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# Outlook on genome editing application to cattle

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## ABSTRACT

In livestock industry, there is growing interest in methods to increase the production efficiency of livestock to address food shortages, given the increasing global population. With the advancements in gene engineering technology, it is a valuable tool and has been intensively utilized in research specifically focused on human disease. In historically, this technology has been used with livestock to create human disease models or to produce recombinant proteins from their byproducts. However, in recent years, utilizing gene editing technology, cattle with identified genes related to productivity can be edited, thereby enhancing productivity in response to climate change or specific disease instead of producing recombinant proteins. Furthermore, with the advancement in the efficiency of gene editing, it has become possible to edit multiple genes simultaneously. This cattle breed improvement has been achieved by discovering the genes through the comprehensive analysis of the entire genome of cattle. The cattle industry has been able to address gene bottlenecks that were previously impossible through conventional breeding systems. This review concludes that gene editing is necessary to expand the cattle industry, improving productivity in the future. Additionally, the enhancement of cattle through gene editing is expected to contribute to addressing environmental challenges associated with the cattle industry. Further research and development in gene editing, coupled with genomic analysis technologies, will significantly contribute to solving issues that conventional breeding systems have not been able to address.

**Keywords:** Breeding; cattle; genetic engineering; genetically engineering cattle; somatic cell nuclear transfer

## INTRODUCTION

Since the 1990s, genetically engineered cattle (GEC) have been produced using several techniques, including deletion and insertion, random integration into the host genome, and selective genome editing [1]. Initially, GEC had been used to produce recombinant pharmaceuticals and understanding human diseases. With increasing global population, we will face into food shortages in 2025. It is necessary to solve the food problem and need to improve productivity in cattle. Coupling with genetic engineering and analysis tools,

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**Conflict of Interest**
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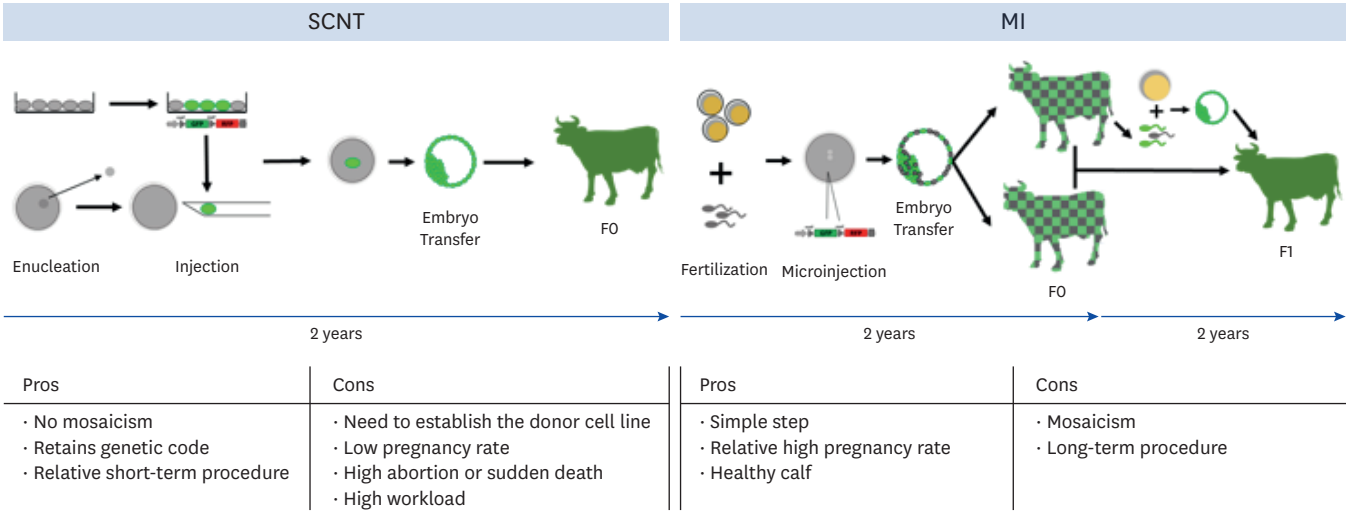
such as next-generation sequencing (NGS) to figure out the gene related to productivity, can maximize productive efficiency more than the conventional breeding system.

