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Analysis of genes involved in cell proliferation, adhesion, and control of apoptosis during embryonic neurogenesis in Induced Pluripotent Stem Cells (iPSCs) from patients with Focal Cortical Dysplasia

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ABSTRACT

Focal cortical dysplasia (FCD) is a malformation of cortical development which is strongly associated with drug-refractory epilepsy. Certain studies have demonstrated an increase in mTOR signaling in patients with FCD on the basis of observation of phosphorylated molecules. The aim of the present study was to verify the differences in genes involved in cell proliferation, adhesion, and control of apoptosis during embryonic neurogenesis in iPSCs derived from the Focal Cortical Dysplasia. Fibroblasts were obtained from the skin biopsies of patients with FCD (n = 2) and controls (n = 2). iPSCs were generated by exposing the fibroblasts to viral vectors that contained the Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC genes) responsible for promoving cell reprogramation. The fibroblasts and iPSCs were tested during different phases of neurodifferentiation for migration capacity and expression of the genes involved in the PI3K pathway. Fibroblasts of patients with FCD migrated with greater intensity during the first two time points of analyses. iPSCs did not exhibit any difference in cell migration between the groups. Fibroblasts, brain tissue, and iPSCs of the patients with FCD exhibited a significant reduction in the relative expression values of 4EBP–1. During neurodevelopment, the iPSCs from patients with FCD exhibited a reduction in the expression of cIAP–1, cIAP–2, PI3K, β-Catenin and 4EBP–1 gene. We suggest that the differences observed in the migration potential of adult cells and in the gene expression related to the fundamental processes involved in normal brain development during the neurodifferentiation process might be associated with cortical alteration in the patients with FCD.

1. Introduction

Focal cortical dysplasias (FCD) are malformation of cortical development and the main clinical manifestation is epilepsy with difficult drug control (Palmini et al., 2004). Around 12–40 % of patients with FCD were submitted to surgery for refractory epilepsy (Arai et al., 2012; Prayson et al., 2002). FCD type IIb is characterized by presenting balloon cells which correspond to large, rounded cells with a single nucleus or multiple nuclei, abundant clear cytoplasm and a poorly defined cell membrane, expressing positive markers for neurons, glial and undifferentiated neural progenitors cells (Kabat and Król, 2019; Taylor et al., 1971). Studies have demonstrated the etiology of FCD might be related to clonal somatic mutations in signaling pathways, however it is still unknown (Kuzniecky, 2015). Some studies have verified an increase in the mechanisms of mTOR signaling in the patients with FCD, observing some phosphorylated molecules such as S6

Abbreviations: FCD, Focal Cortical Dysplasia; iPSC, Induced Pluripotent Stem Cell; PI3K, phosphoinositide 3-kinase; AKT or PKB, protein kinase B; PUCRS, Pontifical Catholic University of Rio Grande do Sul; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; BDNF, Brain Derived Neurotrophic Factor; KSR, KnockOut Serum Replacement; CNS, central nervous system

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ribosomal proteins. Studies suggested that mTOR signaling was increased in 80–90% of the balloon cells and giant neurons in the cerebral cortex (Hsu et al., 2011). Studies using dysplasia tissue evidenced phosphorylation of molecules associated to the phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT or PKB) pathways (Zhou et al., 2009). Events that control cell growth, cell cycle, cell migration, and cell survival can stimulate the phosphorylation of proteins related to the PI3K pathway (Cantley, 2002).

D'Gama et al. (2017) investigated the cell type-specificity of abnormal mTOR activation in dysplasia pathogenesis, confirming the association of FCD with mutations in AKT1, AKT3, DEPDC5, MTOR, NPRL2, PIK3CA, TSC1, and TSC2. The authors demonstrated, through the use of sequencing, the activation of mTOR in the neurons in all the lesions. Induced activation of PIK3CA in the cortex of mice resulted in the activation of mTOR in the excitatory and glial neurons, though not in the interneurons, which is required for the abnormal cortical overgrowth. The study demonstrated that the activation of mTOR in dorsal telencephalic patients could be related to cortical dysplasia D'Gama et al. (2017). Currently, there is an estimative that cortex formation suffers 33 mitotic cycles for producing all nervous cells that will form the neocortex. Same alteration (somatic mosaicism) associated to mTOR pathway were described for FCD type II and hemimegaloencephaly (HME). The difference between these two conditions is in the mitotic cycle occurrence of the somatic mosaicism, whereas for the FDC type II it happens in late cycle (e.g., cycle 32) for HME it occurs in early cycle (e.g., cycle 6), appearing to be the same disease (Sarnat and Flores-Sarnat, 2014). A study by Avansini et al. in 2018 associated the abnormal gene regulation in FCD type II with microRNAs–hsa-let–7f, hsa-miR–31, and hsa-miR34a. Negative regulation of hsa-miR–34a induced a positive regulation of NEUROG2 gene and the super-expression of RND2 gene, leading to a defective coupling in the mechanisms of differentiation and neuronal migration, which may explain the presence of aberrant cells and complete dyslamination in FCD type II (Avansini et al., 2018).

Marinowic et al. (2017) generated induced pluripotent stem cells (iPSCs) from skin fibroblast of patients with FCD type IIb using Yamanaka factors and recently, Majolo et al. (2018) showed difference in expression of genes involved in Notch signaling pathway during embryonic neurogenesis. Also, the same author Majolo et al. (2019) found alterations in genes that may contribute to delays in synaptogenesis. The utilization of iPSCs derived from the patients with cortical malformation diseases may assist in illuminating the embryonic neurogenesis process, cortical formation alterations, and pathophysiology factors present during embryonic development. In the present study, the generation of iPSCs from patients with Focal Cortical Dysplasia Marinowic et al. (2017) and the differences involved in the mechanisms during the neurodevelopment of iPSCs derived from the FCD patients were demonstrated.

