MOLECULAR MANIPULATION OF INHIBITORY PATHWAYS FOR IPS INDUCTION INCREASE.

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BACKGROUND

The pluripotency and development potential of human embryonic stem cells (hESC) has been of great interest for regenerative medicine in the last fill years. Despite the remarkable features of these cells, ethical and immune-genetic compatibility issues are barriers to their use on regenerative therapies. Alternatively, recent studies have developed induced pluripotent stem cells (iPS) that triggers the use of hESC. iPS are fibroblasts modified by retrovirus-mediated transgene expression with hESC-like phenotype. The great interest on iPS cells is the possibility to generate patient-specific cells for therapy purposes, without the issues mentioned above. However, the low efficiency of the induction process and the drawbacks genetic modifications by retrovirus integration, are new barriers that must be overcome to further advance in the field. Thus, further understanding of molecular basis of the reprogramming phenomenon is a task that must be pursued.

AIM

In order to further increase the reprogramming efficiency, we first focused on molecular modulation of WNT/GSK3β/βCatenin and PI3K/PTEN/AKT pathways in different cell types. Likewise, were studied the downregulation of two DNA methyltransferases genes, *DNMT1* e *DNMT3b*, in human fibroblasts BJ1. DNMTs mediate oncogene transcription repression by methylation of their transcription promoters.

METHODS

Human cells: foreskin fibroblasts BJ1, colon tumor cells HCT116 and HCT116/DKO, hESC H1. Optimization protocols for siRNA transfection were analyzed by FACS. siRNA Cy3, siRNA FITC, pré and anti-miRs FAM and Cy3 were used for this procedure. For transfections were used electroporation and lipo-cationic methods. Real-time PCR was used to quantify transcriptional levels of the siRNA targets downregulation (βCatenin, GSK3β, pTEN, AKT1, DNMT1 and DNMT3b), as so as βCatenin targets: cMyc, CyclinD1, AKT1, STAT3 and Axin2; and an pluripotency gene, OCT4. β-Actin was used for normalization and relative expression was calculated using siRNA negative control transfected cells as reference samples.

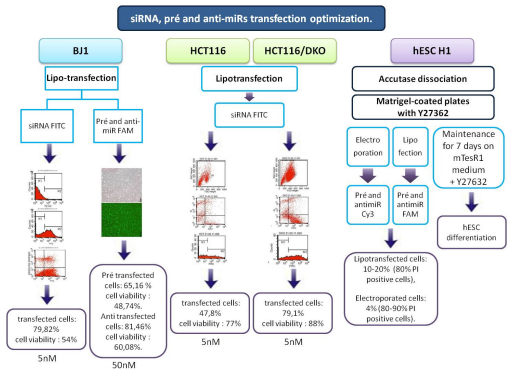

Figure 1. Preliminary experiments design for transfection optimization efficiency. First, for BJ1 cells siRNA, pré and anti-miR transfection, lipofectamine 2000 (invitrogen) was chosen. Second, siRNA lipotransfection was also chosen for HCT116 and HCT116/DKO cells. Third, for hESC H1, we choose two different protocols: lipotransfection by lipofectamine 2000 and Neon electroporation. However, about 80-90% of hESC transfected cells died. Yet, for colonies expansion 2.10^5/well of accutase-dissociated hESC cells were maintained on matrigel-coaetd plates with Y27632; but 7 days after, we could only find morphological fibroblastoid cells. The transfection efficiency and cell viability obtained are indicated by purple squares. All experiments were analyzed by FACS.

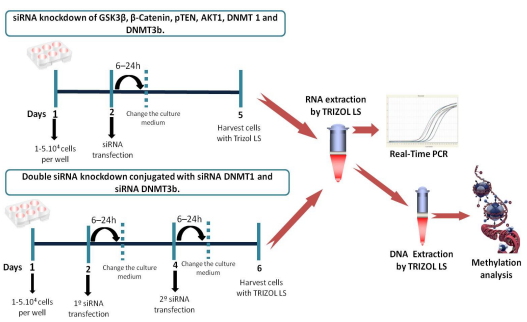

Figure 2. Simple and double siRNA lipotransfection designs on BJ1 cells. 24h before simple siRNA transfection, 5.10^4/well of BJ1 cells were plated on 6 well plate. About 24h after lipotransfection, the culture medium was replaced. After 72h of siRNA transfection, cells were harvested by TrizolLS protocol. For double siRNA lipotransfection, an second set of lipofectamine procedure was added to the experiment design 24h after the first treatment. After 48h of 2º set of siRNA transfection, these cells were also harvested by Trizol LS protocol. Total mRNA extraction was conducted, followed by DNA extraction and finaly, methylation analysis (data not shown).

RESULTS

❖ *GSK3β*, *βCatenin*, *pTEN* and *AKT1* transcription levels of BJ1, HCT116 and HCT116/DKO transfected cells. Inhibition efficiency varied between 70–98% (Figure 3). Also, *DNMT1* and *DNMT3b* siRNA knockdown on BJ1 cells were 86 and 35% respectively (Figure 4).

