POR F.S.E 2014/2020
Per il finanziamento di attività in "rete" a sostegno di un maggiore e migliore raccordo tra Alta Formazione e mercato del lavoro

Azione: Dottorati Internazionali - Borse di Studio Pegaso
Corsi di Dottorato di Ricerca - XXX ciclo
con sede amministrativa presso l'Università degli Studi di Firenze

Corso di Dottorato in DOTTORATO TOSCANO DI NEUROSCIENZE

Coordinatore Prof. RENATO CORRADETTI

Dipartimento di Neuroscienze, Psicologia, Area del Farmaco e Salute del Bambino (NEUROFARBA)

Il sottoscritto Dott. STEFANO DOCCINI

beneficiario di una borsa per la frequenza al dottorato in DOTTORATO TOSCANO DI NEUROSCIENZE erogata dall'Università degli Studi di Firenze a valere su fondi POR F.S.E 2014/2020, messi a disposizione dalla Regione Toscana, visti e recepiti gli obiettivi formativi che si intendono conseguire al termine dei tre anni di frequenza al corso, **presenta**, come previsto dall'Avviso della Regione Toscana, emanato con Decreto Dirigenziale n. 2027 del 14 maggio 2014, **la seguente** relazione sulle attività svolte

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Report of activities
2nd year

Research activity

The overall aim of the research program during this PhD course is to investigate the pathogenetic mechanisms underlying different forms of NCL in order to study common molecular signatures for the identification of biomarkers and modifying factors involved in the CLN5 disease.

During this second year of my studies I had the opportunity to investigate the role *in vitro* of human CLN5 developing a knock-out CLN5 HEK293T model, adopting the CRISP-Cas9 technology, a new approach to interrogate gene function by a versatile platform for RNA-directed genome editing in mammalian cells.
We programmed the endonuclease enzyme CRISPR associated protein 9 (Cas9) to induce DNA double strand breaks at specific genomic loci through a synthetic single guide RNA, which targeting specific coding regions of *CLN5* genes can create in-del mutations that result in a frameshift and loss-of-function allele.

Together with a proper characterization of the cellular KO model generated, we began to study differential proteomic expression in health and disease conditions to outline protein pathways involving CLN5p and its function within the intricate NCL molecular network.

The working hypothesis is that pCLN5 exerts its function at lysosome and mitochondrial levels and that the study of cells lacking CLN5 might help to identify the pCLN5 function.

Methods and Results achieved in the second year

<u>Generation of CLN5 -/- HEK 293T model adopting the CRISP-Cas9 technology</u>

The generation of cellular models follows three basic steps: i) plasmid design targeting Cas9, the appropriately designed sgRNA and the specific site of mutation; ii) transient transfection of plasmids into HEK 293T cells followed by a proper selection of clones that have successfully been modified by CRISPR nucleases; iii) verification of the genomic modification in transfected cells by standard sequencing methods.

The first step is totally computer-assisted. The web tool for CRISPR/Cas9-directed

mutagenesis (http://chopchop.cbu.uib.no/) takes a genomic sequence of interest and identifies suitable target sites able to generate a double-stranded breaks (DSBs) inside target gene. Top and bottom strands of oligos generated for each target site (sgRNA) are phosphorylated and annealed in a thermocycler and plasmid is subjected to a preparative restriction digest for its linearization. The commercial plasmid for this purposes, often presents a restriction site at the exact location where the oligos is incorporated. By a ligation reaction we can obtain the site-specific incorporation. The plasmid is than transformed into a competent *E. coli* strain. Plasmid replicate in cells and also express antibiotic resistance. Then, cells are plating on a select LB agar that it make possible to select only cells that contains the plasmid. Some colonies are picked to isolate the plasmid DNA and to check the correct insertion of the oligo duplex by amplification of the plasmid fragment of interest.

Transfection optimization was performed in a 6-well plate format varying the cell density, transfection reagent amount, and the concentration of exogenous DNA. The transfection occurred following standard procedures. One day post transfection cells were grown at confluence in a media supplemented with the same antibiotic of plasmid cassette in order to select cells which had incorporated the exogenous DNA.

