Please cite this article in press as: Huma T et al. Kisspeptin-10 treatment generated specific GnRH expression in cells differentiated from rhesus monkey derived Lyon NSCs. Neuroscience (2017), http://dx.doi.org/10.1016/j.neuroscience.2017.03.004

Neuroscience xxx (2017) xxx–xxx

KISSPEPTIN-10 TREATMENT GENERATED SPECIFIC GNRH EXPRESSION IN CELLS DIFFERENTIATED FROM RHESUS MONKEY DERIVED LYON NSCS

TANZEEL HUMA, [a,b,d*] **XINTIAN HU,** [a,c] **YUANYE MA,** [c] **ANDREW WILLDEN,** [c] **JOSHUA RIZAK,** [a] **MUHAMMAD SHAHAB** [b] **AND ZHENGBO WANG** [a,c*]

[a] *Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, PR China*

[b] *Laboratory of Reproductive Neuroendocrinology, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan*

[c] *Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, PR China*

[d] *Institute of Molecular Biology and Biotechnology, University of Lahore, Lahore, Pakistan*

Abstract—Embryonic stem cells (ESCs) have enormous potential as novel cell-based therapies, but their effectiveness depends on stem cell differentiation and specific signaling regulators, which remain poorly understood. In this study, a kisspeptin peptide (KP-10) was used at different dosages to determine whether rhesus macaque-derived tau GFP-Lyon ES cells underwent kisspeptin-specific neuronal differentiation. It was found that KP-10 exhibited an anti-proliferative effect on the cells and led to morphological changes and cellular differentiation consistent with neuronal stem cell (NSC) development. The cells differentiated into Gonadotrophin Releasing Hormone (GnRH) neuronal-like cell types in response to the KP-10 treatment. There has been a previously observed connection between kisspeptin signaling, GnRH neurons and their dysfunction found in congenital disorders like idiopathic hypogonadotropic hypogonadism (IHH). Although therapeutics are a still a far-off goal, the formation and development of GnRH-positive neuronal-like cells following the application of KP-10 to Lyon NSC cells opens the door for future NSC-based therapies to treat specific reproductive disorders.

Key words: Lyon ES, kisspeptin, GnRH, neural stem cells, proliferation.

*Corresponding authors. Addresses: Quaid-i-Azam University, Islamabad, Pakistan. Fax: +92-51-2601-176 (T. Huma). Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, PR China. Fax: +86-871-65197002 (Z. Wang).
E-mail addresses: tanzeelhuma@gmail.com (T. Huma), wangzb@mail.kiz.ac.cn (Z. Wang).

Abbreviations: EBs, embryonic bodies; ESCs, Embryonic stem cells; GnRH, Gonadotrophin Releasing Hormone; ICC, immunocytochemistry; IHH, idiopathic hypogonadotropic hypogonadism; KP-10, kisspeptin peptide; KPs, Kisspeptins; NSC, neuronal stem cell; OD, optical density; OECs, olfactory ensheathing cells; SHH, Sonic Hedgehog.

http://dx.doi.org/10.1016/j.neuroscience.2017.03.004
0306-4522/

INTRODUCTION

Embryonic stem cells (ESCs) are capable of indefinite self-renewal and multi-lineage, multifunctional segregation and differentiation into differing cell types (Evans and Kaufman, 1981; Martin, 1981; Svendsen et al., 1999; Czyz and Wobus, 2001; Kuai et al., 2009). These features have increasingly made ESCs a critical focus of research (Byrne et al., 2006; Wobus and Loser, 2011; de Peppo and Marolt, 2012) in the development of novel cell-based therapeutics. However, there have been no approved treatments employing ESCs to date. Advancing stem cell-based therapeutics has been limited by the challenge of controlling cell differentiation, which is particularly difficult when working with pluripotent neural stem cells (NSCs). NSCs require specific signals to control cell differentiation, but if the signaling is not precisely controlled, the cells differentiate non-specifically. Nonetheless, the hallmark of stem cell research continues to be the use of NSCs, which subsequently differentiate into neurons, in tissue transplantation therapy to treat nervous system disorders (Temple, 2001), such as the congenital disorder idiopathic hypogonadotropic hypogonadism (IHH). IHH is a neuroendocrine disorder related to a Gonadotrophin Releasing Hormone (GnRH) deficiency (Young, 2012).

