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Knockout mouse production assisted by *Blm* knockdown

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Abstract. Production of knockout mice using targeted embryonic stem cells (ESCs) is a powerful approach for investigating the function of specific genes *in vivo*. Although the protocol for gene targeting via homologous recombination (HR) in ESCs is already well established, the targeting efficiency varies at different target loci and is sometimes too low. It is known that knockdown of the Bloom syndrome gene, *BLM*, enhances HR-mediated gene targeting efficiencies in various cell lines. However, it has not yet been investigated whether this approach in ESCs is applicable for successful knockout mouse production. Therefore, we attempted to answer this question. Consistent with previous reports, *Blm* knockdown enhanced gene targeting efficiencies for three gene loci that we examined by 2.3–4.1-fold. Furthermore, the targeted ESC clones generated good chimeras and were successful in germline transmission. These data suggest that *Blm* knockdown provides a general benefit for efficient ESC-based and HR-mediated knockout mouse production.
Key words: *Blm*, Embryonic stem cells (ESCs), Gene targeting

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To elucidate roles of any particular gene or genetic element in higher-order biological processes, a general method(s) for genome editing and creation of such genome-modified species is essential. Therefore, the approach of genome editing in mouse embryonic stem cells (ESCs) and ESC-based genome-modified animal production is commonly used. For gene targeting via homologous recombination (HR), a targeting vector possessing a drug-resistant gene plus 5' and 3' homology sequence arms is introduced into the cells. Although there is a well-established general protocol and many genes are already targeted by this method in ESCs, the targeting efficiency varies at different target loci and sometimes is too low.

Recently, other genome editing technologies such as zinc finger nuclease (ZFN) [1, 2], TAL effector nuclease (TALEN) [3, 4] and CRISPR/Cas9 [5, 6] systems have been developed. If specific and highly competent nuclease can be designed, all these systems would work well for gene targeting in mammals [7, 8]. The CRISPR/Cas9 system is the newest of the systems, but it is extremely useful. Unlike the ZFN and TALEN systems, the CRISPR/Cas9 system uses RNA as a guide molecule and a 20-nt RNA sequence specifies the target DNA site. Therefore, preparation of specific targeting materials is much easier and simpler. Furthermore, if Cas9 mRNA is delivered along with guide RNA into mouse zygotes, genome-edited mice can be easily obtained. However, even for the CRISPR/Cas9 system, some technical challenges still exist. An obvious one is the off-target mutagenesis risk due to the 20-nt sequence restriction of the target specificity. Furthermore, because homology-directed repair (HDR) is less efficient in mammals, targeted gene replacement or insertion mediated by HDR is inefficient and the zygote injection method with Cas9 RNA, guide RNA and a targeting template construct is generally not practical for creating such genome-edited mice.

For genome editing using a standard targeting vector, various trials have been applied to improve gene targeting frequencies by HR [9]. Among them, knockdown of the Bloom syndrome gene, *BLM*, has been shown to enhance gene targeting efficiencies in various human cell lines [10]. *BLM* encodes RecQ type DNA helicase [11] and plays a role in the suppression of HR [12]. However, it has not yet been investigated whether this approach in ESCs is applicable for knockout mouse production. Therefore, in this study, we targeted multiple different gene loci with or without *Blm* knockdown in ESCs and used the targeted ESC clones obtained with *Blm* knockdown for chimeric mouse production and germline transmission.

For *Blm* knockdown in ESCs, we designed three different *Blm* siRNAs (siBlm1-3). At 48 h post transfection, the amount of *Blm* mRNA was significantly decreased in ESCs transfected with all independent *Blm* siRNAs (Fig. 1a). Western blot analysis also showed significant reduction of the Blm protein amount specifically by *Blm* siRNA treatment (Fig. 1b). Among them, siBlm-2 and siBlm-3 induced higher knockdown efficiency than siBlm-1. Therefore, we selected siBlm-2 and siBlm-3 and combined them for further *Blm* knockdown experiments (Fig. 1c).

