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Research Article

UPREGULATION OF NEUROGLOBIN PROMOTES TM3 LEYDIG CELL VIABILITY

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Abstract: Neuroglobin (NGB) is a globin family member protein expressed in the nerve system and prevents oxidative stress and apoptosis induced death in neurons. The anti-oxidant role of NGB against oxidative stress and reactive oxygen species (ROS) brings up its promising protective role in other tissues. The overexpression of NGB in testosterone producing Leydig cells might be a solution to hypoxia related male infertility. In the current study, NGB was overexpressed in Leydig cells by using viral transduction methods and the cell proliferation, gene expression and anti-oxidant enzyme levels were analyzed. Upregulation of NGB increased anti-apoptotic Bcl-2, cell proliferation and anti-oxidant enzyme levels and decreased the expression of apoptotic genes BAX, p53 and caspase 3. In addition, NGB transduced cells proliferated and expressed less apoptotic genes after H_2O_2 exposure. In conclusion, NGB might be a target for androgen deficiency related male infertility and could be used in clinics in the future.

Key words: Neuroglobin, Leydig cell, oxidative stress, anti-oxidant enzymes, male fertility.

Özet: Globin ailesi proteinlerinden bir tanesi olan Neuroglobin (NGB) sinir sisteminde ifade edilmektedir ve oksidatif stres ve apoptoz kaynaklı nöron ölümünü engellemektedir. Oksidatif stres ve reaktif oksijen türlerine karşı gözlemlenen anti-oksidan aktivite, NGB'nin diğer dokulardaki potansiyel koruyucu rolünü gündeme getirmektedir. Testosteron üreten Leydig hücrelerinde NGB'nin fazla ifade edilmesi hipoksi ile ilişkili erkek infertilitesi için bir çözüm olabilecektir. Bu çalışmada, NGB proteinin viral aktarım metodu ile Leydig hücrelerinde aşırı ifade ettirilmiş ve hücre çoğalması, gen anlatımı ve anti-oksidan enzim seviyeleri analiz edilmiştir. Artan NGB, anti-apoptotik Bcl-2, hücre çoğalması ve anti-oksidan enzim seviyelerini arttırmış, BAX, p53 ve kaspaz 3 gibi apoptoz genlerinin ifadesini azaltmıştır. Ek olarak, H_2O_2 uygulaması sonrasında NGB aktarılan hücreler çoğalmış ve apoptotik genlerin ifadesi azalmıştır. Sonuç olarak, NGB androjen eksikliğine bağlı erkek infertilitesi için hedef olabilir ve gelecekte klinikte kullanılabilir.

Introduction

Neuroglobin (NGB) was identified as a vertebrate nerve globin in neural tissues by Burmester *et al.* (2000). The NGB protein consists of 150 amino acids and is similar to myoglobin structurally (Burmester *et al.* 2000, Dewilde *et al.* 2001). Expression of NGB is observed in central and peripheral nervous system and some endocrine tissues (Reuss *et al.* 2002, Burmester *et al.* 2000).

The neuroprotective activity of NGB through prevention of apoptosis and oxidative stress is well documented (Amri *et al.* 2017). NGB is able bind to O_2, CO or NO and prevents oxidative stress (Dewilde *et al.* 2001). The potential pathways and regulatory roles of NGB are providing O_2 supply, preventing reactive oxygen species (ROS) damage and hypoxia (Burmester & Hankeln 2009). NGB acts as a respiratory protein with an O_2 binding affinity which resembles to myoglobin oxygen-binding capacity (Hundahl *et al.* 2006). Localization of NGB in specific tissues and cellular regions that are metabolically active supports its O_2 binding ability (Schmidt *et al.* 2003). Interaction of NGB with O_2 enables the regulation of NGB expression in hypoxic conditions (Schmidt-Kastner *et al.* 2006). NGB acts as NO-dioxygenase when O_2 levels are low and react with NO_2 to form NO (Petersen *et al.* 2008). NGB expression in human cell cultures is not only triggered by hypoxia (Haines *et al.* 2012) but also induced by H_2O_2 (De Marinis *et al.* 2013) indicating the regulatory role in O_2 metabolism. 17β-estradiol (E2) induced NGB upregulation in neurons has been shown to be neuroprotective (De Marinis *et al.* 2013). Moreover, NGB was shown to translocate into mitochondria upon hormone and H_2O_2 stimulation and prevent cytochrome C release to help overcoming stress induced programmed cell death (De Marinis *et al.* 2013, Brittain *et al.* 2010). NGB overexpression in transgenic animals prevented heart and brain injury after ischemia or stroke indicating the tissue protective role of NGB after oxidative stress (Sun *et al.* 2003, Khan *et al.* 2006, Jin *et al.* 2010). Endogenous NGB in neural tissues exerted

neuroprotective roles against oxidative stress indicating the possible protective role of NGB in neurodegenerative disorders (Ye *et al.* 2009, Fiocchetti *et al.* 2013).

