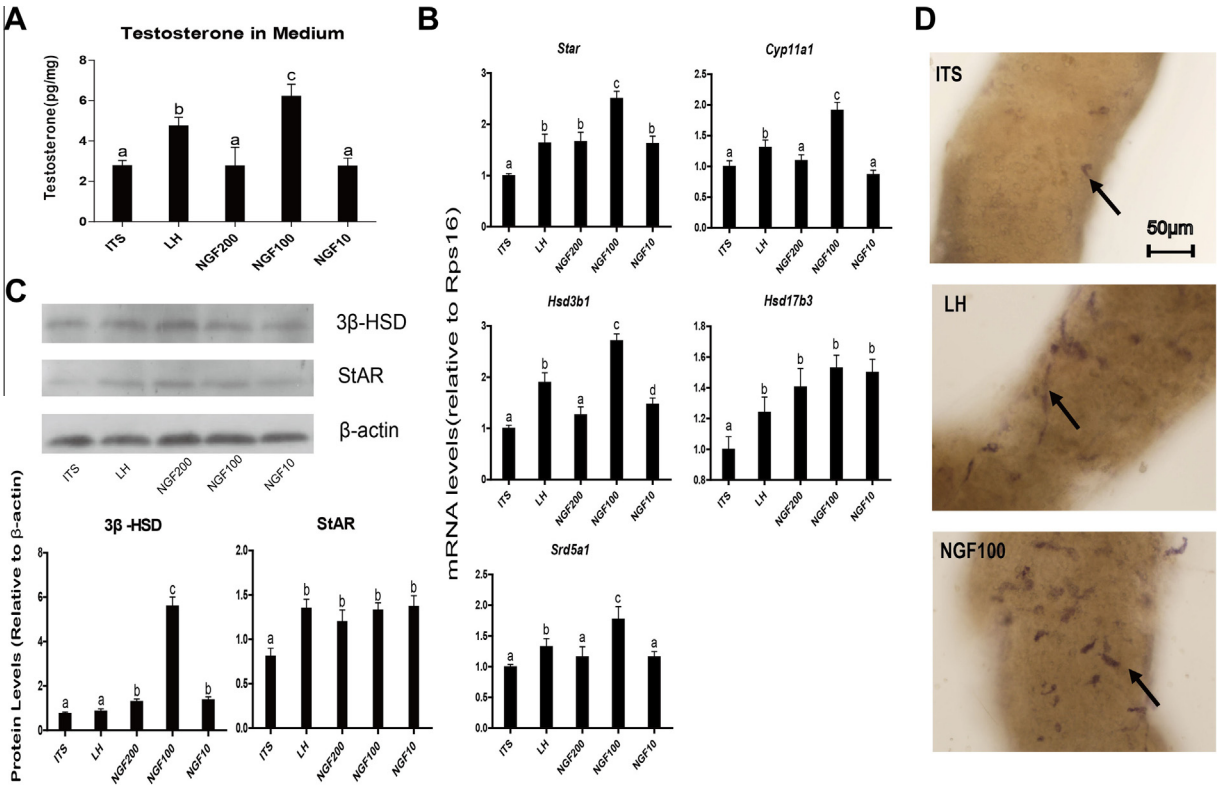

Fig. 3. Analysis of testosterone production in culture medium and the seminiferous tubules at the mRNA, protein and morphological levels on day 21. (A) Testosterone concentrations in culture medium. The concentrations of testosterone were measured using I^{125}-based RIA; (B) mRNA analysis of steroidogenic genes and the transcription factor Star in seminiferous tubules after treatment with NGF and LH ($n = 9$); (C) protein expression analysis in seminiferous tubules. The western blot analysis was performed as described in the materials and methods ($n = 4$); (D) immunolabeling of seminiferous tubules for 3β-HSD and Leydig cells is indicated by arrows. The data are presented as the mean ± SEM. Different letters indicate significant differences ($P < 0.01$).

