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Method of Inducible Knockdown of Essential Genes in OSC Cell Culture of *Drosophila melanogaster*

S. V. Marfina[a, b], E. A. Mikhaleva[a], N. V. Akulenko[a, *], and S. S. Ryazansky[a, **]

[a] *Kurchatov Institute National Research Center, Moscow, 123182 Russia*

[b] *Mendeleev University of Chemical Technology, Moscow, 125047 Russia*

**e-mail: n.akulenko11@gmail.com*

***e-mail: s.ryazansky@gmail.com*

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Abstract—An RNA interference-based method was proposed to achieve an inducible knockdown of genes essential for cell viability. In the method, a genetic cassette in which a copper ion-dependent inducible metallothionein promoter controls expression of a siRNA precursor is inserted into a genomic pre-integrated transgene by CRIPSR/Cas9 technology. The endogenous siRNA source allows the gene knockdown in cell cultures that are refractory to conventional transfection with exogenous siRNA. The efficiency of the method was demonstrated in *Drosophila* ovarian somatic cell culture (OSC) for two genes that are essential for oogenesis: *Cul3*, encoding a component of the multiprotein ubiquitin-ligase complex with versatile functions in proteostasis, and *cut*, encoding a transcription factor regulating differentiation of ovarian follicular cells.

Keywords: knockdown, siRNA, CRISPR/Cas9, *Drosophila*, OSC

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INTRODUCTION

Genetic technologies to inhibit gene expression are classified as knockouts, where mutations are introduced in the reading frame or regulatory regions, and knockdowns, where mRNA production is decreased without affecting the gene sequence. Among knockout methods, the CRISPR/Cas9 technology is distinguished by its relative simplicity and efficiency and is rightfully rated as a universal genome editing tool [1, 2]. In this technology, nuclease Cas9 in complex with a single guide RNA (sgRNA) binds to a genome region complementary to the sgRNA and makes a double-strand break. Its repair by the non-homologous end joining cell system leaves microdeletions or insertions in the breakpoint site. When homologous recombination repair takes place in the presence of an exogenous donor DNA template with arms homologous to the breakpoint-flanking sequences, the template can be inserted in the breakpoint site. Either mechanism makes it possible to edit the sequence of a gene with its complete silencing.

Classical RNA interference (RNAi) is the main approach to perform gene knockdowns [3]. In RNAi, a mRNA is cleaved by the nuclease Argonaute (Ago), which recognizes the target mRNA with the help of an Ago-associated small interfering RNA (siRNA) complementary to the target. To achieve RNAi in a cell culture, cells are transfected with a siRNA or a long precursor double-stranded RNA (dsRNA), which is cleaved to yield siRNA by a cell processing system. Transfection can also utilize the plasmids that encode double- or single-stranded RNA with a hairpin secondary structure element (short hairpin RNA (shRNA)) as a siRNA precursor. The use of certain pre-miRNAs as templates to design the shRNA secondary structure makes it possible to substantially accelerate the shRNA processing to siRNA [4]. Another knockdown technique utilizes dead Cas9 (dCas9), which is devoid of nuclease activity, but is still capable of binding with a sgRNA and a genome region complementary to the sgRNA. Fusion proteins wherein dCas9 is combined with inhibitory domains of transcription factors act in complex with sgRNAs complementary to promoter regions and thus inhibit transcription of the respective genes [5]. Cassettes with the dCas9 gene controlled by an inducible promoter are developed for integration into the genome [6]. Other knockdown methods, such as those utilizing Cas13a, become more common as well [7].

Certain drawbacks are characteristic of the methods of CRISPR/Cas9 knockouts and RNAi knockdowns. For example, CRISPR/Cas9 mutagenesis is generally unsuitable for editing polyploid genomes, which are often found in cell cultures [8, 9]. Compensatory mutations can additionally accumulate in the edited genome over time due to adaptive evolution,

thus complicating a phenotypic analysis [10]. In addition, the irreversibility of knockouts makes sometimes desired short-term suppression of genes impossible. Finally, a knockout of vital genes, which are of particular interest, leads to the formation of cell cultures heterozygous for the mutation and only slightly reduces the expression level of the gene under study [10]. A main problem with RNAi is that dsRNA transfection is inefficient. An extremely low transfection efficiency is observed in a number of cell cultures [11], including *Drosophila* ovarian somatic cell (OSC) cultures.