2. Methods

2.1. Ethics statement

This study was approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) trough of number 17943213.9.0000.5336. All patients enrolled in this study signed the written informed consent.

2.2. Patients

In Marinowic et al. (2017) is described all patients features, the obtaining of fibroblasts from biopsies and histologic analysis of dysplastic tissue, and the reprogrammation of hiPSCs. Skin residual fragment from adult patient were obtained to compose the control group. Briefly, the demographic characteristics of the patients have been listed in Table 1. Control #1 [41-year-old] and Control #2 [50-year-old] were healthy women without any infection and neurological diseases, who attended the Program of Plastic Surgery in São Lucas Hospital, PUCRS. The skin samples were collected from the residual tissue of abdominal plastic surgery. The cerebral tissue RNA of control was previously extracted in another project of research group.

2.3. Cell migration assay

Fibroblasts and iPSCs were cultured in FluoroBlok 8.0 cell culture inserts (BD Biosciences). A volume of 600 μL of the culture medium (DMEM for fibroblasts and E8 for iPSCs) supplemented with 30 % serum was added to the lower chamber (culture dish), while in the upper chamber (insert), 5 × 104 cells were cultured with the specific culture medium supplemented with 10 % serum. Fibroblasts were analyzed after 24 h, 48 h, and 72 h, while the iPSC cells were analyzed after three days and seven days calculated from the beginning of the assay. In each of the aforementioned evaluations, the inserts were removed, washed twice with DPBS, fixed with 4 % paraformaldehyde for 10 min, and stained with rhodamine. For each evaluation, 20 visual fields were randomly captured using the 20x objective lens of the microscope. The images were quantified using the markup area parameter in the Image-Pro Plus 7.0 software.

2.4. Neurodifferentiation of iPSCs

The dissociated iPSC clones were cultured in Neurobasal Medium N5 (Thermo Fisher Scientific, Massachusetts, EUA) supplemented with 20 ng/mL BDNF (Sigma-Aldrich, St. Louis, Missouri, EUA), 1X B27 (Thermo Fisher Scientific, Massachusetts, EUA), and 10 % KSR (Thermo Fisher Scientific, Massachusetts, EUA), under humid conditions in an oven at 37 °C and 5 % CO2 atmosphere for a period of 35 days. Light-field image captures were performed on the 12th, 25th, and 35th days of neurodifferentiation. The evaluation of neurodifferentiation and the identification of neuronal structures were performed using an immunofluorescence assay involving FluoroPan Neuronal Marker antibody (Millipore, Burlington, Massachusetts, EUA).

2.5. Molecular analysis using qrt-pcr

RNA was extracted using SV-Total RNA kit (Promega, Madison, Wisconsin, EUA), following which cDNA synthesis was performed as directed by the manufacturer using the SuperScript VILO MasterMix (Thermo Fisher Scientific, Massachusetts, EUA). The cDNA was quantified using Qubit 2.0 fluorometer (Thermo Fisher Scientific, Massachusetts, EUA) by following the manufacturer's instructions.

Real-time PCR was performed using StepOne Plus (Thermo Fisher Scientific, Massachusetts, EUA) equipment. The samples were amplified from the initial amount of 20 ng of cDNA for each sample. Assays were performed in separate plates for each gene, using the GAPDH gene as the endogenous expression control. In order to analyze the phosphorylation of the PI3K/AKT pathway, the primers complementary to the mRNA sequences of the following genes were used: CIAP–1, CIAP–2, PI3K, β-catenin and 4-EBP1. The primer sequences used and the biological functions of the aforementioned genes are listed in Tables 2 and 3, respectively.

3. Results

3.1. Neurodifferentiation of iPSCs

Subsequent to 20 days of neurodifferentiation induction, the iPSCs began to exhibit morphological characteristics similar to nerve cells (Fig. 1). Post 35 days of neurodifferentiation induction, the cells presented morphological characteristics considerably similar to nerve cells, including the possible formation of neural networks (Fig. 2). In the immunofluorescence assay, iPSCs from patients with FCD and controls

Table 1
Characteristics of patients.

Patient	Age	Genre	Refractory	EEG region	MR region	Histopathology
FCD #1	Male	Yes	Right frontal	Right frontal	Cortical dyslamination	Dysplastic neurons balloon cells
FCD #2	Female	Yes	Left frontal	Left orbitofrontal	Cortical dyslamination	Dysplastic neurons balloon cells

exhibited positive staining for the FluoroPan Neuronal Marker antibody (Fig. 1).

3.2. Cell migration assay

Post 24 h of induction of cell migration, the marked area for migrating fibroblasts observed for the control patients was 33.2 mm^2 (SEM 13.6), while that observed for the FCD patients was 154.2 mm2 (SEM 18.2) ($p < 0.0001$). Post 48 h of the induction of cell migration, the area of cell migration observed for the control group was 65.5 mm^2 (SEM 21.4), and the area observed for the FCD patients group was 160.2 mm2 (SEM 41.7) ($p < 0.001$). Post 72 h of cell migration, the marked area of cells that had migrated was considerably similar in both the groups, without a significant difference, i.e., 221.8 mm^2 (SEM 61.8) for the control group and 202.1 mm2 (SEM 53.8) for the FCD group (Fig. 1).

