수의학박사 학위논문

Advanced technology based on gene engineering in cattle industry: diverse strategies for new superior cattle breed

축산업의 유전자공학 기반 고도화 기술: 우수한 소 형질 개량을 위한 다양한 전략

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지도 교수 장 구

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김 경 민

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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 purpose 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.

Gyeong Min Gim

Advanced technology based on gene engineering in cattle industry: diverse strategies for new superior cattle breed

Gyeong Min Gim

(Supervisor: Goo Jang, D.V.M., ph.D.)

Theriogenology and Biotechnology

Department of Veterinary Medicine, Graduate School

Seoul National University

ABSTRACT

This study aimed to produce specific gene mutated (*PRNP* and *MSTN*) cattle through cytoplasmic microinjection based on the CRISPR/Cas9 system and to verify that mutant traits are transferred to the next generation by germline transmission.

First, to produce *PRNP*-mutated cattle, piggyBac transposon and CRISPR/Cas9 were used. A transposon vector with Cas9, GFP, and sgRNA for *PRNP* was applied to bovine somatic cells and embryos. Cas9 and sgRNA were inserted into the bovine genome and *PRNP* mutation was induced. Then, GFP-expressing blastocysts were selected and transferred into 18 surrogates. Finally, 7 calves were successfully born. Among them, 6 calves (#P1, #P3, #P4, #P5, #P6, and #P7) showed vector insertion (Cas9, sgRNA for *PRNP*), and their mutation rates were 4.1%, 48.3%, 0.2%, 0.0%, 99.6%, and 94.4%, respectively. However, GFP expression and Cas9 activity were observed in only 4 calves (#P1, #P3, #P4, and #P7). To verify germline transmission, #P3 and #P7 germ cells were cultured in vitro, and *PRNP* mutation was detected in their blastocysts. As further gene editing, *GGTA1* mutation was introduced into the embryos using electroporation. Using germ cells (#P3 and #P7), 7 F1 calves

became pregnant. In F1 cattle, the gene of interest in All-in-one DNAs (Cas9, GFP, and sgRNA for *PRNP*) was identified, and *PRNP* mutation was detected.

In addition, to study *PRNP* function in detail, conditional *PRNP*-mutant cattle were produced based on the Cre/loxP system. After Cre treatment, the somatic cells of the cattle expressed Cas9, but showed no *PRNP* mutation. As a result of germline transmission of the conditional PRNP male cattle, transgene integration and GFP expression were observed in blastocysts fertilized with semen.

Second, to generate *MSTN*-mutant cattle, cytoplasmic microinjection based on the CRISPR/Cas9 system was used. Through this, *MSTN*-mutant calves were successfully produced. The *MSTN* mutation pattern was the same with 12-base pair deletion, and, in case of calf #17, enhanced muscle growth was observed. Furthermore, blood analysis results showed no abnormalities in the *MSTN*-mutant cattle.

Next, whether *MSTN* mutation was transferred to the next generation (F1) was confirmed. For this purpose, oocytes and semen were collected after sexual maturation of the *MSTN* cattle (#6 and #17), and embryos produced by in vitro fertilization were analyzed. In addition, the embryos were

subjected to additional gene (PRNP) editing using electroporation. Embryos produced by in vitro fertilization with the *MSTN* male and female cattle were transferred to a surrogate, and 1 calf was successfully born. *MSTN* heterozygous mutation was observed on sequencing of the F1 calf, which had no health issues. As a further experiment, using electroporation, the additional gene-edited embryos fertilized with the *MSTN* male sperm showed high *PRNP* mutation rate (86.2 ± 3.4%).

In conclusion, this study is the first to produce *PRNP*-mutant cattle using transposon and the CRISPR/Cas9 system and *MSTN*-mutant cattle without exogenous gene integration. In addition, germline transmission was confirmed. The CRISPR/Cas9 system can be used to produce specific gene-mutant cattle with high efficiency and can be applied in various fields, such as livestock industry and veterinary medicine.

Key words: Cattle, CRISPR/Cas9, Cytoplasmic microinjection, Electroporation, Germline transmission

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LIST OF ABBREVIATIONS

Ad	Adenovirus
BLG	Beta−lactoglobulin
BET	Bromodomain and extra−terminal domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Cycle threshold
CASA	Computer assisted sperm analysis program
CBC	Complete blood count
COCs	Cumulus oocyte complexes
CRISPR−EZ	CRISPR RNP Electroporation of Zygotes
CSN2	Casein Beta
CFTR	Cystic fibrosis transmembrane conductance regulator
DNA	Deoxyribonucleic Acid
DSB	Double−strand break
DMEM	Dulbecco’s Modified Eagle Medium
ES	Embryonic stem
FGF2	Fibroblast growth factor 2
FBS	Fetal bovine serum
FSH	Follicle−stimulating hormone
GEEP	Gene editing by electroporation of Cas9 protein
GFP	Green fluorescent protein
GOI	Gene of interest
gDNA	Genomic DNA
GAPDH	Glyceraldehyde 3−phosphate dehydrogenase
GGTA1	α1,3−galactosyltransferase
hpi	Hours post insemination

HR	Homologous recombination
HDR	Homology−directed repair
HITI	Homology−independent tarted insertion
HMEJ	Homology−mediated end−joining
HBD3	Human beta−defensin
hBSSL	Human bile salt±stimulated lipase
IgG	Immunoglobulin G
IARS	Isoleucyl−TRNA Synthetase 1
iPS	Induced pluripotent stem
iPSCs	Induced pluripotent stem cells
lncRNA	Long non−coding RNA
IVM	In vitro maturation
IVF	In vitro fertilization
IVC	In vitro culture
Klf4	Kruppel Like Factor 4
MSTN	Myostatin
MMEJ	Microhomology−mediated end−joining
mRNA	messenger RNA
NAs	Nucleic acids
NANOS2	Nanos C2HC−Type Zinc Finger 2
ZFX	Zinc Finger Protein X−Linked
NHEJ	Non−homologous end−joining
NEAA	Non−essential amino acids
NGS	Next generation sequencing
OCT4	Octamer−binding transcription factor 4
PAM	Protospacer adjacent motif

PRNP	Prion protein
PAEP	Progestagen Associated Endometrial Protein
PB	piggyBac
P/S	Penicillin/streptomycin
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
RNAi	RNA interference
RAG1	Recombination activating gene 1
RVD	Repeat variable residue
RFP	Red fluorescent protein
SCNT	Somatic cell nuclear transfer
SP110	SP110 nuclear body protein
SRY	Sex Determining Region Y
SB	Sleeping Beauty
Sox2	SRY−Box Transcription Factor 2
sgRNA	Single guide RNA
TALENs	Transcription activator like effector nucleases
TILD	Targeted integration with linearized dsDNA
Tb4	Thymosin beta−4
TSS	Transcription start site
TALP	Tyrode's albumin lactate pyruvate
T7E1	T7 endonuclease 1
VCN	Vector copy number
YFP	Yellow fluorescent protein
ZFNs	Zinc finger nucleases
2−ME	β−Mercaptoethanol

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PUBLICATION PAPERS

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PART I. LITERATURE REVIEW

1. Production of transgenic cattle

In livestock, genetically engineered animals are used for recombinant pharmaceutical drug production [1], organs for xenotransplantation [2, 3], increased disease resistance [4], and increased frequency of alleles or polymorphism associated with favorable traits (heat tolerance, milk, or meat production/composition) [5-7]. In addition, the animals can also be applied to human genetic disease models because livestock (pig, cattle, sheep, goat) genome is more similar to the human genome than rodents [8]. In particular, genetically modified cattle can be used for large-scale, recombinant pharmaceutical drug production from milk. However, production of transgenic livestock is not as easy as that of a transgenic rodent because germline-transmittable embryonic stem cell of livestock construction is not well developed. Diverse trials have attempted to establish embryonic stem cells, but have not succeeded in producing chimeric animals [9-11].

Figure 1 shows the timeline of important events related to the production of transgenic cattle. At the beginning, transgenic cattle that produce milk including human lactoferrin was induced by microinjection. Although transgenic cattle were produced

several times using microinjection at first, the efficiency rate was very low (~12%) [12-14]. When somatic cell nuclear transfer (SCNT) was first introduced to livestock, numerous types of transgenic cattle could be produced. In addition, with the development of engineered nucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, the production of specific gene edited animals is easier than before. However, for livestock, further studies are needed to efficiently produce genetically modified animals.

Starting from the next section, microinjection, SCNT, and electroporation, which are currently the most used technologies to produce transgenic cattle, will be introduced.

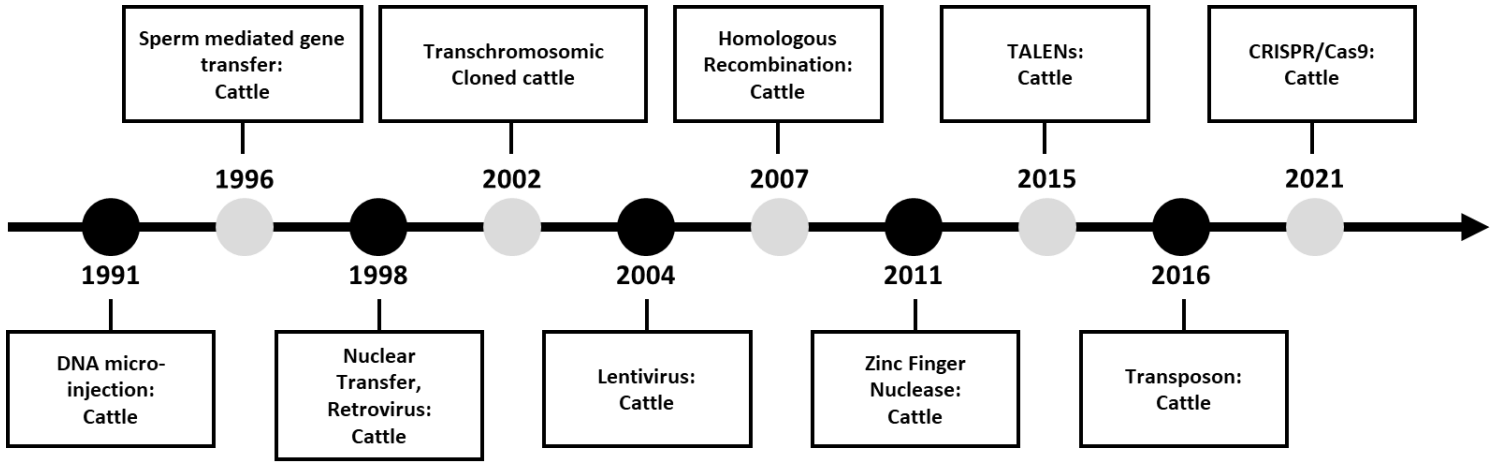

Figure 1. Milestones in the production of transgenic cattle.

1-1. Microinjection

Microinjection is a major method for producing transgenic animals by microinjecting nucleic acids (NAs) into the cytoplasm of fertilized eggs at the zygote stage (Fig. 2A). In the 1990s, genetically modified cattle were attempted by microinjection with vectors or viruses encoding the gene of interest. The trials were successful, but the birth rate of transgenic cattle was very low (~12%) [12-14]. In addition, microinjection resulting in the mosaic phenomenon was unsuitable for farm animals with costly, long gestation period, small litter size, and low transgene integration rate [13]. To improve the efficiency, there were some trials such as perivitelline space injection [15] and lentivirus injection [16]. Recent studies have shown that a solution that can improve the gene engineering bovine embryo is the use of transposon systems such as piggyBac and Sleeping Beauty [17, 18]. Further studies found no health problems during long-term monitoring and germline transmission was stable [19]. However, random insertion of an interesting gene has several potential risks. First, targeted gene insertion into the host genome is not available. Second, gene insertion could affect the expression of endogenous genes. Third, several copies of the insertion gene

can be randomly inserted into the host genome. These reasons show that the stable production of recombinant protein from transgenic cattle milk is difficult.

With the development of the nucleases, ZFNs, TALENs, and CRISPR/Cas9, specific gene editing in cattle is available using microinjection [20-22]. Microinjection combined with nucleases can produce not only a specific gene knock-out cattle but also a knock-in one [23]. The highly efficient gene engineering technology of microinjection outperforms SCNT, which has low efficient healthy calves. In addition, a recent study has shown that microinjection into bovine zygote was not detrimental in pre-implantation embryo development competence [24]. However, microinjection still shows the mosaic phenomenon. To overcome this issue, the use of Cas9 protein instead of Cas9 mRNA reduces the mosaic rate from 100% to 94.2% and increases the gene-editing efficiency from 68.5% to 84.2% [25]. In other trials, microinjection timing (0 hpi, 10 hpi, 20 hpi) was controlled in bovine embryos. The study showed that early delivery groups (0 hpi, 10 hpi) showed reduced mosaic than conventional 20-hpi microinjection [26].

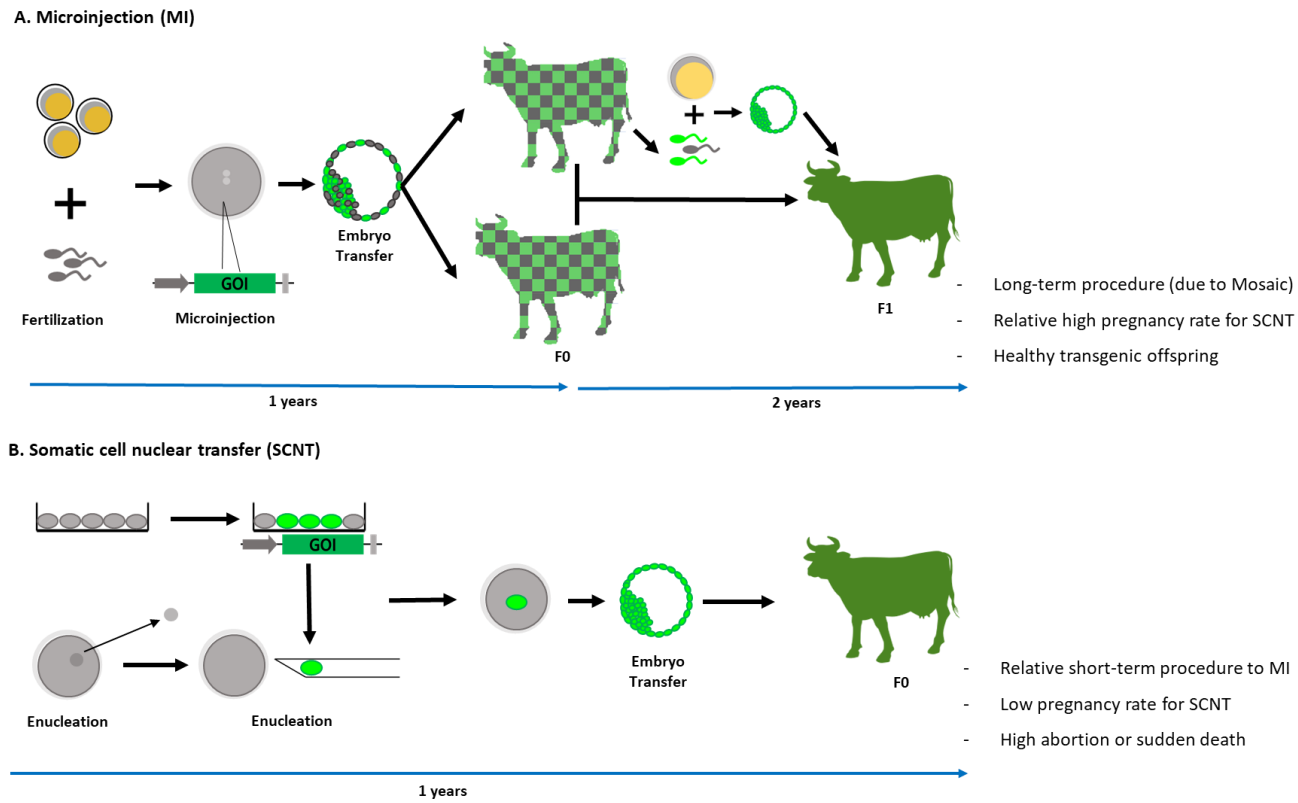

Figure 2. Representative methods for producing transgenic cattle: Micro-injection and Somatic cell nuclear transfer.

1-2. Somatic cell nuclear transfer

SCNT was the most powerful tool to produce transgenic animals in livestock (Fig. 2B). Somatic cell cloning technology is a method of producing a transformed individual by selecting a donor cell with a desired mutation, replacing the nucleus of the donor cell with the nucleus of a mature oocyte, and transplanting the embryo. Transgenic cattle with recombinant protein from milk were produced by SCNT [27, 28] for disease resistance [29, 30] and improved welfare [31].

However, SCNT has low efficiency in obtaining healthy calves because of abnormal reprogramming and epigenetic gene regulation. To overcome this limitation, many efforts have been made to increase the low efficiency [32], among which one aimed to increase the efficiency through donor cells. Although studies have been conducted according to cell cycle alignment [33], cell age [34, 35], cell origin [36], cell passage [36], and cell type [37], the efficiency of somatic cell replication has not increased significantly. However, damage or mutations in chromosomes can be reduced if cells in the G0 or G1 phase, long telomeres, or early passage are used [38]. Despite the disadvantage of low efficiency, it is easy to select donor cells that have the desired

gene or mutation before SCNT, relatively easy to analyze when testing in cells, and easy to select cells with a high expression of transgene. SCNT is still widely used due to the advantage of being able to produce transgenic cattle [39, 40].

Recent studies have devised an electroporation-based method that induces partial dissolution of the zona pellucida, which can introduce sgRNA/Cas9 into mouse and pig embryos. Electroporation, called GEEP or CRISPR-EZ, can overcome the disadvantages of SCNT and microinjection, which require harsh laborious, expensive equipment and skilled person. Initially, many attempts were made using Cas9 mRNA, but now it is used as an RNP together with Cas9 protein to reduce mosaicism and increase efficiency [41-45]. Although studies have shown that electroporation has a negative effect on embryo survival or embryo development, recent studies have shown that mice and pigs have no negative effect on embryos [45-47].

In cattle, gene editing using electroporation was first introduced in 2019. This report showed that bovine *NANOS2* was edited with high efficiency, which means that electroporation is an applicable gene-editing tool that allows direct editing at the bovine zygote stage [48]. In addition, other bovine genes such as *OCT4*, *ZFX*, and *MSTN* were also shown to be edited with high efficiency in other studies [49, 50]. However, it is unclear whether the efficiency for live offspring with desired edits and other species is applicable.

2. Gene engineering tools

2-1. Random integration (viral-based integration and non-viral-based integration)

The gene delivery method used to produce genetically modified animals is largely divided into viral and non-viral methods in the early days of genetically modified livestock. Representative viruses for gene delivery are lentivirus, adenovirus, adeno-assisted virus, and retrovirus. The virus-based method has the advantage of being able to deliver genes to cells by itself without additional equipment and processing and has high efficiency. However, there are disadvantages in that the size of a relatively transferable DNA fragment is limited, and it has the potential to induce an immune response and carcinogenesis. In addition, the cost of packing viruses is also high. On the other hand, the non-viral method of delivering naked DNA to an individual has no restriction on the DNA fragment length, and it is relatively stable because there are no factors that can cause an immune response or carcinogenesis in the viral delivery method. However, it requires additional equipment or processing, and gene transfer efficiency is low compared with the viral method. Transposons have been used to overcome the low

efficiency of non-viral gene transfer methods with high stability and are recognized as a useful method for non-viral gene transfer methods in animal cells.

Based on random integration (viral-based integration, non-viral-based integration), especially DNA fragment, retrovirus, and lentivirus, genetically modified cattle were produced by microinjection into an embryo from 1990 to the early 2000s [12-16, 51]. The main reason for producing genetically modified cattle was to produce recombinant proteins (human lactoferrin, human erythropoietin, hepatitis B surface antigen, human alpha-lactalbumin) from milk. However, the efficiency of producing transgenic cattle with the desired traits was extremely low. With the advent of SCNT, the efficiency has significantly improved since early 2000s [27, 29, 30, 52-61]. The achievement of transgenic cattle produced by random integration is shown in Table 1.

Table 1. Transgenic cattle lists produced by random integration

Year	Overexpression Knock-down Knock-out	Gene	Method of transgenesis	Method of embryo manipulation	Target gene	Reference
1991	Overexpression	Human lactoferrin	DNA fragment	Microinjection	Random	[12]
1994	Overexpression	Human erythropoietin	DNA fragment	Microinjection	Random	[13]
1998	Overexpression	Hepatitis B surface antigen	Retrovirus	Microinjection	Random	[15]
1999	Overexpression	Human alpha-lactalbumin	DNA fragment	Microinjection	Random	[14]
2002	Overexpression	Human lactoferrin	DNA fragment	Microinjection	Random	[51]
2003	Overexpression	β- and κ-casein	DNA fragment	SCNT	Random	[61]

2004	Overexpression	Fluorescent gene	Lentivirus	Microinjection	Random	[16]
2004	Overexpression	anti-human CD28, anti-human melanoma specificity (r28M)	DNA fragment	SCNT	Random	[60]
2005	Overexpression	lysostaphin	DNA fragment	SCNT	Random	[29]
2006	Overexpression	Human growth hormone	DNA fragment	SCNT	Random	[27]
2007	Knock-out	None	DNA fragment	SCNT	PRNP	[30]
2008	Overexpression	Human lactoferrin	DNA fragment	SCNT	Random	[59]
2009	Overexpression	Human Albumin	DNA fragment	SCNT	Random	[58]
2009	Overexpression	Human IgG	DNA fragment	SCNT	Random	[57]
2011	Knock-down	None	DNA fragment	SCNT	PRNP	[56]

2012	Knock-down	None	DNA fragment	SCNT	BLG	[55]
2016	Overexpression	Fluorescent gene	Sleeping Beauty	Microinjection	TA	[18]
2016	Overexpression	Human beta-defensin (HBD3)	DNA fragment	SCNT	Random	[54]
2016	Overexpression	Fluorescent gene	piggyBac	Microinjection	TTAA	[17]
2017	Overexpression	hBSSL	DNA fragment	SCNT	Random	[53]
2017	Overexpression	Human lactoferrin	DNA fragment	SCNT	Random	[52]

2-2. Specific gene targeting system

Before the specific gene targeting system, genes were modified randomly as mentioned above, which was an ineffective method to produce transgenic livestock and was expensive. In addition, there were safety problems with malfunction of the original gene or gene regulation. The gene-editing system needs to improve to increase the efficiency and precisely control the gene.

Gene editing is a powerful tool for identifying specific genetic functions and providing valuable information on genes. However, target gene inactivation, addition, and replacement through a conventional tool that relies on homologous recombination (HR) had limitations of efficiency, time and labor costs, and random integration into the genome. Also, the efficiency of gene editing differed depending on the cell type. RNAi, which can be synthesized faster than HR and has a reasonable cost and high throughput, was introduced to identify genetic function with high efficiency but was not perfect; different results were obtained in the same experiment, and an off-target effect was observed [62].

With the emergence of endonucleases as a gene-editing tool, the abovementioned problems significantly improved. Representative endonucleases as a gene-editing tool are meganucleases, ZFNs, TALENs, and CRISPR/Cas9. These tools can recognize the target sequence and induce a double-strand break (DSB) on the target gene, which stimulates the DNA repair mechanism (non-homologous end-joining, homology-directed repair) [63] and improves efficiency in targeted genetic modification [64].

2-2-1. Meganucleases

Meganucleases with a large recognition site (14–40 bp) are known as homing endonucleases, which are divided into five families based on sequence and structure motifs (LAGLIDADG, GIYYIG, HNH, His-Cys box, and PB-(D/E) XK). Large recognition sites and low cytotoxicity are characteristics of meganucleases that are applied as a genome editing tool [65, 66]. Moreover, due to its relatively small size, it is easy to deliver into cells, and it has a 3' overhang after DNA cleavage [67]. For example, I-CreI, which recognizes the target sequence (5'-CAAACGTCGTGAGACAGTTG-3'), was used to create mice and rats with recombination activating gene 1 [68]. However, in the mammalian genome, the meganuclease target sequence does not naturally exist, so a disadvantage is that the meganuclease target sequence must be inserted before use. In addition, it is difficult to separate the DNA binding and DNA cleavage domains, making it very difficult to redesign the meganuclease. This modification of meganuclease is time-consuming and expensive.

2-2-2. Zinc-finger nucleases

ZFNs were discovered in the 1980s, and the first specific ZNF was reported in 15 years. Individual ZFN consists of around 30 amino acids with two anti-parallel beta-sheets opposing an alpha-helix [69]. The alpha-helix can bind to three specific bases located in the DNA major groove [70]. ZFNs have two domains: site-specific zinc-finger DNA-binding domain and nonspecific cleavage domain of FokI endonuclease. Two or more ZFN molecules are required to modify a specific gene. In general, ZFNs can recognize 9-18 bp of DNA sequence that can be allowed for specific editing, and DSB is caused by dimerized FokI. Through DSB, a specific gene can be blocked by non-homologous end joining (NHEJ), or a desired DNA sequence can be inserted into a specific gene by HDR. Before ZFN emergence, the length of the homologous arm was 6-7 kb [71], but after their appearance, the length of the homologous arm decreased to 0.5-1.5 kb [72]. The history of transgenic cattle produced by ZFNs is shown in Table 2.