To overcome the problem of inefficient transfection, we developed a method of inducible gene knockdown in OSC culture. A cassette utilized in the method contains the inducible metallothionein gene promoter to control the expression of a RNA precursor of siRNA, which is capable of folding into a pre-miRNA-like hairpin structure. The cassette is integrated into a particular genomic site via CRISPR/Cas9 mutagenesis. Copper ions added into the culture medium trigger shRNA expression and target gene silencing. Our method allows to knock down vital genes in the cell cultures that show a low transfection efficiency. The efficiency of our method was demonstrated with the example of the *Cul3* gene, which encodes a component of the multiprotein cullin-RING ligase (CRL) complex. CRL complexes regulate many molecular processes, including cell division, signal transmission, transcription, metabolism, proteostasis, differentiation, and development [12]. The efficiency of our method was additionally demonstrated with the example of *cut*, which encodes a transcription factor that is necessary at various stages of differentiation of somatic follicular cells during *Drosophila* oogenesis [13, 14]. Our method provides an alternative to inducible knockdowns achieved using dCas9 or Cas13. Our study is of importance because *Drosophila* is still broadly used as a model to study the general biological regularities of development and because cultured cells of other species, including humans, can be modified using our approach.

EXPERIMENTAL

Cell culture. The *Drosophila* OSC[Cas9+] line with a genomic insert of pAc-sgRNA-Cas9 (Addgene, #49330) [15] was obtained previously using an OSC culture kindly provided by M. Siomi (University of Tokyo, Japan) [16]. The OSC[Cas9+] was grown in the Shields and Sang M3 medium (Sigma-Aldrich, United States, #S3652) supplemented with 10% fetal bovine serum (FBS) (Gibco, United States, #10270106), 10% fly extract, 10 μg/mL insulin (Sigma-Aldrich, United States, #I9278), 0.6 mg/mL L-glutathione (Sigma-Aldrich, United States, #G6013), 50 units/mL penicillin, 50 g/mL streptomycin, and 1 μg/mL puromycin at 25°C. To obtain a fly extract, 1 g of 3- to 7-day-old flies was homogenized in 7 mL of the M3 medium. The homogenate was centrifuged at 1500 *g* for 15 min; the supernatant was heated at 60°C for 10 min and cleaned by centrifugation at 1500 *g* at 4°C for 1.5 h; and the supernatant was centrifuged again at 4000 *g* at 4°C for 15 min. Plasmid transfection was carried out using FuGENE-HD (Promega, United States, #E2311) according to the manufacturer's protocol. Cells in which a cassette with the hygromycin or blasticidin resistance gene was integrated in the genome were selected in the medium supplemented with 200 μg/mL hygromycin (Calbiochem, United States, #400050) or 10 μg/mL blasticidin (Applichem, Germany, #A3784). Expression of MT-shRNA-bsd was induced by adding a $CuSO_4$ solution to a final concentration of 0.5–2 mM; the expression level was assessed 3 days after the induction.

Knockouts of *Cul3* and *EloB* in OSC cultures. To introduce double-strand breaks in *Cul3* and *EloB*, sgRNA sequences were designed using the TargetFinder program [17]. Synthetic oligonucleotides (sgRNA^{Cul3}: 5'-CTTCGATAAAACACAGCGTTGATAT and 5'-AAACATATCAACGCTGTGTTTTATC; sgRNA^{EloB}: 5'-CTTCGTCAGGACAACGATGTCATGG and 5'-AAACCCATGACATCGTTGTCCTGAC) were annealed, phosphorylated at the 5' end, and cloned in pU6-BbsI-chiRNA (Addgene, United States, #45946) [17], which was preliminarily linearized at the BbsI sites. A gene knockout in OSC[Cas9+] cells was performed using Cas9 according to a published protocol [18]. A donor DNA fragment containing the *hyg* gene under the control of its own promoter was PCR amplified from pMH4 (Addgene, United States, #52529) [19] with the following primers: hyg_Cul3: 5'-CTTCTTTTTTAAGCAACTACTGTATTTATGCCTTCCCCTTCGAATAGGAACCGCCTATATGGATCT-TCCGGATGGCTCGAG and 5'-GAGCTGGGTGCATCTTTATCCTTTTCACTTTCAACTGCCTTGCGGCCATAAAACACAGCGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCATAT and hyg_EloB: 5'-ATCGCAGGCATACTAAAGGTGCAGCCCGTGGACCAGCGGTTATACAATCAGGCAACGATGGATCTTCCGGATGGCTCGAG and 5'-CTGCGCCTTGGCCGTGGACACCGTCACGCCGTAGTCCTGCAACGTGCTGTCGTCCTCCTGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCATAT. The 5'-terminal region of the primers was complementary to the 60-bp gene sequence downstream or upstream of the breakpoint, and the 3'-terminal region (underlined) was complementary to the pMH4 sequence. A mixture of the PCR-amplified fragment and pU6/sgRNA was used to transfect OSC[Cas9+] cells as described above.