No statistically significant difference was observed between the iPSCs from the patients with FCD and those from the control patients for both the time points chosen for cell migration induction. The evaluation on the seventh day demonstrated a reduction in cell migration in patients with FCD in comparison to the cell migration in the control patients after the same period, although this difference was not statistically significant (Fig. 1).

3.3. Molecular analysis

Dysplastic brain tissue from the FCD patients presented relative expression values considerably similar to the brain control (2.55-times less) (Fig. 2). During the neurodifferentiation protocol of the control iPSCs, a 17.9, 27.2, and 17.8-times increase in the cIAP–1 gene expression was observed, in comparison to non-differentiated iPSC cells, after 14, 22, and 35 days of induction of neurodifferentiation, respectively. Small differences in the cIAP–1 gene expression were observed in the iPSCs from patients with FCD throughout the neurodifferentiation protocol. In the 35 days of neurodifferentiation protocol, there is a reduction in 8.6-times de cIAP-1 gene expression in comparison to control. (Fig. 3).

The brain dysplasia tissue showed an increase of cIPA-2 gene expression of 13.8-times in comparison of brain control (Fig. 2). During neurodifferentiation, a small alteration was observed in the expression of cIAP–2 gene in all the periods analyzed, in control group. The reduction in the expression of cIAP–2 gene observed after 14 days of neurodifferentiation in the FCD patients was greater (32.7-times reduction) than the control patients (4.4-times reduction) (Fig. 3).

The cerebral tissues that were analyzed presented similar values of a relative PI3K gene expression (Fig. 2) and during the iPSC neurodifferentiation of the control group (Fig. 3). In FCD patients, a reduction of 5.8-times, 2.5-times, and 5.2-times occurred after 14, 22, and 35 days of neurodifferentiation, respectively (Fig. 3).

β-Catenin expression in the brain tissue from both control patients and patients with FCD was considerably similar (Fig. 2). The iPSCs from the control group, compared to the undifferentiated iPSCs, exhibited a 5,16-times, 7.36-times and 4.46-times increase in the β-Catenin gene expression on the 14th, 22nd, and 35th days respectively during the neurodifferentiation protocol (Fig. 3). In the FCD patients also, an increase in the β-Catenin expression was observed with the initiation of the neurodifferentiation protocol; however, the gene expression increased only 2.37-times, in comparison to the expression in the undifferentiated iPSCs, after 14 days of the neural induction. On the 22nd day of neurodifferentiation induction, the iPSCs from the patients with FCD exhibited an increase of 8.28-times in the gene expression, and a subsequent reduction of 1.55-times less in the gene expression after 35 days of the neurodifferentiation analysis (Fig. 3).

The brain tissue dysplasia exhibited a highest decrease in the relative expression values of the 4EBP–1 gene (37.3 times) (Fig. 2). During the neurodifferentiation protocol, iPSCs from the control group exhibited a 7.5-times increase in the 4EBP–1 gene expression on the 14nd day of neurodifferentiation induction. On day 22 and 35, the relative expression remained similar to the expression in the undifferentiated control iPSCs. In the dysplasia group, the neurodifferentiated cells exhibited a 20.7-times and 44.1-times reduction in the 4EBP–1 expression on the 14th and 35th day of differentiation, respectively (Fig. 3).

4. Discussion

The diseases related to central nervous system (CNS) have important physical and psychosocial repercussions and cost high to the individual as well as to the society as a whole. While significant advances have been achieved in recent decades in neuroimaging and genetics research, as well as in the clinical and/or surgical treatment approaches in certain pathologies, it is unfortunate that effective clinical treatments are limited even to this day (Ichida and Kiskinis, 2015).

Advances in the use of iPSCs and the subsequent possibility of specific differentiation in order to develop cellular models for various diseases allow a novel approach of studying the mechanisms associated with embryonic neurodevelopment and personalized pathological investigation for each patient in consideration of the patient's unique genetic personality (Ichida and Kiskinis, 2015).

In the present study, a cellular model for the investigation of embryonic neurogenesis was developed from patients with drug-refractory epilepsy and focal cortical dysplasia. Considering that the fibroblasts of control patients are derived from abdominoplasty, environmental exposure, such as the sun or whether the person is bald or not, is directly

Table 2
Primers sequences.

Primer	Foward	Reverse
CIAP-1	5'-GAAGGTGAAGGTCGGAGTCAAC-3'	5'-AGAGTTAAAAGCAGCCCTGGT-3'
CIAP-2	5'-GAAGGTGAAGGTCGGAGTCAAC-3'	5'-AGAGTTAAAAGCAGCCCTGGT-3'
PI3K	5'-GAAGGTGAAGGTCGGAGTCAAC-3'	5'-AGAGTTAAAAGCAGCCCTGGT-3'
β-catenin	5'-CCCGTGAGCGATGGAACT-3'	5'-CCTGCCTCCTCCCAACTCAT -3'
4-EBP1	5'-GGAGCAGCATGGAGCCTTCG-3'	5'-TCCCCTGCAAACTTCGTCCTC- 3'
GAPDH	5'-GAAGGTGAAGGTCGGAGTCAAC-3'	5'-AGAGTTAAAAGCAGCCCTGGT-3'

Table 3
Biological function of genes.