Of the genetic engineering tools currently available, genome editing is remarkably powerful to edit bovine endogenous genes. The development of various 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 made genome editing highly convenient and precise. Through the technologies, the cattle with improvement of disease resistance, and enhancement of the frequency of alleles or polymorphisms that are associated with favorable traits (e.g., lack of horns, heat tolerance, and efficient milk or meat production/composition) were produced [2-6]. In addition, gene editing can overcome the challenge of random integration into the host genome, which cause several risks. This review introduces the need of gene editing technology in cattle industry, explaining a general background of gene editing technology and production of cattle with superior traits using gene editing and gene analysis. In addition, our review showed DNA-based genome selection for improving cattle breeds, which involves analyzing the entire cattle genome to identify genetic markers associated with desirable traits, is more accurate and can improve the speed of genetic progress [7].

## PAST TECHNOLOGIES FOR PRODUCING GEC

To date, most GEC have been produced through microinjection and somatic cell nuclear transfer (SCNT). Before the development of SCNT in the 1990s, most GEC were generated through microinjection, in which nucleic acids are microinjected into the cytoplasm of fertilized eggs (zygote). In the 1990s, GEC were produced by microinjecting plasmids or viruses carrying the gene of interest. Although the trials were successful, birth rates of GEC were very low, at about 12% [8-10]. Moreover, microinjection resulted in genetic mosaicism and was unsuitable for use on farm animals because of its high cost, long gestation period, small litter sizes, and low transgene integration rates (**Fig. 1**) [9].




| Pros | Cons |
|---|---|
| · No mosaicism<br>· Retains genetic code<br>· Relative short-term procedure | · Need to establish the donor cell line<br>· Low pregnancy rate<br>· High abortion or sudden death<br>· High workload |

| Pros | Cons |
|---|---|
| · Simple step<br>· Relative high pregnancy rate<br>· Healthy calf | · Mosaicism<br>· Long-term procedure |

**Fig. 1.** Representative techniques for the generation of genetically engineered cattle. SCNT is inefficient at generating healthy calves, while MI is time-consuming because of the emergence of mosaicism.
SCNT, somatic cell nuclear transfer; MI, microinjection.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Since the 2000s, SCNT has been the most powerful tool for GEC production. This method involves the selection of a donor cell with a desired mutation. Subsequently, the nucleus of the donor cell is replaced with that of a mature oocyte following which the embryo is transplanted (**Fig. 1**). SCNT led to the development of GEC that express recombinant proteins in milk [2,3] and are disease resistant [4,5], and also improved animal welfare [6]. However, SCNT-derived calves have numerous health complications because of abnormal reprogramming and epigenetic gene regulation [11,12]. Although several efforts have been made [13-19] to address these limitations, some issues remain. Nonetheless, SCNT has several advantages, including easy selection of donor cells that have the desired gene or mutation, analysis when testing in cells, and selection of cells exhibiting high transgene expression. Thus, before the development of genome-editing tools (ZFNs, TALENs, and CRISPR/Cas9), SCNT was preferred over microinjection.

Before the development of genome-editing tools, GEC were developed through the random insertion of the gene of interest into the host genome (**Table 1**). However, random insertion has several potential drawbacks [13,14]. First, the targeted host gene insertion locus can only be known after analyzing the transgenic animals using PCR and DNA sequencing. Second, gene insertion can alter the expression of endogenous genes. Third, several copies of the gene can be inserted into the host genome.

