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2016-08-09


http://hdl.handle.net/10138/167151
https://doi.org/10.1016/j.stemcr.2016.06.007

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Development of Gonadotropin-Releasing Hormone-Secreting Neurons from Human Pluripotent Stem Cells

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http://dx.doi.org/10.1016/j.stemcr.2016.06.007

SUMMARY

Gonadotropin-releasing hormone (GnRH) neurons regulate human puberty and reproduction. Modeling their development and function in vitro would be of interest for both basic research and clinical translation. Here, we report a three-step protocol to differentiate human pluripotent stem cells (hPSCs) into GnRH-secreting neurons. Firstly, hPSCs were differentiated to FOXG1, EMX2, and PAX6-expressing anterior neural progenitor cells (NPCs) by dual SMAD inhibition. Secondly, NPCs were treated for 10 days with FGF8, which is a key ligand implicated in GnRH neuron ontogeny, and finally, the cells were matured with Notch inhibitor to bipolar TUJ1-positive neurons that robustly expressed GNRH1 and secreted GnRH decapeptide into the culture medium. The protocol was reproducible both in human embryonic stem cells and induced pluripotent stem cells, and thus provides a translational tool for investigating the mechanisms of human puberty and its disorders.

INTRODUCTION

The onset of puberty is regulated by a small population of hypothalamic gonadotropin-releasing hormone (GnRH) neurons, which secrete GnRH decapeptide to the hypophyseal portal system. Unlike other neuroendocrine cell types that reside in the hypothalamus, GnRH neurons are born outside the CNS in the frontonasal area. The olfactory pit, which is formed by the olfactory placodes (OP) and surrounding mesenchyme, provides a niche for GnRH neuron specification. Prenatally, GnRH neurons migrate from the frontonasal mesenchyme along with olfactory axons to the forebrain and into the hypothalamus, where their final maturation occurs (Wray, 2010). The early events that lead to GnRH neuron specification and the origin of their progenitors in the olfactory pit are currently poorly understood. Many existing data support OP as the source of GnRH neurons (Kim et al., 1999; Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a, 1989b), whereas others have proposed that at least some GnRH neurons arise from multiple sources, such as the neural crest, and adenohipophyseal or CNS progenitor cells (Forni et al., 2011; Markakis et al., 2004; Salvi et al., 2009; Whitlock et al., 2003).

GnRH neuron specification occurs under explicit spatiotemporal conditions, involving fibroblast growth factor 8 (FGF8) signaling and bone morphogenetic protein/transforming growth factor β (BMP/TGF-β) pathway inhibition (Forni et al., 2013; Kawauchi et al., 2005; Rawson et al., 2010). The indispensable role of FGF8 signaling through fibroblast growth factor receptor 1 (FGFR1) in this process has been established in various animal models (Chung and Tsai, 2010; Sabado et al., 2012). These findings are mirrored by reports in humans: mutations in FGF8 and FGFR1 are found in patients with congenital hypogonadotropic hypogonadism, a rare genetic disease that causes GnRH deficiency (Dode et al., 2003; Falardeau et al., 2008).

Human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), allow in vitro differentiation of specialized cell types, including neurons (Chambers et al., 2009; Davis et al., 2012; Hay et al., 2008). Here we report a protocol for the generation of GnRH-expressing neurons from hPSCs.

RESULTS

A schematic of the protocol is presented in Figure 1A. In the first step, we employed dual SMAD inhibition on hPSCs by blocking BMP and TGF-β/activin signaling pathways with dorsomorphin (DM) and SB431542 (SB), respectively, for 10 days to produce neural progenitor cells (NPCs) (Chambers et al., 2009). This was followed by 10-day treatment with FGF8, a key ligand in GnRH neuron development, and 4–8 days of treatment with both FGF8 and
Figure 1. Differentiation Protocol Schematic, and Expression of Anterior Neural Progenitor Markers after Neural Induction

(A) Schematic representation of the protocol. For the first 10 days the cells were treated with dual SMAD inhibition (dSMADi) using dorsomorphin and SB431542. FGF8 was added at d11, and Notch inhibitor DAPT was added at d21.

(B) Real-time qPCR results at d10 showing an increased expression of pan-neural marker SOX1 and forebrain- and olfactory placode-associated genes EMX2 and FOXG1. Preplacodal markers SIX1 and EYA1 remained low. Expression levels are relative to d0 hPSCs (HEL11.4 and H9 representative experiments, n = 6, mean ± SEM).

