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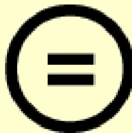
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수의학박사학위논문

Generation and analysis of transgenic cattle with germline transmission via transposon

트랜스포존 시스템을 이용한 생식선 전이 능력이 있는 형질전환소의 생산 및 분석

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Generation and analysis of transgenic cattle with germline transmission via transposon

by Soo Young Yum

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
in
Theriogenology and Biotechnology

Department of Veterinary Medicine, Graduate School
Seoul National University

We accept this thesis as confirming to the required standard

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Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Soo Young Yum

Generation and analysis of transgenic cattle with germline transmission via transposon

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ABSTRACT

Transgenic cattle can provide a powerful model to understand a basic genetic function or performance in livestock. However, the production of transgenic cattle is restricted due to the low efficiency of gene integration, the cost of producing transgenic cattle, and the low rates of embryo survival and embryo transfer. One way to solve this problem is

to apply a gene delivery system with high efficiency gene integration such as a transposon system.

Transposon (non-viral) gene delivery is a simple and efficient transgenic tools, which can be utilized for a variety of species. The transposon system is more effective than any other non-viral system, with a higher integration frequency. The system has a low toxicity, because transposase cuts a transposon and inserts it into a preferred sequence in a recipient sequence which avoids a coding sequence. Several studies have reported the birth of transgenic animals via the transposon gene delivery system. However, this gene transfer system has not yet been investigated in cattle.

Here, I efficiently generated transgenic cattle using two different types of DNA transposon gene transfer system, *Sleeping Beauty* and *PiggyBac*, which deliver ubiquitous expression (SNU-SB-1, Female), conditional expression by rox-Dre recombinase (SNU-PB-1, male), and tissue-specific expression (SNU-PB-2, female), and their transgene integration sites and genome instability were analyzed by next-generation sequencing. The founder cattle have grown up (over 37 months) without any health issues to date. In genomic instability and blood analysis, there were no significant differences between wild type and founder cattle. To investigate genomic variants by the transgene transposition, whole genomic DNA were analyzed by NGS. All of the integration sites were identified that preferred transposable integration (TA or TTAA) in their genome. Even though multiple-copies (i.e. fifteen) were confirmed, there was no significant difference in genome instabilities.

Next, germ-line transmission of the transposon-mediated transgene integrations, and ubiquitous and stable expression of transgene was confirmed in the second generation of offspring (F1). SNU-SB-1 was naturally crossed with SNU-PB-1 and delivered F1. The F1 was born without any assistance and expressed GFP in the eyes without UV light. The ubiquitous expression of GFP was detected in skin fibroblast from the ear tissue and confirmed by genomic DNA PCR, which suggest that the transgene from the paternal transgene was successfully transmitted. Unfortunately, no transgene from SNU-SB-1 was identified. To confirm the transgene integration site, the genomic DNA from the blood was extracted and performed next-generation sequencing. The transgene was integrated in chromosome 4 (two copies), and 6. As a result, a total of two copies of paternal transgene transmitted into the F1. All the integrated positions were not related with coding regions and there was no significant difference in genomic variants between transgenic and non-transgenic cattle.

In conclusion, this is the first report about the generation of transgenic cattle via transposon gene transfer system. In addition to this, germline transmission has been confirmed in F1 by transgene detection and next-generation sequencing. As transposon gene delivery system will be effectively utilized in generating transgenic cattle for stable transgene expression and genome instability. Those transgenic cattle will be a valuable resource for many fields of biomedical research and agricultural science.

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Key words: Germline transmission, *Sleeping Beauty*, *PiggyBac*, Transgenic cattle, Next-generating sequencing

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LIST OF ABBREVIATIONS

ALT	**Alanine transaminase**
AST	**Aspartate transaminase**
β-ME	**Beta-Mercaptoethanol**
bIGHM	**Bovine immunoglobulin mu heavy-chains,**
Bi-scFv	**Bivalent single-chain variable fragments**
BLG	**β-lactoglobulin**
BUN	**Blood urea nitrogen**
CA	**Caggs promoter**
CBC	**Complete Blood Count**
cDNA	**Complementary DNA**
CNV	**Copy number variations**
COCs	**Cumulus-oocyte complexes**
CRISPR	**Clustered Regularly Interspaced Short Palindromic Repeats**
Cryaa	**Lens cell-specific crystallin A alpha promoter**
CSN2	**β-casein gene**
DNA FISH	**DNA *in situ* hybridization**
DMEM	**Dulbecco's modified Eangel medium**
dsDNA	**Double stranded DNA**
FBS	**Fetal bovine serum**
FDA	**Food and drug administration**
FSH	**Follicle Stimulating Hormone**
Gbp	**Giga base pairs**
gDNA	**genomic DNA**
GFP	**Green fluorescence protein**
GNAI1	**Alpha Inhibiting Activity Polypeptide 1**
HBSS	**Hanks' Balanced Salt Solution**

hIGH	**Human immunoglobulin (Ig) heavy-chain**
hIGK	**Human immunoglobulin (Ig) kappa-chain**
hIGL	**Human immunoglobulin (Ig) lambda-chain**
hLYZ	**Human lysozyme**
IGV	**Integrative Genomics Viewer**
INDEL	**Insertion and deletion**
IVC	***in vitro* culture**
IVF	***in vitro* fertilization**
IVM	***in vitro* maturation**
MAQ	**Mean mapping quality**
mRNA	**messenger RNA**
MSTN	**Myostatin**
N/C	**Negative control**
NEAA	**Non-essential amino acids**
NFW	**Nuclease free water**
NGS	**Next-generation sequencing**
PB	**PiggyBac**
PBS	**Phosphate-buffered saline**
PCR	**Polymerase Chain Reaction**
PRNP	**Prion protein**
P/S	**Penicillin/streptomycin**
RBC	**Red blood cells**
RFP	**Red fluorescence protein**
RGRR	**rox-GFP-rox-RFP**
RT-PCR	**Reverse transcription polymerase chain reaction**
SB	**Sleeping Beauty**
SB100X	**hyperactive Sleeping Beauty recombinase**
SCNT	**Somatic cell nuclear transfer**
SNP	**Single nucleotide polymorpism**

SNV	**Single-nucleotide variants**
SP110	**Nuclear body protein**
SSR	**Site-specific recombination**
SV	**Structural variations**
TES	**Transposable elements sequences**
TCM	**HEPES-buffered tissue culture medium**
Tg	**Transgenic**
VSV-G	**Glycoprotein of vesicular stomatitis virus**
WBC	**White blood cells**
WT	**wild type**
YFP	**Yellow fluorescence protein**

PUBLICATION LISTS

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PART I.

LITERATURE REVIEW

This part is based on the publication; Yum SY et al: Development of genome engineering technologies in cattle: from random to specific. *J Anim Sci Biotechnol* 2018, 9:16.

1. Transgenic cattle

1.1. Cattle for animal model

Livestock is very important to human because it provides meat, milk, and other by-products, such as leather. Several group have been generating transgenic by producing new proteins and improving animal genetic features. Consequently, initial studies using transgenic mice to produce milk with improved manufacturing properties have been tested and a great deal of research has been conducted to date [1-4]. Cattle are well known as the best animals to produce large amount of milk (Table 1). Because the cows have a very specialized system for flexible milk production, relatively simple purification and large-scale milk volume, the milk produced by cattle can be modified by genome editing of milk protein gene promoters such as by changing the increase some nutrients or protein composition [5, 6]. Additionally, human or animal bio-pharmacological proteins can be produced on a large scale. This concept of modifying bio-pharmacological proteins from transgenic animals has existed for a long time and three recombinant proteins (Aytrin® from goats, Ruconest® from rabbits and Knuma® from chicken) have been approved for clinical use by the FDA in the USA. The

recombinant proteins were produced via random mutation of animals. Transgenic cattle are considered an attractive resource for producing valuable recombinant proteins and pharmaceuticals. Another application of transgenic cattle is to improve genetic features using genetic engineering. Bovine genome sequencing revealed characteristic traits in bulls and traits introduced via random mutation and natural selection such as increased myostatin or dehorning were identified at the genomic level. Increased myostatin and dehorned cattle have already been born and grown into adults with the expected phenotypes (larger muscles and no horns) [7, 8]. Dehorning cattle is a low risk procedure because transgenic cattle receive dehorning genes from naturally hornless cattle. Additionally, they are utilized for research on assisted reproduction technologies such as superovulation, in-vitro fertilization, embryo transfer, microinjection (MI), somatic cell nuclear transfer (SCNT), and cryopreservation, which help us to understand basic or advanced embryology in animals as well as in humans. Recently, the introduction of new genomic technologies such as whole genome sequencing or genome manipulation in cattle, has opened a new era for industrial applications.

1.2. History of transgenic cattle

Transgenic cattle production has progressed relatively slowly for livestock (Figure 1, Table 2). In the initial stage of transgenic cattle production, plasmids including exogenous recombinant DNAs are microinjected into in-vitro fertilized embryos like mice. In other words, transgenic founder cattle are produced through the MI of recombinant DNAs into the pronucleus of fertilized embryos and transgenesis is verified

by detecting the gene [9]. Because mosaicism is observed in founder offspring, complete genetically modified mice can be produced by breeding genetically modified males or females. However, research on DNA MI into bovine zygotes has progressed slowly or has been limited due to difficulties with detecting the pronucleus of fertilized embryos. For research on bovine zygotes, centrifugation of the denuded zygotes enables clear visualization of the pronucleus. Bovine transgenic blastocysts produced with mechanical treatments (centrifugation and MI) are transferred into the recipient cow to produce transgenic cattle. Unfortunately, the MI approach is impractical for the production of transgenic cattle because of transgene mosaicism, low DNA delivery efficiency, long gestational periods (280 days) and puberty (around 14 months), and single pregnancy in cattle. As an alternative to MI, high integration of the targeted foreign gene to produce transgenic cattle using a viral gene delivery system was introduced [10], and indeed, transgenic cattle successfully engineered via retro- or lentivirus-mediated integration have been born and grown into adults [11, 12]. However, the virus-dependent transgenic approach still has limitations with regard to safety.

As a complementary procedure to MI of the target DNAs or virus-infection, SCNT, that is, a somatic cell, is injected into enucleated oocytes, then fused, activated, and cultured in-vitro to blastocysts [13]. Scientists think that transgenic can be produced relatively easily because genome-modified somatic cells can be reprogrammed into the pre-implantation stage. In other words, because only genetically modified cells are selected for SCNT, there is no doubt that the pre-implantation embryos and offspring will be positive for transgenesis without mosaicism. Indeed, several transgenic cattle

have been produced via SCNT [14]. However, with SCNT, the success rate of live cloned offspring is very low and abortions and abnormalities occur with high frequency due to abnormal reprogramming [15], leading to slow progress in transgenic cattle. In terms of genomic instability in transgenic cattle, MI is preferred over SCNT.

Table 1. Relative merits of animal species.

Species	Volume of milk	Time to milk production	Ease of generating founders
Rabbit	+	++++	++++
Sheep	+++	+++	++
Goat	+++	+++	++
Pig	+	+++	++++
Cow	++++	++	+

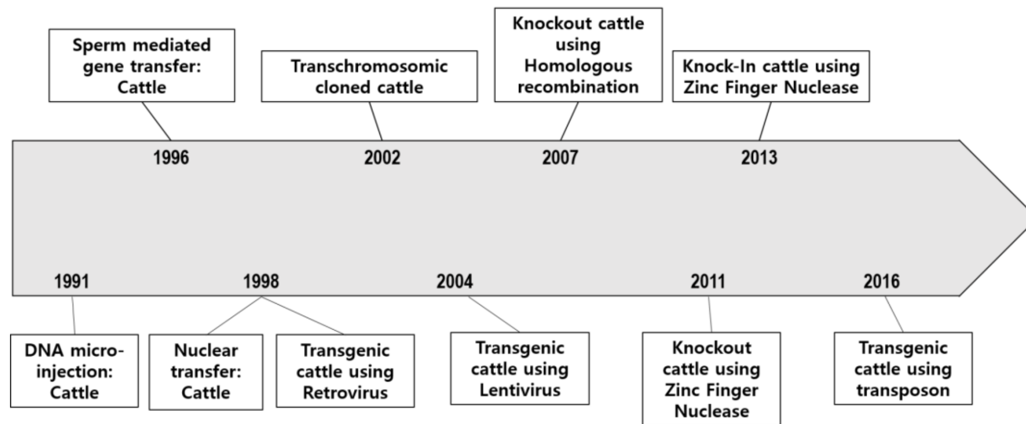


Figure 1. Milestones in the production of transgenic cattle.

Table 2. List of transgenic research in cattle.

Method of transgenesis	Year	Target gene		Recombinant protein	Method of embryo manipulation	References
	1991			lactoferrin	MI	[9]
	1994	αS1-casein promoter		dam-methylated gene	MI	[16]
Retro viral vector	1998			VSV-G-pseudotyped RNA*	MI	[17].
Simian virus 40	1999			human alpha-lactalbumin	MI	[18]
Non-viral vector	2002	αS1-casein promoter		human lactoferrin	MI	[19]
Non-viral vector	2003			β and κ casein	SCNT	[20]
Non-viral vector	2004			human Bi-scFv r28M**	SCNT	[21]
Lenti viral vector	2004			GFP	MI	[11]
	2005			GFP + peptidoglycan hydrolase (lysostaphin)	SCNT	[22]
Non-viral vector	2006			hGH	SCNT	[23]
shRNA	2006	PRNP***	Knock-down		SCNT	[24].
conventional HR	2007	PRNP	(KO)		SCNT	[25]
BAC	2008			human lactoferrin	SCNT	[26]
conventional HR	2009	bIGHM and bIGHML1****	(KO)		SCNT	[27]
			(KI)	hIGH, hIGK and hIGL*****		

Non-viral vector				Human albumin	SCNT	[28]
ZFN	2011	β-lactoglobulin (BLG)	(KO)		SCNT	[29]
Lenti viral vector	2013			EGFP	MI	[12]
ZFN	2014	Myostatin (MSTN)	(KO)		SCNT	[30]
ZFN	2014		(KI)	human lysozyme (hLYZ)	SCNT	[31]
		β-casein gene (CSN2)	(KO)			
TALEN	2015		(KI)	mouse SP110*******	SCNT	[32]
Non-viral vector	2016	MUC1 promoter		human β-defensin 3	SCNT	[33]
Sleeping Beauty	2016			YFP	MI	[34]
PiggyBac				Dre-rox system	MI	
PiggyBac		β-casein gene (CSN2)-promoter		Human IL2	MI	
Sleeping Beauty	2016			GFP +Cryaa*******-tdTomato	MI	[35]
Sleeping Beauty				GFP +pCryaa-tdTomato +pCasein-glycoprotein	MI	
BAC	2017			human lactoferrin	SCNT	[36, 37]

* Glycoprotein of vesicular stomatitis virus (VSV-G)

** Human bivalent single-chain variable fragments

*** Prion protein

**** Bovine immunoglobulin mu heavy-chains, bIGHM and bIGHML1

***** Human immunoglobulin (Ig) heavy-chain (hIGH), kappa-chain (hIGK), and lambda-chain (hIGL)

****** SP110 nuclear body protein

******* Lens cell-specific crystallin A alpha promoter

2. Transposon gene delivery system

The production of transgenic cattle via genome engineering for the gain or loss of gene functions is an important undertaking. In the initial stages of genome engineering, DNA MI into one-cell stage embryos (zygotes) followed by embryo transfer into a recipient was performed because of the ease of the procedure. However, transgenic cattle will remain a hurdle. This limitation includes the cost of producing transgenic cattle, the low efficiency of gene integration, and low rates of embryo survival and embryo transfer. To overcome these several disadvantages in cattle, the transposon gene delivery system was introduced and successfully used to produce transgenic cattle. This transposon system improves the occurrence of mosaicism and transgene integration (Fig. 4). Indeed, several transgenic cattle have been produced via DNA transposon [i.e. *Sleeping Beauty* (SB) and *PiggyBac* (PB)] (Table 2) [34]. The transposon, non-viral system consists of two components, a transposon containing a gene of interest expression cassette and transposase. The general principle of this system is "cut-and-paste". Transposase specifically binds the recognition sequences of the transposon and "cuts" expression cassette of a transposon vector and "pastes" it into the specific site of the genome.

2.1. Sleeping Beauty

SB transposon is one such gene transfer system which has been developed to perform gene transfer in vertebrates. SB was derived from multiple inactive *Tc1/mariner* element in fish. SB transposon consists of the expression cassette flanked by two long inverted

terminal repeat sequences (LTRs). Transposase recognizes TA sequence in the host genome and each ends of the transposon, and the TA sequence is duplicated in the host genome after integration. SB has been applied in a variety of animal models [34, 35, 38-40]. However, TA sequence left in the target sites (foot print) after excised via SB transposase. It can cause unwanted abnormal phenotypes and mutations.

2.2. PiggyBac

PB transposon system is an efficient gene transfer system similar to SB [34, 41-43]. The PB transposon contains the expression cassette with LTRs. PB transposase recognized LTR and each ends of the PB transposon and then transfers the expression cassette into the TTAA sites of the host genome with high efficiency. PB is more safe because it does not leave a footprint when re-exciting via PB transposase unlike SB. Therefore, PB is an effective transposon system for the generation of transgenic animals and is applicable to preclinical gene therapy research among several transposon gene delivery systems.

Transposon gene delivery system is integrated DNA elements into specific position. Transposons have been integrated into the intron region in several studies, indicating that the procedure is not harmful to cells, embryos or animals, because it does not affect the coding region [34].

2.3. History of transposon-mediated transgenesis

Transposon gene delivery systems have been actively studied for transgenesis in zebrafish, mice, rats, pig and cattle [34, 35, 44-47] *in vivo*. In addition, transposon-mediated transgenesis successfully developed with application including germline trangenesis and cancer research in variant animals [38, 40, 45, 48-50]. The transposon system has various advantage as mentioned above. However, transposon-mediated transgenesis could be the happened that multi copies of transgene can be integrated into the multi sites in host genome, and this may cause very high expression of transgenes and affect the genome stability in transgenic animal [51]. Both SB and PB systems have similar principles. Nevertheless, while integration of SB transposon occurs in a fairly random manner and when introduce transposition or excision remain footprint [52-54], PB transposon can be integrated to transcriptionally active regions and PB cassette can be re-transposition or re-excision without footprint in the genome [55, 56].

Katharina Katter et al. [57] have demonstrated that hyperactive SB recombinases (SB100X) protocol applied in three different animal models using transgenes of different sizes (3.2 kb ~ 10.2 kb) and a variety of promoters with SB and PB transposon vector system. The SB100X-mediated transgenesis were less prone to gene silencing and genetic mosaicism as compared to either the pronuclear MI of linear DNA transgenes or lentivirus-mediated approach in mouse, rat and rabbit. Similarly, porcine transgenesis was generated by cytoplasmic injection of zygotes with SB100X system without the necessity of an antibiotic selection marker using the cre-loxP recombination system [38].

In recent study, SB and PB was demonstrated to efficiently generated three types of transgenesis, stable expression, time dependent expression and tissue specific expression of transgene, in cattle. MI of transposon has resulted in multi-copies gene integration into the cattle genome and successfully confirmed the ability of germline transmission without any health issues or genomic instability [34].

The transposon system has been able to address the several bottleneck problems of nonviral gene transfer including toxicity, limitation of packaging capacity, low gene transfer efficiency, genome instability, germline transmission and gene silencing in transgenic animal production. The quality of transposon-mediated transgenic animal was much better than the classical pronuclear injection and viral approach. Therefore, the transposon gene delivery system combined with inducible and tissue specific system may prove to be a valuable tool for transgenesis studies.

PART II.

GENERAL METHODOLOGY

1. Reagents

All reagents were purchased from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise specified.

2. General cell culture

2.1. primary cell culture

Primary culture was performed using ear skin from whole-body of fetus neonatal calves or neonate calves using biopsy punch. The tissue pieces were two or three times washed with Phosphate-buffered saline (PBS) containing 10% Penicillin/streptomycin (P/S) (Gibco) and minced into 3 to 4 mm pieces using surgical blade. The tissue pieces were washed several times with Hanks' Balanced Salt Solution (HBSS) and incubated in 100 U/ml collagenase in HBSS at a temperature of 38 °C, in a 5 % CO2 overnight. The homogenized tissues were washed three times with HBSS and collected by centrifugation at 2000 rpm, for 2 min. The collected pellets were cultured in 60-mm culture dishes with 2ml Dulbecco's modified Eangel medium (DMEM) (Gibco, Carlsbad, California, USA) with 1% P/S, 10% Fetal bovine serum (FBS) (Gibco), 100 mM β-Mercaptoethanol (β-ME) and 1% Non-essential amino acids (NEAA) (Gibco).

2.2. Cell culture of bovine fibroblasts

Bovine cells were cultured at 39 ℃ in humidified incubator supplied with in DMEM supplemented with 1% P/S, 10% FBS, 100 mM β-ME and 1% NEAA. In subculture process, cells were washed 1 time with PBS and treated 1ml 0.25% trypsin-EDTA (Gibco) in 38 ℃ in humidified incubator for 3min. The Cells were suspended and added 2ml DMEM with 10% FBS and removed from the dish and centrifuged at 2000 rpm, for 2 min. Pallets resuspended in DMEM with 1% P/S, 10% FBS, 100 mM β-ME and 1% NEAA and seeded at a 1:4-6 split ratio in a dish, and remained cells were cryopreserved and stored at -196 ℃.

3. Oocyte collection and *in vitro* maturation (IVM)

Ovaries were collected from a local abattoir into saline at 35 ℃ and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18 gauge needle attached to a 10 ml disposable syringe. COCs with evenly-granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected and washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, 2 mM NaHCO3, and 1% P/S (v/v). For IVM, COCs were cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton-Dickinson Ltd., Plymouth, UK) for 22 h in 450 μ L TCM-199 supplemented with 10% FBS, 0.005 AU/ml Follicle Stimulating Hormone (FSH) (Antrin, Teikoku, Japan), 100 μ M Cysteamine, and 1 μ g/ml β -estradiol at 38 ℃ in a humidified atmosphere of 5% CO2.

4. *In vitro* fertilization (IVF)

A Percoll discontinuous gradient (45–90%) for 15 min at 1500 rpm. The 45% Percoll solution was prepared with 1 mL of 90% Percoll (Nutricell, Campinas, SP, Brazil) and 1 mL of capacitation-TALP (Nutricell). The sperm pellet was washed twice with capacitation-TALP by centrifugation at 1500 rpm for 5 min. The active motile spermatozoa from the pellet were used for insemination of matured oocyte (At 24 h of IVM). Oocytes were inseminated (day 0) with 1–2 × 106 spermatozoa/mL for 18 h in 30 μ L microdrops of IVF-TALP medium (Nutricell) overlaid with mineral oil at 39 ℃ in a humidified atmosphere of 5% CO2.