The harmful effects of oxidative stress are not only observed in neural tissues but also disrupt functions in many tissues of the systems of the body one of which is the reproductive system. Oxidative stress induced DNA damage in sperm function is one of the most important reasons for male fertility (Bisht *et al.* 2017). In addition to spermatogenesis, Leydig cell steroidogenesis is also sensitive to oxidative stress. A number of internal and external factors, e.g. age, infection, diabetes, temperature, testis diseases, toxin exposure and hormonal changes cause ROS production in the testes leading to male fertility problems (Asadi *et al.* 2017). The antioxidant defense system in Leydig cells protects testicular tissue and provides appropriate testosterone production and sperm generation (Aitken & Roman 2008). Leydig cells are the primary sources for testosterone production which is required for male reproduction. Therefore, identification of new protective mechanisms that control oxidative stress mechanisms in Leydig cells might be valuable for future therapeutic applications. Although large amounts of NGB were detected in the nervous system and brain, endocrine organs including testis and pituitary and adrenal glands also express NGB (Burmester *et al.* 2000, Reuss *et al.* 2002, Zhang *et al.* 2002). Because NGB is expressed in testis tissue, we hypothesized that overexpression of NGB might protect testis cells against stress. Protective activity of NGB against oxidative stress in tissues such as that of the nervous system where high amounts of localization are observed gave rise to the idea of potential protective effect of NGB in other tissues. In the current study, the protective role of NGB in TM3 mouse Leydig cells was evaluated by using a genetic manipulation approach.

Materials and Methods

Cell line

TM3-Leydig cells #CRL-1714 were purchased from ATCC (Rockville, MD). Cells were incubated in a humidified chamber at 37 °C and 5% CO_2 in 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's Medium (Invitrogen, Gibco, UK) supplemented with 5% horse serum, 2.5% fetal bovine serum (FBS, Invitrogen, Gibco, UK) and 1% Penicillin/Streptomycin/ Amphotericin (PSA, Invitrogen, Gibco, UK).

Viral production

The coding sequence of mouse NGB was ligated into pLenti-III-2A-GFP (Abm, Richmond, CA, USA, Fig. 1A). pLenti-III-2A-GFP was used as the control vector. Lenti viral vector stocks for pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP expressing vectors were produced by calcium phosphate transfection of 293T cells. pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP were transfected into 293T cells together with pCMVDR8.2DVPR (Addgene, Cambridge, MA, USA) and pMD2.G (VSVG, Addgene, Cambridge, MA, USA) for packaging and viral supernatants were collected at 24, 48 and 72 h post-transfection (Fig. 1B). Lenti viral supernatants were filtered and concentrated by ultracentrifugation. HeLa cells were transfected for viral titer calculation and GFP positive cells were analyzed by flow cytometry (FACS Aria, Becton Dickinson, San Jose, CA). $4x10^4$ transducing units/mL was determined as titer of pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors.

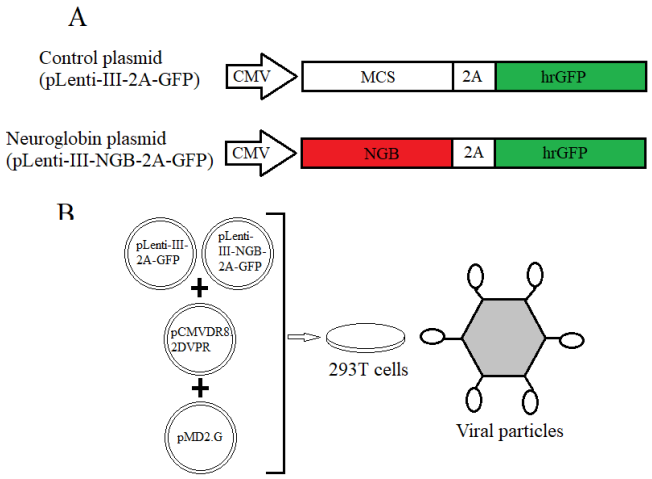

Fig. 1. Plasmid constructs and viral production method. (A) Structure of pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors. (B) Packaging process of pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors in 293T cells.

