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Site-specific modification of genome with cell-permeable Cre fusion protein in preimplantation mouse embryo

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ABSTRACT

Site-specific recombination (SSR) by Cre recombinase and its target sequence, *lox*P, is a valuable tool in genetic analysis of gene function. Recently, several studies reported successful application of Cre fusion protein containing protein transduction peptide for inducing gene modification in various mammalian cells including ES cell as well as in the whole animal. In this study, we show that a short incubation of preimplantation mouse embryos with purified cell-permeable Cre fusion protein results in efficient SSR. X-Gal staining of preimplantation embryos, heterozygous for *Gtrosa26*^{tm1Sor}, revealed that treatment of 1-cell or 2-cell embryos with 3 μM of Cre fusion protein for 2 h leads to Cre-mediated excision in 70–85% of embryos. We have examined the effect of the concentration of the Cre fusion protein and the duration of the treatment on embryonic development, established a condition for full term development and survival to adulthood, and demonstrated the germ line transmission of excised *Gtrosa26* allele. Potential applications and advantages of the highly efficient technique described here are discussed.

Introduction

The Cre recombinase derived from bacteriophage P1 induces SSR between two 34-bp *lox*P sites [1]. The Cre/*lox*P recombination system has played a key role in manipulating mouse genome and contributed extensively to the analysis of mammalian gene function *in vivo* [2]. Thus far, transient expression of Cre recombinase in embryonic stem (ES) cells has been the mainstay in removing selection markers, and inserting or inversing specific genes [2]. For understanding specific gene function in development and in diseases, various strategies for elaborating expression patterns of Cre *in vivo* have been developed. This has led to a continuous accumulation of transgenic mice lines that express Cre in a spatiotemporally specific manner and to the development of techniques to induce Cre expression or control Cre activity [2,3].

Manipulating genome in early developing mouse embryos is advantageous, since the embryo can differentiate into all of the specialized embryonic tissues including germ cells. Direct expression of Cre recombinase in mouse 1-cell embryo can be achieved by the microinjection of plasmid into pronucleus or of *in vitro* transcribed mRNA into cytoplasm [4–6]. However, technical difficulties of microinjection have limited the broad use of Cre/*lox*P system in preimplantation embryos. Recently, genomic manipulation was successfully performed both in cultured cells and in the whole mouse using recombinant Cre recombinase, modified with 6xHis-tagging for purification and with additional motifs to promote cellular uptake and nuclear localization [7–9]. Importantly, demonstration of germ line competency of mouse ES cells treated with recombinant Cre recombinase broadened the potential applications of Cre/*lox*P in manipulating the mouse genome [10]. The essence of this strategy is the use of the protein transduction domain (PTD) which mediates the transport of macromolecules across the cell membrane without any specific receptor [11,12].

In the present report, we develop a method that induces efficient SSR in mouse genome by simple incubation of 1-cell or 2-cell stage embryos with purified recombinant Cre protein. Furthermore, we show that Cre-treated eggs were able to develop to term and germ line transmission occurred normally. These results indicate that *in vitro* treatment of preimplantation embryo with cell-permeable Cre is a simple and efficient way of modifying genome in the mouse.

Materials and methods

His-TAT-NLS-tagged Cre (HTNCre) expression vector and purification of HTNCre. pTriEx-HTNC vector [9] was transformed into TUNER(DE3)pLacI *Escherichia coli* strain (Novagen), and induction of HTNCre fusion protein expression was performed as described previously [9]. Bacterial cell pellet was suspended in NPT buffer (100 mM NaH_2PO_4, pH 8, 10 mM Tris–HCl, pH 8, 300 mM NaCl) containing 10 mM imidazole, 2 mg/ml lysozyme, 62.5 U/ml benzonase at 4 ml/g wet weight and incubated on ice for 30 min. After centrifugation for 20 min at 13,000 rpm at 4 °C, 4 ml of cleared lysate was mixed gently with 1 ml of 50% Ni–NTA slurry (Qiagen)

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at 4 °C for 1 h. Mixed slurry was packed into a column and washed twice with five bed volumes of wash buffer (NPT buffer with 20 mM imidazole). Recombinant proteins were eluted with two bed volumes of elution buffer (NPT buffer with 250 mM imidazole, 5% glycerol) and dialyzed against dialyzing buffer (20 mM Hepes, pH 7.4, 500 mM NaCl, 5% glycerol). Aliquots of dialyzed and filtered Cre protein were kept at −20 °C for long term storage. The aliquot was further concentrated with Microcon centrifugal filter devices (Ultracel YM-10, Millipore), and protein concentration was determined by the Bradford assay (Bio-Rad). Molecular weight calculation (Vector NTI) indicates that 1 mg/ml of HTNCre is equivalent to 21.8 μM.