5. Microinjection

After culture of IVF-embryos for 18h *in vitro*, the cumulus cells were removed using 1.2 mg/ml hyaluronidase in TCM-199 by pipetting. Transposon DNAs were microinjected into the cytoplasm by microinjector machine (Femtojet®, Eppendorf, Germany). The removing the cumulus cells of fertilized oocytes were placed into a drop of TCM-199 with mineral oil. Single zygote was held with holding pipette, and injected vectors (100 ng/ul, 1:1 ratio of transposon and transpoase) using an injection pipette. The microinjected embryos were cultured into piqued micro-drops of sequential DI/DII medium overlaid with mineral oil. After 7 days, GFP expressing pre-implantational stage embryos were chosen and transferred into the surrogate cow.

6. *In vitro* culture of embryos (IVC)

Microinjected embryos were cultured in two-step chemically defined culture medium (DI-DII) overlaid with mineral oil (Sigma–Aldrich). The composition of the DI/DII culture medium was provided in the previous study [58], and this medium was modified to be appropriate for the different stage of the embryos (DI for the stage of one-cell to morula and DII for the stage of morula to blastocyst). The embryos were cultured in the DI for the first 4 days followed by transfer to the DII. Cleavage rates were recorded on Day 2 and embryonic development was monitored according to the stages of the International Embryo Transfer Society (IETS). All incubations were done at 38 ℃ in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

PART III.

GENERATION OF TRANSGENIC CATTLE

Chapter I. Identification of Dre-rox recombination system in bovine fibroblasts using transposon system

1. Introduction

One of the key issue of transgenic livestock production is efficiently integrated transgene and stable expression in transgenic animal. At present, the most powerful method to generate transgenic animal is the transposon system. The transposon system has been widely used to stable gene expression in a variety of mammalian cells and animal models after the SB transposon system was isolated from the fish in 1997 [59]. The non-viral DNA transposon is effective system to transfer transgene between chromosomes via a "cut-and-paste" mechanism. The PB transposon is also plasmid-based gene delivery system, which is originally defined in the Cabbage Looper [60] and successfully active in various cell types [46, 61, 62]. Although several transposon system is simple and efficient and have been established in many research, this system have limitation. Constantly overexpressed transgene can lead to unwanted toxicity, early embryonic lethality and physiological disorder in transgenic animal. One of the best solutions for this problem is regulated temporal control of gene expression using inducible gene expression system. Because of these reasons, an increasing number of involving studies on conditional gene expressions such as Cre-loxP and Tet-on/off have been widely applied to mimic the disease or gene function in animal models [63-67].

Cre-loxP is a site-specific recombination (SSR) technology used to introduce deletion, insertion, translocation and reversal at specific sites in the genome of cells. Recently, another site-specific recombinase, Dre, isolated from the P1-like phages. Like Cre, Dre recombinase recognizes the specific sequence, rox, and causes excision of the flanked gene. Although Dre recombinase has a similar structure to Cre, it does not recognize loxP sequences, indicating that there is no crossover-recombination between Cre-rox and Dre-lox [68]. However, Dre-rox recombination in cattle has not yet been investigated. Here, I hypothesized that using PB transposon approaches could efficient gene integration and to determine if the SSR system, Dre-rox, is applicable in bovine fibroblasts.

2. Materials and Methods

2.1. Construction of vectors

The transposase plasmids for SB (pCMV(CAT)T7-SB100X) and PB-transposase (pCy43) were purchased from Addgene (http://www.addgene.org, Plasmid#34879) and provided by Sanger Institute (Hinxton, UK). GFP was amplified using PCR and inserted into destination vector, pT2/BH for SB (http://www.addgene.org/, #26554) and PB-CA for PB (http://www.addgene.org/, #20960) using gateway PCR cloning kit (MultiSite Gateway® Pro Plus, Invitrogen, 12537100, Life Technologies, Carlsbad, CA, USA), repectively. rox-GFP-polyA and rox-RFP were amplified by gateway PCR cloning and inserted into destination vector, PB-CA (Table 3 and Figure 2).

Table 3. Primer sequences of gateway cloning.

Primer name	Sequence (5' to 3')
Gateway sequence	F: ggggacaagtttgtacaaaaaagcaggcttc (attB1) R: ggggaccactttgtacaagaaagctgggtc (attB2)
Gateway-YFP	F: ggggacaagtttgtacaaaaaagcaggcttcATGGTGAGCAAGGGCGAGGA R: ggggaccactttgtacaagaaagctgggtcTTACGTTTCTCGTTCAGCTT
Gateway-GFP	F: ggggacaagtttgtacaaaaaagcaggcttcATGGTGAGCAAGGGCGAGGA R: ggggaccactttgtacaagaaagctgggtcTTACTTGTACAGCTCGTCCA

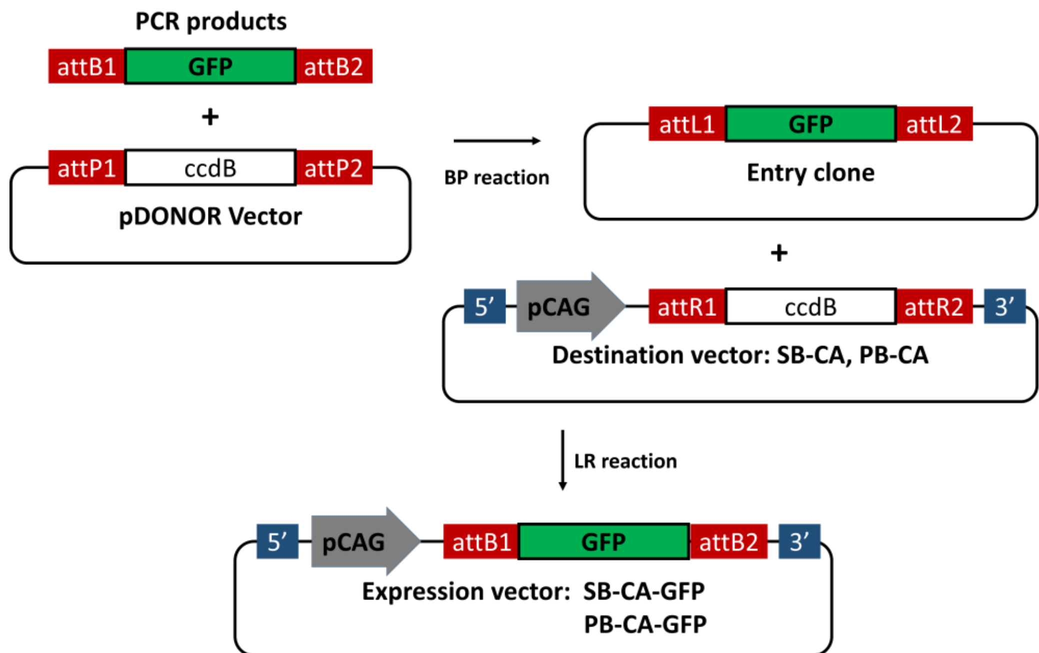


Figure 2. Gateway cloning for construct of PB transposon vectors.

GFP was amplified by PCR using gateway specific primer and purified PCR products recombined with the pDONOR vector by BP reaction. The attB sites recombined with attP sites and changed the attL site that contained the entry vector. In LR reaction, the attL sites in entry clone was reactivated with attR sites that was exist in destination vector. As a result, an expression vector with the GFP flanked by attB sites is generated.

2.2. Transfection

One day before transfection, the bovine fibroblasts cells were seeded by thawing or sub-culturing on to 60mm dish with 5mL DMEM, supplemented with 1% P/S, 1% NEAA, 100mM B-ME and 10% FBS. The cells were transfected with 1ug of plasmid DNA and transposase pCy43 using electroporation systems (Neon®, Invitrogen, voltage:1400 v, pulse width range: 40 ms, pulse number: 2). After 4 hours of incubation, the culture dish was washed 2 times and re-changed with flesh medium.

2.3. Genomic DNA sample preparation

Genomic DNA was extracted from blood or primary cells with DNA extraction kit, following the manufacturer's protocol. Genomic DNA was analyzed by Qubit fluorometer dsDNA assay Kit (Invitrogen, CA) as well as Infinite F200 Pro NanoQuant (TECAN, Männedorf) to verify the quality (O.D. 260/280 ratio is 1.8–2.0 and O.D. 260/230 ratio greater than 1.6) and quantity (1 ug for library construction).

2.4. PCR

About 100 µg of genomic DNA and 10pmol designed primer set were added to Maxime PCR PreMix Kit (iNtRON) for detection of transgene integration with CAG and RFP detectable primer set (5' to 3'; F: GCTCTAGAGCCTCTGCTAA, R: TTGTGGATCTCGCCCTTCAG) following the reaction conditions: 94 ℃ for 2 min, 35 cycles of 94 ℃ for 20 sec/ 60 ℃ for 10 sec/ 72 ℃ for 30 sec, and finally 72 ℃ for 5 min.

3. Results

3.1. Efficiency of transgene delivery into bovine fibroblasts using transposon

To test of efficiency of transgene delivery into bovine fibroblasts, plasmid DNA (pcDNA3.1-GFP) and two transposons (SB-GFP and PB-GFP) were transfected; delivery efficiency of GFP was shown in Figure 3). The expression ratio of GFP at 24 h, 48 h, 72 h, 96 h and 144 h after transfection without antibiotic selection in PB-GFP (12.8, 32.9, 41.7, 44.6 and 24.3%, respectively) was higher than in pcDNA3.1-GFP (1.4, 8.6, 18.7, 0.0 and 0.0%, respectively) and SB-GFP (4, 8.8, 2.6, 3.5, and 2.3%, respectively) (Figure 3).

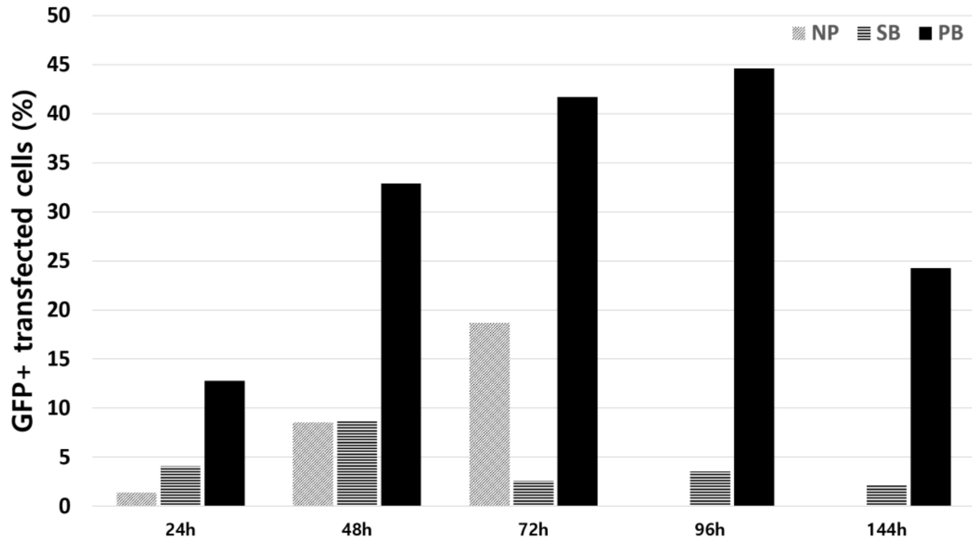


Figure 3. GFP expression ratio after transfection of normal plasmids (NP), *Sleeping beauty* (SB) and *PiggyBac* (PB) into bovine fibroblasts.

After transfection, I counted the GFP positive cells at every 24 h (from 24 h to 144 h), repectively.

3.2. Conditional expression of transgene using Dre-rox system

To investigate Dre-rox system, the expression vector carrying rox flanked (froxed) GFP followed by the RFP (PB-CA-RGRR) and transposase were transfected into bovine fibroblasts by nucelofection. This vector was designed to express GFP before and RFP after Dre recombination (Figure 4). The transfected cells were cultured and collected GFP expressed cells. The Dre mRNA were transfected into the GFP expressing cells. After transfection, RFP signal was detected in the cells, indicating that froxed site was deleted by Dre recombination. At the cellular level, established Dre-rox cell line and excision was confirmed by genomic DNA PCR (Figure 5).

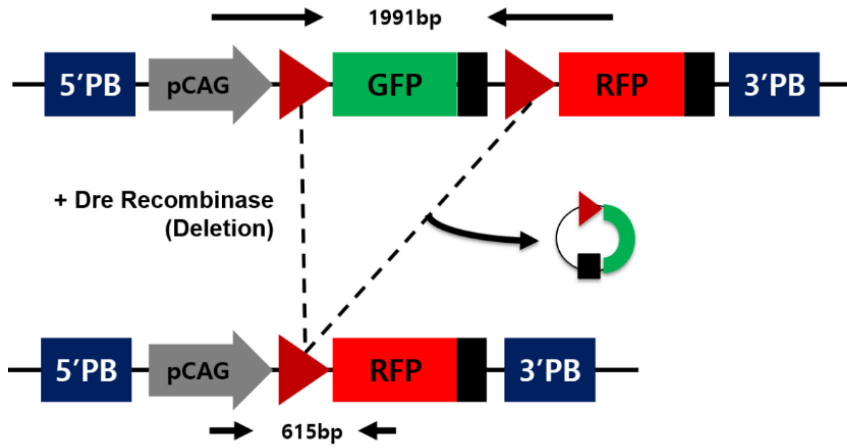
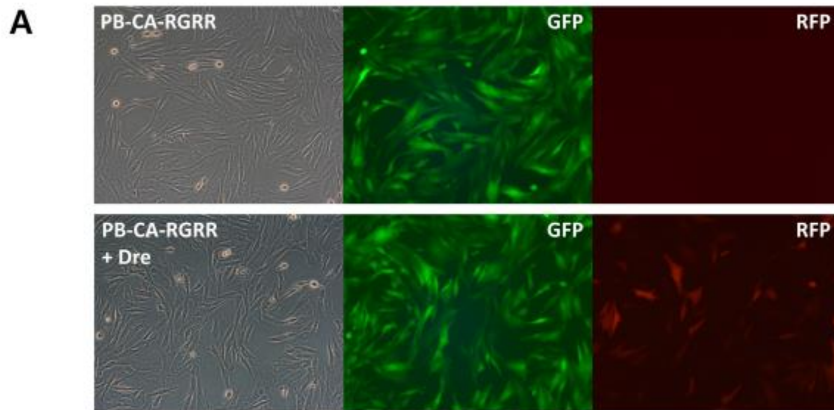


Figure 4. Illustration of Dre-rox recombination used for this study.

DNA for before (upper) and after (lower) of the Dre recombination. In figure, size for arrow indicates the region of genomic amplified DNA. Red triangle, rox site; Black box, poly A.



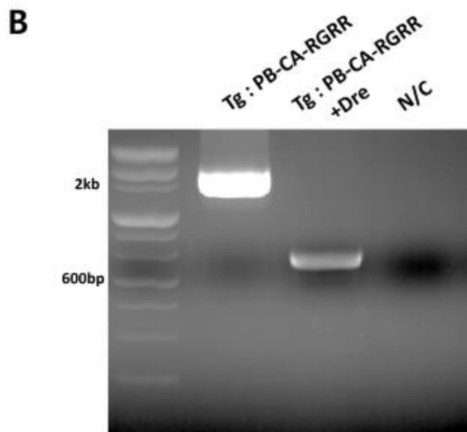


Figure 5. Identification of Dre-rox system in bovine fibroblasts.

(A) Expression of GFP in bovine fibroblasts after transfection. Before recombinase mRNA transfection, the cells with the green-to-red reporter line resulted in GFP expression. In contrast, recombinase mRNA transfection resulted in RFP expression. (B) PCR analysis of the Dre-rox recombination.

4. Discussion

The generation of transgenic cell line and animals for stable transgene expression has been a user-dependent and time-consuming process. Therefore, the integration efficiency of DNA is very important. For this reason, lenti-, adeno associated-, or retro viral vectors have been used in various cell line and animal models to date. Viral vector systems are valuable for gene transfer, because the system has developed the ability of to deliver nucleotides to target organisms by infection. However, some viral vectors have several disadvantages: 1) the cargo capacity is limited. 2) the preparation of viral infection is difficult and costly. 3) random insertion into the host genome causes oncogenic activation, resulting in potential tumorigenesis. To address these issues, I have generated a stable cell line based PB transposon.

I have investigated the integration efficiency of transposon-mediated gene delivery systems (SB and PB) and used PB transposon system for established SSR cell line in bovine fibroblasts. My results suggest that PB has a more highly integrated efficiency into the host genome than SB.

My results also demonstrated that the SSR system, Dre-rox, can be applied in bovine fibroblasts with high efficiency using PB transposon, and that it supports conditional gene expression of a transgene. To evaluate recombination system in bovine cells, I generated a cell line of froxed GFP followed by RFP, and the GFP expressing cells were transfected with recombinase mRNA. 2 days after transfection, RFP signal was detected in the cells, indicating that recombination must be worked in bovine fibroblasts.

Taken together, for the first time, a Dre recombinase dependent cell line was established via PB transposon with transposase in bovine fibroblasts. A transposon gene delivery system can be an alternative and effective way to produced transgenic cattle and Dre might be applied in site specific and conditional target gene expression in generating bovine biomedical models.

Chapter II. Efficient generation of transgenic cattle via transposon

This chapter is based on the publication; Yum SY et al: Efficient generation of transgenic cattle using the DNA transposon and their analysis by next-generation sequencing. *Sci Rep.* 2016, 6:27185 [34].

1. Introduction

Transgenesis is an important tool to understand gene function in mammals. Based on isolation of embryonic stem cells in rodents via germline transmission, transgenic mice have been accelerated in genetic models. Unlike mice with germ-line competent embryonic stem cells, development of transgenic livestock has been hampered to date. Early studies in transgenic livestock depended on microinjecting DNA into pronuclear stage embryos. After improving DNA delivery, several transgenic techniques such as virus- or sperm-mediated gene transfer and somatic cell nuclear transfer (SCNT) with transgenic somatic cells have been applied. Recently, SCNT has been heralded as a promising approach for generating transgenic livestock. Even though abnormalities derived from SCNT are reported, a few transgenic cattle via SCNT have been generated. However, there is still low efficiency due to insufficient reprogramming and high

frequency of abnormalities in the SCNT approach. An alternative approach for producing transgenic livestock is the use of viral vectors. Among several viral vectors, lentivirus-mediated gene transfer has successfully been applied to transgenic cattle. Although viral gene delivery has advantages for efficient genome integration, viral infection may cause activation of proto-oncogene, resulting in potential of tumorigenesis [69, 70]. Recently, in addition to simple plasmid and viral gene delivery, DNA transposons including PB, SB, Tol2 or Tn5 have been successfully used for transgenesis in several studies [61, 71-73]. The basic principle of transposon is that transposase recognizes transposable elements sequences (TES), cut the inside DNA of TES and paste it into the other genome position. When TES moved into another region, they preferred some specific sequences like TA and TTAA for SB and PB, respectively [74]. Furthermore, when the transposase cuts and pastes the transgene, multi-copies integration into genome is possible (i.e. over 60 copies) [75]. Also, as integration of transposon has preference for low-risk chromosomal regions such as intronic sequences [74], it could be safer than viral gene delivery. Due to stable integration with high expression by transposon DNA delivery, transposons are applied to several species as mentioned above. Although there have been several research publications regarding transgenesis in cattle using transposon, live cattle has not been produced. Here, I generated transgenic cattle using two transposons (SB and PB), which deliver ubiquitous expression, conditional expression by Dre-rox recombinase, and tissue-specific expression. Additionally, those were analyzed by next-generation sequencing (NGS) for genome integration site, number of transgenes and genomic variants (Figure. 6).

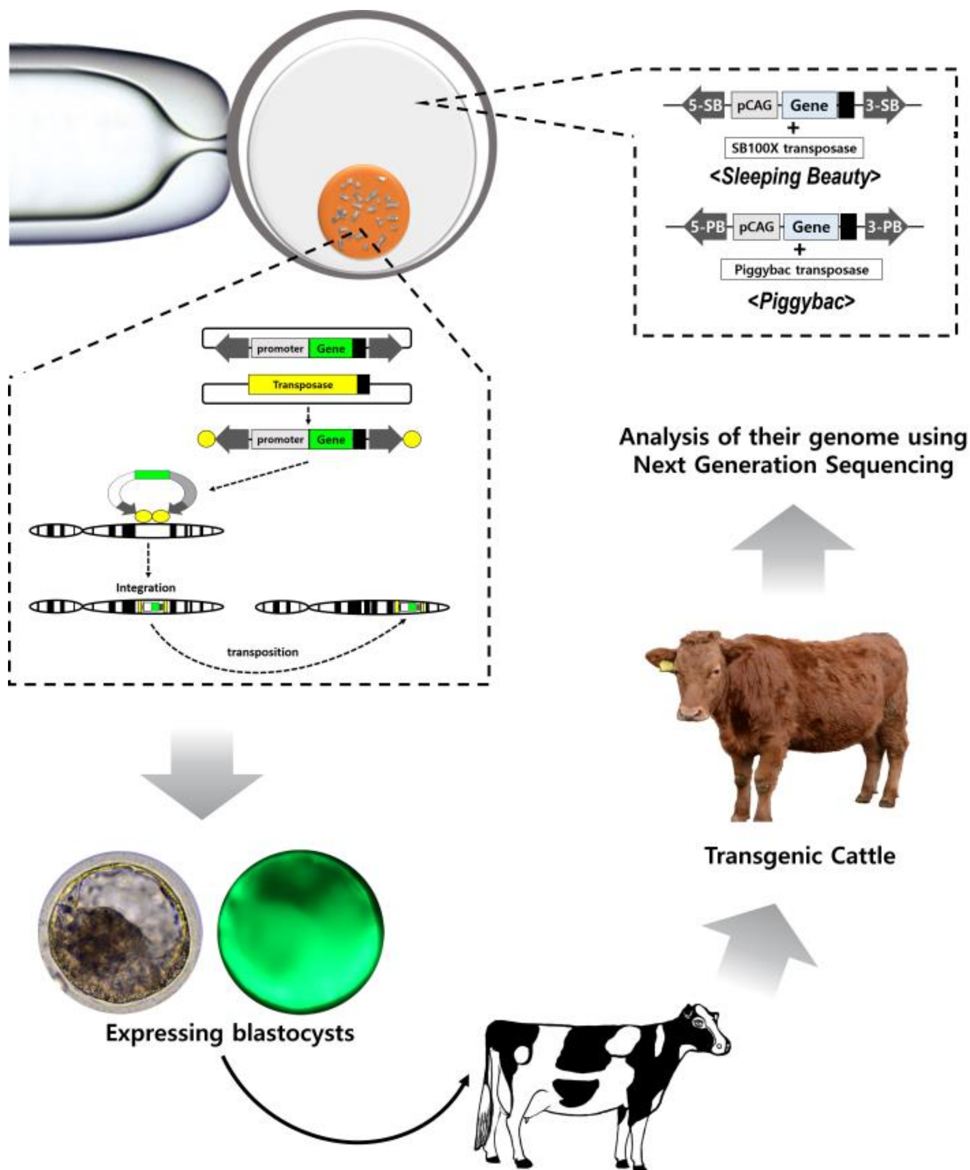


Figure 6. Illustration of this experiments.