Cell transduction

TM3 cells were transduced with the viral supernatants of pLenti-III-2A-GFP and pLenti-III-NGBB-2A-GFP in the presence of 8µg/mL of polybrene for 24h. GFP positive cells were sorted by flow cytometry and NGB overexpression was confirmed by qPCR analysis. Cell populations were referred as TM3-NGB and TM3-GFP for further experiments.

Cell viability (MTS) analysis

Cell viabilities of TM3-NGB and TM3-GFP cells and H_2O_2 applied TM3-NGB and TM3-GFP cells were measured by the 3-(4,5-di-methyl-thiazol- 2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)- 2H-tetrazolium (MTS)-assay (CellTiter96 Aqueous One Solution; Promega, Southampton, UK) as described previously (Doğan *et al.* 2014). Briefly, $5x10^3$ TM3-NGB and TM3-GFP cells were seeded onto 96-well plates and cell viability was measured by MTS assay for 24, 48, and 72 hours. Absorbances were measured at 490 nm by using an ELISA plate reader (Biotek, Winooski, VT).

Determination of H_2O_2 toxic dose

TM3 Leydig cells were seeded onto 96-well plates at a cell density of 5000 cells/well. One day later, cells were treated with various concentrations (100µM, 200µM, 300µM, 400µM) of H_2O_2 to determine the toxic dose for further experiments. Cell viability was measured by MTS assay and absorbance was measured at 490nm using an ELISA plate reader.

TUNEL assay

TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed in TM3-NGB and TM3-GFP cells by using a TUNEL Assay kit (Roche Molecular Biochemicals, Indianapolis, IN) to detect the apoptotic cell number. Briefly, transduced cells were collected and suspended in 50µl of TUNEL reaction mixture (Labeling solution + Enzyme solution, supplied with the kit), incubated at 37°C and suspended in PBS for flow cytometry analysis using Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometry system.

Gene expression analyses

Quantitative RT-PCR experiments were conducted according to the previously described protocol (Apdik *et al.* 2015). Primer sequences for NGB, p53, caspase3, Akt, BAX, Bcl-2 and β-actin were designed by IDT primer Quest software. β-Actin was used as housekeeping gene for normalization of the data. Total RNA was isolated form transduced cells and H_2O_2 administered cells by using a RNA-easy plus mini kit (Qiagen, Hilden, Germany) and cDNA was synthesized using High Fidelity cDNA synthesis kit (Roche, USA). qPCR experiments were conducted by SYBR Green using the CFX96 RT-PCR system (Bio-Rad, Hercules, CA).

SOD and GPx activity measurements

SOD (19160, Sigma, MO, USA) and GPx (CGP1, Sigma, MO, USA) enzyme activities were determined in TM3-NGB and TM3-GFP cells according to the manufacturer's instructions. Protein samples were isolated from TM3-NGB and TM3-GFP cells by RIPA buffer and used for enzyme activity analysis. Absorbances of SOD and GPx assays were measured at 450nm and 340nm, respectively by using an ELISA plate reader.

Statistical analysis

Results are expressed as mean ± standard deviation. Standard errors and t-test values were calculated using the GraphPad Prism 5 (GraphPad, La Jolla, CA) software. Differences were considered to be statistically significant at P values of less than 0.05 ($P < 0.05$).

Results

Cell transduction and proliferation analysis

TM3 cells were successfully transduced by pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP lentiviral vectors. pLenti-III-2A-GFP transduction was conducted as control of viral transduction. GFP expressing cells were visualized by a fluorescence microscope (Fig. 2A). NGB expression was approximately 10 fold higher in TM3-NGB-GFP cells (10±0.0197) compared to TM3-GFP (1±0.0056) cells demonstrating the successful overexpression by lentiviral vectors (Fig. 2B). In order to determine the effect of NGB upregulation on cell proliferation, MTS analyses were performed. Cell proliferation of TM3-NGB-GFP cells was significantly higher for 24, 48 and 72 h compared to TM3-GFP cells (Fig. 2C). Although absorbance values for TM3-GFP at 490 nm were 0.290±0.0051, 0.350±0.0167 and 0.410±0.0197; absorbances of TM3-NGB-GFP were 0.367±0.0201, 0.520±0.0198 and 0.630±0.0265 for day 1, day 2 and day 3 respectively.

