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Transgenic RNAi in mouse oocytes: The first decade☆

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ABSTRACT

RNA interference (RNAi), a sequence-specific mRNA degradation induced by double-stranded RNA (dsRNA), is a common approach employed to specifically silence genes. Experimental RNAi in plant and invertebrate models is frequently induced by long dsRNA. However, in mammals, short RNA molecules are used preferentially since long dsRNA can provoke sequence-independent type I interferon response. A notable exception are mammalian oocytes where the interferon response is suppressed and long dsRNA is a potent and specific trigger of RNAi. Transgenic RNAi is an adaptation of RNAi allowing for inducing sequence-specific silencing upon expression of dsRNA. A decade ago, we have developed a vector for oocyte-specific expression of dsRNA, which has been used to study gene function in mouse oocytes on numerous occasions. This review provides an overview and discusses benefits and drawbacks encountered by us and our colleagues while working with the oocytes-specific transgenic RNAi system.

1. Introduction

RNA interference (RNAi) is one of several pathways, which employ small RNAs as sequence-specific guides for inhibition of gene expression. RNAi (reviewed in Nejepinska et al., 2011) is initiated by long double-stranded RNA (dsRNA) that is processed by the RNase III endonuclease Dicer into 21-22 nucleotide long RNA molecules. These small interfering RNAs (siRNAs) are loaded onto Argonaute-containing effector complexes, usually referred to as the RISC (RNA-Induced Silencing Complex), where they serve as guides for sequence-specific recognition and degradation of cognate mRNAs. The typical outcome of RNAi is sequence-specific endonucleolytic cleavage of cognate mRNA in the middle of the duplex formed between mRNA and siRNA. This cleavage is mediated by Argonaute 2 (AGO2) and requires formation of a perfect or nearly perfect RNA duplex (reviewed in Sontheimer, 2005).

In most mammalian cells, exogenous long dsRNA (>30 base pairs) triggers the interferon I response resulting in a global inhibition of protein synthesis and general RNA degradation (reviewed in Wang and Carmichael, 2004). The specific dsRNA-mediated RNAi response without sequence-independent effects was observed only in a few cell types, such as oocytes, early embryos, embryonic stem cells and a few others (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000; Billy et al., 2001; Yang et al., 2001; Gan et al., 2002). Mammalian oocytes are probably a privileged cell type, in which long dsRNA preferentially induces RNAi. This is supported by apparently suppressed interferon response in mouse oocytes (Stein et al., 2005) and observation that long dsRNA expression induces readily RNAi in oocytes of transgenic mice but not in other tissues (Nejepinska et al., 2011). Furthermore, mammalian endogenous siRNAs, markers of functional RNAi pathway, have been cloned only from mouse oocytes (Tam et al., 2008; Watanabe et al., 2008).

Given its ability to selectively block gene expression, RNAi was rapidly adopted as an experimental tool. In mouse oocytes, the most common types of RNAi experiments involve microinjection of dsRNA (or siRNA) and transgenic RNAi. Since the microinjection is usually

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performed in fully-grown oocytes and 1-cell embryos, studying gene function during oocyte growth and/or during oocyte-to-embryo transition is difficult if not impossible using the microinjection. A solution of this problem is expression of dsRNA from a transgene, which was developed in order to provide a tool allowing for extending the exposure to RNAi (Stein et al., 2003; Yu et al., 2004). Transgenic RNAi also offers several advantages over conditional mutagenesis because transgenic RNAi is less technically demanding, produces phenotypes faster, and it is cheaper. Technical aspects of using both RNAi methods in oocytes have been discussed in detail elsewhere (Stein et al., 2003; Svoboda and Stein, 2009). Here, we will focus on summarizing our experience with transgenic RNAi based on oocyte-specific expression of long RNA hairpin, in which dsRNA is formed upon folding of an inverted repeat into an intramolecular duplex (Stein et al., 2003).

2. Design of a transgene for transgenic RNAi

Transgenes for expression of long dsRNA in the oocyte mostly follow a previously developed design (Fig. 1A) where dsRNA hairpin is embedded in the 3′UTR of an EGFP-encoding mRNA (Svoboda et al., 2001). Expression is controlled by an oocyte-specific post-meiotic zona pellucida 3 (*Zp3*) promoter. The use of a *Gdf9* promoter allows for the induction of RNAi in pre-meiotic oocytes. Detailed protocols for designing a transgene for RNAi in oocytes have been described elsewhere (Svoboda, 2009b,d,c,a; Svoboda and Stein, 2009).

