REVIEW

Embryo-mediated genome editing for accelerated genetic improvement of livestock

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Abstract Selecting beneficial DNA variants is the main goal of animal breeding. However, this process is inherently inefficient because each animal only carries a fraction of all desirable variants. Genome editing technology with its ability to directly introduce beneficial sequence variants offers new opportunities to modernize animal breeding by overcoming this biological limitation and accelerating genetic gains. To realize rapid genetic gain, precise edits need to be introduced into genomically-selected embryos, which minimizes the genetic lag. However, embryo-mediated precision editing by homology-directed repair (HDR) mechanisms is currently an inefficient process that often produces mosaic embryos and greatly limits the numbers of available edited embryos. This review provides a summary of genome editing in bovine embryos and proposes an embryo-mediated accelerated breeding scheme that overcomes the present efficiency limitations of HDR editing in bovine embryos. It integrates embryo-based genomic selection with precise multi-editing and uses embryonic cloning with elite edited blastomeres or embryonic pluripotent stem cells to resolve mosaicism, enable multiplex editing and multiply rare elite genotypes. Such a breeding strategy would enable a more targeted, accelerated approach for livestock improvement that allows stacking of beneficial variants, even including novel traits from outside the breeding population, in the most recent elite genetic background, essentially within a single generation.

Keywords animal breeding, cattle, cloning, CRISPR/ Cas9, cytoplasmic injection, embryo, genome editing, germline chimaeras, HDR, livestock improvement, TALENs

1 Introduction

Existing natural variation in the gene pool of domesticated animals has been successfully used to improve livestock, such as cattle, through conventional breeding by artificial selection. Although this strategy has been proven highly successful, progress is limited to small incremental steps at each generation, typically amounting to annual genetic gains of 1% to 3%, which become more substantial over longer time frames[1].

However, conventional breeding is slow. As every breeding event generates new gene combinations, which cannot be directed or controlled, each animal only carries a fraction of all potentially desirable sequence variants. To accomplish genetic improvement, it is important to select the best animals that will become the parents for the next generation of improved animals. Genomic selection, using genome-wide single-nucleotide polymorphism (SNP) chip arrays or whole genome sequencing, provides a powerful means for the identification of animals with the most favorable genotypes that assemble the overall best combination of sequence variants in an individual animal[2]. This concept, first introduced in 2001[3], has allowed unprecedented advances in commercial breeding in the past 15 years, including a doubling of dairy cattle improvement per generation compared to traditional selection. However, the potential for genetic improvement can be compounded by genetic linkages that result in the concurrent inheritance of desirable and non-desirable sequence variants. Depending on how tight the genetic linkages are, passenger mutations may not be easily removed by breeding into subsequent generations, even if their identity is known.

The development of programmable nucleases allows for the precise site-specific editing of livestock genomes[4]. With genome editing technology, it is now possible to uncouple genetic linkages and directly introduce individual beneficial variants, even from outside the natural

breeding population, into elite genetic backgrounds. Further, it facilitates the assembly of multiple high-impact sequence variants in individual genomes which can be very difficult or impossible to achieve with conventional breeding strategies. Presently, genome editing targets are still limiting due to a low number of known causative sequence variants with relatively high impact on phenotypic production traits. Decreasing cost for whole genome sequencing is now allowing the sequencing of large numbers of animals which can greatly facilitate the fine-mapping and identification of causative variants[5]. Likewise, the inclusion of increasing numbers of causative sequence variants is also expected to increase the accuracy of genomic selection.

Genome editing, in combination with genomic selection, offers a novel opportunity that could change the scope and speed for improving efficiency of livestock production and completely overhaul current livestock breeding practices. For increased speed of genetic gain of large animals with long generation times, such as dairy cattle, it is paramount that selection and editing is undertaken as early as possible, using appropriately elite baseline genetics.

Here, we review the current approaches of embryo-mediated genome editing in cattle, outline its short-comings, suggest solutions to overcome these issues and present a model for an improved breeding strategy. We propose to combine embryonic genomic selection, genome editing and multiplication into a breeding platform that captures embryo-derived germlines as clones or germline chimaeras. This generates a contemporary breeding strategy offering large scale dissemination and accelerated genetic gain that is superior to currently implemented procedures.

2   Genome editing for livestock breeding

Programmable nucleases have provided the molecular tools to efficiently introduce defined sequence variants into livestock genomes. This enables the direct introgression of beneficial traits essentially within a single generation, which offers scope for improvements of animal breeding by directed and fast genetic gain.