Gene	Official symbol	Full name	Biological functions
cIAP–1	BIRC2	Baculoviral IAP repeat containing 2	Apoptosis induced by serum deprivation and menadione, a potent inducer of free radicals
cIAP–2	BIRC3	Baculoviral IAP repeat containing 3	Inhibits apoptosis induced by serum deprivation
PI3K	PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	Cell growth and division (proliferation), cell migration, production of new proteins, transport of materials within cells, and cell survival
β-catenin	CTNNB1	Catenin beta 1	Creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells
4-EBP1	EIF4EBP1	Eukaryotic translation initiation	Cellular target of mTOR kinase activity that mediates the mTOR function in the control of mammalian cell translation factor 4E binding protein 1

influencing, contrasting with the child. No doubt this should be taken into account in obtaining this material. The patients affected by the disease had their fibroblasts obtained from the surgical incision, presenting a totally different environmental exposure, as well as the patients' age. One individual lived with solar radiation for 12 years and the other for 45 years. The piece of fibroblast we get from the surgeries is really small. If during fertlization, when the first cells are generated, the insult is capable of genetically altering the embryo in formation, this insult will be present in the iPSCs and the fibroblast. But if it is an insult with a change at the epigenetic level or at a temporal level, there will be a normal embryology. The present study is very preliminary so that an opinion can be formed in relation to a more molecular and genetic or environmental origin.

Considering the opposite age and gender of the two patients affected by the disease from which fibroblasts were obtained (male of 45 years and female of 12 years), different responses can be found in the young patient in relation to the adult. The sample size is very small because they are surgical patients due to drug refractoriness, so, we decided not to explore the analysis and use the results as suggestive of a change without any more conclusive statement. These results are original and fundamental as a pilot study of two patients and justify new studies from a larger number of patients for statistical analysis, also considering the pairing of age and gender.

When we treat a non-hereditary mutation, fibroblasts are not the best alternative, however, there is only speculation regarding the pathogenesis of FCD. It is not known whether somatic mutations occur due to a fragility or even when they occur (embryonic development at a later stage - corticogenesis, for example, up to about 30 weeks' gestation). Exactly for this we used this model, it would not surprise us if there were no differences in relation to the controls, but even so, we would describe something inedited. Moreover, there is the mosaicism of the brain that must be considered, another important point when we think of reprogramming. These findings are fundamental to a better understanding of the levels of expression, action and involvement of these genes during the development of the nervous system, including neural migration. Thus, the pattern of cortical malformation and the site where the cortex can be influenced by these changes in the CNS (Kuzniecky, 2015).

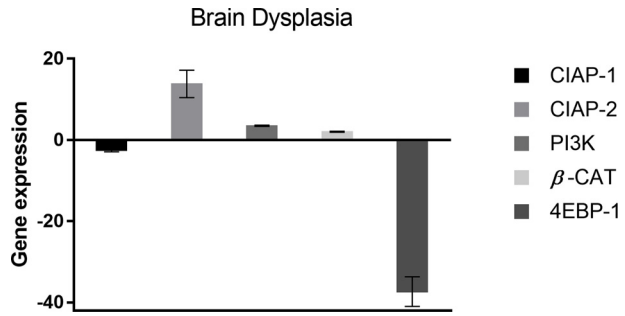

Fig. 2. Relative quantification through qRT-PCR of the genes in brain tissue of FCD patients. The results are showing the relative genes expression in FCD brain compared with brain control.

In Fig. 4, we showed the possible molecular mechanisms involved in cortical malformation. Recently, a few studies have demonstrated an association between genetic alterations and several types of cortical malformations, relating in an individualized way to the main stages of development of the central nervous system (Kuzniecky, 2015). More than 100 genes have been associated with different types of cortical malformations (Guerrini and Dobyns, 2014). Majority of these genes, and as a consequence, certain signaling pathways related to the cerebral cortex malformation, are involved in the control of apoptosis, cell proliferation, cytoskeletal structure, cell migration, and neurodifferentiation. These changes may have a variable impact on, in addition to the pattern of cortical malformation, the regions where the cortex may

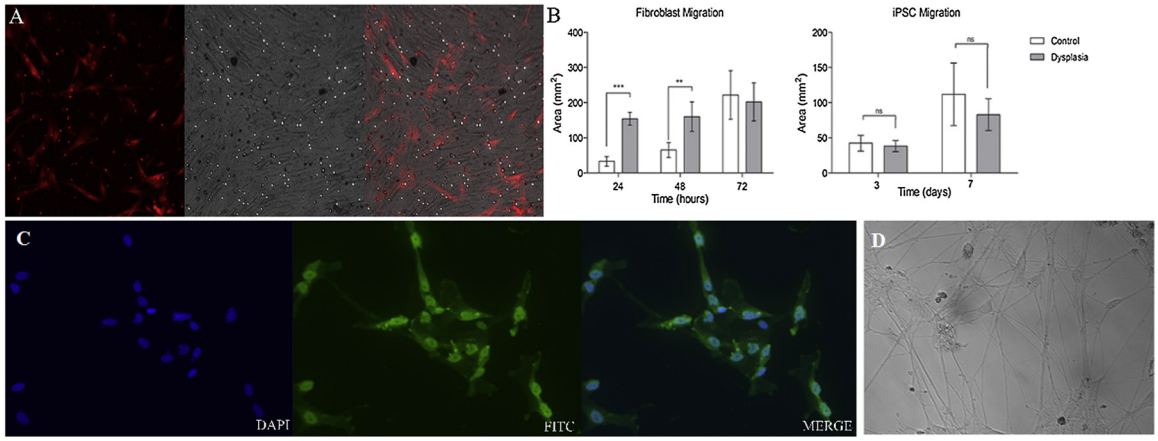

Fig. 1. A Immunofluorescence of cell migration assay and **B** results of quantification of fluorescence area of cell migration after 24, 48 and 72 h for fibroblasts and 3 and 7 days for iPSC migration. **C** Image capture of iPSC cells after 35 days of neurodifferentiation and immunofluorescence staining. Magnification, x200. **D** Image capture of clear field showed a cell extension after 35 days of neurodifferentiation protocol.

