

RNA interference rescue by bacterial artificial chromosome transgenesis in mammalian tissue culture cells

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RNA interference (RNAi) is a widely used method for analysis of gene function in tissue culture cells. However, to date there has been no reliable method for testing the specificity of any particular RNAi experiment. The ideal experiment is to rescue the phenotype by expression of the target gene in a form refractory to RNAi. The transgene should be expressed at physiological levels and with its different splice variants. Here, we demonstrate that expression of murine bacterial artificial chromosomes in human cells provides a reliable method to create RNAi-resistant transgenes. This strategy should be applicable to all eukaryotes and should therefore be a standard technology for confirming the specificity of RNAi. We show that this technique can be extended to allow the creation of tagged transgenes, expressed at physiological levels, for the further study of gene function.

off-target effects | loss-of-function experiment | interferon response | gene knockdown

RNA interference (RNAi) has become a widely used tool for functional genomic studies in vertebrates and invertebrates (1). RNAi works by silencing a gene through homologous short interfering dsRNAs (siRNAs), which trigger the destruction of corresponding mRNA by the RNA-induced silencing complex (RISC) (2). The ease, speed, and cost-effectiveness have made it the method of choice for loss-of-gene function studies. However, RNAi has produced a new set of problems in determining the specificity of the altered phenotype. Recent publications reported off-target effects that in addition to the targeted genes led to changes in the expression of other genes on both mRNA and protein level (3–5). Also, several authors reported the induction of genes involved in the IFN response machinery (6–9), further challenging the reliability of RNAi in loss-of-function studies.

Control experiments are therefore important to confirm the specificity of an RNAi phenotype (10). The ultimate way to be sure of the specificity of a loss-of-function phenotype is a rescue experiment (11). To perform such an experiment in mammalian cells, the reintroduced gene must be resistant to the trigger dsRNA. Ideally, this rescue gene should also be expressed within the physiological range. For other important model organisms such as yeast, rescue experiments can be easily achieved by using homologous recombination, thereby ensuring physiological expression of the rescue construct. The lack of efficient homologous recombination in mammalian cells makes this approach unpractical in this experimental system.

One approach to generate an RNAi-resistant construct is to create silent point mutations in the target site of the coding sequence or to target a sequence in the 3' UTR of a cDNA, which is replaced in the rescue construct (12). Although this approach can work to achieve rescue of an RNAi phenotype, it has certain limitations (Table 1). First, this approach requires the availability of full-length high-quality cDNAs, and, although the list of full-length cDNA clones is growing, a clone may not be available for any given gene. Second, the cloning procedure to introduce

a point mutation in the coding sequence or the replacement of the 3' UTR is time consuming and expensive, especially for large transcripts. This shortcoming may be avoided by using a cross-species cDNA construct. Third, cDNAs do not allow the expression of alternatively spliced transcripts. Fourth, expression from vectors carrying cDNA inserts depends on the promoter used to drive the transgene expression. Most of these promoters are derived from viral or model vertebrate promoters that do not recapitulate physiological expression of most transgenes. This point may be critical for many rescue experiments, because inappropriate expression levels of a particular protein would either not rescue or could cause artifactual effects (10). Thus, a cDNA-based approach is not generally applicable and reliable for RNAi rescue experiments.

We propose a technology circumventing the problem of inefficient homologous recombination in mammalian cells by expressing an orthologous gene from a closely related species, including its regulatory sequences carried on a bacterial artificial chromosome (BAC). In contrast to cDNA expression constructs, the transfer of these large segments of genomic DNA speeds up the generation of transgenic cell lines and allows physiological expression and the generation of alternatively spliced variants of the transgene. Mouse geneticists have successfully used these advantages of large constructs for the generation of transgenic animals. For many years, cDNA constructs have been used to generate transgenic mice. Because of position effects, many founder lines needed to be tested to identify one with the appropriate expression pattern. The development of BAC technology for generation of transgenic mice has alleviated this problem to a large extent. Coupled with the development of methodology to specifically mutate large DNA molecules (13), now termed recombineering (14, 15), BACs are now the preferred choice to generate transgenic animals (16, 17).

