Short Term Mobility Program – CNR 2016

Relazione Finale

Fruitore: Dr. Agnese Re

Proponente: Dr. Antonella Farsetti

Istituzione: IBCN-CNR, Roma

Titolo del programma: Generation of CRISPR/Cas9 vectors to knockout eNOS in murine ES cells

Descrizione dettagliata dell'Istituzione ospitante: Division of Cardiovascular Epigenetics, Medicine Clinic III, Department of Cardiology, Goethe University, Frankfurt-am-main, Germany (Prof. Carlo Gaetano)

During my stay at the Goethe University of Frankfurt, Department of Cardiology (4-25 October 2016) I could contribute in obtaining very promising results, summarized in the following paragraphs:

1. Identification, isolation and characterization of the ESNO+/- subpopulations.

In vivo studies indicated the production of nitric oxide (NO) as important for the early stage embryo implantation in uterus. However, NO synthesis or responsiveness, well documented in murine embryonic stem cells (mES) cultured in standard medium (SM), occurs relatively late during the differentiation process. In fact, mES cultured in SM are unable to synthesize NO in their undifferentiated state or during the very early phases of their differentiation process in association with a relatively low level of Nitric oxide synthase (NOS) expression and function. On the contrary, cGMP synthesis occurring early in naïve mES after release from ground state medium (GS), suggested for the possibility that a mES subpopulation could be responsible for the rapid NO synthesis. To identify these cells, at the Goethe laboratory where I spent my STM program, a series of cell sorting experiments were already performed in the presence of the NO-activated 4-Amino-5-methylamino-2′,7′-difluorescein (DAF) probe. Figure 1, panel a, shows a DAF+ mES population (depicted in blue color) detectable as early as 2h after release from stemness (ESNO+) (Figure 1, panel a, upper and lower right graphs). This condition allowed cells to be sorted at high efficiency, according to the presence or absence of the DAF signal. ESNO+ cells, plated for 24h in differentiation medium (DMGS) to allow recovery, did not show any significant morphological difference compared to controls (ESNO-) (Figure 1, panel b). After that, the expression of mesodermal markers was analyzed in both cell populations. Figure 1, panel c left graph, shows that ESNO+ cells expressed higher levels of eNOS and the other mesendoderm-associated markers, compared than ESNO- cells.

