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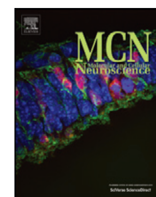

hVGAT-mCherry: A novel molecular tool for analysis of GABAergic neurons derived from human pluripotent stem cells

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ABSTRACT

Background: GABAergic synaptic transmission is known to play a critical role in the assembly of neuronal circuits during development and is responsible for maintaining the balance between excitatory and inhibitory signaling in the brain during maturation into adulthood. Importantly, defects in GABAergic neuronal function and signaling have been linked to a number of neurological diseases, including autism spectrum disorders, schizophrenia, and epilepsy. With patient-specific induced pluripotent stem cell (iPSC)-based models of neurological disease, it is now possible to investigate the disease mechanisms that underlie deficits in GABAergic function in affected human neurons. To that end, tools that enable the labeling and purification of viable GABAergic neurons from human pluripotent stem cells would be of great value.
Results: To address the need for tools that facilitate the identification and isolation of viable GABAergic neurons from the in vitro differentiation of iPSC lines, a cell type-specific promoter-driven fluorescent reporter construct was developed that utilizes the human vesicular GABA transporter (hVGAT) promoter to drive the expression of mCherry specifically in *VGAT*-expressing neurons. The transduction of iPSC-derived forebrain neuronal cultures with the hVGAT promoter-mCherry lentiviral reporter construct specifically labeled GABAergic neurons. Immunocytochemical analysis of hVGAT-mCherry expression cells showed significant co-labeling with the GABAergic neuronal markers for endogenous VGAT, GABA, and GAD67. Expression of mCherry from the VGAT promoter showed expression in several cortical interneuron subtypes to similar levels. In addition, an effective and reproducible protocol was developed to facilitate the fluorescent activated cell sorting (FACS)-mediated purification of high yields of viable VGAT-positive cells.
Conclusions: These studies demonstrate the utility of the hVGAT-mCherry reporter construct as an effective tool for studying GABAergic neurons differentiated in vitro from human pluripotent stem cells. This approach could provide a means of obtaining large quantities of viable GABAergic neurons derived from disease-specific hiPSCs that could be used for functional assays or high-throughput screening of small molecule libraries.
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1. Introduction

The use of human pluripotent stem cells (hPSCs) has proven to be a powerful approach for studying human development and disease. The impact of hPSC-based differentiation strategies has been of particular importance in modeling diseases of the central nervous system (CNS), including many neurodevelopmental, neuropsychiatric and neurodegenerative disorders, due to the lack of readily available primary tissues from affected individuals. Where available, this tissue is restricted to post-mortem samples. A great deal of progress has been made in the development of methods for the differentiation of hPSCs into specific neuronal cell types (Chambers et al., 2009). Although effective at enriching for the specific neuronal type, these protocols result in the production of heterogeneous cultures containing multiple neuronal cell types, as well as, underlying supporting cells such as astrocytes and glial cells (Bilic and Izpisua Belmonte, 2012; Maherali and Hochedlinger, 2008; Narsinh et al., 2011). As a result, approaches are needed for the identification and isolation of specific cell types of interest from the complex mixture of cells.

Recent studies have supported an important role for inhibitory neurons, such as cortical interneurons (GABAergic neurons), in the development of the cortex. In addition, impairment in this cell

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population has been linked to neuropsychiatric disorders such as autism and schizophrenia (Acosta and Pearl, 2003; Lewis and Levitt, 2002; Rubenstein and Merzenich, 2003; Spencer et al., 2004). Although GABAergic interneurons make up a relatively small fraction of the total number of cells in the neocortex, even small changes in the balance of excitation and inhibition could have profound effects on key neurological functions, including cognition, sensory perception, language and spatial reasoning (Lui et al., 2011). This suggests that GABAergic interneurons play an important role in not only regulating the degree of excitation of the neocortex but also in the fine-tuning of neural networks and the quality of information processing across the different regions of the cortex.

The vesicular γ-aminobutyric acid (GABA) transporter (VGAT; also called SLC32A1) is a well-known marker for GABAergic neurons and is specifically expressed in inhibitory neurons (Gasnier, 2000; Sagne et al., 1997). GABA is the principal inhibitory neurotransmitter in the mammalian CNS and functions by binding to specific transmembrane receptors on both pre- and post-synaptic neuronal processes (Watanabe et al., 2002). It is synthesized from glutamate through the actions of two glutamate decarboxylases (GAD65 and GAD67) and loaded into synaptic vesicles by the vesicular GABA transporter (VGAT or SLC32A1) (Jin et al., 2003). In transgenic mouse and rat lines, the selective fluorescent labeling of nearly the entire GABAergic neuron population in the neocortex (>95%) has been accomplished using bacterial artificial chromosome (BAC) constructs that allow the expression of the fluorescent marker Venus, to be driven from the VGAT gene promoter (Uematsu et al., 2008; Wang et al., 2009). Given the results of these studies, we reasoned that a human VGAT promoter-driven fluorescent reporter construct might serve as a useful tool in the identification and isolation of the population of GABAergic cortical neurons generated in vitro through the differentiation of hiPSCs.