Cre-mediated excision in vitro using purified HTNCre fusion protein. The plasmid, pOneloxP, containing single *lox*P in pBluescript II (Stratagene) was linearized and diluted to 0.1 μg/μl in TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA). Sense and antisense *lox*P oligonucleotides (100 pmol each) were annealed in a total 0.1 ml of annealing buffer (10 mM Tris–HCl, pH 7.5, 100 mM KCl) to bring the final concentration of duplex *lox*P oligonucleotides to 1 pmol/μl, and annealing was confirmed by polyacrylamide gel electrophoresis. To analyze Cre activity *in vitro*, total 10 μl reaction mixtures containing 0.1 μg of linear pOneloxP, 1 pmol of *lox*P, and purified Cre in 1× reaction buffer (50 mM Tris–HCl, pH 7.5, 33 mM NaCl and 10 mM $MgCl_2$) were incubated at 37 °C for the indicated times. After heating at 80 °C for 5 min, the extent of recombination was analyzed by 0.8% agarose gel electrophoresis.

HTNCre-mediated excision in primary mouse embryo fibroblast (PMEF) cells. ROSA26-PMEF cells were isolated from embryos heterozygous for *Gtrosa26*^{tm1Sor} [13] at 16.5-dpc (days postcoitus) as described previously [14] and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Ten thousand ROSA26-PMEF cells were passaged onto 24-well plate 1 day before HTNCre treatment. The cells were washed twice with DMEM and incubated with 0.2 ml of DMEM containing indicated concentration of purified HTNCre at 37 °C in a humidified atmosphere with 5% CO_2. Cre-treated cells were washed twice with culture media and further cultured for 2 days before passaging onto 35 mm dish. The fraction of cells that have undergone recombination was determined by X-Gal staining for *lacZ* activity after 1 day of culture.

Treatment of fertilized eggs with purified HTNCre fusion protein. C57BL/6J female mice at 3 weeks of age were superovulated [14], and subsequently mated with male mice homozygous for *Gtrosa26*^{tm1Sor} to obtain heterozygous embryos. One-cell embryos were collected at 20–22 h post-hCG injection from the oviduct, and removal of cumulus cells was carried out with 0.1% hyaluronidase in PBSP buffer (1× PBS, pH 7.4, 0.1% PVP-40). The 2-cell embryos were collected from oviducts by flushing with M2 medium (Sigma–Aldrich) at 30–32 h post-hCG injection. Embryos were washed several times with KSOM media supplemented with amino acids (KSOM+AA, Chemicon). The embryos were incubated in microdrop of HTNCre diluted in DMEM at 37 °C for indicated times. The embryos were subsequently washed several times with KSOM+AA, and cultured in microdrop of KSOM+AA under mineral oil at 37 °C for 2.5–3.5 days in a humidified atmosphere of 5% CO_2 in air [14] prior to X-Gal staining as described below. Another group of Cre-treated embryos were cultured overnight and transplanted into the oviduct of 0.5-dpc pseudopregnant female.

X-Gal staining. PMEF cells were rinsed three times with PBS and fixed with 0.25% glutaraldehyde in 1× PBS at 4 °C for 10 min. After rinsing in 1× PBS several times, the cells were incubated at 37 °C overnight in X-Gal staining solution (0.04% X-Gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide in 1× PBS, pH 7.4). X-Gal staining of preimplantation embryos was carried out as described previously [15] by incubating the embryos at 37 °C for 3 h in X-Gal staining solution. For postimplantation embryos, embryos were fixed in the fixer solution (0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$ in 1× PBS, pH 7.4) at 4 °C for 30 min and rinsed with detergent-containing solution (2 mM $MgCl_2$, 0.02% Igepal CA630, 0.01% sodium deoxycholate in 1× PBS, pH 7.4) three times for 10 min each. The embryos were incubated at 37 °C for 3 h in X-Gal staining solution containing 0.02% Igepal CA630 and 0.01% sodium deoxycholate.

