CRISPR/Cas9 generation of HADHA KO PANC-1 cells to study the role of HADHA in pancreatic cancer stemness

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Introduction

CRISPR/Cas9 has rapidly changed the genome editing field and it has been widely applied to generate gene knockout cell lines, useful to understand the biological consequences of gene silencing in selected cell lines. The technique relies on the use of guide RNAs (gRNAs) and an endonuclease (Cas9) that mediate double strand break on a specific gRNA-targeted genomic region[1,2]. Here, we select the Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Alpha (HADHA) gene as target to knockout in human pancreatic cancer cells (PANC-1). The gene was found to be overexpressed in pancreatic cancer stem cells (PCSCs)[3]. Therefore, CRISPR/Cas9 was used to generate HADHA KO PANC-1 cells. The knockout cell line was then used to obtain pancreatic cancer stem cells (PCSCs) and the effect of HADHA absence was evaluated on the ability to form tumourspheres. While HADHA knockout did not alter the stemness of pancreatic cancer cells, HADHA KO-PCSCs display the formation of denser tumourspheres than WT-PCSCs.

Workflow

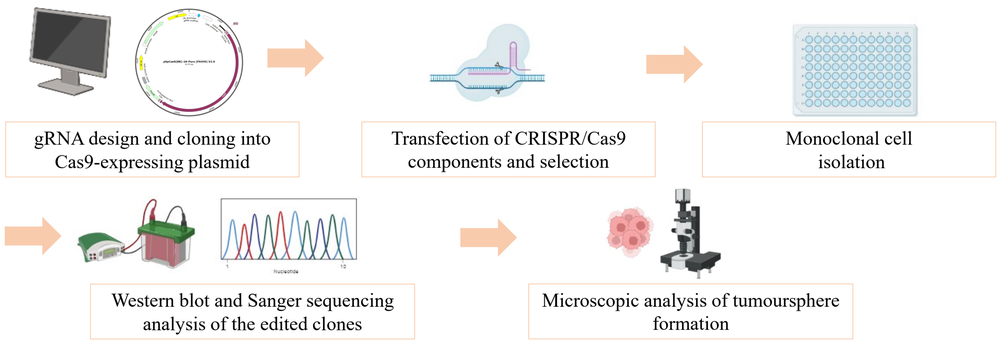

Figure 1. Experimental workflow.

Materials and methods

CRISPR/Cas9 generation of knockout cells

Two gRNAs targeting exon 1 of the HADHA gene were designed by using CRISPRscan and separately cloned into Cas9-expressing plasmids. GFP transfection was firstly used to assess transfection efficiency with Lipofectamine3000, Transit-LT1 and nucleofection. Both gRNA/Cas9 plasmids were then transfected into parental PANC-1 (P) cells by Lipofectamine3000, after optimization of the DNA:reagents ratio. After selection with puromycin, single cell cloning was performed by seeding 0.3 cells/well in 96-well plates. Individual clones were then tested by Western blot with anti-HADHA monoclonal murine antibody. Genomic DNA was extracted and Sanger sequencing was performed on the PCR-amplified gRNA-targeted region. The TOPO® TA Expression Kit was used to clone PCR products into the TOPO® vector and, following bacterial transformation, Sanger sequencing was performed on six isolated colonies. Final sequences were aligned with the reference WT sequence by EMBOSS Needle software. For each gRNA, the most probable off-target sites were selected by COSMID web tool. These top-ranked off-target genomic sites were then PCR-amplified, Sanger sequenced and aligned to the reference WT sequence.

Tumoursphere formation assay

PCSCs were obtained by culturing P cells in a specific “stem selective medium”. Images of tumoursphere formation were taken after 5, 10, 15 days using the EVOS FL Imaging System. Only cell clusters >40µm were considered.

Results

1. Transfection optimization on PANC-1 cells

Transfection efficiency was tested by transfecting pEGFP plasmid into PANC-1 cells with Lipofectamine3000, Transit-LT1 and nucleofection. Lipofectamine3000 displays the best transfection efficiency, even if moderate (Fig.2).