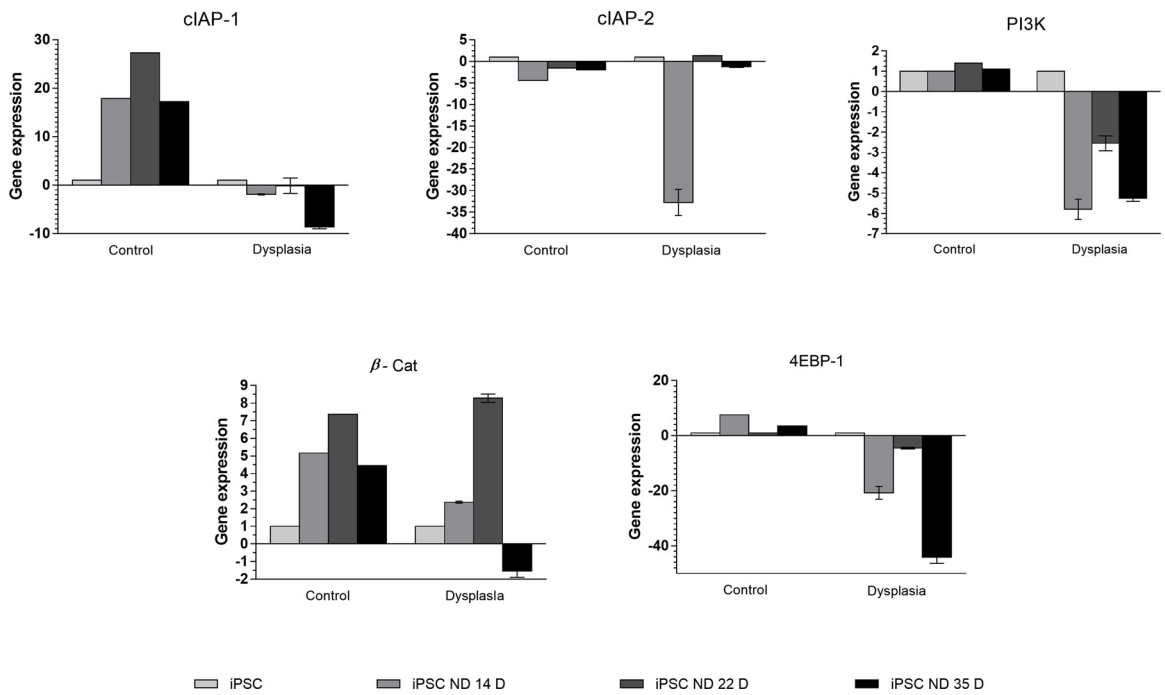

Fig. 3. Relative quantification through qRT-PCR of the genes during the neurodifferentiation of iPSCs. The results are presented using undifferentiated iPSCs of the calibrator.

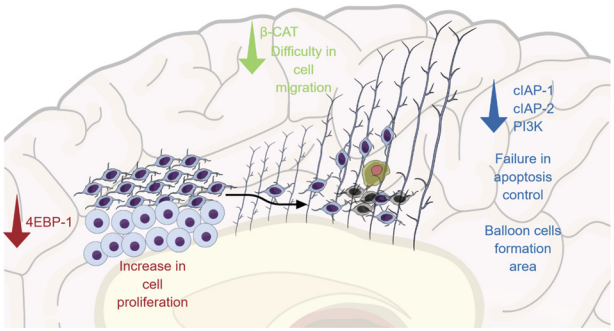

Fig. 4. Molecular alterations during brain cortex formation associated with FCD. The reduction of 4EBP-1 gene expression can increase the proliferation of neural precursor cell from the subventricular zone and increase the number of neuroblast before the migration stage. The excessive number of neuroblast disturbs the normal cell migration process. The reduction of β-catenin increases the difficulty of cell migration, impeding the cells from reaching specific area during the cortex formation. The apoptosis control is defective leading several cells to death. The reduction of PI3K gene expression, possibly altering different processes during neurodifferentiation, generating big cells undifferentiated or balloon cells in the areas of cortex with space mediated from the apoptosis failure.

be affected (Kuzniecky, 2015).

Around 80 %–90 % of the balloons cells present in the cortex of patients with FCD type IIb exhibit an increase in phosphorylation in the mTOR pathway. A few cases of FCD type IIb also exhibit an increase in the PI3K and AKT activity (Hsu et al., 2011; Majolo et al., 2018; Kuzniecky, 2015; Zhou et al., 2009). Signaling of the PI3K/AKT/mTOR pathway was observed to increase in the FCD type IIa and IIb even in the absence of genetic mutation and was attributed to the common mechanisms associated with other pathologies (Jansen et al., 2015).

In the present study, during the neurodifferentiation protocol, no difference was observed in the behavior and morphology of the iPSCs from the patients with FCD when compared to the iPSCs from the control patients. The structural formation of the cells generated by the neurodifferentiation protocol did not appear to be influenced by the presence of the pathology in the adults. It was demonstrated that the ability to generate neural cells, in terms of their structure, polarization, and the presence of specific neuronal markers, was not affected by FCD, and it was possible to assume that this pathology does not interfere with the morphological state of a neuron developed from the iPSCs derived from the patients with FCD.