## GEC PRODUCTION USING GENOME-EDITING TOOLS (ZFNs, TALENs, AND CRISPR/Cas9)

Before the development of targeted genome-editing methods, the genome was modified randomly, which was ineffective and expensive for the development of GEC. In addition,

**Table 1.** GEC 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 | [8] |
| 1994 | Overexpression | Human erythropoietin | DNA fragment | Microinjection | Random | [9] |
| 1998 | Overexpression | Hepatitis B surface antigen | Retrovirus | Microinjection | Random | [65] |
| 1999 | Overexpression | Human alpha-lactalbumin | DNA fragment | Microinjection | Random | [10] |
| 2002 | Overexpression | Human lactoferrin | DNA fragment | Microinjection | Random | [66] |
| 2003 | Overexpression | β- and κ-casein | DNA fragment | SCNT | Random | [67] |
| 2004 | Overexpression | Fluorescent gene | Lentivirus | Microinjection | Random | [68] |
| 2004 | Overexpression | anti-human CD28, anti-human melanoma specificity (r28M) | DNA fragment | SCNT | Random | [69] |
| 2005 | Overexpression | lysostaphin | DNA fragment | SCNT | Random | [4] |
| 2006 | Overexpression | Human growth hormone | DNA fragment | SCNT | Random | [2] |
| 2007 | Knock-out | None | DNA fragment | SCNT | PRNP | [5] |
| 2008 | Overexpression | Human lactoferrin | DNA fragment | SCNT | Random | [70] |
| 2009 | Overexpression | Human Albumin | DNA fragment | SCNT | Random | [71] |
| 2009 | Overexpression | Human IgG | DNA fragment | SCNT | Random | [72] |
| 2011 | Knock-down | None | DNA fragment | SCNT | PRNP | [73] |
| 2012 | Knock-down | None | DNA fragment | SCNT | BLG | [74] |
| 2016 | Overexpression | Fluorescent gene | Sleeping Beauty | Microinjection | TA | [75] |
| 2016 | Overexpression | Human beta-defensin (HBD3) | DNA fragment | SCNT | Random | [76] |
| 2016 | Overexpression | Fluorescent gene | piggyBac | Microinjection | TTAA | [77] |
| 2017 | Overexpression | hBSSL | DNA fragment | SCNT | Random | [78] |
| 2017 | Overexpression | Human lactoferrin | DNA fragment | SCNT | Random | [79] |

SCNT, somatic cell nuclear transfer.