(legend continued on next page)
Notch inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), to induce terminal maturation of the neurons (Borghese et al., 2010). We used hiPSC line HEL11.4 (Mikkola et al., 2013), and hESC line H9 (Thomson et al., 1998; WiCell) for differentiation experiments, and the main results were repeated in hiPSC line HEL24.3 (Trovik et al., 2015). Both hiPSC lines have been established from healthy donor fibroblasts.

**Dual SMAD Inhibition Followed by FGF8 Treatment Produces Highly Proliferating Neural Progenitor Cells that Retain Anterior Identity**

Ten days of dual SMAD inhibition induced early pan-neural marker SOX2 and neural progenitor markers PAX6, EMX2, and FOXG1 (Figure 1B), which are expressed in the developing forebrain and the OP (Duggan et al., 2008; Forni et al., 2011; Simeone et al., 1992; Zhang et al., 2010). In contrast, the expression of preplacodal genes EYA1 and SIX1 (Ikeda et al., 2007; Schlosser et al., 2008) remained low (Figure 1B). Immunocytochemical analyses confirmed the expression of neural progenitor markers including PAX6, FOXG1, and SOX2, whereas preplacodal marker SIX1 was undetectable (Figure S1A). The expression levels of ventral forebrain marker NKX2.1 and caudal markers PAX5 and GBX2 (Kirkeby et al., 2012; Maroof et al., 2013) were low (Figures 1C and S1B). These results indicate that dual SMAD inhibition with DM and SB for 10 days efficiently induces anteriorly patterned NPCs.

During the subsequent 10 days, the anteriorly primed NPCs were treated with FGF8. At day 20 (d20), FGF8-treated cells had further increased the expression of EMX2 and FOXG1, as well as DLX2 and DLX5, which are also anteriorly expressed; in the frontonasal area, the OP, and the anterior basal forebrain (Merlo et al., 2007; Simeone et al., 1994) (Figures 1C and S1B). A high expression of these anterior markers was sustained throughout the protocol, whereas the expressions of NKX2.1, PAX5, and GBX2 remained low (Figures 1C and S1B). Between d10 and d20, the cells formed neural rosettes that abundantly expressed both SOX2 and FOXG1 (Figure 1D). Overall, an average of 93% (±0.9%, n = 5) of cells were FOXG1 positive at d21. FGF8-treated cells expanded more rapidly than the controls (Figure S1C). Proliferation occurred predominantly in the rosette structures, as judged by abundant Ki-67 expression (Figure 1D). Neural-specific TUJ1-positive cells, which were consistently negative for Ki-67, were located at the periphery and around the neural rosettes, indicating neuronal maturation and cell-cycle exit (Figure 1D).

**FGF8 and Subsequent Notch Inhibition Induces GnRH Expression**

To induce the terminal differentiation of NPCs, we inhibited Notch signaling with DAPT from d21 onward. After 4–8 days, the GNRH1 mRNA level was highly increased in cells supplemented with FGF8, but not in the control cells (Figures 2A and S2A). Immunofluorescence showed presence of GnRH-positive cells mostly in the peripheral regions of condensed cell clusters (Figure 2Ba). The GnRH-positive cells were bipolar and expressed neural marker MAP2 (Figure 2Bb). Concurrently, the number of proliferating (Ki-67 positive) cells was low, and, importantly, no GnRH and Ki-67 double-positive cells were observed (Figure 2C). This indicates that the GnRH-positive cells had exited the cell cycle. The neural identity of the GnRH-expressing cells was further confirmed by double staining with TUJ1 (Figures 2D and S2B). A proportion of the GnRH-positive cells also expressed FOXG1 (Figure 2E). On average, 15.1% (±1.6%, n = 4) of the HEL11.4-derived cells and 12.2% (±4.2%, n = 3) of the H9-derived cells were GnRH positive.

**Secretion of GnRH Decapeptide**

Human GnRH is translated as a 96-amino-acid prohormone, which is processed by intracellular proteases to form the secreted decapetide. The GnRH decapetide was clearly detectable in the culture medium of the FGF8-treated cells, but not of the control cells from d25 onward, with the peak concentrations measured on d27 (Figures 3A and S3A). High-magnification images of GnRH immunopositive cells showed a punctate staining pattern in the majority of the cells, indicating vesicular packaging of GnRH decapetide (Figures 3B and S3B). Activated caspase-3 (Porter and Janicke, 1999) was not present in the GnRH-positive cells (Figure 3C), suggesting that the release of GnRH was not due to cell death. FGF8-treated cells readily responded to KCl-induced depolarization by 1.97-fold higher GnRH decapetide release (95% confidence interval, 1.62–2.31) when compared with vehicle-treated cells.