Table 2. Gene edited cattle lists produced by Zinc-finger nuclease

Year	Knock-out Knock-in	Inserted gene	Method of transgenesis	Method of embryo manipulation	Target gene	Reference
2011	Knock-out	None	ZFNs mRNA	SCNT	BLG	[73]
2014	Knock-out Knock-in	Human lysozyme	ZFNs	SCNT	BLG	[74]
2014	Knock-out	None	ZFNs	SCNT	MSTN	[75]
2018	Knock-out	None	ZFNs	SCNT	BLG	[76]

2-2-3. Transcription activator-like effector nucleases

TALENs, which can infect various plant species, are naturally produced in the plant pathogenic gram-negative bacteria *Xanthomonas*. TALENs consist of DNA-binding domains with 33–35 amino acid repeat domains and nonspecific endonuclease Fok1. Amino acids positioned at 12, 13 of DNA-binding domains called repeat variable residue (RVD) can recognize single base pairs. One RVD specifically binds to one nucleotide in genomic DNA [77, 78]. The DNA-binding sequence of TALENs should be initiated with thymidine, and target sequence length is generally 30–40 base pairs. DNA-binding domain can be modified to edit the endogenous sequence of certain cells, and DNA repair mechanism (NHEJ, HDR) can be induced through the nonspecific endonuclease Fok1 [73, 79-81].

There are some differences between ZFNs and TALENs; 1) the TALEN repeat is 3–4 times larger than the ZFN repeat. Moreover, because TALENs recognize one nucleotide, they are more sophisticated than ZFNs, which recognize three nucleotides. 2) The modification of ZFNs requires a high-level design because DNA recognition may not be successful due to crosstalk

between each finger [82]. 3) TALENs are easier to design than ZFNs, as they are simpler and can be produced quickly and cheaply in terms of production efficiency. 4) TALENs have less off-target effect than ZFNs. 5) TALENs have higher efficiency in genome editing through cytoplasmic injection in livestock embryos than ZFNs [83].

TALEN is a powerful gene-editing tool for generating gene knock-out in rats and zebrafish [84-86]. In addition, it can be applied to a wide range of animals by efficiently mutating desired genes in cattle, sheep, and pigs [21, 87]. The history of transgenic cattle produced by TALENs is shown in Table 3.

Table 3. Gene edited cattle lists produced by TALENs

Year	Knock-out Knock-in	Inserted gene	Method of transgenesis	Method of embryo manipulation	Target gene	Reference
2015	Knock-in	Mouse SP110	TALENs	SCNT	Chr28	[88]
2015	Knock-out	None	TALENs mRNA	Microinjection	MSTN	[21]
2016	Knock-in	P_C *POLLED*	TALENs	SCNT	POLLED locus	[31]
2018	Knock-out	None	TALENs	Microinjection	BLG	[20]

2-2-4. Clustered Regularly Interspaced Short Palindromic Repeats/Cas9

CRISPR was discovered in the 1980s, and its key role in bacteria and archaea is an adaptive immunity system that protects from an invasion of bacteriophages [89-92]. It induces RNA-guided DNA cleavage. CRISPR/Cas9 is widely used as a genetic tool that can cause DSB on a specific target site. The CRISPR/Cas9 system consists of a Cas9 endonuclease that recognizes the protospacer adjacent motif located downstream of a target sequence and causes DSB, and a single guide RNA that interacts with Cas9 to provide target recognition [93]. CRISPR/Cas9 is easier to design to target new genes compared with existing gene-editing tools (ZFNs, TALENs) and forms an RNA-protein complex, unlike ZFNs and TALENs, which form a DNA-protein complex. More than anything, CRISPR/Cas9 can simultaneously induce DSBs to more than one gene. CRISPR/Cas9 has also contributed to the production of gene-edited farm animals [94, 95]. In addition, by introducing Cas9 mRNA into early embryos, one-step method for producing gene-editing animals was introduced in zebrafish [96, 97], rats [98, 99], mice [100-103], rabbits [104], pigs [95, 105, 106], sheep, and cattle [21, 107]. Adaptation of CRISPR/Cas9 as a gene-editing tool has contributed to the efficiency of gene editing

in mammalian animals and made genetically modified animals more sophisticated [100, 108, 109].

However, although the efficiency of generating genetically modified animals greatly increased with CRISPR/Cas9, there is still a limit in inserting foreign genes. Inserting foreign genes by HDR is challenging [110-112], especially inserting large cargoes requires large population cell sorting or selection [113]. Many trials have attempted to improve knock-in efficiency; long single-stranded DNA [114], homology-independent targeted insertion [115, 116], homology-mediated end-joining (HMEJ) [110, 117], microhomology-mediated end-joining (MMEJ) [118], and targeted integration with linearized dsDNA [119]. Through further studies, knock-in efficiency in mammalian animals will be improved. The history of transgenic cattle produced by CRISPR/Cas9 is shown in Table 4. Table 5 showed comparison of three classes of molecular scissors.

Table 4. Gene edited cattle lists produced by CRISPR/Cas9

Year	Knock-out Knock-in	Inserted gene	Method of transgenesis	Method of embryo manipulation	Target gene	Reference
2015	Knock-in (Embryo level)	GFP	CRISPR/Cas9	Microinjection	NANOG	[120]
2016	Knock-in (Embryo level)	Human FGF2	CRISPR/Cas9	SCNT	Beta-casein	[121]
2016	Knock-out (Embryo level)	None	CRISPR/Cas9	Microinjection	PRNP	[122]
2017	Knock-in	Correct IARS gene, GFP	CRISPR/Cas9	SCNT	IARS	[123]
2019	Knock-out (Embryo level)	None	CRISPR/Cas9	Microinjection	PAEP CSN2	[26]

2019	Knock-out (Embryo level)	None	CRISPR/Cas9	Electroporation	NANOS2	[48]
2019	Knock-out (Embryo level)	None	CRISPR/Cas9	Electroporation	MSTN	[50]
2020	Knock-in (Embryo level)	SRY	CRISPR/Cas9	Microinjection	Chr17	[124]
2020	Knock-out (Embryo level)	None	CRISPR/Cas9	Microinjection	POLLED H11 ZFX1	[25]
2020	Knock-out (Embryo level)	None	CRISPR/Cas9	Electroporation	OCT4 ZFX	[49]
2021	Knock-in	SRY	CRISPR/Cas9	Microinjection	Chr17	[23]
2022	Knock-out	None	CRISPR/Cas9	Microinjection	MSTN	[22]

Table 5. Comparison of three classes of molecular scissors

	ZFN	TALEN	CRISPR/Cas9
Targeting domain	Zinc−finger proteins	Transcription activator−like effector	CRISPR RNA or single−chain guide RNA
Nuclease	Fok I	Fok I	Cas9/Fok I
Biallelic knockout achieved	Yes	Yes	Yes
Average mutation rate	++	+++	+++
Length of recognition domain (bp)	18−36	30−40	20
Restriction in target site	G−rich	Start with T and end with A	Protospacer adjacent motif (NGG or NAG) at end of target sequence
Complexity to design vector	+++	+	+
Off−target events	Variable	Low	Variable, to be determined

Cytotoxicity	Variable to high	Low	Low
Number of plasmids necessary	2	2	1 (2 in case of a CRISPR/Fok I construct)
Costs	+++	++	+

(transgenic Res. 2015; 24:381−96 [125])

3. PiggyBac transposon

3-1. Characteristics of piggyBac transposon

As mentioned earlier, there are representative transposons used in mammalian cells; piggyBac was derived from the cabbage looper moth *Trichoplusia ni* and Tcl-like transposons. Sleeping Beauty (SB) and Frog Prince were reconstructed from inactivated transposons in fish and frog genomes, respectively, and hAT-like Tol2 was a naturally activated vertebrate transposon isolated from the Japanese medaka fish genome. Among these transposons, piggyBac and SB have the highest activity in mammalian cells [126]. The strength of piggyBac is that it can accommodate large cargoes, but in the case of viruses, its ability to carry DNA fragments >10 kb is limited. Li et al. [127] successfully delivered and integrated a 100-kb DNA fragment into mouse embryonic stem cells using piggyBac, and the expression was also confirmed [127]. In addition, because it can be used in diverse species, it is widely used in the basic science research and biomedical field [128, 129].

In the case of SB, TA is recognized as an integration site, and the part is changed after excision, whereas in piggyBac, TTAA is an integration site, and the original sequence is returned

after excision [126]. This phenomenon is called "seamless" excision and is consequently considered to be "transgene-free or genetically unmodified cells" [130]. This ability to integrate and remove chromosomes could be an advantage for researchers to elaborately evaluate the role of GOIs in vivo or in vitro. Figure 3 shows the simple integration mechanism of piggyBac transposon.

Furthermore, piggyBac tends to have a relatively higher expression rate than other transposons. Wu et al. confirmed transposition activity among piggyBac, SB, and Tol2 in mammalian cells, and piggyBac was the highest measured [131]. Therefore, studies showed that piggyBac transposon is often inserted into the transcription unit and has strong expression [131-137]. In addition, the piggyBac transposon avoids the G/C-rich position and transgene concatemerization, thereby preventing the silencing of the transposition transgene [138, 139].

piggyBac can accommodate a large cargo, transgenesis of various mammalian cell lines, and has a continuous expression and relatively strong expression compared with other transposons, which are very useful in producing transgenic animals. In the next chapter, I will look at cases of piggyBac applied to the production of transgenic livestock.

3-2. History of transgenesis in livestock through piggyBac

3-2-1. Pigs

Early studies using piggyBac in pigs showed transposition activity in porcine cells and that gene regulation was possible by Cre, Dre, and Flp recombinase [140, 141]. Kim et al. [11] produced iPS-like cells using doxycycline-inducible piggyBac encoding four porcine transcription factors. The pluripotent characteristics were demonstrated through self-renewal, high proliferation, pluripotent markers, and aggregation formation rate. Using these cells, SCNT was performed to produce cloned blastocysts with an increased total cell number than wild type.

PiggyBac can be used to accommodate multiple genes and express them in a single cell, which is important for producing domestic animals with strong traits. Masahiro et al. [39] simultaneously expressed seven genes in porcine embryonic fibroblasts using the piggyBac transposon. Five genes were drug resistance proteins and two were GFP and RFP. Cloned blastocysts expressing both RFP and GFP were produced through the colonies through selection. Zhang et al. [142] successfully produced transgenic pigs expressing three microbial enzymes—beta-glucanase, xylanase, and phytase in the salivary gland—to improve the digestive capacity of pigs. The digestive capacity of the plant was increased, and the growth rate also increased by

23.0% for gilts and 24.4% for boars. Wang et al. (2020) successfully produced pigs expressing beta-glucanase, xylanase, and phytase, and then additionally pigs expressing pectinase and cellulase. Through this, it was demonstrated that the insertion and expression of several genes using piggyBac is possible and that it can be sufficiently used as a transgenesis tool of donor cells for SCNT.

There is difficulty in using piggyBac as a therapeutic application due to low DNA delivery efficiency. To overcome this limitation, Ashley et al. [143, 144] used piggyBac/adenovirus (Ad) to treat the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, effectively delivering the gene and showing that persistent expression is possible. PiggyBac/Ad was injected into the pig's airway via aerosolization and showed a wide range of delivery efficiency in the pulmonary distribution of vectors. Approximately 30–50% of large and small airway epithelial cells in non-CF pigs expressed GFP. This result showed treatment using *CFTR* pigs by complementing the disadvantage of low efficiency of piggyBac and decrease of episomal expression of AAV [145].

3-2-2. Cattle and goat

Fluorescent gene was delivered to bovine fibroblast cells using PiggyBac and expression was observed. Using this cell as a donor cell, it was confirmed that fluorescent protein was also expressed in blastocysts [146], and gene regulation by doxycycline is also possible [147]. Transgenic cattle were successfully produced by transplanting bovine blastocysts delivered with piggyBac transposon by microinjection. As a result of whole-genome sequencing, it was observed that the gene was specifically inserted into the TTAA site. Although it had several copy numbers, genome stabilities showed no significant difference [17]. Additionally, no abnormalities were observed in blood tests and appearances in transgenic cows aged approximately 36 months, which did not appear to be affected by long-term gene expression. It was also confirmed that germline transmission was performed normally in the next generation. In this founder animal, no significant changes in normal blood levels and overall genomic stability were observed [148].

In mice, embryonic stem cell culture system is well developed, and it is possible to purchase and use them commercially, whereas it is still under development for livestock. *Oct3/4, Sox2, Klf4*, and *cMyc* are important genes in induced pluripotent stem cells, and bovine iPSCs were produced with

these genes using the piggyBac transposon. iPSCs thus produced could be cultured up to passage 50 and showed a dome shape similar to that of normal karyotype and mouse embryonic stem cells. The rate of formation of single cell-derived bovine iPSCs by this method was 40% [149].

In goats, Ding et al. (2012) [150] experimented on the transposition of the PiggyBac transposon into cashmere goat fetal fibroblast cells. Compared to random integration, the expression level of GFP in the PiggyBac transposon increased by 7.78-fold. Shi et al. (2017) [151] produced Tb4-overexpressing cashmere goats using cells in which the thymosin beta-4 (Tb4) gene was overexpressed by piggyBac transposon to increase cashmere production in cashmere goats. As a result, it was observed that the number of secondary hair follicles producing cashmere (fine fiber) increased compared with that in the wild type [151].

3-3. Refinement of piggyBac

PiggyBac is a powerful gene engineering tool that can efficiently deliver transgenes to humans, mice, rats, pigs, cattle, and sheep without barriers between species and can also deliver transgenes regardless of cell types. Through many studies, the efficiency of piggyBac has been continuously increased. Transposon efficiency means that it stably passes through the

cell membrane and is inserted into the host genome. To increase this, three consecutive events are required. Transposon vectors are excised from the plasmid backbone, and the overall stable transfection efficiency and averages of vector copy number per cell determine the efficiency of piggyBac [152]. Past studies have significantly improved the efficiency of piggyBac through various methods, including piggyBac transposase modification, piggyBac transposase mRNA, Inverted Terminal Repeat modification, use of insulators, use of matrix attachment regions, and combination with viral vectors. In particular, as the hyperactive piggyBac transposase [153] was developed by Yusa et al., it showed that the integration efficiency was increased 9-fold compared with the existing transposase in mouse ES cells. In addition, recently, using adenovirus and lentivirus together, the transient expression of the virus was reduced and the piggyBac gene delivery efficiency was increased, thereby showing potential for in vivo therapy.

However, since piggyBac does not specifically deliver transgene through host genome integration, safety concerns are always accompanied. Through research, it was found that when piggyBac is integrated into the host genome, it tends to be easily inserted into genes containing locus or transcription start sites [154]. Therefore, a recent study reported that piggyBac

interacts with cellular BET protein and transposase to guide the TSS site [155], which means that piggyBac is very similar to gamma-retrovirus murine leukemia virus [155-157]. Intergenic integration may be detrimental to the host genome, and in the case of TSS, it may be involved in oncogene expression, so improvement instability is required. To overcome this, for the specific integration of piggyBac, the fact that it can accept protein domain well was utilized (amenable). For example, the DNA domain Gal4 DNA-binding domain [158] and TAL domain [159] were used, and the efficiency was 24% and 0.01%, respectively. This specific integration of piggyBac can improve stability problems, but so far, many improvements are needed to increase efficiency.

Since the insertion region with a high preference for piggyBac is the TSS or intergenic locus, a study to confirm the potential genotoxicity by the long-term expression effect is also important. Nakazawa et al. [160] confirmed the potential clonal outgrowth in human T lymphocytes to confirm the long-term effect of piggyBac. Saha et al. [161] showed no clonal expansion in human foreskin fibroblasts. Saridey et al. [162] also reported that tumor formation was not observed even in 1-year-old mice modified by piggyBac. In addition, in our experimental results, even cattle that are currently expressing GFP using piggyBac

have been living without problems for more than 7 years, and germline transmission has also been confirmed. However, more studies are needed to determine the potential genotoxicity of long-term expression.

3-4. Biomedical field (meat, milk, and bioproduction)

In livestock, the increase in food production efficiency and production of recombinant protein through milk are especially important to research. PiggyBac stably delivers cassettes, and the size of the delivered cassettes is up to 200 kb, and it can even be applied to various cells and is provided as a toolbox in many fields. Potential areas such as knock-out screening, transgenic production [163], iPSC production [164], and somatic cell therapy [165] are becoming increasingly possible. These capabilities of piggyBac can be applied to food production and recombinant protein production in milk.

Experiments to produce recombinant protein using mammary glands have been conducted in cattle and goats. In the past, many transgenic cattle were produced using viruses. However, there have been problems with the method using viruses or random insertion, including the risk of tumorigenesis, insertion of a backbone excluding the desired gene, and gene silencing. If an individual is produced using piggyBac, problems

caused by viruses can be minimized. Using piggyBac, there was no health problem in the individual cattle for 7 years [19], and the gene was normally expressed and the production of monoclonal antibody through the cell line was possible [166]. It is also possible to produce a bioreactor from milk produced from goats.

In addition, the gene delivery ability of piggyBac can be utilized to increase livestock feed efficiency or food production. In one study, the growth rate was increased due to the increase of specific digestive enzymes including beta-glucanase, xylanase, and phytates in pigs, and the feed conversion rate was also increased compared with the wild-type. In a recent study, endogenous gene overexpression became possible through the combination of piggyBac and CRISPR/Cas9. This technique can produce animals with increased food production by overexpressing long non-coding RNA (lncRNA) that affects meat or milk [167].

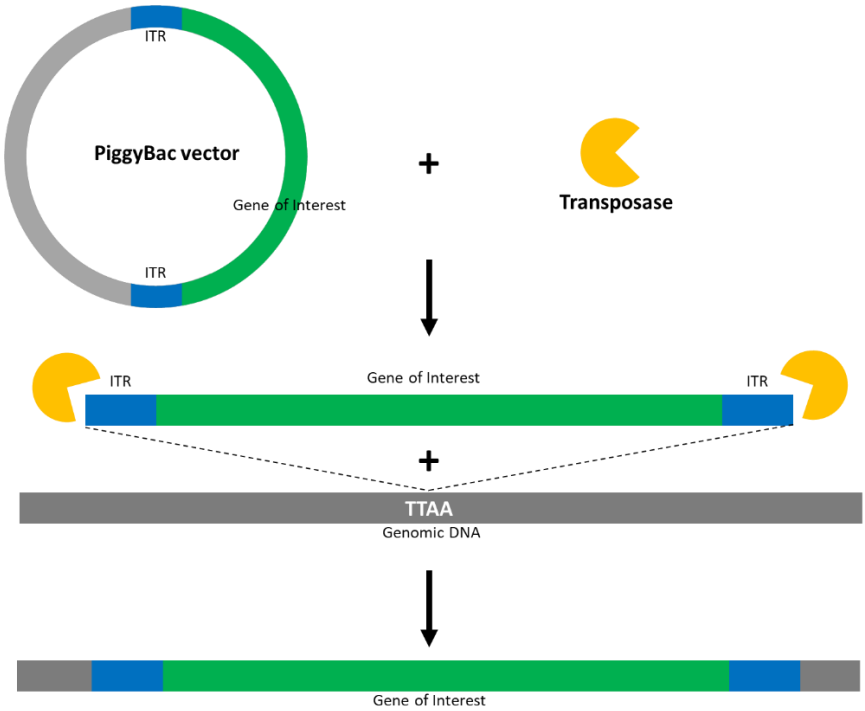

Figure 3. Mechanism of transposition of piggyBac transposon.

PART II. GENERAL METHODOLOGY

1. Reagents

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

2. Primary cell culture

Primary cells were obtained from a biopsy of the ear skin of calves. The ear skin was chopped into small pieces with a sterile scalpel and then washed several times and incubated at 38.5 ℃ for 4-18 h in Hank's balanced salt solution (Gibco, 14175095) supplemented with collagenase (Collagenase type I, Gibco, 17-100-017). The following day, the dispersed cells were washed several times in Dulbecco' s Modified Eagle Medium (DMEM; Gibco, 21068028) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, GIB-16000-044), 1% penicillin/streptomycin (Gibco, 15140148), 1% non-essential amino acids (Gibco, 11140050), and 100 mM β-mercaptoethanol (M3418).

3. In vitro maturation

Ovaries were obtained from a local slaughterhouse and

delivered to the laboratory within 2 hours. The ovaries were aspirated with an 18-gauge needle to obtain cumulus-oocyte complexes (COCs) from follicles 2-8 mm in diameter. The COCs with more than three layers of cumulus cells and evenly distributed cytoplasm were sorted in the study. For in vitro maturation (IVM), the COCs were cultured in chemically defined TCM-199 supplemented with 0.005 IU/mL follicle-stimulating hormone (FSH, F2293), 1 μg/mL 17β-estradiol (E4389), 100 μM cysteamine (M6500), and 10% FBS in a humidified atmosphere of 5% CO_2 at 38.5 ℃.

4. In vitro fertilization and in vitro culture of embryo

Motile spermatozoa were selected using the Percoll gradient method. Briefly, frozen-thawed cattle semen at 35 ℃ was filtered by centrifugation on a Percoll discontinuous gradient (45-90%) at 1680 rpm for 15 min. To produce the 45% Percoll solution, 1 mL of capacitation- Tyrode's albumin lactate pyruvate (TALP) medium was added to 1 mL of 90% Percoll. The sperm pellet was washed two times by the addition of 3 mL of the capacitation-TALP medium and was subsequently

centrifuged at 1680 rpm for 5 min. Washed, motile spermatozoa were used for in vitro fertilization (IVF). Spermatozoa (1-2 × 10^6 sperm/mL) were incubated with mature oocytes for 18 h in 50 ㎕ microdrops of IVF-TALP medium covered with mineral oil (Nidacon, NO-100) in a humidified atmosphere of 5% CO_2 at 38.5 ℃. After 18 h of co-incubation, cumulus cells were removed from presumptive zygotes. The zygotes were cultured in a two-step chemically defined culture media that was covered in mineral oil in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 38.5 ℃.

PART III.
GERNERATION OF GENETICALLY ENGINEERED CATTLE

Chapter Ⅰ. The production of *PRNP* gene mutated cattle by CRISPR/Cas9 and piggyBac transposon

1. Abstract

Even though the incidence of bovine spongiform encephalopathy (BSE) has decreased, developing bovine models is an important point for basic understanding of prions in cattle. This study investigated the possibility that PRNP knockout cattle (F0) are born by two strategies: microinjecting all-in-one DNAs and conditional PRNP vectors into in vitro fertilized zygotes. As a result, 7 calves were successfully born by microinjecting all-in-one DNAs. Among them, 4 claves (#P1, #P3, #P6, and #P7) showed PRNP mutation. In addition, GFP expression and Cas9 activity were observed in the calves (#P1, #P3, #P4, and #P7). To verify germline transmission, #P3 and #P7 germ cells were cultured in vitro and *PRNP* mutation was observed in their blastocysts. In PRNP F1 cattle, the gene of interest in all-in-one DNAs (Cas9, GFP, and sgRNA for *PRNP*) was identified and *PRNP* mutation was observed. To study the detail function of *PRNP*, conditional *PRNP* mutant cattle was born based on the Cre/loxP system. In the somatic cells of the cattle, after Cre treatment, the cells expressed Cas9 protein but did not show *PRNP* mutation. As a result of germline transmission of the conditional *PRNP* male cattle, transgene integration and GFP

expression were observed in blastocysts fertilized with the male cattle semen. In conclusion, these data demonstrated that *PRNP*-mutant cattle were born, germline transmitted to the next generation and survived up to date. Those *PRNP*-mutant cattle and their germ cells will be valuable resources for studying prion diseases.

2. Introduction

Prion diseases, such as bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and Creutzfeldt-Jakob disease (CJD) in humans, are fatal neurodegenerative diseases. Clinical symptoms include abnormal behavior, trouble walking, and weight loss in BSE-infected cattle, and it has long incubation periods (4-5 years) for onset of signs. Even seriously, BSE has also been transmitted to humans, resulting in the CJD variant. For this reason, despite being a very rare disease, incidence of BSE has been highlighted to the world.

The pathology of BSE is known to develop when normal cellular prion proteins are converted into abnormal pathologic prion, and it is not elucidated exactly how the normal form becomes pathologic. It is also known that normal prion proteins have several functions [168], such as stemness [169], glutamate receptor function [170], calcium homeostasis [171], memory formation [172], and neuroprotection [173]. However, most functions of prion have been identified in in vivo or in vitro mice models or human cells, not sheep and cattle. To date, studies on the functions of prion in cattle with host-specific diseases have been very limited, especially in vivo models.

Therefore, even though bovine prion models are eagerly required for exact prion function and BSE, producing live cattle with mutated prion gene is very limitedly carried out and only one is reported [174] to date. There are several reasons why generating live genetically engineered cattle is difficult. Several reasons for mutated prion in cattle have technical issues such as high cost, long gestation term, and single pregnancy. Another issue, for producing genetically engineered cattle, mostly SCNT is employed to date because there are no germline competent embryonic stem cells such as mice. While SCNT is known as a powerful tool for generating genetically engineered cattle, its progress in establishing genetically engineered cattle with germline transmission is slow due to abnormal reprogramming of somatic cells after genome engineering. In our previous study [175], while efficient *PRNP* (encoding prion protein) knockout cells are isolated, for the reasons described above, it was very difficult for us to produce prion-mutated bovine models via SCNT.