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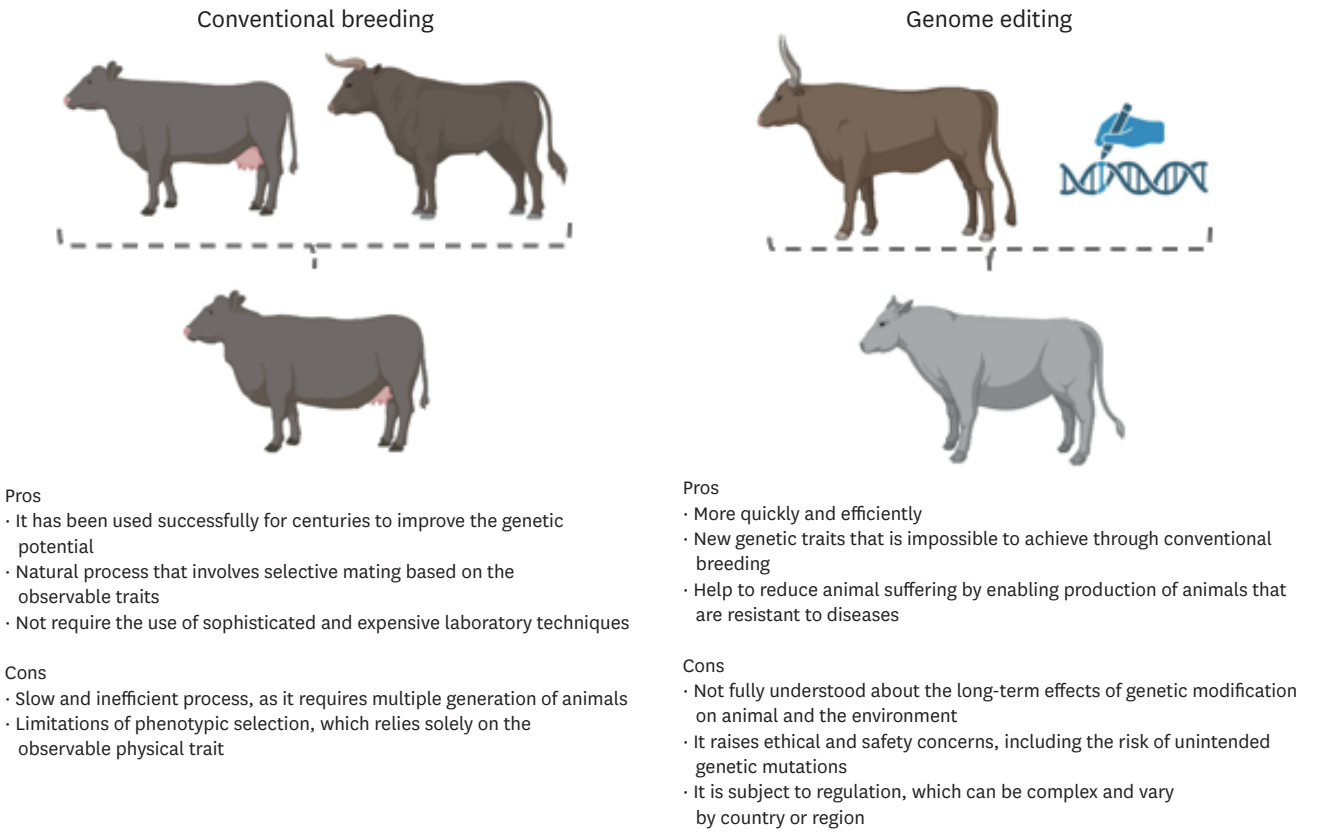


**Fig. 2.** Breeding methods: conventional breeding vs. genome editing. Although conventional breeding, which is based on observable traits has been used for centuries, it is time-consuming because it requires multiple animal generations. Modern breeding techniques that use genome editing can introduce new genetic traits that are impossible to transfer through conventional breeding. However, this requires a complete understanding of the long-term effects of genome editing and raises ethical and safety concerns.

there were safety concerns about malfunctioning original genes or effects on gene regulation [13,14]. However, the emergence of endonucleases as genome-editing tools helped significantly overcome these challenges. The endonucleases that are used for genome editing, including ZFNs, TALENs, and CRISPR/Cas9, recognize specific target gene sequences and induce double-strand breaks (DSBs), which trigger DNA repair via non-homologous end-joining (NHEJ) or homology-directed repair (HDR) [15], thereby markedly increasing the efficacy of targeted genetic modifications [16].

Because of its ability to accurately edit target loci, genome editing leads to quicker and more effective livestock improvements when compared with conventional breeding methods (**Fig. 2**). However, the long-term effects of genome editing are not fully understood and there are concerns that although it is less risky than conventional genetic engineering methods, it may endanger animal welfare. Barring some minor issues, current genome-editing tools allow quick investigation of superior traits, such as productivity, disease resistance, and resilience to climate change.

## ZFNs

ZFNs were discovered in the 1980s and the first specific one was reported 15 years later. A ZFN is comprised of 30 amino acids, with two anti-parallel beta-sheets opposing an alpha-

**Table 2.** GEC 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 | [20] |
| 2014 | Knock-out Knock-in | Human lysozyme | ZFNs | SCNT | BLG | [80] |
| 2014 | Knock-out | None | ZFNs | SCNT | MSTN | [81] |
| 2018 | Knock-out | None | ZFNs | SCNT | BLG | [21] |

GEC, genetically engineered cattle; ZFN, zinc-finger nuclease; mRNA, messenger RNA; SCNT, somatic cell nuclear transfer.