Here, we establish BACs as rescue constructs for RNAi in mammalian tissue culture cells. The use of BACs carrying the orthologous gene from a closely related species confers RNAi resistance to the transgene. The cross-species strategy also abolishes the need for introducing point mutations or replacing the 3' UTR. We establish this approach for human tissue culture cells by the use of mouse BACs. This experimental concept provides a standardized platform to check the specificity of any particular RNAi experiment. In addition, this technology mimics homologous recombination by depleting an endogenous gene by means of RNAi and replacing it with a transgene while maintaining normal gene expression. Therefore, our approach helps to turn mammalian tissue culture cells into a real genetic system

Abbreviations: *mSNW1*, mouse *SNW1*; *hSNW1*, human *SNW1*; *mSPD2*, mouse *SPD2*; siRNA, short interfering dsRNA; esiRNA, endoribonuclease-prepared siRNA; RNAi, RNA interference; BAC, bacterial artificial chromosome; RFP, red fluorescent protein.

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Table 1. Comparison of the cDNA and transgenic BAC approaches for generation of RNAi rescue constructs

Method	Modification of full-length cDNA clone inserts	Use of transgenic BACs
Availability of rescue constructs	Limited number of full-length ORF cDNAs	High Bac coverage of the mouse/human genome
Expression level	Dependent on artificial promoter Often not physiological	Dependent on natural promoter Physiological range
Transcriptional regulation	No	Yes
Alternative splicing	No	Yes

that should be useful for protein localization studies, structure/function analyses, and the purification of protein complexes.

Methods

BAC Engineering. The BACs RP23-285E19 [harboring mouse *SNW1* (*mSNW1*)], RP24-181C3A [harboring mouse *DNAJA3* (*mDNAJA3*)], and RP24-351L1 [harboring mouse *SPD2* (*mSPD2*)] were obtained from the BACPAC Resources Center (<http://bacpac.chori.org>). Neo/Kan^r-dsRed and EGFP-IRES-Neo cassettes were PCR amplified with primers carrying 50 nucleotides of homology to the targeting sequence. Recombinering of the BACs was performed as described (18) (Gene Bridges, Dresden, Germany).

BAC Transfection. HeLa cells were seeded 16 h before transfection into 6-cm dishes with a density of 700,000 cells per well in 5 ml of medium (DMEM/10% FBS/2 mM glutamine/100 units/ml penicillin/100 μ g/ml streptomycin). Transfection was performed with Effectene (Qiagen, Valencia, CA) by using 1 μ g of supercoiled BAC DNA, purified with the large-construct kit (Qiagen). The cells were transferred 24 h later on 10-cm dishes and cultivated in selection medium containing 750 μ g/ml geneticin (GIBCO).

Detection of *DNAJA3* and *SNW1* Expression. RNA was extracted from transgenic HeLa cells by using the RNeasy Mini Kit (Qiagen). cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen), and expression of the transgenes was detected by PCR by using the primers 5'-AGTCACCCACA-CAAGCACTG-3'/5'-AAGCTGTAAGCCGGGTCTTT-3' (*DNAJA3*) and 5'-TGACCAAAGGCTCTTCAACC-3'/5'-CTGGACAAGGACATGTATGGTG-3' (*SNW1*). The *SNW1* PCR product was digested with *Sfa*NI (New England Biolabs), and products were separated on a 3% agarose gel.

Endoribonuclease-Prepared siRNA (esiRNA) Synthesis. We generated esiRNA against a 3' UTR fragment from human *SNW1* (*hSNW1*) tagged with T7 promoter sequences on both sides (Fig. 5, which is published as supporting information on the PNAS

web site). The template for *in vitro* transcription of firefly luciferase was generated as described elsewhere (19). EsiRNA was synthesized as described (20) with a modified purification procedure (21).

Quantification of *hSNW1* Knockdown. WT HeLa cells and cells expressing *mSNW1* were seeded 16 h before transfection into 12-well plates with a density of 20,000 cells per well in 1 ml of medium (DMEM/10% FBS/2 mM glutamine/100 units/ml penicillin/100 μ g/ml streptomycin). Transfection was performed with Oligofectamine (Invitrogen) by using 0.5 μ g of esiRNA targeting *hSNW1* or firefly luciferase. Cells were harvested 48 h after transfection, and RNA extraction and cDNA synthesis were performed as described above.

hSNW1 mRNA expression was quantified by quantitative PCR by using the Brilliant SYBR Green system and the Mx4000 Multiplex Quantitative PCR system (Stratagene), by using the primers 5'-TCCTAATCCTCGACTTCCA-3'/5'-GGGC-CATATCTTTACCACCTC-3'. Expression levels of *hSNW1* in cells transfected with *hSNW1*-specific esiRNA were normalized against the expression level of cells transfected with esiRNA targeting firefly luciferase.