Here, I reported that for understanding prion diseases, two types of *PRNP*-mutant cattle were produced. One was born by microinjecting all-in-one DNAs into bovine zygotes, not

SCNT. The other was production of conditional *PRNP*-mutant cattle based on the Cre/loxP system. In addition, I also verify the germline transmission of those PRNP-mutant cattle (all-in-one DNAs and conditional PRNP) to the next generation.

3. Materials and methods

3.1 Vector cloning

The transposase plasmid for PB (pCy43) were provided by Sanger Institute (Hinxton, UK). The PB-CAG/EF1a-Cas9-GFP were prepared by inserting PCR-amplified sequence of CAG/EF1a-Cas9-GFP into the restriction enzyme site (NheI/BglII) of PB-CAG vector (http://www.addgene.org/, #20960) via In-Fusion Cloning (In fusion HD cloning kit, Clontech, 639644, California, US). spCas9 is under the expression of a CAG or EF1a promoter respectively, with SV40 nuclear localization signal (NLS) on the N terminus and linked to the GFP using 2A sequence followed by a rabbit beta-globin poly(A) signal. Single guide RNA (sgRNA) target sequence within exon 3 of the bovine *PRNP* gene (sg*PRNP*) was designed based on the Cas RGEN tool(http://www.rgenome.net). U6 promoter with a sg*PRNP* was synthesized and cloned into the PB-CAG/EF1a-Cas9-GFP using the XhoI restriction site to create final expression vector (Fig. 4A).

3.2 Microinjection

All-in-one DNAs (50 ng/㎕) and transposase vectors (50 ng/㎕) were microinjected into the cytoplasm by microinjector machine (Femtojet, Eppendorf, Germany) after removing the cumulus cells of presumed zygote. Concentration of injected DNAs was 100 ng/mL (1:1 ratio of transposon and transposase). After 7 days, GFP expressing pre-implantational stage embryos were chosen and transferred into the surrogate cow.

3.3 Embryo recovery

For embryo recovery, animals with random estrous were treated with an intravaginal progesterone device (Repro360, Cue-mate) in the vagina and 2.0 mg intramuscular of estradiol benzoate. The animal was injected with 400 mg of FSH (Kawasaki Pharm, Antorin R-10) divided into eight times every 12 hours on Day 6, 7, 8 and 9. On day 8, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Bremer Pharma GMBH, Synchromate-S). Approximately 60 and 72 hours after $PGF_{2\alpha}$ injection, the animal was artificial inseminated with frozen-thawed semen.

Embryos were recovered non-surgically using a 3-way catheter (IMV technologies, REF. 006355). Both uterine horns were flushed with pre-warmed solution (Euroflush, IMV technologies). Recovered embryos were observed under a stereomicroscope.

3.4 Semen collection

The semen was collected from bull using an electro ejaculation. (3 times per bull). Before semen collection, the preputial hairs were clipped, and the orifice was washed with clean water and then dried with clean paper towel to minimize contamination. Electro ejaculation was accomplished using a manually controlled electro-ejaculator, ElectroJac6 (Ideal Instruments Neogen Corporation, Lansing, MI, USA) attached with a 6.5-cm-diameter rectal probe with three ventrally oriented electrodes approximately 1 cm apart and was placed completely in the rectum with the electrodes facing ventrally. The number of electrical stimuli was increased until the bull ejaculated. Each stimulus lasted 8-10 s and then paused for approximately 2.0 s before the next stimulus was applied. When

the seminal discharge turned cloudy, a collection tube was placed over the penis to collect the semen. The ejaculated semen was transported to the laboratory at 25 ℃ within 30 min.

3.5 Semen cryopreservation and thawing

The semen samples were used for cryopreservation when they exhibited at least 60% general motility. The semen samples were extended with Optixcell (IMV Technologies) at 37 ℃. The extended semen was equilibrated at 4 ℃for 3 h before placed into 0.5-ml straws. Filled straws were arranged on a special rack, 5 cm above liquid nitrogen and exposed to liquid nitrogen vapour for 15 min, and then plunged into a cryogenic tank filled with liquid nitrogen (-196 ℃). The cryopreservation sperms were thawed in a water bath at 37 ℃ for 45 s.

3.6 Single guided RNA (sgRNA) design

Single guide RNA (sgRNA) targeting bovine *PRNP*, *NANOG,* and *GGTA1* were designed by Cas-Designer software (http://www.rgenome.net/cas-designer/) that showed sgRNA

candidates for the target genes (Table 6). Following the details of kit manual, the sgRNA was synthesized using Precision gRNA synthesis Kit (ThermoFisher, A29377).

3.7 T7 endonuclease I (T7E1) assay

Gene mutation was confirmed through the T7 endonuclease (T7E1) assay. For this, genome DNA was extracted by Kit (Qiagen, 69504). The PCR primers (Table 7) for target loci (*PRNP*, *NANOG* and *GGTA1*) was designed using PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4. 0/) and the target sequence was amplified by polymerase chain reaction (PCR) at 94◦C for 5 min, 35-40 cycles at 94◦C for 20 s, at 57◦C for 30 s, at 72◦C for 35 s, and 72◦C for 5 min. The PCR product from each sample was treated with T7E1 enzyme (NEB, M0302L) to detect gene mutations. Digested and undigested mixes were observed on a 1% agarose gel. The estimated gene modification was calculated as described previously.

3.8 Electroporation

Genome Editor electroporator (BEX, GEB 15) and electrode (gap: 1.0 mm, volume: 40 ㎕) (BTX, 45-0104) were used for electroporation. The electrode was connected to the electroporator and was set under a stereoscopic microscope. Before electroporation, bovine zygotes were washed with Opti-MeM I (ThermoFisher, 31985062). At one time, 30-40 bovine zygotes were electroporated. Zygotes cultured in IVF medium were washed with Opti-MEM I three times to remove the serum in the medium, placed in a line in the electrode gap filled with 10 ㎕ of Opti-MEM I which is containing 200 ng/㎕ Cas9 protein (ThermoFisher, A36499) and 100 ng/㎕ sgRNA for *GGTA1*, and subjected to electroporation. The electroporation condition was 15 V (3 msec ON+ 97 msec OFF) × 7 times. After electroporation, the zygotes were immediately collected from the electrode chamber and subjected to four washes with TCM-199 based medium followed. The embryos were then cultured in chemically defined medium at 38.5 ℃, 5% CO2, and 5% O2 in an incubator.

3.9 Primary cell culture and genomic DNA extraction

Primary cells were obtained by biopsy of the ear skin of cattle. The ear skin was chopped into small pieces with a sterile scalpel and then washed several times and incubated at 38.5 ℃ for 4-18 h in Hank's Balanced Salt Solution (Gibco, 14175095) supplemented with collagenase (Collagenase type I, Gibco, 17-100-017). The following day the dispersed cells were washed several times in DMEM (Gibco, 21068028) and cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco, 15140148), 1% non-essential amino acids (Gibco, 11140050), and 100 mM β-mercaptoethanol (M3418). Genomic DNA from primary cells was extracted using a DNA extraction kit (Qiagen, 69504).

3.10 Analysis of *PRNP* off-target effect

The potential off-target effects caused by CRISPR-Cas9 in the three *PRNP* mutant calves were assessed using Cas-OFFinder software (http://www.rgenome.net/cas-offinder/). This software offers a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases.

In the *PRNP* target site, the candidates of five loci targeting the whole genome of cattle were found by adjusting the mismatch number to three. A primer targeting each sequence (Table 8) was prepared to confirm the off-target effect through T7E1 assay.

3.11 Deep sequencing

Target sites were first amplified to a size of ~1000 bp from extracted genomic DNA using Maxime™ PCR PreMix i-StarTaq (Intron biotechnology, 25167). The 1st PCR amplicons were amplified again to a size of ~220 bp through 2nd primers having custom index sequence. And 2nd PCR products also were amplified for adding adaptor sequence to NGS (Mini-seq, Illumina). Primers used in this study are listed in Table 9.

Then, 3rd PCR amplicons were pooling and purified using a PCR purification kit (MN, Gel and PCR clean up, 740609). This purified library was used for NGS according to the illumina manual. The sequencing results of Mini-seq was saved as fastaq files and it could be analyzed through Cas-analyzer(www.rgenome.net).

3.12 Western blotting

Protein from the birth claves fibroblast cells was isolated using ProEX™ CETi Lysis Buffer with Inhibitors (TransLab, Daejeon, Korea, TLP-121.1) to the manufacturer' s protocol. Protein concentration was measured using the Bradford method (Quick Start™ Bradford Protein Assay Kit 1, Bio-rad, California, USA, 5000201).

To conduct sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), equivalent amounts of protein samples were used to fill a 12% SDS-PAGE gel (12% Mini-PROTEAN® TGX™ Precast Protein Gel, 4561043). Protein samples were incubated and loaded at 96 ℃ for 7 min for denaturing (5x SDS-PAGE, Translab, TLP-102.1 / Precision Plus Protein Dual Color Standards, Bio-rad, 1610374). The gel was allowed to run for 30 min at 90 V, and 60 min at 60 V (10X Tris-Glycine-SDS Buffer, Translab, TLP-104.2). After that, the transfer process was initiated at 385 mA for 50 min using a PVDF membrane (ProNA™ Transfer Buffer(10X), Translab, TLP-110.1 / ProNA™ Pre-Cut Transfer Paper, Translab, TLP-130). Ponceau staining was then conducted to confirm that the protein had been transferred (ponceau S solution, Translab,

TLP-113). After removing staining with distilled water (DW), the blocking process was performed for 1 h (Protein-Free 5X General-Block, Translab, TLP-115.1G). Later, the membrane was washed with DW and allowed to react with the primary antibody at 4 ℃ overnight. To remove unbound antibody, the membrane was washed with TBS-T media for 1 h. The secondary antibody was allowed to react at room temperature for 90 min, then washed. The band was identified by exposure to ECL (Bio-Rad Clarity™ Western ECL Substrate, 1705061). For running and transfer, Biorad's western blot tank (Mini-PROTEAN® Tetra cell, 1658036) was used. The gel was washed with TBS and TBS-T (Translab, TLP-118.1, TLP-118.3). The primary antibodies used to detect Cas9 were the Anti-Cas9 Antibody (7A9-3A3, Cell Signaling, Massachusetts, USA, 14697S). The secondary antibodies were Anti-mouse IgG HRP-linked (Cell signaling, 7076S).

Table 6. CRISPR/Cas9 target sequences

Gene	Target Sequence
PRNP	GAGGTGTTCGTTCGTTTTTC
NANOG	CCACTGTCCCCGTCTGTGG
GGTA1	TGGCCCAGCTACAAGCCTGG

Table 7. PCR primer sequences

Gene	Forward	Reverse	Size (bp)
PRNP	AAAGCCACATAGGCAGTTGG	GTGCATGACTGTGTCAACAT	571
NANOG	CAAAACCGCTTCCCAGCAAC	TGAACCTCAGCTACAAGCAG	515
GGTA1	ATGTCTCCAGGATGCCTTTG	GCCTACCTGCGGATATTAAGC	546
Cas9-GFP	CATCAAGCTGCCCAAGTACA	CACATGAAGCAGCACGACTT	915
U6-sgPRNP	TTATGAAGATCCCTCGACCTG	AACTTGAAAAAGTGGCACCG	510
GFP-Cas9	CCAACTGGGGTAACCTTTGA	CCACAGCATCAAGAAGAACC	1339

Table 8. *PRNP* gene off-target PCR primer sequences

Gene	Forward	Reverse	Size (bp)	Chromosome
Off-target 1	TTGAGTGTTTGCATTCTGGA	GAATGAATGGATGGGGAATG	572	3
Off-target 2	CCCACTCCAGTCTGCTCATT	TGAGAGTCATGCGTGTGACC	531	5
Off-target 3	GGCTTTGTTCTCAGGTGGAG	CAATGAGTTGGCTTTTTGCA	509	7
Off-target 4	TCACCCTTTGGTTCTGATGC	CCTTGGCTTGTGGGATCTTA	547	19
Off-target 5	GTAATGGAAACTGGGCAAGC	CTGTGGGGAAGGACATGGTA	599	24

Table 9. Deep-sequencing primer information

Gene	Sequence
PRNP-1st-F	catgggcatatgatgctgac
PRNP-1st-R	aacaggaaggttgcccctat
PRNP-2nd-F	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GCAACCGTTATCCACCTCAG
PRNP-2nd-R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CCAGCATGTAGCCACCAAG

4. Results

4.1 Introducing all-in-one DNA to bovine fibroblast and embryos

To cause prion mutation, piggyBac transposon vectors consisting of all-in-one DNA (Cas9-2A-GFP and U6-sgRNA for *PRNP* exon3), which have two types of promoters (CAG and EF1a) to express Cas9-2A-GFP, were constructed (Fig. 4A). One of the vectors and transposase were transfected into bovine fibroblast cells to assess the feasibility of all-in-one DNA. After extracting genome DNA of the cells with transfected, *PRNP* mutation was observed through T7E1 assay result (Fig. 4B, Fig. 5A). Then, the vector and transposase were microinjected into zygotes as well. On day 7, GFP-expressing blastocysts were observed (Fig. 4C, Fig. 5B) and *PRNP* mutation in randomly selected GFP blastocysts (Fig. 4D). These results showed that two types of vectors can make GFP expression and *PRNP* mutation in both bovine fibroblast cells and embryos.

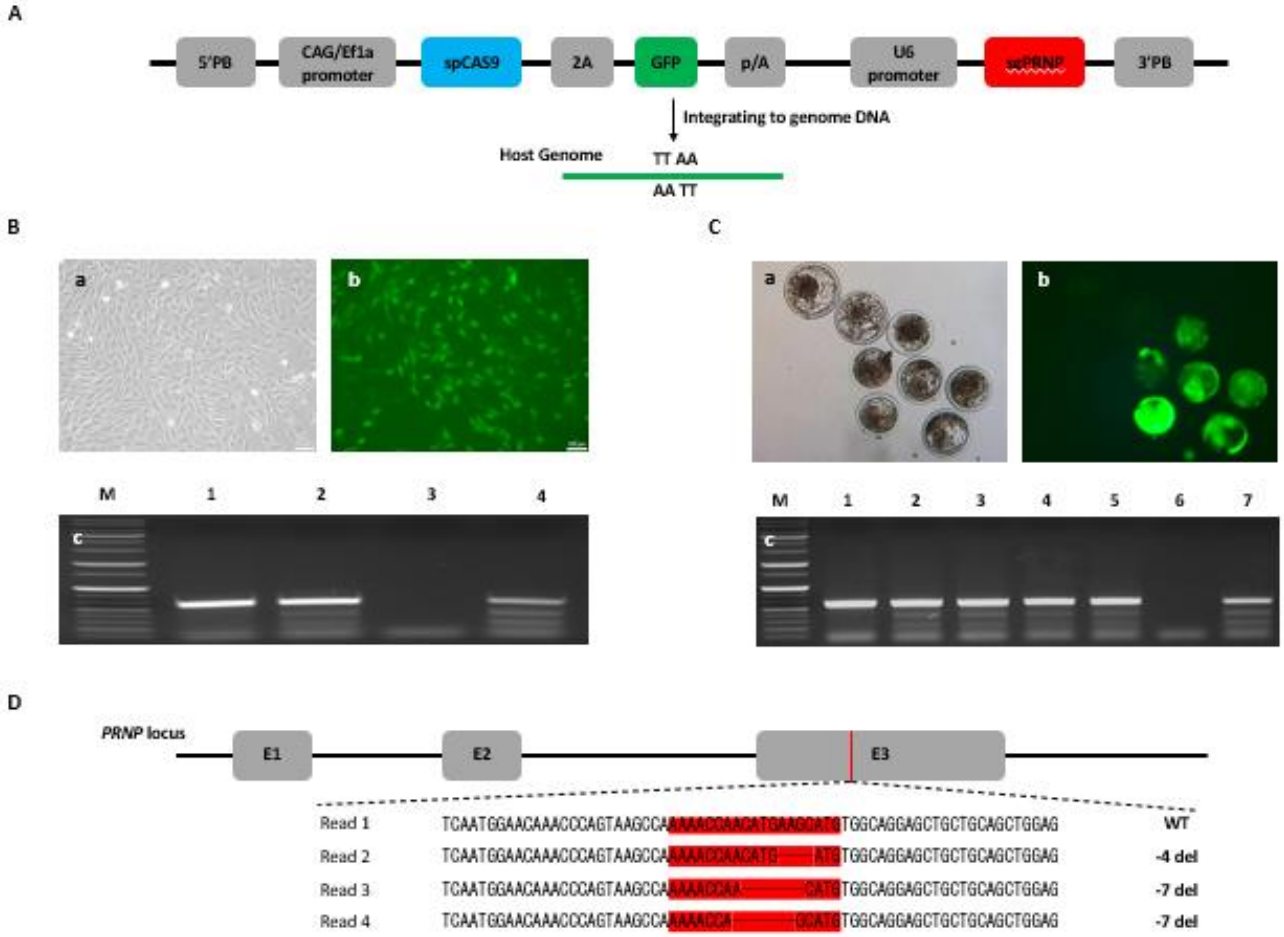
A
5'PB
CAG/Ef1a promoter
spCAS9
2A
GFP
p/A
U6 promoter
spPRNP
3'PB
Integrating to genome DNA
Host Genome
TT AA
AA TT
B
a
b
M
1
2
3
4
c
C
a
b
M
1
2
3
4
5
6
7
c
D
PRNP locus
E1
E2
E3
Read 1 TCAATGGAACAAACCCAGTAAGCCAAAAAGCAAGATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAG WT
Read 2 TCAATGGAACAAACCCAGTAAGCCAAAAAGCAACATG ATGTGGCAGGAGCTGCTGCAGCTGGAG -4 del
Read 3 TCAATGGAACAAACCCAGTAAGCCAAAAAGCAA CATGTGGCAGGAGCTGCTGCAGCTGGAG -7 del
Read 4 TCAATGGAACAAACCCAGTAAGCCAAAAAGCA GCATGTGGCAGGAGCTGCTGCAGCTGGAG -7 del

Figure 4. Validation of All-in-one DNAs to bovine somatic cells and embryos.

A. The construction of All-in-one DNAs vector (CAG promoter) and principle of PiggyBac transposon. B. All-in-one DNAs vector transfection into bovine somatic cells; a) Under bright light, b) Under blue light (488 nm), c) The T7E1 assay result about targeted *PRNP* gene region (M: marker, 1: non-transfected cells, 2: Transfected cells, 3: Negative control, 4: T7E1 assay positive control). C. Microinjection with All-in-one-DNAs into bovine embryos; a) Under bright light, b) Under blue light (488 nm), c) The T7E1 assay about targeted *PRNP* gene region (1: Wild type blastocyst, 2-5: GFP expressing blastocysts, 6: Negative control, 7: T7E1 assay positive control). D. The sequencing result of targeted *PRNP* region in GFP expressing blastocysts (Red box: target sequence).

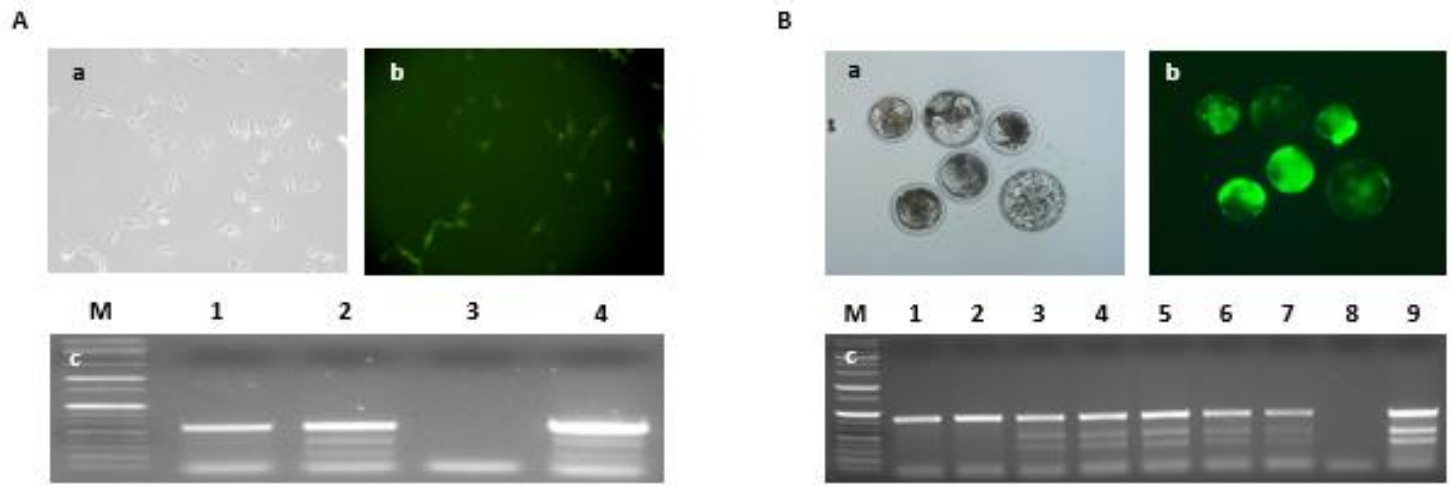

Figure 5. Validation of All-in-one DNAs(Ef1 α promoter) in bovine somatic cells and embryos.

A. All-in-one DNAs vector (Ef1 α promoter) transfection into bovine somatic cells; a) Under bright light, b) Under blue light (488 nm), c) The T7E1 assay result about targeted *PRNP* gene region (M: marker, 1: non-transfected cells, 2: Transfected cells, 3: Negative control, 4: T7E1 assay positive control). B. Microinjection with All-in-one-DNAs into bovine embryos; a) Under bright light, b) Under blue light (488 nm), c) The T7E1

assay about targeted *PRNP* gene region (1−2: Wild type blastocyst, 3−7: GFP expressing blastocysts, 8: Negative control, 9: T7E1 assay positive control).

4.2 Birth of *PRNP*-mutant cattle

To produce *PRNP*-mutant cattle, all-in-one DNAs were microinjected into 424 zygotes, and 84 blastocysts were formed (19.9 ± 5.2%). Eighteen of GFP-positive blastocysts (37.9 ± 13.2%) were selected and transferred into 18 recipients. In 18 recipients, pregnancy was confirmed, and 7 calves were successfully born (Table 10). As a result of PCR, 6 calves (#P1, #P3, #P4, #P5, #P6, and #P7) showed cassette integration-derived PiggyBac transposon (Fig. 6A). The calves showed mutations on *PRNP* at 4.1%, 48.3%, 0.2%, 0.0%, 99.6%, and 94.4%, respectively (Fig. 6B, Table 11). In addition, they were not observed any off-target effects through T7E1 assay (Fig. 7). However, though fluorescence-activated cell sorting (FACS), GFP expression was observed in 4 calves (#P1, #P3, #P4, and #P7), except #P5 and #P6, and the expression percentages were 87.8%, 42.0%, 16.0%, and 15.4%, respectively (Fig. 8).

In terms of Cas9 expression, Cas9 protein was detected in 3 calves (#P1, #P3, and #P4) through western blotting (Fig. 9A). However, when sgRNA (additional target genes; *MSTN* or *NANOG*) was transfected, the calves (#P1, #P3, and #P4) adding #7 calf showed mutation on target sites (Fig. 9B). This

result was consistent with the FACS results (Fig. 8).

Table 10. Development of bovine embryos produced under microinjection with All-in-one DNAs

Group	No. injected oocyte	No. Cleavage (%)	No. Blastocyst (%)	No. GFP Blastocyst (%)	No. recipient	No. offspring
CAG	223	136 (60.7±4.5)	48 (21.4±7.7)	16 (34.4±19.6)	15	5
Ef1 α	201	99 (51.3±7.6)	36 (18.5±1.8)	15 (41.4±4.0)	3	2

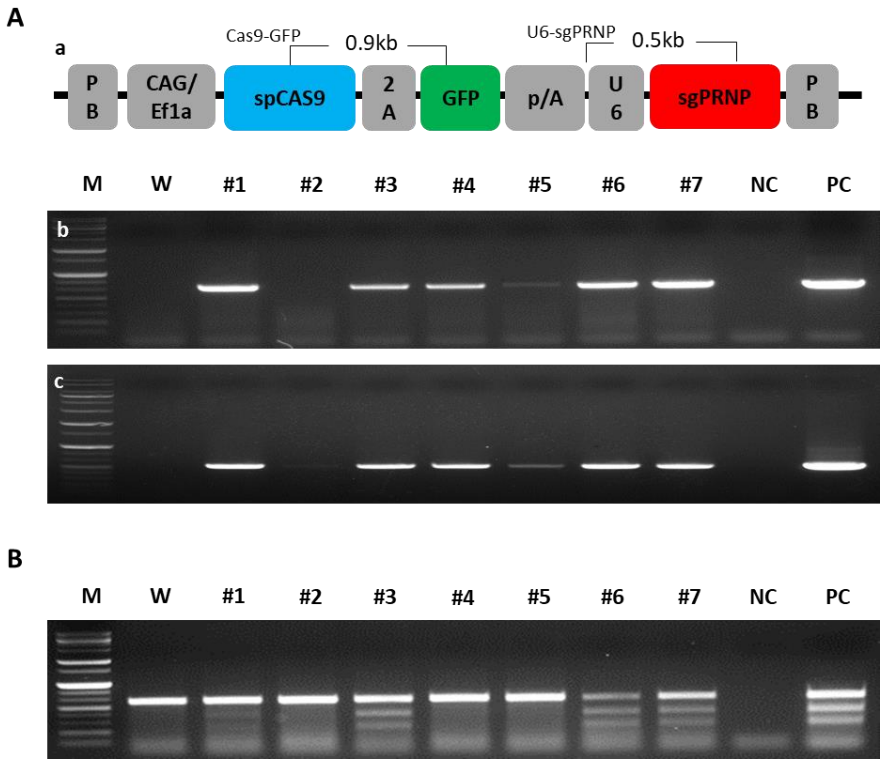

Figure 6. The birth of *PRNP* mutated calves.