TUNEL assay

The apoptotic status of NGB overexpressing cells were detected by TUNEL assay. Although 27% of the TM3-GFP cells were apoptotic after transduction, only 12% of the TM3-NGB-GFP cells were positively stained (Fig. 2D).

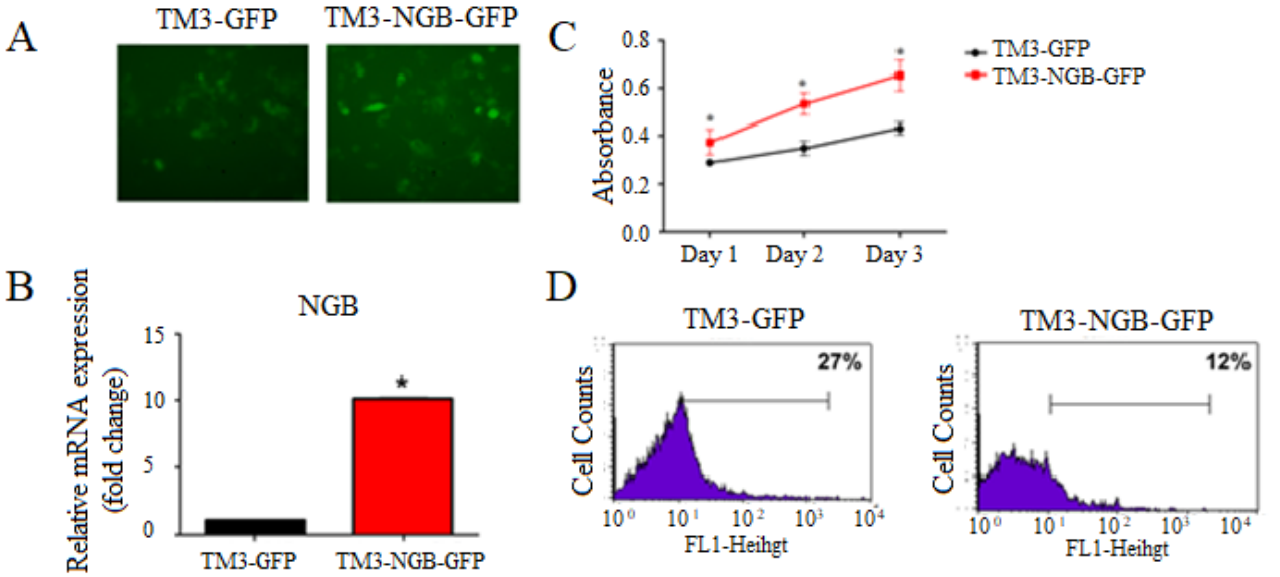

Fig. 2. Transduction of TM3 cells with pLenti-III-2A-GFP and pLenti-III-NGB-2A-GFP vectors and viability analysis. (A) Successful transduction of TM3 cells. Cells were GFP positive under fluorescence microscope indicating the viral transduction. (B) Confirmation of overexpression of NGB by qPCR analysis. (C) Cell proliferation of TM3-NGB-GFP and TM3-GFP cells. (D) TUNEL staining of TM3-NGB-GFP and TM3-GFP cells. *P < 0.05.

Gene expression analyses

Pro-apoptotic and anti-apoptotic gene expression levels were detected in NGB overexpressing TM3 cells. The apoptotic genes BAX, Caspase-3 and p53 were downregulated in TM3-NGB-GFP indicating the protective role of NGB in TM3 cells. Bcl-2 as an anti-apoptotic gene was upregulated in NGB overexpressing TM3 cells (Fig. 3). The expression in TM3-NGB-GFP expressed almost 2-fold higher Bcl-2 (1.964±0.0298) compared to TM3-GFP (1±0.1390). BAX expression was not significantly different in TM3-NGB-GFP (0.764±0.0299) and TM3-GFP (1±0.1470) cells. Caspase-3 and p53 were downregulated in TM3-NGB-GFP (0.56±0.0210 and 0.58±0.0231) compared to GFP expressing TM3 cell line.

