International Journal of Developmental Neuroscience 69 (2018) 112–118

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

Notch signaling in human iPS-derived neuronal progenitor lines from Focal Cortical Dysplasia patients

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ARTICLE INFO

Keywords:
Focal cortical dysplasia type II
Drug refractory
Induced puluripotent stem cell (iPSCs)
Neurodifferenciation
Cortical malformation

ABSTRACT

Focal Cortical Dysplasia (FCD), a common type of Malformations of cortical development, may result from an early disturbance in the migration and final arrangement of the cortical architecture of immature neurons. FCD type II is now known to be due to a post-zygotic somatic mutation that involves the mTOR and AKT pathways. The aim of the present study was to investigate the possible differences in neurogenesis and neurodifferentiation of iPSCs (induced pluripotent stem cells) from fibroblasts of individuals affected by FCD type II (2) and normal individuals (2). iPSCs were generated from skin fibroblasts of FCD individuals and healthy individuals. The reprogramming was done through the fibroblasts exposure to viral vectors containing the OCT4, KLF4, SOX2, and c-MYC genes and the clones were characterized by immunohistochemistry. iPSCs were neurodifferentiated and analyzed at the 14th, 22nd and 35th days. We also analyzed the cerebral cortex tissue, fibroblasts and iPSCs cells from the individuals. Through qRT-PCR, the expression of 4 genes involved in Notch signaling process were quantified. In general, individuals with dysplasia presented increase and decrease in the relative quantification in the most genes analyzed compared to control individuals in all processes and study groups. We suggest that, during embryonic neurogenesis, the neural precursor cells of FCD type II individuals present increase and decrease in gene expression in the Notch signaling pathway causing cortical formation disorders and can be seen as a candidate for the developmental changes observed in the cerebral cortex of individuals with FCD type II. This altered gene expression may be related to brain formation with dysplasia.

1. Introduction

Focal Cortical Dysplasia (FCD) is one of the most frequent forms of malformations of cortical development (MCD), and the pathology underlying a significant portion of partial epilepsy refractory to drug treatment, seizures usually begin in childhood (Palmini et al., 2004). Previous studies have shown that the pathogenesis of MCD in general is multifactorial since several factors influence corticogenesis, among them genetic mutations, pre and postnatal injuries (Pascual-Castroviejo et al., 2012; Sisodya, 2004). Both genetic and acquired factors may be involved in the pathogenesis of cortical dysplasia, sharing a phenotype and a possible origin with other cortical malformations. FCD may result from an early disturbance in migration and final arrangement of the cortical architecture of immature neurons (Rowland et al., 2012), but the pathogenesis of these malformations has not yet been fully elucidated. FCD type II, an isolated lesion characterized by cortical dyslamination and dysmorphic neurons without (FCD IIa) or with balloon cells (FCD IIb) (Blümcke et al., 2011), is now known to be due to a post-zygotic somatic mutation that involves the mTOR and AKT pathways (Lee et al., 2012; Lim et al., 2015; Poduri et al., 2012; Blümcke et al., 2015).

Notch signaling represents another molecular pathway that is integral to cortical development. It is responsible for regulating neuronal self-renewal and differentiation (Breunig et al., 2007), the survival (Saura et al., 2004) and neuronal plasticity (De Bivort et al., 2009; Zhang et al., 2014). Its activity has a strong impact on the morphology of developing neurons (Whitford et al., 2002a; Whitford et al., 2002b). This signaling pathway promotes an asymmetric division of progenitor cells into a neural stem cell and a differentiated neuron through lateral inhibition (Imayoshi and Kageyama, 2011). Rapid activation of cytoplasmic signaling thorough PI3 kinase-Akt and mammalian target of rapamycin (mTOR) mediates the notch-1 activation signaling that promotes the survival of neural stem cell (Lathia et al., 2008).

In this study, iPSC (induced pluripotent stem cells)-derived neurons were obtained from FCD type II and control patients by reprogramming fibroblasts using a Sendai Reprogramming kit containing the four

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https://doi.org/10.1016/j.ijdevneu.2018.07.006
Received 19 May 2018; Received in revised form 18 June 2018; Accepted 17 July 2018
Available online 23 July 2018

transcription factors OCT4, SOX2, KLF4, and CMYC. A gene profiling analyses of these differentiated neurons was performed using qRT-PCR. Genes activity associated with Notch signaling were quantified during different periods of neurodifferentiation of pluripotent stem cells (iPSCs) from affected and control patients and cerebral cortex tissue.

2. Methods

2.1. Patients enrolled

The human fibroblasts were obtained from biopsies from two patients compatible with FCD type II, free from infectious diseases, undergoing surgical treatment for medically refractory epilepsy at the Epilepsy Surgery Program at São Lucas Hospital after signing the informed consent form. As control, were obtained skin residual fragment from two adult pacient, healthy, free of infectious and neurological disease treated at the Plastic Surgery Program at São Lucas Hospital after signing the informed consent form. Procedures were performed in accordance with protocol number 915.598 (CAAE: 37977114.3.0000.5336) approved by the Ethics Committee for Analysis of Research Projects of São Lucas Hospital at PUCRS. The entire characterization of the patients as well as the production of fibroblasts from skin biopsies, histologic analysis of dysplastic tissue and generation of iPSCs are published in Marinowic et al. (2017). Healthy “brain” tissue was obtained from RNA already extracted from another Project.

