ESC First Contact Initiative Grant Final Report

10/11/2023

Dear Council Members,

I would like to thank the European Society of Cardiology for awarding me with the First Contact Initiative Grant (FCIG). This grant allowed me to visit the Amsterdam UMC/VUMC Center in the Netherlands for a period of three months and to work under the supervision of Professor De Man on the role of the long non-coding RNA (lncRNA) Down Syndrome Critical Region 9 (DSCR9) in pulmonary arterial hypertension (PAH). During my stay, I could learn and apply specific protocols to obtain endothelial cells (EC) derived from human induced pluripotent stem cells (hiPSC), extract and culture primary human pulmonary arterial endothelial cells (hPAECs) from pulmonary artery samples, and perform lentiviral overexpression. Moreover, this experience allowed me to collect useful data and samples to continue the research on DSCR9 involvement in PAH. In the present report, I have the pleasure to inform you about the outcome of the initiative. However, due to the fact that the data generated during the FCIG are still unpublished, the results will not be presented extensively.

PAH is a rare condition that is characterized by an impairment in nitric oxide (NO) production. This condition is responsible for the remodelling of the vascular tone and the vasoconstriction of the pulmonary artery, leading over time to right ventricular hypertrophy and heart failure. However, the exact mechanism underlying the NO deficiency is still unclear. To date, it was demonstrated only the miRNAs involvement in NO deficiency in PAH[1], while no information has been reported yet about the possible involvement of lncRNA in NO deficiency typically observed in PAH.

At the Amsterdam UMC/VUMC Centre, I could investigate DSCR9 expression *in vitro*, using primary hPAECs extracted from the pulmonary arteries of both female with idiopathic PAH (iPAH) and donors, as well as in ECs derived from hiPSC carrying the BMPR2 mutation (iBMPR01 line) and the isogenic counterpart (iso01BMPR01).
Primary hPAECs were extracted from the lung artery, cultivated in plates coated with gelatine 0.1% in complete endothelial cell growth medium (cECM), and were used for the analysis from passage 4 to passage 6 after the selection for VE-Cadherin expression marker.
To obtain hiPSC-ECs I applied Orlova's protocol [2]. Mesodermal Induction (MI) was induced on day 0 by adding B(P)EL medium supplemented with CHIR. Vascular specification (VS) was induced on day 3 by adding to the B(P)EL medium SB431542 and the recombinant human Vascular Endothelial Growth Factor (rhVEGF). Endothelial cell expansion was performed on days 6 and 9 from the beginning of the differentiation. The EC expansion medium has the same composition as the VS medium. On day 10, differentiated cells were purified by selecting for VE-Cadherin expression marker and cultured in cECM. Total RNA samples from the various specific time points of the differentiation process were collected and RT-qPCR analysis was performed to ensure the proper commitment in endothelial cell differentiation.
To assess the type of cells obtained the following genes were considered: NANOG and SOX2 as markers of pluripotency, VE-Cadherin (CD144), PECAM1 (CD31), NOS3, as typical endothelial cell markers, and von Willebrand Factor (vWF) as a marker of maturity, SOX17, JAG1 as arterial marker, NRP2 as a venous marker, SM22 as smooth muscle marker. RNA pool of primary hPAEC from donors was used to compare the gene expression level in all the differentiation steps. PCR data were normalized on RPL27, as the most stable gene expressed during the differentiation and to ensure the arterial nature of the hiPSC-