helix [17]. The alpha-helix can bind three specific bases located in the major groove of DNA [18]. ZFNs have a site-specific zinc-finger DNA-binding domain and nonspecific *Fok*I endonuclease cleavage domain. Two or more ZFN molecules are required to modify a specific gene. In general, ZFNs recognize 9–18 bp that can be specifically edited. Dimerized *Fok*I induces DSBs, which trigger NHEJ, through which a specific gene can be blocked, or HDR, through which a desired DNA sequence can be inserted into a specific gene. Originally, the lengths of homologous arms were 6–7 kb but decreased to 0.5–1.5 kb with the emergence of ZFNs [19]. Several GEC produced by ZFN are listed in **Table 2**. Notable among these was the successful mutation of the beta-lactoglobulin (BLG) gene in cattle [20]. *BLG* protein is not present in human milk but is present in significant quantities in whey and is an allergen in cow milk. Importantly, the *BLG* gene modification was stably inherited by subsequent generations. Interestingly, milk obtained from the *BLG*-mutated cows was found to be digested by pepsin approximately 30 times faster than normal milk containing *BLG* [21].

## TALENs

TALENs are naturally produced by *Xanthomonas*, a genus of gram-negative bacteria that infect several plant species. TALENs are comprised of DNA-binding domains with 33–35 amino acid repeat domains and the nonspecific endonuclease, *Fok*I. The repeat variable di-residue (RVD), the amino acid residues on the 12th and 13th positions of the DNA-binding domains, recognize single base pairs, with each RVD specifically binding a single genomic DNA nucleotide [22,23]. The DNA-binding sequence of TALENs begins with thymidine and the target sequence length is 30–40 bp. The DNA-binding domains can be modified to target endogenous DNA sequences for cleavage by the nonspecific endonuclease, *Fok*I, thereby triggering DNA repair via NHEJ and HDR [20,24-26].

TALENs have several advantages over ZFNs: 1) the TALEN repeat is 3–4 times longer than the ZFN repeat and because TALENs recognize one nucleotide, they are more sophisticated than ZFNs, which recognize three nucleotides, 2) the ZFN modification requires a high-level design because DNA recognition may not be successful because of crosstalk between the fingers [27], 3) TALENs are easier to design than ZFNs because they are simpler and their production is quicker and more cost-effective, 4) TALENs have less off-target effects than

**Table 3.** GEC 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 | [82] |
| 2015 | Knock-out | None | TALENs mRNA | Microinjection | MSTN | [29] |
| 2016 | Knock-in | Pc *POLLED* | TALENs | SCNT | POLLED locus | [6] |
| 2018 | Knock-out | None | TALENs | Microinjection | BLG | [83] |

GEC, genetically engineered cattle; TALEN, transcription activator-like effector nuclease; mRNA, messenger RNA; SCNT, somatic cell nuclear transfer.

ZFNs, and 5) compared with ZFNs, TALENs are more amenable to genome-editing because they can be injected into the cytoplasm of livestock embryos [28].

TALENs are a powerful tool for knock out genes in rats and zebrafish [77-79] but they have also been used to efficiently mutate genes of interest in cattle, sheep, and pigs [29,30]. **Table 3** summarizes the history of GEC production using TALENs, which was used to develop hornless cattle via the introduction of the Pc Celtic POLLED allele into dairy bulls [6]. Recently, crossing these bulls with horned cows resulted in the birth of six hornless calves and genome analysis confirmed that the calves possess the Pc Celtic POLLED allele without any unintended genomic alterations [31].