A. Genomic PCR results in birth calves; a) All-on-one DNAs construction, b) GFP-Cas9 PCR result, c) U6-sg*PRNP* PCR result. B. The T7E1 assay about targeted *PRNP* region (W: wild type, #P1-P7: birth calves, NC: negative control, PC: positive control).

Table 11. Deep−sequencing results from birth calves (#P1−P7: birth calves)

ID	Mutation rate	Mutation pattern	
#P1	4.1%	GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT	WT
		GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCCATGTGGCAGGAGCTGCTGCAGCT	+1
		GGAACAAACCCAGTAAGCCAAAAACCAA-------CATGTGGCAGGAGCTGCTGCAGCT	-7
#P2	0.0%	GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT	WT
#P3	48.3%	GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT	WT
		GGAACAAACCCAGTAAGCCAAAAACCAACATG----ATGTGGCAGGAGCTGCTGCAGCT	-4
		GGAACAAACCCAGTAAGCCAAAAACCAA-------CATGTGGCAGGAGCTGCTGCAGCT	-7
#P4	0.2%	GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT	WT
		GGAACAAACCCAGTAAGCCAAAAACCAACAT----------GAGCTGCTGCAGCT	-14
		GGAACAAACCCAGTAAGCCAAAAACCAA-------CATGTGGCAGGAGCTGCTGCAGCT	-7
		GGAACAAACCCAGTAAGCCAAAAACCAACATGAA-------GCAGGAGCTGCTGCAGCT	-7

#P5	0.0%	**GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT**	WT
#P6	99.6%	**GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT**	WT
		GGAACAAACCCAGTAAGCCAAAAACCAA-------CATGTGGCAGGAGCTGCTGCAGCT	-7
		GGAACAAACCCAGTAAGCCAAAAACCAA----------TGCTGCAGCT	-21
		GGAACAAACCCAGTAAGC----------CAGGAGCTGCTGCAGCT	-24
#P7	94.4%	**GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT**	WT
		GGAACAAACCCAGTAAGCCAAAAACCAA-------CATGTGGCAGGAGCTGCTGCAGCT	-7
		GGAACAAACCCAGTAAGCCAAAAACCAACATGAA-------GCAGGAGCTGCTGCAGCT	-7
		GGAACAAACCCAGTAAGCCAAAAACCAACATGAAG-ATGTGGCAGGAGCTGCTGCAGCT	-1

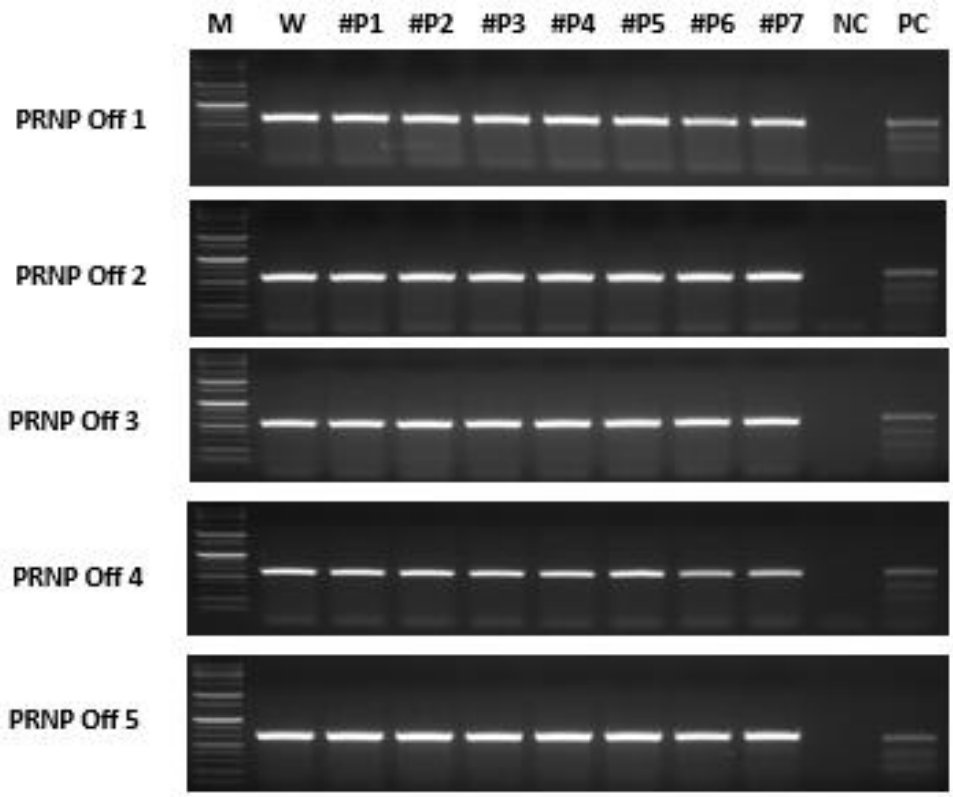

Figure 7. T7E1 assay result about off-targeting effect of targeted *PRNP* gene.

(*PRNP* Off 1-5: PRNP off-targeting candidate site, W: wild type, #P1-P7: birth calves genomic DNA, NC: negative control, PC: positive control).

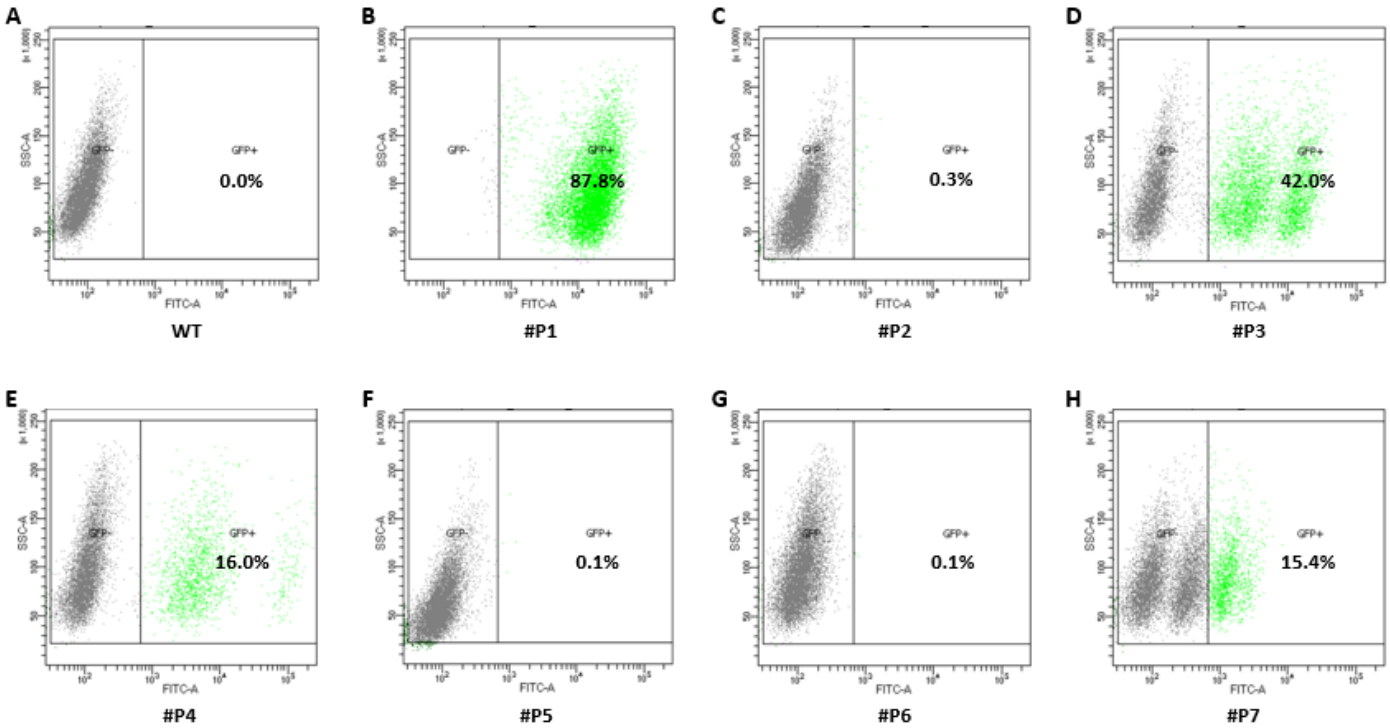

Figure 8. FACS (fluorescence-activated single cell sorting) results from birth calves.

(A: wild type, B: #P1 calf, C: #P2 calf, D: #P3 calf, E: #P4 calf, F: #P5 calf, G: #P6 calf, H: #P7 calf).

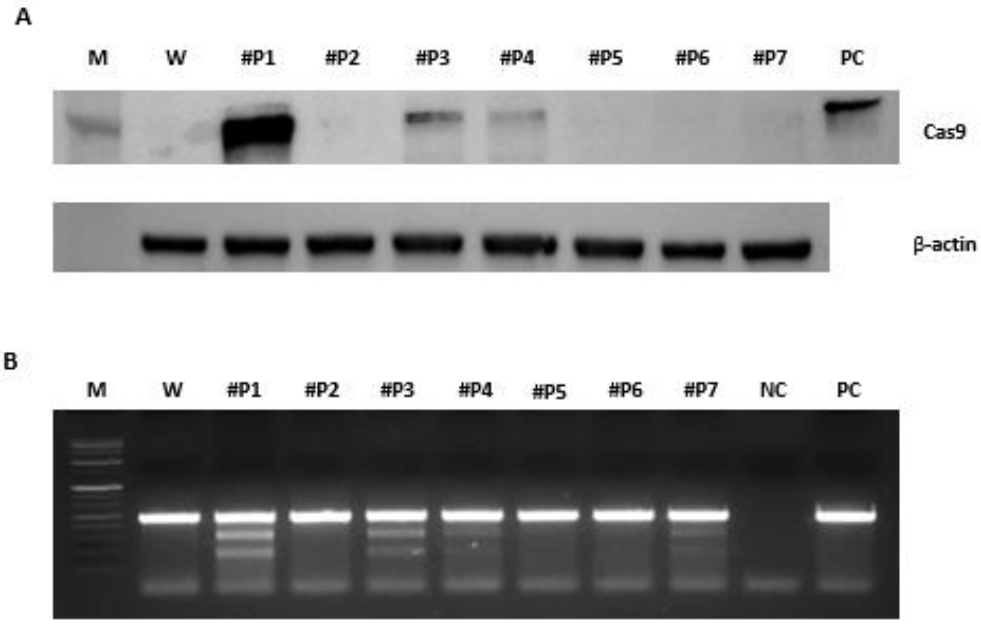

Figure 9. Cas9 protein detection and activity from birth calves.

A. Cas9 protein western-blotting in the claves (upper panel: Cas9 western blotting, downer panel: β-actin western blotting). B. T7E1 assay result about cas9 activity derived from birth calf somatic cells. (W: wildtype, #P1-P7: birth calves genomic DNA, NC: negative control, PC: positive control).

4.3 Germline transmission of *PRNP*-mutant cattle

Two calves (F0-female and -male) that have prion mutations are growing well to date. A female (#P3) has been grown up to date (current age: 29 months old) and for confirming the germline transmission, the oocytes by ovum pick up (OPU) were collected, matured in vitro, and fertilized with wildtype frozen-thawed semen. The presumptive zygotes were cultured in chemically defined media, which are used in our previous publication. Fourteen of the 49 collected oocytes were developed to blastocyst stage (9 times). Nine blastocysts were transferred to 9 recipients (single blastocyst/recipient). In 3 recipients, pregnancy was confirmed by rectal palpation and ultrasound. Three continued the pregnancy to term. Leftover developmental embryos were used for T7E1 mutation assay and knockout was positive (Fig. 10A).

A F1 calf was born, and primary cells were isolated. In the primary cells, GFP expression was positive (Fig. 10B), but *PRNP* mutation was negative through T7E1 assay and deep sequencing (Fig. 11, Table 12). In western blotting of Cas9, the protein was detected (Fig. 12A) and Cas9 activity was observed in the primary cells (Fig. 12B). Then, Cas9 and sgRNA for genomic

DNA from F1 offspring were positive (Fig. 13).

The F0 male (#P7) has reached puberty, and semen was collected by electro-ejaculation. Frozen-thawed semen was subjected to genomic mutation assay. The target locus of genomic DNA from the semen was positive for mutation. Then, the oocytes from the wildtype cow were fertilized with frozen-thawed semen from F0 male and cultured for 7 days. All blastocysts were positive for *PRNP* gene mutation. The blastocysts were transferred into 5 recipients, and 1 pregnancy was identified. In F1 calf, *PRNP* mutation was observed in T7E1 assay (Fig. 14). Another gene knockout using electroporation was subjected to in vitro fertilized embryos from the F0 male (#P7) semen. In 8 blastocysts, *GGTA1* knockout was 62.5% and *PRNP* was 100% (Fig. 15).

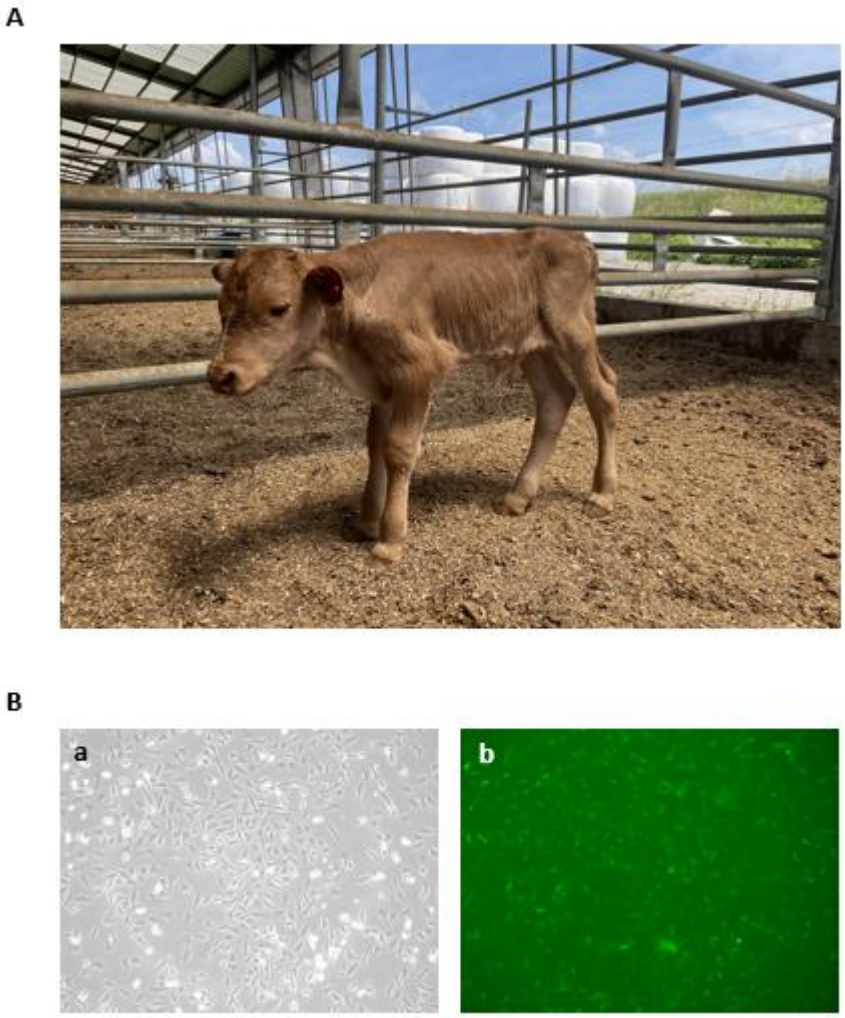

Figure 10. Germline transmission of *PRNP* mutated cattle.

A. 3-month prion mutated #P3-F1 calf. B. F1 fibroblast cells derived from ear tissue; a) under bright light, b) under blue light.

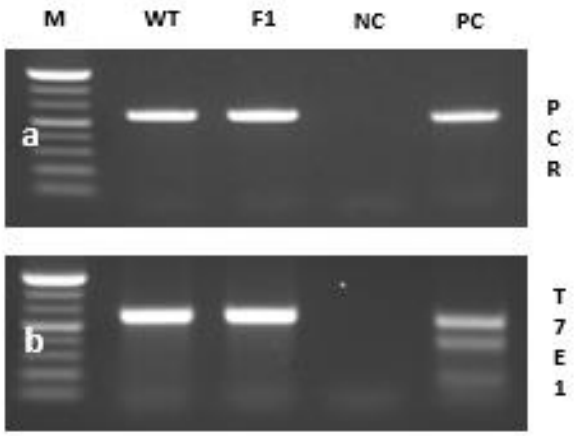

Figure 11. T7E1 assay result about *PRNP* gene.

a) PCR band, b) T7E1 band (M: marker, WT: DNA derived from wild type fibroblast cells, F1: DNA derived from F1 calf fibroblast cells, NC: negative control, PC: positive control).

Table 12. F1 calf deep-sequencing result for *PRNP*

ID	Mutation rate	Mutation pattern	
F1 calf	0.0%	**GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT**	**WT**

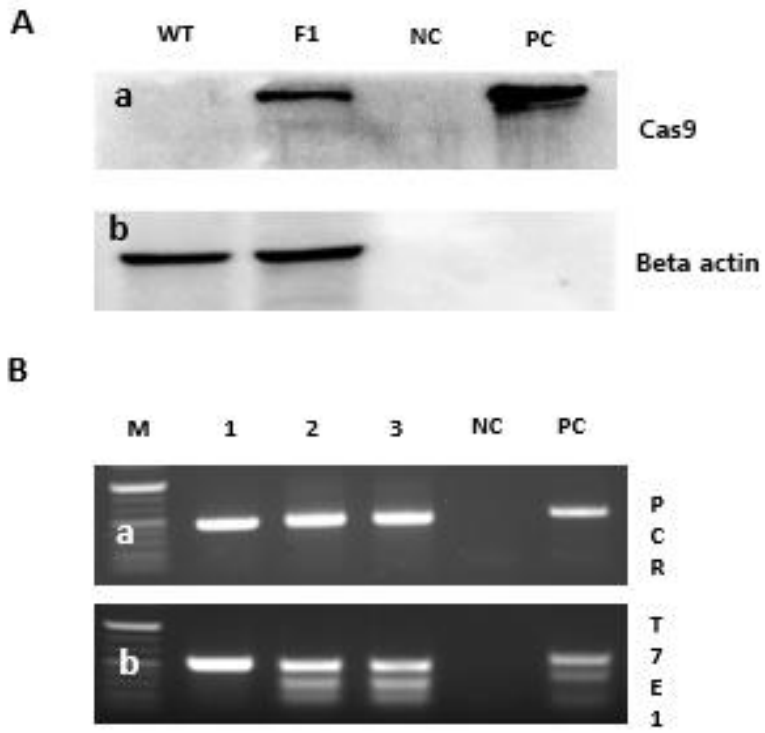

Figure 12. Cas9 protein detection and activity.

A. Western-blotting for Cas9 and beta actin protein; a) Cas9 protein (160 kDa), b) beta actin protein (43 kDa) (WT: wild type, F1: F1 calf, NC: negative control, PC: Cas9 protein 0.075 ng). B. T7E1 assay about Cas9 activity from F1 fibroblast cells; a) PCR band, b) T7E1 band (1: F1 fibroblast cells, 2: Transfection with sgRNA for *NANOG*, 3: Transfection with Cas9 protein and sgRNA for *NANOG*, NC: negative control, PC: positive control).

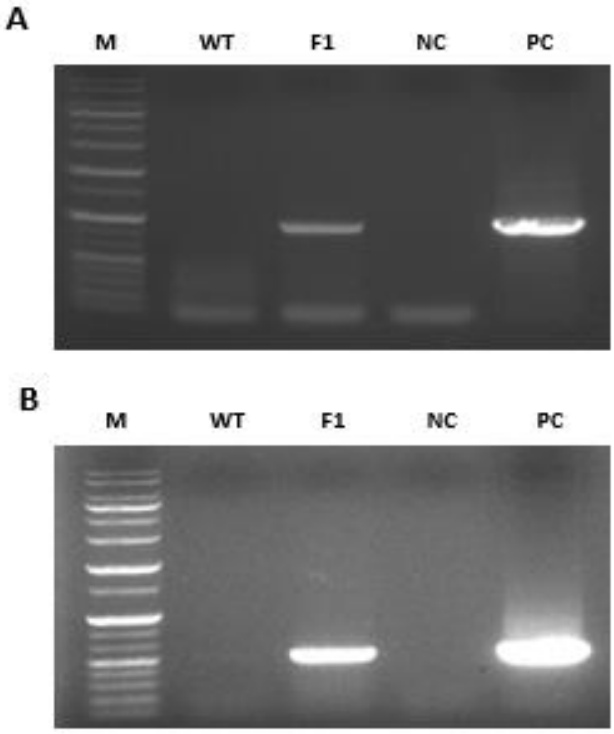

Figure 13. Genomic PCR result for F1 calf.

A. Cas9-GFP site PCR result. B. U6 promoter-sgRNA site for *PRNP* PCR result. (M: marker, WT: DNA derived from wild type fibroblast cells, F1: DNA derived from F1 calf fibroblast cells, NC: negative control, PC: positive control).

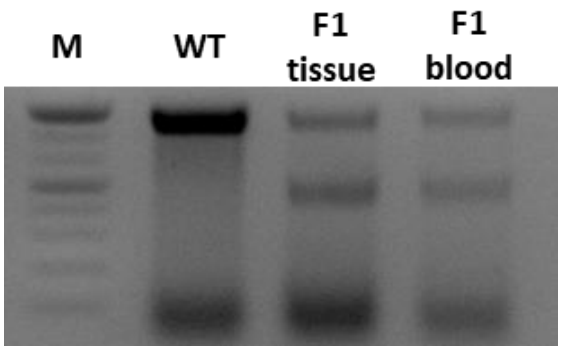

Figure 14. *PRNP* T7E1 assay result in *PRNP* F1 calf.

(M: marker, WT: DNA derived from wild type fibroblast cells, F1 tissue: DNA derived from F1 calf ear skin tissue, F1 blood: DNA derived from F1 calf blood).

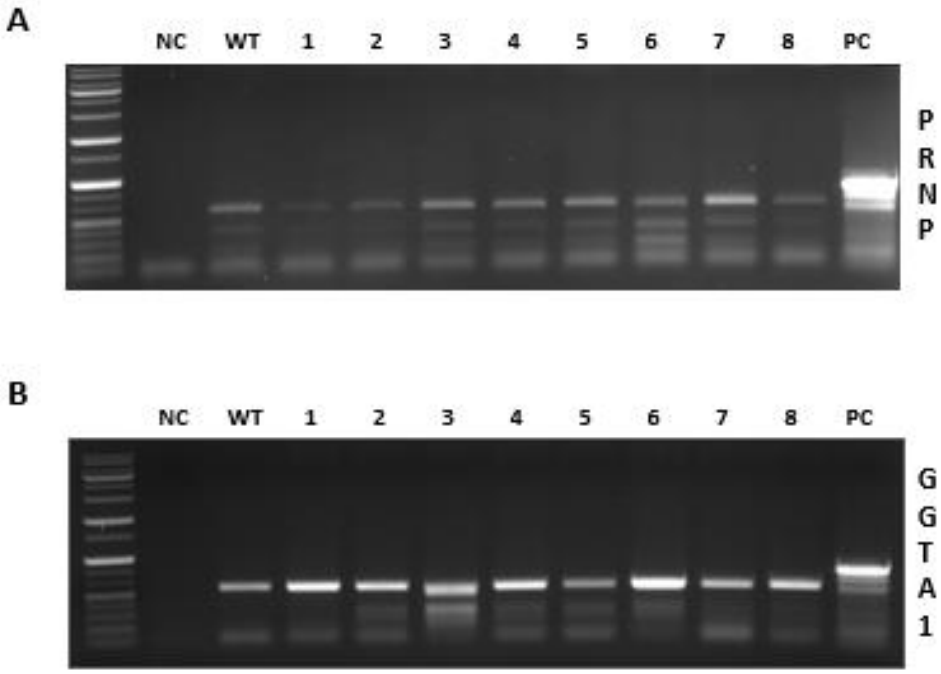

Figure 15. Further gene editing using electroporation.