The cultivation of NSCs *in vitro* to control, improve and assure cell fate, including the differentiation into specific cell types, their proliferation and migration has been the subject of much research. Many earlier studies have shown that cytokines, chemicals (retinoic acid), selected proteins (eg., nanog, laminin), Chinese medicines, electrical stimulation, hormones, and low calorie environmental factors affect the differentiation of NSCs with varying outcomes (Boyer et al., 2005; Chambers et al., 2003; Flanagan et al., 2006; Hwang et al., 2004; Okada et al., 2004; Wang et al., 2006a,b; Dong et al., 2012). Other novel approaches have been used to improve NSC proliferation and differentiation, such as inducing excessive nephroblastoma gene expression (Shi et al., 2006). Utilizing growth medium inured with olfactory ensheathing cells (OECs) was found to promote axonal regeneration and functional recovery after transplantation (Barnett and Riddell, 2004; Pellitteri et al., 2009) and the use of carbon nanotube ropes as guide structures, in conjunction with electrical stimulation, supported the differentiation of NSCs into neurons (Huang et al., 2012). However, these approaches, including the more recent nanotechnology-based methods, to control

NSC differentiation all exhibited significant drawbacks, including, but not limited to, chemical toxicity, insufficient cell-type differentiation specificity and glial scar formation after transplantation (Rossi and Cattaneo, 2002).

Intrinsic and extrinsic factors have been suggested to play key roles in regulating neural stem cell differentiation and proliferation (McKay, 1997; Okano, 2002). While intrinsic factors that control pro-neural neurogenesis, such as basic/helix-loop-helix/leucine (b-HLH) transcription factors, have been more clearly defined in their role in determining neuronal fate (Sommer et al., 1996; Sun et al., 2001; Nieto et al., 2001), no extrinsic factor has been conclusively evaluated for their role in regulating NSC development, especially with respect to neuroendocrine cell differentiation.

Kisspeptins (KPs), peptides encoded by the *KISS1* gene (Lee et al., 1996), are endogenous ligands for the G-protein-coupled receptor GPR54, also known as hOT7T175 or AXOR12 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). The C-terminal end of KPs binds to and activates GPR54 signaling (Kotani et al., 2001), which has many downstream effects. Kisspeptin/GPR54 signaling has been shown to inhibit cellular motility, proliferation, invasion, chemotaxis and metastasis (Kotani et al., 2001; Ohtaki et al., 2001). The activation of GPR54 by KPs is also involved in cell growth and differentiation. Kisspeptin-10 (KP-10), a 10-amino acid segment of the KP C-terminal binding domain, was reported to play antiproliferative roles in a R366.4 cell line and to lead to cellular differentiation into more specific cell types (Huma et al., 2013). KP treatments have also been shown to increase GnRH mRNA levels and protein expression in the GT1-7 cell line in a dose-dependent manner (Terasaka et al., 2013; Sukhbaatar et al., 2013) and KP-10 treatments have been reported to effect GnRH neurite outgrowth *in vitro* (Fiorini and Jasoni, 2010). Collectively, these results suggest that KPs may play a previously unknown role in inducing GnRH expression in pluripotent NSCs.

In this study, different doses of KP-10 were administered to a GFP-labeled Lyon ES cell line at the NSC stage to examine the role of KPs on stem cell growth and cellular differentiation with respect to GnRH neuronal cell fate. Different exposure times and doses of KP-10 were used to measure the effect of kisspeptin on neuronal stem cell proliferation, differentiation, morphological changes and expression of GnRH (Fig. 1A).