## CRISPR/Cas9

CRISPR, which was discovered in the 1980s, provides adaptive immunity that protects bacteria and archaea from bacteriophage invasion [32-35]. CRISPR mediates RNA-guided DNA cleavage. The CRISPR/Cas9 system is widely used to induce targeted DSBs. It contains a Cas9 endonuclease that causes DSBs after recognizing the protospacer adjacent motif downstream of a target sequence and a single-guide RNA that interacts with and directs Cas9 to the target DNA sequence [84]. The CRISPR/Cas9 system is much more efficient at targeting genes of interest than the older gene editing tools (ZFNs and TALENs). Moreover, unlike ZFNs and TALENs, which form DNA-protein complexes, CRISPR/Cas9 forms an RNA-protein complex (**Table 4**). Importantly, CRISPR/Cas9 can simultaneously induce DSBs in more than one target gene. CRISPR/Cas9 has also been used to generate gene-edited farm animals [36,37]. In addition, a one-step method of generating gene-edited animals was developed by introducing Cas9 messenger RNA (mRNA) into early zebrafish [38-40], rats [40,41], mice [42-45], rabbits [46], pigs [37,47,48], sheep, and cattle embryos [29,49]. The adoption of CRISPR/Cas9 as a gene-editing tool has improved the efficiency of gene editing in mammals and allowed the generation of highly sophisticated genetically engineered animals [42,50,51].

**Table 4.** Comparison of three classes of molecular scissors [84]

| Category | ZFN | TALEN | CRISPR/Cas9 |
|---|---|---|---|
| Targeting domain | Zinc-finger proteins | Transcription activator-like effector | CRISPR RNA or single-chain guide RNA |
| Nuclease | FokI | FokI | Cas9/FokI |
| 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/FokI construct) |
| Costs | +++ | ++ | + |

ZFN, zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats.

**Table 5.** GEC lists produced by CRISPR/Cas9

| Year | Knock-out Knock-in | Inserted gene | Method of transgenesis | Method of embryo manipulation | Target gene | Reference |
|---|---|---|---|---|---|---|
| 2017 | Knock-in | Correct IARS gene, GFP | CRISPR/Cas9 | SCNT | IARS | [85] |
| 2021 | Knock-in | SRY | CRISPR/Cas9 | Microinjection | Chr17 | [65] |
| 2022 | Knock-out | None | CRISPR/Cas9 | Microinjection | MSTN | [62] |

GEC, genetically engineered cattle; CRISPR, clustered regularly interspaced short palindromic repeats; SCNT, somatic cell nuclear transfer.


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Although CRISPR/Cas9 has greatly increased the efficiency of generating genetically engineered animals, there are still limitations to the insertion of foreign genes. For instance, inserting foreign genes by HDR is challenging [52-54], especially when large cargoes that require extensive cell sorting or selection are involved [55]. Several techniques, including the utilization of long single-stranded DNA [56], homology-independent targeted insertion [57,58], homology-mediated end-joining (HMEJ) [52,59], microhomology-mediated end joining (MMEJ) [60], and targeted integration with linearized double strand DNA [61], have been used to enhance knock-in efficiency. However, further studies are required to improve knock-in efficiency in mammals. The history of CRISPR/Cas9-generated GEC is summarized in **Table 5**. One of the most recent studies on the generation of GEC using CRISPR/Cas9 involved the targeted editing of the *MSTN* gene, which doubled the muscle mass [62]. It has been confirmed that the cattle can transmit the *MSTN* mutation to the next generation, indicating stable germline transmission of the trait [63].

## GENOMIC SELECTION

The recent increase in CRISPR/Cas9 use has led to the production of specific gene-targeted cattle [62,64]. Even in cattle, germline transmission of the specific gene mutation was proven. The increasing popularity of specific gene targeting has led to increased research on the genomic selection of single nucleotide polymorphisms. In the case of DNA-based genomic selection, the improvement rate is relatively objective compared with that of the existing method, which is based on phenotypic selection and cannot always be a reliable indicator because of the influence of environmental factors (**Fig. 2**). In addition, the use of genomic selection has advanced cattle