Genotyping. Yolk sacs from 10.5-dpc embryos or toe clips from 2-week-old pups were used to extract DNA for PCR genotyping. The presence of the *Gtrosa26*^{tm1Sor} allele and the excised *Gtrosa26*^{tm1Sor} allele (named *Gtrosa26*^{lacZ}) was determined by PCR using three primers together: F1295, 5′-ggttgaggacaaactcttcgc-3′; Pgk-As, 5′-ctgactaggggaggagtagaag-3′; lacZ-As, 5′-gctgcaaggcgattaagttgg-3′. The F1295 and Pgk-As primers amplify a 337-bp product specific for

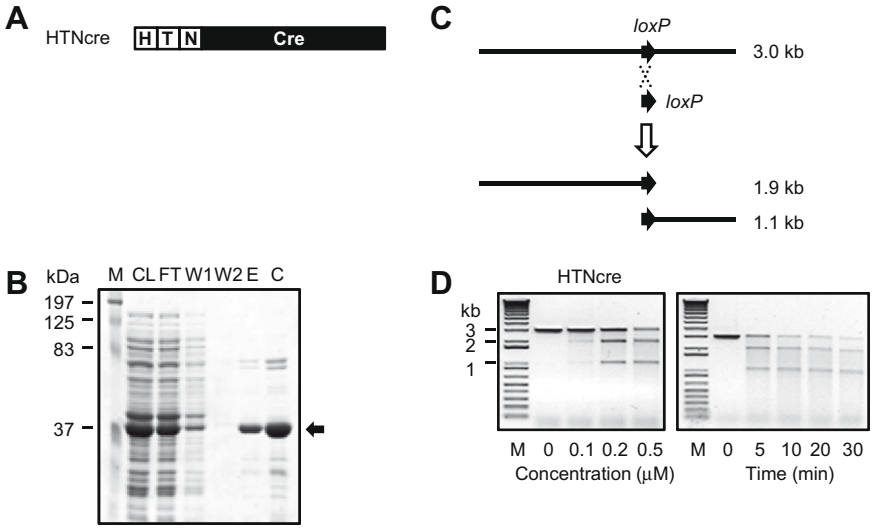

Fig. 1. Purification of Cre fusion protein and analysis of Cre activity *in vitro*. (A) Modular structure of recombinant Cre. H, 6xHis; T, a protein transduction peptide; N, a nuclear localization sequence. (B) SDS–PAGE analysis of purified HTNCre. M, Kaleidoscope molecular weight marker (Bio-Rad); CL, cleared lysate; FT, flow through; W1, wash fraction 1; W2, wash fraction 2; E, eluted fraction; C, Microcon concentrate. The arrow indicates purified Cre. (C) The diagram shows Cre-mediated excision where Cre induces excision reaction between two *lox*P (arrows), one in the linear plasmid and the other in the annealed *lox*P. (D) Cre activity was analyzed by incubation at 37 °C for 20 min with indicated concentrations of HTNCre (left); Cre activity over time was analyzed by incubating the substrates with 0.5 μM of HTNCre at 37 °C for the indicated times (right). The cleaved DNA products were separated by agarose gel electrophoresis. M, 1 kb Plus DNA marker (Invitrogen).

the *Gtrosa26*^{tm1Sor} allele, and primers F1295 and lacZ-As amplify a 255-bp product specific for the *Gtrosa26*^{lacZ} allele. PCR products were resolved and visualized by 1.2% agarose gel electrophoresis.