ECs obtained. The analysis of gene expression during the differentiation showed a decrease in NANOG and SOX2 expression, assessing the iPSC commitment in the differentiation process, and an increase in the endothelial surface marker (CD144 and CD31). NOS3 is more induced during the vascular specification and endothelial cell expansion due to the activation of PI3K pathways[3] . Moreover, the NOS3 is increased during the vascular specification and the endothelial cell expansion allows the expression of VE-Cadherin and PECAM1 in the later phase of the differentiation[4]. The vWF increases its expression during the vascular commitment and endothelial cell expansion. However, the vWF level remains low in comparison to primary hPAEC suggesting that the iPSC-ECs are not fully mature at the end of the differentiation process[5]. SOX17 is a transcription factor and a well-known arterial marker. The increase of SOX17 and JAG1 during the differentiation process suggests the arterial nature of iPSC-ECs[5,6]. The NRP2 expression level is higher in comparison to PAEC cells expressing a plasticity characteristic[7]. Along with gene expression, I performed immunofluorescence staining to determine the effective presence of arterial endothelial markers, to establish the percentage of iPSC-EC cells obtained, and to exclude the presence of smooth muscle cells as a side product of the differentiation[8]. Thus iPSC-EC cells were stained for VE-Cadherin, SOX17, vWF, and αSMA (representative images- Figure 1 and Figure 2). The images were acquired with confocal microscopy and analyzed with ImageJ Software. According to RT-qPCR data, only a few cells expressed the vWF (4%) indicating that they are not completely mature. The percentage of endothelial cells obtained by calculating the number of cells expressing VE-Cadherin is 88,61% of which 94,5% are expressing SOX17, indicating that the iPSC-ECs obtained have an arterial nature. 11.39% of cells are expressing αSMA.

The iPSC-EC from the iBMPR01 and iso01BMPR01 lines were then analyzed for DSCR9, and the other genes of interest in the eNOS pathway. iPSC-ECs from iBMPR01 displays a 2-fold increase in DSCR9 expression, however, no significant difference was detected.
RT-qPCR analysis performed on primary hPAECs showed an increase of 93-fold in DSCR9 expression. Along with RNA samples, protein samples and culture medium were collected for further analysis.
To assess DSCR9's possible involvement in the eNOS pathway, DSCR9 overexpression was performed in primary PAEC of both iPAH patients and control.
As a first step, DSCR9 plasmid, containing human DSCR9 sequence fused with the green fluorescence protein (DSCR9-GFP) under the control of the human cytomegalovirus promoter, as well as the Chloramphenicol and Puromycin antibiotic-resistances for bacterial and mammalian selection, was amplified using as host the E.coli STBL3 cells. Chloramphenicol antibiotic was added to the Terrific Medium plates to enable the bacterial selection. A negative control was generated to verify the success of bacterial selection and to exclude any potential bacterial contamination. Lentiviral particles were then produced using HECK-293T cells and adding three packaging plasmids (pMD2.G, pMDLg/pRRE, pRSV-REV) when the cell confluence was between 40% and 70%. The lentiviral particles carrying the construct of interest were then tested to assess the vector's quality and the dynamic of DSCR9 overexpression on primary hPAEC.
Thus, three different time points were selected: 24 hours, 48 hours and 7 days after the Puromycin selection. The latter time point was chosen to carry on the analysis since the RT-qPCR analysis displays the most significant fold-change for DSCR9 expression.
Therefore, primary female hPAECs from both donors and iPAH patients were subjected to a 16-hour exposure period to the lentivirus vector. Following this, the selection process using Puromycin was conducted, and the percentage of cells expressing DSCR9 was determined with immunofluorescence staining against the GFP protein, pictures were acquired with a confocal microscope (Figure 3). Upon the conclusion of a 7-day period since lentiviral and Puromycin exposure, RNA samples, protein and culture medium were collected for further analysis related to the eNOS pathway and NO production after DSCR9 overexpression (the last two will be evaluated at the University of Genova).
Following DSCR9 overexpression, RT-qPCR data displays a significant reduction (15-fold change) in one of the upstream activators of eNOS.

Results obtained on primary hPAECs from donors display the same behaviour for the three genes considered, however, no differences were detected, suggesting that other mechanisms could be involved in the pathological process.

To conclude, this collaboration helped me to study the role of DSCR9 in other two models of disease, and to confirm the data previously obtained.