A. T7E1 result for *PRNP* gene. B. T7E1 result for *GGTA1* gene (NC: negative control, WT: wild type, 1−8: blastocysts produced by electroporation with RNP (Cas9 protein and sgRNA for *GGTA1*), PC: T7E1 positive control).

4.4 Conditional *PRNP* vector design and validation in vitro

To produce conditional *PRNP*-mutated cattle, a conditional PiggyBac transposon vector based on Cre/loxP system was designed (Fig. 16). The vector was transfected into bovine somatic cells and the cells were observed with GFP expression (Fig. 17). After Cre treatment, GFP expression disappeared from the cells and loxP-flocked GFP cassette excision was observed (Fig. 18.). Using T7E1 assay, *PRNP* mutation was observed in the Cre treatment group (Fig. 19).

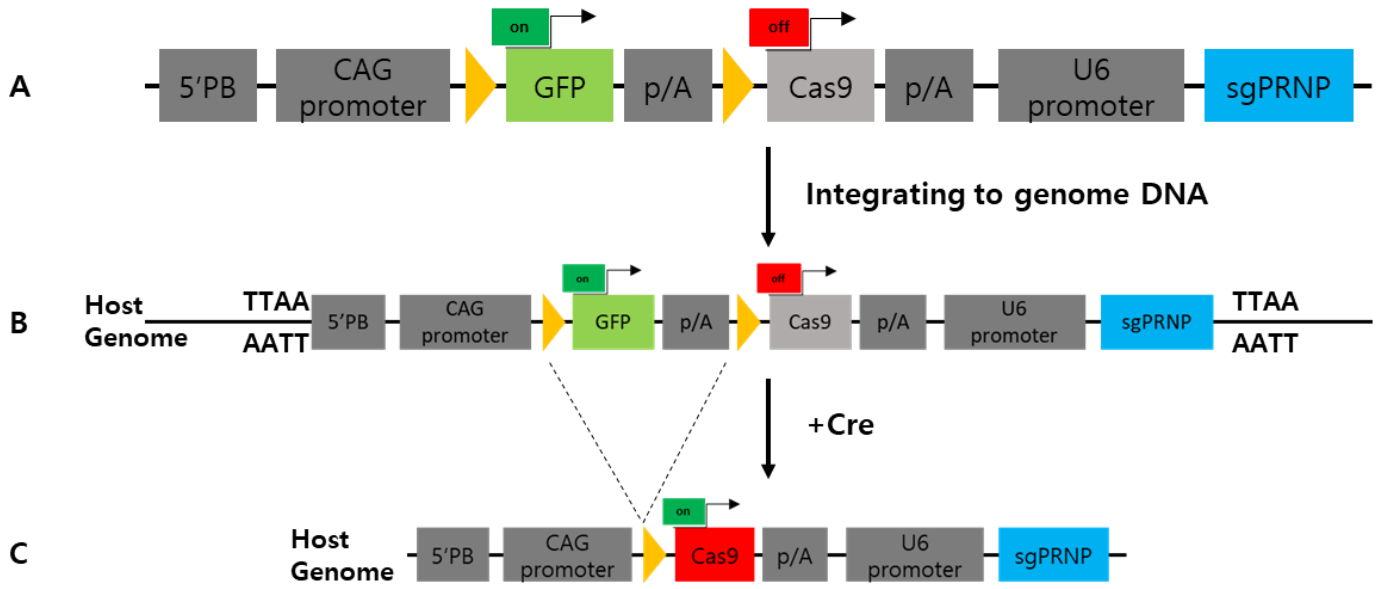

Figure 16. The construction of conditional *PRNP* vector.

A. The construction of conditional PRNP vector. B. Host genome after integration into genome DNA. C. Host genome after Cre protein treatment.

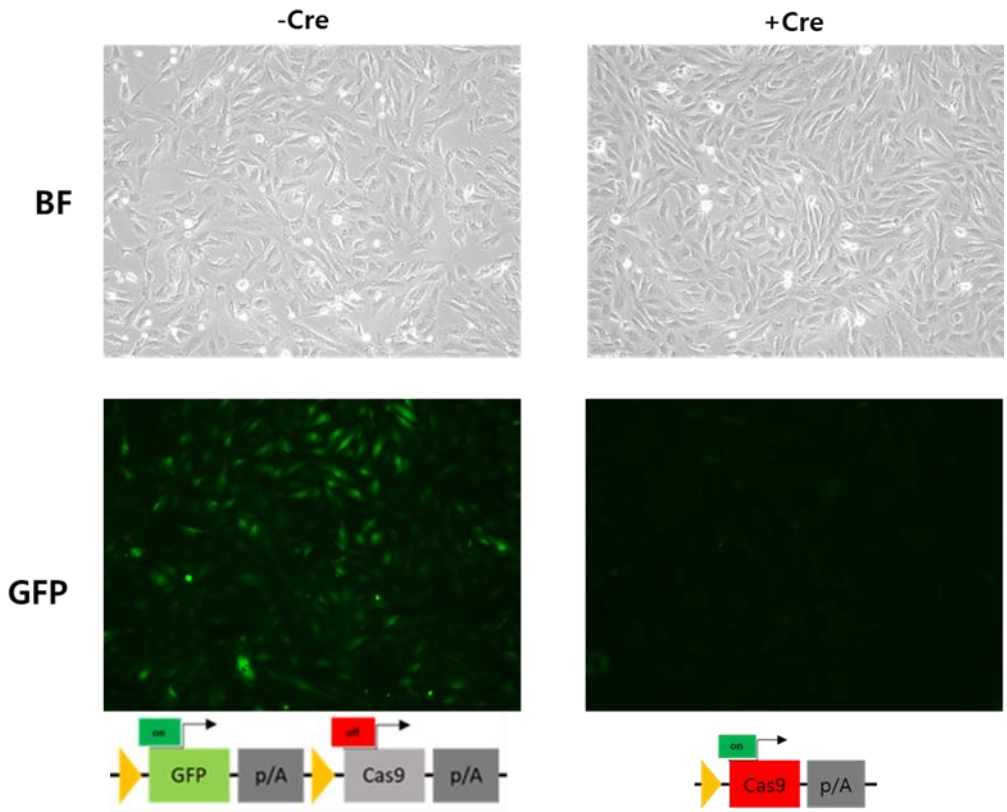

Figure 17. Conditional *PRNP* vector transfection into bovine somatic cells.

(BF: bright field, GFP: under blue light, −Cre: Cre negative, +Cre: Cre treatment).

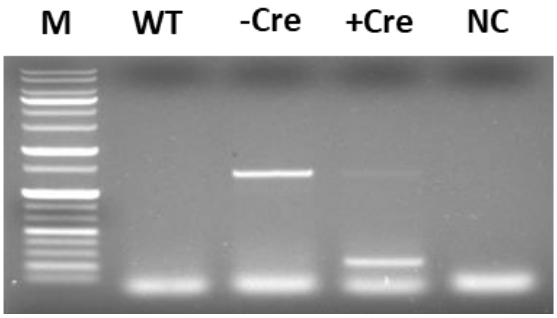

Figure 18. PCR for loxP-flocked GFP excision.

(WT: wild type, -Cre: Cre negative, +Cre: Cre treatment, NC: negative control).

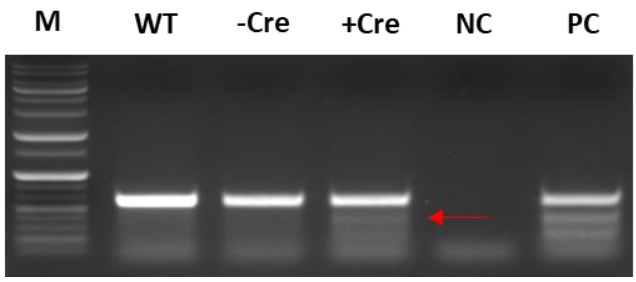

Figure 19. *PRNP* mutation after Cre treatment.

(Red arrow: cleaved *PRNP* PCR band, WT: wild type, −Cre: Cre negative, +Cre: Cre treatment, NC: negative control, PC: positive control).

4.5 Birth of conditional *PRNP*-mutated calves

Bovine zygotes were microinjected with conditional *PRNP* vector and GFP expressing blastocysts were transferred into 5 surrogates. Two surrogates were pregnant on 55 day, and two calves (#CP1 and #CP2) were born (Fig. 20). In FACS results, their GFP expression rate in somatic cells derived from their ear was 46.1% and 61.6%, respectively (Fig. 21), and transgene integration was observed (Fig. 22). After Cre treatment to the cells, loxP-flocked GFP cassette was excised (Fig. 23A) and Cas9 protein was detected in western blot (Fig. 23B). However, *PRNP* mutation was not observed in the Cre treatment group in T7E1 assay and deep-sequencing result (Fig. 23C, Table 13).

To find out whether there is problem with Cas9 or with sgRNA, Cas9 activity was confirmed. Only GFP cells in two calves were sorted and then Cre treatment and GFP negative cells were sorted. GFP-negative cells were transfected with sgRNA for *PRNP*. In T7E1 and Sanger sequencing, *PRNP* mutation was 6.9% and 20%, respectively (Fig. 24).

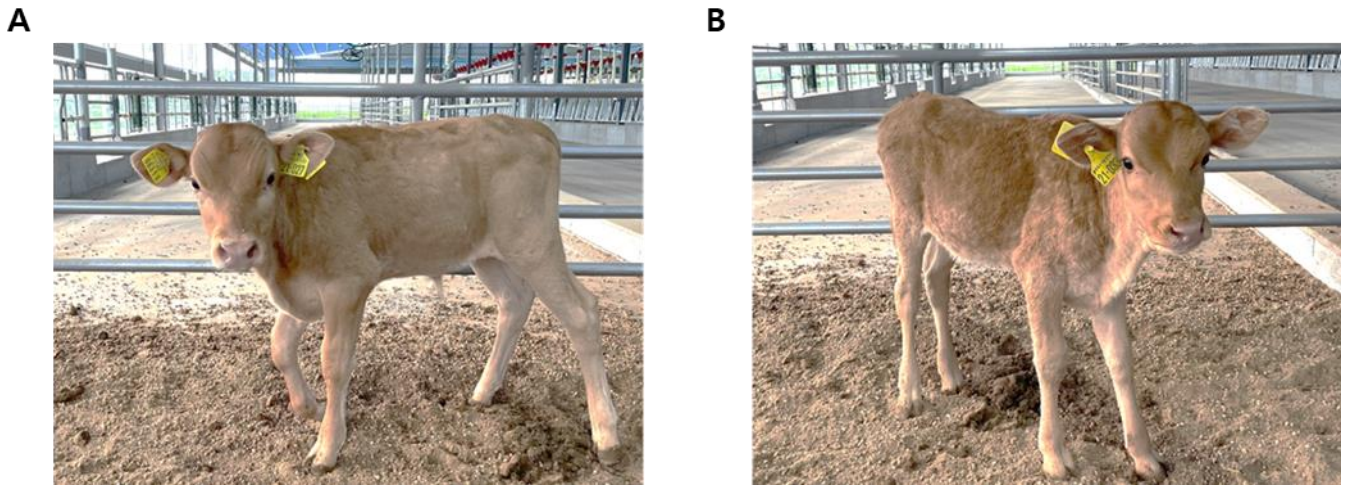

Figure 20. Conditional *PRNP* mutated calves.

(A: conditional *PRNP* male calf, B: conditional *PRNP* female calf).

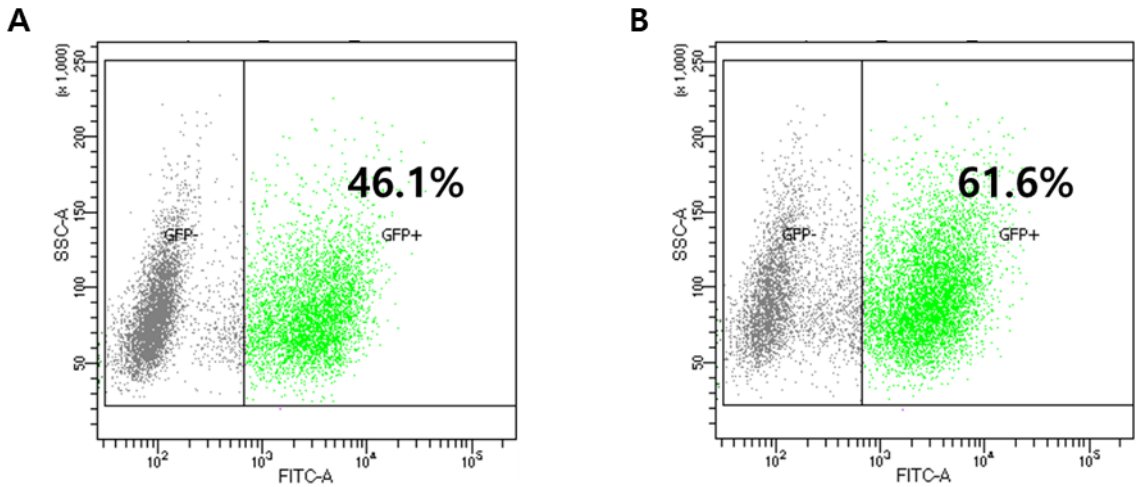

Figure 21. FACS result of conditional *PRNP* mutated calves.

(A: conditional *PRNP* male calf, B: conditional *PRNP* female calf).

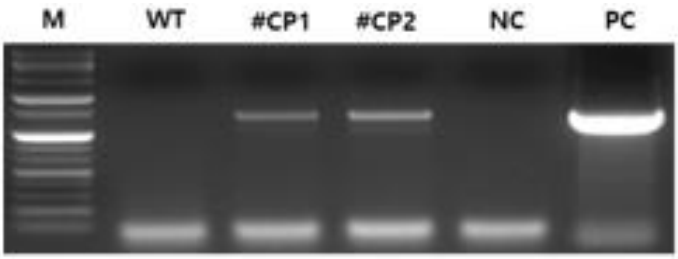

Figure 22. PCR for transgene integration.

(WT: wild type, #CP1: conditional *PRNP* male calf, #CP2: conditional *PRNP* female calf, NC: negative control, PC: positive control).

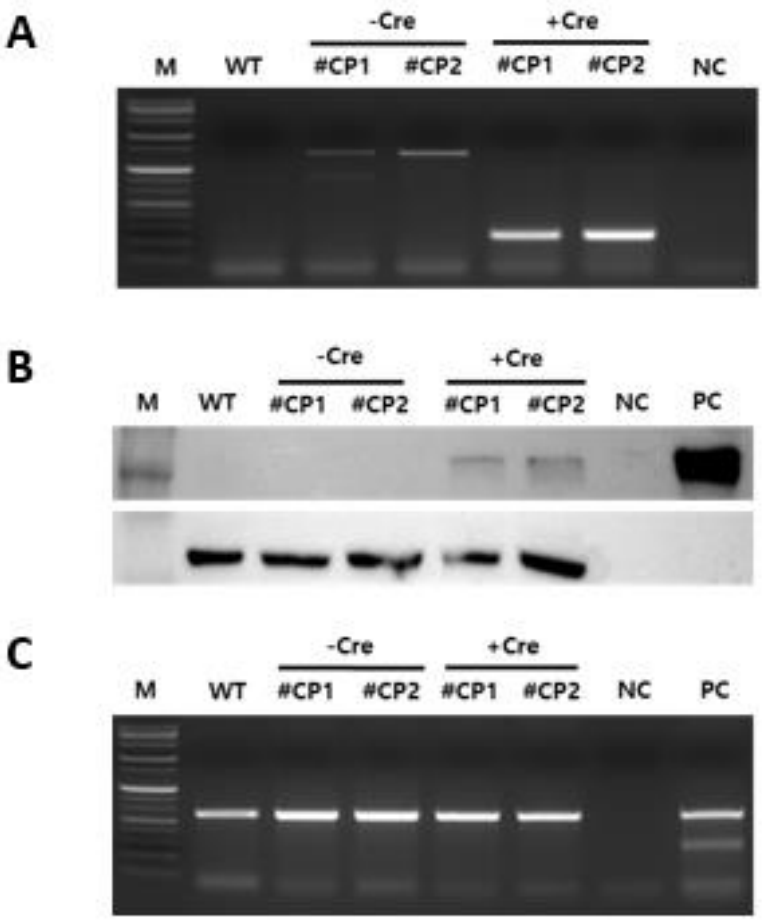

Figure 23. Cre treatment to conditional *PRNP* mutated calves.

A. PCR for loxP-flocked GFP cassette excision. B. Cas9 protein western blot. C. T7E1 assay result for *PRNP*. (WT: wild type, #CP1: conditional *PRNP* male calf, #CP2: conditional *PRNP* female calf, NC: negative control, PC: positive control).

Table 13. *PRNP* conditional calves deep-sequencing result for *PRNP*

ID	Mutation rate	Mutation pattern	
#CP1 +Cre	0.0%	**GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT**	**WT**
#CP2 +Cre	0.0	**GGAACAAACCCAGTAAGCCAAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCT**	**WT**

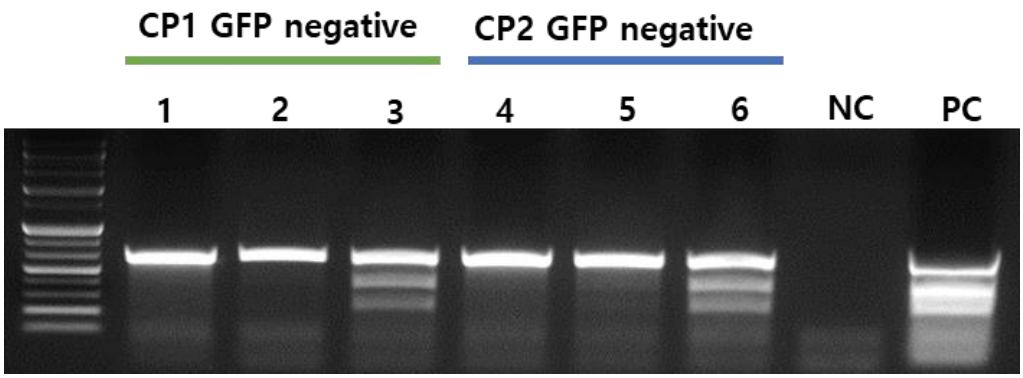

Figure 24. Cas9 activity of conditional *PRNP* calves.

(1: #CP1 GFP negative, 2: #CP1 GFP negative + sgRNA for *PRNP*, 3: #CP1 GFP negative + Cas9 protein + sgRNA for *PRNP*, 4: #CP2 GFP negative, 5: #CP2 GFP negative + sgRNA for *PRNP*, 6: #CP2 GFP negative + Cas9 protein + sgRNA for *PRNP*, NC: negative control, PC: positive control)

3.6 Germline transmission of conditional *PRNP* cattle

After sexual maturation of conditional *PRNP* male cattle, semen was collected and frozen. To validate the germline transmission, 118 oocytes were fertilized with the semen. The cleavage rate was 61.0% and the blastocyst formation rate was 25.4% (Fig. 25A-a). Among the blastocysts, GFP expression was observed in 6 blastocysts (Fig. 25A-b). In PCR result, the GFP blastocysts showed same transgene derived from *PRNP* F0 male cattle (Fig. 25B).

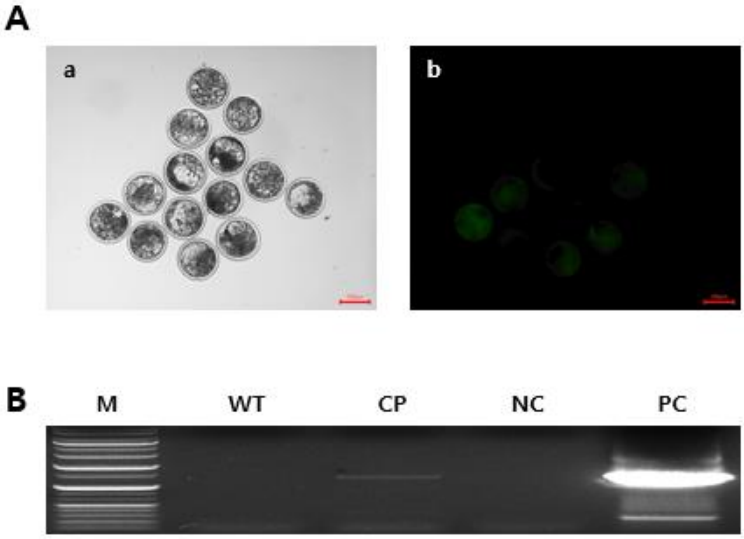

Figure 25. Germline transmission of conditional *PRNP* male cattle.

A. Blastocysts fertilized with conditional *PRNP* male semen; a. under the bright field, b. under the blue light field. B. PCR for transgene (WT: wild type, CP: conditional *PRNP* male semen, NC: negative control, PC: positive control).

5. Discussion

In our previous study, *PRNP*-knockout somatic cells were efficiently isolated by genome editing tools TALEN and CRISPR-Cas9, and those cells were reprogrammed into pre-implantation stage embryos [175]. As an extension of the study, several knockout blastocysts were selected and transferred into 10 recipients. Three pregnancy was confirmed at around 50 days, but they were all absorbed because of probably abnormal reprogramming.

As an alternative to this, here I approached IVF embryos through microinjection of all-in-one DNAs, which contained Cas9, GFP, and sgRNA. After microinjection of all-in-one DNAs, the blastocysts with GFP expression were analyzed and every single blastocyst had mutation in the targeted region of *PRNP*. Embryos were transferred into the 18 recipients, and 7 calves were born. All embryos previously transplanted into SCNT embryos were absorbed during pregnancy, but all-in-one DNAs embryos were successfully maintained until the gestation period. Among the born individuals, two calves (female and male) with the confirmed *PRNP* mutation grew well and are still alive (current age: 34 months (female), 24 months (male), and

germline transmission can be confirmed. The male is currently growing well with no health problems, but the female has observed gas bloating symptoms at 12 months, which is maintained through diet control and rumen cannulation. At this time, it is not possible to determine whether the primary gas distension is related to the all-in-one DNAs because the male is growing well and healthy with no symptoms in the rumen.

An F1 calf from F0 female (#P3) was successfully born and, in the genome, Cas9, GFP, and sgRNA were identified (Fig. 13). Unfortunately, mutation on the *PRNP* locus was negative. Because the mutation was observed after transfection of the same plasmids (U6 promoter and sgRNA for *PRNP*) on the primary cell from F1 calf, it was assumed that U6 promoter was silenced in embryonic development and sgRNA was not transcripted. However, Cas9 activity was observed in the cells, which will be useful for another genetic engineering model in *in vitro* bovine model. A calf is currently growing healthy and will be analyzed for further study.

In the case of the male (#P7), mutations were observed in sperm, and all the blastocysts derived from the semen had *PRNP* mutation. In addition, *GGTA1*, which was developed through gene

mutation (Fig. 15), also knocked out, so if cattle without *GGTA1* antigen and prion protein are born in the future, it can be applied as a resource for heart valve replacement [176].

Through this study, I found two things. One is that using the all-in-one DNAs, prion knockout cattle could be effectively produced, and these transgenes were even transmitted to the next generation. The all-in-one DNAs system can be applied to various animal models in the future. However, in case of conditional *PRNP* knock-out system based on the Cre/loxP system, *PRNP* mutation did not occur. Second is about gene silencing. In this study, three promoters (CAG and EF1α for Cas9 and GFP; U6 for sgRNA) were employed in the vectors. In our previous transgenic study [17, 19, 148], silencing has not been observed, but in this study, in all-in-one DNAs, Cas9 and GFP were not observed due to inactivation of the CAG promoter. Perhaps the CAG promoter was not transcribed because the promoter has many CG dinucleotides that could cause epigenetic shutdown [177]. Some calves produced by vectors (all-in-one DNAs and conditional *PRNP* vectors) did not show *PRNP* mutation. One reason is that the CMV enhancer region can cause U6 promoter silencing due to methylation issues [178]. Reports

on the silencing of the U6 promoter are very limited, so further studies are required.

In conclusion, these data demonstrated that *PRNP*-mutated cattle were successfully born and has survived to up to date, and germline was transmitted to the next generation. The *PRNP*-mutated cattle and their germ cells will be valuable resources for studying prion diseases.

Chapter II. The production of *MSTN* gene mutated cattle by CRISPR/Cas9

This chapter is based on the publication; Gim,GM.,et al. (2022). "Production of MSTN-mutated cattle without exogenous gene integration using CRISPR-Cas9." Biotechnol J: e2100198.

1. Abstract

Many genome-edited animals have been produced using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology to edit specific genes. However, there are few guidelines for the application of this technique to cattle. The goal of this study was to produce trait-improved cattle using the genome-editing technology CRISPR-Cas9. Myostatin (MSTN) was selected as a target locus, and synthetic mRNA of sgRNA and Cas9 were microinjected into fertilized bovine embryos in vitro. As a result, 17 healthy calves were born, and three of them showed *MSTN* mutation rates of 10.5%, 45.4%, and 99.9%, respectively. Importantly, the offspring with the 99.9% *MSTN* mutation rate had a biallelic mutation (-12 bps) and a double muscling phenotype. In conclusion, we demonstrate that the genome-editing technology CRISPR-Cas9 can produce genetically modified calves with improved traits.