To begin to identify inhibitory GABAergic interneurons from complex hPSC-derived neuronal cultures, an ~1800 base pair region of the VGAT promoter was cloned upstream of the fluorescent marker mCherry. Lentiviral-mediated delivery of the VGAT promoter driven fluorescent construct (pLV-hVGAT-mCherry) resulted in sustained expression of mCherry in GABAergic interneurons that co-localized with endogenous VGAT expressing cells. Although variable levels of expression were observed, the majority of hVGAT promoter-mCherry positive cells stained positively for GAD67 and GABA. In addition, these VGAT promoter mCherry positive cells could be purified by fluorescent activated cell sorting (FACS) using a novel protocol resulting in a highly pure and viable population of mCherry-expressing cells. These hVGAT-mCherry expressing cells stained positively for GABA and survived for over 3 weeks post-FACS purification. This population of hVGAT positive cells included a variety of GABAergic interneuron subtypes with unique developmental origins.

2. Methods

2.1. Development of the human inhibitory neuron-specific hVGAT-mCherry fluorescent reporter construct

To target human stem cell-derived GABAergic neurons in heterogeneous populations of differentiated cells, a lentiviral-based fluorescent reporter system was developed that uses the human VGAT (solute carrier family 32 (GABA vesicular transporter), member 1, SLC32A1) gene promoter to drive the expression of the fluorescent protein mCherry (Fig. 1). A 1865 bp region of the VGAT gene including 262 bp downstream of the transcription start site (TSS) and 1603 bp upstream of the TSS was PCR amplified from genomic DNA purified from foreskin fibroblast cells. This region overlaps with peaks for several markers of promoter activation, histone H3K4 monomethylation (H3K4me1), histone H3K4 trimethylation H3K4me3, and RNA polymerase II (Pol2) binding (Fig. 1A). The purified PCR product was digested with BamHI and EcoRI and ligated into the corresponding sites in the pENTR4 no ccdB vector (Addgene plasmid 17424) into which the mCherry gene had been previously cloned (Campeau et al., 2009). The inserted region of the VGAT promoter was confirmed by Sanger sequencing and the VGAT promoter mCherry cassette was transferred to the lentiviral vector pLentiX1 puro DEST (Addgene plasmid 17297) using the LR Clonase II enzyme mix (Invitrogen) to produce the pLV-hVGAT-mCherry vector. The pLV-hSYN-RFP vector was obtained from Addgene (Addgene plasmid 22909) (Nathanson et al., 2009).

2.2. Production of lentiviral expression particles

The pLV-hVGAT-mCherry was packaged by cotransfection with the psPAX2 lentiviral packaging plasmid (Addgene plasmid 12260) and the Vesicular stomatitis virus envelope glycoprotein expressing pCMV-VSV-G plasmid (Addgene plasmid 8454) in Lenti-X 293T cells (Clontech) using jetPRIME® (Polyplus-transfection™). The culture supernatant was harvested after 48 h and the viral particles concentrated using the Lenti-X™ concentrator (Clontech) according to the manufacturer's protocol. The concentrated virus was resuspended in DMEM:F12 media, aliquoted, and stored at −80 °C. The well characterized reporter construct expressing red fluorescent protein (RFP) from the neuron-specific synapsin I gene promoter (pLV-hSynapsin-RFP, hSYN-RFP) lentiviral construct has been used throughout as a positive control for neurons. Lentiviral transduction of the reporter constructs was carried in culture medium supplemented with 4 μg/mL polybrene using a multiplicity of infection (MOI) of 5.

2.3. Culture of hiPSCs and in vitro differentiation of ventral forebrain-like neurons

Human iPSCs (System Biosciences) were maintained on mitomycin c treated mouse embryo fibroblasts (MEFs) (EmbryoMax® Primary Mouse Embryo Fibroblasts, Strain CF1; Millipore) in mTESR1 culture medium (STEMCELLTechnologies) supplemented with 10 μM CHIR99021 (STEMGENT®), 1 μM PD325901 (STEMGENT®), 1 μM thiazovivin (STEMGENT®), and 10 μM Y27632 (STEMGENT®). The culture medium was changed daily and hiPSC colonies were enzymatically passaged with StemPro® Accutase® Cell Dissociation Reagent (Thermo Fisher Scientific) at a 1:4–1:6 split ratio every 4 to 7 days. If identified, spontaneously differentiated cells were mechanically removed prior to passaging. All cultures described here were kept in a 37 °C incubator with 5% CO_2.

Prior to inducing neural differentiation, hiPSCs were isolated from MEF feeder layer cells through magnetic column separation using MEF-specific antibodies (anti-mEF-SK4) coupled to paramagnetic beads (Miltenyi Biotec). Magnetic separation of the cells was carried out according to the manufacturer's instructions with few exceptions. Briefly, hiPSC colonies were dissociated into a single cell suspension through a 10-min treatment with the StemPro® Accutase® Cell Dissociation Reagent. In lieu of MACS buffer, mTESR1 containing 2 μM thiazovivin and 20 μM Y27632 was used for both incubation of the cells in MEF-specific antibodies and for column washes during magnetic separation.

Cortical inhibitory neuron differentiation of hiPSCs was carried out using a chemically defined system similar to the previously described B27 + 5F method (Nicholas et al., 2013). The concentration of recombinant growth factors and small molecule compounds used in our differentiation scheme, in addition to the days that they were added to the differentiation medium are listed in Table S1. The diagram shown in Fig. 2A outlines the methods used for cortical inhibitory neuron differentiation. Neural induction was initiated through the formation of neural aggregates using AggreWell™800 plates (STEMCELLTechnologies) according to the manufacturer's protocol. Briefly, 3–4.5 × 10^6 cells (10,000 to 15,000 cells per neural aggregate) were added to each well of the AggreWell™800 plate in neural induction media (NIM; STEMCELLTechnologies) supplemented with 10 μM Y27632, 10 μM

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