Results

Purification of HTNCre fusion protein and HTNCre-mediated excision in vitro

Cell-permeable Cre recombinase (HTNCre) was purified as described previously [9] with a slight modification and concentrated by centrifugation with Microcon (Fig. 1A). The Microcon centrifugation which allows concentration of HTNCre without increasing glycerol concentration in purification buffer apparently improved the survival of preimplantation embryos after HTNCre protein treatment. Although the binding of HTNCre to Ni–NTA was not highly efficient, we were able to routinely obtain 10–20 μM of HTNCre after elution, and 40–90 μM after Microcon concentration (Fig. 1B). Cre activity was analyzed *in vitro* by measuring the amount of cleaved DNA fragments by SSR that occurred between two *lox*P sites *in trans*. Cre-mediated excision of 3.0-kb linear DNA resulted in 1.9-kb and 1.1-kb DNA fragments as predicted (Fig. 1C). Cre-mediated excision took place in a concentration-dependent manner (Fig. 1D), and the cleaved DNA appeared rapidly within minutes after the initiation (Fig. 1D).

HTNCre protein-mediated excision in PMEF cells

HTNCre activity in cells was examined using ROSA26-PMEF cells wherein Cre-mediated excision of *Gtrosa26*^{tm1Sor} allele results in a strong expression of *lacZ* [13]. X-Gal staining of ROSA26-PMEF cells incubated with HTNCre readily demonstrated an efficient Cre-mediated excision of *Gtrosa26*^{tm1Sor} allele (Fig. 2A). The fraction of X-Gal-stained cells increased along with the concentration of HTN-Cre and with the duration of the treatment (Fig. 2B). Over 50% of

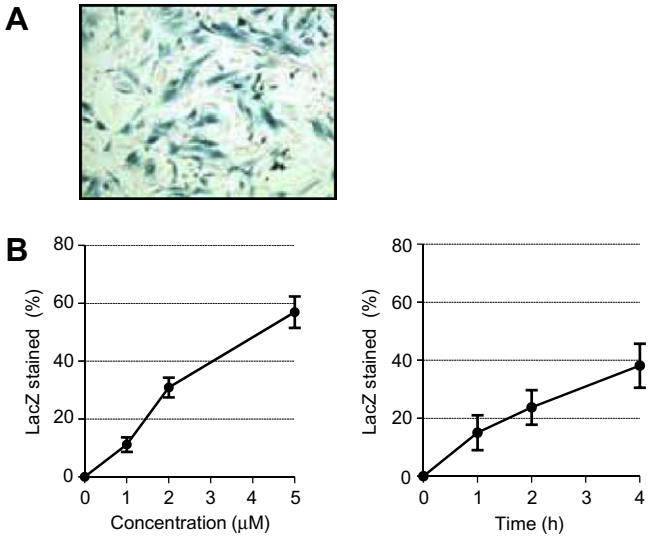

Fig. 2. Transduction of Cre protein into ROSA26-PMEF cells. (A) The PMEF cells treated with 3 μM of HTNCre for 2 h were cultured for 2 days before X-Gal staining. Shown are representative PMEF cells stained with X-Gal. (B) The PMEF cells treated for 2 h with indicated concentrations of HTNCre (left) or for indicated times with 2 μM of HTNCre (right) were passaged 2 days later and further cultured for 1 day before X-Gal staining. The number of stained cell was counted from randomly selected visual fields with 100× magnification.