2. Introduction

Many animal product (milk and meat) studies have focused on improving the performance traits of cattle because cattle contribute to 45% of the global animal protein supply for human consumption [179, 180]. Significant effort has been made to improve the traits of cattle using advanced reproductive technologies [181, 182] based on genotyping and phenotyping analysis. One application of genotyping and phenotypic breeding is the selection of breeds with a large amount of muscle. Belgian Blue and Piedmontese cattle are the most representative double-muscled cattle breeds [183]. Genetic analysis identified the mutation in myostatin (*MSTN*), or growth and differentiation factor 8, as the causative factor behind enhanced muscle development. Mutations in the gene have also been observed in dogs [184], sheep [185], pigs [186], and humans [187]. However, the incidence of these natural mutations is very low, and selecting and breeding these individuals to establish an independent breed is time consuming and costly.

The development of genome-editing tools, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short

palindromic repeats (CRISPR-Cas9), has provided new and powerful tools for functional mutation studies in various biotechnological industries. Applying these genome-editing tools to livestock will contribute to improvements in cattle traits and a better understanding of how to prevent and treat cattle diseases [188]. Proof-of-concept gene-edited cattle—using bovine somatic cell nuclear transfer (SCNT)— with enhanced traits for disease resistance, allergen removal, production, and welfare have been produced [174, 189-192]. Because SCNT is used to produce cloned offspring with precise gene-edited cells, it has been employed to generate valuable cattle models [193, 194]. However, abnormal reprogramming results in low efficiency and a low survival rate, limiting the application of SCNT. An alternative approach to increase the efficiency of gene editing is through the microinjection of in vitro fertilized embryos [195]. One study used TALENs to edit the *MSTN* gene for in vitro microinjection in fertilized cattle embryos [196]. However, there are no reports of a liveborn genome-edited calf in which CRISPR-Cas9 was used on the *MSTN* locus. Accordingly, the aim of the present study is 1) to develop a method to produce gene-edited bovine pre-implantation embryos using microinjection with Cas9 mRNA

and 2) to produce *MSTN*-mutated calves.

3. Materials and methods

3.1 Single guide RNA design and testing on bovine fibroblast cells

To produce the *MSTN*-mutated cattle, single guide RNA (sgRNA) for *MSTN* targeting exon 2 was designed by CHOPCHOP software (https://chopchop.cbu.uib.no/) that selected sgRNA candidates for the target genome (Fig. 26A). Then, the sgRNA for *MSTN* and Cas9 protein were co-transfected on bovine fibroblast cells. After 3 days of transfection, they were harvested for the extraction of genome DNA. Through the T7E1 assay, whether the target *MSTN* gene was mutated was confirmed.

3.2 Microinjection

When performing the microinjections, to determine the most appropriate condition, the different concentrations of Cas9 mRNA (CAS9MRNA) and sgRNA were carried out into four groups. (CB: only TE microinjection; RNA1X: 100 ng/μl Cas9 mRNA and 50 ng/μl sgRNA; RNA2X: 200 ng/μl Cas9 mRNA and 100 ng/μl sgRNA; RNA4X: 400 ng/μl Cas9 mRNA and 200 ng/μl sgRNA). After 18 h of IVF, presumptive zygotes were

injected with Cas9 mRNA and sgRNA synthesized using GeneArt™ Precision gRNA Synthesis Kit (Thermo Fisher, A29377) using a microinjector machine (Eppendorf, Femtojet). The concentration of injected Cas9 mRNA and sgRNA for *MSTN* were different for each group. After 7 days of microinjection, preimplantation-stage embryos were collected, and the mutation of *MSTN* was observed in vitro or transferred in vivo into a surrogate cow (Fig. 26B).

3.3 Detection of *MSTN* gene mutation

Genomic DNA from transgenic primary cells was extracted using a DNA extraction kit (Qiagen, 69504). The *MSTN* primer was designed using PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4.0/, Table 14), and the target sequence was amplified by polymerase chain reaction (PCR) at 94 ℃ for 5 min, 35-40 cycles at 94 ℃ for 20 s, at 57 ℃ for 30 s, at 72 ℃ for 35 s, and 72 ℃ for 5 min. The PCR product from each sample was assessed using T7E1 assay (Toolgene, TGEN_T7E1) to detect *MSTN* mutations.

3.4 Gene expression by real-time PCR

Total RNA was extracted from primary cultured cells using a RNeasy Mini Kit (Qiagen, 74106), and complementary DNA was synthesized from 1 ug of RNA using the RNA to cDNA EcoDry™ Premix (Takara, 639543). Gene expression assay was conducted using SYBR Green on QuantStudio 3 (Applied Biosystems, A28132), and relative cycle threshold (CT) values were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used in this study are listed in Table 15.

3.5 Embryo transfer and pregnancy diagnosis

Blastocysts were stored in TCM-199 based medium supplemented with 20% FBS. A single blastocyst was transferred on day 7 (estrus = day 0 = day of fusion) to the uterine horn of each recipient animal using a non-surgical transcervical method. Pregnancy detection was performed on day 50 post estrus using rectal palpation and ultrasonography. Pregnant cattle were checked by rectal palpation and ultrasonography at regular intervals thereafter.

3.6 Targeted deep sequencing

Target sites were first amplified to a size of ~500 bp from extracted genomic DNA using KAPA HiFi HotStart DNA polymerase (Roche, #KK2502) according to the manufacturer' s protocols. Then, amplicons were amplified again to a size of ~230 bp, after which the amplicons were amplified using TruSeq HT dual index-containing primers to add adaptor and index sequences for Illumina sequencing platforms to each sample [197]. Primers used in this study are listed in Table 16. Pooled PCR amplicons were purified using a PCR purification kit (MGmed) and sequenced on a MiniSeq (Illumina) with paired-end sequencing systems (2x150 bp). The Cas-Analyzer (http://www.rgenome.net/cas-analyzer/#!) was used to quantify the indel frequencies from deep-sequencing data [197].

3.7 Analysis of *MSTN* off-target effect

The potential off-target effects caused by CRISPR-Cas9 in the three *MSTN* mutant calves were assessed using Cas-OFFinder software (http://www.rgenome.net/cas-offinder/). This software offers a fast and versatile algorithm that searches

for potential off-target sites of Cas9 RNA-guided endonucleases. In the *MSTN* target site, the candidates of five loci targeting the whole genome of cattle were found by adjusting the mismatch number to three. A primer targeting each sequence (Table 14) was prepared to confirm the off-target effect through T7E1 assay.

3.8 Blood analysis

To evaluate the general health status of the calves, 5 mL of whole blood was collected from the jugular vein for complete blood count (CBC) analysis (Hemavet 950, Drew Scientific, USA) and serum chemistry analysis (BS-400, Mindray, China). The animals were also monitored by a veterinarian to assess their general health conditions.

3.9 Statistical analysis

All data are presented as mean ± standard error of mean (SEM) and replicated more than three times. Newman-Keuls multiple comparison tests and Tukey's tests in a one-way

analysis of variance (ANOVA) were used to statistically evaluate for differences in embryo development, frequency of mutagenesis, and mRNA expression levels. A value of $P<0.05$ was considered statistically significant. All analyses were performed using the GraphPad Prism program (version 5.01).

Table 14. PCR primer sequences

Gene	Forward	Reverse	Size (bp)
MSTN	GAGGTGTTCGTTCGTTTTTC	CTACCAGTTTCCTGTGCTTA	538
Off-target 1	TCAGCACAGAAAAGGTGAGG	GAGACGGACACAACTGAGCA	515
Off-target 2	TGAGCCCCTACTTTGTGGAC	GTTTTCTGGTAAGGGGTGCA	580
Off-target 3	TTGAAAACCTAGTGGGGAAAAA	GCACTCTCAAACACTGTGGC	591
Off-target 4	TCCTTGCACCTTCCAAAATC	ATCTGCGTGTAACTCCAGCC	520
Off-target 5	TCACCCATTCCAGTCCATTT	CCTCTAATGCCCTCTTGCAG	542

Table 15. qRT-PCR primer sequences

Gene	Forward	Reverse	Size (bp)
MSTN	AACAGCGAGCAGAAGGAAAA	CCAGGCGAAGTTTACTGAGG	124
GAPDH	GGCGTGAACCACGAGAAGTA	CCCTCCACGATGCCAAAGT	120

Table 16. List of primers used for targeted deep sequencing

Primer	Sequence
MSTN−1st−F	gaggtgttcgttcgtttttc
MSTN−1st−R	taagcacaggaaactggtag
MSTN−2nd−F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaacgcaagtggaaggaaaac
MSTN−2nd−R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtgctctgccaaataccagtg

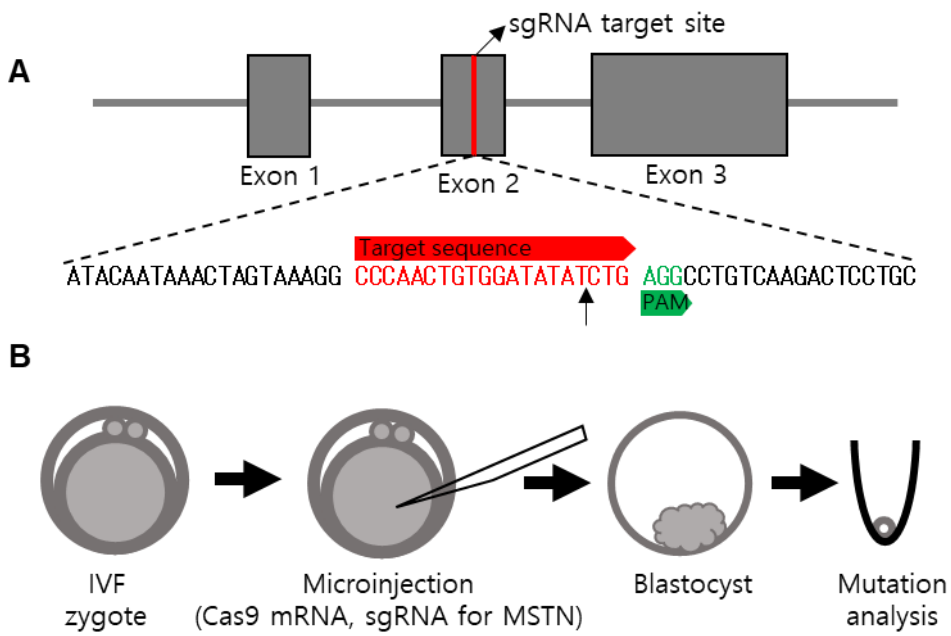

Figure 26. Schematic design for *MSTN* target site (A) and graphical illustration of the analysis of microinjected embryos (B).

The red letters and line represent the *MSTN* target site, and the green letters represent the protospacer adjacent motif (PAM) sequence.

4. Results

4.1 Validating single guide RNA for *MSTN* in bovine fibroblast cells

In bovine fibroblast cells, the selected sgRNA caused a mutation in the target site. The mutation on *MSTN* was detected by T7E1 assay (data not shown) and sanger sequencing. Through the sanger sequencing results, various types of mutation patterns were observed (Fig. 27). These results show that the selected sgRNA worked on the *MSTN* target site.

```
                Target sequence
AAACTAGTAAAGG CCCAACTGTGGATATATCTG AGGCCTGTCAAGAC
                                   PAM

AAACTAGTAAAGG CCCAACTGTGGATATA-CTG AGGCCTGTCAAGAC   -1 bp
AAACTAGTAAAGG CCCAACTGTGG--ATATCTG AGGCCTGTCAAGAC   -2 bp
AAACTAGTAAAGG CCCAACTGTGGATAT--C-G AGGCCTGTCAAGAC   -3 bp
AAACTAGTAAAGG CCCAACT----------CTG AGGCCTGTCAAGAC   -10 bp
AAACTAGTAAAGG CCCAACTG------------ AGGCCTGTCAAGAC   -12 bp
AAACTAGTAAAGG CCCAACTGTGGATATATTCTG AGGCCTGTCAAGAC  +1 bp
```

Figure 27. *MSTN* mutation sequence pattern on pooled cells after transfection with sgRNA and Cas9 protein.

The red letters and line represent the *MSTN* target site, and the green letters represent the PAM sequence.

4.2 Developmental competence and mutation efficiency of microinjection-mediated gene editing

To determine optimal conditions, including Cas9 mRNA and sgRNA concentrations, for producing mutations in bovine blastocysts, four experimental conditions were conducted. Then, developmental competence and mutation efficiency were investigated in the blastocyst stage following each condition (Fig. 28A, B and C). There was no significant difference in cleavage rates across all four groups (data not shown). However, in the RNA4X group, a diminished blastocyst formation rate was observed in comparison to the control group (Fig. 28B). In terms of the mutation rate, the RNA2X group (81.3 ± 17.2%) had the highest mutation rate compared the other three groups (WT: 0%, RNA1X: 33.3 ± 16.0%, RNA4X: 50.0 ± 28.5%) as seen in Fig. 28C. Thus, the RNA2X condition was selected as the optimal condition for the further production of *MSTN*-mutated cattle.

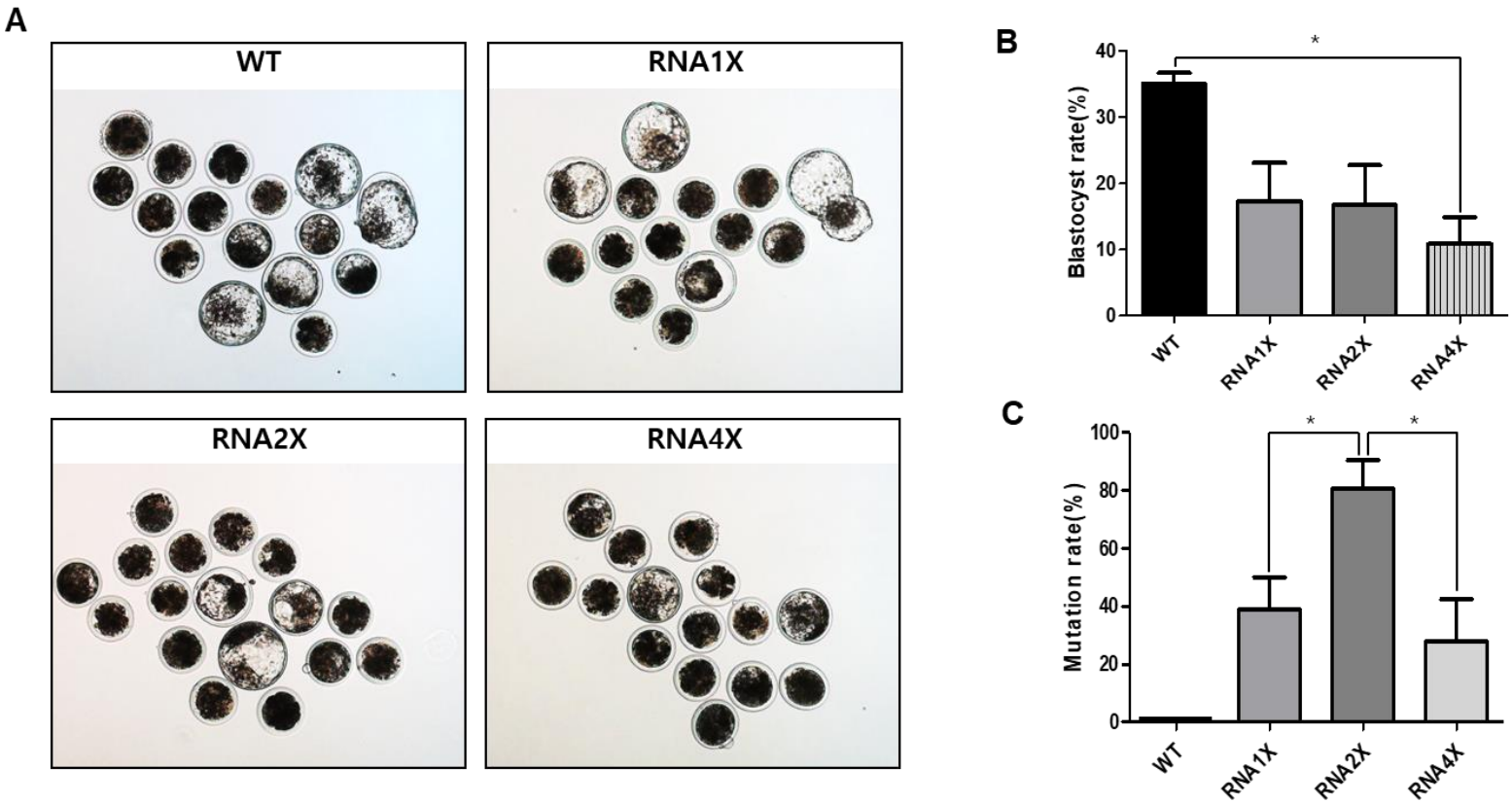
A
WT
RNA1X
RNA2X
RNA4X
B
Blastocyst rate(%)
40
30
20
10
0
*
WT
RNA1X
RNA2X
RNA4X
C
Mutation rate(%)
100
80
60
40
20
0
*
*
WT
RNA1X
RNA2X
RNA4X

Figure 28. Efficient production of *MSTN* mutant blastocysts.

A. Representative pictures of bovine blastocysts on day 7 for each experimental condition after microinjection (WT: microinjection with Tris-EDTA buffer; RNA1X: 100 ng/μl Cas9 mRNA and 50 ng/μl sgRNA; RNA2X: 200 ng/μl Cas9 mRNA and 100 ng/μl sgRNA; RNA 4X: 400 ng/μl Cas9 mRNA and 200 ng/μl sgRNA). B. Blastocyst formation rate after microinjection with each condition. C. The efficiency of *MSTN* mutation in each experimental condition at the blastocyst level. *P < 0.05, **P < 0.01, ***P < 0.001. E) *MSTN* mutation rate in blastocysts at day 7 for each condition.

4.3 Production of *MSTN*-mutated Korean beef cattle

The aim of this study was to produce *MSTN*-mutated Korean beef cattle. Microinjection was performed on fertilized embryos under the RNA2X conditions, and cultured blastocysts were used for embryo transfer. The experiment was repeated four times, and 595 oocytes were used to generate mutant embryos. The cleavage rate was 59.0 ± 21.0%, and 86 blastocysts (14.5 ± 14.0%) were produced. A total of 26 blastocysts with a high-quality morphology were transferred to surrogate mothers (one blastocyst per recipient). Some leftover blastocysts (n = 28) were used to assess the mutation occurrence in *MSTN*, which was determined to be 71.6 ± 44.3%.

After embryo transfer, 19 of the 26 surrogates were pregnant, and 2 of the 19 fetuses were absorbed in the middle of pregnancy. A total of 17 calves (I.D.: #1 ~ #17) were liveborn, and one calf was stillborn (#14) from dystocia. Deep-sequencing analysis showed mutations in three of the 17 animals (Table 17). Their *MSTN* mutation rates were 10.5, 45.4, and 99.9% for #6, #14, and #17, respectively. In addition, *MSTN* mutation was detected in #17 by T7E1 assay, and there were no off-target effects (Fig. 29). Real-time PCR was conducted to

assess the *MSTN* RNA level of primary cells from #17 and #14. In both individuals, the level of *MSTN* RNA was significantly decreased with the wild type (Fig. 30). As a sequence result, #14 and #17 have the same mutation pattern of −12 bp deletion (nt 466 in *MSTN*) compared to the wild type (Fig. 31). Especially in #17 (nt 466, del 12), enhanced muscle growth was observed compared to wild−type calf (Fig. 32). There were no abnormal values in blood tests performed to evaluate the general health status of five offspring (#4, #6, #7, #9 and #17), including the knockout one (#17) at 8 months old (Table 18).

Table 17. Mutation rate of fetuses (labeled #1–17) on *MSTN* target site by deep sequencing

I.D.	Mutation rate
#1…#5	–
#6	10.5%
#7…#13	–
#14	45.4%
#15 and #16	–
#17	99.9%
Summary: 3/17 (16.4%)	

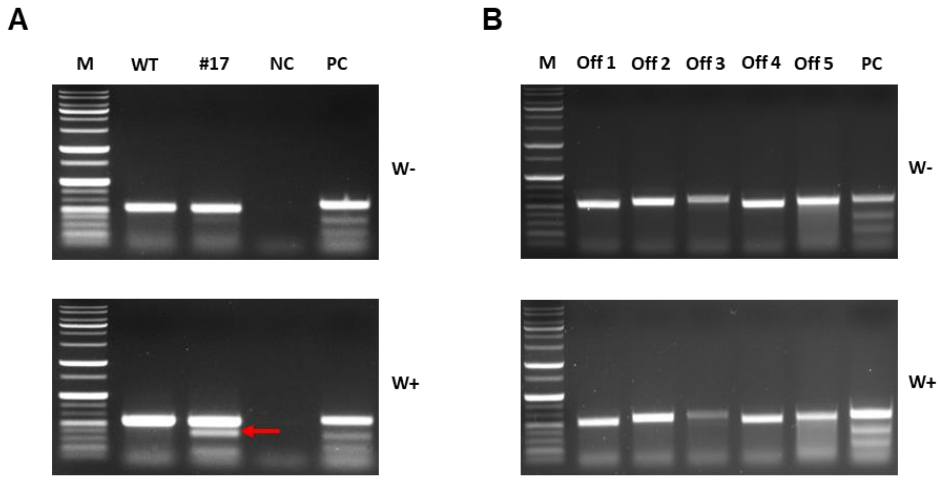

Figure 29. *MSTN* mutation and off-target effect in one calf (#17).

A. T7E1 assay result for #17 calf. WT: wild type, NC: negative control, PC: positive control, W-: T7E1 assay without wild-type genomic DNA, W+: T7E1 assay with wild-type genomic DNA. Red arrow: cleaved *MSTN* PCR band B. Analysis of five candidate genes with possible off-target effects for one calf (#17). Off 1-5: *MSTN* off-targeting candidate site.

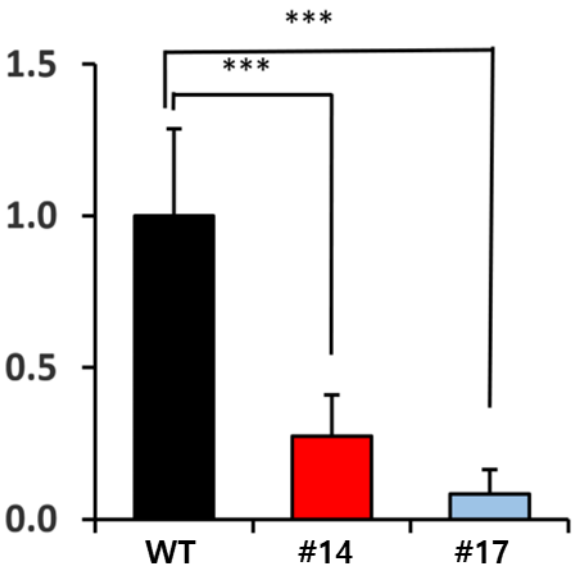

Figure 30. Relative expression of *MSTN* mRNA among wild-type, #14 and #17.

The bar graph represents the fold changes in mRNA levels, and the error bars show SEMs (n=3). *P < 0.05, **P < 0.01, ***P < 0.001.

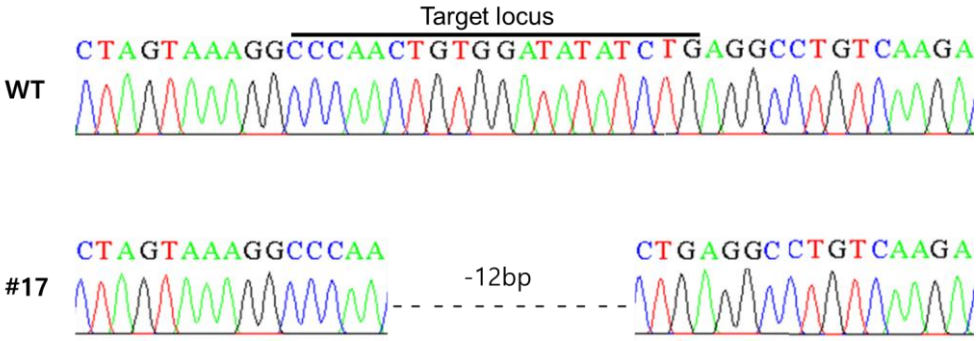

Figure 31. The comparison of *MSTN* sequences between the wild type and #17.