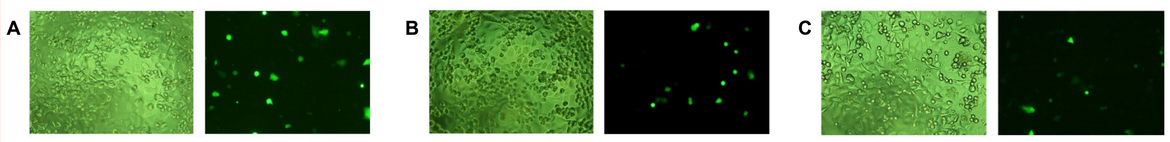

Figure 2. GFP transfection on PANC-1 cells. Lipofectamine3000 (A) shows a greater transfection efficiency than Transit-LT1 (B) and nucleofection (C).

Different Lipofectamine3000-based transfection conditions were evaluated in order to find the optimal one (Fig.3). Cells transfected with conditions 6 (1.5:4:1, ratio Lipofectamine 3000 – p3000 – DNA, with 5 µg DNA) and 8 (1.25:2:1, ratio Lipofectamine 3000 – p3000 – DNA, with 3 µg DNA) exhibit the best survival rate after puromycin selection and they were therefore subjected to single cell cloning, along with conditions 7-9 (pooled together) that display a lower survival percentage.

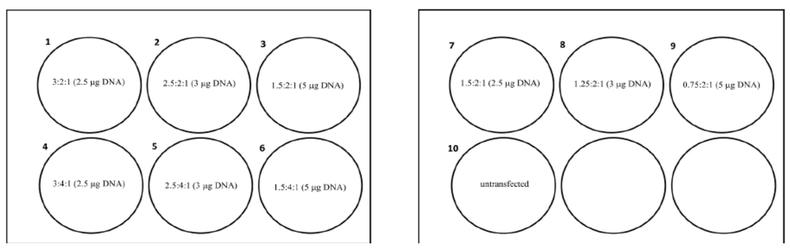

Figure 3. Lipofectamine3000 transfection optimization. *In each ratio, the first value refers to the amount of Lipofectamine3000 reagent, the second value to the amount of p3000 reagent, and the third value to the amount of DNA. In parentheses, the total amount of DNA is expressed in µg.*

2. Generation of HADHA KO PANC-1 cells

Western blot showed absence of the HADHA protein band in one (C8E12) of the tested clones (Fig.4).

The knockout was confirmed by Sanger sequencing, showing a 124-bp frameshift deletion on the first allele removing part of exon 1 and intron 1 and a 78-bp deletion on the second allele disrupting almost entirely exon 1 (Fig.5). Off-target analysis was conducted by selecting the two most probable off-target sites for each gRNA. All the off-target sites in the KO clone (C8E12) correspond to the WT sequence, meaning that no unintended off-target events have occurred in the predicted off-target sites (Fig.6).

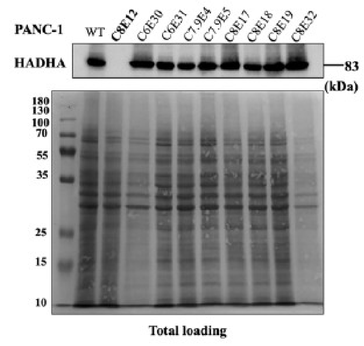

Figure 4. WB on the isolated clones. *Only one clone (C8E12) showed absence of the HADHA protein.*

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WT          51 AGGGCTGCGTCTCCGGGCTGGGGCAGGCGGCAGCGAAAGGGAGACGCGGC  100
               ||||||||||||||||||||||||||||
Allele1     51 AGGGCTGCGTCTCCGGGCTGGGGCAGG-----------------------   77

WT         101 TCCGGGGCCACCCCAGTCCCGGCAGGAGTTCTGCCCGGAGGTCTGGGATG  150
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Allele2    101 TCCGGGGCCACCCCAGTCCCGGCAGGAGTTCTGCCCGGAGGTCTGGGATG  150

WT         101 TCCGGGGCCACCCCAGTCCCGGCAGGAGTTCTGCCCGGAGGTCTGGGATG  150
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Allele1     78 -----------------------------------------------------   77

WT         151 CCCTCGGCCAGGCCTCACCTCGGGAGCGGAGGATCCTGAAGGCAGAAAAG  200
               ||||||||||||||||||
Allele2    151 CCCTCGGCCAGGCCTCACC-----------------------------------  169

WT         151 CCCTCGGCCAGGCCTCACCTCGGGAGCGGAGGATCCTGAAGGCAGAAAAG  200
               ||||||||||||||||||
Allele1     78 -----------------------------------------------------   77