There are certain limitations to cell differentiation, especially for the neural lines. Neurodifferentiation protocols require longer periods of culture in order to obtain mature neurons (Eiraku et al., 2008; Nityanandam and Baldwin, 2015). The longer culture periods may promote an increase of cell heterogeneity in the culture environment, thereby increasing the possibility of obtaining cells at various stages of differentiation or various sub-classifications, forming a mosaic-like environment (Sandoe and Eggan, 2013).

The migration of pluripotent cells derived from patients with FCD and controls subsequent to three and seven days of induction did not exhibit any difference in the quantified area of cells that migrated, through the membrane, to the attraction site. If any change regarding the cell migration had to occur during the embryonic neurodevelopment for the group comprising patients with FCD, this alteration would not probably affect the embryonic cells and would occur at the other stages during the onset of corticogenesis. However, the fibroblast cell migration assay demonstrated a higher cell migration capacity in the first two periods (24 h and 48 h) in the group comprising patients with FCD, when compared to the control group.

It was observed in this assay that the adult cells (fibroblasts) derived from the patients with FCD presented a greater potential for cell migration, which did not occur in the pluripotent cells. This observation may indicate that a possible alteration in the cell migration process might be occurring post tissue differentiation, and the altered state remains even after the final maturation of a given tissue line. This finding may indicate that if the neurogenic cortex neuroblasts from the FCD patients begin their migration phase before the physiologically typical period, then these cells might not be able to migrate, or the structure of the radial scaffold might not be completely developed for the process, thereby hindering the arrival of these cells to their precise destination.

4EBP–1 is a cellular target for mTOR kinase activity that mediates the mTOR function in the control of translation in mammalian cells. The molecular mechanism of mTOR associated with 4EBP–1 is not yet completely understood. The 4EBP-1 is responsible for controlling the levels of translation in cells, and its activity is controlled by mTOR. When 4EBP–1 is activated, translation levels are generally decreased in the cells. Its activity is regulated through the phosphorylation of mTOR (Hay and Sonenberg, 2004). Since 4EBP–1 serves an inhibition factor in protein synthesis, its decline symbolizes an overall increase in the protein synthesis (Liu et al., 2007).

The neurodifferentiation protocol altered the 4EBP–1 expression levels differently for the iPSCs from the FCD patients and the iPSCs from the control group. Again, the cells from the FCD patients exhibited reduced gene expression, mainly at the 14th and 35th days of the neurodifferentiation protocol, indicating an increase in the protein synthesis within these cells during the induction of neurodifferentiation; the same did not occur in the control patients. Neurodifferentiated iPSCs from the FCD patients appeared to exhibit higher levels of protein synthesis compared to the controls, due to the reduction in the 4EBP–1 expression. This finding could be related to a possible metabolic increase in both fibroblasts and iPSCs from the patients with FCD when conducting the neurodifferentiation.

A possible cause of cortical malformation in the patients with FCD may be related to an excessive increase in the levels of cell division, resulting in an excessive increase in the neuroblast population at the beginning of the embryonic neurodifferentiation, thereby impeding the normal course of migration and differentiation. It may even be related to a potential mechanism of marked migration, as observed in the fibroblast migration assay, which could trigger a migration that occurs faster or before the exact maturation time of the migratory cells or the radial formation that is fundamentally important for the success of cell migration.

β-Catenin is a protein with a role to perform in the regulation of cell-to-cell adhesion. It is a subunit of Caderin protein that also acts on the intracellular signal in a well-known pathway in neurodevelopment, the WNT pathway. WNT proteins are from a family of signaling molecules that participate in various developmental events during embryogenesis and homeostasis of different tissues (Haegel et al., 1995; Hofsteen et al., 2016; Logan and Nusse, 2004).

During the neurodifferentiation protocol, there was an increase in β-Catenin expression and they remained stable throughout the analyzed range for the control group. In patients with FCD, there was also an increase in β-Catenin expression in the first two time points (14 and 22 days), however in 14th it is much less expressive when compared to the increase occurred in the control group. In addition, for patients with FCD, on the 35th day of neurodifferentiation there was a reduction of 1.5-times in β-Catenin expression.

During embryonic development, adequate β-Catenin levels are essential for the formation of the entire embryo, especially the development of the central nervous system. When compared to the control group, β-Catenin levels expressed during the neurodifferentiation of the iPSC from patients with FCD presented a great reduction when compared to the levels detected in the controls and may be related to possible insufficient amounts of β-Catenin during corticogenesis. These insufficient amounts of β-Catenin in the different phases of the brain neurodevelopment may be related to the impairment of fundamental mechanisms for normal neurodevelopment.

The neuroblasts from patients with FCD may not be performing migration through an appropriately radial framework due to difficulty in adhesion between the migrating neurons and the radial glia, leading to alteration in the migratory course or rendering it difficult to reach the cortical plate, resulting in the generation of retarded neurons that do not reach their destination, consequently forming aberrant cortex with the presence of giant or balloon cells in the white matter.

The lack of lamination present in the FCD type II may also be attributed to β-Catenin deficiency during the formation of the central nervous system. The neurons that reach the cortex to form different cortical layers require cell-to-cell contact, as the formation of these layers is dependent on the temporal arrangement of the migratory neurons, which form laminations in the ventricle–cortical direction, and it is required for the cells that arrive for the formation of the cortical layers to be able to cross the neurons that are already present in the ventricular layers.

Although cIAP–1 and cIAP–2 perform similar roles inside the cells, their expressions are regulated differently. The control of cIAP–2 expression is mediated by transcription of NF-κB (Wang et al., 1998); whereas, cIAP–1 expression is controlled at the level of protein synthesis. This complex regulatory network reflects the differences in the temporal and spatial states of the cIAP–1 and cIAP–2 proteins generated in response to various physiological conditions (Zhao et al., 2009).