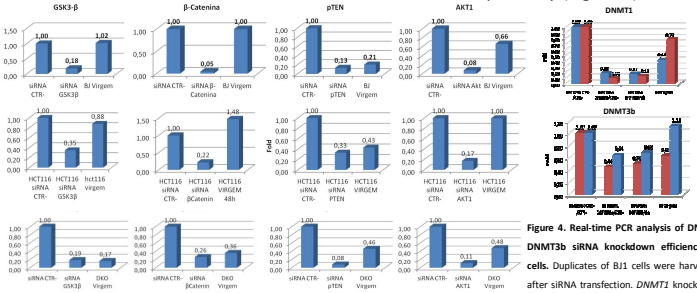

Figure 3. Real-time PCR analysis of *GSK3-ß*, *ß-Catenin*, *pTEN* and *AKT1* siRNA knockdown efficiency on BJ1, HCT116 and HCT116/DKO cells. BJ1 cells were colected 72h after siRNA transfection, while HCT116 and HCT116/DKO were colected only 48h post-transfection. *GSK3-ß* expression varied between 18-35%, *ß-Catenina* expression was between 5-26%, pTEN expression was between 8-33%, and AKT1 expression was between 8-17%.

Figure 4. Real-time PCR analysis of DNMT1 and DNMT3b siRNA knockdown efficiency on BJ1 cells. Duplicates of BJ1 cells were harvested 72h after siRNA transfection. *DNMT1* knockdown was 83% and 86%, and *DNMT3b* knockdown was 27% and 35% respectively. On conjugated siRNA inhibition, *DNMT1* expression was 17% and 13%, whereas *DNMT3b* expression was 50% and 68%.

❖ Transcript levels of βCatenin targets *c-Myc, Cyclin-D1*, *AKT1*, *STAT3* and *Axin2* were increased upon 2 fold on BJ1 siRNA GSK3-β knockdown (Figure 5).

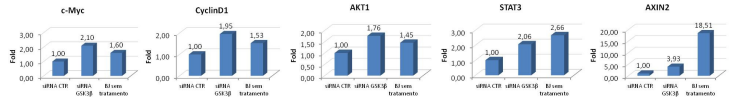

Figure 5. βCatenin transcription targets expression after 72h of siRNA lipotransfection. Real-time PCR analysis of *c-Myc, Ciclina D1, AKT1, STAT3* e *Axin2* genes, the relative expression ($2^{-\Delta\Delta Ct}$) was obtained in relation to siRNA CTR- treated BJ1 cells.

❖ Next, conjugated lipotransfection of siRNA DNMT1/3b followed by siRNAs target-specific were analyzed on BJ1 cells. Slight increase of *AKT1* and *CyclinD1* were noted in siRNA DNMT1/3b/GSK3β downregulation (Figure 6). These results were next compared to HCT116 and HCT116/DKO cells. We found highly increased expression of *MYC*, *OCT4* and *AKT1* on HCT116 cells lineages (Figure 7).

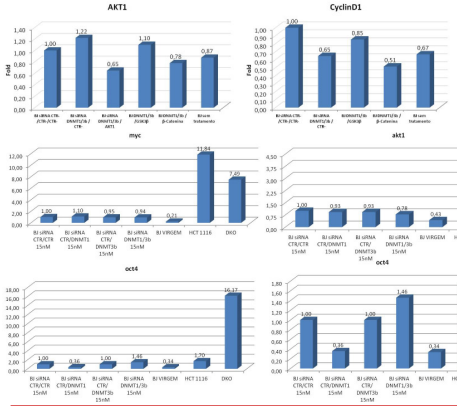

Figure 6. Real-time PCR analysis of AKT1 and CyclinD1 genes after conjugated lipotransfection of siRNAs DNMT1/3b followed by siRNAs specific targets on BJ1 cells. These genes expression were increased upon 2 fold on siRNA DNMT1/3b/GSK3β, compared to the relative expression ($2^{-\Delta\Delta Ct}$) obtained in relation to siRNA CTR- BJ1 cells.

Figure 7. Real-time PCR analysis of βCatenin target genes after conjugated lipotransfection of siRNAs DNMT1/3b followed by siRNAs target-specific on BJ1 cells compared to HCT116 and HCT116/DKO without treatment. Myc was highly expressed on HCT116 and DKO cells (12 and 7,5 fold respectively), followed by AKT1 expression, 2-4 fold higher on HCT116 and DKO cells. Finaly, OCT4 expression was found 1,7 to 16 fold increased on HCT116 and DKO cells. All results were compared to the relative expression ($2^{-\Delta\Delta Ct}$) obtained in relation to siRNA CTR- BJ1 cells.

PERSPECTIVES

➢ Finish the methylation analysis by DNA bisulfite conversion technique to access the effects of treatments described here.

➢ Re-conduct the preliminary optimization protocol on hESC H1, to allow the efficient delivery of synthetic pre/anti-miRs. Once we achieve this optimization, specific microRNA modutations will be held on hESC, looking for early differentiation or maintenance of pluripotencial state.

➢ In a next step, microRNAs screening analysis will be performed on two lentiviral reporters cells lineages NTera2 and BJ1, searching for reprogramming improvement points, differentiation punctual starts and increasing of pluripotency state (this project has been jointly developed with students Danuta Sastre and Ildercilio Lima).

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