WT         201 CGGCTGAGGATGCCAATCGCCCGGCAGGCCACCATCTTGAGCTGAAGAGG  250
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
Allele2    170 -----------------------------------------------------AGG  172

WT         201 CGGCTGAGGATGCCAATCGCCCGGCAGGCCACCATCTTGAGCTGAAGAGG  250
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
Allele1     78 -GGCTGAGGATGCCAATCGCCCGGCAGGCCACCATCTTGAGCTGAAGAGG  126

WT         251 ACAGCAGTGGAGAGCGCCTCTAACGGGTGCGGCCGAGCGGAGGACTTTTC  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
Allele2    173 ACAGCAGTGGAGAGCGCCTCTAACGGGTGCGGCCGAGCGGAGGACTTTTC  222
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Figure 5. Alignment of the edited sample to the WT*. Allele 1 shows a 124bp-deletion, while allele 2 has a 78bp-deletion.*

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WT#1        CCTCTCCCGAGGTGAGTGTGCC    WT#2        CCACTCCCGAGGTGCTGCTGCC    WT#3        TGAAGCCACAAAAGCGGCTGTGG    WT#4        CCCCAGCCGCTTTGCAGCCTGCC
C8E12#1     CCTCTCCCGAGGTGAGTGTGCC    C8E12#2     CCACTCCCGAGGTGCTGCTGCC    C8E12#3     TGAAGCCACAAAAGCGGCTGTGG    C8E12#4     CCCCAGCCGCTTTGCAGCCTGCC
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Figure 6. Alignment of off-target sites of KO clone to the WT*. No off-target events occurred in the selected genomic sites.*

3. Tumoursphere formation of HADHA KO PCSCs

After induction of dedifferentiation, HADHA KO clone still retains the ability to grow in suspension and to remain undifferentiated, demonstrating that the absence of HADHA does not affect the stemness of this pancreatic cancer cell line. Microscopic analysis shows that KO-PCSCs tend to form larger and more compact tumourspheres than the WT-PCSCs (Fig.7).

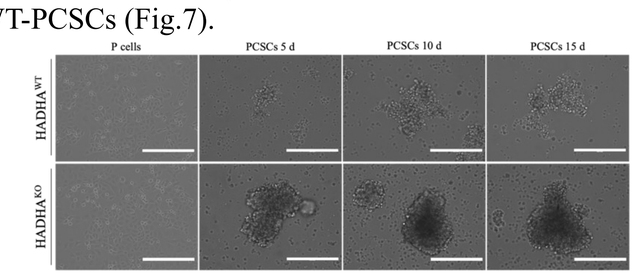

Figure 7. *Bright-field microscopic images of WT and HADHA KO P cells and PCSCs, at different days (5-10-15).*

Discussion

Since HADHA gene was found to be overexpressed in PCSCs, we generated HADHA KO PANC-1 cells to evaluate the effect of HADHA absence on pancreatic cancer stemness. PANC-1 cells appeared difficult to transfect, with Lipofectamine3000 yielding the best transfection efficiency results, albeit moderate. Among several tested clones, one resulted HADHA KO, as confirmed by Western Blot analysis and Sanger sequencing. KO cells do not lose the ability to undergo undifferentiation, meaning that the absence of HADHA does not alter the stemness of the pancreatic cancer cell line. However, the HADHA KO-PCSCs form denser and more compact structures than the WT-PCSCs. Starting from these preliminary results at the morphological level, the effect of the absence of HADHA will be next evaluated on proliferation, chemoresistance and tumour migration of PCSCs to better define HADHA role in PCSCs biology or its potential involvement as therapeutic target in pancreatic cancer.

References

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[2] Giuliano, C. J. *et al.* (2019) ‘Generating Single Cell–Derived Knockout Clones in Mammalian Cells with CRISPR/Cas9’, *Current Protocols in Molecular Biology*, 128(1). doi: 10.1002/cpmb.100

[3] Di Carlo, C. *et al.* (2021) ‘Integrated lipidomics and proteomics reveal cardiolipin alterations, upregulation of HADHA and long chain fatty acids in pancreatic cancer stem cells’, *Scientific Reports*, 11(1), pp. 1–13. doi: 10.1038/s41598-021-92752-5.

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