Protective role of NGB against H_2O_2 toxicity

Four different doses (100 µM, 200 µM, 300 µM, 400 µM) of H_2O_2 were applied to determine the toxic concentration for cell viability and qPCR analysis. Significant toxicity was observed starting from 200µM H_2O_2 (0.164±0.0080) compared to the control group (0.669±0.0205). 200 µM H_2O_2 significantly reduced cell viability and was selected for further analysis (Fig. 4A). TM3-NGB-GFP cell viability was higher compared to TM3-GFP cells for 24, 48 and 72h. The cell viability showed a time dependent increase in TM3-NGB-GFP cells for 3 days and was 2 fold higher compared to TM3-GFP by day 3 (TM3-NGB-GFP: 0.205±0.0148, TM3-GFP: 0.093± 0.0265) (Fig. 4B). BAX, Caspase-3 and p53 genes were downregulated in TM3-NGB-GFP indicating the protective role of NGB in of TM3-NGB-GFP for p53, BAX, Bcl-2 and Caspase-3 were detected as 0.864±0.0299, 0.800±0.0100, 1.100±0.0300 and 0.812±0.0400, respectively and no statistically significant difference was measured for these values (Fig. 4C).

SOD and GPx enzyme activity

Potential role of NGB overexpression on antioxidant enzyme activities was detected by SOD and GPx activity measurements. NGB overexpression increased the antioxidant enzyme activities significantly compared to TM3-GFP cells. TM3-NGB-GFP cells exerted approximately 2 fold higher antioxidant enzyme activity. SOD and GPx enzyme activities of TM3-GFP cells were determined as 60%±3.1 and 57%±3.2, respectively while activities of both were 100% in TM3-NGB-GFP cells (Fig. 5).

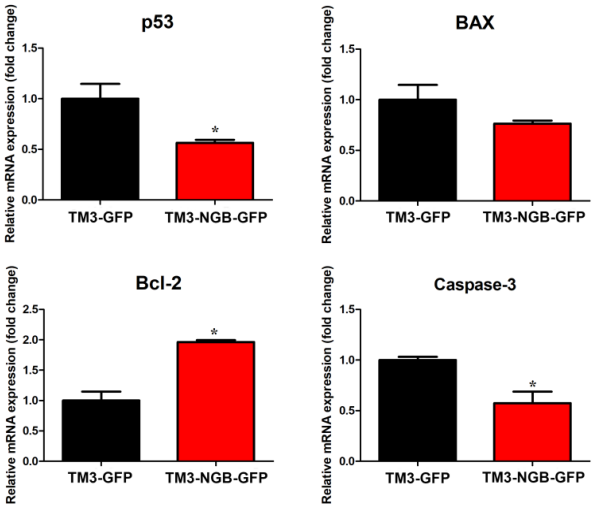

Fig. 3. Gene expression analysis of TM3-NGB-GFP and TM3-GFP cells for pro- and anti-apoptotic genes. *P < 0.05.

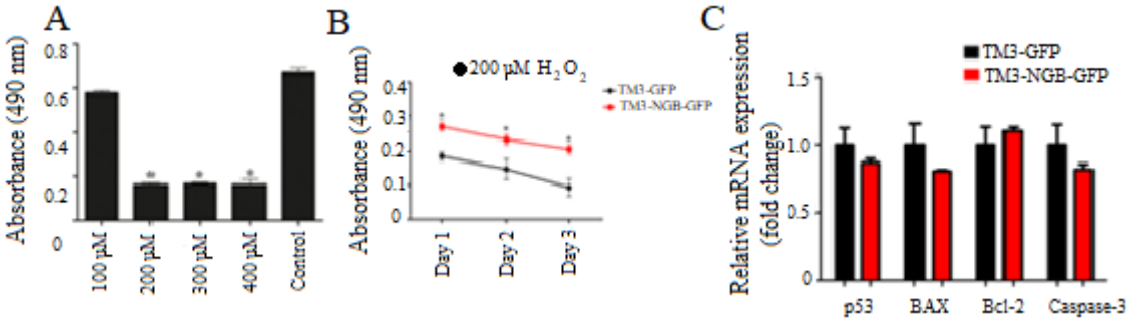

Fig. 4. Protective role of NGB against H_2O_2 stress. (A) Determination of H_2O_2 toxic dose based on cell viability. (B) Cell proliferation of TM3-NGB-GFP and TM3-GFP cells after 200µM H_2O_2 exposure. (C) Expression levels of pro- and anti-apoptotic genes in TM3-NGB-GFP and TM3-GFP cells after 200 µM H_2O_2 exposure. *P < 0.05.