To validate how *Blm* knockdown affects gene targeting efficiency in ESCs, we targeted three different gene loci, namely, *Prdm5* on

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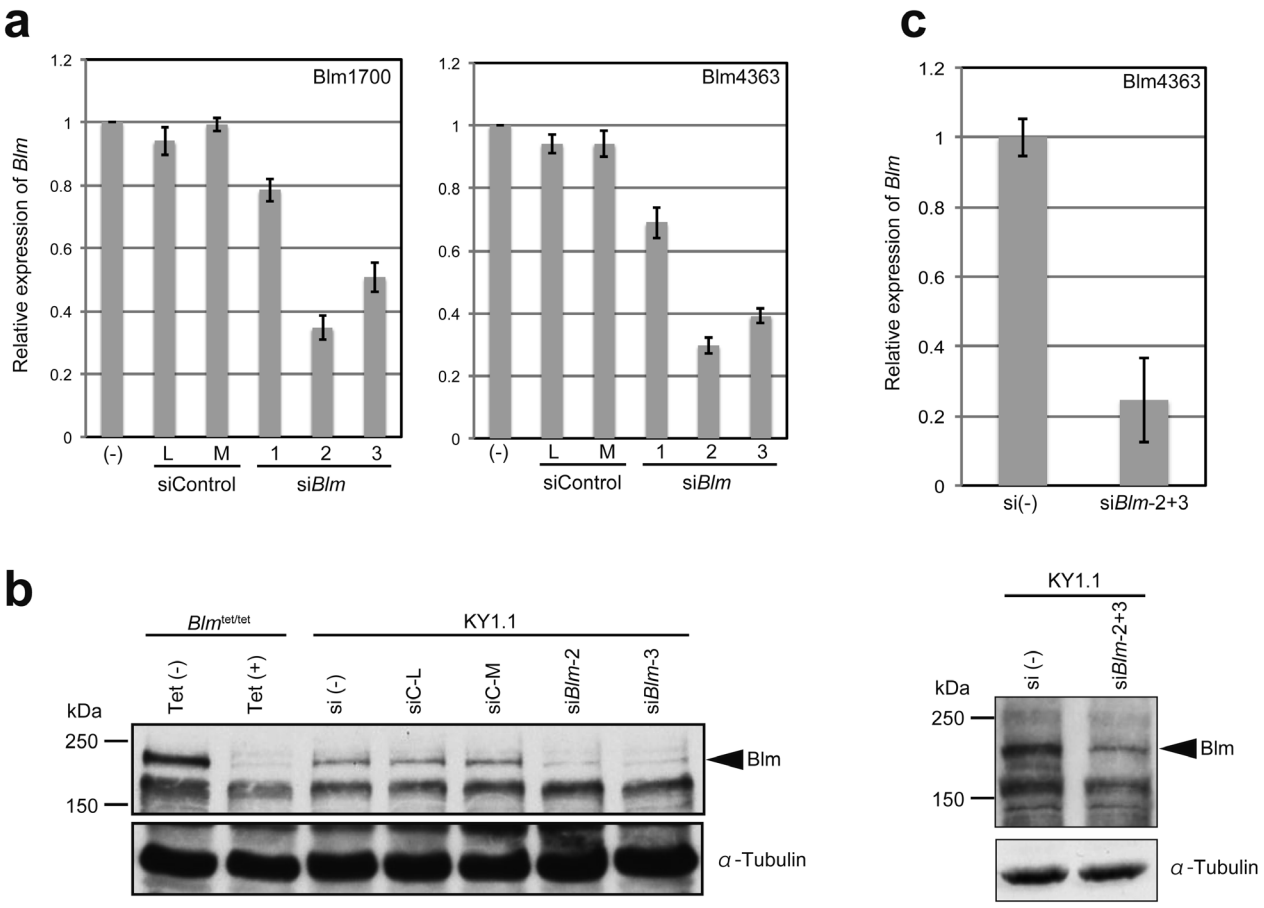