3.6. NGF up-regulated Hsd17b3 mRNA levels and LH-induced Cyclin D1 gene expression in PLCs

To confirm whether NGF had an effect on PLCs, we treated PLCs with NGF for 24 h. *Star* was not affected by either LH stimulation or NGF stimulation alone. *Hsd3b1* gene expression was similar to that of *Star*. For *Cyp11a1* and *Srd5a1*, LH increased mRNA expression significantly. *Hsd17b3* mRNA was induced by either NGF or LH stimulation. However, the mRNA levels of *Star, Hsd3b1, Cyp11a1*, *Srd5a1* and *Hsd17b3* in cells treated with both NGF and LH were lower than in cells treated with NGF or LH alone. The mRNA levels of *Cyclin D1* in cells stimulated with NGF alone were similar to the control, yet LH-stimulated cells showed significantly increased expression of *Cyclin D1* mRNA compared with the control. Thus, LH and NGF were both able to induce *Cyclin D1* mRNA expression.

3.7. NGF up-regulated Cyclin D1 mRNA and inhibited LH-induced steroidogenic genes expression in ILCs

In ILCs, LH significantly induced *Star*, *Cyp11a1* and *HSD3b1* mRNA expression compared with the control. However, NGF suppressed the increase in expression induced by LH stimulation to different degrees (Fig. 4B). The mRNA expression of *HSD17b3* was not affected by LH stimulation alone but was inhibited by exposure to NGF and LH. For *Cyclin D1*, there was about a 2-fold increase in mRNA expression after NGF stimulation, relative to the control and the LH-stimulated group. However, LH inhibited the induction of *Cyclin D1* mRNA expression by NGF. NGF had no effect on *Srd5a1* mRNA expression.

4. Discussion

There have been few reports on the activity of NGF during Leydig cell development. The aim of the present study was to investigate this process. By day 7 after EDS treatment, the ALCs are completely eliminated, SLCs start to proliferate and differentiate, and a new ALC population begins to form [26]. At this time point, NGF mRNA levels were significantly increased, with no further significant change occurring in subsequent days. These results indicate that NGF may act during the development stage in which SLCs differentiate into LCs. In this model, when SLCs begin to differentiate, NGF would be active, and once SLCs have differentiated into PLCs, NGF function would attenuate or disappear.

To further investigate whether NGF is active during SLCs differentiation, we used the established seminiferous tubule culture system to assess the role of NGF. 200 ng/ml NGF could induce SLCs to proliferate significantly, and this result raised the question of whether all EdU-labeled cells were SLCs. In fact, the EdU-labeled cells were not all SLCs but mainly came from the SLCs and spermatogenic lineages. These cells were located in different positions on the seminiferous tubules; the SLCs were located on the outer surface of the seminiferous tubules, and the spermatogonia were on the inner side of the seminiferous tubules, at the base of the lamina propria [27]. Moreover, the two types of cells showed different configurations, with SLCs being spindle-shaped and spermatogonium, round-shaped.

Additionally, the mRNA expression of *Cyclin D1* in PLCs and ILCs demonstrated the obvious proliferative effect of NGF. During normal LC development, PLCs undergo multiple rounds of division, but ILCs undergo a single round of division to form the adult Leydig