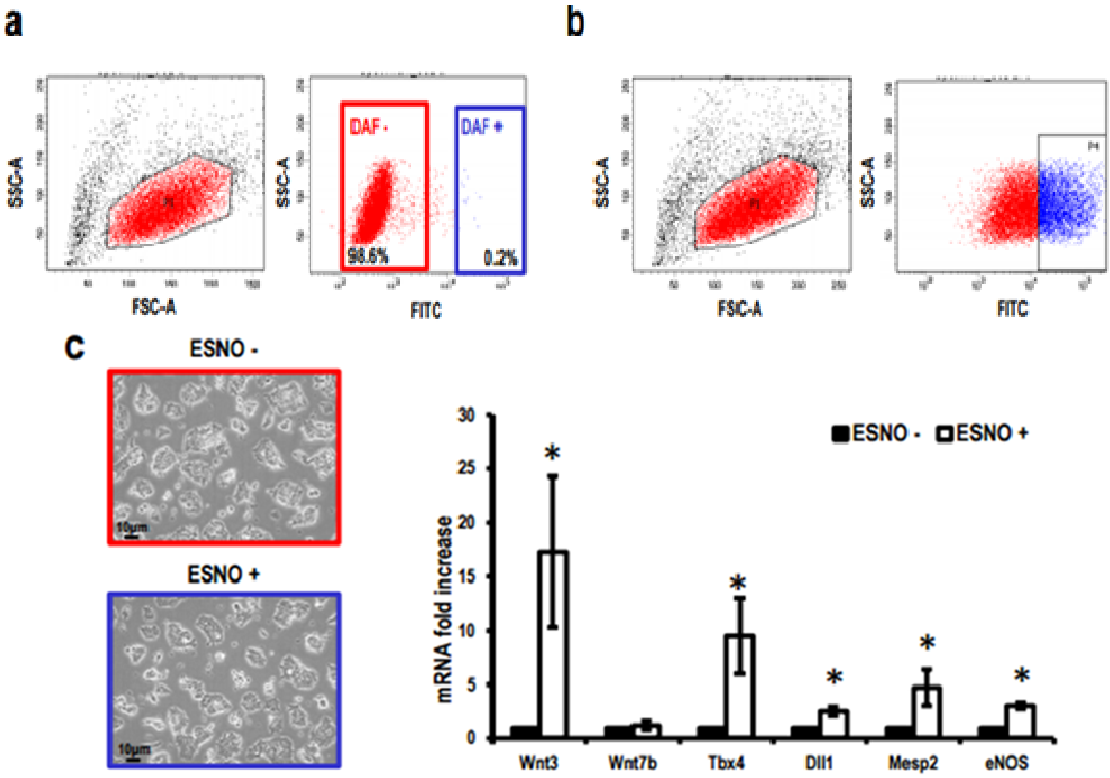

Figure 1. (a and b) Fluorescence activated cell sorter analysis. a) Left cytogram: representative scatter plot showing the forward (FSC-A) and side scatter (SSC-A) distribution of the mES population cultured in DMGS for 24 h in absence of the DAF fluorescent probe. Right cytogram: representative dot plot showing the fluorescein isothiocyanate (FITC) and SSC-A distribution of the mES population cultured in DMGS for 24 h in absence of the DAF fluorescent probe. b) Left cytogram: representative scatter plot showing FSC-A and SSC-A distribution of the mES population in cultured in DMGS for 24 h in the presence of the DAF fluorescent probe. Right cytogram: representative dot plot showing the FITC and SSC-A distribution of the mES population cultured in DMGS for 24 h in the presence of the DAF fluorescent probe. In each experiment, gating to separate ESNO+ (DAF+; blue dots) from ESNO- (DAF-; red dots) cells was established manually (n=3). (c) Left panel: Representative phase contrast microscopy images of sorted ESNO- (red upper panel) and ESNO+ (blu lower panel) cells cultured in DMGS for 24 h after sorting. Right panel: qRT-PCR analysis of Wnt3, Wnt7b, Tbx4, Dll1, Mesp2 and eNOS transcripts in ESNO+ (white bar) compared to ESNO- (black bar) cells at the 24 h time point after sorting. Data are shown as the mean of three independent experiments ± SE represented as fold increase compared to ESNO- cells after subtraction of the housekeeping gene p0 signal (*$p < 0.05$).

2. Functional characterization and gene expression analysis of ESNO+/- subpopulations.

On the basis of the above characterization performed at the host laboratory, I tested the capability of the ESNO+/- subpopulations to differentiate into mature cardiomyocytes using the hanging-drop embryoid body (EBs) technique. Figure 2, left panel, shows representative images of EBs taken at different time-points. The ESNO+ and - populations were both able to generate EBs (Figure 1, panel a) although ESNO+ cells grew larger. Figure 1, panel a , shows that after plastic adherence (time point t0+6), EBs from both cell types demonstrated the ability to further grow and differentiate (time point t0+6+1). Remarkably, in the ESNO+ population (open triangles) the number of beating EBs steadily increased from time-point t6+3 to time point t6+10 arriving to >60% efficiency in 7 days (Figure 2, panel b). Conversely, the formation of detectable beating areas was negligible in EBs generated by ESNO- cells (closed squares).