This study contributes to giving new insight into a new possible actor in PAH pathology and NO production giving a substantial contribution to the literature about NO deficiencies in PAH. However, this study contains some limitations. In detail, the hiPSC-ECs obtained display immature and mixed phenotypes, as revealed from the vWF detection and the RNA level of the venous marker NRP2. For this reason, considering that the lncRNA DSCR9 is mainly expressed during the embryonic development it is not surprising that there are no significant differences between the two lines analysed. Another issue could be the variation observed after each differentiation. Thus, to establish if DSCR9 is effectively differentially expressed would be worth it to try a protocol to increase the maturity level of the hiPSC-ECs and to increase the number of differentiations in both lines. Another limitation is represented by the static condition the various models had been exposed to. Thus, a future perspective could be to study the role of DSCR9 in a model exposed to pulsatile pressure in order to resemble the condition observed in PAH, since the shear stress is the main stimulus for the NO release.

Considering the amount of the First Contact Initiative Grant, it covered completely the rent of a room in a shear flat in the Netherlands for the full period of three months.

Yours Sincerely,

Nadia Bernardi

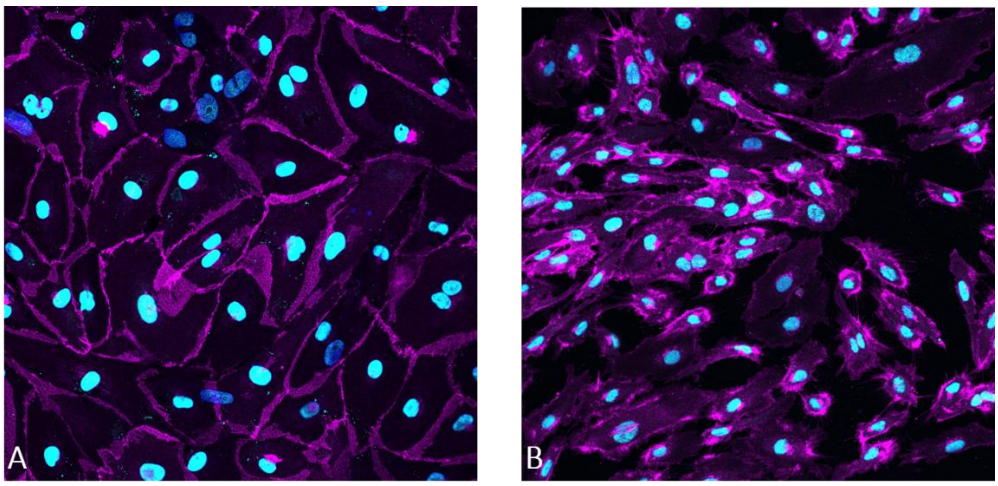

Figure 1. representative images of iPSC-ECs expressing VE-Cadherin (purple),SOX17 (light-green), and αSMA (white) (A), human Pulmonary Artery Endothelial Cells (PAEC) used as internal control for the positivity of the staining (B). Nuclei were stained with HOECHST (blue).

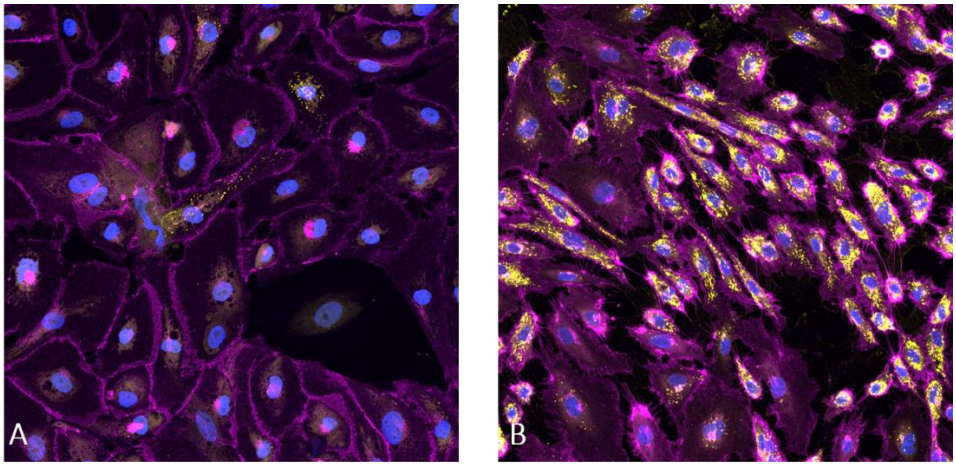

Figure 2. representative images of iPSC-EC expressing von Willebrand Factor (vWF) (yellow) (A) compared with primary PAEC (B).