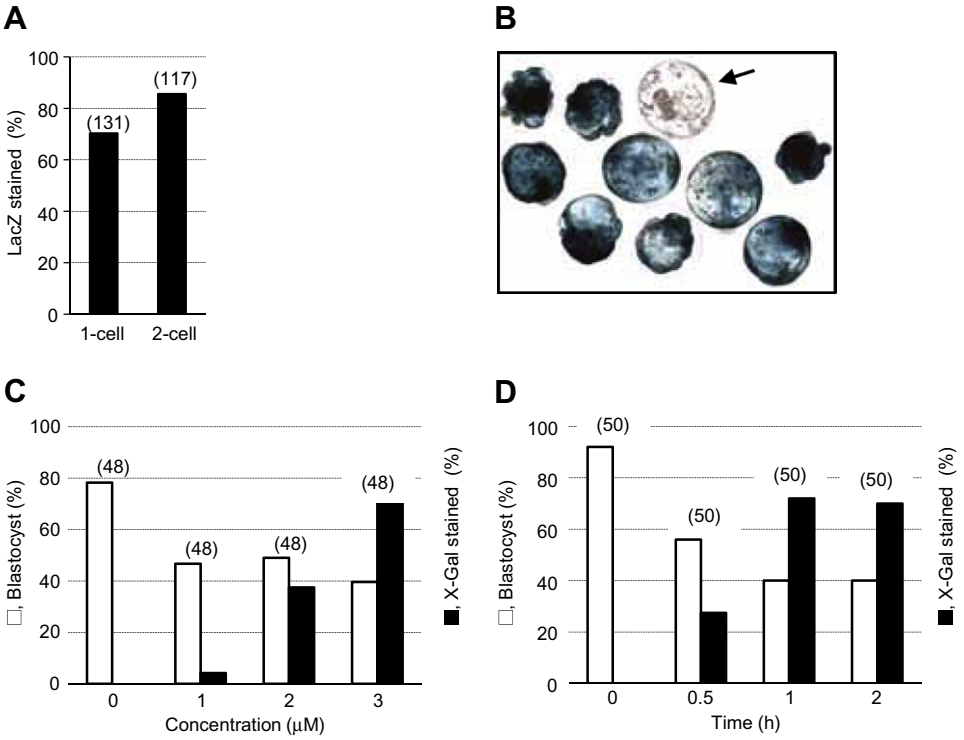

Fig. 3. Transduction of Cre protein into preimplantation embryos. (A) The *Gtrosa26*$^{tm1Sor/+}$ embryos at the indicated stages were treated with 3 μM of HTNCre for 2 h. The Cre-treated embryos were washed, cultured for 2.5 days and then X-Gal stained. (B) Picture shown is representative X-Gal-stained and un-stained embryos treated with 3 μM of HTNCre for 2 h at 2-cell stage and cultured for 3.5 days. The arrow indicates un-stained blastocyst. (C) The 2-cell embryos were treated with different concentrations of HTNCre for 2 h, or (D) treated with 3 μM of HTNCre for the indicated times. Cre-treated embryos were further cultured for 3.5 days until X-Gal staining. The open bars in (B) and (C) indicate embryonic development to blastocyst stage *in vitro*, and the closed bars indicate X-Gal-stained embryos. Data from three independent experiments were pooled, and the total numbers of embryos examined are indicated in parenthesis.