Transposon DNAs were mincroinjected into fertilized embryos and the blastocysts with transgene were transferred into recipient cow. Some transgenic cattle were born, and NGS analysis was used for their genome variants.

2. Materials and Methods

2.1. Vector construct

The SB-YFP transposon plasmid was provided by the University of Minnesota [76]. The transposases, SB100X, PB (pCy43) transposase, and PB-CA-RGRR were described in the previous chapter. β-Casein promoter and hIL2 cDNAs were amplified by PCR and inserted into PB-GFP by Infusion Cloning (In fusion HD cloning kit, Clontech, 639644, California, US).

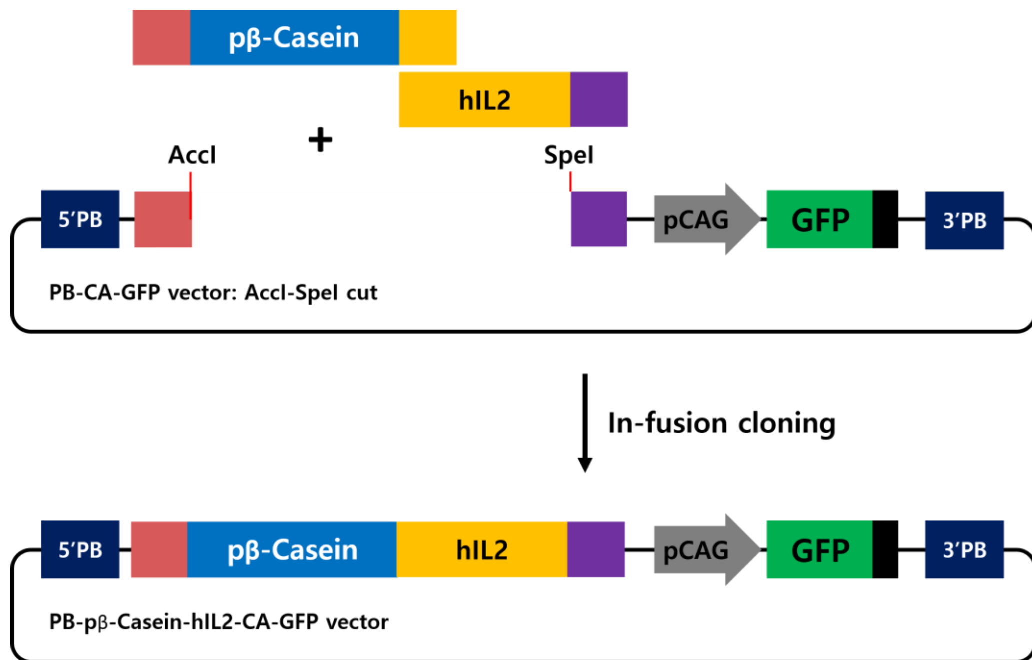


Figure 7. Illustration of PB-pβ-Casein-hIL2-CA-GFP vector with In-fusion cloning.

β-casein promoter and hIL2 was amplification by PCR using designed gene-specific primers with 15bp extensions homologous to vector ends, identical to the sequences surrounding the use restriction enzyme sites (AccI-SpeI). The two fragments and restricted PB-CA-GFP vector were fused by in-fusion enzyme.

2.2. Microinjection

The microinjection into IVF-embryos was carried out as described in the general methodology section.

2.3. RT-PCR

To confirm expression of mRNA or DNA integration, PCR and RT-PCR were carried out. Genomic DNA was extracted from blood or cells using DNA extraction kit (DNeasy Blood&Tissue kit 69506, Qiagen, Limburg, Netherlands). Total RNAs were extracted using an RNA extraction kit (Easy spin total RNA extraction kit, Cat no. 17221, iNtRON, Seongnam-si, Korea). One ug total RNAs were used for synthesizing cDNA using a cDNA synthesis kit (RNA to cDNA EcoDry™ Premix Kit, PT5153-2, Clontech, California, US). Amplification of the target DNA using specific PCR primers was performed by PCR machine (Eppendorf Vapo Protect Mastercycler, Eppendorf, Germany).

2.4. Embryo transfer and pregnancy diagnosis

All experiments with live animals were performed in accordance with the relevant laws and institutional guidelines of Seoul National University and Seoul Milk Coop, and institutional committees of Seoul Milk Coop have approved the experiments. A GFP-expressing blastocyst in PBS supplemented with 20% FBS was transferred to the uterine horn of each recipient cow by a transcervical method on Day 7 (estrus = Day 0 = day of fusion) by non-surgical approach. In order to determine embryo survival and regnancy,

cows were examined by rectal palpation and ultrasonography on Day 45 post estrus. Pregnant cows were monitored by rectal alpation and ultrasonography at regular intervals thereafter.

2.5. Genomic DNA sample preparation

Genomic DNA was extracted from blood or primary cells with DNA extraction kit, following the manufacturer's protocol. Genomic DNA was analyzed by Qubit fluorometer dsDNA assay Kit (Invitrogen, CA) as well as Infinite F200 Pro NanoQuant (TECAN, Männedorf) to verify the quality (O.D. 260/280 ratio is 1.8–2.0 and O.D. 260/230 ratio greater than 1.6) and quantity (1 ug for library construction).

2.6. Library construction and sequencing

1 μ g of genomic DNA for a 350 bp insert size was fragmented using a Covaris S2 Ultrasonicator. DNA sequencing libraries were constructed using the TruSeq DNA PCR-Free Sample Preparation Kit from Illumina (San Diego, CA). They were prepared according to the manufacturer protocol by eliminating PCR amplification steps to removes typical PCR-induced bias and streamlines. The final library size and quality were evaluated electrophoretically with an Agilent High Sensitivity DNA kit (Agilent Technologies, Santa Clara).

Sequencing was done on Illumina HiSeq 2500 using the TruSeq Paired End Cluster Kit v3 and the TruSeq SBS Kit v3-HS (FC-401-3001), generating 2 × 100 bp reads at TheragenEtex Bio Institute, Korea. Image analyses were performed using the HiSeq

control software (Version 2.2.58). Raw data was processed and base calling performed using the standard Illumina pipeline (CASAVA version 1.8.2 and RTA version 1.18.64).

2.7. Sequencing data quality control

Over about four hundred million pass-filter reads were generated per each sample. Quality control analysis of the sequencing reads was conducted using the FastQC software29 and In-house script. During data analysis, the raw reads obtained from sequencing were trimmed for low quality ends with the Sickle software (version 1.33) 30, using a Phred quality threshold of 20. All reads shorter than 50 bp after the trimming were discarded.

2.8. Variant analysis

Multi-sample calling of single-nucleotide variants (SNV) and indels was performed on processed, sample-level BAM files with the GATK Unified Genotyper32. After multi-sample calling, variants were first filtered for confident calls using a quality score cutoff of 30. The SnpEff software33 together with the UMD 3.1/bosTau Ensembl annotation was used to predict the functional effects of the variants detected.

2.9. Identification of copy number variations (CNVs) and structural variations (SVs)

To identify copy number changes in cattle, I used the Control-FREEC software 34. FREEC calculates ploidy for the regions of interest as the copy number value in each 50

kb window in the region of interest after GC content read count normalization, given a normal autosomal ploidy of 2. SVs (deletions, tandem duplications, inversions and translocations) called at nucleotide resolution with split-read support using Delly software35 that uses diploid genotype likelihoods and the best likelihood determines the final genotype. I use the 3 criteria of the precision filter as follow. First, I use the PRECISE/IMPECISE creteria. PRECISE are structural variant calls at nucleotide resolution with split-read support. I select only PRECEISE structural variant call. Second, I select > = 20 the paired-end support of the structural variant. Third, the mean mapping quality (MAQ) has to be > = 60. To compare calls between transgenic and wild type, I used bedtools software36 intersect requiring 80% reciprocal overlap (-r -f 50). If this condition is satisfied more than 2 transgenic, this SV consider the same things. And then this compared to wild type for identifying transgenic-specific SVs. Transgenic-specific CNV was called in the same way.

2.10. Transgene insertion site detection.

With mapping data BAM (aligned format) generated by BWA, I analyzed the insertion site of transgene. BWA meant that some nucleotides at either extreme of the read could be omitted (that is, “soft trimmed” or “soft clipped”) as determined by a Smith-Waterman like scoring scheme. By checking the mapped pattern of soft-clipped sequence, I inferred the insertion candidate sites. In parallel, I also used Delly to detect whether genome structural variation can be a candidate for the insertion site of transgene. Lastly, the candidate sites were also manually inspected using the IGV software.

2.11. Calculation telomere length using whole genome sequence

Whole genome data are mined for reads that are rich in telomere sequence, and relative length is determined. Using TelSeq11, I examined the frequency of reads from transgenic and control with different number of copies of TTAGGG.

3. Results

3.1. Transgene expression in somatic cells, embryos and calf

A total of nine recipients were pregnant and nine transgenic calves were naturally delivered. One died due to respiratory distress with delayed delivery, another was suffered from severe diarrhea, and died one month later and the other had been attacked by another cattle, suffered serious injury and had to be euthanized (Table 4). The remained transgenic cattle have normally grown up without any morphological abnormality to date.

Primary cells were isolated from all the transgenic calves and expressed fluorescence protein. Number of fluorescence-positive cells was calculated and summarized.

Table 4. Summary of birth of transgenic cattle using MI of DNA transposon.

I.D.	DNA	Transposase	Breed	Gender	Expression(%)*	Age
SNU-SB-1	SB-CA-YFP	SB	Beef cattle (Han-Woo)	Female	100	51 months
SNU-PB-1	PB-CA-RGRR	PB	Beef cattle (Han-Woo)	male	99	44 months
SNU-PB-2	PB-pβ-casein-hIL2-CA-GFP	PB	Beef cattle (Han-Woo)	Female	99	Died after 37 months
SNU-PB-3	PB-pβ-casein-hIL2-CA-GFP	PB	Beef cattle (Han-Woo)	male	77	Died after birth
SNU-PB-4	PB-pβ-casein-hIL2-CA-GFP	PB	Beef cattle (Han-Woo)	male	96	35 months
SNU-PB-5	PB-pβ-casein-hIL2-CA-GFP	PB	Beef cattle (Han-Woo)	male	26	Died after 1 month
SNU-PB-6	PB-pβ-casein-hIL2-CA-GFP	PB	Dairy Cattle (Holstein)	male	56	33 months
SNU-PB-7	PB-pβ-casein-hIL2-CA-GFP	PB	Dairy Cattle (Holstein)	Female	91	33 months
SNU-PB-8	PB-pβ-casein-hIL2-CA-GFP	PB	Dairy Cattle (Holstein)	male	53	33 months

* Expression percentage was calculated by ration of GFP positive cells in primary cells.

3.1.1. Transgenic cattle via SB transposon system

SB-CA-YFP and SB100X were microinjected into 191 fertilized embryos. Twenty blastocysts were formed and one of them expressed YFP without mosaicism (Figure 8). YFP expressing blastocyst were transferred into 1 recipient. A female from SB-YFP were born and is apparently healthy to date (current age: 51 months) (Figure 9).

Primary cell from ear skin biopsy of the calf expressed YFP under confocal microscopy. Transgene detected in various sample such as placenta, ear skin, and blood. This data confirmed by genomic DNA PCR (Figure 10). Primary cells were subjected to be reconstructed via SCNT to determine whether these cell lines could maintain the establishment of stable gene expression in cloned blastocysts. The cloned embryos expressed YFP signals, and blastocysts development was observed from day 6-7 after reconstruction (Figure 11).

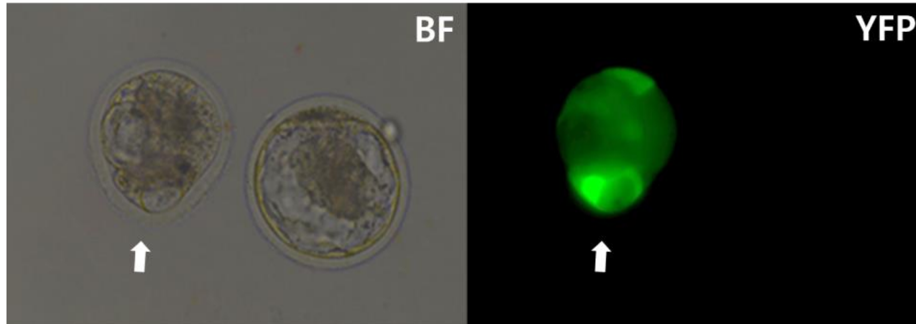


Figure 8. ***In vitro*** **development of YFP transgenic embryo via MI.**

The embryo expressed YFP via MI were developed to pre-implantation stage without abnormalities (arrow). BF: bright field.

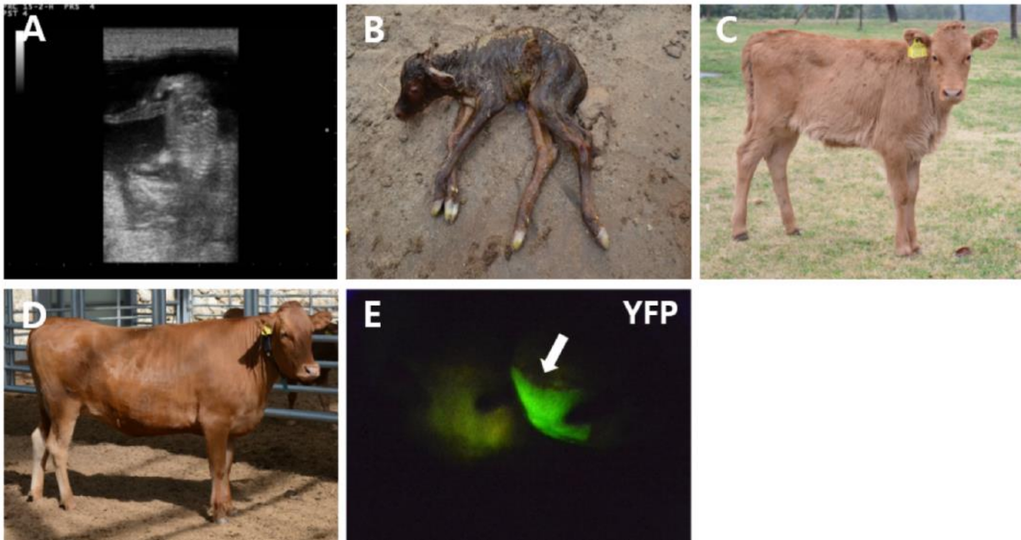


Figure 9. Birth of a transgenic cow with the YFP gene via *Sleeping beauty*.

(A) After 60 days of embryo transfer, pregnancy was confirmed by ultrasonography. The calf was delivered without assistant (B) and grew to 5-months (C) and 16 months (D) old without any health issue. (E) When ultraviolet light was exposed to nose of tg cattle, YFP expression was found (arrow).

Figure 10. Identification of SB-CA-YFP integration and expression in tissues.

To determine YFP expression in primary skin and endometrial cells, the cells were cultured and captured by confocal image equipment (A) skin cells from a wild type, (B) skin cells from a tg cattle, (C) endometrial cells from a tg cattle, upper: brightness, lower: fluorescence). The transgene integration was confirmed by PCR (D) and sequencing (E). tg: transgnecic cattle. N/C: Negative control; NFW

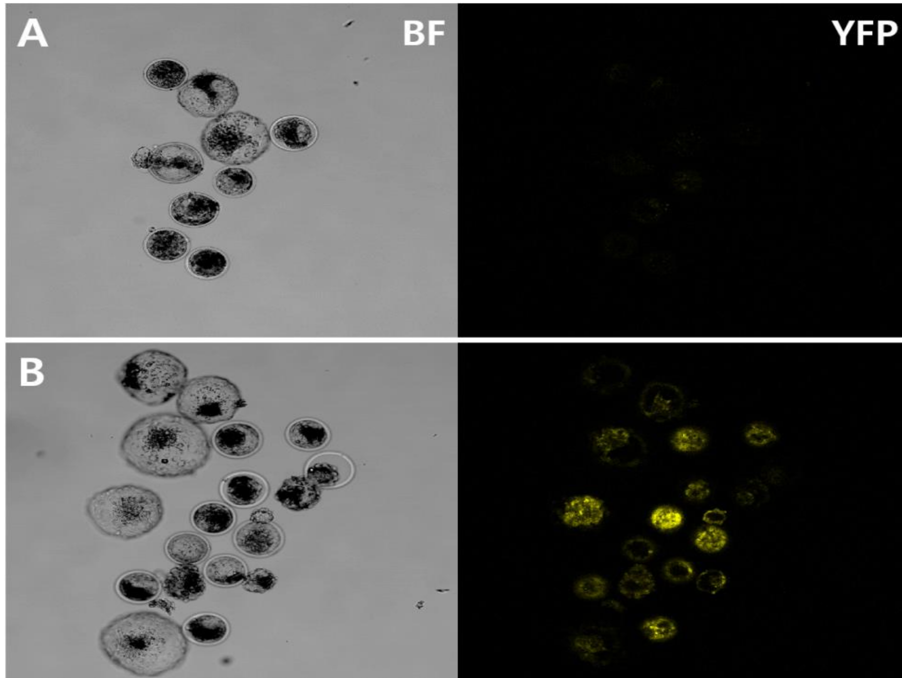


Figure 11. The cloned embryos derived from the fibroblasts of SNU-SB-1.

The primary skin cells from tg or non-tg were reprogrammed and developed into blastocysts (A: blastocysts from skin cells of non-tg cattle, B: blastocysts from skin cells of the tg cattle; left: brightness, right: fluorescence).

3.1.2. Transgenic cattle via PB transposon system

3.1.2.1. Dre-rox system in transgenic cattle

PB-CA-RGRR and transposase were microinjected into 560 fertilized embryos, and 93 blastocysts (11 GFP expression) were formed. Homogenously GFP expressing blastocyst were transferred into surrogate mother. A male from PB-CA-RGRR was born (Figure 12). To evaluate recombination in tissues and cells from the transgenic calf, GFP expressing tissues and primary fibroblasts from SNU-PB-1 were transfected with mRNA of Dre recombinase. 2 days after transfection, recombination reaction was confirmed by RFP expression via GFP gene excision and genomic DNA PCR amplification, indicating that Dre-rox recombination system must be worked in transgenic calf (Figure 13)

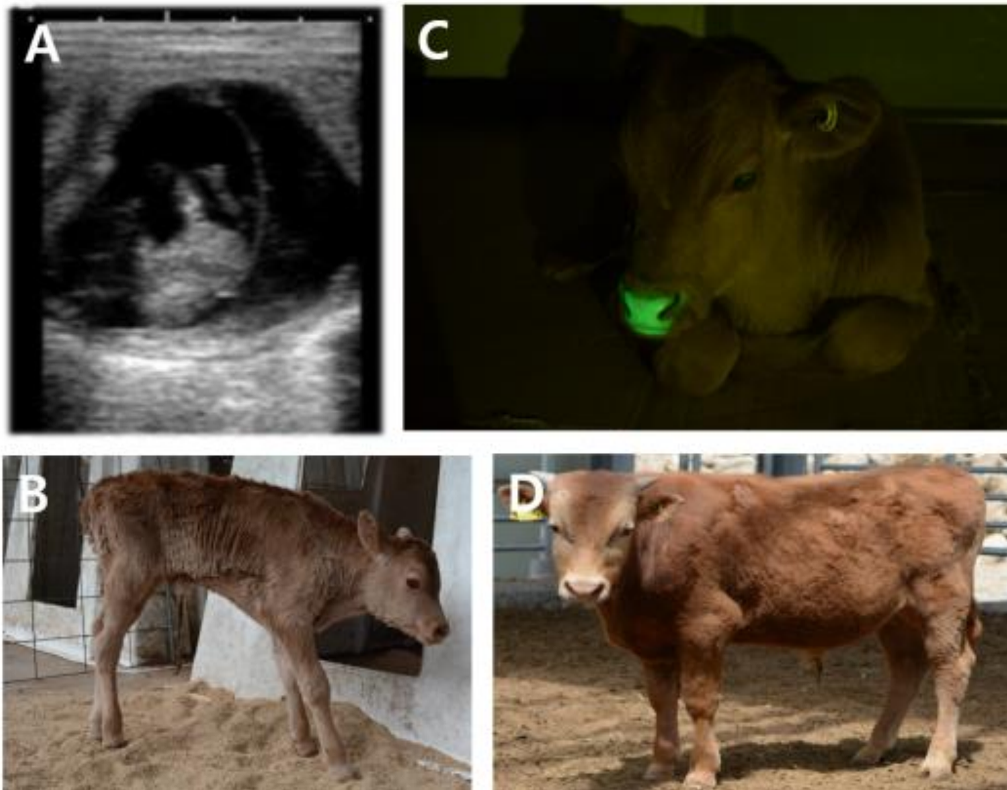


Figure 12. Birth of a transgenic cattle with the RGRR gene via *PiggyBac*.

(A) After 45 days of embryo transfer, pregnancy was confirmed by ultrasonography. (B) The calf was delivered without assistant. (C) When ultraviolet light was exposed to nose of tg cattle, GFP expression was strongly observed. And the tg cattle grew up to 12 months old without any healthy issue (D).