**Migration of GnRH-Expressing Cells**

GABA_A receptor stimulation has been shown to decelerate the migration of embryonic mouse GnRH neurons (Casoni et al., 2007; Oinuma et al., 2008). Notch inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), to induce terminal maturation of the neurons (Borghese et al., 2010). We used hiPSC line HEL11.4 (Mikkola et al., 2013), and hESC line H9 (Thomson et al., 1998; WiCell) for differentiation experiments, and the main results were repeated in hiPSC line HEL24.3 (Trovik et al., 2015). Both hiPSC lines have been established from healthy donor fibroblasts.
Figure 2. GnRH-Expressing Cells Emerge after Treatment with FGF8 and DAPT

(A) Relative expression of GNRH1 at the end of the protocol in HEL11.4-derived cells (n = 4), and H9-derived cells (n = 6) treated with or without FGF8 (mean ± SEM). **p < 0.01, ****p < 0.0001 (paired ratio t test).

(B–E) Immunocytochemistry in HEL11.4-derived cells at the end of the protocol. (B) GnRH-positive neurons appeared mostly in the periphery of condensed node-like structures. These cells appeared to be interconnected by long cellular projections (a), which stained positive for MAP2 (b). (C) The GnRH-expressing cells were not proliferating, as indicated by the absence of colocalization with Ki-67 staining. (D) The GnRH-positive cells also expressed neuron-specific marker TUJ1. See also Figure S2B. (E) At the end of the protocol, the majority of the cells expressed FOXG1. Some of the GnRH-positive cells were also FOXG1 positive. All scale bars represent 50 μm.
et al., 2012). Therefore, we investigated the migratory properties of our FGF8-treated cells with a gap-closure assay, whereby d25 cells were allowed to migrate to an empty area in the middle of the culture wells in the presence or absence of GABA<sub>A</sub> receptor agonist muscimol (0.1 mM). After 50 hr, significantly fewer cells had migrated to the empty area in muscimol-treated wells than in control wells (paired ratio t test, n = 4; p < 0.05).

Detection of GnRH-Expressing Neurons in FOXG1-Positive Frontonasal Tissues
As some GnRH cells were positive for FOXG1 in vitro, we investigated whether GnRH neurons express FOXG1 also in vivo. Immunohistochemistry on an 8-gestational-week human fetus showed migrating GnRH neurons that appeared in typical cell clusters, called the migratory mass (Miller et al., 2010; Tarozzo et al., 1995; Wray et al.,
The cell clusters contained GnRH neurons, other neurons, and glial cells that delaminated from the olfactory epithelium and migrated toward the forebrain (Figure 4A). Indeed, FOXG1 was expressed in the GnRH neurons, as well as in other cells of the migratory mass and the olfactory epithelium (Figure 4A). Many GnRH-positive cells appeared also to stain with TUJ1 (Figure 4B).

**DISCUSSION**

According to current understanding, GnRH neurons are first formed during early embryogenesis in the olfactory placode area, from where they migrate to the hypothalamus via the vomeronasal/terminal nerve fibers (Wray, 1994). Once at their final destination, they mature and become an integral part of the hypothalamic-pituitary-gonadal axis. While the OP have been generally accepted as a source of GnRH neurons, the exact origin of GnRH neuron progenitors has remained controversial. Our results show that GnRH-secreting cells can be efficiently differentiated from NPCs that express high levels of anterior neural progenitor markers. The induction of neural commitment in hPSCs by using dual SMAD inhibition leads by default to anterior neural cell fates (Chambers et al., 2009; Kirkeby et al., 2012; Pankratz et al., 2007). Indeed, our dual SMAD inhibition-treated NPCs showed high expression of FOXG1 and EMX2. Expressed in the frontonasal area, where GnRH neurons first emerge, FOXG1, DLX2, and DLX5 have been suggested to be involved in GnRH neuron