The results of the present study suggested that during the neurodifferentiation of the control iPSCs, the anti-apoptotic effect was promoted through an increase in the cIAP–1 expression, resulting consequently in an inhibition of caspases in the cells of the control group. During neurodifferentiation, cIAP–2 levels in the control group remained considerably similar to the levels expressed in the undifferentiated iPSCs. In the FCD group, a 32.7-times reduction in the cIAP–2 expression was observed on the 14th day of iPSC neurodifferentiation. The inhibition of programmed cell death during neurodifferentiation might be having a role in regulating the amount of precursor cells present during the cell proliferation phase as well as in the other stages of brain formation.

Apoptosis occurs extensively during the normal development of the mammalian nervous system and has been observed in distinct cellular populations, such as in the neural precursor cells, differentiated postmitotic neurons, and glial cells (Buss et al., 2006; Jacobson et al., 1997; Oppenheim, 1991). Such programmed cell deaths are important for the establishment of neural and glial populations in correct proportions. In normal mammalian development, generally, about one-third of the cells die due to apoptosis during the first two weeks post birth (Kristiansen and Ham, 2014).

Cellular signaling through PI3K has different roles in the diversified attributions of the human body, regulating essential functions such as cell growth, cell migration, cell differentiation, and cell survival (Gross and Bassell, 2014). PI3K may be related to the vital function of cell survival, and the activity of the PI3K pathway also holds an important place in the regulation of neuronal functions. The PI3K pathway transmits signals from the cell-surface receptors to the AKT/mTOR pathway, which is essential for synapsis, development of dendritic spines, as well as for the durable form of synaptic plasticity which forms the basis of memory and learning (Chan and Ye, 2012; Cuesto et al., 2011; Jaworski et al., 2005; Lee et al., 2011; Tang et al., 2008).

The neurodifferentiation protocol of iPSCs demonstrated a significant reduction in the expression of PI3K gene in the cells of the affected patients in comparison to the expression in the undifferentiated iPSCs. In the control patients, PI3K expression levels during the different phases of neurodifferentiation were considerably similar to those observed in the undifferentiated iPSCs. The decline in the PI3K expression may promote changes of various orders during the formation of embryonic tissues. Particularly in the CNS, substantial functions involved in the process of accurate brain development may get affected by the consequences triggered by the alterations occurred in the PI3K pathway, affecting all the processes essential for cortical formation and encompassing all the speculated dysfunctions of each of these processes which may be involved in the development of various brain diseases, especially the cortical dysplasias.

The decline in the PI3K levels observed in the iPSCs derived from the patients with FCD may be responsible for promoting the changes in brain formation through the modification of any of the crucial stages of neurodevelopment, and may influence the proliferation phase of the precursor cells during the migration phase of neuroblasts, neuroblasts in the developing cortex, differentiation in specific neurons, growth of

the axonal extensions and formation of dendrites, and the control of programmed cell death.

The global understanding of neurodevelopment and normal brain functioning depends on the broad knowledge regarding brain formation, the pattern of connections between the neurons and between the regions of the brain, as well as the synaptic communications that form these connections.

5. Conclusion

The present study provides the beginning of the solution, of a great speculation regarding the understanding of embryonic neurodevelopment in relation to focal cortical dysplasia, a disease with its genesis not well defined to date. The generation of iPSCs from skin fibroblasts was successfully established in the present study, enabling further studies using the generated cells, as well as confirming the feasibility of reprogramming the cells of interest from the patients affected by the other diseases of the central nervous system.

The results of the present study demonstrated that the differences in the migration potential of the adult cells and in the expression of genes related to the fundamental processes of normal brain development in the iPSCs and during the neurodifferentiation process may be associated with the cortical alteration in the patients with FCD.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DRM, FM, GGZ and IP carried out the cell culture, molecular genetic studies and drafted the manuscript. EPN, ALFP, DCM and JCC conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) trough of number 17943213.9.0000.5336.

Consent for publication

Not applicable

Declaration of Competing Interest

The authors declare that they have no competing interests.