A long hairpin RNA ensures efficient dsRNA formation because an intramolecular RNA hairpin forms more efficiently than a duplex of two complementary molecules. Inverted repeats can be produced in two configurations with respect to the cognate mRNA sequence: antisense-loop-sense (A-S) or sense-loop-antisense (S-A). Both configurations have been successfully used in transgenic RNAi in mouse oocytes (Stein et al., 2003; Yu et al., 2004). The S-A configuration would be an advantage if activation of a cryptic polyadenylation site in the antisense sequence would occur since a partial hairpin could be still formed from S-A configuration, but not from the A-S one (Fig. 2).

3. Cognate sequence selection

We recommend to select a unique mRNA sequence at least 0.5 kb in length. Location of the dsRNA with respect to the mRNA sequence does not appear to be a critical factor. We and others achieved efficient RNAi knockdowns with sequences targeting the coding sequence as well as the 3′UTR. When the 3′UTR is targeted, a concern may be that the cleaved mRNA could still undergo translation. However, given the efficiency of RNAi and likely inefficient translation of a message lacking the poly A tail, this should not be a significant problem. However, if one needs rescue experiments or the studied gene has close paralogues, targeting the 3′UTR is a better option as one can perform a rescue experiment with a different 3′UTR fused to the coding sequence of the inhibited gene. One should only pay attention to select a non-redundant target sequence, which would not show sequence identity in stretches longer than 20 nt with other transcripts. This can be easily resolved with a BLAST sequence search. In addition, one should avoid canonical polyA site sequence (AAUAAA) in both directions.

4. Cloning of an inverted repeat

Hairpin RNA expression requires cloning an inverted repeat into a vector. Several strategies available for this task have been described in detail elsewhere (Svoboda, 2004, 2009b). Briefly, one strategy uses PCR products with appropriate restriction sites, which are ligated in vitro and the inverted repeat is then inserted into a plasmid of interest. It is important to avoid AATAAA motif in both arms of the inverted repeat as it could function as a cryptic polyadenylation site. One of the PCR products can be a bit longer at the side that will be in the center of the inverted repeat, so the additional sequence creates a spacer, which enhances efficiency of cloning the inverted repeat. Based on our experience a short spacer (20–50 bp long) is sufficient for successful cloning and does not have a negative impact on RNAi efficiency. The spacer can be even longer as successful RNAi was reported with a spacer as long as 700 bp (Paddison et al., 2002). Another strategy is sequential insertion of inverted repeat arms into a plasmid. Finally, one can sequentially clone an inverted repeat with a large spacer (1–2 kb) that is later removed. Another possibility is to clone a head-to-tail tandem array of two fragments where one is flanked with loxP sites allowing for inverting it by Cre recombinase (Paddison et al., 2002). This strategy overcomes the problem of inserting an inverted repeat into a plasmid, which may be a difficult task because transformation sometimes results in a high background of empty plasmids. This is presumably caused by the presence of an inverted repeat that may interfere with plasmid replication and create a strong negative selection.

5. Outcomes of transgenic RNAi in mouse oocytes

Here, we will focus on additional considerations, which emerged recently. Altogether, we have information about fifteen transgenic RNAi experiments, including ten published ones (Table 1). None of these experiments encountered serious problems with cloning an inverted repeat and preparing a transgenic construct. Occasional delays in cloning an inverted repeat with a short spacer were solved by growing bacterial clones at room temperature in suitable bacterial strains (e.g. Stbl4 (Invitrogen) or Sure (Stratagene)). All transgenic experiments yielded transgenic lines with a strong (80% or more) mRNA knockdown. An average time from the transgene cloning to the first phenotype analysis typically ranged from 6 to 9 months.

One of the problems of RNAi experiments in somatic cells are unwanted non-specific effects which must be kept under control (reviewed in Echeverri et al., 2006; Svoboda, 2007). The most common non-specific effects in RNAi experiments are the induction of an interferon response as well as off-target effects whereby siRNAs down-regulate partially complementary transcripts. These two effects pose little problem in using RNAi in mouse