**Figure 4. GnRH, FOXG1, and TUJ1 in an 8-Gestational-Week Human Fetus**

(A) Immunohistochemistry of coronal sections in a human fetus. GnRH was expressed in the frontonasal area between the olfactory epithelia (oe) and the nasal medial cartilage (nmc), in migrating cells also known as the migratory mass. These cells were FOXG1-positive, including GnRH-expressing cells. The arrow in the top panel indicates the area magnified in the middle panel. GnRH-FOXG1 double-positive cells were also detected in the ventral forebrain. (B) TUJ1 was expressed in the migratory mass cells, including GnRH-expressing cells. Arrow indicates the area magnified on the right. cp, cribriform plate; fb, forebrain; lv, lateral ventricle; nmc, nasal medial cartilage; oe, olfactory epithelia; tn, terminal nerve.
development (Duggan et al., 2008; Givens et al., 2005; Iyer et al., 2010). Indeed, all of these markers were upregulated along our differentiation protocol. Placodal markers SIX1 and EYA1 (Schlosser, 2014) were, however, not upregulated at any time during this protocol.

Our results show that anterior NPCs can give rise to GnRH-expressing cells when cultured with FGF8, which has been shown to promote progenitor cell survival in the anterior neural structures (Forni et al., 2013; Kawauchi et al., 2005). In mouse anterior neural ridge, FGF8 has been shown to positively regulate Foxg1 expression (Shimamura and Rubenstein, 1997), and mouse embryos that carry hypomorphic and conditional Fgf8 mutations display reduced telencephalons, which is suggested to result from a decreased Foxg1 expression (Storm et al., 2006). In our model, FOXG1 expression persisted following FGF8 treatment, and importantly, FOXG1 was also expressed in migrating GnRH neurons of a human fetus.

The final step in our protocol comprised Notch inhibition, which induces neuronal maturation from progenitor cells (Borghese et al., 2010; Imayoshi et al., 2010; Noisa et al., 2014; Yoon and Gaiano, 2005). As expected, the blocking of Notch in our FGF8-treated cells after d20 decelerated cell proliferation, increased the expression of neuron-specific markers TUJ1 and MAP2, and induced GNRH1 expression, with up to 15% of the cells being immunopositive for GnRH. This efficiency is high, given that humans only have few thousand GnRH neurons. Two recent publications have reported hPSC differentiation to hypothalamic-like neuroendocrine cells, which is suggested to result from a decreased Foxg1 expression (Storm et al., 2006). In our model, FOXG1 expression persisted following FGF8 treatment, and importantly, FOXG1 was also expressed in migrating GnRH neurons of a human fetus.

Analysis of GnRH Expression and Secretion
RNA extraction and real-time qPCR were performed using standard protocols. The expression levels of target genes were normalized to cyclophilin G, and compared with undifferentiated hPSCs. For immunocytochemistry, cells were fixed by 4% paraformaldehyde and immunostainings were performed using standard protocols. FOXG1 and GnRH immunopositive cells were quantified by manually counting the cells in images taken with a 40x objective. Secreted GnRH was quantified by a competitive fluorescent enzyme immunoassay.

Biomedical Ethics
A human fetus (8 gestational weeks) was obtained from a voluntarily terminated pregnancy with the parent's written informed consent. Permission to utilize human fetal tissues was obtained from the French agency for biomedical research (Agence de la Biomedecine, Saint-Denis la Plaine, France, protocol no. PFS16-002). Tissues were made available in accordance with French bylaws. Tissues were fixed with 4% paraformaldehyde and immunostaining was performed on 20-μm cryosections. Immunostaining was performed according to standard procedures (INSERM).

Statistical Analyses
When used, “n” always stands for number of independent experiments. Statistical comparisons were performed by using paired ratio t test in Prism 5.0 (GraphPad). p < 0.05 was accepted to indicate statistical significance.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.06.007.
ACKNOWLEDGMENTS

We thank the Biomedicum Stem Cell Center and the Biomedicum Imaging Unit for advice and assistance. We thank Professor Erik Harbovszky for the generous gift of GnRH antibody, Professor Timo Otonkoski for valuable comments, Mr. Samuel Malone for the human fetus collection and processing of the samples for immunohistochemical procedures, and M.A. Annika Tarkkanen for linguistic guidance. This work was supported by the Academy of Finland, Foundation for Pediatric Research, Sigrid Juselius Foundation, Novo Nordisk Foundation, Emil Aaltonen Foundation, University of Helsinki, Helsinki University Central Hospital, and Agence Nationale de la Recherche, ANR, France (ANR-14-CE12-0015-01 RoSes and GnRH).

Received: November 26, 2015
Revised: June 13, 2016
Accepted: June 15, 2016
Published: July 14, 2016

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