Extract from the results section

This is a writing example from my PhD thesis "Investigating the differential instructive roles of WT1's isoforms", Giulia Petrovich, University of Edinburgh, 02/07/2016.

Chapter 3. Establishing models to investigate the role of WT1 single isoforms

Overview

The models used to study the functions of WT1 isoforms have so far failed to provide unanimous results, emphasizing the need for further investigation to understand their role in development and cancer. To shed light on the instructive functions of WT1, I established two cell models expressing in an inducible manner the main four murine isoforms of WT1 (with or without the exon 5 and/or the KTS, here referred as: +/+, +/-, -/+ and -/-). In this chapter, I will explain the design and cloning of the different plasmids I used to derive these stable cell lines.

At first, I failed to establish stable clones that constitutively expressed WT1 isoforms fused to a fluorescent protein (FP), possibly because of the toxicity of the constitutive over-expression of WT1. Therefore, I decided to design an inducible model. First, I created a classic Tet-On 3G inducible system, which requires two different constructs, a regulator plasmid and a response plasmid: The regulator plasmid expresses the Tet-On 3G transactivator gene under the control of the CMV promoter, while the response plasmid carries the gene of interest regulated by the doxycycline-inducible promoter pTRE3G. I cloned two pairs of vectors in which the inducible isoforms of WT1 were tagged with two different fluorescent proteins (AmCyan1 and mCherry). To confirm the pattern of FP-WT1 expression, I sequenced and tested the plasmids by western blot (WB) and immunofluorescence (IF) after transient transfection.

Because the classic Tet-On 3G system is based on two plasmids, two steps of integration are needed to derive stable cell clones. To simplify this process, I cloned single vectors (called pSV40-Tet3G-pTRE3G-(FP)-Wt1) that expressed the Tet-On 3G transactivator constitutively, and the (FP)-Wt1 protein upon doxycycline treatment. I designed three sets of vectors: one where the isoform was fused to the AmCyan1 protein, one where it was fused to the mCherry protein, and one where the isoform remained untagged (w/o FP). After confirming the sequence of the plasmids and testing them in transient transfection, I demonstrated that the isoforms were expressed only after treatment with doxycycline and that the signals of the fluorescent proteins mirrored WT1 expression.

Given the crucial role of WT1 in the epithelial-mesenchymal transition (EMT) and in the reverse process (mesenchymal–epithelial transition, MET) [1-5], I chose the MDCK cells to generate stable and inducible clones. In fact, these cells are a widely used model for EMT studies and renal epithelial cells that do not express endogenous WT1. Although the inducible MDCK cells were indeed a valuable system to study the isoforms, they also showed some disadvantages: first, the single clones had different expression levels of WT1 that led to clonal variability issues; and second, even if the murine and canine WT1 are supposed to bind to the same promoter sequence [158], the mouse WT1 may not behave physiologically in a dog cell.

Therefore, I tried to generate a more homogeneous cell system by integrating a single copy of plasmid in a specific chromosomal position. I cloned a new set of vectors, called pGoldiLox, that offered several advantages over the pSV40-Tet3G-pTRE3G-(FP)-Wt1 plasmids: I added ROSA26 homology arms to target the integration of the constructs; I substituted the SV40 promoter for the CAG promoter, as it guarantees an optimal activity in the ROSA26 locus and it is less subjected to silencing in embryonic stem cells; I cloned a constitutively expressed EGFP (enhanced green fluorescent protein) to facilitate the screening of stable clones.

After testing the pGoldiLox-Wt1 vectors in transient transfection, I generated stable and inducible IMCD3 clones. The murine IMCD3 cells are polarized epithelial cells that were derived from the inner medullary collecting duct and express mesenchymal markers in the same way as the cells that express endogenous WT1 [159].

To establish IMCD3 stable clones and integrate the pGoldiLox plasmids into the ROSA26 locus, I transfected each of the pGoldiLox-Wt1 plasmids together with two Zinc Finger-encoding plasmids. I then picked clones and checked the integration of the plasmid and WT1 expression. Unexpectedly, most of the clones expressed WT1 also before induction, raising the issue of using a leaky system in the following studies.

To solve the leaky expression of the pGoldiLox-Wt1 plasmids, I cloned a smaller and simpler set of plasmids, the CAG-Tet3G-TRE3G-Cherry vectors. In this chapter, I will discuss the design and the testing of these plasmids, which I am currently using to derive inducible stable clones of wild-type and *Wt1* knockout embryonic stem cells. These clones will be of crucial importance to address *in vivo* the relevance of the findings observed *in vitro* so far.

(Full text available here)