Rescue Experiment. WT HeLa cells and cells expressing *mSNW1* or *mSPD2* were seeded 16 h before transfection into 96-well plates with a density of 2,000 cells per well in 100 μ l of medium. Transfections were performed by using 50 ng of esiRNA (*hSNW1*), or 40 nM siRNA (*hSPD2*) and Oligofectamine (Invitrogen). The sequences of the *hSPD2* siRNA (Ambion, Austin, TX) are 5'GGAAGACAUUUUCAUCUCUtt-3' and 5'AGAGAUGAAAUGUCUUCtt-3'. Because of three mismatches to this sequence, the mouse transcript is not significantly silenced by this siRNA.

Cell viability was measured 96 h after transfection by using the WST-1 assay (Roche Diagnostics). Mitotic cells were counted 36 h posttransfection.

For visualization of the mitotic spindles, HeLa cells grown on coverslips were transfected as described above. Forty-eight hours after transfection, the cells were fixed and permeabilized in

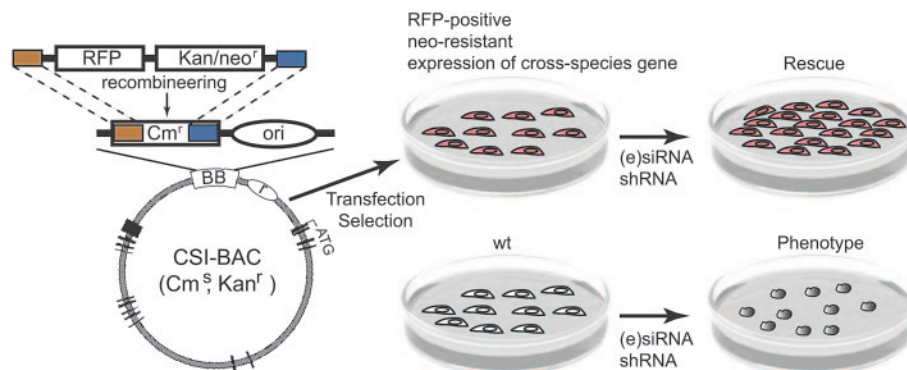


Fig. 1. Experimental strategy of RNAi rescue by BAC transgenesis. RFP, red fluorescent protein; Kan/neo^r, kanamycin/neomycin resistance gene; Cm^r, chloramphenicol resistance gene; ori, origin of replication; BB, BAC backbone; CSI-BAC, cross-species RNAi rescue-BAC.

methanol at -20°C for 8 min. Cells were washed with PBS and incubated 10 min in PBS containing 0.2% fish skin gelatin (Sigma). Cells were incubated for 20 min with a mouse monoclonal antibody against tubulin (DM1, Sigma) either directly labeled with FITC or using a donkey anti-mouse antibody labeled with Texas red and mounted in the presence of DAPI ($1\ \mu\text{g}\cdot\text{ml}^{-1}$) to visualize chromatin. Three-dimensional data sets were acquired on a DeltaVision imaging system (Applied Precision, Issaquah, WA) equipped with an Olympus (Melville, NY) IX70 microscope. Images were computationally deconvolved by using the SOFTWORX software package and shown as two-dimensional projections.

Results and Discussion

Generation of Transgenic Human Cell Lines. The mouse genome-sequencing project has produced overlapping large genomic constructs, including libraries of BACs. These constructs are typically larger than 100 kb, and most genes are available on a single BAC. Therefore, these constructs are well suited to express genes within their physiologically genetic “environment.” The proportion of human genes without any homologue currently detectable in the mouse genome is $<1\%$ (22). Therefore, the described methodology should be generally applicable to almost all human genes. Based on the BAC coverage and gene size in mouse, we estimate that BACs containing the genomic sequence of a gene and 20 kb of upstream sequence should be available for $>90\%$ of all genes. These BACs are available through several public resources and can be rapidly identified with open-access databases.