Construction of the pAc-MT-shRNA-bsd vector. Several vectors were used to construct the donor plasmid for homologous recombination repair of the double-strand breaks introduced by Cas9. First,

pWalium20 was used to obtain the MT-shRNA-bsd cassette, which contained shRNA sequences under the control of the MT promoter and the *bsd* blasticidin resistance gene. To construct the cassette, shRNA-coding oligonucleotides (*Cul3*: 5'-ctagcagtTACGATCATGGATGAGTTTAAtagttatattcaagcataTTAAACTCATCCA-TGATCGTAgcg and 5'-aattcgcTACGATCATGGATGAGTTTAAtatgcttgaatataactaTTAAACTCATCCATGATCGTAactg, *cut*: 5'-ctagcagtAAGGAAGTGAACACACTCAAAtagttatattcaagcataTTTGAGTGTGTTCACTTCCTTgcg and 5'-aattcgcAAGGAAGTGAACACACTCAAAtatgcttgaatataactaTTTGAGTGTGTTCACTTCCTTactg; regions used to excise siRNAs are in capitals) were annealed and cloned in pWalium20 (DGRC, United States, #1472) [4, 20], which was linearized at the EcoRI and NheI sites, yielding pWalium20-shRNA. The sequences of hairpin structures were retrieved from the FlyRNAi database of The Transgenic RNAi Project at Harvard Medical School [20]. The *mini-white* gene was replaced with *bsd* by Gibson cloning (NEBuilder HiFi kit, NEB, United States) of two DNA fragments. One was pWalium20-shRNA linearized at the HindIII sites to excise *mini-white*. The other was a *bsd*-containing fragment PCR-amplified using the primers 5'-CTCGAGATCGATGATATCAAAATAAACATATGCTGTTGG and 5'-CGAATTGGGTACAAGCTCCATATGTTAGAAACAAATTTAT and the pMH4 plasmid (Addgene, United States, #52529) [19] as a template. The resulting plasmid was designated as pWalium20-shRNA-bsd. A major part of the 10×UAS-hsp70 promoter was replaced with the MT promoter by Gibson cloning of two fragments. One was pWalium20-shRNA-bsd linearized at the BglII and BamHI sites to excise 5×UAS-hsp70. The other was a fragment containing the MT sequence and was PCR-amplified using the primers 5'-GGATGTTTTCTAGAACACCTTTAGTTGCACTGAGATGAT and 5'-ATACGAAGTTATGGATCCGTTGCAGGACAGGATGT and pMT-OsTIR1-P2A-H2B-AID-EYFP (kindly provided by C. Lener, University of Zurich, Switzerland) as a template. To construct the final donor plasmid pAc-MT-shRNA-bsd, a fragment with part of *Cas9* was excised from pAc-sgRNA-Cas9 at the SacI sites and the MT-shRNA-bsd cassette was inserted in the remaining vector by Gibson cloning. This was achieved using pAc-sgRNA-Cas9-SacI linearized at the SmaI site and a cassette-containing fragment PCR-amplified using the primers 5'-CGACGTCCCCCGGGAATTAACCCTCACTAAAGG and 5'-CGAGGGTGCGTACGGCATATGTTAGAAACAAATTTATTT and pWalium20-MT-shRNA-bsd as a template. The resulting plasmid pAc-MT-shRNA-bsd can be used as a vector to clone other shRNA sequences into the EcoRI and XbaI sites. To construct the plasmid for expression of $sgRNA^{puro}$ targeting *puro*, synthetic oligonucleotides (5'-CTTCGGCGAGGGTGCGTACGGCCC and 5'-AAACGGGCCGTACGCACCCTCGCC) were annealed; phosphorylated at the 5' end; and cloned in pU6-BbsI-chiRNA (Addgene, United States, #45946), which was preliminarily linearized at the BbsI sites. All constructed plasmids were checked by Sanger sequencing. A mixture of pU6/$sgRNA^{puro}$ and pAc-MT-shRNA-bsd was used to transfect OSC[Cas9+] cells as described above.

