

Characterisation of SilenciX® cell lines as a stable, syngenic and loss-of-function model

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INTRODUCTION

Silencing is today the most adopted method to study gene function. The most classic approaches are based on siRNA expressed in the cell through different channels like transient transfection of RNA duplexes, of plasmids or of virus expressing RNA constructs. While efficient, these strategies suffer from the lack of stability that renders study of some proteins challenging. To overcome this issue, short hairpin RNAs (shRNA) can be used to increase stability but they disrupt genomic integrity. Our solution to this technical challenge is SilenciX®. Derived from our in-house technical knowledge and scientific collaboration, SilenciX® combines RNAi technology, DSIR Program and pEBV (Epstein Barr Virus) derived vector to generate knock-down clones in numerous cell lines covering various fields of research.

To demonstrate the advantages of a stable and functional model to study loss-of-function, we have chosen here the example of Xeroderma Pigmentosum, complementation group C (XPC).

XPC is a major protein of the nucleotide excision repair (NER) pathway involved in damage recognition, open complex formation, and repair protein complex formation. Mutations in this gene or some other NER components result in *Xeroderma pigmentosum*, a rare autosomal recessive disorder characterized by increased sensitivity to sunlight with the development of carcinomas at an early age

MATERIAL & METHODS

Cell line establishment and culture

HeLa cells were transfected using Lipofectamine2000 with SilenciX® vectors(1), (2) containing siRNA for XPC and control sequence. We established XPC SilenciX® cell lines, Control SilenciX® cell lines. Cells were grown with DMEM containing 4.5g/l D-Glucose, 580mg/l L-Glutamine and 110mg/l sodium pyruvate, supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (10,000 U/ml; 10,000µg/ml), and 125µg/ml hygromycin B (Life Technology) was added to the culture media to maintain EBV vectors into the cells.

qRT-PCR

RNA was isolated from both cell lines (control and knock-down) using the NucleoSpin RNA II (Macherey-Nagel) and according to the manufacturer's instructions for mammalian cells. Reverse transcription was realized with RT² first strand kit and RT² SYBR Green Master Mixes according to the manufacturer's instructions. (cat numbers C-03 and 330520 SABiosciences, a Qiagen Company). Real-time PCR in XPC HeLa SilenciX® cells was performed in comparison with Control HeLa using primers set for GAPDH, Actin and XPC (cat number PPH00150E, PPH00073E and PPH01536A, SABiosciences, a qiagen company).

WB analysis

Western-blot was realized as described as follow: total protein equivalent to 600,000 cells were isolated, subjected to 10% SDS polyacrylamide gel electrophoresis and electro-transferred onto nitrocellulose membranes. Antibodies used: mouse polyclonal antibody XPC, monoclonal antibodies Ku70/80 (cl.162), DNA-PKcs (cl.18.2; Lab Vision/Neomarkers, Newmarket, United Kingdom), human kin17 protein (Ig K36 and Ig K58); rabbit polyclonal antibodies directed against XRCC4 (AbD Serotec, Cergy Saint-Christophe, France); ligase IV (Abcam, Cambridge, United Kingdom).

Clonogenic cell survival

For clonogenic growth after UVC irradiation, cells were plated at 500 cells per dish (6cm) and irradiated 24 hours later. Growing clones were fixed with 4% paraformaldehyde and stained with methylene blue after 14 days of culture. Clones containing more than 50 cells were counted. Each point represents the mean of three culture dishes. Colony formation was normalized as a percentage of the control.

RESULTS AND DISCUSSION

EBV-based plasmid vectors carry oriP (origin of replication) and EBNA1 gene. Inside the cells, OriP interacts with EBNA1 to promote episomal maintenance thus keeping the vector extrachromosomally over long periods of time. This property allows the vector to be stably transmitted to daughter cells (reinforced by hygromycin B selection) without impairing genome integrity. By cloning shRNA sequence into the plasmid, we aimed to obtain long term gene silencing in cultured human cell lines.

Thus, genome integrity is ensured though the vector is perfectly maintained.

qRT-PCR Analysis

Our first step to assess silencing efficiency was to perform qRT-PCR experiments.

qPCR analysis shows a decrease of 81% for XPC gene.

These results confirmed the efficiency of our silencing at RNA Level.