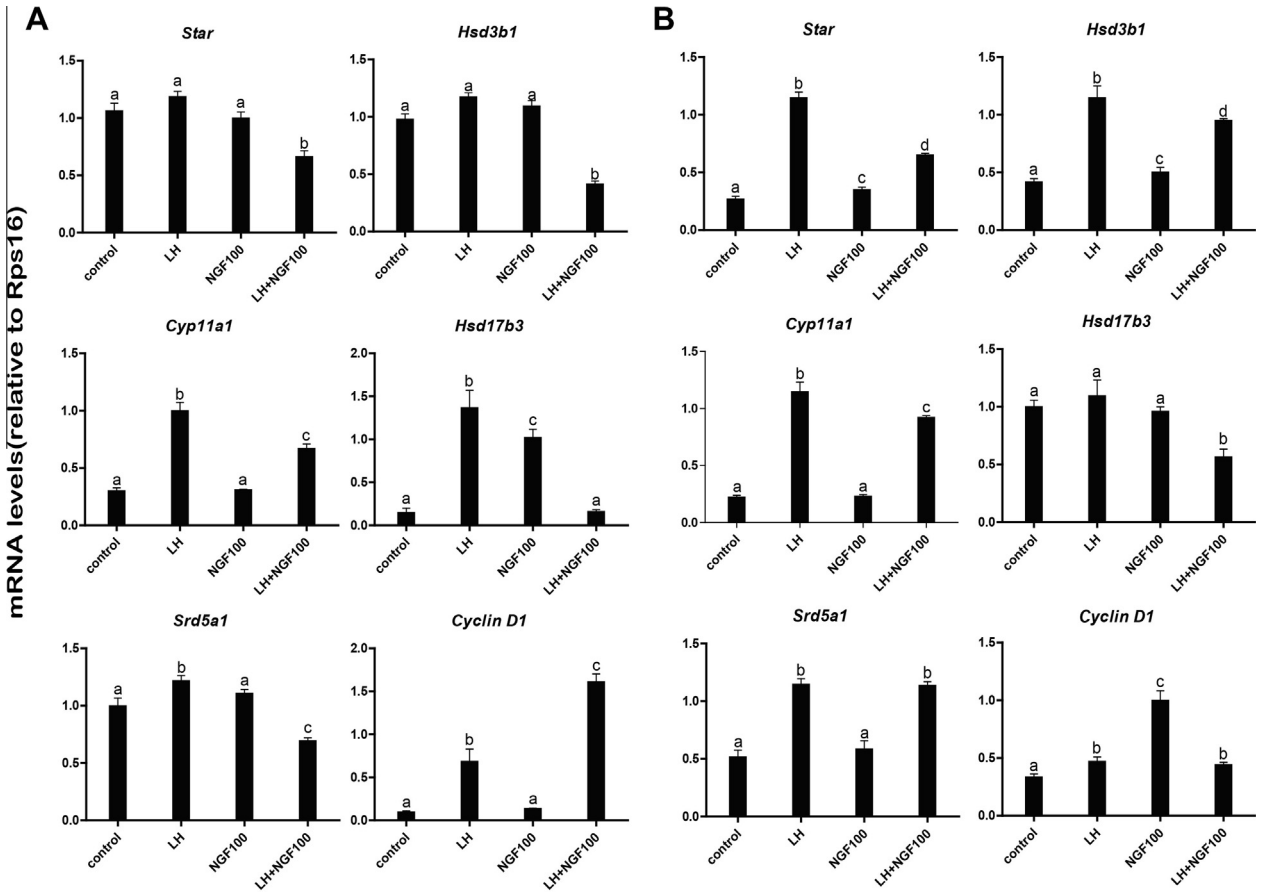

Fig. 4. Real-time PCR analysis for different steroidogenic genes, the transcription factor Star and *Cyclin D1*. (A) Real-time PCR analysis in PLCs after treatment with NGF and LH; (B) real-time PCR analysis in ILCs after treatment with NGF and LH. The data are presented as the mean ± SEM ($n = 4$). Different letters indicate significant differences ($P < 0.01$).

cell population [26]. Given the pattern of NGF expression described previously during LC regeneration, we hypothesized that NGF may affect the number of LCs. This would be consistent with a previous report describing the effects of NGF on neuronal stem cells [28].