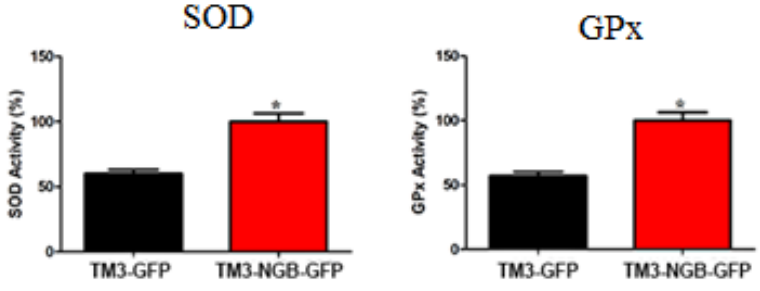

Fig. 5. SOD and GPx enzyme levels in TM3-NGB-GFP and TM3-GFP cells. *P < 0.05.

Discussion

Free radicals generated through cellular metabolism cause oxidative stress in tissues such as testis and induce cell death. Oxidative stress induced ROS disrupts reproductive system, prevents androgen (testosterone) secretion and spermatogenesis (Aitken *et al.* 2008). Leydig cell dysfunction due to membrane lipid peroxidation upon Lipopolysaccharide (LPS) application has been observed previously (Husain & Somani 1998). As Leydig cells produce androgens such as testosterone which binds to the androgen receptor and activates sperm generation (Dohle *et al.* 2003), they are potential targets for oxidative stress related male fertility treatments. Understanding the protective mechanisms against oxidative stress and identifications of new potential pathways are among the recent aims of studies addressing development of new therapeutic targets.

In the present study, we evaluated the role of NGB in Leydig cells by using an overexpressing gene editing approach. Because NGB protects many tissues against oxidative stress and are expressed in testis tissue, overexpression of NGB in Leydig cells could be promising to prevent harmful effects of oxidative stress. Proliferation of NGB overexpressing mouse TM3 Leydig cells were higher compared to control cells indicating the potential protective activity. The activity of NGB on cell proliferation and protection has previously been shown in neurons and cancer cells (Fiocchetti *et al.* 2017, Greenberg *et al.* 2008, Zhang *et al.* 2018) which supports our results. NBG overexpressing transgenic mice can rescue under hypoxic stress and NGB induces neural protection in vitro against hypoxic injury (Greenberg *et al.* 2008). Overexpression of NGB increased cell viability in MCF-7 breast cancer cells against nutrient deprivation through an anti-apoptotic Bcl-2 expression (Fiocchetti *et al.* 2017). Similarly, both overexpression and knockdown of NGB in glioblastoma cells regulated the cell proliferation by activating the PI3K/AKT pathway (Zhang *et al.* 2018). Promoting activity of NGB in cell proliferation and viability might be useful to increase Leydig cell number and to treat oxidative stress related male fertility in the future clinical applications. Additionally, low levels of TUNEL positive cells in NGB transduced cells demonstrated the anti-apoptotic role of NGB in Leydig cells. Knockdown of NGB in neurons increased TUNEL positive cells in arsenite induce toxicity indicating the protective role of NGB against apoptosis (Liu *et al.* 2015). Anti-apoptotic activity was confirmed by gene expression analysis of pro- and anti-apoptotic genes including BAX, p53, caspase-3 and Bcl-2. Enhanced Bcl-2 and low levels of BAX and caspase-3 have been reported after spinal cord injury in NGB overexpressing animals (Lan *et al.* 2014). Because Leydig cells are responsible for testosterone production and highly crucial for spermatogenesis, anti-apoptotic function of NGB to block apoptosis is highly important for potential therapeutic options. Same observations for cell proliferation and gene expression were reported after H_2O_2 exposure indicating the protective effect against ROS and oxidative stress. Induction of SOD and GPx in testis tissue (Kaur *et al.* 2006) and Leydig cells (Baek *et al.* 2007) is a defense mechanism after oxidative stress. Therefore, we evaluated the effect of NGB overexpression on anti-oxidant enzyme activities. NGB overexpression upregulated the SOD and GPx levels as reported in the literature. Although there is not an evidence in the literature for NGB overexpression in Leydig cells, transgenic mice overexpressing NGB showed high SOD and GPx levels in hippocampal tissues (Li *et al.* 2010).

In conclusion, we demonstrated, for the first time, the promising protective role of NGB in Leydig cells against oxidative stress. NGB gene could be a potential target for oxidative stress induced male infertility and might be used for therapy in future. Further experiments explaining the molecular mechanism of NGB in Leydig cells should be conducted both *in vitro* and *in vivo*.

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