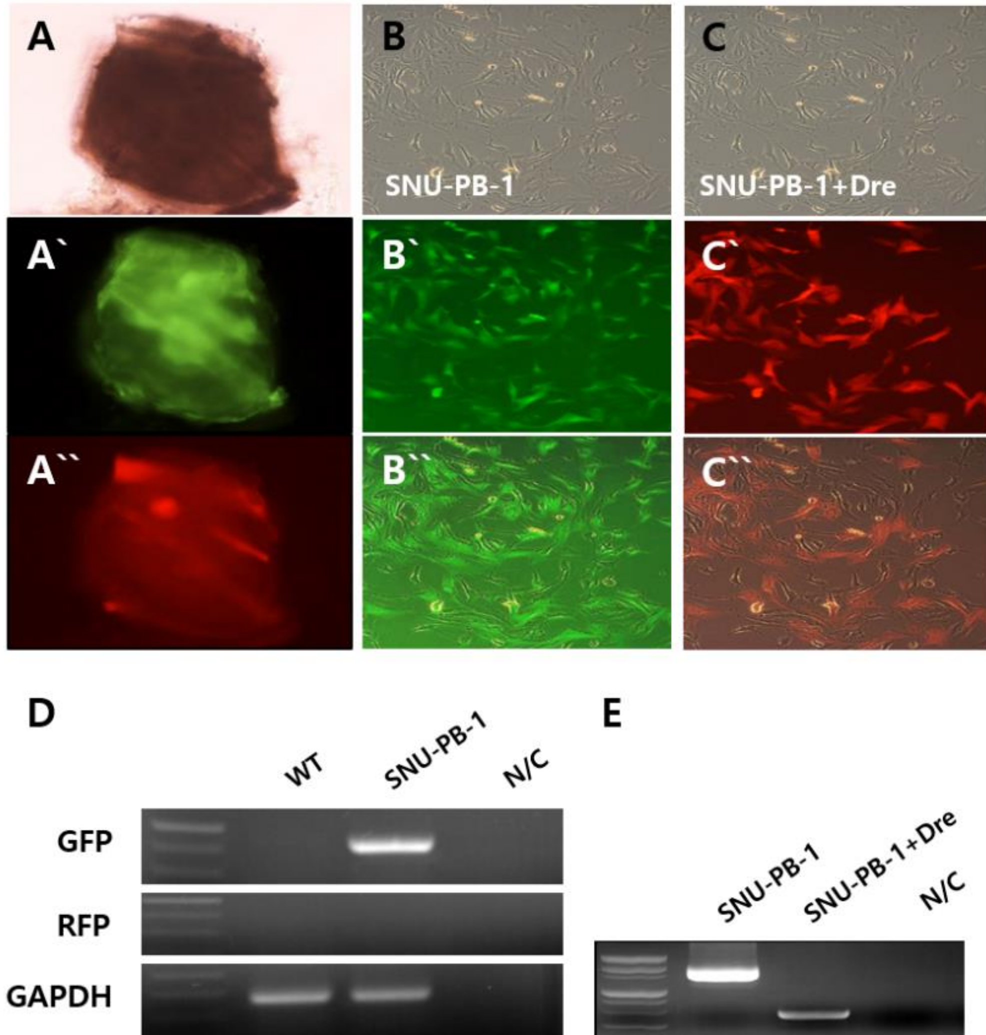


Figure 13. Identification of Dre-rox recombination in SNU-PB-1.

To determine GFP or RFP expression in a piece of tissue or primary skin cells via recombination, the tissue and cells were cultured and transfected with Dre recombinase mRNA by nucleofection (A) a piece of tissue, (B) primary cells from tg cattle brightness, (A`, B`) before Dre recombinase transfection (GFP), (A``, B``) after Dre recombinase

transfection (RFP). (D) The transgene integration confirmed by RT-PCR. (E) After Dre recombinase transfection, GFP excision was confirmed by genomic DNA PCR. N/C: Negative control; NFW

3.1.2.2. Tissue-specific expression of transgene in transgenic cattle

After microinjecting PB-pβ-casein-hIL2-CA-GFP and transposase into 4033 fertilized embryos, 49 of 779 blastocysts expressed GFP. Selected blastocysts with ubiquitous expression were transferred into 17 recipients. 7 transgenic calves were delivered without any assistance. Light or strong green color (fluorescent response) in some organs (the hooves, nose, eyes, lips and tongues) were observed under normal lights in some transgenic cattle (Figure 14-16). SNU-PB-5 which died due to severe diarrhea confirmed transgene expression in several organs, such as intestine, hear, spleen, and liver. The primary cells from the ear skin tissue of a transgenic calf were isolated, and expressed GFP and confirmed by genomic DNA PCR and RT-PCR (Figure 17).

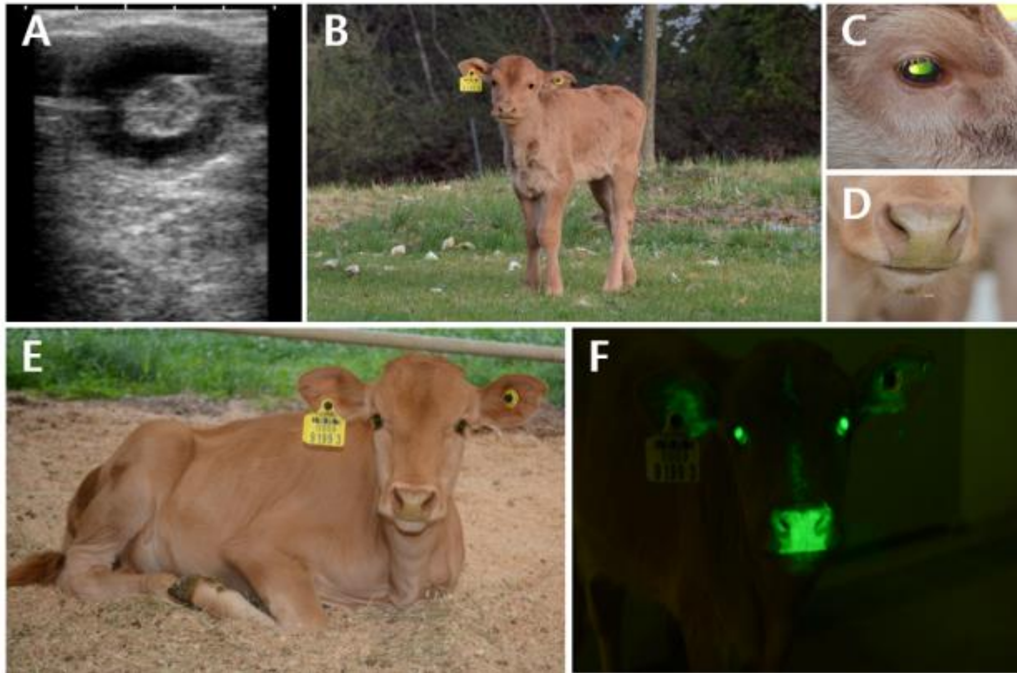


Figure 14. Birth of a transgenic cattle with the pβ-casein-hIL2-CA-GFP gene via *PiggyBac*.

(A) After 45 days of embryo transfer, pregnancy was confirmed by ultrasonography. (B) The calf was delivered without any assistance and grew up to 2 months. Analyzing the calf without ultraviolet light, GFP expression was observed in the eyes (C) and nose (D). The tg cattle have been grown to 5 months old without any health issue (E). When ultraviolet light was exposed to the head, GFP expression was strongly observed (F).

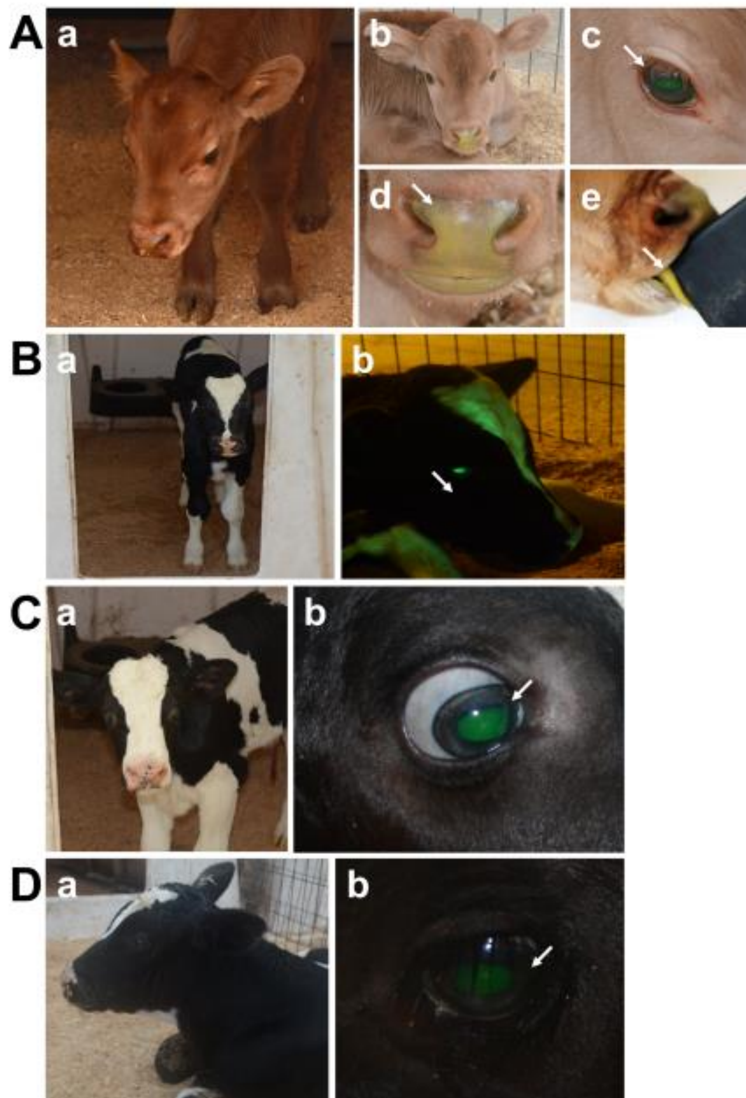


Figure 15. The pictures of four transgenic cattle.

(A) Transgenic cattle (SNU-PB-4): Pictures of the head at neonate (a) and at one month later (b), Strong GFP expression in the nose (c-arrow), eyes (d-arrow) and tongue (e-arrow); (B) Picture of transgenic cattle (SNU-PB-6): the picture of neonate calf (a) and strong expression of GFP in the eyes (b); (C) Picture of transgenic cattle (SNU-PB-7):

the picture of neonate calf (a) and strong expression of GFP in the eyes (b); (D) Picture of transgenic cattle (SNU-PB-8): the picture of neonate calf (a) and strong expression of GFP in the eyes (b).

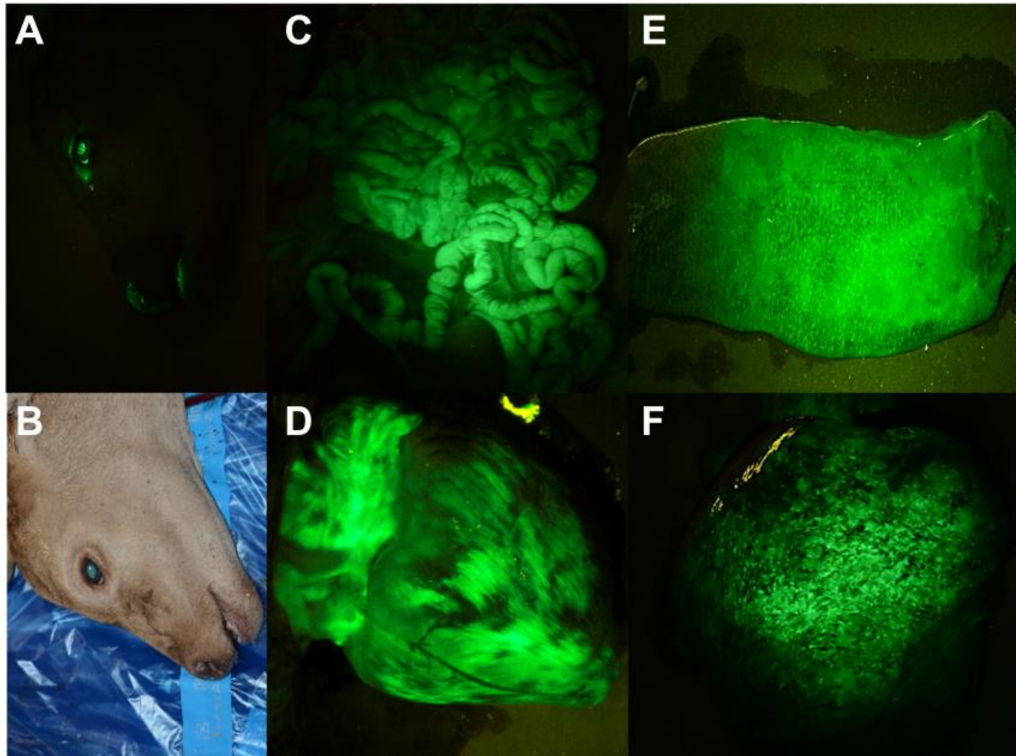


Figure 16. Autopsy of the dead transgenic cattle (SNU-PB-3).

Pictures of the head with fluorescence (A) or brightness (B). The pictures of the intestine (C), heart (D), spleen (E) and liver (F) under the fluorescence.

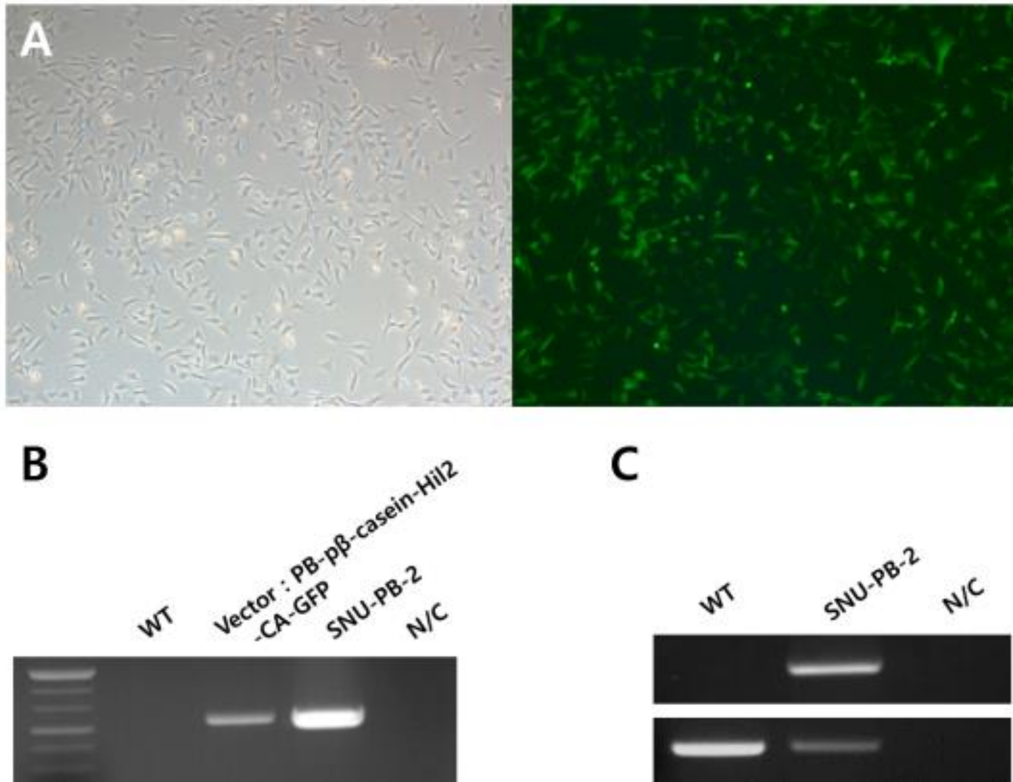


Figure 17. Identification of pβ-casein-hIL2-CA-GFP integration and expression in tissues.

To know GFP in skin cells, the primary skin cells from the tg cattle were isolated and cultured. (A) In over 99% of cells, GFP expression were observed. The transgene integration was confirmed by genomic DNA PCR (B) and RT-PCR using primary cells (D). N/C: Negative control; NFW.

3.2. Copy number and integration site

To detect integration events of transgene, single-nucleotide variants (SNV), structural variation (SV), and copy number variations (CNV), whole genome sequence from three transgenic and wild type cattle blood samples were analyzed. On average, more than 60 giga base pairs (Gbp) per sample were produced (Table 5). Based on the sequencing quality metrics, I estimated about 16-fold coverage of whole genome of cattle with the quality passed and aligned paired-end reads. The average mapping rate to the cow reference genome (UMD3.1) was over 99.73% (Table 5).

For integration site and copy number, all the transgene sites were found by the Integrative Genomics Viewer (IGV) program (https://www.broadinstitute.org/igv/, Broad Institute). All the integrated sites including exact position and 5′ -, 3′ - flanked genes were summarized Table 6 and illustrated in Figure 18. The YFP gene (SNU-SB-1) was integrated in chromosomes 4, 21 and 26. One transgene was integrated in intron between exons 1 and 2 at chromosome 4, locus designed for GNAI1 (Genbank assess NM_174324.2). To evaluate transcripts of GNAI1, RT-PCR was performed and its expression was not shown to be affected (Figure 19).

The RGRR gene (SNU-PB-1) was integrated in chromosomes 1, 2, 3, 4, 5, 6 (two sites), 7, 14, 17, 22, 25, GJ0599801.1, 26 and X. The pβ-casein-hIL2-CA-GFP gene was integrated in chromosomes 3 (two sites), 5 (three sites), 6, 7, 9, 10, 11 (two sites), 15, 18 and X (two sites).

All of the integration sites were confirmed manually by PCR with endogenous and exogenous specific primers (Figure 20-22 and Table 7-8).

Table 5. Summary of sequencing results for transgenic and wild type cattle.

I.D. (DNA; Resources)	Reads	Mapped Reads	Raw coverage*	Analysis coverage*
Wild type (No transgene; Blood)	542,361,156	540,861,561 (99.72%)	22.86	16.06
SNU-SB-1 (SB-CA-YFP; Blood)	532,811,029	531,298,284 (99.72%)	22.44	15.80
SNU-PB-1 (PB-CA-RGRR; Blood)	537,542,347	536,035,830 (99.72%)	22.86	15.78
SNU-PB-2 (PB-pß-casein-hIL2-CA-GFP; Blood)	537,419,066	536,196,898 (99.77%)	21.64	15.88

*Raw coverage corresponds to the sequencing reads generated from machine. Analysis coverage is calculated from quality filtered reads and this dataset is used for insertion site discovery.

Table 6. All integration sites in transgenic cattle.

I.D	No	Chromosome	Insertion site	Orientation	Overlapping gene	Location	5' gene	3' gene
SNU-SB-1	1	4	41,232,050–41,232,051	Reverse	GNAI1	E1-2 intron	GNAT3	PHTF2
	2	21	28,416,682–28,416,683	Forward	—		TRPM1	APBA2
	3	26	48,405,454–48,405,455	Forward	—		MKI67	EBF3
SNU-PB-1	1	1	2,651,736–2,651,737	Reverse	—		MIS184	HUNK
	2	2	31,593,723–31,593,724	Forward	—		SLC38A11	COBLL1
	3	3	54,580,493–54,580,494	Forward	—		GBP5	GBP4
	4	4	95,433,564–95,434,563	Forward	—		TSGA13	MKLN1
	5	5	4,588,449–4,588,450	Reverse	—		ATXN7L3B	CAPS2
	6	6-1	20,085,913–20,086,912	Forward	—		DKK2	GIMD1
	7	6-2	99,730,977–99,731,976	Reverse	PLAC8	E3-4 intron	PLAC8	COQ2
	8	7	31593691–31593728	Forward	—		ERAP2	LNPEP
	9	14	53,149,061–53,149,062	Reverse	—		CSMD3	CSMD3
	10	17	55,906,674–55,907,673	Forward	KDM2B		ORAI1	RNF34
	11	22	43,933,057–43,933,058	Forward	SLMAP	E1-2 intron	bta-mir-2370	DENND6A
	12	25	30,150,644–30,151,643	Forward	—		AUTS2	ENSBTAG00000047342
	13	26	38,489,750–38,490,749	Reverse	—		EMX2	RAB11FIP2

	14	GJ059980.1	21,074–22,073	Forward	—		—	—
	15	X	143,271,631–143,272,662	Forward	UTY		WWC3	DDX3Y
SNU-PB-2	1	3-1	9,538,861–9,539,321	Forward	ENSBTAG00000005796		PEX19	PEA15
	2	3-2	79,749,737-79,750,160	Forward	MGC137454		PDE4B	OB-R
	3	5-1	50,479,229-50,479,653	Reverse			TMEM5	AVPR1A
	4	5-2	55,868,825-55,869,356	Forward			XRCC6BP1	CTDSP2
	5	5-3	75,829,995-75,830,420	Reverse			MPST	KCTD17
	6	6	40,200,341-40,200,867	Reverse			LCORL	SLIT2
	7	7	104,834,424-104,835,058	Reverse			C7H5orf30	NUDT12
	8	9	85,788,192-85,788,618	Forward			STXBP5	SAMD5
	9	10	85,854,063-85,854,558	Forward	LIN52		ALDH6A1	VSX2
	10	11-1	58,260,496-58,260,907	Forward			PTP	LRRTM4
	11	11-2	107,297,269-107,297,597	Forward			PSMD13	
	12	15	36,723,446-36,723,809	Forward	SOX6		SMAP	INSC
	13	18-1	8,775,609-8,776,056	Forward	MPHOSPH6		HSD17B2	CDH13
	14	X-1	91,193,469-91,194,016	Forward			ARAF	SYN1
	15	X-2	80,581,077-80,581,395	Forward			PBDC1	MAGEE2

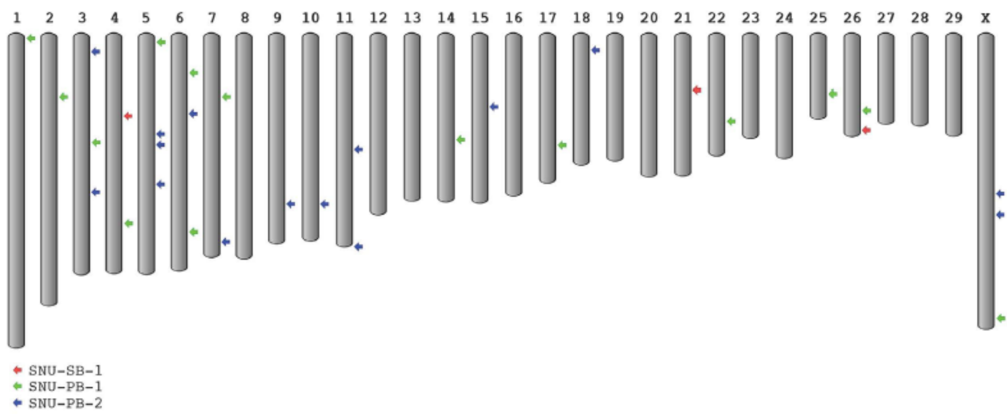


Figure 18. Analysis of transgene integration sites in cattle showed that shared integration of site and unique site existed as integration event.

Each index color showed individual sample.