In addition to proliferation, we found that NGF affected the differentiation of SLCs. In order to demonstrate this, we immunolabeled testicular cells with an anti-3β-HSD antibody. The results of this assay showed that 3β-HSD-positive cells were situated on the outer surface of the seminiferous tubules. However, we did not quantify the number of 3β-HSD-positive cells in the present study (Fig. 3D). A great deal of evidence indirectly supports our findings regarding the role of NGF during Leydig cell development [21,29,30]. Alternative approaches have also been used to study the effects of NGF on Leydig cells. NGF was shown to elicit cellular effects in MA-10 cells, a well-established, gonadotropin-responsive Leydig cell line, and NGF increased cellular steroid production in these cells [12]. However, as an immortalized Leydig cell line, MA-10 cells are differentiated cells that have lost the ability to synthesize testosterone due to a loss of CYP17 (P450c17) expression [31–33]. The MA-10 cell line is a valuable model for the study of Leydig cell physiology, but it is not a suitable model for the study of Leydig cell development.

In the present study, we observed that there were different effects in response to different NGF concentrations. Using the Click-iT EdU cell proliferation assay, 200 ng/ml NGF induced significant proliferation of SLCs; however, 100 ng/ml NGF induced high T production. We reasoned that different concentration of NGF could induce SLCs to differentiate into distinct cell lineages. 100 ng/ml NGF may induce SLCs to differentiate into Leydig cells, whereas 200 ng/ml may stimulate SLCs to differentiate into the peritubular myoid cell lineage [34,35].

NGF interacts with distinct receptors to elicit the proliferation and differentiation of Leydig cells. Interestingly, the data from PLCs and ILCs showed that NGF combined with LH inhibited the expression of steroidogenic genes. To our knowledge, this is the first report of a role for NGF in regulating steroidogenic enzyme gene expression, and this may involve the regulation of the signaling pathways downstream of NGFR and LHR. Previous studies have shown that the inhibitory response of Leydig cells to NGF and LH may involve the Src tyrosine kinase pathway. A cascade involving Src and Ras is a key feature of the signal transduction pathway for NGF. NGF activates Src to induce gene expression [36,37]. Src tyrosine kinase also plays an important role in regulating steroid secretion. Either the inhibition of Src activity or deficiency of Src expression will result in a significant increase in steroid production [38,39]. Therefore, we concluded that NGF and LH may have completely opposite effects on Src, resulting in the inhibition of steroidogenic gene expression.

In this study, we showed that high levels of NGF mRNA expression affected SLCs differentiation into PLCs in EDS-treated rats. Further experiments revealed that NGF induced significant SLCs proliferation and high levels of T production, which may result from high levels of steroidogenic enzyme expression at both the mRNA and protein levels. Moreover, NGF induced PLCs to proliferate and differentiate and also induced ILCs to proliferate. Taken together, these results demonstrate that NGF plays an important role in the regulation of LC development. These observations indicate that the NGF pathway may be a target for new adjuvant therapeutic

tools to treat diseases that are associated with the dysfunction of aged Leydig cells, like Partial Androgen Deficiency of the Aging Male (PADAM).

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grants Nos. 81070477, 31000663 and 31271607) and the Fundamental Research Funds for the Central Universities of China (Grant No. 21611378).