2.1   Double strand break (DSB)-dependent editors

At present, the toolbox contains three main technology platforms. Zinc finger nucleases (ZFNs) were the first available genome editors, combining a programmable DNA binding domain of multiple zinc finger modules with the catalytic domain of the restriction enzyme *Fok*I[6]. They were soon followed by the development of an analogous protein-based editing platform, transcription activator-like effector nucleases (TALENs) where the *Fok*I domain was linked to a modular DNA binding domain

consisting of an array of repeat units whose intrinsic polymorphic sites determine DNA binding specificity[7]. Both platforms were protein-based, which required customization of the DNA binding domain for each editing target, limiting flexibility and making it expensive. This changed with the advent of the much simpler clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system[8]. Rather than entirely protein-based, it is an RNA-guided nuclease system that uses a universal monomeric nuclease (Cas9) and a small programmable guide RNA (gRNA), which guides the nuclease to the editing site through sequence complementarity. The gRNAs can be readily designed for unique genome target sites through various online tools and economically synthesized by commercial suppliers. Given that the Cas9 nuclease can be universally used for any target site, it is now commercially available as mRNA or recombinant protein. This provides unprecedented flexibility and easy access to cost-effective genome editors for any desirable target site.

All site-specific nucleases function by introducing a DSB into the genomic DNA at the programmed target site. A DSB in the genome is typically repaired by the cellular repair machinery via the non-homologous end joining (NHEJ) repair process. NHEJ is an error-prone process. Due to endogenous nuclease activity at the DSB, the repair process often leads to an introduction of small insertions or deletions (indels) at the target site. However, there is no control over how the target site is edited by these indels, which makes this mechanism unsuitable for directed breeding applications. This is different when a pair of editors is introduced which can efficiently delete the fragment between the two DSBs[9]. In this case, the outcome can be more predictable and used to introduce a naturally occurring deletion that is associated with a beneficial trait.

Many of the sequence variants relevant for breeding can be conversions, deletions or insertions of single base pairs. Their introduction requires full control over genome editing outcomes, which can be achieved through an HDR mechanism. The site-specific DSB generated by a genome editor can trigger HDR. In the presence of a homologous repair template provided in *trans*, the cellular repair machinery can then repair the DSB according to the repair template. This can be exploited for the introduction of defined mutations, such as a single base pair change, by supplying a repair template that specifies the intended sequence changes[10].

2.2   DSB-independent editors

Although limited to the change of specific base pairs, a new concept for the introduction of precise changes was developed with base editing. This no longer requires the introduction of a DSB and its subsequent repair. Rather, it

is based on a Cas9 with either one or both of its endonuclease domains inactivated, rendering it a nickase, able to only introduce a nick at the target site, or removing any endonuclease activity. These mutant Cas9 enzymes retain uncompromised target site binding and when fused to a deaminase, enable the direct conversion of a C-G base pair into a T-A base pair and an A-T base pair into G-C base pair[11]. The limitation of base editors for conversion of only a subset of single base pair combinations might already be resolved by the development of a new innovative tool with great potential for precise genome editing[12]. For this novel editor, a Cas9 nickase was fused to a reverse transcriptase. Like Cas9, it is programmed by a single RNA molecule but has additional functional components, compared to a conventional gRNA. The RNA molecule for the new editor combines the function of the gRNA to guide the nickase to the target site with a primer and template for reverse transcription. When encoded in the RNA template section of the RNA molecule, intended edits, including indels and all 12 types of point mutations, can be efficiently introduced into the genome without the need for a DBS or other exogenous DNA molecules as repair template for HDR.

2.3   Potential for unintended off-target edits

Programmable nucleases have high cleavage activity and show some degree of tolerance to mismatches for target-specific binding. This creates the potential for unintended off-target events with the introduction of unwanted mutations somewhere in the genome[13]. In particular, Cas9-based editors have seen rapid improvements to increase binding specificity and associated reduction in off-target activity[14]. As a recent example, prime editors appear to be associated with much lower off-target editing compared to Cas9 nuclease[12].

Systematic identification of all possible cleavage sites *in vitro* and their validation in an *in vivo* mouse model demonstrated that despite efficient editing, no detectable off-target mutations were found when following appropriate design guidelines for the gRNA/Cas9 editor[15]. PCR and sequencing analyses commonly characterize only short genomic regions, which might miss larger deletions and complex rearrangements that can also be introduced through DSB repair triggered by genome editing. This is an issue that is particularly relevant for multiplex applications[16]. Comprehensive unbiased genome-wide assessment of genome-edited sheep and goats showed that the level of *de novo* mutations was comparable to normal animals, with only one and two mutations attributable to off-target events, respectively[17,18]. Most studies to date observed no to negligible off-target effects in genome-edited livestock. Although the risk appears to be very small, the potential for off-target mutations should be considered.