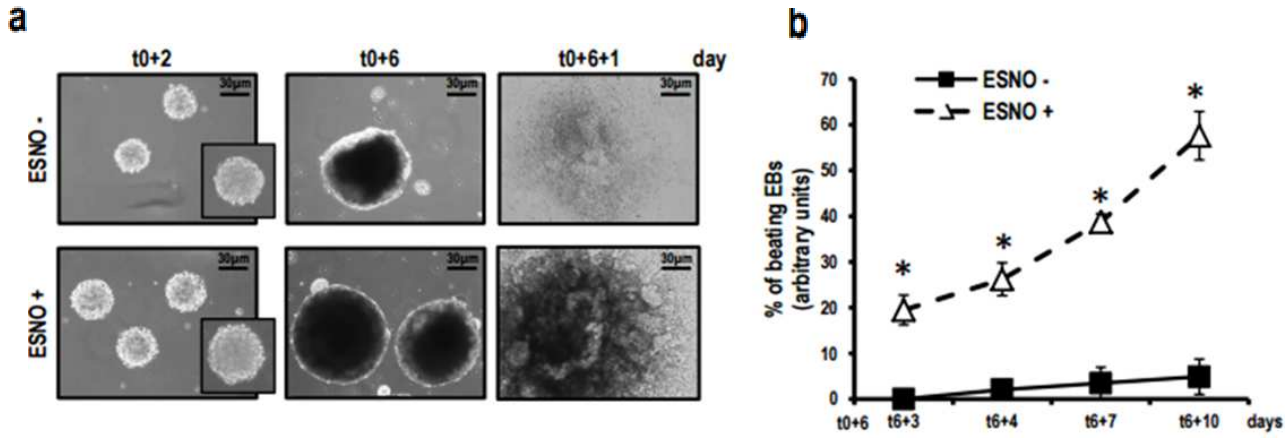

Figure 2. a) Phase contrast microscopy images of ESNO- and ESNO+ derived EBs (upper and lower panels) taken at different stages of the EB formation process after sorting (t0). Specifically, the following time points were considered: 2 days of hanging drop culture after t0 (t0+2), 6 days of hanging drop culture after t0 (t0+6) and the first day after plastic adherence following hanging drop culture (t0+6+1). b) The graph shows the number of beating EBs generated by ESNO+ (open triangles) and ESNO- (closed squares) cells upon a time course from three (t6+3) to ten (t6+10) days after plastic adherence (t0+6). Data were expressed as the mean ± SE of 4 independent experiments (average total number of plated EBs/condition/experiment = 48; *p < 0.05 ESNO+ vs. ESNO-)

qRT-PCR analysis (Figure 3) performed during a time course from time point t0 to t0+6+10 indicated that cardiac-specific markers, including Nkx2.5, Casz1, Acta1, Myh6, Myh7 and Cx30.2, were almost exclusively upregulated in ESNO+ cells. This result is in agreement with the evidence that only the ESNO+ population efficiently formed beating EBs.

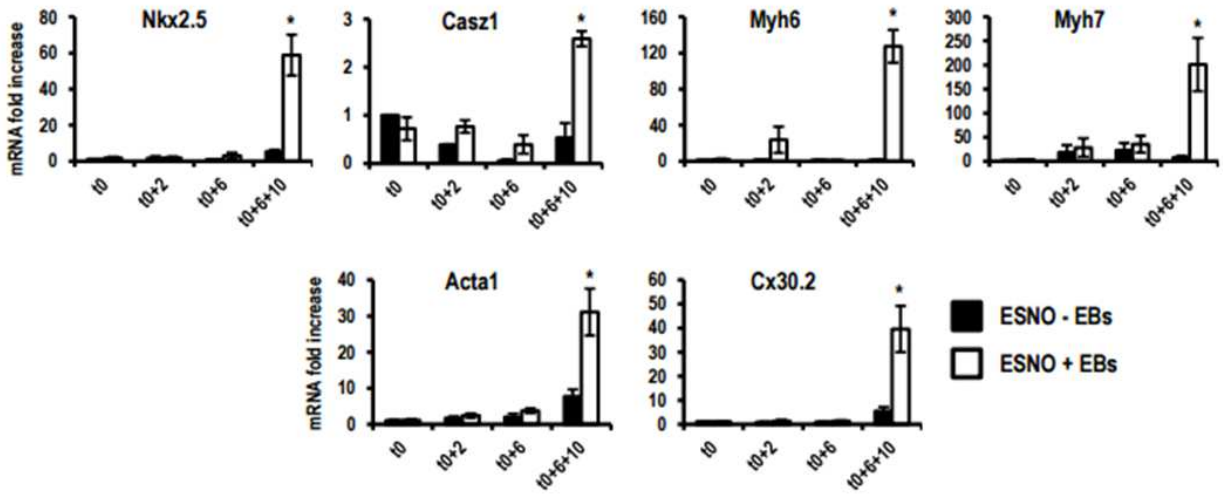

Figure 3. qRT-PCR analysis of cardiac associated markers (Nkx2.5, Casz1, Myh6, Acta1 and Cx30.2) in ESNO- (black bars) and ESNO+ (white bars) derived EBs taken at different time points of the EB maturation process. Data are shown as mean±SE fold increase compared to ESNO- derived EBs after subtraction of the housekeeping p0 gene signal (n=4 each time point; *p <0.05).