Fig. 1. *Blm* was knocked down by siRNAs. a) 20 nM of each *Blm* siRNA was transfected into KY1.1. At 48 h after transfection, the *Blm* mRNA level was measured by quantitative RT-PCR using two primer sets. b) The expression level of Blm protein was determined by western blot. Blm is indicated by an arrowhead. As a control for *Blm* knockdown, the *Blm* conditional knockdown ESC line, $Blm^{tet/tet}$ [14], was used. Tubulin was used as an internal control for protein content. Si (–), no siRNA; siC-L, siRNA Negative Control Low Duplex; siC-M, Medium Duplex; Tet, Tetracycline c. *Blm* was knocked down by a mixture of siBlm-2 and siBlm-3 in KY1.1. This protocol was used for the gene targeting experiments. *Blm* expression was determined by quantitative RT-PCR (upper panel) and western blot (lower panel). All data are presented as the mean ± SE.

chromosome 6, *Prdm8* on chromosome 5 and, *Arl14ep* on chromosome 2, with or without *Blm* siRNAs pretreatment. *Prdm5* and *Arl14ep* are expressed but *Prdm8* is silent in ESCs (not shown). We used standard gene targeting vectors for these three genes (Fig. 2a) [13]. Forty-eight hours before transfection of the targeting vectors, one part of the cells was transfected with *Blm* siRNAs in the condition shown in Fig. 1c. Then, the ESCs transfected with each targeting vector were selected with G418. More than 200 drug-resistant colonies per transfection were screened for proper gene targeting. As summarized in Table 1, the targeting efficiencies of *Prdm5*, *Prdm8* and *Arl14ep* were 8/214 (3.7%), 8/796 (1.0%) and 14/240 (5.8%) for ESCs without *Blm* knockdown, respectively. For the ones with *Blm* knockdown, the targeting efficiency was 29/232 (12.5%) for *Prdm5*, 15/363 (4.1%) for *Prdm8* and 32/240 (13.3%) for *Arl14ep*. Thus, pretreatment with the *Blm* siRNAs pre-treatment enhanced the targeting efficiency for all three gene loci, and the fold activation enrichment was 3.4 for *Prdm5*, 4.1 for *Prdm8* and 2.3 for *Arl14ep*. In another experiment for *Arl14ep* gene targeting, we screened cells transfected with control siRNA (siC-L) in addition to cells treated with *Blm* siRNAs (Table 2). This time, the targeting efficiencies were generally low but treatment with the *Blm* siRNAs gave higher fold activation enrichment than that with siC-L (2.6 for *Blm* siRNAs and 1.4 for siC-L) suggesting that the effect of *Blm* siRNAs is not non-specific.

Then, we examined whether the targeted ESC clones obtained with *Blm* siRNA pretreatment maintained pluripotency, especially for the germline transmission potential. Since sister chromatid exchanges (SCEs) are highly increased in *Blm* knockout or knockdown cells [14–19], we first checked chromosome stability. As shown in Table 3, we performed a karyotype analysis of three targeted ESC clones for *Arl14ep* (#12, #13 and #27) with *Blm* knockdown and a parental ESC, KY1.1, as a control. For all targeted clones examined, the average chromosome number per cell was not changed and remained at ~40.

Finally, we created chimeric mice using the targeted ESC clones obtained with *Blm* knockdown for the three genes, *Prdm5, Prdm8* and *Arl14ep*. As summarized in Table 4, 2/3 to 4/4 of them generated > 80% chimeric mice as judged by the coat color contribution. Furthermore, germline transmission of the targeted allele was confirmed for all