Quantification of gene expression by qRT–PCR. Approximately 1.5 μg of total RNA, which was isolated from 10^6 cells with the Extract RNA reagent (Evrogen, Russia), was treated with DNase I (Ambion, United States). To synthesize cDNA, we used Mint reverse transcriptase (Evrogen, Russia) and a mixture of random hexamers as primers. Quantitative real-time PCR was run on a DT-96 thermal cycler (DNK-tekhnologiya, Russia), using cDNA and primers to *Cul3* (5'-CTTCGATAAAACACAGCGTTGATAT and 5'-GCGTGAAGCCTTTGACATTTTC), *EloB* (5'-AAACCCATGACATCGTTGTCCTGAC and 5'-GAGCTGAAGCGAATGATTGAG), *cut* (5'-CTTCGTTGTTCGGCGAGTCCGTGCT and 5'-CCAGGAACATCTTCATGCGAATG), and *rp49* (5'-ATGACCATCCGCCCAGCATAC and 5'-GCTTAGCATATCGATCCGACTGG). Changes in expression of *Cul3*, *EloB,* and *cut* relative to *rp49* were calculated as $2^{-\Delta\Delta Ct}$ in comparison with their relative expression in original OSC[$Cas9^+$] cells.

Detection of transgene insertion in the genome by PCR. Genomic DNA was isolated from 10^6 OSC[*Cul3-KO*] or OSC[*EloB-KO*] cells by phenol–chloroform extraction and used in PCR with HSTaq polymerase (Evrogen, Russia) and the following primers: 5'-GCGTGAAGCCTTTGACATTTTC (Fig. 1, primer 1), 5'-ATATGTGCTTGCGTGTGGTG (primer 2), 5'-GAAAGGAGGTACCGGTATGAA-AAAGCCTGAACTCACC (primer 3), 5'-AAATTTATTTTAAAGTTTTATTTTTAATAATTTCTATTCCTTTGCCCTCGGAC (primer 4), 5'-GAGCTGAAGCGAATGATTGAG (primer 5), and 5'-CGCCTTGTCCGCTAGATAAC (primer 6).

RESULTS AND DISCUSSION

Inefficient Cul3 Knockout in OSC Cultures

To study the functions of *Cul3* and *EloB*, which encode components of ubiqutin-ligase CRL complexes involved in regulating many cellular processes [12], we tried to knock out the genes in cultured OSC[Cas9+] cells with constitutive *Cas9* expression. A cassette with the hygromycin resistance gene *hyg* was inserted into the coding regions of *Cul3* and *EloB* in OSCs genome (Fig. 1a). Cells with the cassette inserted in the genome were selected in the presence of hygromycin. The respective gene transcripts were quantified in the resulting cultures by qRT–PCR, and a knockout was observed to decrease the gene expression levels. However, the transcript amount was only halved in the case of *Cul3-KO*, while a tenfold