WT: wild type

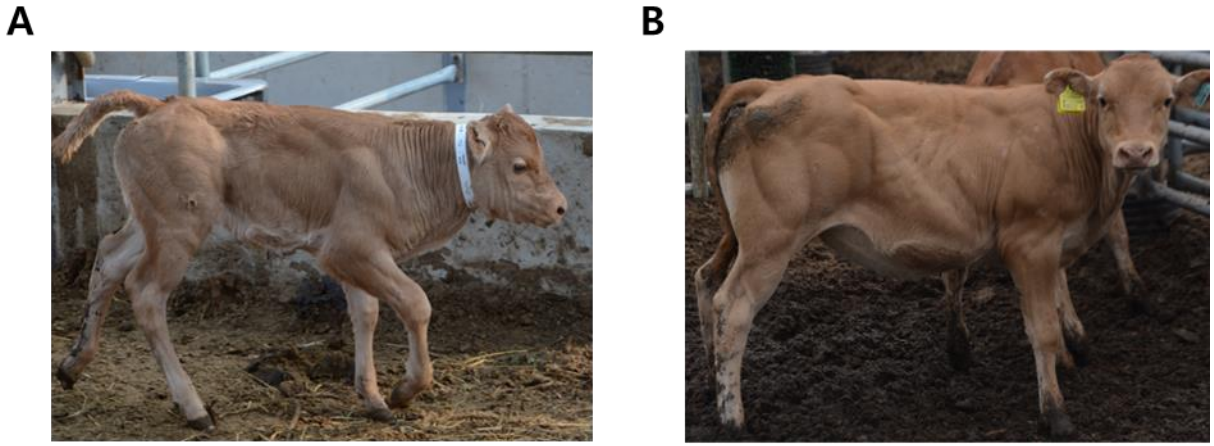

Figure 32. Representative pictures of the *MSTN* knockout calf (#17) along with age.

(A: 1−month age, B: 4−month age).

Table 18. Blood analysis in the offspring

Blood items	References	Offspring				
		#4	#6	#7	#9	#17
WBC	4.0 ~ 12.0(10^3/mm^3)	7.02	8.80	8.16	10.36	8.20
Neutrophil	0.6 ~ 4.1(10^3/mm^3)	3.12	4.36	3.55	5.79	3.66
Lymphocyte	2.5 ~ 7.5(10^3/mm^3)	2.42	2.53	2.65	3.48	3.30
RBC	5.0 ~ 10.0(10^3/mm^3)	8.14	10.09	9.56	8.83	8.62
HGB	8.0 ~ 15.0 (g/dL)	11.20	12.80	11.60	11.20	10.70
HCT	24.0 ~ 46.0 (%)	26.30	29.30	27.70	25.10	27.20
PLT	200 ~ 800(10^3/mm^3)	441.00	286.00	431.00	352.00	395.00
AST	53 ~ 162	55.00	78.00	63.00	75.00	68.00
ALP	77~ 265	209.00	321.00	331.00	390.00	343.00
BUN	10 ~ 25	21.00	12.00	13.00	12.00	12.00
Creatinine	0.4 ~ 2.0	0.90	1.10	0.90	1.00	1.70
Total Protein	7.2 ~ 9.0	7.40	8.30	8.20	7.90	7.60
Albumin	3.2 ~ 4.2	3.80	4.20	4.10	4.00	4.10
Ca (mg/dl)	8.3 ~ 10.4	9.40	9.55	9.77	9.35	9.99
P (mg/dl)	4.2 ~ 7.7	9.02	7.76	9.64	9.22	9.23
Total Cholesterol	73 ~ 280	144.00	112.00	110.00	104.00	152.00
Glucose	31 ~ 77	78.00	84.00	90.00	75.00	87.00
Triglyceride	7 ~ 323	15.00	10.00	14.00	12.00	30.00
Total Bilirubin	0.01 ~ 0.5	0.029	0.026	0.031	0.023	0.028

5. Discussion

CRISPR-Cas9-mediated genome editing is a powerful biological technology that has widespread applicability. However, its application to livestock has been slow [198]. In this study, by applying genome editing without the integration of the transgene, an effective knockout condition was established at the embryo level, and liveborn edited offspring were produced. The *MSTN* knockout was used as a proof of concept because of its clear double-muscling phenotype.

The *MSTN* gene consists of three domains (a signal sequence, a pro-peptide, and a mature region). After transcription, translation, and two cleavage events (pro-peptide convertase by furin and tolloid protease by BMP-1 metalloprotease), the released mature *MSTN* protein dimer regulates the inhibition of skeletal muscle growth [199]. In more detail, the first cleavage occurs at the 266th position by furin followed by cleavage at the 76th by BMP-1/tolloid metalloproteinase, and finally, the released active *MSTN* protein dimer binds to the receptor (ActRIIB), resulting in the inhibition of muscle growth [199-201]. Two representative cattle breeds, Belgian Blue and Piedmontese, show natural mutations in this

gene, 11 bps deletion, and one base mutation on the mature *MSTN* domain, respectively. Additionally, various mutations have been observed at various genomic positions in several cattle breeds [202]. These cattle breeds phenotypically indicate that mature *MSTN* domain mutations contribute to muscle growth [183, 202]. To mimic or reproduce these natural mutations using genome-editing technologies, in a previous study, ZFNs disrupted the exon 1 locus (signal sequences region), and subsequently, the mature *MSTN* domain was broken [75]. In another study, the mature domain locus was directly targeted and mutated in microinjected embryos via TALENs [196]. In both studies, the phenotype was observed after the mutation of the mature locus of *MSTN*.

In my study, it was assumed that the disruption of the mature *MSTN* locus might occur by applying effective sgRNA on the pro-peptide locus using CRISPR-Cas9 as happens in natural mutations [202]. Microinjected embryos were transplanted, and the double-muscling phenotype was observed in one calf (#17). Thus, we thought that gene editing on the pro-peptide locus region worked well and predicted that the sequence of the mature domain locus might be mutated by CRISPR-Cas9. However, one

interesting finding was observed as a result of sequencing. This was an in-frameshift (-12 bps deletion) mutation in the target locus that did not disrupt the mature *MSTN* domain region or amino acids of two cleavage regions. In other words, the 266th and 76th amino acids for furin and proteinase were respectively conserved. Thus, the active *MSTN* protein dimer may be formed, and muscle production would be normally suppressed, and finally a wild-type offspring should be born. Interestingly, muscle outgrowth, a typical phenotype, was observed in calf #17, especially in the shoulder and hip, and the expression of the *MSTN* mRNA was decreased (Fig. 30). Because there have been no reports of phenotyping with this type of mutation [202], it is hard to explain why this phenomenon occurred. One possibility is that the nt 466-477 position can be thought of as another molecular biological function in addition to the previously known two cleavage events. Similarly, in-frameshift mutation of the *MSTN* pro-peptide in mice showed a muscle-gain phenotype [203]. Importantly, the blood test results of the mutated calves were normal (Table 18), and the calves showed no issues in their general health. In the future, I will monitor their growth, including germline transmission, and investigate how this mutation may

have affected the function of *MSTN*.

Microinjections commonly result in mosaic F0 founder animals that are then screened for the exact knockout/knock-in in the F1 generation following subsequent breeding. This technique is very effective in rodent experiments but is not suitable for cattle because of their long gestational periods and single pregnancies. A cattle F0 and F1 system would take more than three years and require high costs. Consequently, most genome-edited cattle are produced using a SCNT approach. However, live, healthy calf offspring are limited when SCNT is employed because of abnormal reprograming during embryogenesis. In my study, microinjection was used to produce live, healthy genome-edited calves. Randomly selected blastocysts were analyzed in vitro by sgRNA/Cas9 mRNA, and an 81.3 ± 17.2% knockout efficiency rate was found. Embryo transfer was performed, and a lower *MSTN* mutant cow generation rate of 17.6% was found in vivo. It is possible that non-mutated blastocysts were selected during the randomly selected process. In the future, to improve the efficiency in producing mutated offspring, a portion of the blastocysts could be biopsied prior to transfer to identify possible mutations [204].

Genotyping analysis showed another interesting result. When the mRNA of sgRNA and Cas9 was introduced into cells and embryos (blastocysts), various mutant pattern (−12, −10, −3, −2, −1, and +1; Fig. 27) were shown, but only one mutant pattern (−12 bps) was observed in genome−edited calves. It is difficult to explain why only one pattern was observed in all *MSTN*−mutated calves. One possible theory is that the cells with the other mutated pattern may have been embryonically lethal at some time after the point of embryo transfer. Further studies should determine how mutant patterns other than −12 bps affects embryonic development.

In conclusion, I demonstrated, for the first time, that the microinjection of Cas9 mRNA and sgRNA for *MSTN* into embryos fertilized in vitro can produce health, genome−edited Korean beef calves—including one calf with a biallelic mutation. These calves will serve as a model for the future development of CRISPR-Cas9 technology in the agricultural industries.

Chapter III. Stable germline transmission from the *MSTN* gene mutated cattle

This chapter is based on the publication; Gim, G. M., et al. (2022). "Germline transmission of *MSTN* knockout cattle via CRISPR-Cas9." Theriogenology 192: 22-27.

1. Abstract

Although the production of several founder animals (F0) for gene editing in livestock has been reported in cattle, very few studies have assessed germline transmission to the next generation due to the long sexual maturation and gestation periods. The present study aimed to assess the germline transmission of *MSTN* mutations (-12bps deletion) in *MSTN* mutant F0 male and female cattle. For this purpose, oocytes and semen were collected after the sexual maturation of *MSTN* cattle, and embryos produced by in vitro fertilization were analyzed. In addition, the embryos were subjected to additional gene (PRNP) editing using electroporation. Embryos produced by in vitro fertilization with *MSTN* male and female cattle were transferred to a surrogate, and one calf was successfully born. *MSTN* heterozygous mutation was shown by sequencing of the F1 calf, which had no health issues. As a further experiment, using

electroporation, additional gene-edited embryos fertilized with the *MSTN* male sperm showed a high mutation rate of *PRNP* (86.2 ± 3.4%). These data demonstrate that the cattle produced through gene editing matured without health issues and had transmitted *MSTN* mutation from the germ cells. Also, additional mutation of embryos fertilized with the *MSTN* male sperm could enable further mutagenesis using electroporation.

2. Introduction

Throughout history, people have been interested in studying the production of high-performing animals. With the development of artificial insemination, semen samples of males with excellent traits have helped to reduce the generation interval and increase the chances of offspring with desired traits. However, the current breeding method is time consuming, especially for cattle, as the breeding interval is longer than that of other livestock, such as pigs and sheep. Moreover, the chances of obtaining desired breeders with superior traits remain low. Recently developed gene editing methods, such as zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) methods, are being evaluated as sophisticated tools to change specific target loci. Thus, gene editing is known to play a groundbreaking role in improving agricultural performance, particularly that of livestock. Indeed, these gene editing methods have been adopted to produce disease-resistant pigs [205] and productivity-enhanced lambs [196] and pigs [206, 207], and their potential has been highlighted [208]. *MSTN*-knockout cattle with

enhanced productivity [75, 196], NRAMP-1-knockin cattle with tuberculosis resistance [209], and SRY-GFP-knock-in cattle [210] have been produced, and their demand is increasing dramatically.

Although several studies have assessed the genetic modification of cattle, besides our investigations [211, 212], studies on germline transmission and long-term monitoring are very limited. Previously, I reported the successful generation of *MSTN* mutant cattle by CRISPR-Cas9 and microinjection; the offspring matured as expected without any health issues [213]. However, in a previous study, *MSTN* mutations derived from ear tissue and blood samples were observed. The evaluation of the germline transmission of *MSTN* mutant cattle was impossible due to the long sexual maturation period. Hence, the present study aimed to assess the health condition and germline transmission of *MSTN* mutant founders to increase the number of gene-edited cattle and to evaluate whether the embryos derived from the semen of *MSTN* mutant cattle could be subsequently used to introduce new genetic traits (Fig. 33).

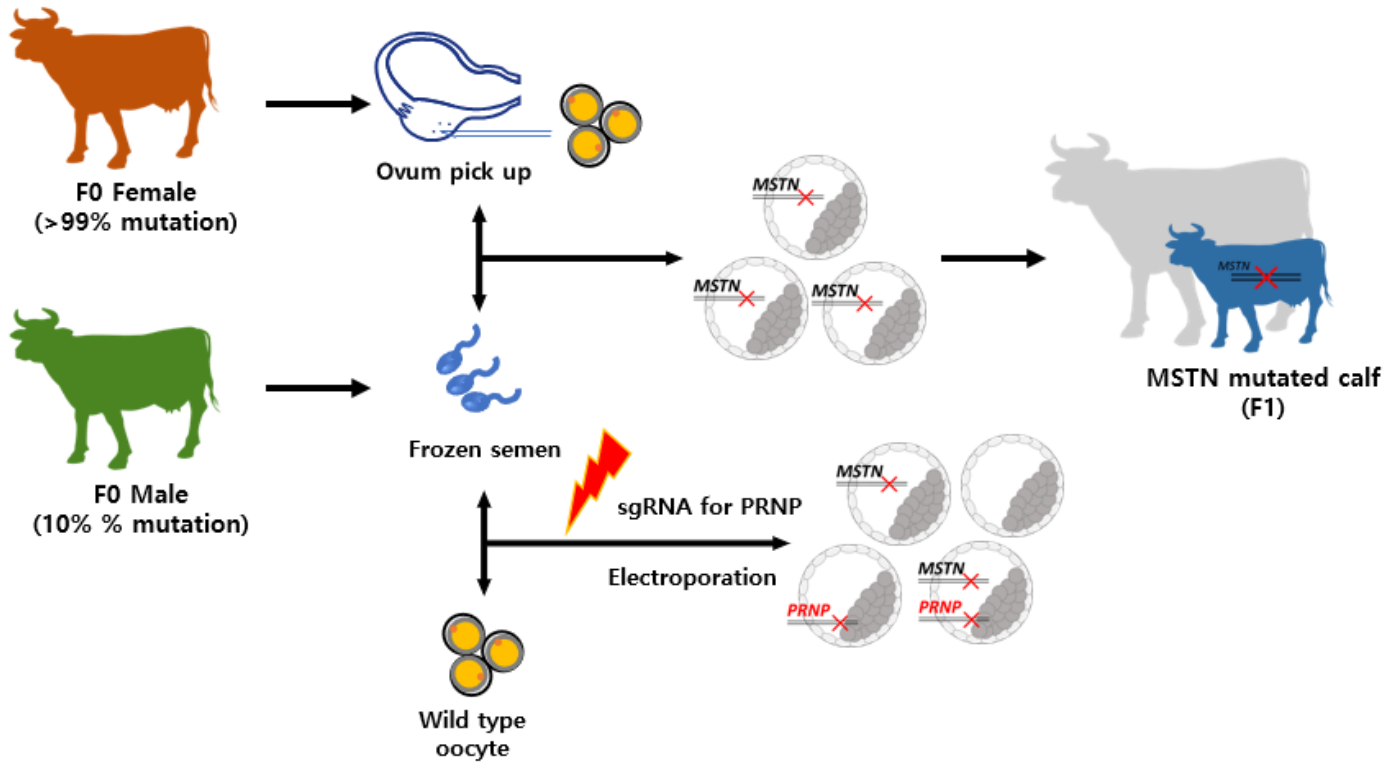

Figure 33. Illustration of generating the new breed cattle with *MSTN* mutant and further specific gene editing using electroporation.

3. Materials and methods

3.1 Donor management and Ovum Pick Up (OPU)

MSTN mutant donor heifer with random estrous cycle inserted with an intravaginal progesterone device (Repro360, Cue-mate) in the vagina and 2.0 mg intramuscular of estradiol benzoate was injected intramuscular. The donor was received 200 mg of FSH (Kawasaki Pharm, Antorin R-10) divided into four times (57, 57, 43, and 43 mg) every 12 hours on Day 4 and 5. The progesterone device was removed on Day 7 immediately before OPU.

For OPU, donor heifer was refrained in cattle crush. Epidural anesthesia was carried out by 5 ml, 2% lidocaine (Daihan, DAIHAN Lidocaine, South Korea). The ovary was hold and stayed to the probe of ultrasound device by transrectal manipulation approaches. A single trained OPU technician performed the OPU procedures using ultrasonic device (Esaote, mylab one) combined with a 7.5 MHz transrectal transducer probe with follicular aspiration guide (WTA, 10283). The follicular puncture was conducted with 18G OPU treaded needle (WTA, 17927) and follicle fluid was collected into 50 ml tube.

The oocytes in the follicle fluids were collected under the stereomicroscope and used for in vitro production. Leftover follicular debris was used for primary culture.

3.2 Semen collection

The semen was collected from *MSTN* mutant bull using an electro ejaculation. (3 times per bull). Before semen collection, the preputial hairs were clipped, and the orifice was washed with clean water and then dried with clean paper towel to minimize contamination. Electro ejaculation was accomplished using a manually controlled electro-ejaculator, ElectroJac6 (Ideal Instruments Neogen Corporation, Lansing, MI, USA) attached with a 6.5-cm-diameter rectal probe with three ventrally oriented electrodes approximately 1 cm apart and was placed completely in the rectum with the electrodes facing ventrally. The number of electrical stimuli was increased until the bull ejaculated. Each stimulus lasted 8-10 s and then paused for approximately 2.0 s before the next stimulus was applied. When the seminal discharge turned cloudy, a collection tube was placed over the penis to collect the semen. The ejaculated semen was

transported to the laboratory at 25 ℃ within 30 min.

3.3 Semen cryopreservation and thawing

The semen samples were used for cryopreservation when they exhibited at least 60% general motility. The semen samples were extended with Optixcell® (IMV Technologies) at 37 ℃. The extended semen was equilibrated at 4 ℃ for 3 h before placed into 0.5-ml straws. Filled straws were arranged on a special rack, 5 cm above liquid nitrogen and exposed to liquid nitrogen vapour for 15 min, and then plunged into a cryogenic tank filled with liquid nitrogen (-196 ℃). The cryopreservation sperms were thawed in a water bath at 37 ℃ for 45 s.

3.4 Sperm motility analysis

To analyze and quantify sperm motility, IVOS-II computer-assisted sperm analysis (CASA) program system was used as instruction from manufacturer. Briefly, frozen semen was thawed, incubated, and purified using the same protocol as used in in vitro fertilization (IVF). Thereafter 3ul of sperm was loaded

to sperm analysis chamber (Leja slides) and analyzed by CASA. For normalization, frozen straws from three different bulls were used. Each semen was analyzed three times to preclude technical error, and the average values of CASA results were used for statistical evaluation.

3.5 Single guided RNA (sgRNA) design and detection of gene mutation

Single guide RNA (sgRNA) targeting bovine PRNP exon3 was designed by Cas-Designer software (http://www.rgenome.net/cas-designer/) that showed sgRNA candidates for the target genome. Following the details of kit manual, the sgRNA was synthesized using Precision gRNA synthesis Kit (ThermoFisher, A29377).

Gene mutation was confirmed through the T7 endonuclease (T7E1) assay. For this, genome DNA was extracted by Kit (Qiagen, 69504). The PCR primers (Table 19) for target loci (*PRNP* and *MSTN*) was designed using PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4. 0/), and the target sequence was amplified by polymerase chain reaction (PCR) at

94◦C for 5 min, 35–40 cycles at 94◦C for 20 s, at 57◦C for 30 s, at 72◦C for 35 s, and 72◦C for 5 min. The PCR product from each sample was treated with T7E1 enzyme (NEB, M0302L) to detect gene mutations. Digested and undigested mixes were observed on a 1% agarose gel. The estimated gene modification was calculated as described previously.

3.6 Electroporation

Genome Editor electroporator (BEX, GEB 15) and electrode (gap: 1.0 mm, volume: 40 ㎕) (BTX, 45-0104) were used for electroporation. The electrode was connected to the electroporator and was set under a stereoscopic microscope. Before electroporation, bovine zygotes were washed with Opti-MeM I (ThermoFisher, 31985062). At one time, 30–40 bovine zygotes were electroporated. Zygotes cultured in IVF medium were washed with Opti-MEM I three times to remove the serum in the medium, placed in a line in the electrode gap filled with 10 ul of Opti-MEM I which is containing Cas9 protein (ThermoFisher, A36499) and sgRNA, and subjected to electroporation. The electroporation condition was 15 V (3 msec

ON+ 97 msec OFF) × 7 times. After electroporation, the zygotes were immediately collected from the electrode chamber and subjected to four washes with TCM-199 based medium followed. The embryos were then cultured in chemically defined medium at 38.5 ℃, 5% CO2, and 5% O2 in an incubator.

3.7 Blood analysis

To evaluate the general health status in *MSTN* mutant male and female, 5 mL of whole blood was collected from the jugular vein for complete blood count (CBC) analysis (Hemavet 950, Drew Scientific, USA) and serum chemistry analysis (BS-400, Mindray, China). The animals were also monitored from a veterinarian to assess their general health condition.

3.8 Primary cell culture and genomic DNA extraction

Primary cells were obtained by biopsy of the ear skin of F1 calf. The ear skin was chopped into small pieces with a sterile scalpel and then washed several times and incubated at 38.5 ℃ for 4-18 h in Hank's Balanced Salt Solution supplemented with

collagenase. The following day the dispersed cells were washed several times in DMEM and cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids, and 100 mM β-mercaptoethanol. Genomic DNA from primary cells was extracted using a DNA extraction kit (Qiagen, 69504). Extracted DNA was used for T7E1 assay and Sanger sequencing.

Table 19. PCR primer sequences

Gene	Forward	Reverse	Size (bp)
MSTN	GAGGTGTTCGTTCGTTTTTC	CTACCAGTTTCCTGTGCTTA	538
PRNP	AAAGCCACATAGGCAGTTGG	GTGCATGACTGTGTCAACAT	571

4. Results

4.1 Germline transmission of *MSTN* mutations and further gene editing of F0 semen

The male founder matured without any health issues (Fig. 34A), and semen was collected by electroejaculation from a male bull (F0) with 10.5% mutation [11]. The F0 semen was frozen for storage and in vitro fertilization. CASA revealed significant differences in progressive cells (%), VCL, ALH, and BCF between the F0 and wild-type semen. However, LIN and STR revealed no significant differences between the F0 and wild-type semen (Table 20). Oocytes obtained from a slaughterhouse were fertilized with the frozen/thawed F0 semen, cultured, and developed into blastocysts. A total of 335 oocytes (replication number = 3) were used. Of these, 261 (78.9 ± 10.8%) were cleaved, and 166 blastocysts (50.5 ± 6.8%) were formed (Fig. 35A). The total cell number of the blastocysts was 81.3 ± 20.6 (n = 20). An analysis of *MSTN* mutations in 117 blastocysts revealed the presence of a mutation in 15 blastocysts (12.7 ± 3.1%) (Fig. 36).

Using electroporation, a knockout experiment (target gene: *PRNP* exon 3) was performed on the embryos derived from the F0 semen. In total, 373 oocytes were fertilized, and 99 blastocysts (26.0 ± 0.5%) were formed (Fig. 35B). Among the 81 blastocysts tested for a *PRNP* mutation, 70 blastocysts showed diverse mutation patterns of *PRNP* (86.2 ± 3.4%, Fig. 37A and C). Of 98 blastocysts, 8 showed a *MSTN* mutation (7.7 ± 4.0%, Fig. 37B). The double-positive mutation (*PRNP* and *MSTN*) rate was 6.0 ± 3.6%. These results confirm that the frequency of *MSTN* mutation in germ cells of an F0 male was similar to that observed in blood tissue in my previous study. Semen from the F0 male had no detrimental effect on fertilization and enabled further gene editing using electroporation. The F0 male showed no significant change in the blood analysis (Table 21).

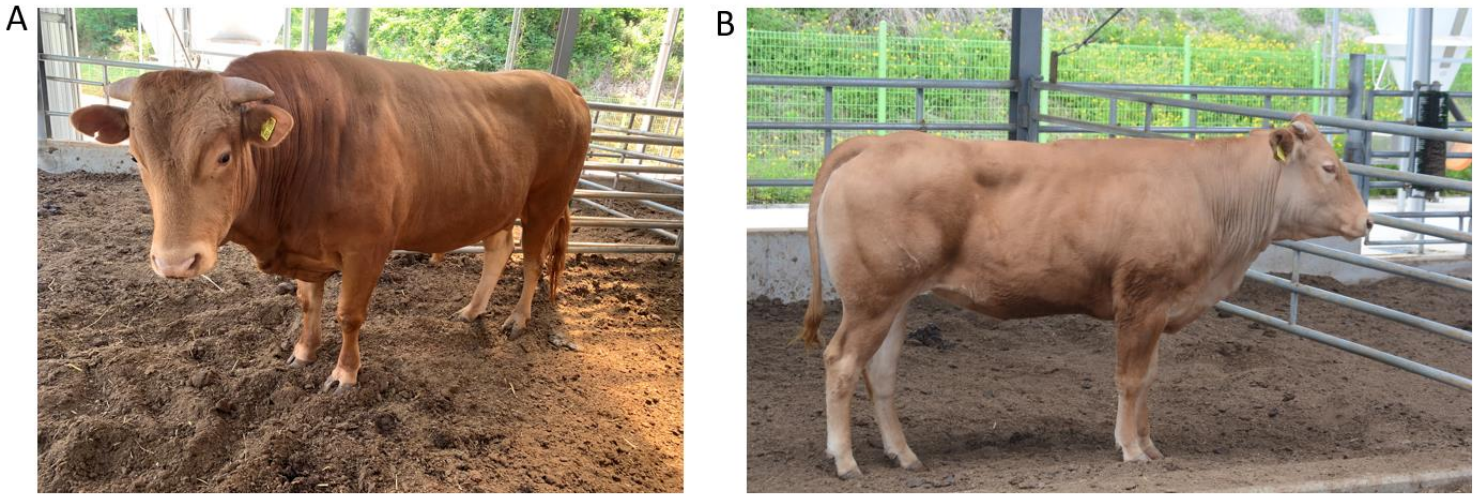

Figure 34. Representative pictures of *MSTN* mutated founder male (A) and female (B) at current age (25-month-olds).