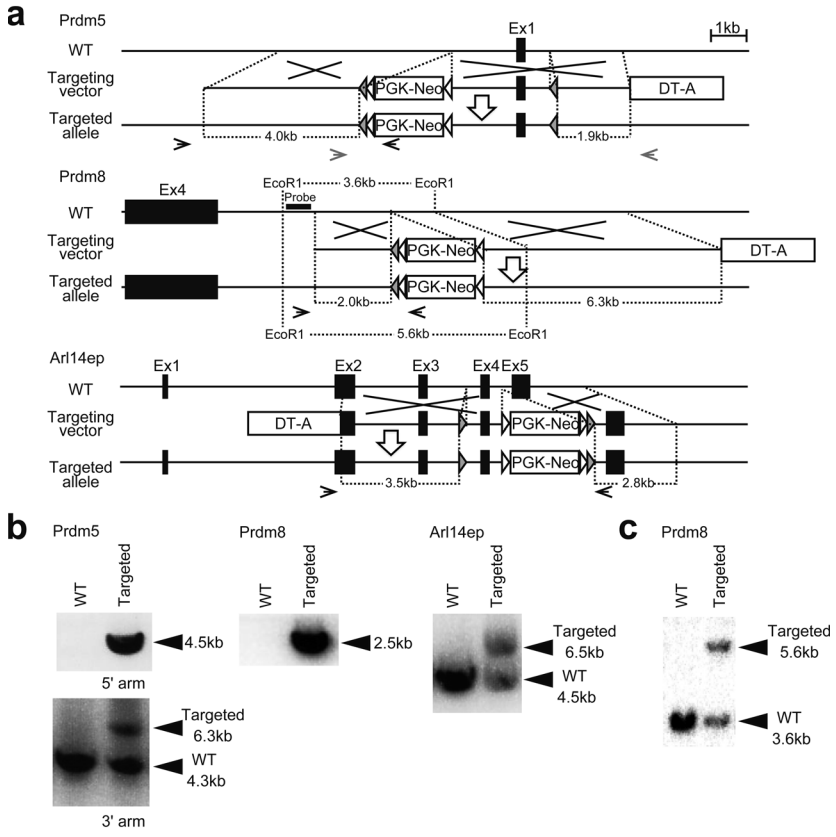

Fig. 2. Schematic diagram for gene targeting. a) *Prdm5* targeting: lox P (shaded triangle)-frt (open triangle)-PGK-Neo-frt and another lox P site were introduced into upstream and downstream of exon (Ex) 1, respectively. *Prdim8* targeting: lox P-frt-PGK-Neo-frt was introduced downstream of exon 4. *Arl14ep* targeting: lox P site and frt-PGK-Neo-frt-lox P were introduced upstream and downstream of exon 4, respectively. Arrows described below indicate the targeted allele or above the wild type allele indicate primers used for the screening of correctly targeted clones. For knockout of *Prdm8*, the *Prdm8* targeting vector was transfected into the ESC clone already possessing another lox P site in exon 2 of *Prdm8*. b) Genotyping of a correctly targeted clone by PCR. c) Genotyping of *Prdm8* targeted clone by Southern blot.

Table 1. Gene targeting efficiency with or without *Blm* knockdown

Targeted gene	Used ES cell line (genetic background) / genetic origin of homology arms of the targeting vector	Bloom siRNA	The number of colonies		Targeting efficiency (%)	Fold activation enrichment (+/–)
			Screened	Targeted		
*Prdm5**	KY1.1 (B6 × 129F1) / B6	–	214	8	3.7	3.4
		+	232	29	12.5	
*Prdm8***	M1 (B6 × 129F1) / B6	–	796	8	1.0	4.1
		+	363	15	4.1	
*Arl14ep****	KY1.1 (B6 × 129F1) / B6	–	240	14	5.8	2.3
		+	240	32	13.3	

* Screened by PCR for the expected 5' arm and 3' arm recombinations. ** Screened by PCR or Southern blot for the expected 5' arm recombination.
*** Screened by PCR for the expected 5' arm recombination.

Table 2. Influence of siRNA on gene targeting

siRNA	The number of colonies		Targeting efficiency (%)	Fold activation enrichment (+/–)
	Screened	Targeted		
-	212	3	1.4	
Control	201	4	2.0	1.4
Bloom	218	8	3.7	2.6

Arl14ep was targeted.

Table 3. Karyotype analysis of the established targeted ESC lines with *Blm* knockdown

Cell		Average chromosome number (n = 12)
KY1.1		39.8 ± 0.11
Arl14ep targeted	#12	39.8 ± 0.16
	#13	39.8 ± 0.11
	#27	39.8 ± 0.13

Table 4. Production of chimeric mice and germline transmission by the targeted ESC lines with *Blm* knockdown