It is important to underline that transfection is transient and that both the Cas9 activity and the antibiotic resistance work only during the first replicative steps of cells. For this reason the antibiotic treatment must be time limited. The transfected cells encode for the Cas9 nuclease and use the sgRNA like a template to drive the cut on genome DNA. Moreover the expression of a fluorescent protein allows to verify the transfection efficiency. Certainly the most complicated and long step for cell editing is the proper selection of clones and their classification in edited and not edited clones.

After transfection and a proper selection of cells, the transfection pools was used to isolate single clones by "limiting dilution“ technique. Cells are diluted sufficiently enough so that when one plates a certain volume of the cell suspension per well, there is sufficient statistical assurance that a few wells contain only a single cell. The remaining cells can be kept and used for genotyping and evaluate the overall modification efficiency. The clone selection was performed in a 96-well plate format varying the cell density. After 2-3 weeks, the single clones were expanded to evaluate the genotype by Sanger sequencing and to confirm the knockick-down of the gene product by Western blotting analysis. Forty clonal lines (from three independent transfection experiments) were grown and assayed by Sanger sequence analysis. Seve of the total clonal lines characterized (17.5%) showed gene edited with high efficiency (>80%)

Assessment of mitochondrial function in *CLN5* -/- model

In three different KO clones and in vector transfected control cells line, we assayed the mitochondrial energy metabolism by Seahorse XF Analyzers measuring the oxygen consumption rate (OCR) in living cells. The WT and two (out of three) KO cell lines were measured simultaneously in each experiment with seven wells per cell line. Then the entire experiment was repeated three times. To ensure equal cell numbers across the four lines, cells were seeded in an XF24 cell culture plates coated with poly-D-lysine hydrobromide (Sigma) at 4 x 10^4 cells/well and incubated at 37°C for one hour in unbuffered, serum-free DMEM media prior to analysis. Bioenergetics profiling was performed by monitoring basal oxygen consumption for 25 minutes followed by the sequential injection of the following inhibitors, with the final concentrations indicated in parentheses: oligomycin (2 uM), cyanide p-trifluoromethoxy-phenylhydrazone (FCCP; 1.5 uM), and rotenone (0.5 uM) plus antimycin A (0.5 uM) . Basal respiration, proton leak-linked respiration, ATP-linked respiration and non-mitochondrial respiration were calculated.
Basal oxygen consumption as well as the ATP-linked respiration was decreased in all three CLN5 KO cell lines compared to vector transfected control cells. Also the rate of oligomycinin-sensitive oxygen consumption, which reflects proton leakage across the inner mitochondrial membrane, was lower in all three CLN5 KO cell lines. By contrast, no significant differences were observed in non-mitochondrial respiration. These features remark the possible mitochondrial involvement in the pathogenesis of the disease.

Label-free proteomics experiments on *CLN5* -/- cell model

In collaboration with researchers at the Meilahti Clinical Proteomics Core Facility, Institute of Biomedicine/Biochemistry and Developmental Biology, University of Helsinki we analyzed differential proteomic alterations between CLN5-mutant and wild-type cells. The high replicative capacity of HEK293 genetic background makes it possible to separe the lysosomal and mitochondrial compartments for a more targeted and accurate detection of the different degrees of protein expression.
To this end, cells are lysed preventing protein aggregation or are fractionated to isolate single cellular compartments (mitochondria and lysosomes). Samples are then processed by filter assisted sample preparation (FASP) proteolytic digestion which combines both purification and digestion of proteins. Ultrafiltration spin columns are used to retain the protein sample while removing impurities, as well as for alkylation and subsequent

digestion. Sample proteins and peptides are then concentrate, desalt and purify by C_{18} micro-scale reverse phase column-like (tips). Peptides are separated by nano-UPLC and then directed to the mass spectrometry (MS) analysis.
Data analysis and interpretation are still ongoing and no results on this issue are now available.