EXPERIMENTAL PROCEDURES

Lyon ES cells

The rhesus monkey-derived tau GFP-Lyon ES cells used in this study were gifted by the Lyon Stem Cell Research Institute. The GFP-labeled Lyon ES cells were cultured in Knockout-DMEM (10829018, ThermoFisher Scientific Inc., Beijing, China) containing 20% knockout serum replacer (KSR, (10829028, ThermoFisher Scientific Inc.) supplemented with 1% nonessential amino acids (NEAA, 11140050, ThermoFisher Scientific Inc.), 2 mM L-glutamine (25030081, ThermoFisher Scientific Inc.), 0.1 mM β-mercaptoethanol (β-ME, Sigma–Aldrich China Inc., Shanghai, China) and 5 ng/ml basic fibroblast growth factor (bFGF) (13256029, Thermo-Fisher Scientific Inc.). Mechanical passaging of the undifferentiated colonies was performed manually every 5–7 days by cutting the colonies into large clumps using a flamed-pulled Pasteur pipette and then plating them onto a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts (CF-1-MEF; ATCC, Manassas, USA). CF-1 (mouse embryonic fibroblast cells) were grown in DMEM (11995065, ThermoFisher Scientific Inc.) with 2 mM L-glutamine supplemented with 15% Fetal Bovine Serum (FBS) (10099141, ThermoFisher Scientific Inc.).

Formation of embryonic bodies (EBs)

A previously established protocol (Zhang et al., 2001) with further modifications (Conti et al., 2005) was used to form EBs. Briefly, GFP-labeled Lyon ES cell colonies were digested with dispase (1 mg/ml) (D4818-2MG, Sigma-Aldrich China Inc., Shanghai, China) at 37 °C for 5–8 min, then washed with phosphate-buffered saline (PBS, 10010023, ThermoFisher Scientific Inc., Beijing, China) to remove dispase and then suspended in the N/M medium (1:1 DMEM/F12 (11320082, ThermoFisher Scientific Inc.): Neural Basal Medium (A2477501, ThermoFisher Scientific Inc.) containing 1xN2 supplement (17502048, ThermoFisher Scientific Inc.), 1xB27 (12587010, Thermo-Fisher Scientific Inc.) and 2 mM glutamine (G5792-100G, Sigma-Aldrich China Inc.). Cells were plated on 15 × 30-mm wells coated with 2% agar (A1296-500G, Sigma-Aldrich China Inc.) and allowed to aggregate for 4 days to form EBs (Fig. 1B).

Neural progenitor cell induction

After aggregation, EBs were selected and cultured in Neuronal Progenitor Media (NPM) (1:1 DMEM/F12: 1X ITS-x (51500056, ThermoFisher Scientific Inc.) containing 2 μg/ml heparin (H3149-500KU-9, Sigma-Aldrich China Inc.) and 2 mM glutamine in 4 well plates coated with Extracellular Matrix Media (ECM, E1270, Sigma-Aldrich China Inc.) for 3–4 days until rosettes appeared (Fig. 1C). Rosette-Neural Stem Cells (R-NSC) represent the first characterized neuronal stem cell stage at which cells are responsive to patterning cues that direct differentiation to specific neuronal subtypes (Darmon et al., 1981). A detailed experimental design is outlined in Fig. 1A.

Kisspeptin-10 treatment

On day 12 (early rosettes stage; Fig. 1C), NSCs were treated with vehicle (ddH_20), 0.1 nM, 1 nM, 10 nM, or 100 nM kisspeptin-10 (Phoenix Biotech Co. Ltd., Beijing, PR China). Dosages were based on findings from previous studies (Cho et al., 2009; Fiorini and Jasoni, 2010; Ramaesh et al., 2010; Milton et al., 2012; Huma et al., 2013; Terasaka et al., 2013). Media were changed daily and KP-10 solution was added immediately after

Please cite this article in press as: Huma T et al. Kisspeptin-10 treatment generated specific GnRH expression in cells differentiated from rhesus monkey derived Lyon NSCs. Neuroscience (2017), http://dx.doi.org/10.1016/j.neuroscience.2017.03.004