	ID number of injected clones	Number of chimeric mice generated					Number of clones with germline transmitted / examined for germline transmission
		100%*	> 60%	>30%	0%	Unknown	
Prdm5	#3	1	0	0	0	0	2/3
	#10	0	0	0	0	0	
	#17	3	0	0	0	0	
	#31	9	4	1	0	2	
Prdm8	#11	11	0	0	0	1	2/2
	#28	4	0	0	0	4	
	#118	2	6	1	5	0	
	#443	13	1	0	0	3	
Arl14ep	#6	4	0	1	1	0	2/2
	#27	0	3	2	0	0	
	#28	0	0	0	0	0	

For each clone, 70–100 cells were injected into 60–80 blastocysts. They were transferred into 3 pseudopregnant mice.
* Percentage of chimerism judged by coat color.

three gene loci from those good chimeric mice (more than two lines for each gene). Therefore, we concluded that *Blm* siRNA pretreatment does not have clear negative effects on chromosomal stability or germline transmission potential of the obtained targeted ESC clones.

In conclusion, *Blm* knockdown provides a general benefit for efficient ESC-based and HR-mediated knockout mouse production.

Methods

Targeting vector construction

Targeting vectors *for Prdm5, Prdm8 and Arl14ep* were constructed using a BAC recombineering system (kindly provided by Dr Neal G Copeland) [13, 20].

Mouse ESC lines

ESC lines M1 and KY1.1 (B6 and 129 F1 hybrids) were used for the gene targeting experiments. M1 was obtained from Dr. Haruhiko Koseki and KY1.1 and *Blm*$^{tet/tet}$ ESC lines [16] were obtained from Dr. Junji Takeda. They were cultured in D-MEM (D6429, Sigma Aldrich, MO, USA) containing 15% FCS (for M1) or 15% KnockOut Serum Replacement (KSR) (Gibco, NY, USA) (for KY1.1), MEM Non-Essential Amino Acids (Gibco), 100 μM 2-mercaptoethanol and 10^3 U/ml ESGRO (Merck-Millipore, MA, USA) on feeder cells.

Blm knockdown

Stealth RNAi^{TM} siRNA for *Blm* was designed using BLOCK-iT^{TM} RNAi Designer (Invitrogen, CA, USA). The three selected candidate sequences for *Blm* siRNA were as follows: GCUUCGCCAGAAGUUUCCUUCUGUU, sense, and AACAGAAGGAAACUUCUGGCGAAGC, antisence, for siBlm-1; CCUCAGGUGUUUAGCAUGAGCUUUA, sense, and UAAAGCUCAUGCUAAACACCUGAGG, antisence, for siBlm-2; and CCAGACUGAAGAGACUUAUAAUGAU, sense, and AUCAUUAUAAGUCUCUUCAGUCUGG, antisence, for siBlm-3. Stealth RNAi^{TM} siRNA Negative Control Low GC Duplex #2 (catalog no. 12935-110) (siC-L) and Medium GC Duplex (catalog no. 45-2001) (siC-M) (Invitrogen) were used as negative controls. For the initial validation experiment, individual siRNAs were transfected into 2×10^5 ESCs with 5 μl of Lipofectamine RNAiMAX Reagents (Invitrogen) in one well of a 6 well plate. The final concentration of siRNA was 20 nM in 2.5 ml of medium. The medium was changed the next day, and the cells were harvested to analyze the knockdown efficiency at 48 h post transfection. For the gene targeting experiment, the mixture of siBlm-2 and siBlm-3 or siC-L was transfected into $0.8–1.0 \times 10^6$ ESCs with 30 μl Lipofectamine RNAiMAX Reagent in a 10-cm dish. The final concentration of each siRNA was 10 nM in 15 ml of medium. Transfection efficiency of siRNA oligo into mouse ES cells was validated by transfection with BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (Invitrogen) and it was > 95%.

ES cell targeting and mouse generation

At 48 h after Blm KD, the ESCs were harvested. Ten micrograms of the targeting vector was linearized and transfected into $1–2 \times 10^7$ ESCs by electroporation. The next day, drug selection with G418 (0.3 mg/ml; Nacalai Tesque, Kyoto, Japan) was initiated. More than 200 G418-resistant colonies were analyzed for each transfection, and the targeted ESC lines were injected into 8-cell stage embryos to create chimeric mice. Chimeric mice with ESC contributions of more than 80% were used for the germline transmission experiment.