Western Blot

There is today no direct correlation between mRNA and protein levels of expression within the cell.

With this in mind, we performed WB experiments on control and silenced cells.

WB analysis was performed on HeLa cell and control (3)

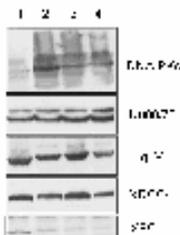


Figure 1 Western Blot Analysis:

- 1, control (day 94);
- 2, XPC^{KD} cl.4 (day 315);
- 3, XPC^{KD} cl.21 (day 267);
- 4, XPC^{KD} cl.24 (day 264);

Functionality

As with RNA and protein levels, there is no direct correlation between a protein down-expression and a loss of function.

To demonstrate the correlation between down regulation and loss of function we performed ultra-violet (UVC) irradiation experiment.

UVC induces a wide variety of mutagenic and cytotoxic DNA lesions like cyclobutane, pyrimidine dimers, 6-4 photoproducts and DNA Strand Breaks. Organisms counteract these lesions with highly conserved repair mechanisms like base excision repair (BER), Nucleotide Excision Repair (NER) and Mismatch Excision Repair (MMR)

Being a part of NER, XPC is activated when cells are submitted to UVC and improves their survival after irradiation.

For XPC silenced cells, we expected an improper DNA Repair leading to UVC inducing cell death.

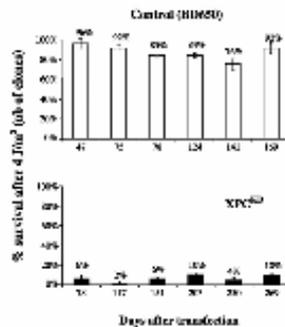


Figure 2 : Clonogenic Cell Survival Experiments

(4J/m²) control (BD650) and XPC^{KD}

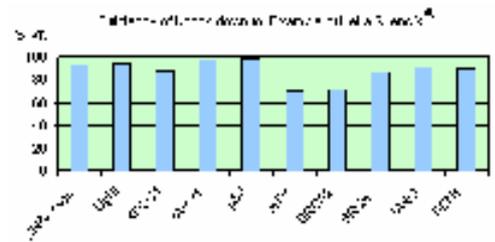
Figure 2 shows cell survival experiments after UVC radiation (4J/m²) in control and XPC^{KD} cells. Even 269 days after irradiation, XPC^{KD} cells still display a 15 fold increase in cell death when compared to the control. The expected phenotype (sensitivity to UVC and increased cells death) is observed in silenced cells and maintained overtime.

These results demonstrate the validity of the XPC SilenciX® cell line as a loss-of-function model for DNA Repair.

OTHER CELLS LINES AVAILABLE

Other SilenciX® cell lines have been established and more than one hundred cell lines are already available.

For instance:



Target HeLa SilenciX®	cat. N°	% KD
DNA-PKcs	01-00005	93
LigIII	01-00009	94
XRCC1	01-00017	87
PARP1	01-00019	97
p53	01-00024	98
ATR	01-00025	70
BRCA2	01-00028	72
BRCA1	01-00041	86
PARG	01-00085	91
FEN1	01-00089	90

It also shows that the other proteins tested (XRCC4, Ligase IV, Ku70/80) were expressed normally in silenced cells. Furthermore, the different times tested (up to 315 days) demonstrate the stability of the EBV-Based plasmid vector in a silencing strategy.

Taken together, qRT-PCR and WB experiments show that we have generated a cell line presenting a long term stable down regulated expression of XPC.

Figure 2 shows that silenced cells (here noted XPC^{KD}) from different clones at different times of cultures all display a significant reduction in XPC protein expression when compared to control cells.

SilenciX® technology has been shown to be **compatible with numerous mammalian cell types** (MRC5, HCT116,...) Custom Cell lines development on request.(5)

In this study, we demonstrate why XPC SilenciX® cell line is a functional model to study loss of function effect.

Our technology, a combination of RNAi Technology and pEBV-derived vector creates a syngenic model which allows to mimic human diseases in a comparable genetic background. Our technology brings Stability, efficiency and functionality. The long-term maintenance of gene silencing in syngenic cells may open new areas of research, in particular in the field of cancer to improve target validation in a synthetic lethality approach or to evaluate efficiency of inhibitors.

REFERENCES

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