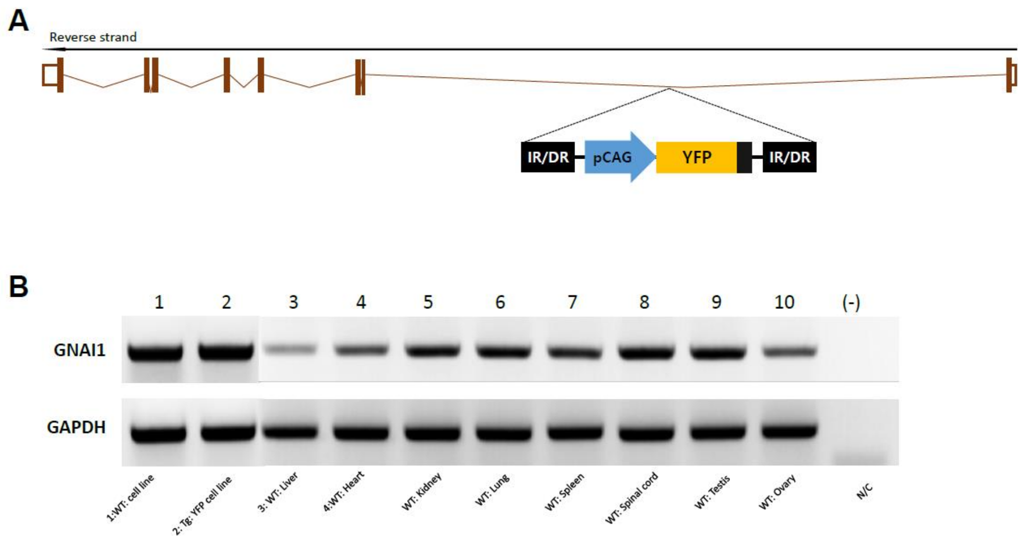
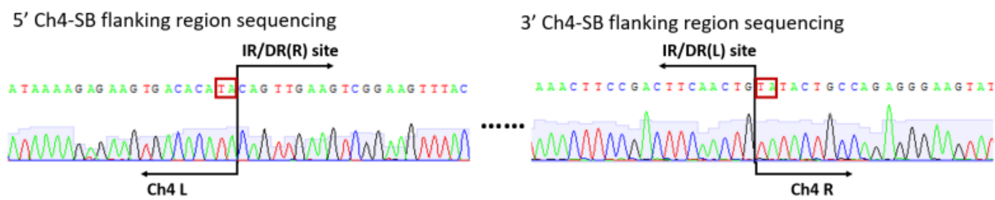


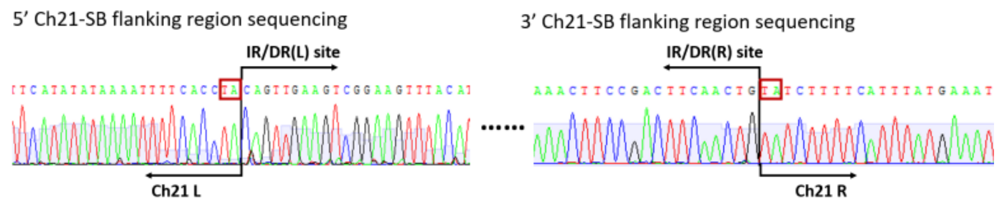
Figure 19. Guanine Nucleotide Binding Protein (G Protein), Alpha Inhibiting Activity Polypeptide 1 (GNAI1) expression in various organ.

(A) Schematic representation of insertion for GNAI1 in the Tg cattle. (B) RT–PCR analysis for GNAI1 mRNA in Skin Fibroblasts from a wild-type (WT), Transgenic cattle and various organs of WT.

Ch4: 41,232,050 – 41,232,051 (Reverse-GNAI1)



Ch21: 28,416,682 – 28,416,683 (Forward)



Ch26: 48,405,454 – 48,405,455 (Forward)

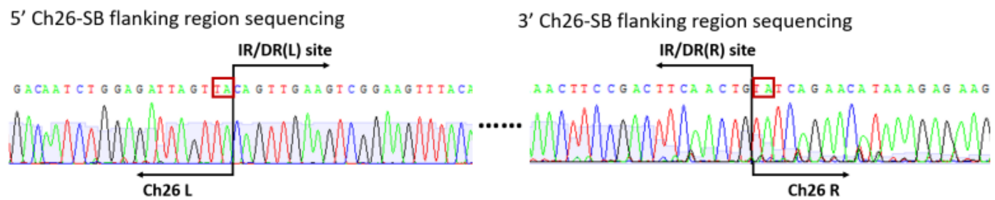
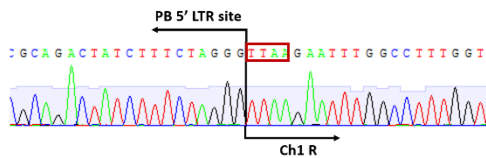


Figure 20. Identification of integration and TA duplication in the insertion sites in SNU-SB-1 by sequencing.

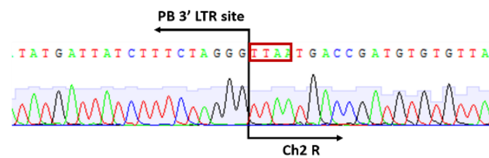
Ch1: 2,651,736 – 2,651,737 (Reverse)

3' Ch1-PB flanking region sequencing



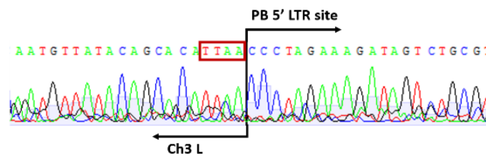
Ch2: 31,593,723 – 31,593,724 (Forward)

3' Ch2-PB flanking region sequencing



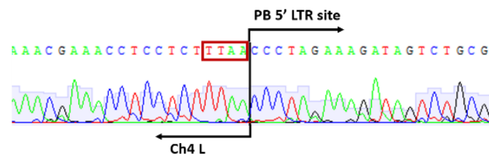
Ch3: 54,580,493 – 54,580,494 (Forward)

5' Ch3-PB flanking region sequencing



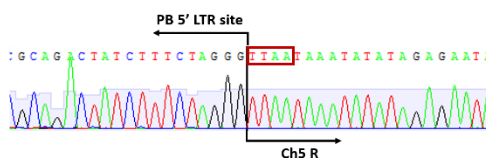
Ch4: 95,433,564 – 95,434,563 (Forward)

5' Ch4-PB flanking region sequencing



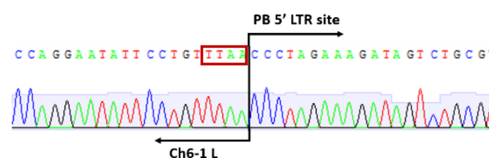
Ch5: 4,588,449 – 4,588,450 (Reverse)

3' Ch5-PB flanking region sequencing



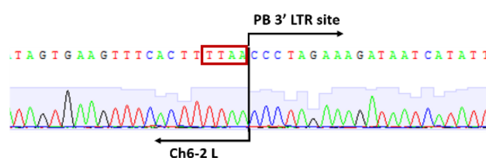
Ch6-1: 20,085,913 – 20,086,912 (Forward)

5' Ch6-1-PB flanking region sequencing



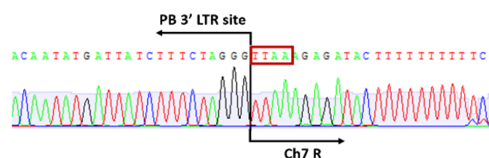
Ch6-2: 99,730,977 – 99,731,976 (Reverse)

5' Ch6-2-PB flanking region sequencing



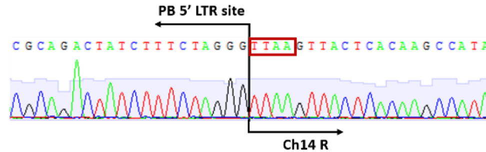
Ch7: 31,593,691 – 31,593,728 (Forward)

3' Ch7-PB flanking region sequencing



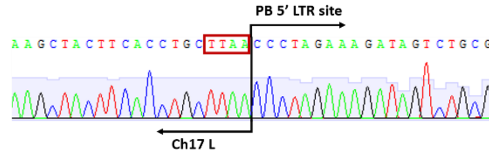
Ch14: 53,149,061 – 53,149,062 (Reverse)

3' Ch14-PB flanking region sequencing



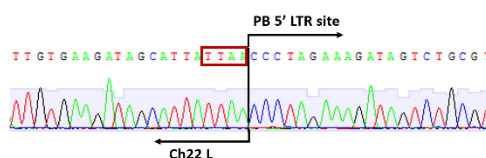
Ch17: 55,906,674 – 55,907,673 (Forward)

5' Ch17-PB flanking region sequencing



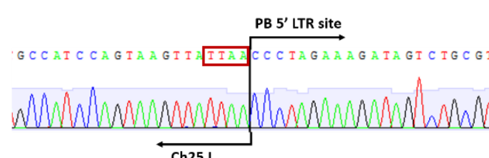
Ch22: 43,933,057 – 43,933,058 (Forward)

5' Ch22-PB flanking region sequencing



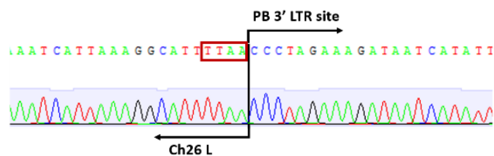
Ch25: 30,150,644 – 30,151,643 (Forward)

5' Ch25-PB flanking region sequencing



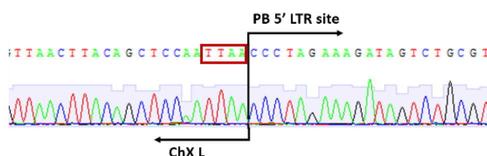
Ch26: 38,489,750 – 38,490,749 (Reverse)

5′ Ch26-PB flanking region sequencing



ChX: 143,271,631 – 143,271,852 (Forward)

5′ ChX-PB flanking region sequencing



GJ059980.1 21,074 – 21,525 (Forward)

5′ GJ059980.1-PB flanking region sequencing

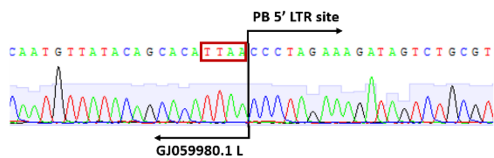
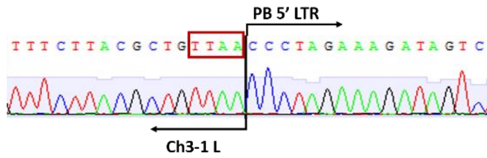


Figure 21. Identification of integration and TTAA duplication in the insertion sites in SNU-PB-1 by sequencing.

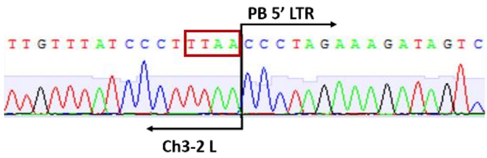
Ch3-1: 9,538,861-9,539,321 (Forward)

5′ Ch3-1-PB flanking region sequencing



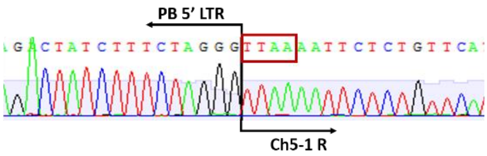
Ch3-2: 79,749,737-79,750,160 (Forward)

5′ Ch3-2-PB flanking region sequencing



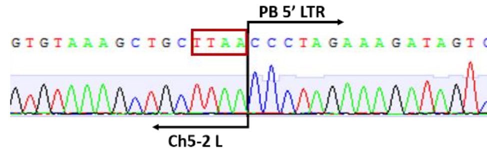
Ch5-1: 50,479,229-50,479,653 (Reverse)

3′ Ch5-1-PB flanking region sequencing



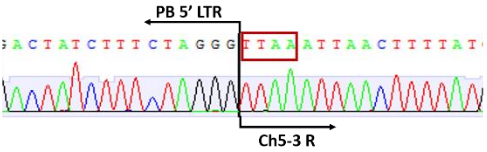
Ch5-2: 55,868,825-55,869,356 (Forward)

5′ Ch5-2-PB flanking region sequencing



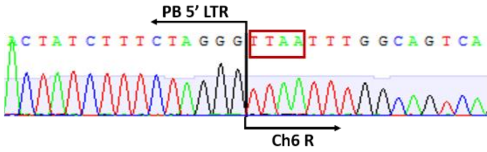
Ch5-3: 75,829,995-75,830,420 (Reverse)

3′ Ch5-3-PB flanking region sequencing



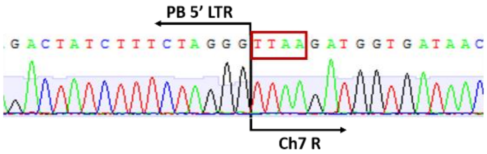
Ch6: 40,200,341-40,200,867 (Reverse)

3′ Ch6-PB flanking region sequencing



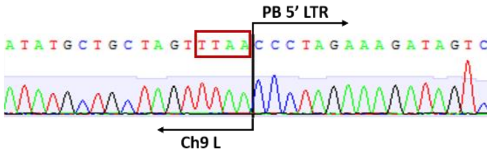
Ch7: 104,834,424-104,835,058 (Reverse)

3′ Ch7-PB flanking region sequencing



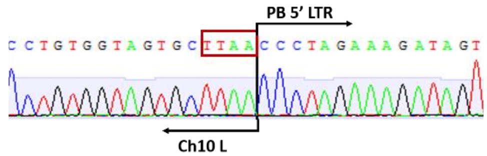
Ch9: 85,788,192-85,788,618 (Forward)

5′ Ch9-PB flanking region sequencing



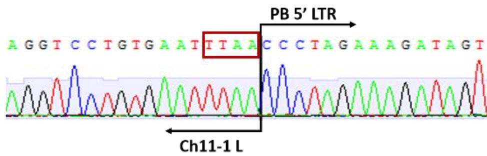
Ch10: 85,854,063-85,854,558 (Forward)

5′ Ch10-PB flanking region sequencing



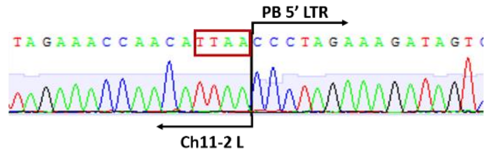
Ch11-1: 58,260,496-58,260,907 (Forward)

5′ Ch11-1-PB flanking region sequencing



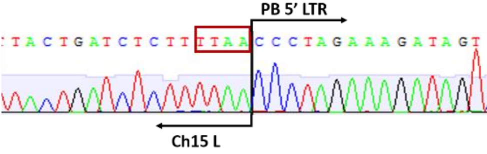
Ch11-2: 107,297,269-107,297,597 (Forward)

5′ Ch11-2-PB flanking region sequencing



Ch15: 36,723,446-36,723,809 (Forward)

5′ Ch15-PB flanking region sequencing



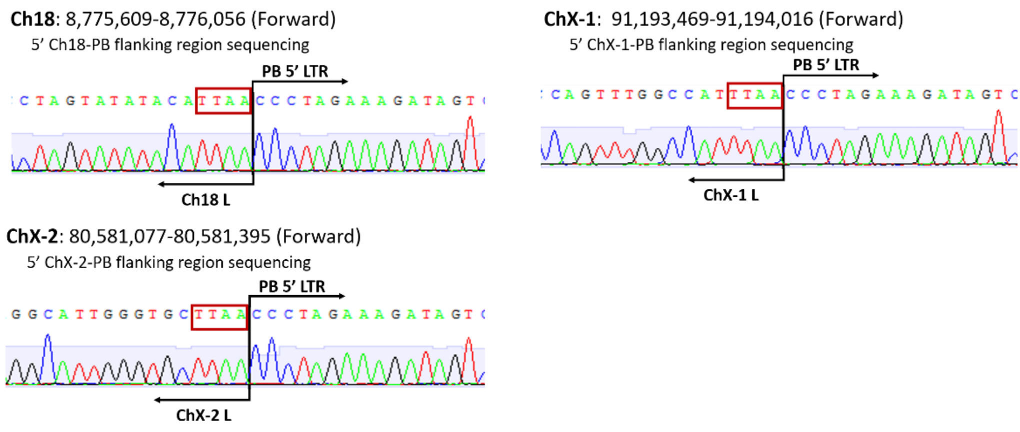


Figure 22. Identification of integration and TTAA duplication in the insertion sites in SNU-PB-2 by sequencing.

Table 7. Primer sequence of manual PCR on transgene integration site.

I.D.	No.	Chromosome		Primer sequences(5' - 3')
SNU-SB-1	1	4	F	TGGAGTCTGCAAACAGGTGT
			R	CTCTAGATGGCCAGATCTAG
	2	21	F	GTACACTGGCAACAACCACA
			R	AGGCACAGTCAACTTAGTGT
	3	26	F	CGTGTTTACAGTCAGTGCAG
			R	AGGCACAGTCAACTTAGTGT
SNU-PB-1	1	1	F	TTTTGACTCACGCGGTCGTT
			R	ATGGTTAACAAAGGGAATGTGG
	2	2	F	GCTTGGATCCCTCGAGTTAA
			R	CATGAACCAAAGATTCTTCAGGC
	3	3	F	GCTAACACCTATCCTGCTCA
			R	TTTTGACTCACGCGGTCGTT
	4	4	F	TCATCTTGGTGGTGGTGATT
			R	TTTTGACTCACGCGGTCGTT
	5	5	F	TTTTGACTCACGCGGTCGTT
			R	TTGCACACACCTTGAGTAAG
	6	6-1	F	CCATGGTCCCAGGAAATACA
			R	GAGAGGATATGCTCATCGTC
	7	6-2	F	TGTGGTGTCAGAATGTCTGA
			R	GCCAAAGTTGTTTCTGACTG
	8	7	F	GCTTGGATCCCTCGAGTTAA
			R	CTGCTGTTTCCTAACAGGAG
	9	14	F	TTTTGACTCACGCGGTCGTT
			R	GGCAGTCCATTTGAACTATTGA
	10	17	F	GCTGGATTCTAGAAACACCG
			R	TTTTGACTCACGCGGTCGTT
	11	22	F	TAGAGGAGTGGTGGACATGT
			R	TTTTGACTCACGCGGTCGTT
	12	25	F	GAGAGAAGGCTCCTGATCCA
			R	TTTTGACTCACGCGGTCGTT
	13	26	F	CAATCTCTTGAGCAGCCTCT
			R	GCCAAAGTTGTTTCTGACTG
	14	GJ059980.1	F	TCATTCTATGAGGCCACCAT
			R	TTTTGACTCACGCGGTCGTT
	15	X	F	ATACGCAAAGGCGAGATCGA
			R	TTTTGACTCACGCGGTCGTT
SNU-PB-2	1	3-1	F	GAGTTGGGGTATTGTTCTTGG
			R	TTTTGACTCACGCGGTCGTT

	2	3-2	F	AATGATGGCAAGCCCTCATC
			R	TTTTGACTCACGCGGTCGTT
	3	5-1	F	TTTTGACTCACGCGGTCGTT
			R	ATGAATCCTGCCCTAAAGTG
	4	5-2	F	CTTCACGGAATTTGGGGTAT
			R	TTTTGACTCACGCGGTCGTT
	5	5-3	F	TTTTGACTCACGCGGTCGTT
			R	CCACTCCAGTATTCTTGCCT
	6	6	F	CTTGGCTGCTTATCGGATTG
			R	GCTTGGATCCCTCGAGTTAA
	7	7	F	TTTTGACTCACGCGGTCGTT
			R	TACAGAGCATGCCCTAGTTC
	8	9	F	CACCCTGAGTTCTGTATTCC
			R	TTTTGACTCACGCGGTCGTT
	9	10	F	GTTGGTTGGTTTGAATCAGGA
			R	TTTTGACTCACGCGGTCGTT
	10	11-1	F	AGGCAATGTAACAACCTTCT
			R	TTTTGACTCACGCGGTCGTT
	11	11-2	F	GACACTGAGATCTGACTGTC
			R	TTTTGACTCACGCGGTCGTT
	12	15	F	TGCTTCCACTCCCACTTAAG
			R	TTTTGACTCACGCGGTCGTT
	13	18	F	CATCAACCAAGCTTAGAGGT
			R	TTTTGACTCACGCGGTCGTT
	14	X-1	F	GCACCGAGCAACAAAAAAGG
			R	TTTTGACTCACGCGGTCGTT
	15	X-2	F	TTCCACTGGGGTCTAACTCT
			R	TTTTGACTCACGCGGTCGTT

Grey box: for exogenous (transgene) gene, White box: for endogenous gene.

Table 8. Primer sequence of genomic DNA PCR and RT-PCR for transgenic cattle.

Genomic DNA-PCR		
SNU-SB-1	1F	TCTGCTAACCATGTTCATGCC
	1R	AAGAAGATGGTGCGCTCCTG
	2F	GGGCAACGTGCTGGTTATTG
	2R	ACTTGAAGAAGTCGTGCTGC
SNU-PB-1	F	GCTCTAGAGCCTCTGCTAA
	R	TTGTGGATCTCGCCCTTCAG
SNU-PB-2	F	CAGCCAAGGTCTGTAGCTAC
	R	CACTTCCTCCAGAGGTTTGA
RT-PCR		
GFP	F	AACGGCCACAAGTTCAGCGT
	R	TCACCTTGATGCCGTTCTTC
RFP	F	AACGGCCACGAGTTCGAGAT
	R	TTGTGGATCTCGCCCTTCAG
GAPDH	F	TGCACCACCAACTGCTTGGC
	R	CACGTTGGGAGTGGGGACGC

3.3. Identification of transgenic variants compared to wild type

In transgenic and wild type, overall, about 8.1 million SNVs and 1.0 million insertions and deletions (Indels) were identified (Table 9). Using this data, I investigated the transgenic-specific SNV. The number of transgenic-specific SNV, as "high impact" by SnpEff software (http://snpeff.sourceforge.net/, version 4.2) were 315 (Table 10). Furthermore, I also identified the transgenic-specific SV and CNV were 65 and 38, respectively. The SV event was consisted of 49 deletions, 2 duplications, 8 inversions and 6 translocations. In the case of CNVs, there were 33 gains and 5 losses. In my analysis, SNP density of chromosome 12 and 23 in all samples were very high compared to other chromosomes (Fig. 23).

Table 9. Statistics of SNP and INDEL.