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References

Arai, A., et al., 2012. Abnormal maturation and differentiation of neocortical neurons in epileptogenic cortical malformation: unique distribution of layer-specific marker cells of focal cortical dysplasia and hemimegalencephaly. Brain Res. 1470, 89–97.
Avansini, S.H., et al., 2018. Dysregulation of NEUROG2 plays a key role in focal cortical dysplasia. Ann. Neurol. 83 (3), 623–635.
Buss, R.R., Sun, W., Oppenheim, R.W., 2006. Adaptive roles of programmed cell death during nervous system development. Annu. Rev. Neurosci. 29, 1–35.
Cantley, L.C., 2002. The phosphoinositide 3-kinase pathway. Science 296 (5573), 1655–1657.
Chan, C.B., Ye, K., 2012. Phosphoinositide 3-kinase enhancer (PIKE) in the brain: is it simply a phosphoinositide 3-kinase/Akt enhancer? Rev. Neurosci. 23 (2), 153–161.
Cuesto, G., et al., 2011. Phosphoinositide-3-kinase activation controls synaptogenesis and spinogenesis in hippocampal neurons. J. Neurosci. 31 (8), 2721–2733.
D'Gama, A.M., et al., 2017. Somatic mutations activating the mTOR pathway in dorsal telencephalic progenitors cause a continuum of cortical dysplasias. Cell Rep. 21 (13), 3754–3766.
Eiraku, M., et al., 2008. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell 3 (5), 519–532.
Gross, C., Bassell, G.J., 2014. Neuron-specific regulation of class I PI3K catalytic subunits and their dysfunction in brain disorders. Front. Mol. Neurosci. 7, 12.
Guerrini, R., Dobyns, W.B., 2014. Malformations of cortical development: clinical features and genetic causes. Lancet Neurol. 13 (7), 710–726.
Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K., Kemler, R., 1995. Lack of beta-catenin affects mouse development at gastrulation. Development 121 (11), 3529–3537.
Hay, N., Sonenberg, N., 2004. Upstream and downstream of mTOR. Genes Dev. 18 (16), 1926–1945.
Hofsteen, P., Robitaille, A.M., Chapman, D.P., Moon, R.T., Murry, C.E., 2016. Quantitative proteomics identify DAB2 as a cardiac developmental regulator that inhibits WNT/beta-catenin signaling. Proc. Natl. Acad. Sci. U. S. A. 113 (4), 1002–1007.
Hsu, P.P., et al., 2011. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. Science 332 (6035), 1317–1322.
Ichida, J.K., Kiskinis, E., 2015. Probing disorders of the nervous system using reprogramming approaches. EMBO J. 34 (11), 1456–1477.
Jacobson, M.D., Weil, M., Raff, M.C., 1997. Programmed cell death in animal development. Cell 88 (3), 347–354.
Jansen, L.A., et al., 2015. PI3K/AKT pathway mutations cause a spectrum of brain malformations from megalencephaly to focal cortical dysplasia. Brain 138 (Pt 6), 1613–1628.
Jaworski, J., Spangler, S., Seeburg, D.P., Hoogenraad, C.C., Sheng, M., 2005. Control of dendritic arborization by the phosphoinositide-3'-kinase-Akt-mammalian target of rapamycin pathway. J. Neurosci. 25 (49), 11300–11312.
Kabat, J., Król, P., 2019. Focal cortical dysplasia - review. Polish J. Radiol. 77 (2), 35–43.
Kristiansen, M., Ham, J., 2014. Programmed cell death during neuronal development: the sympathetic neuron model. Cell Death Differ. 21 (7), 1025–1035.
Kuzniecky, R., 2015. Epilepsy and malformations of cortical development: new developments. Curr. Opin. Neurol. 28 (2), 151–157.
Lee, C.C., Huang, C.C., Hsu, K.S., 2011. Insulin promotes dendritic spine and synapse formation by the PI3K/Akt/mTOR and Rac1 signaling pathways. Neuropharmacology 61 (4), 867–879.
Liu, Y., Hidayat, S., Su, W., Deng, X., Yu, D., Yu, B., 2007. Expression and activity of mTOR and its substrates in different cell cycle phases and in oral squamous cell carcinomas of different malignant grade. Cell Biochem. Funct. 25 (1), 45–53.
Logan, C.Y., Nusse, R., 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20, 781–810.
Majolo, F., Marinowic, D.R., Machado, D.C., Da Costa, J.C., 2018. Notch signaling in human iPS-derived neuronal progenitor lines from Focal Cortical Dysplasia patients. Int. J. Dev. Neurosci. 69, 112–118.
Majolo, F., Marinowic, D.R., Palmini, A.L.F., DaCosta, J.C., Machado, D.C., 2019. Migration and synaptic aspects of neurons derived from human induced pluripotent stem cells from patients with focal cortical dysplasia II. Neuroscience 408, 81–90.
Marinowic, D.R., et al., 2017. Induced pluripotent stem cells from patients with focal cortical dysplasia and refractory epilepsy. Mol. Med. Rep. 15 (4), 2049–2056.
Nityanandam, A., Baldwin, K.K., 2015. Advances in reprogramming-based study of neurologic disorders. Stem Cells Dev. 24 (11), 1265–1283.
Oppenheim, R.W., 1991. Cell death during development of the nervous system. Annu. Rev. Neurosci. 14 (1), 453–501.
Palmini, A., et al., 2004. Terminology and classification of the cortical dysplasias. Neurology 62 (6 Suppl 3), S2–8.
Prayson, R.A., Spreafico, R., Vinters, H.V., 2002. Pathologic characteristics of the cortical dysplasias. Neurosurg. Clin. N. Am. 13 (1), 17–25 vii.
Sandoe, J., Eggan, K., 2013. Opportunities and challenges of pluripotent stem cell neurodegenerative disease models. Nat. Neurosci. 16, 780.
Sarnat, H.B., Flores-Sarnat, L., 2014. Morphogenesis timing of genetically programmed brain malformations in relation to epilepsy. Prog. Brain Res. 213, 181–198.
Tang, X., et al., 2008. Netrin-1 mediates neuronal survival through PIKE-L interaction with the dependence receptor UNC5B. Nat. Cell Biol. 10 (6), 698–706.
Taylor, D.C., Falconer, M.A., Bruton, C.J., Corsellis, J.A., 1971. Focal dysplasia of the cerebral cortex in epilepsy. J. Neurol. Neurosurg. Psychiatry 34 (4), 369–387.
Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., Baldwin, A.S.J., 1998. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281 (5383), 1680–1683.
Zhao, T.T., et al., 2009. hnRNP A1 regulates UV-induced NF-kappaB signalling through destabilization of cIAP1 mRNA. Cell Death Differ. 16 (2), 244–252.
Zhou, J., et al., 2009. Pharmacological inhibition of mTORC1 suppresses anatomical, cellular, and behavioral abnormalities in neural-specific Pten knock-out mice. J. Neurosci. 29 (6), 1773–1783.