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Project title: Brain gene regulation in 3D: long-range promoter-enhancer functional interaction networks in mouse brain-derived neural stem cells, and their relevance for neurodevelopmental disease

Abstract (max 150 words)

We aim to define the impact of 3D genome organization in mouse neural cells on gene regulation during normal development and in neurodevelopmental disease (NDD).
We previously defined genome-wide long-range interaction maps, connecting gene promoters to multiple distal DNA regions, representing putative enhancers.
We will test the effects of multiplex targeted inactivation, via CRISPR/Cas9-mediated approaches, of specific enhancers onto the activity of the connected gene(s). We will focus on enhancers whose human homologs are potentially involved in human NDD, based on genome-wide linkage analysis and copy-number variation associated to NDD. We will also look at genes mediating the pathological effects of Sox2 deletion in NSC and neurons, using a variety of functional tests; we will also define a functional gene network controlling key NSC functions, such as self-renewal. The project will define basic issues on gene regulation, as well as functionally characterized enhancers, for diagnostic and therapeutic perspectives.

Background, aims and significance of the proposed work (max 300 words)

It has recently become clear, through genome-wide studies, that mammalian genomes present a highly organized three-dimensional (3D) organization within the cell nucleus, involving long-range interactions that connect gene promoters to distal DNA regions. These interactions, involving RNA polymerase II (RNApolII), connect promoters to other promoters, or to DNA regions carrying epigenetic marks of transcriptional enhancers (Zhang et al 2013; https://www.encodeproject.org/). Such interactions, identified at the genome-wide level by Chromatin interaction analysis via paired-end tagging (ChIA-PET; Zhang et al 2013; https://www.encodeproject.org/), collectively form a complex 3D network (Wei et al, 2019); the genome-wide perspective allows to appreciate that most interactions do not connect a given putative enhancer to the nearest gene, but skip one or more intervening genes. Further, multiple putative enhancers are often connected to a given gene promoter (Zhang et al 2013; Bertolini et al, 2019).

The function of this 3D interaction network in gene regulation just begins to be explored. A central, unanswered question is the relevance of individual putative enhancers for the function of the connected gene, regarding gene transcription, as well as its consequences on cell function (e.g. cell proliferation and differentiation).

CRISPR/Cas9-related methods have revolutionized our ability to produce targeted modifications (mutations; epigenetic modifications with repressive/activatory effect on transcription) within any genome (Doudna 2020).

We will use CRISPR/Cas9-based targeted modifications of putative enhancers involved in interactions with disease-relevant genes, alone or in combination, to understand their role in i) gene transcription; ii) gene function, i.e. cell proliferation and differentiation. This will shed light on general mechanisms of gene regulation, and provide a new perspective on pathogenetic mechanisms. For NDD caused by increased gene copy number (Down syndrome; triplications leading to autism), enhancer silencing might further provide a future therapy hypothesis.

Experimental plan (max 1000 words)

Neural stem cells (NSC) cultured from the mouse developing brain represent a model of the cell proliferation and differentiation events that give rise, during embryogenesis, to the mature brain (constituted of neurons and glia), in the normal situation, as well as in NDD. We identified, by ChIA-PET in mouse NSC, a 3D network of long-range interactions, and showed that its functional integrity requires the transcription factor SOX2 (Bertolini et al, 2019). Sox2 is required to maintain NSC (Favaro et al 2009), and its mutation in man causes NDD. SOX2-bound DNA regions (ChIPseq) are overrepresented within interactions, pointing to a direct role in their maintenance and function; indeed, we identified, among genes controlled by SOX2 via long-range interactions, important SOX2 effectors in defects of the cerebellum (connected to ataxia; Cerrato et al, 2019), and the thalamus (connected to defects of the visual system; Mercurio et al, 2019). Notably, among SOX2 targets within interactions, we find abundant putative enhancers connected to genes whose mutation, in man, causes NDD, with phenotypic overlap with SOX2-dependent disease (Bertolini et al, 2019; Wei et al, 2019).

We identified, in mouse NSC, multiple putative enhancers, connected to genes important for normal and pathological (NDD) brain development (Bertolini et al, 2019).