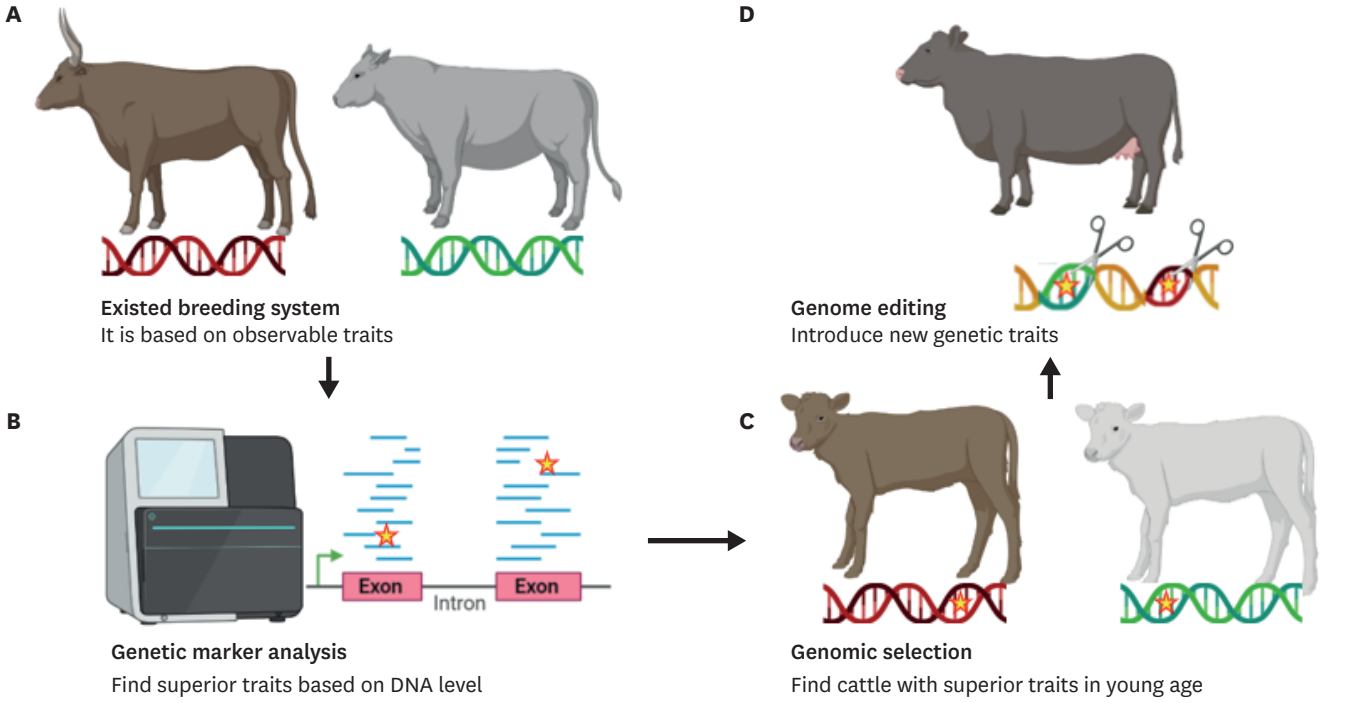


**Fig. 3.** An improved breeding system for the future. (A) Cattle generated using an observable traits-based breeding system. (B) Based on DNA sequences, genetic marker analysis can detect superior traits that cannot be influenced by the environment. (C) When compared with conventional breeding techniques based on physical characteristics, genetic markers can quickly and accurately identify cattle with superior production traits and disease resistance. (D) Genome editing can produce genetically engineered cattle with new genetic traits that cannot be found in existing breeds.

breeding by enabling breeders to identify and select animals with superior genetic traits at a younger age, which increases efficiency and productivity in the cattle industry.

## DISCUSSION

Cattle are known to be a major source of animal protein in the world. With the increasing global population, enhancing cattle production efficiency is crucial. While conventional breeding systems have improved cattle breeds, gene editing technology can create new cattle breeds with higher productivity that conventional breed system cannot achieve. In addition, advances in genetic analysis technology have led to a shift from phenotype-based selective breeding to DNA-based selective breeding. The combination of genomic selection and genome editing can enhance superior traits more efficiently and with high accuracy (**Fig. 3**), especially in areas that have traditionally required prolonged periods for improvement. Further development of new breeding systems will contribute to solve the problems that conventional breeding systems have previously been unable to address.

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