To allow rapid assessment of transfection efficiency and selection for stable integration of the mouse BAC in human cells, we designed a universal cassette carrying a neomycin/kanamycin selection marker and a red fluorescent protein (RFP) marker that carries homologous sequences to replace the chloramphenicol cassette on the BAC backbone by recombineering (14, 15). The incorporation of this cassette replaces the internal chloramphenicol resistance gene, thereby allowing a simple selection scheme to obtain successfully modified BACs, monitoring kanamycin resistance and chloramphenicol sensitivity (Fig. 1). All positive colonies analyzed had integrated the cassette at the intended locus, demonstrating the efficiency and simplicity of this approach. By using the universal cassette, the BAC modification can be streamlined and performed within 2 days, allowing rapid processing of many different BACs.

The large size of BAC constructs makes them more difficult to transfect into mammalian cells than cDNA constructs. To monitor and optimize the transfection efficiency, we included the RFP gene in the replacement cassette. Based on the expression of RFP monitored 48 h after transfection, we obtained an average transfection efficiency of $\approx 2\%$ with standard lipofection reagents, which was sufficient to obtain many clones that stably express the transgene (Fig. 6, which is published as supporting information on the PNAS web site).

We modified two BACs carrying the mouse orthologues of the two human genes *SNW1* and *DNAJA3*. *SNW1* is a transcription coactivator and pre-mRNA splicing factor (23) that we identified recently to be essential for cell division in human cells by a large scale RNAi screen (21). We showed that RNAi-mediated depletion of *SNW1* in HeLa cells resulted in spindle and cytokinesis defects, suggesting a link between splicing and cell division. To test the specificity of this provocative knockdown phenotype, we generated a BAC transgenic cell line that expresses the mouse *SNW1* gene. To test for alternative splicing of a murine gene in human cells, we chose the mouse orthologue of the human gene *DNAJA3*, a heat shock protein for which alternatively spliced transcripts have been reported (24).