References

[1] H. Chen, R.-S. Ge, B.R. Zirkin, Leydig cells: from stem cells to aging, Mol. Cell. Endocrinol. 306 (2009) 9–16.
[2] R.S. Ge, Q. Dong, C.M. Sottas, V. Papadopoulos, B.R. Zirkin, M.P. Hardy, In search of rat stem Leydig cells: identification, isolation, and lineage-specific development, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 2719–2724.
[3] E. Stanley, C.Y. Lin, S. Jin, J. Liu, C.M. Sottas, R. Ge, B.R. Zirkin, H. Chen, Identification proliferation, and differentiation of adult Leydig stem cells, Endocrinology 153 (2012) 5002–5010.
[4] F. Gaytan, C. Bellido, E. Aguilar, N. van Rooijen, Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats, J. Reprod. Fertil. 102 (1994) 393–399.
[5] K. Teerds, M. Veldhuizen-Tsoerkan, F. Rommerts, D. De Rooij, J. Dorrington, Proliferation and Differentiation of Testicular Interstitial Cells: Aspects of Leydig Cell Development in the (pre) Pubertal and Adult Testis, Molecular and Cellular Endocrinology of the Testis, Springer-Verlag, New York, 1994 (37–65).
[6] J.M. Bartlett, J.B. Kerr, R.M. Sharpe, The effect of selective destruction and regeneration of rat Leydig cells on the intratesticular distribution of testosterone and morphology of the seminiferous epithelium, J. Androl. 7 (1986) 240–253.
[7] R. Molenaar, D.G. de Rooij, F.F. Rommerts, P.J. Reuvers, H.J. van der Molen, Specific destruction of Leydig cells in mature rats after in vivo administration of ethane dimethyl sulfonate, Biol. Reprod. 33 (1985) 1213–1222.
[8] D.R. Kaplan, F.D. Miller, Neurotrophin signal transduction in the nervous system, Curr. Opin. Neurobiol. 10 (2000) 381–391.
[9] M.V. Chao, Neurotrophins and their receptors: a convergence point for many signalling pathways, Nat. Rev. Neurosci. 4 (2003) 299–309.
[10] H. Sariola, The neurotrophic factors in non-neuronal tissues, Cell Mol. Life Sci. 58 (2001) 1061–1066.
[11] H. Chen, I. Huhtaniemi, B.R. Zirkin, Depletion and repopulation of Leydig cells in the testes of aging brown Norway rats, Endocrinology 137 (1996) 3447–3452.
[12] D. Muller, M.S. Davidoff, O. Bargheer, H.J. Paust, W. Pusch, Y. Koeva, D. Jezek, A.F. Holstein, R. Middendorff, The expression of neurotrophins and their receptors in the prenatal and adult human testis: evidence for functions in Leydig cells, Histochem. Cell Biol. 126 (2006) 199–211.
[13] M. Benedetti, A. Levi, M.V. Chao, Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 7859–7863.
[14] B.L. Hempstead, D. Martin-Zanca, D.R. Kaplan, L.F. Parada, M.V. Chao, High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor, Nature 350 (1991) 678–683.
[15] K. Seidl, A. Buchberger, C. Erck, Expression of nerve growth factor and neurotrophin receptors in testicular cells suggest novel roles for neurotrophins outside the nervous system, Reprod. Fertil. Dev. 8 (1996) 1075–1087.
[16] K. Seidl, A.F. Holstein, Evidence for the presence of nerve growth factor (NGF) and NGF receptors in human testis, Cell Tissue Res. 261 (1990) 549–554.
[17] E.F. Wheeler, M. Bothwell, Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis, J. Neurosci. 12 (1992) 930–945.
[18] M.A. Russo, T. Odorisio, A. Fradeani, L. Rienzi, M. De Felici, A. Cattaneo, G. Siracusa, Low-affinity nerve growth factor receptor is expressed during testicular morphogenesis and in germ cells at specific stages of spermatogenesis, Mol. Reprod. Dev. 37 (1994) 157–166.
[19] D. Djakiew, B. Pflug, C. Dionne, M. Onoda, Postnatal expression of nerve growth factor receptors in the rat testis, Biol. Reprod. 51 (1994) 214–221.
[20] W. Jin, K.Y. Arai, K. Shimizu, C. Kojima, M. Itoh, G. Watanabe, K. Taya, Cellular localization of NGF and its receptors trkA and p75LNGFR in male reproductive organs of the Japanese monkey, *Macaca fuscata fuscata*, Endocrine 29 (2006) 155–160.
