UNIVERSITÀ DEGLI STUDI DI MILANO

D-MEM PhD-UNIMI | Doctorate program Milan EXPERIMENTAL MEDICINE

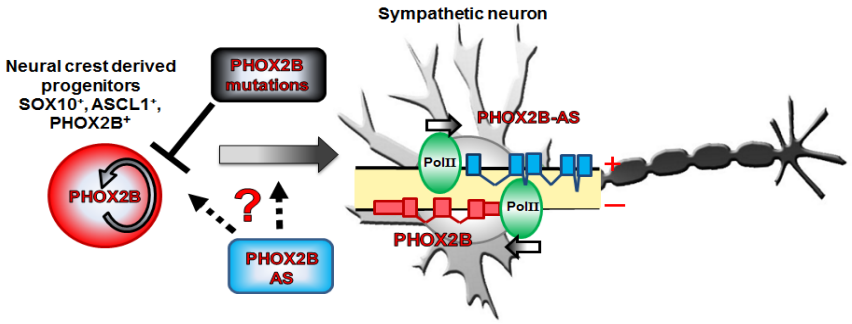
Study of the role of lncRNAs and transcriptional dysregulation in the pathogenesis of Congenital Central Hypoventilation Syndrome using patient-derived Induced Pluripotent Stem Cells (iPSCs).

TUTOR: Diego Fornasari; Co-Tutor: Simona Di Lascio

Laboratory of molecular and cellular pharmacology, Dept. Medical Biotechnology and Translational Medicine (BioMeTra), Università degli Studi di Milano

Contacts to be used in case of interest in our project: diego.fornasari@unimi.it; simona.dilascio@unimi.it

Rationale and main objectives of this project



Congenital central hypoventilation syndrome (CCHS) is a genetic disorder affecting the **Autonomic Nervous System** (ANS) and central chemosensitivity, due to heterozygous poly-alanine triplet expansions (PARM, 95%) and frameshift mutations (NPARM, 5%) in the ***PHOX2B* gene**, a transcription factor that drives the development of the autonomic visceral circuits. Alveolar hypoventilation is the hallmark of the disease, that is generally more severe during sleep than during wakefulness, which can be manifest since birth or adulthood (late-onset CHS, LO-CHS). The disease can be isolated or associated with a spectrum of non-respiratory symptoms, among which seizures and other conditions that reflect a more global ANS dysfunction, including cardiac arrhythmias, ocular disorders, Hirschsprung's disease and neural crest tumors. **No diagnosis, a mis-diagnosis or inadequate treatment can be responsible for either fatal consequences or very severe neurological damage due to episodes of apnoea and cerebral hypoxia.** Neurocognitive impairment has been reported in children with CCHS, and intervention from early infancy are essential to limit cognitive defects.

In vivo and *in vitro* studies suggest that a **loss of function mechanism** and in particular **transcriptional dysregulation** may be an important mechanism in CCHS pathogenesis; moreover, poly-alanine proteins show **protein aggregation and toxic effects** and has a dominant-negative effect on the transcriptional activity of the wild-type protein.

No pharmacological intervention is currently available for treating the disease or to treat symptoms. Fortuitous clinical observation that the progestin desogestrel can improve respiratory functions in two CCHS patients, opened the possibility for a relief of the respiratory symptoms and a reduction of risks of death during sleep in CCHS patients. *In vitro* and *in vivo*, we showed that desogestrel down-regulates the expression of both wild-type and mutant PHOX2B and some of its target genes thus suggesting that the clinical effect