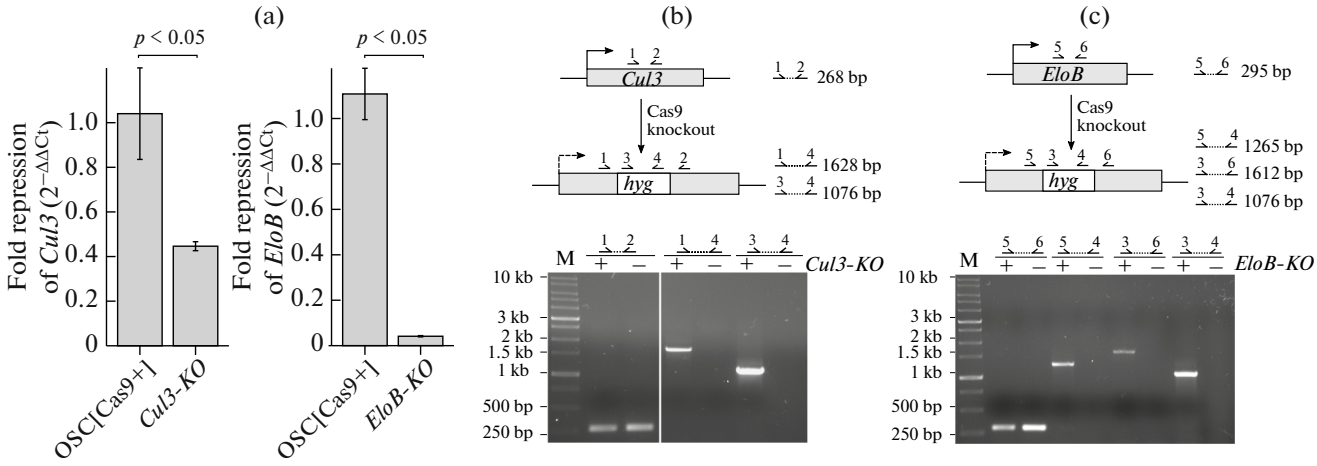

Fig. 1. Results of knocking out of *Cul3* and *EloB* in OSCs. (a) Changes in *Cul3* and *EloB* expression levels in knockout cells were assessed relative to the original OSC[Cas9+] line. Expression levels measured by qRT–PCR were normalized to *rp49* expression. The results were averaged over two biological replicates, each including three technical replicates. Whiskers show the standard error of the mean. The statistical significance of differences were tested by the Wilcoxon test. (b, c) Genomic PCR analysis of (b) *Cul3-KO* and (c) *EloB-KO* cells showed that both of the lines carried both the wild-type allele and the mutant allele with a *hyg* insert. The positions of the primers specific to the gene sequences and amplicon sizes are shown along with the PCR results (agarose gel electrophoresis).

decrease was detected in the case of *EloB-KO* (Fig. 1b). It is possible to assume that the *Cul3-KO* cell line was heterozygous for the mutant gene in contrast to the *EloB-KO* line. However, a PCR analysis analysis of their genomes showed that the intact wild-type alleles were preserved in both of the cell lines (Figs. 1b, 1c). Two situations could therefore be assumed to explain the greater extent of silencing by a knockout in the case of *EloB* compared with *Cul3*, i.e., either a smaller cell fraction is heterozygous in the *EloB-KO* cell line, or *EloB-KO* is a dominant mutation.

Diploid cells are mostly found in OSC cultures, but polyploid cells are also present [21]. To knock out all gene copies in a diploid genome, two cassettes with different antibiotic resistance genes can be used in place of a single cassette [22] with only one cassette being integrated in each of the respective chromosomes. Subsequent selection for resistance to the two antibiotics yields cells whose genome carries both of the cassettes with different resistance genes in different chromosomes. In an approach developed for polyploid cells, a cassette integrated into the genome encodes one or more sgRNAs targeting the gene of interest [23]. In this case, Cas9 loaded with continuously expressed sgRNA introduces double-strand breaks in a genome copy as long as the wild-type gene copy is present there.

Heterozygosity arose in the *Cul3-KO* line possibly because *Cul3* is vital, precluding selection of the cells that are homozygous for the mutation. A complete knockout of a vital gene is impossible to achieve even with two cassettes that express different antibiotic resistance genes and are integrated into different alleles [22] or a cassette that ensures continuous sgRNA expression [23], or the resulting line will be unstable. A knockdown with dCas9 or RNAi provides a more efficient means to inactivate a gene in such cases. However, classical RNAi with dsRNA transfection fails to ensure appreciable gene silencing in *Drosophila* OSC and similar cultures. We therefore developed a method wherein a knockdown is triggered by shRNA whose gene is integrated into the genome and expressed under the control of an inducible promoter. A cassette with the shRNA-coding sequence is integrated into the genome by the CRISPR/Cas9 technique.