2.2. Neurodifferentiation of iPSCs

Neurodifferentiation was performed according to a protocol described by Song and Sanchez-Ramos (2008). Briefly, clones dissociates as monolayers were grown on Neurobasal Medium N5 (Gibco) supplemented with 20 ng/mL of Brain Derived Neurotropic Factor (Sigma-Aldrich), B27 1X (Gibco) and 10% de KnockOut Serum Replacement. Cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 with daily medium change. Cells were cultured for the periods of 12, 25 and 35 days. Light field image captures were performed using a microscope Axiovert 25 (Zeiss) in different stages of the neurodifferentiation protocol.

2.3. Evaluation of neurodifferentiation by immunofluorescence

The evaluation of neurodifferentiation and identification of neuronal structures was performed by immunofluorescence assay. Briefly, cells were fixed with 4% paraformaldehyde for 15 min. The cultures were then washed with DBPS buffer and 1 ml per well of blocking solution (Fetal Bovine Serum and Bovine Serum Albumin) (Life Technologies), incubated for one hour at room temperature. After incubation, the antibody FluoroPan Neuronal Marker (Chemicon) conjugated to fluorescein isothiocyanate (FITC) was added, diluted in blocking buffer (1:100) for characterization of cytoarchitecture and neuronal polarization. The cultures were incubated at room temperature for two hours. Samples were washed twice with DPBS buffer and 0.01% DAPI added for nuclear labeling. Cell images were captured by Zeiss LSM-5 exciter confocal microscope using excitation wavelength of 488 nm for antibody labeling and 405 nm for nuclear labeling by DAPI.

2.4. RNA extraction and cDNA synthesis

Molecular analysis of genes related to neurogenesis in cerebral cortex tissue, fibroblasts, iPSCs cells, during different periods of neurodifferentiation and in neurodifferentiated iPSC cells from affected patients and control patients were performed. RNA was extracted using SV-Total RNA kit (Promega). The samples were exposed to a lysis buffer, heated at 70 °C for 3 min and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was collected and 200 µL of 95% alcohol was added. The contents were transferred to a silica column and centrifuged at 12,000 *g* for 1 min. 600 µL of buffer was added and centrifuged again at 12,000*g* for 1 min. Diluted DNAse enzyme was added in buffer and manganese chloride (MgCl2) and incubated for 15 min at room temperature. Enzyme blocking buffer was added and the columns were again centrifuged at 12,000*g* for 1 min. The columns were washed with wash buffer and the silica-retained RNA was eluted with 100 µL of RNAse-free water by centrifugation at 12,000*g* for 2 min. The extracted RNA was quantified using the Qubit 2.0 fluorometer (Life Technologies) according to manufacturer's guidance. Aliquots of 5 µL of each extracted RNA sample were added to 195 µL of buffer containing an Assay RNA HS Assay (Life Technologies) and reading for quantification. The cDNA synthesis was performed using SuperScript VILO MasterMix (Life Technologies) as directed by the manufacturer. 16 µL of RNA was added to 4 µL of the SuperScript VILO master mix and exposed to thermal cycles (25 °C for 10 min, 42 °C for 60 min and 85 °C for 5 min) using Verit thermal cycler (Life Technologies). DNA complementary to the extracted RNA was quantified using the Qubit 2.0 fluorometer (Life Technologies) as directed by the manufacturer. Aliquots of 1 µL of each cDNA sample were added to 199 µL of buffer containing an Assay ssDNA probe (Life Technologies) and read out for quantification.

2.5. Molecular analysis through qRT-PCR

For analysis of gene expression, a real-time Polymerase Chain Reaction (PCR) technique was performed using SpetOne Plus (Life Techologies) equipment. Amplifications were performed from 20 ng of cDNA for each sample. Assays were performed on individual plaques for each gene using the GAPDH gene as an endogenous expression control. Below the primer sequences used (Table 1).

3. Results

3.1. Patients enrolled

Both individuals were affected by FCD type II, more specifically, IIb (Fig. 1). The diagnosis was made through imaging and EEG exams, and confirmation of homogeneity and type of FCD through histopathological analysis, allowing the pairing of those involved. The participants are of opposite sexes and have a great difference of age, being an adult patient and the other pediatric patient. At the time of collection of the skin biopsy, the male individual was 45 years old (Indiv. 01), and the female, 12 years old (Indiv. 02). Both patients underwent resection of

Table 1
RT-PCR and qPCR primers for iPSC and differentiated neuron gene markers.

	Forward	Reverse
HEY1	5′-CGAAATCCCAAACTCCGATA-3′	5′-TGGATCACCTGAAAATGCTG-3′
NOTCH1	5’-GGT CAA TGA GTG CAA CAG CA-3′	5’-GGG TCA CAG TCG CAC TTG TA-3′
HES1	5′-AGTGAAGCACCTCCGGAAC-3′	5′-TCACCTCGTTCATGCACTC-3′
PAX5	5′-CTGATCTCCCAGGCAAACAT-3′	5′-TTGCTCATCAAGGTGTCAGG-3′
GAPDH	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	5′-CATGTGGGCCATGAGGTCCACCAC-3′

HEY1 (hes related family bHLH transcription factor with YRPW motif 1); *NOTCH1* (Notch homolog 1); *HES1* (Hes family bHLH transcription factor 1); *PAX5* (Paired box 5).