might be mediated by the limitation of the toxic effect of the mutant protein. Therefore, a potential treatment for CCHS may be reached by the allele-selective lowering of mutant PHOX2B.
The possible approach to modulate the expression of the *PHOX2B* gene and thereby inhibiting all downstream toxic effects induced by mutant proteins is limited by the fact that the transcriptional and post-transcriptional regulation of this gene represents a largely unexplored aspect.
Emerging evidence suggests that most of the human genome is transcribed into **non-protein coding RNAs (ncRNAs)**, that are key regulators of protein-coding gene expression. One major class of regulatory RNA genes contains the long non-coding RNAs (lncRNAs), of which **natural antisense transcripts (NATs)** represent a subgroup. NATs are transcribed from the opposite DNA strand to that responsible for sense transcripts, partially overlap with sense genes or their regulatory regions, and positively or negatively regulate gene expression at different levels by means of multiple mechanisms.
Bioinformatics analyses of the *PHOX2B* locus region and data in our laboratory predict a five exons antisense transcript ***(PHOX2B-AS1),*** that partially overlaps the first exon of *PHOX2B,* conserved in both human and murine tissues. *PHOX2B-AS1* is expressed in different PHOX2B^{+}, but not in PHOX2B^{-} cell lines and tissues, thus suggesting a positive correlation with *PHOX2B* expression. Two splicing variants have been detected in cells and human tissues, and *PHOX2B-AS1* silencing experiments in NB cells suggest a role of positively regulating PHOX2B protein translation.
However, our understanding of the pathogenesis of CCHS and the consequent possibility of developing new and effective means of treating it is limited by the unavailability of CCHS mice models; in fact, heterozygous mice bearing the most frequent polyalanine expansion (+7 alanine) show phenotypes resembling the most severe cases of CCHS and die of respiratory failure soon after birth. To overcome this issue, we have recently generated **iPSC lines from two CCHS patients** with *PHOX2B* PARM mutation (20/25 genotype), that differ for the disease-onset being the UMILi027-A from LO-CHS and UMILi028-A from early onset CCHS manifestation, respectively. Gene expression analysis on iPS cells from the LO-CHS patient (UMILi028-A) has shown an ectopic expression of *PHOX2B* and *PHOX2B-AS1* already at the undifferentiated level, despite the expression of the pluripotency markers *NANOG* and *OCT4,* including both alleles, thus confirming that dysregulated *PHOX2B-AS1* and *PHOX2B* transcription at earlier developmental stages may be involved in CCHS pathogenesis. The putative *PHOX2B-AS1* promoter lies within the region encompassing the 20-alanine tract in PHOX2B. Our preliminary data in NB cells showed that the presence of repeat expansions increases the activity of the *AS1* promoter, and support the hypothesis that alanine expansions may alter the epigenetic signature of the *PHOX2B* locus, leading to dysregulated *PHOX2B-AS1* and *PHOX2B* transcription
The **aim of this project** is to assess the role of *AS1* in the expression of *PHOX2B*, the effect of mutations on this molecular mechanism, thus shedding light on CCHS pathogenesis and explore the possibility of *PHOX2B-AS1* therapeutic targeting, taking advantage of the disease-derived iPSCs cellular model.

Most relevant publications for the project

1. Cuadros Gamboa AL, Benfante R, Nizzardo M, Bachetti T, Pelucchi P, Melzi V, Arzilli C, Peruzzi M, Reinbold RA, Cardani S, Morrone A, Guerrini R, Zucchi I, Corti S, Ceccherini I, Piumelli R, Nassi N, Di Lascio S, Fornasari D. (2022) Generation of two hiPSC lines (UMILi027-A and UMILi028-A) from early and late-onset Congenital Central hypoventilation Syndrome (CCHS) patients carrying a polyalanine expansion mutation in the PHOX2B gene. Stem Cell Res. 61:102781.

2. Di Lascio S, Benfante R, Cardani S, Fornasari D. (2021) Research Advances on Therapeutic Approaches to Congenital Central Hypoventilation Syndrome (CCHS). Front Neurosci. 14:615666.

3. Di Lascio S, Belperio D, Benfante R, Fornasari D. (2016) Alanine Expansions Associated with Congenital Central Hypoventilation Syndrome Impair PHOX2B Homeodomain-mediated Dimerization and Nuclear Import. J Biol Chem. 291(25): 13375-93.

4. Di Lascio S, Bachetti T, Saba E, Ceccherini I, Benfante R, Fornasari D. (2013) Transcriptional dysregulation and impairment of PHOX2B auto-regulatory mechanism induced by polyalanine expansion mutations associated with congenital central hypoventilation syndrome. Neurobiol Dis. 50: 187-200.

5. Cargnin F, Flora A, Di Lascio S, Battaglioli E, Longhi R, Clementi F, Fornasari D. (2005) PHOX2B regulates its own expression by a transcriptional auto-regulatory mechanism. J Biol Chem. 280(45): 37439-48.

Candidate specific requirements

Basic cellular and molecular biology techniques (PCR, RNA extraction, RT-qPCR, western blot), mammalian cell culture and immunofluorescence.