We will focus, in particular, on:

1) Genes controlling brain-derived NSC proliferation and differentiation. We know that Sox2-deleted NSC initially proliferate like the wild type, but then fail to self-renew long term (Favaro et al 2009; Bertolini et al 2019), and produce, by differentiation, reduced numbers of neurons (M. Pagin, in preparation). The combined ChIA-PET, RNAseq and SOX2 ChIPseq analysis of Sox2-mutant versus control NSC identified genes downregulated in Sox2-deleted NSC, that are directly bound by SOX2 on distal enhancers, connected to the gene promoters (Bertolini et al, 2019). The re-introduction, via lentiviral vectors, of specific Sox2-regulated target genes, encoding Suppressor of cytokine signaling 3 (Socs3; Bertolini et al, 2019), or the transcription factor Fos, rescues NSC self-renewal (Bertolini et al, 2019; Pagin et al, submitted to Stem Cells, https://www.biorxiv.org/content/10.1101/2020.03.17.995621v1; Bikshapathi .. Pagin, Nicolis et al, submitted to Science). Further, Fos lentiviral re-expression leads to Socs3 reactivation, defining a small Sox2-regulated gene regulatory network key to NSC self-renewal (Pagin et al, submitted). Finally, Fos lentiviral transduction of Sox2-deleted NSC also leads to the recovery of NSC ability to differentiate to neurons (Pagin et al, in preparation).

2) Genes important for NDD. SOX2 binds to distant enhancers connected to genes, whose mutation causes NDD of various types (microcephaly; Autism-related diseases such as Angelman syndrome, Rett syndrome; Down syndrome, eye genetic diseases, and others; Bertolini et al, 2019; Wei et al, 2019). We will focus, in particular, on genes involved in copy number variation (CNV) causing NDD, such as e.g. Down syndrome (DS), caused by three copies (instead of two) of a defined tract of human chromosome 21 (or the corresponding, synthenic DNA region of the mouse genome), or genes causing Autism spectrum syndromes by loss-of-function mutations, but also by triplication (three functional gene copies), such as MeCP2 and FoxG1. Among DS-related genes, Olig1 and 2 (important in embryogenesis), Dyrk1a (important postnatally), and APP (also involved in Alzheimers' disease), are genes whose individual contribution (by their triplication) to important aspects of DS has been demonstrated (Nguyen et al, 2018, and references therein).

We will:

a) epigenetically silence specific putative enhancers, connected (ChIA-PET) to the above mentioned gene, alone or in combination, in NSC. To this aim, we will generate lentiviral vectors, transducing a gene encoding the nuclease-defective Cas9 (dCas9), fused to a transcription repression domain (KRAB), together with a guide-RNA, directing dCas9-KRAB to a specific putative enhancer (vector available from J. Shendure, Gasperini et al, 2019; Doudna, 2020);

b) examine the effect of enhancer(s) silencing on the transcription of the connected gene, by qRT-PCR on undifferentiated NSC, and on NSC undergoing differentiation to neurons and glia;

c) examine the effect of enhancer(s) epigenetic silencing on undifferentiated NSC proliferation (by cell counts, and immunofluorescence for markers of dividing cells), and on neuronal and glial NSC differentiation (by immunofluorescence with cell-type-specific markers, onto NSC subjected to a differentiation protocol).

d) use enhancers identified in a)-c), whose silencing affects the activity of the connected gene, to generate reporter transgenes, connecting them to a minimal promoter and eGFP, and characterizing their activity in differentiating brain organoids (in collaboration with J. Mariani, Yale), and in transgenic zebrafish (in collaboration with P. Bovolenta, Madrid).

e) in perspective, we may sequence interesting enhancers emerging from a)-c) in patients affected by NDD but not carrying mutations in protein-coding gene sequences (in collaboration with U. Cavallari, Ospedale Niguarda Milano, and S. Russo, Istituto Auxologico Italiano, Cusano Milanino);

f) in perspective, we may use the silencing of effective enhancers as a therapy approach, by repressing disease genes within animal genetic models (Park et al, 2019), in an attempt to validate a therapy approach based on quantitative, cell-type-specific downregulation of disease-relevant genes (such as Dyrk1a in DS, whose postnatal modulation in DS in mouse models mitigates aspects of the disease, Nguyen et al, 2018).

Feasibility and financial support (single statement)

To support this research, I have applied for grants from the European Community (ERC, first selection step passed, outcome of second selection step expected by August 2020, final outcome expected by November 2020; Eranet Neuron, first selection step passed, outcome of final selection step expected by October), from AIRC (outcome expected by November 2020), and I am applying for a Telethon grant (outcome expected by December 2020-January 2021).

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