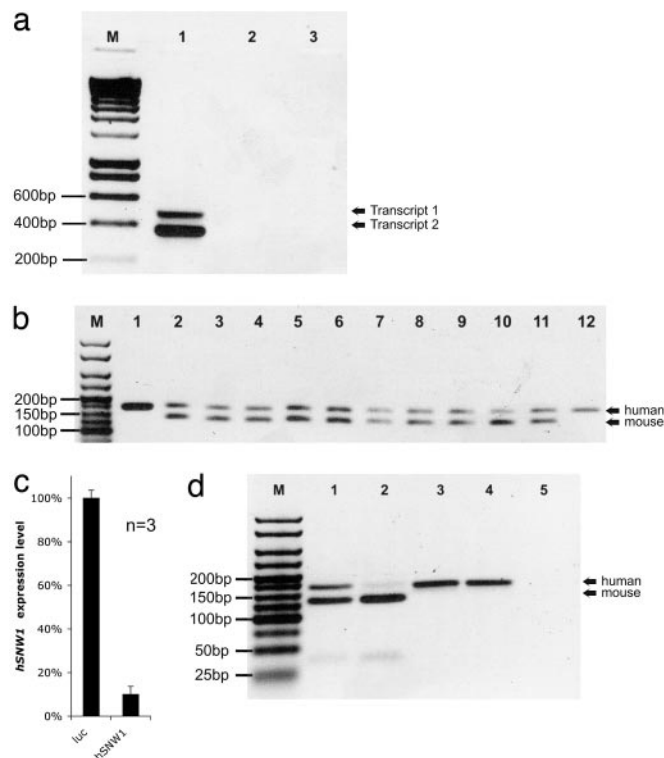


Fig. 2. Expression and RNAi resistance of mouse transgenes. (a) Detection of expression and alternative splicing of *mDNAJA3* by RT-PCR. Lane 1, the two bands (495-bp and 377-bp) amplified from cDNA of HeLa cells transfected and selected for a BAC carrying *mDNAJA3* representing the two splice isoforms; lane 2, negative control (cDNA from WT HeLa cells); lane 3, non-template control. M, marker. (b) Expression levels of *hSNW1* and *mSNW1* in transgenic cells. The comparison of the band intensities indicates relative expression levels of *hSNW1* and *mSNW1*. Lane 1, undigested 161-bp fragment from the same clone depicted in lane 2; lanes 2–11, *Sfa*NI digestion products of a 161-bp fragment amplified from cDNA of 10 clones from HeLa cells transfected and selected for a BAC carrying *mSNW1* (the upper band represents the uncut human-specific fragment, the lower band the mouse-specific fragment); lane 12, digested fragment generated from cDNA of WT HeLa cells. M, marker. (c) Knockdown of *hSNW1* in transgenic HeLa cells. *hSNW1* expression after transfection of esiRNAs targeting *hSNW1* and firefly luciferase. *hSNW1* mRNA expression was quantified 2 days after transfection. Expression levels were normalized against *hSNW1* expression of cells transfected with esiRNA targeting firefly luciferase. (d) Expression levels of *hSNW1* and *mSNW1* in transgenic HeLa cells. Lanes 1 and 2, *Sfa*NI digestion products of the 161-bp fragment amplified from cDNA of transgenic HeLa cells transfected with esiRNA targeting firefly luciferase (lane 1) and *hSNW1* (lane 2) (note the change of relative band intensities indicating the specific knockdown of *hSNW1*); lanes 3 and 4, undigested 161-bp fragment amplified from cDNA of transgenic HeLa cells transfected with esiRNA targeting firefly luciferase (lane 3) and *hSNW1* (lane 4); lane 5, non-template control. M, marker.

Alternative Splicing and Physiological Expression of Murine Transgenes in Human Cells. To test whether alternative splicing of the murine *DNAJA3* gene (*mDNAJA3*) would take place in human HeLa cells, we designed a primer pair specific for two isoforms, which should generate two PCR fragments of different length. RT-PCR using cDNA derived from HeLa cells that have stably integrated a BAC carrying *mDNAJA3* revealed the expression of two products with the expected length for both splice isoforms, indicating successful alternative splicing of murine genes in human cells (Fig. 2a).

To analyze and compare the expression levels of an endogenous human gene with the mouse transgene in human cells, we developed a PCR-based assay for *SNW1*. We chose a single primer pair that perfectly matches to both human (*hSNW1*) and

schemes have also begun providing insights into the localization of the mammalian proteome (35). In these studies, cDNA constructs, whose expression is typically driven by viral promoters, are used, and, as a consequence, the GFP-tagged genes are typically expressed at nonphysiological levels, which can lead to phenotypes and mislocalization (36–38). Furthermore, the expression of cDNAs precludes the visualization of alternatively spliced variants, which may differ in their subcellular localization. However, for the reasons mentioned earlier, the use of modified BACs may overcome these limitations (Fig. 4a).

To test whether BAC-GFP tagging can be combined with cross-species RNAi rescue and thereby allow functional expression of the tagged protein in the absence of the endogenous protein, we inserted a GFP tag at the C terminus of the mouse homologue of the *C. elegans* SPD2 protein (39). Consistent with the localization pattern of SPD2 in *C. elegans* and its role in spindle assembly in the early embryo (39, 40), the human homologue of SPD2 (*hSPD2*) also localizes to the centrosome (41).

As was observed for the *mSNW1* clones, most *mSPD2* clones expressed the mouse transgene at equivalent levels to that of the endogenous human gene, and transfection of human-specific siRNA resulted in predominant silencing of the human transcript (data not shown). Immunofluorescence analysis of *mSPD2* transgenic lines revealed that SPD2-GFP fluorescence was re-

stricted to the mitotic spindle poles, suggesting that the tagging does not interfere with the subcellular localization of SPD2 (Fig. 4b). We next wanted to determine whether the murine SPD2-GFP BAC was able to complement functionally the human SPD2 gene. Consistent with its role in spindle assembly in *C. elegans*, the depletion of SPD2 in HeLa cells by RNAi leads to an increase in the mitotic index of cells from $\approx 5\%$ in control transfected cells (data not shown) to $\approx 25\%$ in cells treated with siRNA against SPD2 (Fig. 4c). In contrast, in cells expressing the *mSPD2-GFP* transgene, the mitotic index dropped considerably toward WT levels (Fig. 4c). These results demonstrate that tagging by BAC recombination can be combined with RNAi to mimic homologous recombination in mammalian tissue culture cells. In particular the ability to introduce tagged transgenes on their own promoter, and to remove the endogenous gene function, heralds the era of mammalian tissue culture cell genetics.

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