Construction of Vector for Inducible Gene Knockdown

A cassette coding for particular shRNA under the control of the inducible metallothionein promoter was constructed on the basis of the pWalium20 vector, which has been designed to integrate into the genome and to express shRNA in particular *Drosophila* tissues [20]. The shRNA encoded in the vector is based on the sequence of pre-miR-1, a precursor of the *Drosophila* microRNA miR-1 with a hairpin-like secondary structure. Expression of this RNA is controlled by the *hsp70* minimal promoter and the UAS regulatory element. Insulators flank the UAS-hsp70-shRNA sequence in pWalium20 to allow efficient shRNA expression from the transgene in various genome regions regardless of the local heterochromatization level.

A scheme used to construct the cassette is shown in Fig. 2a. The *mini-white* gene, which has originally been included in pWalium20 to allow in vivo studies and to select transgenic flies by eye color, was replaced

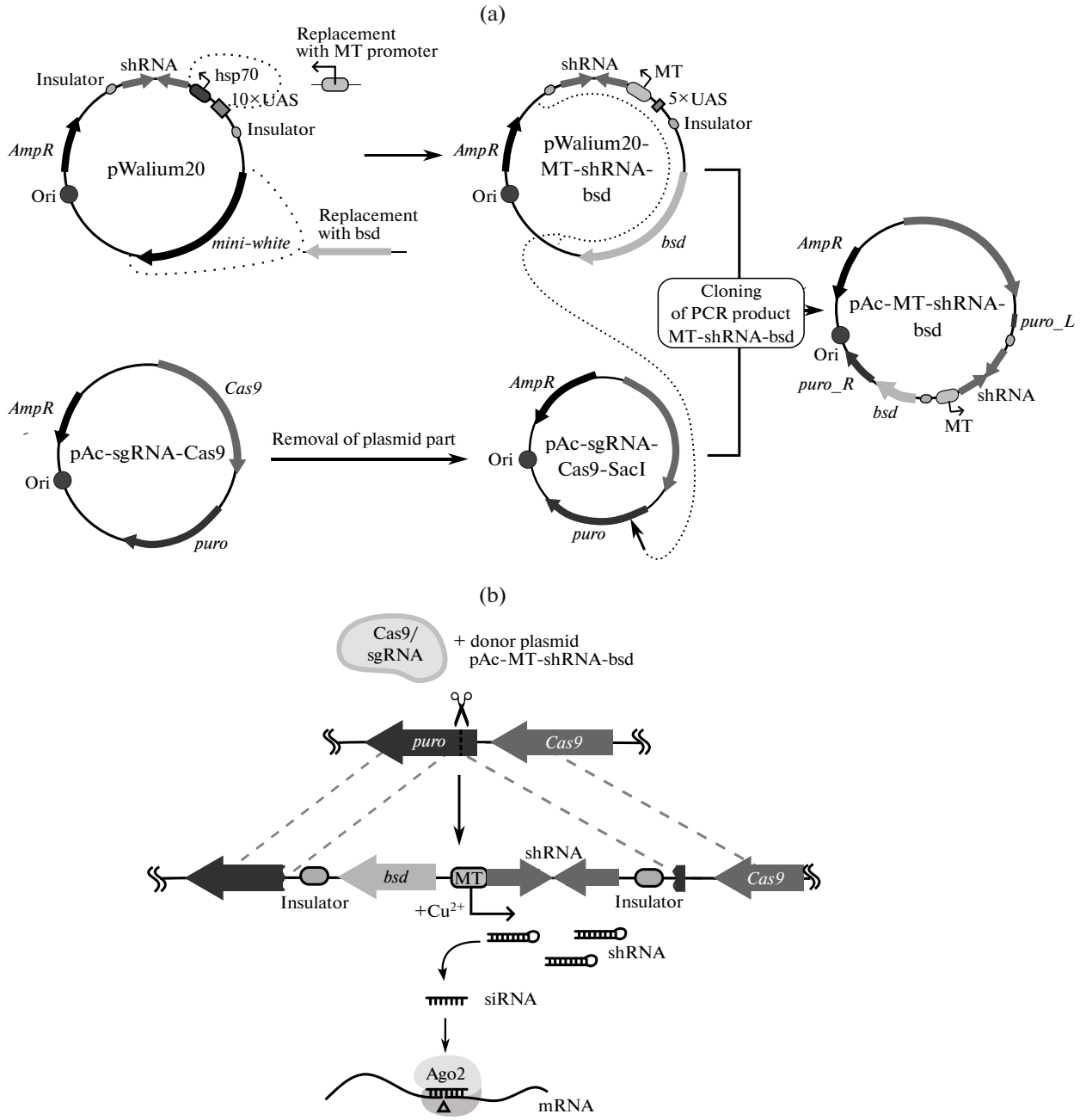

Fig. 2. (a) Construction of the donor plasmid pAc-MT-shRNA-bsd, which contained the MT-shRNA-bsd cassette for inducible shRNA expression. See text for details. *puro_R* and *puro_L* are the *puro* fragments included in pAc-MT-shRNA-bsd to serve as arms in homologous recombination. (b) The MT-shRNA-bsd cassette of the donor plasmid is inserted in the pAc-sgRNA-Cas9 pre-integrated transgene (a transgene part with *puro* and *Cas9* is shown) with the help of Cas9 and is activated by adding copper ions. The shRNA expressed from the cassette is processed to siRNA, which acts in complex with *Drosophila* Ago2 to bind to the complementary mRNA and to cause its degradation via the RNAi mechanism.