3. Generation and characterization of eNOS knockout (eNOS-/-) clones by CRISPR/Cas9:sgRNA gene engineering technology.

To explore the role of eNOS in the gene expression regulation of mES cultured in GS or DMGS the CRISP/Cas9 technology was adopted to inactivate its expression, taking advantage of the expertise and tools available at the host Institution. Specifically, to knock out eNOS two different target-specific sgRNAs were cloned into LentiCRISPR2 vector using the GoldenGate protocol. I could therefore transformed both plasmids (LCv2_eNOS_1 and LCv2_eNOS_2) into NEB 5-alpha Competent E. Coli and purify DNA by EZNA Fastfilter Endo-Free Plasmid DNA Maxi Kit (Omega Bio-Tek). Nucleofection was performed in mES cultured in GS using Amaxa P3 primary cell 4D Nucleofector Kit (Lonza). Briefly, 10^6 mES were resuspended in 100 µl of prewarmed (RT) nucleofector solution/supplement mixture containing 6µg of DNA. After a short incubation this cell/DNA mixture was aliquoted and transferred into a dedicated nucleofection cuvette and exposed to electroporation. After nucleofection mES cells were cultured for 48h and then selected by 1.5µg puromycin. After recovery from selection, mES were tested for eNOS knockout by western blot and qRT-PCR (Fig. 4 a and b). Upper panels of figure 4, shows the effect of two different CRISP/Cas9 vectors designed for eNOS in order to reduce off-target effects. In all cases, the expression of the targeted genes analysed in puromycin-selected bulk populations was significantly reduced (Fig. 4 a-b). Consistently, the down modulation of eNOS expression in undifferentiated cells determined the prevention to activate mesendoderm-associated genes including Wnt3a, Wnt7b and Dll1 once in differentiation conditions (Fig. 4c).

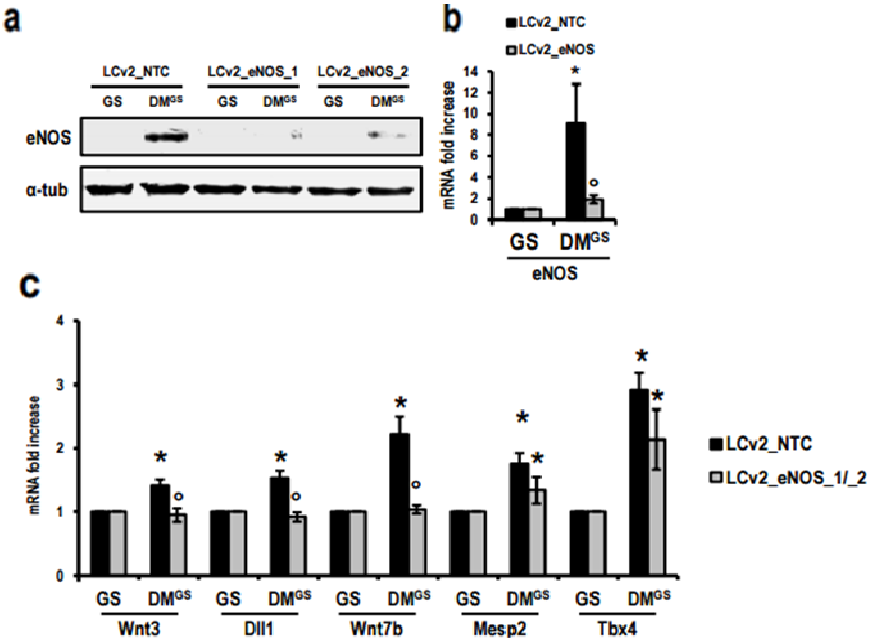

Figure 4. a) Representative image of western blotting analysis showing the result of eNOS inactivation by two independent CRISP/Cas9 vectors (DMGS-LCv2_eNOS_1 and DMGS-LCv2_eNOS_2) compared to control (GS-LCv2_NTC). Extracts were probed with an anti-eNOS antibody. α-tubulin was used as a loading control for each. b and c) mRNA expression analysis of eNOS, Wnt3, Dll1, Wnt7b, Mesp2 and Tbx4 in mES cultured in GS and DM^{GS} after control vector expression (black bars) or CRISP/Cas9-dependent eNOS inactivation (gray bars). Data are shown as the mean of 3 independent experiments ± SE represented as fold increase compared to mES cultured in GS and engineered with the CRISP/Cas9 control vector after subtraction of the housekeeping gene p0 signal (*p<0.05 vs GS; °p<0.05 vs GS NTC).

In summary, during my stay at the Goethe University, I could accomplish the following experimental points: i) the identification of a NO-dependent transcription profile in mES released from naïve state; ii) the generation and validation of two vectors obtained with the CRISP/Cas9 technology with the aim of knocking down the eNOS expression in puromycin-selected bulk populations.

Functional analyses using the hanging-drop embryoid body (EBs) technique started during my stay at the Goethe University and currently in progress, will allow us to validate the inability of puromycin-selected eNOS-/- clones to differentiate into mature cardiomyocytes, as compared to wild type mES.

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Dr. Agnese Re