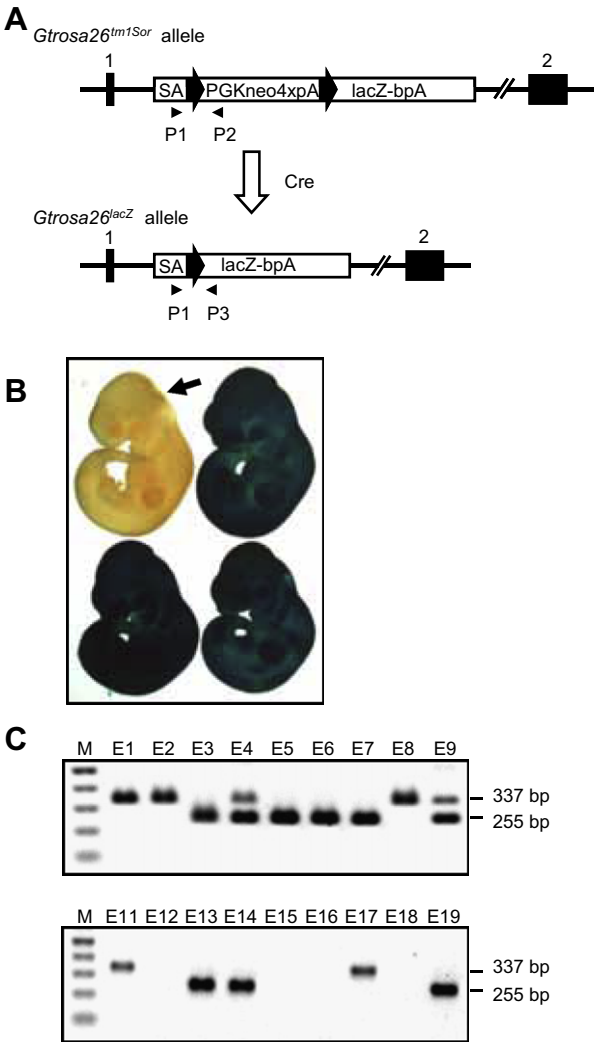

Fig. 4. Postimplantation embryonic development of HTNCre-treated eggs. (A) Shown at the top is *Gtrosa26*^{tm1Sor} allele targeted into *ROSA26* locus with a splice acceptor sequence (SA), neomycin transphosphorylase expression cassette (PGK-neo-4xpA) flanked by *lox*P sites (arrows), a *lacZ* gene and a polyadenylation (bpA) sequence. At the bottom is the same locus after Cre-mediated SSR. The exons of *ROSA26* are labeled 1 and 2. Arrowheads indicate PCR amplification primers used for genotyping. (B) The HTNCre-treated (3 μM for 1 h) heterozygous 1-cell or 2-cell embryos were further developed *in utero*, and the 10.5-dpc embryos were stained with X-Gal. Shown are representative X-Gal-stained embryos with un-stained embryos (arrow). (C) The genotype of 10.5-dpc embryos was determined by PCR. (Top) E1–E9 indicate individual embryos derived from HTNCre-treated 1-cell embryos. (Bottom) One of the founder male mice was bred to wild type mouse and the genotype of 10.5-dpc embryos was determined by PCR. E11–E19 indicate individual F_1 embryos. PCR product is not produced from wild type embryos. M, 1 kb Plus DNA marker; *Gtrosa26*^{tm1Sor} allele, 337-bp PCR product; *Gtrosa26*^{lacZ} allele, 255-bp PCR product.

cells have apparently undergone SSR after incubation with 5 μM of HTNCre for 2 h (Fig. 2B).

HTNCre-mediated excision in preimplantation mouse embryos

To determine whether purified Cre fusion protein can induce *lox*P-dependent SSR in preimplantation embryos, we treated 1-cell or 2-cell stage embryos with 3 μM of HTNCre for 2 h *in vitro* and assayed for SSR by X-Gal staining. The result showed that SSR was very effective in both 1-cell and early 2-cell embryos with 70% and 86% of embryos staining, respectively (Fig. 3A). Since SSR was more effective in 2-cell embryos, effects of Cre protein concentration and treatment duration were determined using 2-cell embryos. Cre-treated embryos were cultured to blastocyst stage before X-Gal staining to examine embryonic development more accurately. X-Gal staining showed that Cre-mediated excision increased with the concentration of HTNCre (Fig. 3B and C). Unexpectedly, micromolar changes in HTNCre concentration resulted in dramatic differences in outcome (1 μM, 4%; 2 μM, 38%; 3 μM, 73%). At the same time however, embryonic development to blastocyst stage was apparently compromised by HTNCre treatment. Contrary to sharp increase in proportion of X-Gal staining embryos, effect of HTNCre concentration on development was relatively consistent, as 40–49% embryos developed to blastocysts compared to control group (Fig. 3C).

Treatment period-dependent X-Gal staining revealed that only a half hour incubation was sufficient to induce SSR in 25% embryos (Fig. 3D). Although SSR frequency increased along with incubation time, the proportion of successful preimplantation development decreased. There was no difference in the intensity of X-Gal staining between normal blastocysts and fragmented embryos. Most embryos exhibited ubiquitous staining of X-Gal, but some embryos were mosaic for X-Gal staining. The purified HTNCre stored at 4 °C resulted in consistent SSR for at least a month, indicating that no significant loss of Cre activity occurred under this condition. After a month, however, SSR efficiency decreased gradually accompanied by loss of Cre activity *in vitro*. Interestingly, Cre fusion protein without TAT and NLS also gave rise to SSR in preimplantation embryos (data not shown), but the efficiency was extremely low (1.0%, treatment with 7 μM for 4 h) compared to HTNCre.