with the *bsd* blasticidin resistance gene to allow selection of cells with a necessary insert on a selective medium. The UAS-hsp70 promoter was replaced with the MT metallothionein promoter, which is induced by Cu^{2+} ions. To integrate the resulting MT-shRNA-bsd cassette into the genome via CRISPR/Cas9 mutagenesis, it was necessary to choose the integration site and to construct a donor plasmid wherein the cassette-flanking arms are homologous to regions near the site of cassette integration, which is expected to proceed by the mechanism of homologous recombination repair of double-strand breaks. The *puro* puromycin resistance gene was chosen as an integration site. The *puro* gene is a component of the pAc-sgRNA-Cas9 plas-

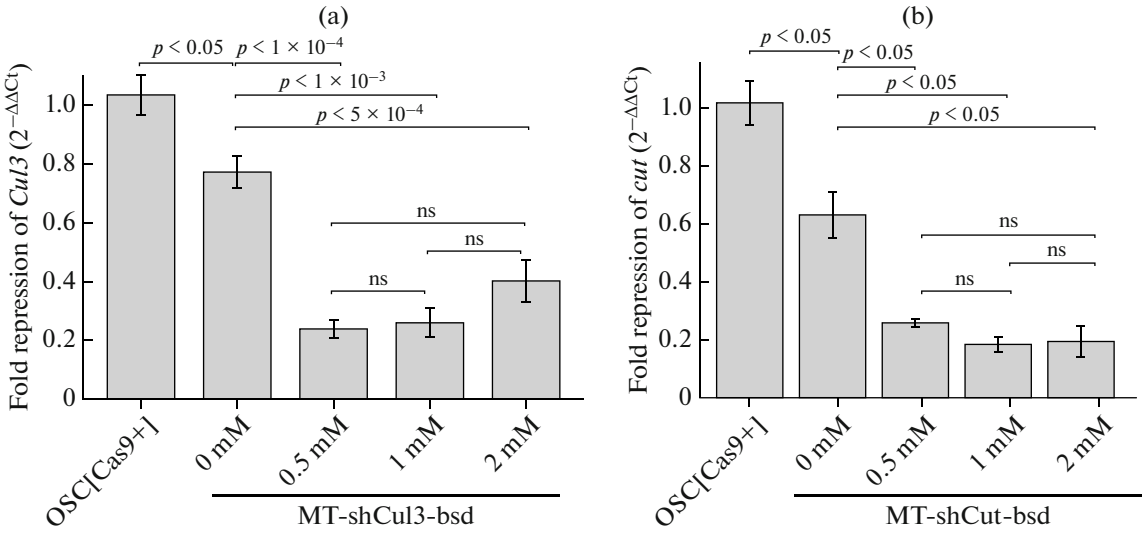

Fig. 3. Changes in (a) *Cul3* and (b) *cut* expression in the presence of 0.5, 1, and 2 mM Cu^{2+} in cultured cells carrying the corresponding shRNA expression cassette integrated in the genome as compared with expression in OSC[Cas9+]. Expression levels measured by qRT–PCR were normalized to *rp49* expression. The results were averaged over two or three biological replicates, each including three technical replicates. Whiskers show the standard error of the mean. The statistical significance of differences were tested by the Wilcoxon test; ns, nonsignificant difference.