I.D. (DNA)	The number of SNP	The number of INDEL
Wild type (No transgene)	8,113,244	1,141,867
SNU-SB-1 (SB-CA-YFP)	8,194,444	1,156,313
SNU-PB-1 (PB-CA-RGRR)	8,146,673	1,166,927
SNU-PB-2 (PB-pß-casein-hIL2-CA-GFP)	8,127,879	1,142,798

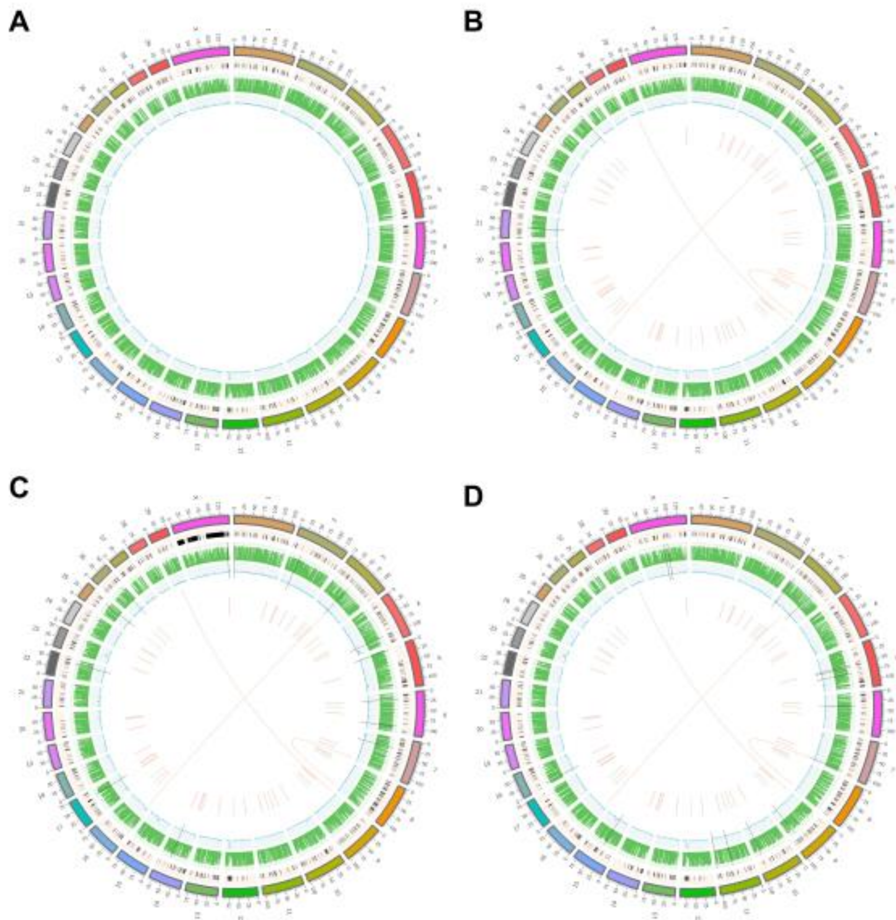


Figure 23. Overview of genomic variation in cattle.

Reference chr (stands for chromosome) containing from chr1 to X chr is colored in a variety of different colors in peripheral boxes. And, copy number variation (CNV), coverage and histogram indicating SNP density of sample plotted per 10-kb windows are colored in black, green and blue colors, respectively. Structural variations (SVs) including deletion (red), translocation (orange), inversion (green) and duplication (blue)

are indicated by lines and links. Black lines passed through the coverage (green) and the histogram (blue) refers to the integration sites of transgenes in the reference chr. (a) Wild type, (b) A transgenic cattle using SB transposon (SNU-SB-1), (c) A transgenic cattle using PB (SNU-PB-1), (d) A transgenic cattle using PB (SNU-PB-2).

Table 10. Statistics of SNP and INDEL in comparison of transgenic cattle to wild type.

Type	SNP	INS	DEL	Total
All variants	2,016,456	90,135	108,135	2,214,992
High impact only*	177	65	73	315

*Using cattle genomes (UMD3.1.78), SnpEff was applied to predict high-impact single nucleotide variant (SNV) resulting in gain/loss stop codon, frame-shift, splice site changes (donor or acceptor) or loss of start codon in these elite natural variants.

3.4. Telomere length analysis

Telomeric sequences (TTAGGG) were measured by analysis software, used as in a previous study [77]. Its length was described in Table 11 (SNU-SB-1: 6.59, SNU-PB-1:7.26, SNU-PB-2: 6.98, Wild type: 5.69).

Table 11. Relative telomere lengths in cattle.

Sample (DNAs; Resources)	Estimated telomere length
Wild Type : 24 months old (No transgene; Blood)	5.68661
SNU-SB-1 : 10months old (SB-CA-YFP; Blood)	6.59096
SNU-PB-1 : 4 months old (PB-CA-RGRR; Blood)	7.26370
SNU-PB-1 (PB-CA-RGRR; Primary cells)	7.61535
SNU-PB-2 : 2 months old (PB-β-casein-hIL2-CA-GFP; Blood)	6.98291

4. Discussion

Transgenic cattle in agriculture fields have been of interest due to basic embryology and genetic models. Although several trials to generate transgenic cattle have been carried out, the number of live transgenic cattle and germ line transmission of transgene into NGS have been hampered to date. While live transgenic cattle and germ line transmission using lentiviral-mediated transgenesis has been applied successfully [78], the issue that viral gene delivery may cause oncogenic activation remains. As an alternative approach, nuclear transfer is considered. It has several disadvantages such as very low efficiency, abnormalities and sudden deaths. To overcome those issues on transgenic cattle, here, I reported efficient production of transgenic cattle using the transposon system. Furthermore, transgene integration and genome variants were analyzed by NGS for genomic stability of transgenic cattle. DNA transposon is well established to generate target gene overexpression in rodents, particularly gene function via mutagenesis [79] or cancer study [75]. Additionally, in human cells, SB or PB delivery have been used for gene therapy [55, 80, 81]. Unlike mice, development of gene function via transgene delivery in mutant live offspring in livestock have been slow to garner to attention due to low efficiency or severe mosaicism of MI and nuclear transfer [82] at greater costs. Recently, SB transposon has been successfully applied to generate transgenic pigs and its germline transmission [38]. However, progress of transgenic cattle has relatively been very slow due to long-term gestational periods (around 280 days) and single calf pregnancy even though several transgenic cattle using nuclear

transfer has been born with low efficiency. Here, I introduced two DNA transposons (SB and PB) for generating transgenic cattle.

I produced three kinds of transgenic cattle. First, a transgenic cattle expressing transgene (YFP) under the ubiquitous promoter was born via a SB transposon method. Second, a transgenic cattle with conditional gene expression by Dre recombinase was born via a PB transposon. In this cattle, ordinarily GFP transgene was expressed in the whole body. Furthermore, after Dre recombinase treatment, GFP gene excision occurred and sequentially RFP gene was expressed. Lastly, several transgenic cattle with tissue specific promoter (β-casein)-human gene (IL2) with reporter gene (GFP) via PB transposon were born. During this study, I did not find either any miscarriage or stillbirths in recipient cow after diagnosing pregnancy or health problems in growing cattle.

One of the most important issues in transgenic animals is integration-number, -site and expression of the transgene because it may affect the lethality or gene silencing [8, 32]. Theoretically, when target gene by transposons (jumping gene) move into another site, it has moved into its preference sequences (TA for SB and TTAA for PB) [15]. In this study, to confirm transposon preference and genome instability (copy number variation, structure variation and telomere lengths), the genome from blood of these transgenic cattle was analyzed by NGS. As expected, the genes by SB and PB were integrated into TA or TTAA position, respectivel. While a few transgene copies were inserted intron of coding gene, most transgene were integrated in non-coding region. Even though

transgene were integrated in intron of coding gene (exons), its transcriptional expression was not changed.

In the previous reports on transgenic animals or plants generated by transposons and plasmids, they did the integration or expression based on conventional PCR approaches [13, 83, 84]. Its disadvantage is to find out only amplified products with primer conditions, indicating that not all the transgenes can be identified. Transgene insertion site is not typically characterized because traditional methods for transgene insertion site discovery are either expensive and/or offer low resolution (DNA FISH) or are complicated by the multi-copy nature of the inserted sequences (inverse PCR). However, whole genome sequencing enables us to find out all the integration details with high specificity at single-nucleotide resolution and also provided information on the chromosomal location and transgene copy number [85, 86]. Indeed, in my study NGS analysis provided transgene integration number and position with single nucleotide resolution. Furthermore, I hypothesized that as the transposon moved initial site into another position, the genome variants such as SNP, SV, CNV and telomere lengths might be affected in these transgenic cattle. When I analyzed the genome variants in 5′- and 3′-region (1 kbps) of the transgene integration positions, there were no significant genomic variants. On chromosomes 12 and 23, on the other hand, I found high variable regions as previously reported [87]. The result indicated that it was breed-specific characteristics, not transgenic cattle.

Using NGS analysis, relative telomere lengths, which is co-related to age of individual were measured on the transgenic cattle to know if transposition of transgenes might

affect the telomere length or not. Although there were no considerable changes in telomere length, only one transgenic cattle showed shorter size telomere compared to other individuals. To figure out whether senescence changes could be identified for the transgenic cattle, its development to adult will be monitored.

PART IV.

GERMLINE TRANSMISSION IN TRANGENIC CATTLE

Chapter I. Health and germline transmission of transposon-mediated gene transfer in cattle

This chapter is based on the publication; Yum SY et al: Long-term health and germline transmission in transgenic cattle following transposon-mediated gene transfer. *BMC genomics*. 2018, 19:387

1. Introduction

Transposon gene delivery is a valuable system for usage in gene therapy, ex vivo gene delivery, *in vitro* cell lines, and animal models [88-91]. Among various DNA transposons, SB and PB have been used to deliver exogenous genes into cell lines and generate animals. In mice and rats, transposon-mediated transgenic offspring have been born and used for studies. Transposons such as SB and PB have even been successfully used to produce transgenic livestock in pigs, sheep, goat, and cattle [34, 35, 38, 92-94], and germline transmission was particularly verified in pigs [95].

The efficient integration of transgenes into the genomes of target cells or animals and their stable expression is a valuable property of the transposon system. Transposase specifically binds the recognition sequences of the transposon and induces the 'cut and paste' transposition of a transposon vector and its site-specific integration into the

genome free from vector backbone DNA (SB integrates at TA, PB integrates at TTAA) [96]. This system can be easily operated with various non-viral delivery systems such as chemical-based transfection, electroporation, or MI. Compared with viral delivery systems, transposons have higher security and safety [97, 98]. For this reason, the SB transposon has been approved by the NIH-OBA and FDA for testing in humans [99-101].

Cellular-based studies show that a preferable random insertion of SB and PB could introduce insertional mutagenesis [88, 102]. To date, because transposon-based gene transfers can occur in non-coding region sites, geno-toxicity safety issues are much lower than in viral-mediated delivery. In the same line, my study on analyzing transgene-integrating sites in transgenic cattle showed that all the transgenes were integrated in the non-functional region [34]. However, there may be a difference between genomic analysis and general clinical data. Although several transgenic cattle generated via transposon-, viral vector-, or SCNT-mediated gene transfers have been born, there has been no report on long-term monitoring of the next generation and health issues. Therefore, I hypothesize that multi-copy transgene integration in transgenic cattle (founders) may not affect long-term survival (over 3 years) and germline-transmission in transgenic cattle.

2. Materials and Methods

2.1. Cell isolation and culture

The primary cultures of fibroblasts were derived from ear skin biopsies of transgenic cattle. Ear skin was minced into 3–4 mm pieces with a sterile scalpel. Each biopsy was washed several times and incubated at 37 ℃ for 16 h in the HBSS with collagenase (Collagenase type IV, Gibco). The dispersed cells were washed in HBSS and cultured in DMEM that contained 1% P/S, 10% FBS, 100 mM β-ME and 1% NEAA.

2.2. Semen collection and freezing

Semen from a male transgenic founder was collected using an artificial vagina (Fujihira Industry, Tokyo, Japan) containing warm water at 50–55 ℃. After collecting the semen, it was transported immediately into the laboratory for freezing. The semen was diluted 50%:50% using OPTIXcell (IVM technologies, France) and kept at room temperature for 10 minutes. Thereafter, the first diluted semen was diluted again 50%:50% and a sperm concentration of 5.0 × 107/ml was kept at 4 ℃ for 2 h. The concentrated sperm was loaded into a 500 ul semen straw (IMV technologies, France) and sealed with straw powder (Fujihira Industry, Tokyo, Japan). The straw was frozen above 5.0 cm from liquid nitrogen surface for 30 minutes and then plunged into a liquid nitrogen tank.

2.3. Blood analysis and Veterinary Care

A veterinarian collected 5 ml whole blood samples from the jugular vein for blood analysis and monitored regularly general health condition. Some were used for Complete Blood Count (CBC) (Hemavet 950, Drew Scientific, USA) and the others were used for serum chemistry analysis (BS-400, Mindray, China). One transgenic cow, which was fell with attack of other cattle, was suffered from severe respiratory distress and euthanized by just treatment with a general anesthesia reagent, Xylazine (0.15mg/kg, BAYER) via intravenous approach

2.4. Library preparation for massively parallel sequencing

Purified genomic DNA was randomly sheared to yield DNA fragments of an average 350 bp in size using a Covaris S2 Ultrasonicator. Library preparation was performed following the Illumina TruSeq DNA PCR-free preparation kit. Adaptor enrichments were performed using PCR according to the manufacturer's instructions. The final library size and quality were evaluated electrophoretically with an Agilent High Sensitivity DNA Kit. The 150 bp paired-end reads were sequenced on an Illumina HiSeq 4000 platform. Further image analysis and base calling were performed with RTA 2.7.3 (Real Time Analysis) and bcl2fastq v2.17.1.14.

2.5. Identification of genome instability

In order to investigate genome instability, I classified SNPs and INDELs into three groups (RefHom (homozygous reference genotype), Hetero (heterozygous genotype), and AltHom (homozygous alteration genotype)) according to the maternal and paternal

samples. Classified mutations are then filtered out with the following conditions: (1) if the genotype is RefHom, the selection of the ratio of the reference allele depth is more than 90%; (2) if the genotype is Hetero, the selection of the ratio of the reference allele depth is more than 40% and less than 60%; (3) if the genotype is AltHom, the selection of the ratio alteration allele depth is more than 90%; (4) I removed the mitochondrial genome, X chromosome, and unanchored scaffolds. Finally, I extracted de novo mutations without inherited genotype.

2.6. Measure fluorescence intensity

To quantify the fluorescence intensities of SNU-F1-1 and SNU-F1-2, cells of the same passage and density were acquired. Using ImageJ (v1.50, NIH), the same-size region was selected using a square of the drawing/selection tools and the measurements set (area, mean gray value, and integrated density). The integrated density for each cell was calculated in the region of interest.

3. Results

3.1. Health monitoring using blood analysis

Female (SNU-SB-1) and male (SNU-PB-1) transgenic cattle have lived up to 51 and 44 months, respectively, without any health issues (Figure 24). In blood analysis, no significant changes (white blood cells: WBC, red blood cells: RBC, and platelets) were detected in the transgenic cattle (Figure 25). Furthermore, there were no significant changes in various serum chemical parameters, including aspartate transaminase (AST), alanine transaminase (ALT), and blood urea nitrogen (BUN).

A

I.D.	DNA	Transposase	Gender	Age
SNU-SB-1	SB-CA-YFP	SB	Female	51 months
SNU-PB-1	PB-CA-RGRR	PB	male	44 months

B

Figure 24. The current status of transposon-derived transgenic cattle.

A) Brief information on the transgenic cattle. B) A recent photograph of transgenic cattle. Left: SNU-SB-1; right: SNU-PB-1.

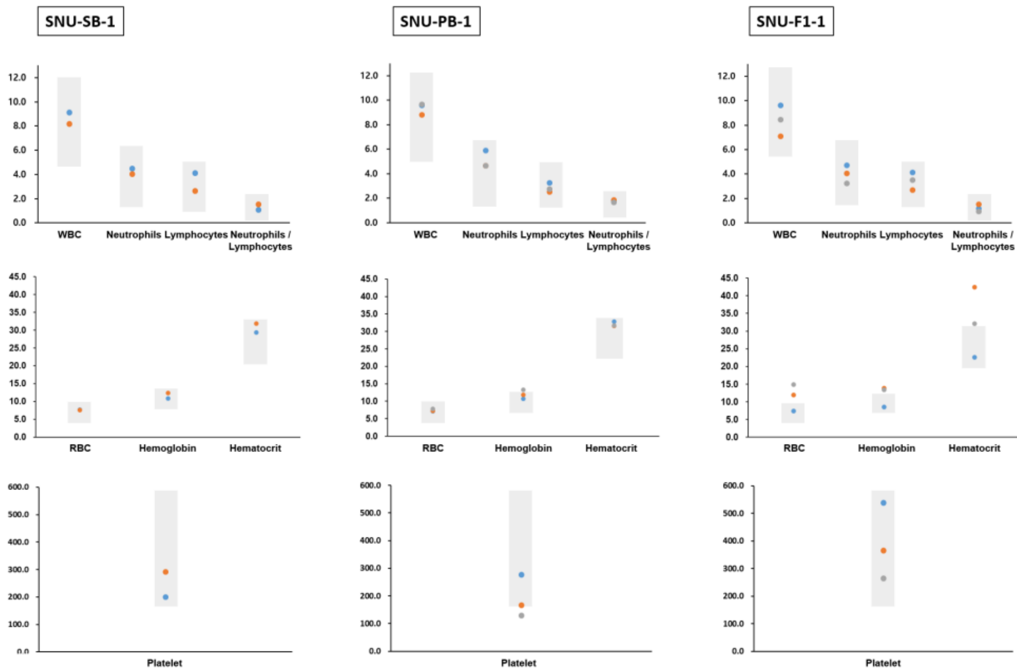


Figure 25. Analysis of blood in three transgenic cattle (SNU-SB-1, SNU-PB-1, and SNU-F1-1).

Samples were collected three times at different ages. In SNU-SB-1, one sample for WBC failed due to blood coagulation, but the serum chemical analysis was fine (Additional file 1). Circles: dates of blood collection and analysis from transgenic cattle; Orange circle: 26/08/2016; Blue circle: 26/10/2016; Gray circle: 27/03/2017; WBC: White blood cells; RBC: Red blood cells; Gray box: reference range.

3.2. Germline transmission of the transposon in germ cells

In SNU-SB-1 (female), I performed superovulation, artificial insemination and embryo collection. I failed to collect viable fertilized embryos. Nine unfertilized oocytes were collected and the transgene were detected by genomic PCR (Figure 26). When collecting the embryos in uterus, some tissues from uterine epithelium were isolated and cultured. All uterine epithelial cells expressed YFP protein (Figure 27). In case of SNU-PB-1 (male), to assess the germline transmission ability of transgenic sperm, sperm was harvested using manual ejaculation and cryopreserved to create freezing semen from SNU-PB-1 after puberty. Over 200 straws were produced and preserved in a liquid nitrogen tank for further application. The motility of frozen-thawed semen was normal, and the semen was employed for *in vitro* fertilization (IVF) with wild-type oocytes. In every IVF replication, around 88% of blastocysts expressed the green fluorescence protein (GFP) (Figure 28). This frozen sperm can be used to rapidly increase the population of transgenic cattle. The results show that germline transmission by the transposon carrying the transgene is possible.

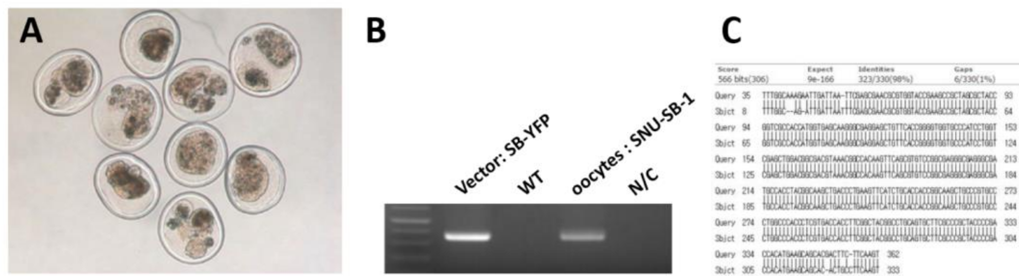


Figure 26. YFP expression in the oocytes.

(A) Picture of nine oocytes after superovulating the transgenic cattle (SNU-SB-1), (B) Identification of transgene in genomic DNA from the oocytes by PCR, (C) Sequencing validation of transgene PCR amplicons.

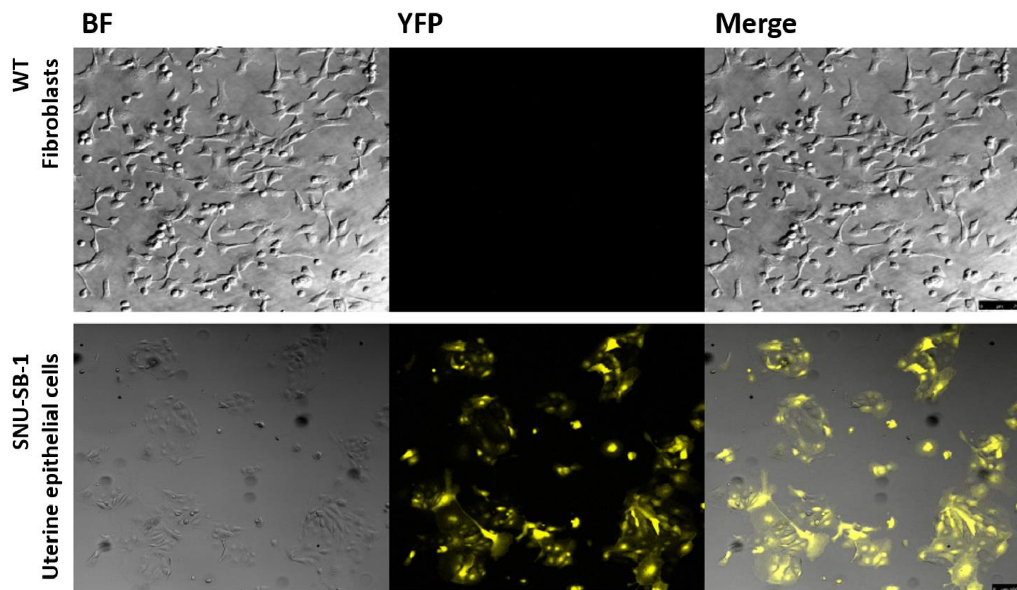


Figure 27. YFP expression in the uterine epithelial cells.

Confocal images of YFP expression in control fibroblasts (upper panel) and uterine epithelial cells (lower panel).