Table 20. Analysis of semen from a *MSTN* male founder by Computer Assisted Semen Analysis

Variable	F0 (repeat time = 3)	Wild type (n=9)	p-value
Motile cells (%)	61.9 ± 4.6	67.3 ± 9.6	0.3802
Progressive cells (%)	26.0 ± 0.9	48.4 ± 13.3	0.0178
Static cells (%)	18.7 ± 1.5	14.6 ± 24.9	0.7844
VAP (㎛/s)	105.5 ± 4.1	108.4 ± 14.4	0.7416
VCL (㎛/s)	214.6 ± 8.5	189.2 ± 7.4	0.0006
VSL (㎛/s)	75.2 ± 1.7	92.8 ± 21.8	0.2074
ALH (㎛)	10.2 ± 0.3	7.9 ± 1.4	0.0178
BCF (Hz)	26.1 ± 0.8	30.9 ± 3.6	0.0496
LIN (VSL/VCL)	36.9 ± 0.7	50.4 ± 10.7	0.0603
STR (VSL/VAP)	71.4 ± 1.2	83.6 ± 9.7	0.059

Data are expressed as mean±s.d.; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight−line velocity; ALH: amplitude of lateral head; BCF: beat cross frequency; LIN: linearity; STR: straightness

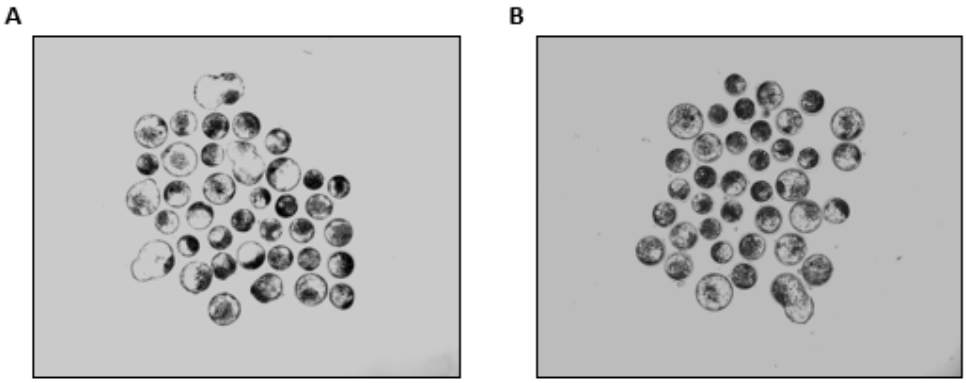

Figure 35. The representative blastocyst pictures: blastocysts produced in vitro fertilized with *MSTN* mutant semen (A) and applying additional gene editing using electroporation (B).

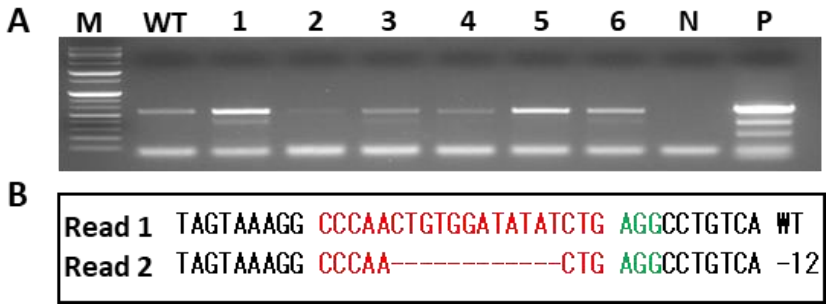

Figure 36. The mutation on *MSTN* gene in blastocyst derived from in vitro fertilized *MSTN* mutant semen.

1−6: randomly selected blastocysts. Upper panel (A) shows the T7E1 result, and bottom panel (B) shows the *MSTN* sequencing results in sample 1, 3, and 6. The red letters and line represent the *MSTN* target site, and the green letters represent the PAM sequence.

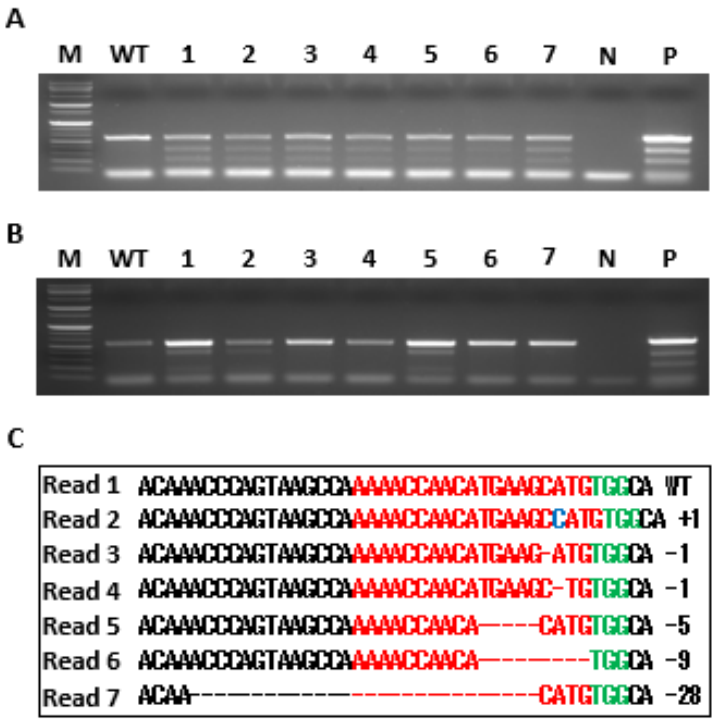

Figure 37. Additional gene editing using electroporation to embryo derived from in vitro fertilized *MSTN* mutant semen.

Upper panel shows the T7E1 results of *PRNP* (A) and *MSTN* (B) gene, and bottom panel (C) shows the *PRNP* sequencing results in sample 1, 2, and 3. 1−7: randomly selected blastocysts (M: marker; WT: wild type; 1: blastocysts derived from *MSTN* mutant female; N: negative control; P: T7E1 positive control). The red letters and line represent the *MSTN* target site, and the green letters represent the PAM sequence.

Table 21. Blood analysis in the F0 *MSTN* mutant cattle

Blood Parameters	References	*MSTN* mutant cattle	
		Male (23-month-old)	Female (22-month-old)
WBC	4.0 ~ 12.0(10^3/mm^3)	7.75	9.47
Neutrophil	0.6 ~ 4.1(10^3/mm^3)	3.57	3.09
Lymphocyte	2.5 ~ 7.5(10^3/mm^3)	3.57	5.17
RBC	5.0 ~ 10.0(10^3/mm^3)	9.35	8.19
HGB	8.0 ~ 15.0 (g/dL)	16.00	13.40
HCT	24.0 ~ 46.0 (%)	45.40	37.60
PLT	200 ~ 800(10^3/mm^3)	282.00	248.00
AST	53 ~ 162	86.00	80.00
ALP	77~ 265	163.00	210.00
BUN	10 ~ 25	11.50	14.20
Creatinine	0.4 ~ 2.0	1.21	2.25
Total Protein	7.2 ~ 9.0	7.41	7.22
Albumin	3.2 ~ 4.2	3.91	3.79
Ca (mg/dl)	8.3 ~ 10.4	9.20	9.10
P (mg/dl)	4.2 ~ 7.7	6.50	5.70
Glucose	31 ~ 77	77.00	76.00
Total Bilirubin	0.01 ~ 0.5	0.03	0.09

4.2 Germline transmission of *MSTN* mutant female cattle

By performing OPU, a total of 66 oocytes were collected (n = 5). After *in vitro* fertilization with wild-type frozen/thawed semen, 66 oocytes were cultured, and seven blastocysts (13.0 ± 9.8%) were formed (Fig. 38A). The T7E1 assay and Sanger sequencing revealed the heterogenous mutation of *MSTN* in the embryos (Fig. 38B). The mutation pattern was the same as that in the F0 female. In addition, the follicular fluid obtained during OPU was cultured, and the same *MSTN* mutation was observed by T7E1 assay and Sanger sequencing (Fig. 39). In a previous study on *MSTN* mutant females, an *MSTN* mutation was identified in somatic cells rather than germ cells due to the long sexual maturation period. In this study, the genotype in the *MSTN* mutant female was successfully transferred to F1 embryos. Moreover, the blood test results showed that there were no health issues and that the F0 female was growing well (Fig. 34B, Table 21).

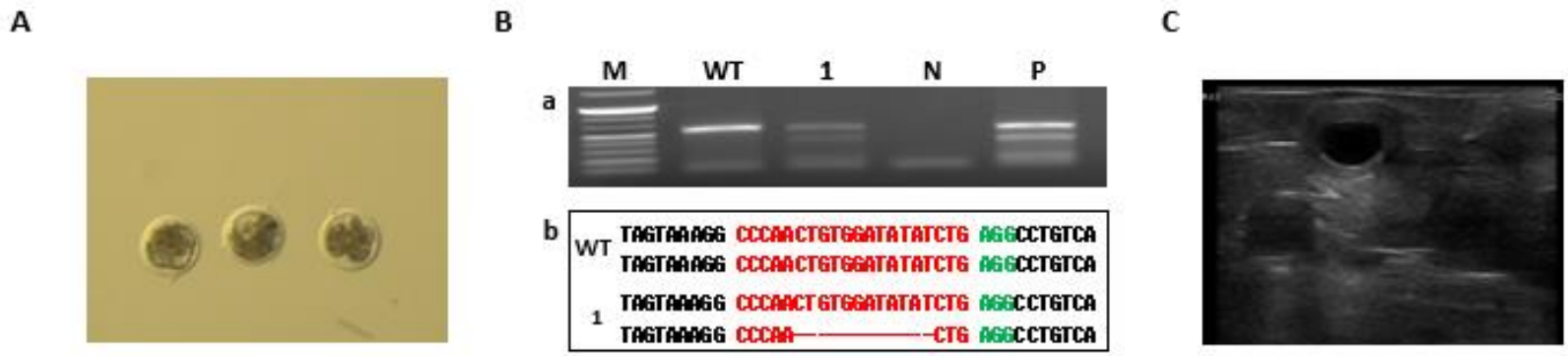

Figure 38. Germline transmission of *MSTN* mutant female cattle.

A. Blastocysts derived from *MSTN* mutant female oocytes. B. T7E1 analysis result (a) and sequencing data (b) from the blastocyst derived from *MSTN* mutant oocytes fertilized with wild-type semen. M: marker; WT: wild type; 1: blastocysts derived from *MSTN* mutant female; N: negative control; P: T7E1 positive control. C. Representative picture of pregnancy diagnosis using ultrasound machine at day 30. The red letters and line represent the *MSTN* target site, and the green letters represent the PAM sequence.

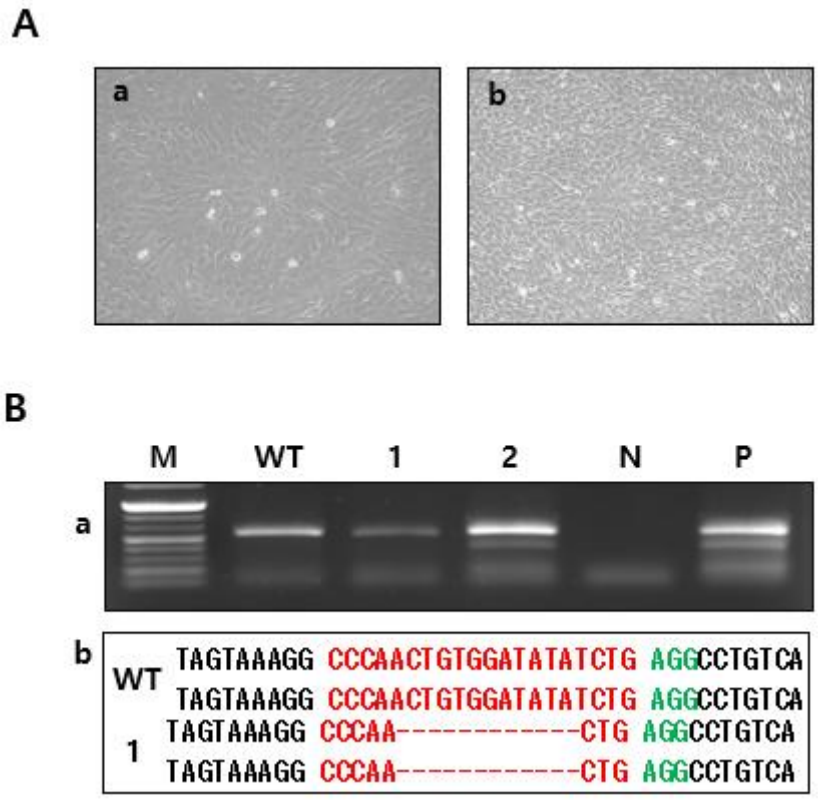

Figure 39. *PRNP* mutation for somatic cells derived from the follicular fluid obtained in the OPU process.

A. Somatic cells derived from OPU fluid. (a): *MSTN* mutant female, (b): wild type. B. T7E1 analysis result (a) and sequencing data (b) from somatic cells from the follicular fluid. 1: PCR from *MSTN* mutant female without wildtype genomic DNA; 2: with wildtype genomic DNA. The red letters and line represent the *MSTN* target site, and the green letters represent the PAM sequence.

4.3 Birth of F1 calf from *MSTN* mutant cattle

To generate the *MSTN* F1 calf, embryos derived from *MSTN* mutant female and male germ cells were transferred to a surrogate. The selected blastocysts were transferred to seven recipients. Pregnancy was confirmed in two recipients by rectal palpation and ultrasound on Day 30 (Fig. 38C). An F1 calf from an *MSTN* mutant cattle was successfully born from one cow, with the other pregnancy at the 6-month point and going well at the time of writing. Through Sanger sequencing of the *MSTN* locus, the wild-type read and the same mutation (-12bps) in F0 were observed from genomic DNA derived from F1 blood, which means the *MSTN* mutant trait from *MSTN* mutant cattle was transferred to the next generation (Fig. 40).

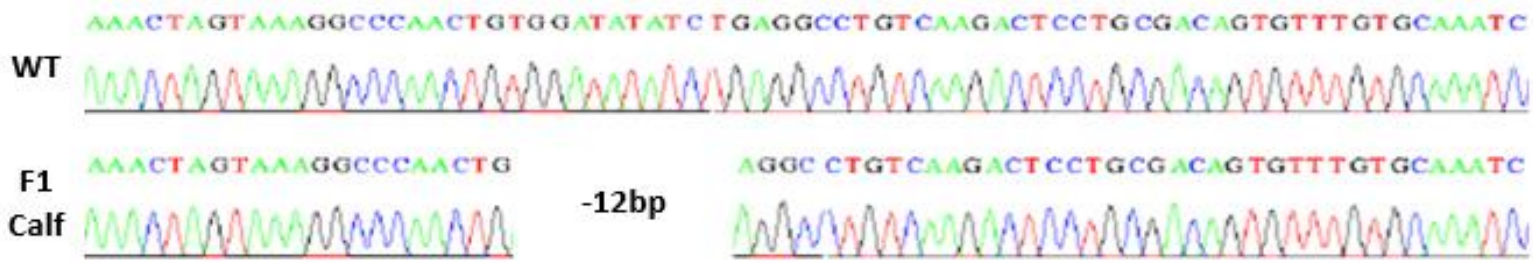

Figure 40. F1 calf of Sanger−sequencing data on *MSTN* target locus.

5. Discussion

Gene editing is a powerful tool for improving the traits of livestock. Several successful studies have been conducted on the use of gene editing methods in goats, sheep, pigs, and cattle [196, 213, 214]. However, although the analysis of germline transmission is particularly important, little related research has been conducted in genetically modified cattle due to the long sexual maturation period (cattle: 12 months vs. pig: 6 months), high cost of maintenance, and long generation period (cattle: average 280 days vs. pig: average 114 days). Very recently, I reported that *MSTN* mutant male and female founders can be produced and grown normally [213]. As a follow-up study to the previous report, here, I assessed the long-term monitoring and germline transmission of their germ cells to increase the number of gene-edited cattle and found that the oocytes and semen had the same mutation as the founder cattle.

This study produced three key findings. First, when *MSTN* mutant F0-derived germ cells were *in vitro* fertilized with wild-type germ cells, they developed to the blastocyst stage. In the case of the F0 male, sperm with 10.5% mutation were fertilized with wild-type oocytes and did not affect the

developmental competence of blastocysts (50.5 ± 6.8%). On the other hand, when oocytes obtained through OPU from the F0 female were fertilized with the F0 male, the blastocyst development rate was low (13.0 ± 9.8%) compared to wild-type *in vitro* fertilized embryonic development competence [214]. *MSTN* (GDF8) plays an important role in female reproductive physiology. In recent studies, myostatin was detected in human follicular fluid, which has attracted interest because of its potential functions in the reproductive system [215, 216]. In bovine studies, *MSTN* was expressed in granulosa cells and small antral follicles [217]. Additionally, when *MSTN* was inhibited in oocyte maturation, major oocyte maturation factors, growth differentiation factor-9 (GDF9), and bone morphogenetic protein-15 (BMP15) [218] were decreased [219]. These studies suggest that *MSTN* plays an important role in oocyte maturation, which can cause a low blastocyst formation rate. Despite low embryonic development, normal implantation occurred when a transferable blastocyst was transplanted, and an F1 calf was successfully born.

Second, only one genotype (-12bps) was observed in germ cells. In general, various genetic mutation patterns are

observed in knockout animals through microinjection [25]. However, in my previous study [22], interestingly, only −12bps was found through deep sequencing in blood, and the same result was observed in the oocytes and semen of the founder animals as well. Because the oocyte and sperm had the −12bps mutation, −12bps was observed in the newborn F1 calf as expected. That is, only the −12bps mutations existed in germ cells. I plan to produce more *MSTN* knockout individuals in the future to investigate whether they repeatedly exhibit the specific −12bps mutation and conduct research to understand the reason.

Lastly, in a previous study, I produced embryos with high knockout efficiency through microinjection, resulting in the production of mutant offspring [213]. However, the microinjection method has the disadvantage of requiring expensive equipment and sophisticated skills. As an alternative, in the present study, I subjected F1 embryos (zygotes) derived from F0 semen to electroporation. As in previous studies [213, 220], high knockout efficiency in the *PRNP* locus was noted in the present study. Interestingly, I also identified blastocysts with double knockout because the semen already had the *MSTN* mutation (Fig. 37C). By transferring the blastocysts obtained

through electroporation into recipients, single- or double-knockout calves can be produced with ease. Further research will be needed to assess the ability to produce healthy calves using electroporation as few studies have produced livestock animals using this method.

In conclusion, the present data indicate that the *MSTN* mutation induced in male and female cattle via genome editing with CRISPR-Cas9 could be transmitted to germ cells in the next generation (F1 calf), which can mature without any health issues. Moreover, additional mutations in embryos derived from F0 semen using electroporation could be useful for further gene editing (*PRNP*). Finally, in terms of practical benefits, these *MSTN*-edited cattle will contribute to improving agricultural productivity in the future.

FINAL CONCLUSION

This study shows that *PRNP*-mutant cattle and *MSTN*-mutant cattle were successfully produced by the CRISPR/Cas9 system. Germline transmission to the next generation (F1) was observed. In addition, this study introduced efficient double knock-out method using F0 germ cells.

Firstly, *PRNP*-mutant cattle were produced by piggyBac transposon and CRISPR/Cas9. *PRNP*-mutant calves were successfully born, germline transmitted to the next generation, and survived up to date. The F1 calf showed Cas9 activity, GFP expression, and *PRNP* mutation. In addition, conditional *PRNP*-mutant cattle was produced based on the Cre/loxP system. In F0 somatic cells, after Cre treatment, the cells expressed Cas9 protein but not *PRNP* mutation. However, the conditional *PRNP* male cattle showed the normal germline-transmission ability.

Secondly, *MSTN*-mutant cattle without exogenous gene insertion were produced by cytoplasmic microinjection based on the CRISPR/Cas9 system. Three of 17 had *MSTN* mutation and their mutation pattern was consistent with 12-bp deletions. Especially in #17, it was observed that the muscles were more developed in the shoulder and hips. In addition, in the blood test, there were no health problems.

Thirdly, germline transmission of *MSTN* mutation from *MSTN* mutant cattle (#6 and #17) to next generation (F1) was confirmed. In #17 female, oocytes were collected using OPU and developed into blastocysts. The blastocysts had the same *MSTN* mutation pattern as #17, and two were pregnant through embryo transfer. One of them was born and showed the same *MSTN* mutation. In #6 male, the sperm had a normal activity and the developmental competence of embryo fertilized with the semen was normal. The embryo had the same *MSTN* mutation pattern as #6. As further experiment, *PRNP* gene mutation was induced by electroporation in zygotes in vitro fertilized with the frozen semen of #6. Produced some blastocysts were observed mutation on both *MSTN* and *PRNP* genes.

Finally, this study shows the efficient production of *PRNP*-mutant cattle and *MSTN*-mutant cattle using the CRISPR/Cas9 system. Germline transmission was achieved. Then, the cattle had no issue on health and were confirmed with normal reproductive abilities. In addition, further gene editing using the F0 germ cell resource was introduced. This study will contribute to the basics and applications of livestock industry and veterinary medicine by producing cattle with desired traits.

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국문 초록

본 연구의 목적은 CRISPR/Cas9 이용하여 특정 유전자(*PRNP, MSTN*)을 타겟팅하여 돌연변이가 유도된 형질전환 소를 생산하는 것과 그 돌연변이가 다음 세대로 정상적으로 생식선 전이가 일어나는 것을 확인하는 것이다.

첫번째 *PRNP* 돌연변이 소를 생산하기 위해서 microinjection과 Piggybac 트랜스포존을 이용하여, *PRNP* 유전자에 돌연변이를 유도하였다. Cas9, GFP, *PRNP* 가이드 RNA가 포함된 트랜스포존 벡터가 소의 체세포와 수정란의 유전체에 정상적으로 삽입이 되고, *PRNP* 유전자에 돌연변이가 발생되는 것을 확인되었다. GFP 발현하는 배반포를 선별하여 18마리의 수란우에 이식하여 7마리의 송아지를 생산하였다. 생산된 7마리 중에서 4마리에서 성공적으로 *PRNP* 돌연변이를 보여주었다.

생식선 전이를 확인하기 위해서 #P3, #P7 소의 정상적인 성성숙 이후 생식세포를 이용하였다. 이들의 생식세포를 이용하여 생성된 배반포에서 *PRNP* 돌연변이를 확인되었고, 수정란 이식을 통해서 성공적으로 F1 송아지에서 *PRNP* 돌연변이가 정상적으로 생식선 전이가 이루어지는 것을 관찰하였다.

PRNP 유전자를 구체적으로 분석하기 위해서, Cre/loxP 시스템을 기반으로 하여, conditional *PRNP* 돌연변이 소를 생산하였다. 하지만, F0의 체세포에서 Cre 단백질 처리이후 Cas9 단백질 발현은 정상적으로 이루어 졌지만, *PRNP* 유전자 돌연변이는 발생되지 않았다. 하지만, conditional PRNP 돌연변이 수컷에서 정상적인 생식전전이를 수

정란 체외배양으로 생산된 배반포에서 관찰이 되었다.

두번째 연구로서, 외래 유전자 삽입이 없는 *MSTN* 돌연변이 소를 생산하기 위해서 Cas9 mRNA와 sgRNA for *MSTN*을 수정란에 세포질 microinjection 하였다. 생산된 배반포는 26마리에 이식을 진행하고, 17마리의 송아지를 얻었다. 그 중 3마리에서 *MSTN* 돌연변이가 관찰이 되었다. 태어난 MSTN 돌연변이 소에서 off-targeting 영향과 혈액검사 결과 건강상 문제가 없음이 확인되었다.

다음으로, #6, #17 *MSTN* 돌연변이가 생식선 전이가 되는지를 수정란 수준에서 확인하였다. #6과 #17의 체외수정으로 생산된 배반포를 수란우에 이식을 하여 성공적으로 F1 송아지를 생산하였다. 태어난 송아지에서는 *MSTN* 돌연변이를 보여주었으며, 건강상에 문제는 관찰되지 않았다.

본 연구는 최초로 CRISPR/Cas9 기반으로 하여 *PRNP* 돌연변이 소와 외부 유전자가 삽입되지 않은 *MSTN* 돌연변이 소를 성공적으로 생산하였다. 또한 생식선 전이를 통해 이들의 돌연변이가 다음 세대로 돌연변이가 전달되는 것을 확인하였다. 이러한 연구 결과는 CRISPR/Cas9 시스템을 이용하여 특정 유전자 돌연변이 소를 높은 효율로 생산할 수 있음을 보여주었으며, 축산업 및 수의학 등의 다양한 분야에서 적용될 수 있을 것이다.

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주요어: 소, CRISPR/Cas9, 세포질 미세주입, 전기천공법, 생식선 전이

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