mid, which has been integrated earlier to allow constitutive expression of Cas9 in OSCs (the OSC[Cas9+] cell line). Thus, pAc-sgRNA-Cas9 integrated into the OSC genome serves as a source of Cas9 and simultaneously provides a platform to insert a shRNA expression cassette. The donor plasmid pAc-MT-shRNA-bsd was constructed using pAc-sgRNA-Cas9, in which *puro* was modified by inserting the MT-shRNA-bsd cassette flanked with insulators. Arms that flank the MT-shRNA-bsd construct in the donor plasmid are as necessary for homologous recombination with the genomic *puro*. A sequence coding for *puro*-targeting sgRNA (sgRNA^{puro}) was cloned in the pU6-BbsI-chiRNA vector.

In our inducible knockdown method, nuclease Cas9 loaded with *puro*-targeting sgRNA^{puro} makes a double-strand break in *puro*. The break is then repaired via homologous recombination with the donor plasmid pAc-MT-shRNA-bsd used as a template (Fig. 2b). The MT-shRNA-bsd cassette is thus integrated into *puro*. Expression of shRNA and, therefore, target gene knockdown are induced by adding Cu^{2+} ions to the culture medium. The hairpin sequence cloned in pAc-MT-shRNA-bsd can be replaced by any other hairpin sequence; i.e. the plasmid can be used as a vector.

Inducible Knockdown of Cul3 and Cut

The *Cul3* gene was used to check the efficiency of our inducible knockdown method. A sequence coding for shRNA directed to the *Cul3* transcript was cloned in pAc-MT-shRNA-bsd. The resulting plasmid was used together with the plasmid carrying the sgRNA^{puro} sequence to transfect OSC[Cas9+] cells. Clone selection on a blasticidin-containing medium yielded a polyclonal blasticidin-resistant cell culture with the MT-shCul3-bsd cassette integrated in the genome. Measurements by qRT–PCR showed that the *Cul3* expression level in cells carrying the MT-shCul3-bsd insert was significantly lower than in the original OSC[Cas9+] cell line even without adding Cu^{2+} to the medium (Fig. 3a). The Cu^{2+} amount in the medium was probably great enough to trigger *shCul3* expression. Then Cu^{2+} ions were added to activate *shCul3* expression and to silence *Cul3*. To determine the maximum extent of gene silencing, the Cu^{2+} concentration was varied from 0.5 to 2 mM (2 mM is the highest concentration at which OSCs still remain viable). *Cul3* expression decreased already in the presence of 0.5 mM Cu^{2+} by a factor of 3–4 relative to expression in cells without induction or 5–6 relative to expression in OSC[Cas9+] (Fig. 3a). An increase in Cu^{2+} concentration to 2 mM did not further increase the extent of *Cul3* silencing. A similar experiment was performed to obtain an inducible knockdown of the *cut* transcription factor gene, and a decrease in target gene transcripts was observed again. Thus, gene silencing is achieved with our induced knockdown method. In the case of *Cul3,* the silencing efficiency was higher (a factor of 5–6) than with a partial knockout in *Cul3-KO* cells (a factor of 2–3).

Note that the possibility of a reversible temporal gene silencing is an advantage provided by the induced knockdown method. To restore gene expression, it is enough to change the medium containing excess cop-

per ions with a standard medium and to determine the time it takes for the shRNA pool to decrease. The system with an endogenous siRNA source can be used with a broad range of cell cultures that are poorly susceptible to transfection with exogenous dsRNAs. In the long view, a cell line with the integrated cassette is possible to maintain in the lab and to use in experiments for a prolonged period of time. Finally, an important feature of our method is that the hairpin-coding cassette is integrated into the particular genomic site. The *puro* sequence contained in a preliminarily integrated transgene was used as an integration site in our case. This allows correct comparisons of silencing efficiency of shRNAs targeted to the same of different genes.

ABBREVIATIONS

shRNA, short hairpin RNA; siRNA, short interfering RNA; OSC, ovarian somatic cell; CRL, cullin-RING ubiquitin ligase complex.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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