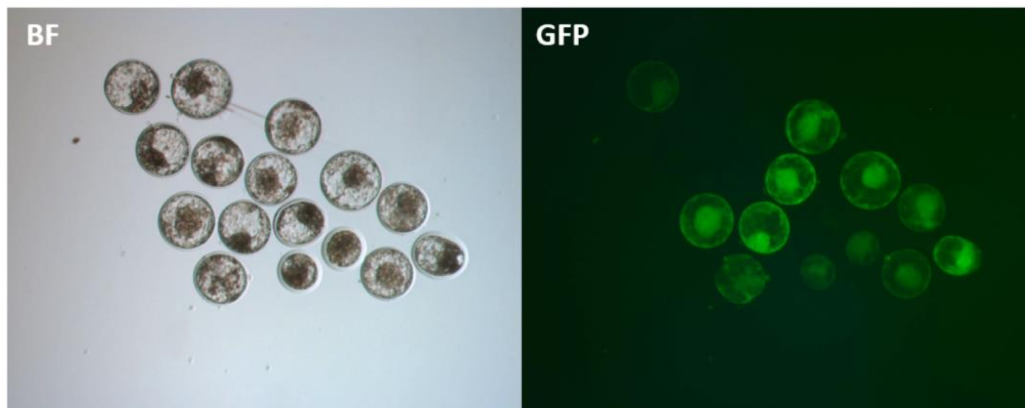


Figure 28. Germline transmission of the multiple transgenes in spermatozoa from SNU-PB-1.

Blastocyst stage embryos express GFP after IVF using frozen-thawed semen from SNU-PB-1. BF: bright field; GFP: GFP field.

3.3. Diversity of the number of transgenes transmitted

To investigate the diversity of transgene transmitted, blastocysts from *in vitro* fertilization with frozen-thawed semen from the transgenic founder SNU-PB-1 were used for the flanking sequence analysis. However, concentration of genomic DNA from blastocyst is limited, I tested only 5 insertion sites of SNU-PB-1. As results, each blastocyst showed various germline transmission pattern (Figure 29).

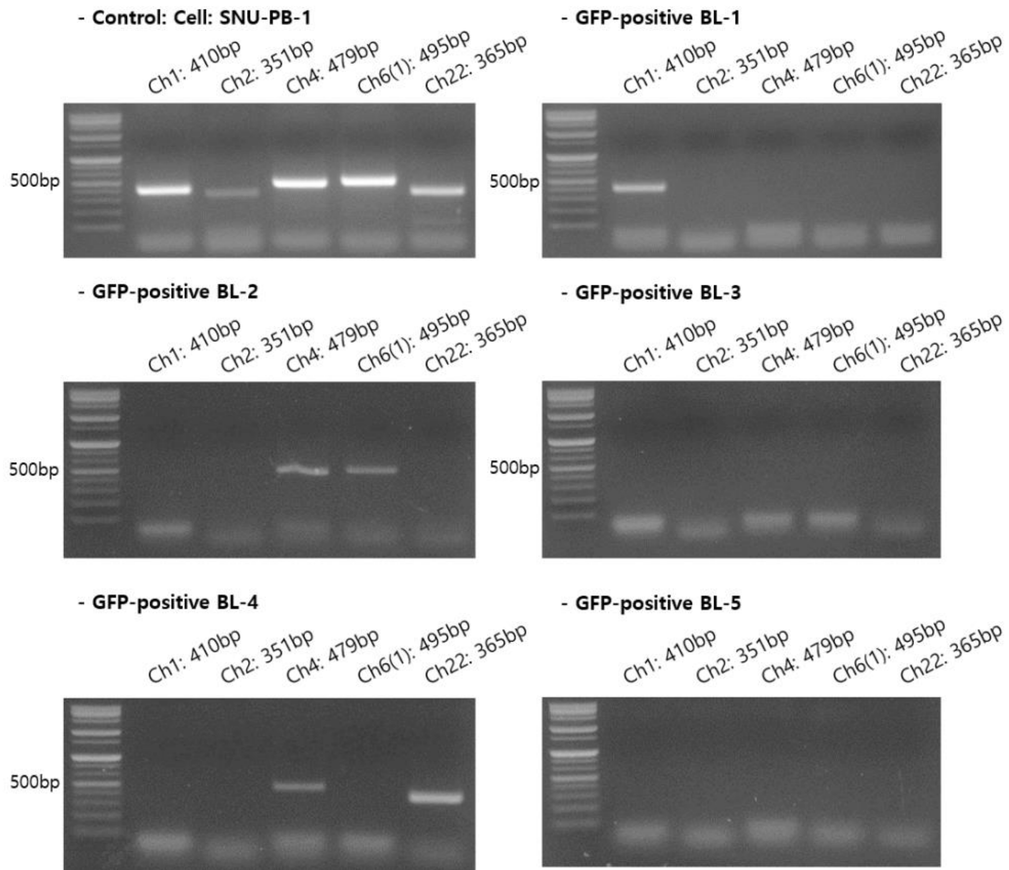


Figure 29. Identification of the diversity of transgene transmitted in embryo.

The diversity of transmitted transgene integration was evaluated in blastocysts. In the 5 different blastocysts, integrated patterns altered according to transgene transmitted.

3.4. Ubiquitous expression of transmitted transgene in embryo

To confirm stable expression of transmitted transgene without silencing, genomic DNA PCR was performed using blastocysts derived from *in vitro* fertilization with frozen-thawed semen from the transgenic founder SNU-PB-1. As expected, there was no finding of transgene in GFP-negative blastocysts and PCR bands were only detected in GFP expressed blastocysts (Figure 30). This data indicated that GFP-negative blastocysts did not silencing of transgene but no-transmission of the transgene from the SNU-PB-1.

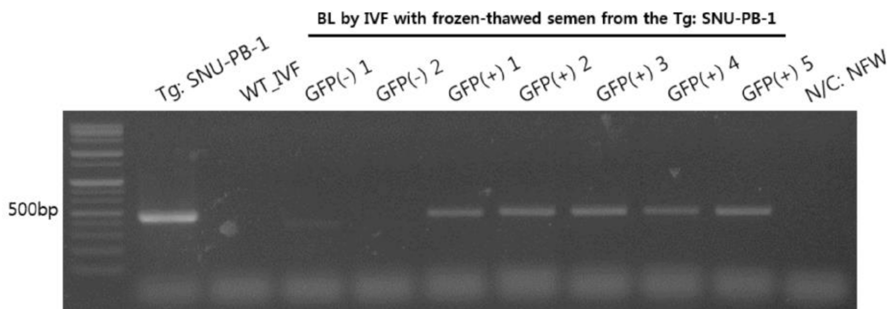


Figure 30. Identification of Ubiquitous expression of transmitted transgene in embryo.

The stable expression of transmitted transgene integration was evaluated in blastocysts.

3.5. Birth of the calf from the transgenic cattle

SNU-SB-1 was naturally crossed with SNU-PB-1 to establish a stable germline transmission of the transgene, and pregnancy was diagnosed in the cow. After the gestation period, the F1 (SNU-F1-1, male) was delivered without any assistance (Figure 31A). No congenital defects were discovered during a physical examination. The GFP was detected in the eyes without the equipment. To investigate the germline transmission, the skin fibroblasts were isolated from the F1, cultured, and expanded for genomic analysis. All the fibroblasts homogenously expressed GFP, and genomic DNA PCR and sequencing analysis of the GFP cells showed that the GFP transgenes were integrated at the genome. The SNU-PB-1 had froxed GFP followed by red fluorescence protein (RFP) [34]. If the transgenes were successfully transmitted to F1, the expression of Dre recombinase excises froxed GFP from the genome, leaving only one rox site and RFP in the genome (Figure 31B). As expected, that Dre recombinase transfection using electroporation was sufficient to introduce the expression of the RFP and the deletion of froxed GFP was confirmed by PCR (Figure 31C). Unfortunately, there was no finding of the maternal gene (yellow fluorescence protein: YFP). This data indicated that the transgenes from the SNU-PB-1 were successfully transmitted. Furthermore, there was no significant change in the blood analysis of the F1 (Figure 25).

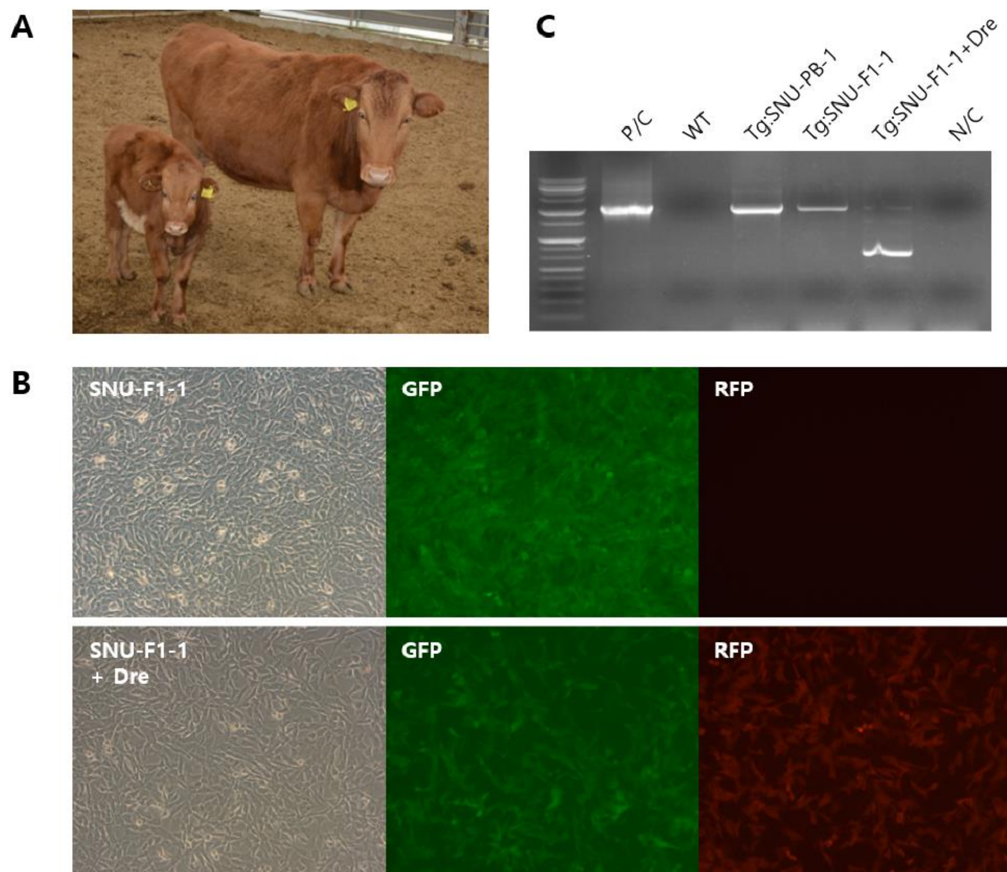


Figure 31. Detection of germline-transmission in SNU-F1-1.

A) A photograph of the SNU-F1-1 (left) from SNU-SB-1 (right). B) The primary cells from ear skin of SNU-F1-1 with the green-to-red reporter line resulting in GFP expression (upper). In contrast, recombinase Dre transfection resulted in RFP expression (lower). C) gDNA PCR analysis of Dre-rox recombination in the SNU-F1-1 cell line. N/C, negative control; nuclease-free water.

3.6. Detection of fluorescence protein in milk

To investigate whether the fluorescence protein would be detected in milk from the SNU-SB-1 after delivery of F1, wild-type milk and transgenic milk were examined by confocal microscopy. As expected, YFP was observed in milk from SNU-SB-1 (Figure 32). This result suggested that transposon-derived cattle can be used as bioreactors for the production of various recombinant proteins.

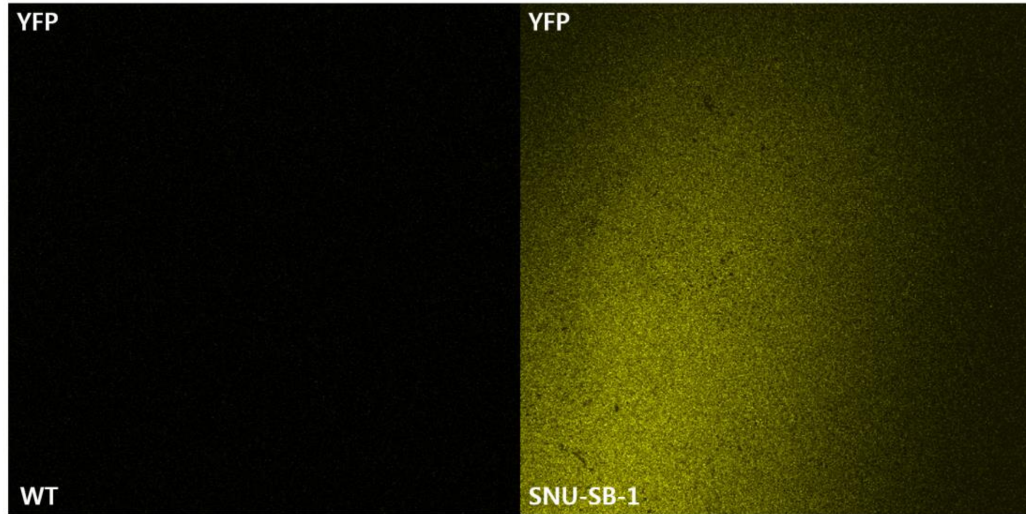


Figure 32. Detection of the expression of YFP in milk from SNU-SB-1 by confocal microscopy.

Images of milk from wild type (left) and SNU-SB-1 (right) in a high-throughput confocal microscope. YFP: YFP field.

3.7. Whole genome sequencing (integration and genome instability)

To determine the integration site of the gene from maternal and paternal genomes, whole genome sequencing was done through next-generation sequencing (NGS). NGS has been widely used for high-throughput genomic analysis such as molecular characterization and structure variation. I then confirmed the transgene insertion sites identified in transgenic cattle [34, 103]. NGS of the SNU-F1-1 was performed to confirm the transgene insertion sites. Results showed a total of three copies of paternal transgene were transmitted into the F1 and I identified the presence of the transmitted transgenes in the non-coding regions (Table 12). Insertion sites with transgenes were verified by 5' junction sequence analysis using a specific primer set that anneals the unique genome-to-transposon junction in chromosome 4 (two sites) and 6 (Figure 33). Additionally, I compared the F1 among parents (F0 samples) and identified 147 heterozygous de novo mutations and 2 homozygous de novo mutations from the maternal and paternal genomes (Table 13). The heterozygous de novo mutations were classified into intergenic (125, 78.61%), intron (33, 20.76%), and exon (1, 0.63%, in the ENSBTAG00000038261 gene), with most occurring in the intergenic and intron regions. In addition, the homozygous de novo mutations were identified as wrong variants because they occurred in the long terminal repeat (LTR) region. The result of the de novo mutations is similar to previous results [104], corresponding to a mutation rate of 5.62 $\times$10-8 per position per generation per genome. SNV, INDELs, and CNVs were visualized (Figure 34).

Table 12. All integration sites in SNU-F1-1.

No.	Chromosome	Insertion site	Orientation	5' gene	3' gene
1	4	95,433,564–95,434,563	Forward	TSGA13	MKLN1
2	4	113,823,097–113,823,101	Forward	ENSBTAG00000001198.5	ENSBTAG00000046257.1
3	6	20,085,913–20,086,912	Forward	DKK2	GIMD1

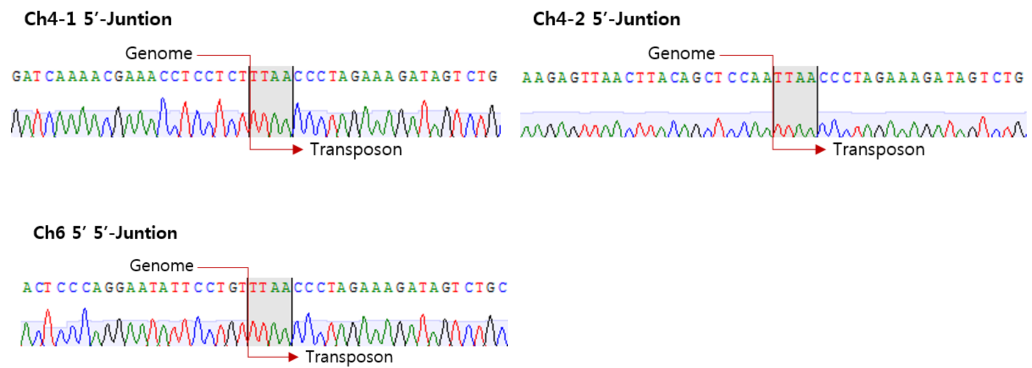


Figure 33. 5' junction sequence analysis of all integration sites in SNU-F1-1.

Sequences showing the junction of genome-to-transposon and integration at TTAA site.

Table 13. Pattern of SNPs and INDELs by parents' DNA.

Father	Mother	SNPs RefHom[1]	SNPs Hetero[2]	SNPs AltHom[3]	INDELs RefHom	INDELs Hetero	INDELs AltHom
RefHom	RefHom	0	120	2	0	7	0
RefHom	Hetero	123,614	93,583	6	10,537	8,154	1
RefHom	AltHom	0	11,903	3	0	809	0
Hetero	RefHom	113,234	75,948	0	9,609	6,448	0
Hetero	Hetero	112,916	227,992	117,673	9,634	20,491	9,905
Hetero	AltHom	0	28,758	43,359	0	1,984	2,994
AltHom	RefHom	5	12,384	0	0	858	0
AltHom	Hetero	2	37,357	499	0	2,738	3517
AltHom	AltHom	0	3	103,686	0	0	9,418

[1] Homozygous reference genotype

[2] Heterozygous genotype

[3] Homozygous alteration genotype

Yellow box : heterozygous *de novo* mutation without parents DNA

Red box : homozygous *de novo* mutation without parents DNA

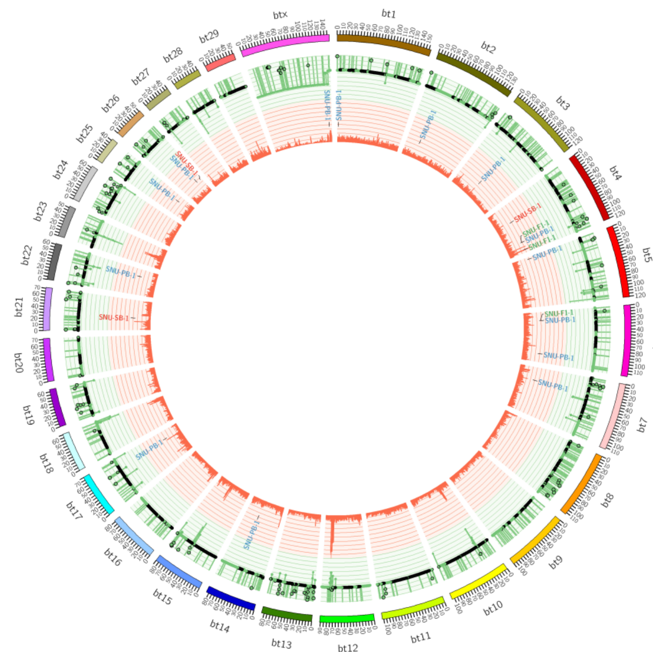


Figure 34. Overview of genomic variation in SNU-F1-1.

Reference chromosome containing 1 to X is colored in a variety of different colors in peripheral boxes. In addition, copy number variation (CNV; the black dot plot in the green area), coverage (the green line plot in the green area), and a histogram indicating SNP density (orange, histogram in orange area) indicating the sample are plotted per 10 kb window.

3.8. The expression of GFP level with transgene copy number

There are several studies in which a transgene copy number has been reported to reflect protein expression [105, 106]. To investigate the correlation between GFP expression level and transgene copy number, two different types of cell lines were used for analysis, the skin fibroblasts from SNU-F1-1 and the fetal fibroblasts, derived from the fetus (SNU-F1-2) from a pregnant SNU-PB-2 [34]. The SNU-PB-2 became pregnant and fell accidentally after being attacked by other cattle, was gravely injured, and had to be euthanized. It was confirmed that the fluorescence was expressed in the recovered uterus, ovary, and oocytes (Figure 35). In the NGS analysis of SNU-F1-2, six integration sites were identified, and there were no changes in genome instability (Table 14 and Figure 36). Both SNU-F1-1 and SNU-F1-2 were generated from a single embryo, and there was no mosaicism. As expected, SNU-F1-2 (six copies) showed approximately 2.2-fold higher expression level of GFP compared to SNU-F1-1 (three copies) (Figure 37). This result indicated that the copy number is an important factor of protein expression in transgenic cattle.

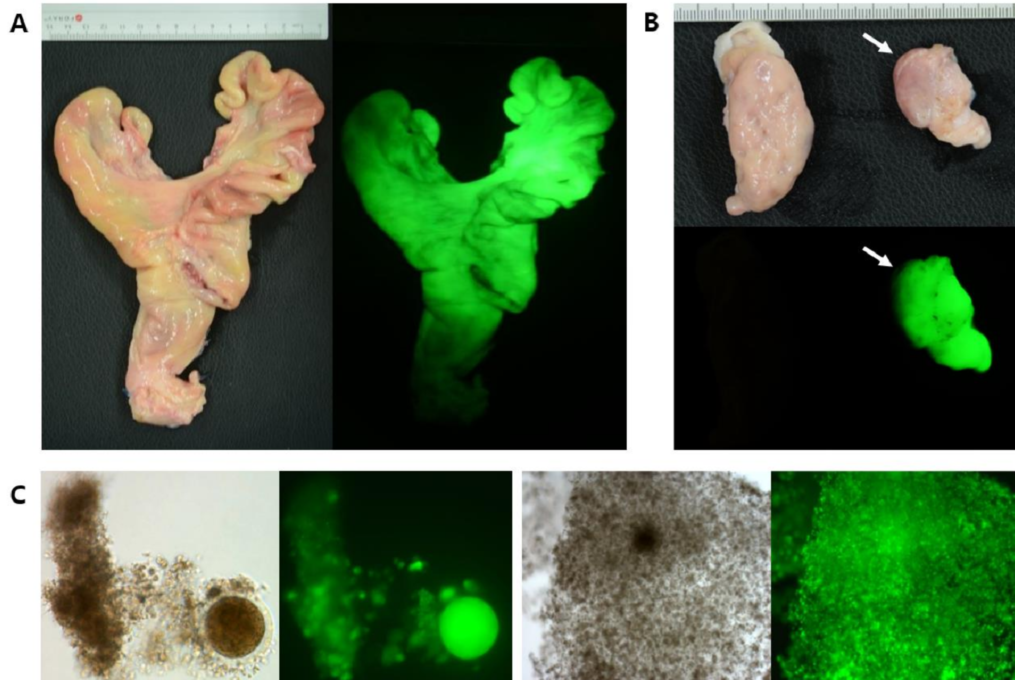


Figure 35. Expressing GFP by germline transmission in uterus, ovary, and oocytes from SNU-PB-2.

A) Photograph of uterus. B) A GFP-expressing ovary from SNU-PB-2 (arrow) and a WT ovary (left side). C) Fluorescence microscope images of GFP expression in oocytes and cumulus cells.

Table 14. All integration sites in SNU-F1-2.