[21] A.S. Cupp, L. Tessarollo, M.K. Skinner, Testis developmental phenotypes in neurotropin receptor trkA and trkC null mutations: role in formation of seminiferous cords and germ cell survival, Biol. Reprod. 66 (2002) 1838–1845.
[22] H. Chen, E. Stanley, S. Jin, B.R. Zirkin, Stem Leydig cells: from fetal to aged animals, Birth Defects Res. C 90 (2010) 272–283.
[23] R.S. Ge, Q. Dong, C.M. Sottas, H. Chen, B.R. Zirkin, M.P. Hardy, Gene expression in rat leydig cells during development from the progenitor to adult stage: a cluster analysis, Biol. Reprod. 72 (2005) 1405–1415.
[24] H. Lin, R.S. Ge, G.R. Chen, G.X. Hu, L. Dong, Q.Q. Lian, D.O. Hardy, C.M. Sottas, X.K. Li, M.P. Hardy, Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 7218–7222.
[25] A.M. Betancourt, S.C. Burgess, R.L. Carr, Effect of developmental exposure to chlorpyrifos on the expression of neurotrophin growth factors and cell-specific markers in neonatal rat brain, Toxicol. Sci. 92 (2006) 500–506.
[26] L. Benton, L.X. Shan, M.P. Hardy, Differentiation of adult Leydig cells, J. Steroid Biochem. Mol. Biol. 53 (1995) 61–68.
[27] M. Nagano, M.R. Avarbock, E.B. Leonida, C.J. Brinster, R.L. Brinster, Culture of mouse spermatogonial stem cells, Tissue Cell 30 (1998) 389–397.
[28] E. Cattaneo, R. McKay, Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor, Nature 347 (1990) 762–765.
[29] H. Wang, Y. Dong, W. Chen, J. Hei, C. Dong, Expression and localization of nerve growth factor (NGF) in the testis of alpaca (*llama pacos*), Folia Histochem. Cytobiol. 49 (2011) 55–61.
[30] M.B. Levanti, A. Germana, F. de Carlos, E. Ciriaco, J.A. Vega, G. Germana, Effects of increased nerve growth factor plasma levels on the expression of TrkA and p75 in rat testicles, J. Anat. 208 (2006) 373–379.
[31] S. Hoelscher, M. Ascoli, Immortalized Leydig Cell Lines as Models for Studying Leydig Cell Physiology, The Leydig Cell, Cache River Press, Vienna, IL, 1996 (523–534).
[32] O.O. Anakwe, A.H. Payne, Noncoordinate regulation of de novo synthesis of cytochrome P-450 cholesterol side-chain cleavage and cytochrome P-450 17 alpha-hydroxylase/C17-20 lyase in mouse Leydig cell cultures: relation to steroid production, Mol. Endocrinol. 1 (1987) 595–603.
[33] S.H. Mellon, C. Vaisse, CAMP regulates P450scc gene expression by a cycloheximide-insensitive mechanism in cultured mouse Leydig MA-10 cells, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 7775–7779.
[34] A.C. Kim, G.D. Hammer, Adrenocortical cells with stem/progenitor cell properties: recent advances, Mol. Cell. Endocrinol. 265–266 (2007) 10–16.
[35] N. Shimizu, K. Yamamoto, S. Obi, S. Kumagaya, T. Masumura, Y. Shimano, K. Naruse, J.K. Yamashita, T. Igarashi, J. Ando, Cyclic strain induces mouse embryonic stem cell differentiation into vascular smooth muscle cells by activating PDGF receptor beta, J. Appl. Physiol. 104 (2008) 766–772.
[36] N.E. Kremer, G. D'Arcangelo, S.M. Thomas, M. DeMarco, J.S. Brugge, S. Halegoua, Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions, J. Cell Biol. 115 (1991) 809–819.
[37] G. D'Arcangelo, S. Halegoua, A branched signaling pathway for nerve growth factor is revealed by Src-, Ras-, and Raf-mediated gene inductions, Mol. Cell. Biol. 13 (1993) 3146–3155.
[38] C.C. Taylor, D. Limback, P.F. Terranova, Src tyrosine kinase activity is related to luteinizing hormone responsiveness: genetic manipulations using mouse MA10 Leydig cells, Endocrinology 137 (1996) 5735–5738.
[39] G. Chaturvedi, K. Arai, P.F. Terranova, K.F. Roby, The Src tyrosine kinase pathway regulates thecal CYP17 expression and androstenedione secretion, Mol. Cell. Biochem. 318 (2008) 191–200.