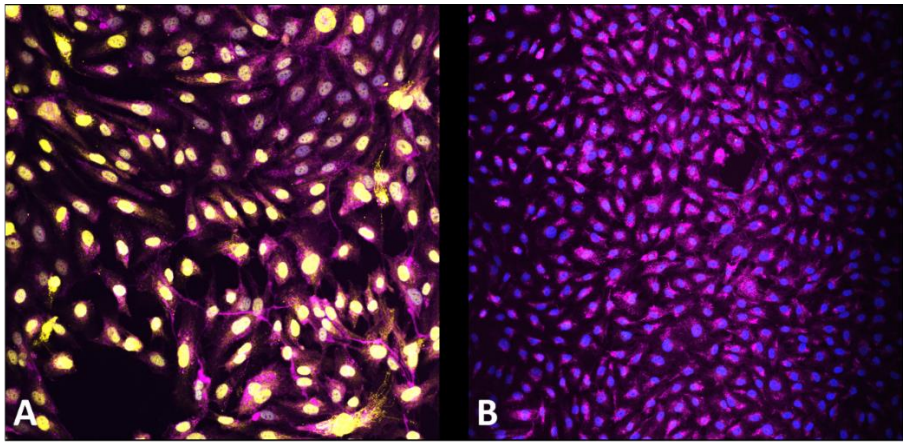

Figure3. representative images of DSCR9 overexpression. PAEC cells were stained with HOECHST for the nuclei visualization, WGA for the membrane visualization, anti-GFP protein to detect the cells that successfully overexpressed DSCR9-GFP. In A. PAEC cells after the lentiviral overexpression with the plasmid carrying the DSCR9-GFP construct, and in B PAEC cells exposed to the negative control plkO.

Bibliography

1. Bernardi, N., Bianconi, E., Vecchi, A. & Ameri, P. Noncoding RNAs in Pulmonary Arterial Hypertension: Current Knowledge and Translational Perspectives. *Heart Fail Clin* **19**, (2023).
2. Orlova, V. V. *et al.* Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nat Protoc* **9**, 1514–1531 (2014).
3. Belt, H. *et al.* Temporal Dynamics of Gene Expression During Endothelial Cell Differentiation From Human iPS Cells: A Comparison Study of Signalling Factors and Small Molecules. *Front Cardiovasc Med* **5**, 316594 (2018).
4. Huang, N. F., Fleissner, F., Sun, J. & Cooke, J. P. Role of nitric oxide signaling in endothelial differentiation of embryonic stem cells. *Stem Cells Dev* **19**, 1617–1625 (2010).
5. Harding, A. *et al.* Highly Efficient Differentiation of Endothelial Cells from Pluripotent Stem Cells Requires the MAPK and the PI3K Pathways. *Stem Cells* **35**, 909–919 (2017).
6. Nakajima-Takagi, Y. *et al.* Role of SOX17 in hematopoietic development from human embryonic stem cells. *Blood* **121**, 447–458 (2013).
7. Olmer, R. *et al.* Differentiation of Human Pluripotent Stem Cells into Functional Endothelial Cells in Scalable Suspension Culture. *Stem Cell Reports* **10**, 1657–1672 (2018).
8. Lu, X., Dunn, J., Dickinson, A. M., Gillespie, J. I. & Baudouin, S. V. Smooth Muscle α-Actin Expression in Endothelial Cells Derived from CD34+ Human Cord Blood Cells. *https://home.liebertpub.com/scd* **13**, 521–527 (2004).