No.	Chromosome	Insertion site	Orientation	5’ gene	3’ gene
1	1	105665894	Forward	ENSBTAG00000025847.3	ENSBTAG00000011051.5
2	3	79750136	Forward	PDE4B	LEPR
3	4	71122343	Reverse	NPVF	C7orf31
4	10	85854536	Forward	ALDH6A1	VSX2
5	12	51221667	Reverse	ENSBTAG00000010680.5	U2
6	X	80581377	Forward	PBDC1	MAGEE2

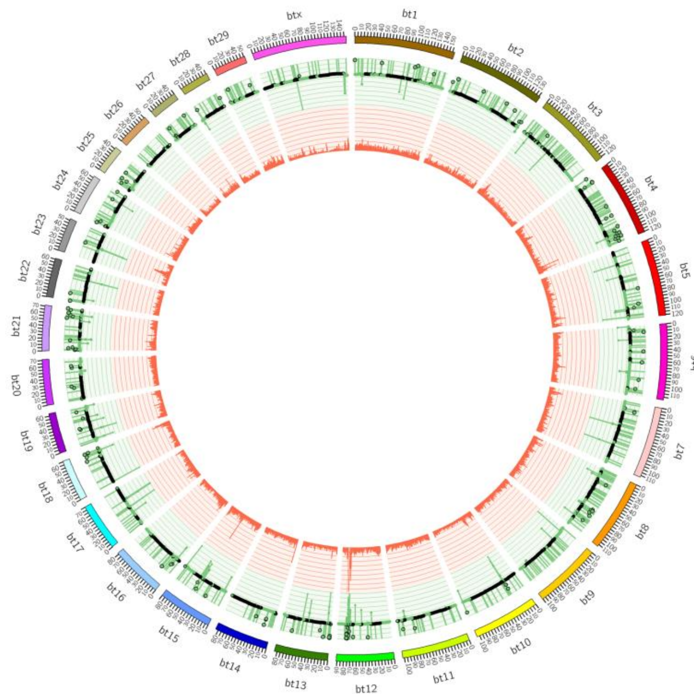


Figure 36. Overview of genomic variation in SNU-F1-2.

A reference chromosome containing 1 to X is colored in a variety of different colors in peripheral boxes. In addition, copy number variation (CNV; the black dot plot in the green area), coverage (the green line plot in the green area), and a histogram indicating SNP density (orange, histogram in orange area) of the sample are plotted per 10 kb window.

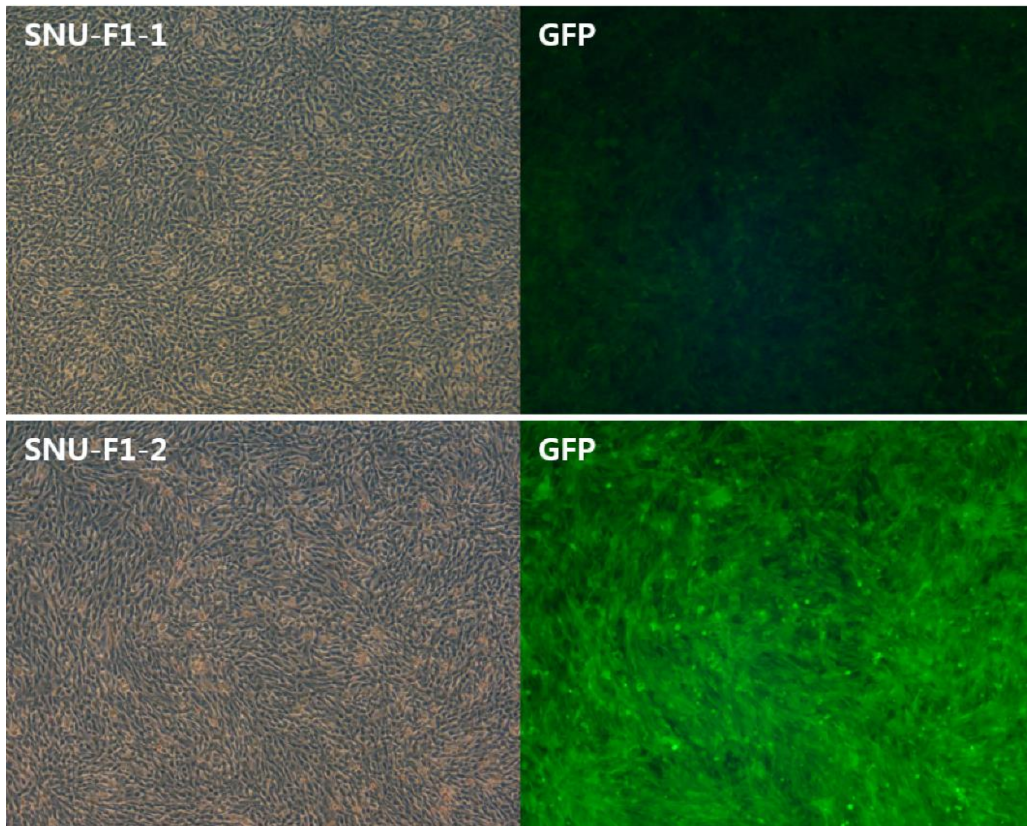


Figure 37. Expression level of GFP-mediated copy number in the SNU-F1-1 and SNU-F1-2.

Fluorescent microscope images of SNU-F1-1 (upper) and SNU-F1-2 (lower).

3.9. Disruption of GFP and Knock-in

Transgene integration positions in the transgenic cattle were considered at the safe target region because they have grown up without health issues to date. Thus, I transfected guide RNA endonuclease for the GFP as a previous study and knock-in donor that included the RFP and Puromycin gene between the homology arms together into the primary cells from SNU-F1-1. After transfection, during three days, the cells were isolated with antibiotic selection, puromycin. On 10 days post-transfection, I found the several colonies expressed RFP without GFP expression only in GFP guide RNA/Cas9 + Donor group. In the other groups (control, GFP guide RNA/Cas9 and Only Donor group), all of the cells were dead (Figure 38).

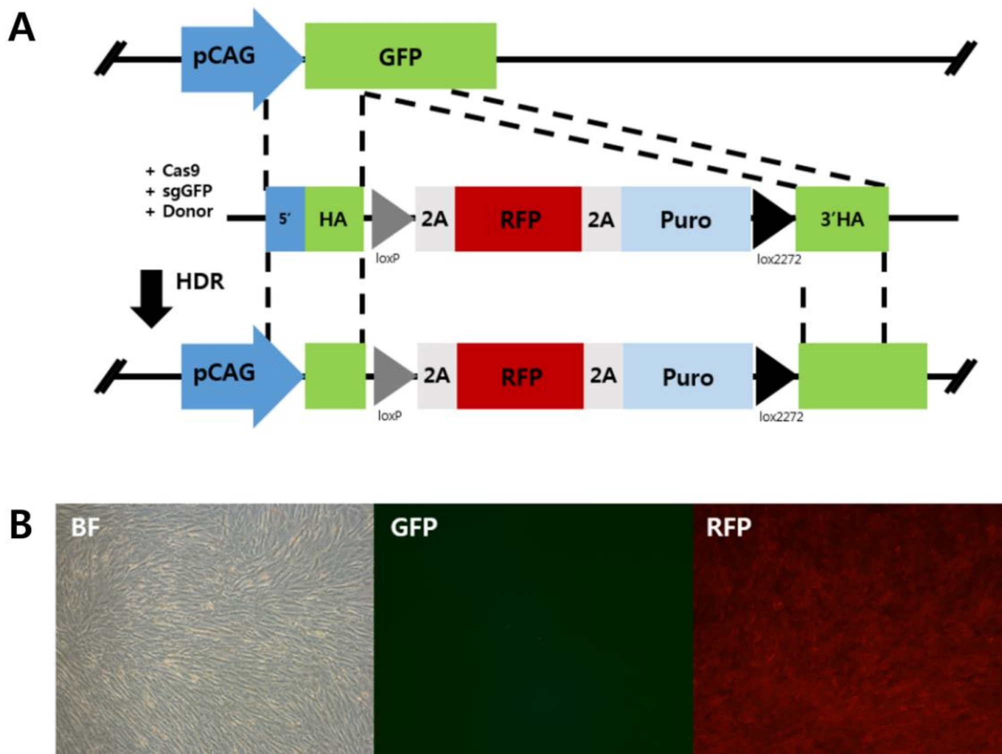


Figure 38. CRISPR/Cas9-mediated KI of SNU-F1-1.

(A) Schematic of CRISPR/Cas9-mediated KI of the donor plasmid. (B) After co-transfection of the donor plasmid with Cas9 and sgRNA for GFP, CRISPR/Cas9-mediated homologous direct repair occurs in the genome of SNU-F1-1 at the sgRNA target site of the GFP, resulting in the expression of the RFP. pCAG: CAGGS promoter; HA: homology arm.

4. Discussion

Since the study of transgenesis in cattle can provide various insights into basic embryogenesis, disease models, and bioreactor production, it has long been of interest as a biotechnology in agriculture and veterinary science. However, low efficiency of gene delivery, abnormal reprogramming in cloned embryos, and a low success rate of transgenic cloned offspring or complications (i.e., early embryonic loss and sudden death) have hampered its progress at the practical level [15, 107].

Transposons have been actively applied when generating animal models for transgenesis and have been successfully utilized in zebrafish [108], mice [61], rats [109], pigs [44], and cattle [35]. Moreover, germline transmission using transposons in transgenic zebrafish [110], rodents [40, 111], and pigs [38, 95] have been achieved. However, in germline transmission of cattle, because they have a long gestational term and puberty, there has been only one study done via lentivirus-mediated transgenesis [78]. In the present study, I proved that both female and male transgenic cattle with multi-copy integration of transposon-derived transgenes can grow up without health issues and that their transgenic germ cells can be transmitted to the next generation.

The cut-and-paste transposition notion of DNA transposon presumed that the transposition of a gene could affect genome instability [91, 112]. In reality, many articles on the application of transposons reported on their usage for safety purposes [38, 57, 109, 113, 114]. Additionally, in my previous report, all transgenes were found to be integrated in non-coding regions in several transgenic cattle with SB and PB. As with previous

reports, there was no significant difference [113] in NGS analysis of the SNU-F1-1 in terms of SNP, CNV, structural variation (SV), and telomere length. Transgene integration sites via NGS analysis in SNU-F1-1 were identified in three loci and the transgenes were only transmitted from the paternal genome (SNU-PB-1). This is because the transgenes in the maternal genome (SNU-SB-1) were heterozygotically integrated and it may be that the non-integrated oocytes were ovulated and fertilized with the sperm with the GFP gene. The transgenes in the offspring (SNU-F1-1) were integrated into PB-preferable sequences, "TTAA", as in my previous report [34]. Two integration sites were identical to that of SNU-PB-1. One was not identified in the previous reports on paternal genome analysis because all of the identified transgene loci in my report were detected in the blood [34], meaning that the transgene-integrated loci did not represent the condition of the whole body, such as the testes.

Additionally, transgenic animals were proven to be potential bioreactors for the secretion of exogenous proteins in milk [115-117]. In case of SNU-SB-1, I identified the YFP was detected in milk. Furthermore, the calf sucked the SNU-SB-1's milk containing fluorescence proteins until weaning for 7 months. Even though milk containing fluorescence proteins were fed into calf for a long period, the calf has not shown any health issues to date. It can be assumed that the feeding of the fluorescence proteins does not affect health. Thus, I believed that the transgene integration sites could be used as the target region (safe harbor regions, such as mROSA26 and hAAVS1) for another useful protein expression using genome-editing technologies. For this approach, RNA-guided endonuclease for GFP was applied and all the GFP regions were disrupted.

Furthermore, recombination knock-in cassette using donor DNAs were integrated in GFP target site. In future, I will add a gene of interest into the target locus by Cre-recombinase-based exchange and used as the donor cells for producing cloned cattle. The transposon-mediated transgenic cattle can be used as a bioreactor for producing various recombinant proteins.

Since the transgene is sometimes expressed or inhibited by the positioning effect, silencing, or mosaicism in germline-transmitted transgenic mice derived from viral mediated gene transfer [118-121], the relationship between the copy number and the expression of the transgene is important. This must also be considered in transposon-based transgenic animals, particularly in cattle, because one of the purposes of producing a transgenic cow is to generate bioreactor models. Germline-transmitted SNU-F1-1 and SNU-F1-2 have three and six copies of transgene integration, respectively; indirect information on copy number and expression levels could be provided. Although only two samples were subjected on the analysis, the expression level of SNU-F1-2 was almost 2.2-fold higher than in SNU-F1-1, indicating that transposon mediated integration was not affected by silencing or mosaicism.

Another issue is long-term stable expression of the fluorescence protein in my transgenic cattle (SNU-SB-1, SNU-PB-1, and SNU-F1-1) because GFP have the potential for immunogenicity and cytotoxicity [122]. Therefore, I assume that multi-copy integration and continuous transgene expression of the fluorescence gene may affect transgene expression or normal growth. The first (SNU-SB-1, female), second (SNU-PB-1, male), and the calf (SNU-F1-1, male) have grown up and reached 51, 44,

and 19 months old, respectively, and have no general health issues (feeding, growth, body weight, urination or defecation). As further evidence of their health, there were no significant changes in regular blood analysis, indicating that multi-gene integration and continuous expression in the bovine genome by transposons did not adversely affect health, such as organ condition. Some RBC, Hemoglobin, and Hematocrit values in SNU-F1-1 were out of the reference range. As in the previous report [123], this was because the blood was collected at the calf stage at 2 and 10 weeks old. These cattle are likely healthy because the genetic variants in SNU-SB-1 and SNU-PB-1 are not significantly different from wild-type cattle, as shown in previous NGS analysis [34], and the genome instability (SNP, INDEL, and telomere length, etc.) of SNU-F1-1 was not significantly different, as mentioned above. I will continue to monitor their longevity and health issues. As far as I know, this is the first report of transposon-medicated transgenic animals with germline transmission surviving this long-term without any health issues.

FINAL CONCLUSION

This thesis was conducted to generate transgenic cattle with germline transmission using transposon gene transfer system *in vivo*. Furthermore, safety of transposon-mediated gene integration for genome instability was evaluated via NGS.

Firstly, a transposon-based gene delivery system confirmed that the transgene was efficiently integrated into the genome in the bovine fibroblasts and PB transposon system had a much higher efficiency then SB.

Secondly, three kinds of transgenic cattle were generated by transposon system. 1) Transgenic cattle expressing fluorescence transgene under the ubiquitous CAG promoter were born via a SB transposon. 2) Transgenic cattle with conditional expression by Dre-rox recombination system were born via a PB transposon. 3) Transgenic cattle with tissue specific promoter (β-casein)-human gene (IL2) with reporter gene via PB transposon were born. A total of nine transgenic cattle were generated by SB or PB, and one of them which died due to severe diarrhea confirmed transgene expression in several organs, such as intestine, heart, spleen, and liver. Insertion site, and genome instability were identified via NGS. Inserted transgene by SB and PB were integrated into TA and TTAA.

Finally, germline transmission of the transgene in calf from transgenic cattle was confirmed. Two of these animals (SNU-SB-1 and SNU-PB-1) reached puberty and showed the transgene insertion in germ cells. Microscopic observation and genomic DNA PCR analysis of oocytes and sperm generated by transgene expression indicated that the transgenic germ cells from SNU-SB-1 and SNU-PB-1 had the ability for germline transmission, respectively. SNU-SB-1 was naturally crossed with SNU-PB-1

and delivered F1. Germline transmission was confirmed by transgene expression in fibroblasts and genomic DNA PCR. The germline-transmitted calf has grown to over 19 months old, and there have been no significant changes of blood analysis and genome instability. Multi-copy integrated SB and PB cattle have survived for more than 3 years without any health issues and their germline transgene was stably transmitted to the next generation.

To my knowledge, this is the first report of transgenic cattle via a transposon-mediated gene transfer system showing not only the transgene expression in skin fibroblasts and organs, but also germline transmission of the transgene, and normal development without any health problems in the transgenic calf.

As the final conclusion, this study provides valuable data about the safety and long-term expression of transgenes in transgenic cattle using transposon-mediated gene modification and applications such as exogenous protein expression. In addition to this, because all the transgene integrations were in intronic sites and no genomic instabilities were identified, the transgene integration sites could be used as target region like safe harbor for useful protein expression using genome-editing technologies. I suggest that those transgenic cattle could be valuable resources for bio-agricultural science.

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국문초록

트랜스포존을 이용한 생식선 전이 능력이 있는 형질전환소의 생산 및 분석

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형질전환소는 가축의 기본적인 유전적 기능이나 성능을 이해하는데 있어 매우 중요한 동물 모델이다. 하지만 형질전환소를 생산·유지하는 기본 비용이 많이 들고, 배아의 생존율과 이식 성공률이 낮기 때문에 형질전환소를 생산하는데 어려움을 겪고 있다. 따라서 가장 기본적이고 초기 단계인 형질전환 배아를 생산할 때 발현시키고자 하는 형질을 안정적이며 높은 효율로 유전체에 전달하는 유전자 전달 기술을 적용하는 것이 중요하다. 유전자 전달 기술로서는 크게 바이러스성과 비바이러성으로

나뉜다. 바이러스성 유전자 전달 장치는 유전자 발현율이 높아 다양한 연구에서 이용되었지만 사용하기 어렵고, 비특이적으로 염색체에 끼어 들어가기 때문에 암을 유발할 수 있는 유전자를 활성화시키는 문제점을 가지고 있다. 비바이러스성 유전자 전달 장치는 바이러스성 유전자 전달 장치에 비해 사용하기 편하고 낮은 독성을 가지지만 유전자 전달 효율이 낮다. 하지만 트랜스포존 시스템를 이용하면 이러한 단점을 해결할 수 있다. 트랜스포존 시스템은 비바이러스성 유전자 전달장치로써, 여러 연구를 통해 실험 방법이 간단하고, 높은 효율로 다양한 종류의 형질전환 동물을 생산할 수 있음이 확인되었다.

외부 형질을 안정적으로 발현하는 형질전환소를 생산하기 위한 첫번째 단계로 트랜스포존이 세포의 유전체에 높은 효율로 삽입되는지를 확인하였다. 이를 위해 전체적으로 녹색 형광을 발현하는 보통의 플라스미드와, 2 가지 종류 (슬립핑 뷰티, 피기백)의 트랜스포존과 이를 유전체에 높은 효율로 삽입시키는 역할을 하는 트랜스포세이즈를 같이 세포에 형질전환시켰다. 그 결과 피기백 트랜스포존이 높은 효율로 세포에 외부 형질을 전달하는 것을 확인하였다.

트랜스포존을 이용하여 외부유전자를 높은 효율로 유전체에 삽입되는 결과를 확인한 후, 두 종류의 트랜스포존 시스템을 이용하여 세가지 종류의 발현 시스템을 가진 형질전환소를 생산하였다. 첫째로 슬립핑 뷰티

트랜스포존을 이용하여 전체적으로 황색 형광 단백질을 발현하는 개체를 생산하였고, 둘째로 피기백 트랜스포존과 Dre-rox 발현 조절 시스템을 이용하여 녹색 형광 단백질과 적색 형광 단백질의 발현을 특이적으로 조절할 수 있는 개체를 생산하였으며 마지막으로 피기백 트랜스포존과 조직 특이적 발현 시스템을 이용하여 유선 특이적으로 특정 단백질을 발현하는 개체를 생산하였다.

세 종류의 형질전환 개체를 만들 수 있는 트랜스포존 시스템을 소의 수정란에 미세 주입하였고, 형광 단백질이 발현되는 배반포를 선별하고 대리모에 이식하여 9 마리의 형질전환소를 생산하였으며 생산된 일부 형질전환소에서 육안으로 형광을 관찰할 수 있을 정도의 매우 강한 형광이 발현되는 것이 확인되었다. 형질전환 개체의 외부 유전자 삽입 위치를 확인하고 그 위치에 따른 다양한 변이가 있는지 확인하기 위해 차세대 유전자 염기서열 분석을 하였다. 그 결과, 개체의 유전체에서 다른 유전자의 발현에 영향을 끼치지 않는 부분에 삽입되는 트랜스포존의 기본 특성대로 안전한 위치에 삽입되었고, 일반소와 비교했을 때 유의할만한 유전체의 복제 수 변이나 구조적 변이가 일어나지 않았다. 또한 생산된 형질전환 개체 중, 성성숙된 암컷 개체에서 과배란을 통해 얻은 난자와 수컷 개체의 정자로부터 얻은 수정란에서 미세 주입된 외부 유전자의 발현을 확인하였다. 이는 트랜스포존 시스템을 이용하여 생산된 형질전환

개체가 획득한 외부 유전자를 다음 세대에 전달할 수 있는 생식선 전이 능력을 가지는 것을 말한다.

그 다음으로 삽입된 외부유전자가 실제로 2 세대 자손으로 유전되는지 확인하였다. 성성숙된 트랜스포존-형질전환소를 자연적으로 교배시켜 2 세대 자손을 획득하였고, 부계의 형질전환 유전자인 녹색 형광을 별도의 장치없이 눈으로 확인 할 수 있었다. 2 세대 자손의 귀 조직으로부터 얻은 피부섬유아세포를 분석한 결과 전체적으로 녹색 형광 단백질의 발현이 확인되었고, 모체 쪽 외부유전자인 황색 형광 단백질은 검출되지 않았다. 이는 부계의 형질 전환 유전자만이 안정적으로 2 세대로 유전되었음을 말한다. 부계로부터 유전된 외부 유전자의 위치를 정확하게 확인하고 전체 유전체의 안정성을 확인하기 위하여 차세대 염기서열 분석을 수행하였다. 그 결과 외부 유전자는 총 3 개가 삽입되었으며 그 중 2 개가 부계의 외부 유전자 삽입 위치와 같은 위치에 있는 것을 확인하였다. 또한 유전체 안정성 검사 결과, 일반소와 형질전환소 간의 유의적인 차이가 없었다. 생산된 형질전환 개체는 길게는 51 개월 이상 건강상의 문제 없이 자라고 있으며 대용량의 혈액과 고기를 보유하고, 우유를 생산하기 때문에 최근 개발된 유전자 편집 기술을 적용하여 확인된 외부 유전자의 삽입 위치에 유용 단백질을 삽입시킨다면 차세대 바이오 리액터로 이용할 수 있다.

본 연구에서는 처음으로 트랜스포존 시스템을 적용하여 다양한 종류의 유전자 발현 시스템과 안정적인 유전체를 가진 형질전환 소를 효율적으로 생산하였다. 이러한 비바이러스성 유전자 전달 기술은 형질 전환 소를 높은 효율로 생산할 수 있을 것이며, 축산업 및 수의학 등의 다양한 분야에서 적용될 수 있을 것이다.

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주요어: 생식선 전이 능력, 트랜스포존, 슬립핑 뷰티, 피기백, 형질전환소, 차세대염기서열분석

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