Future diractions:

We will analyze the organelle-specific proteome in murine tissues derived by *Cln5*-/- and wild-type siblings. This part of the project will involve the Institute for Molecular Sciences, University of Eastern Finland, Kuopio.
Cln5-/- and aged matched control littermate, at two stages, 3 (pre-syntomatic) and 9 months (syntomatic) of age will be used for this purpose. We plan to investigate at least 4 animals per group at the two time points.
Animal samples will also be used to validate the data obtained from MS analysis through Western blotting or inmmunohistochemistry.
Bioinformatics analyses of collected data will allow pinpointing proteins that exhibit a different degree of expression. The proteomic profile of different models of CLN5 (animal and cellular systems) will allow to identify new functional pathways and anticipate the protein role in disease status.

Publications:

- Marchese M, Pappalardo A, Baldacci J, Verri T, **Doccini S**, Cassandrini D, Bruno C, Fiorillo C, Garcia-Gil M, Bertini E, Pitto L, Santorelli FM. Dolichol-phosphate mannose synthase depletion in zebrafish leads to dystrophic muscle with hypoglycosylated α-dystroglycan. Biochem Biophys Res Commun. 2016;477(1):137-43
- **Doccini S**, Sartori S, Maeser S, Pezzini F, Rossato S, Moro F, Toldo I, Przybylski M, Santorelli FM, Simonati A. Early infantile neuronal ceroid lipofuscinosis (CLN10 disease) associated with a novel mutation in CTSD. J Neurol. 2016;263(5):1029-32.
- Mignarri A, Rubegni A, Tessa A, Stefanucci S, Malandrini A, Cardaioli E, Meschini MC, Stromillo ML, **Doccini S**, Federico A, Santorelli FM, Dotti MT. Mitochondrial dysfunction in hereditary spastic paraparesis with mutations in DDHD1/SPG28. J Neurol Sci. 2016;362:287-91
- Criscuolo C, Procaccini C, Meschini MC, Cianflone A, Carbone R, **Doccini S**, Devos D,

Nesti C, Vuillaume I, Pellegrino M, Filla A, De Michele G, Matarese G, Santorelli FM. Powerhouse failure and oxidative damage in autosomal recessive spastic ataxia of Charlevoix-Saguenay. J Neurol. 2015;262(12):2755-63

- **Doccini S**, Meschini MC, Mei D, Guerrini R, Sicca F, Santorelli FM. Mitochondrial respiratory chain defects in skin fibroblasts from patients with Dravet syndrome. Neurol Sci. 2015;36(11):2151-5

Educational activities:

Abstract and poster presentation

27° Congresso Nazionale della Società Italiana di Neuropsichiatria dell'Infanzia e dell'Adolescenza (SINPIA); October 7-9, 2016 – Alghero, Italy. Poster presentation.
Doccini S , Sartori S, Maeser S, Pezzini F, Rossato S, Moro F, Toldo I, Przybylski M, Santorelli FM, Simonati A. Early infantile neuronal ceroid lipofuscinosis associated with a novel mutation in CTSD

27° Congresso Nazionale della Società Italiana di Neuropsichiatria dell'Infanzia e dell'Adolescenza (SINPIA); October 7-9, 2016 – Alghero, Italy. Poster presentation.
Doccini S, Pezzini F, Moro F, Santorelli FM, Simonati A. Molecular genetics results in a national NCL reference center. A 3-year experience

Carrying out of the period of study/research abroad

- Meilahti Clinical Proteomics Core Facility, Institute of Biomedicine/Biochemistry and Developmental Biology, University of Helsinki. May-July 2016. Conduct of research activities/international PhD program (Pegaso scholarship). Collaboration in the project "Proteomic analysis and molecular pathways in models of CLN5 disease".
- Meilahti Clinical Proteomics Core Facility, Institute of Biomedicine/Biochemistry and Developmental Biology, University of Helsinki. September-December 2016. Conduct of research activities/international PhD program (Pegaso scholarship). Collaboration in the project "Proteomic analysis and molecular pathways in models of CLN5 disease".

Satisfaction level of the PhD program

I am fully pleased with how this PhD program is enriching my scientific background and provides the required tools for the advancement of my research project.

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Firenze, 03 Ottobre 2016

Il dottorando, Dott. *STEFANO DOCCINI*

Il tutor, prof. *FILIPPO M. SANTORELLI*

Il coordinatore del dottorato, prof. *RENATO CORRADETTI*

La presente relazione andrà trasmessa per posta

AL SEGUENTE INDIRIZZO

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