Normal embryonic development after HTNCre-mediated SSR

To analyze the developmental capacity of HTNCre-treated embryos, *Gtrosa26*$^{tm1Sor/+}$ embryos treated at 1-cell or 2-cell stage were cultured overnight and then transferred into pseudopregnant females to further develop *in utero*. The presence of *Gtrosa26*^{tm1Sor} allele and *Gtrosa26*^{lacZ} allele can be confirmed by PCR genotyping with three primers together (Fig. 4A). The X-Gal staining (Fig. 4B) and the genotyping (Fig. 4C, top) of postimplantation embryos were carried out with 10.5-dpc embryos. Approximately 75% embryos treated with 3 μM of HTNCre for 1 h showed either complete or partial Cre-mediated excision (Table 1). X-Gal staining of whole embryos matched genotype of embryos perfectly. In most cases allelic mosaic embryos also showed strong X-Gal staining indistinguishable from that of the embryos with *Gtrosa26*^{lacZ} allele only.

Table 1
Postimplantation development of HTNCre-treated 1-cell embryos.

Treatment	Collection time	X-Gal stained/examined	Genotype		
			ROSA26^{tm1Sor}	*ROSA26*^{lacZ}	Mosaic[c]
3 μM, 2 h	10.5-dpc	10/10	0	10	0
3 μM, 1 h	10.5-dpc	24/32	8	17	7
3 μM, 1 h	P14[a]	ND[b]	18	6	7

[a] 14-day-old pup.
[b] ND, not determined.
[c] Mosaic indicates mixed genotype including *ROSA26*^{tm1Sor} and *ROSA26*^{lacZ} alleles.

The embryo size and gross morphology were similar between non-stained and stained embryos.

Some transferred embryos developed to term and were born without gross morphological abnormality, but one third of pups were found dead within 1 day after birth. The genotype analysis revealed that most dead pups (12/15) had *Gtrosa26*^{lacZ} allele only. Approximately 42% of 2-week-old pups either were pure *Gtrosa26*^{lacZ} animals or mosaics (Table 1). Some pups having *Gtrosa26*^{lacZ} allele exhibited moderate growth retardation but reached to normal size after 2 months (data not shown). The genotyping of 10.5-dpc embryos from wild type female bred to founder mice that had undergone SSR revealed the presence of *Gtrosa26*^{lacZ} allele indicating successful germ line transmission of the modified genome (Fig. 4C, bottom).

Discussion

In vitro manipulation of preimplantation mouse embryo has become indispensable in mammalian genetics [14]. Breeding with Cre transgenic mice has also been established as a key tool for conditional gene targeting [2,3]. Cre-mediated gene deletion in preimplantation embryo is typically achieved by crossing the transgenic mouse strains that express Cre under the control of human cytomegalovirus promoter [16] or adenovirus EIIa promoter [17]. Recent introduction of genomic modification by cell-permeable Cre in the mouse tissues offers a rapid and efficient alternative means to manipulate genome *in vivo*, where the translocation of Cre is achieved by tagging the peptides from Kaposi fibroblast growth factor [7]. A variety of cell-permeable peptides have been successfully used to deliver heterologous cargoes into cells [18]. In our study, a very high rate of SSR in ROSA26 locus by Cre tagged with a basic peptide derived from HIV TAT transactivator was observed. This indicates that the uptake of Cre into preimplantation mouse embryos occurred efficiently despite being surrounded by thick porous coat, the zona pellucida. This strategy reduces the time and the cost compared to the conventional way which involves crossing with Cre transgenic mouse strains.

Along with the expanded use of Cre/*lox*P system in mammalian genetics, unexpected and undesirable side effects of Cre actions are beginning to be reported. After first report in Cre-dependent chromosome rearrangement in transgenic mouse spermatids [19], Cre-mediated cell damages have been widely observed both in cultured cells and in the various tissues of Cre transgenic mouse strains [20–22]. These results suggest that high levels of Cre result in DNA rearrangement between pseuo-*lox*P sites distributed genome-wide [23,24] and in subsequent cell death. A similar observation in our study, the reduction of developmental capacity in some HTNCre-treated 1-cell embryos, indicates adverse effect of Cre on embryonic development and post-natal growth. Nevertheless, the germ line transmission of *Gtrosa26*^{lacZ} allele by founder mice derived from Cre-treated 1-cell embryos strongly supports that simple incubation of preimplantation embryo with low levels of HTNCre is a feasible approach for examining gene function in early development and an alternative way of efficient genome modification in mouse.

Acknowledgments

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