Screening for gene targeting

Genomic DNAs were isolated by the high salt preparation method [21] and used for the PCR and Southern blot analysis. The following primer sets and Taq polymerases were used for PCR screening: 5'-TCCCAGCCTGACCTATCATT-3' (forward) and 5'-CGCATCGCCTTCTATCGCCTTCTTGACGAG-3' (reverse ; POL2) primer set with KOD FX Neo (Toyobo Life Science) for targeting of the 5' arm of *Prdm5*, 5'-TGAACCTGTGAGCCAAAACA-3' (forward) and 5'-TGACTTACCATCAGCCGCCAG-3' (reverse) primer set with KOD FX Neo for targeting of the 3' arm of *Prdm5*, 5'-GATGGGTCCTGCGTAGGATCTCT-3' (forward) and 5'-CGCATCGCCTTCTATCGCCTTCTTGACGAG-3' (reverse ; POL2) primer set with TaKaRa EX Taq (Takara Bio) for targeting of *Prdm8* and, 5'-TGGATCCGTGTTCAGTTGG-3' (forward) and 5'-AGGTCAATTCAGAGCTGCAT-3' (reverse) primer set with KOD FX Neo for targeting of *Arl14ep*. The expected PCR product and size

for the wild type (WT) and targeted allele of them are shown in Fig. 2b. Some *Prdm8* targeting was also screened by Southern blot. Genomic DNAs were digested by EcoR1, separated in TAE gel, blotted on nylon membrane and hybridized with the probe indicated in Fig.2a.

Quantitative RT-PCR analysis

Total RNAs were purified by Sepasol-RNAISuper G (Nacalai Tesque). cDNAs were synthesized with an Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) and the expression level of *Blm* mRNA was measured with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and a StepOnePlus system using the following two primer sets: 5'-CTGTGGGGCATCCTAATAAAG-3'(forward) and 5'-AGTGGTGGTGGGTAAACATTCC-3' (reverse) for Blm1700 and 5'-AATGTCAGCCACCCATAAGC-3' (forward) and 5'-TGATGTTGCCTGAGAAGCAC-3' (reverse) for Blm4363. The obtained data were analyzed by the ΔΔ-CT method using StepOne Software 2.1 (Applied Biosystems). Gapdh (5'-CATCTTCTTGTGCAGTGCCA-3' (forward) and 5'-CGTTGATGGCAACAATCTCC-3' (reverse)) was used as an internal control.

Western blot analysis

Cells were harvested, washed with PBS and lysed with lysis buffer containing 420 mM NaCl, 20 mM HEPES-KOH (pH 7.5), 1.5 mM $MgCl_2$, 0.1% NP40 and protease inhibitors (Nacalai Tesque). They were kept on ice for 30 min and centrifuged for 15,000 rpm for 10 min. The supernatants were collected, and 30 μg was separated by electrophoresis and transferred to a nitrocellulose membrane (Pall). After blocking with 5% skim milk-TBST for 1 h, anti-BLM antibody (A300-570A, Bethyl Laboratories, TX, USA) and anti-α-Tubulin antibody (T5168, Sigma-Aldrich) were used as primary antibodies and ECL Anti-Mouse IgG HRP Antibody (NA931, GE Healthcare, Buckinghamshre, UK) and ECL Anti-Rabbit IgG HRP Antibody (NA931, GE Healthcare) were used as secondary antibodies. Western Lightning Plus ECL (PerkinElmer, MA, USA) was used to detect signals.

Karyotype analysis

Cells were treated with 0.1 μg/ml KaryoMAX Colcemid Solution in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) for 1 h, harvested and washed twice with PBS. They were suspended in ice-cold 0.075 M KCl solution, incubated at RT for 10 min and centrifuged. Ice-cold Carnoy's fluid was added slowly and gently into the pellets, which were kept at –20°C for more than 20 min. They were then centrifuged, resuspended in the Carnoy's fluid and dropped onto slide glasses. After drying, the samples were embedded with VECTASHIELD (Vector Laboratories) containing 10 μg/ml of DAPI and the total number of mitotic chromosomes per cell was counted by fluorescence microscope analysis.

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