2.4   Editing somatic vs. embryonic cells

Genome editing of cattle can be approached by two alternative experimental strategies. First, genome editors can be introduced into cultured primary somatic cells to effect site-specific sequence changes of their genomes. Following the isolation and characterization of cell clones, individual cells from cell clones that possess the intended edits can then be used as donor cells for somatic cell transfer (SCT) (Fig. 1(a)). This approach has so far been the method of choice for the generation of genome-edited cattle. It allows accurate characterization of the edited genotype, including monoallelic or biallelic modification and the potential introduction of any unintentional mutations from off-target events. Hence, it provides the ability to only generate cattle with intended genotypes and avoids the wasteful production of unwanted cattle. However, this approach is also associated with some major disadvantages. SCT is notoriously encumbered by low production efficiencies of viable offspring due to faulty or incomplete epigenetic reprogramming of the donor cell genome. This causes the commonly observed high rates of losses during pregnancy and can also affect the viability of live-born calves[19]. From a breeding perspective, adult somatic cloning increases the genetic lag between nucleus and commercial populations by one generation interval (equivalent to two years in cattle), compared to embryonic clones.

The second approach introduces genome editing tools into embryos, which are subsequently transferred into recipient cows for development to term and production of live genome-edited calves (Fig. 1(b)). As with somatic cells, not all embryos will be edited. To screen and identify edited embryos, there is the option to take a small biopsy which can provide a good representation about the editing status of the embryo and resulting animal[20]. The injection and biopsy do not compromise the developmental potential of the embryos.

Importantly, the same biopsy sample can be used for genomic selection, using SNP genotyping on different density bovine SNP BeadChips[21]. Following imputation with parental genotypes, there was high concordance (≥95%) between biopsied and vitrified *in vitro* produced (IVP) embryos and their respective calf genotypes[21]. Applying this approach to dairy cattle, where vitrified IVP embryos are commonly used in breeding programs without loss of viability, would offer two advantages. First, it enables more intense selection of favorable genotypes within full-sib IVP embryos and second, it reduces the costs for yearling bulls and heifers because embryos of low genetic merit would not be transferred into surrogate mothers and reared to breeding age[22]. Genome editing can effectively integrate with this embryo-based breeding platform, strongly decreasing the time to fixation for a desired allele compared to both genomic selection alone[23]
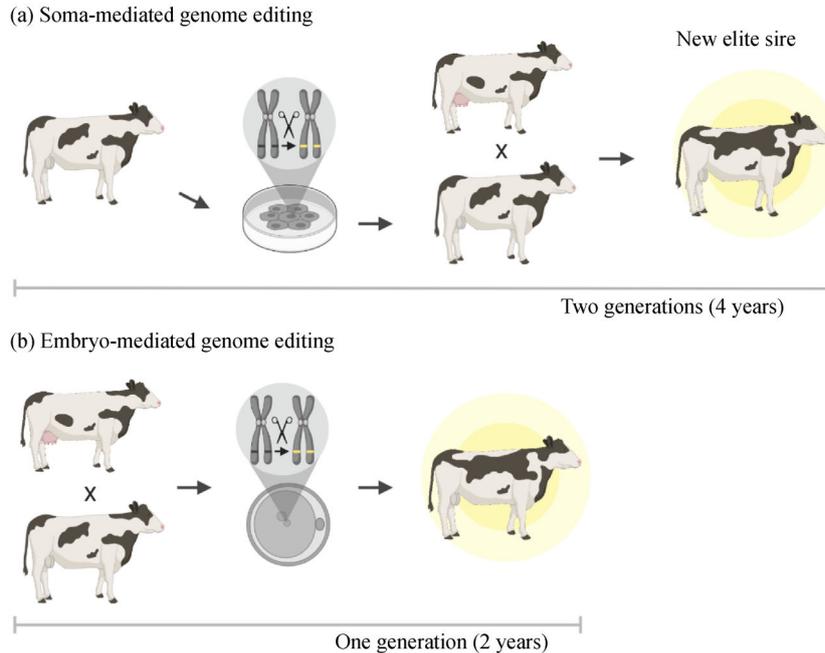
Fig. 1 Soma- and embryo-mediated genome editing approaches in cattle. (a) Somatic cells isolated from an elite sire are genome edited (locus black to gold) and cloned to introduce the modification on the same genetic background. A new elite sire with a higher breeding worth—and carrying the genomic modification—is produced after crossing the cloned sire with an elite dam. (b) For embryo-mediated genome editing, the elite sire and dam are crossed, and the genome editing carried out in the zygote, introducing the genomic modification in the higher breeding worth genetic background in a single generation.

and to somatic clones, which require another generation interval[24]. In contrast to the inefficient SCT route, embryo-mediated genome editing can therefore rapidly produce live genome-edited calves comparable to industry standards for conventional cattle. Thus, it fulfills an important prerequisite for a breeding application.

A major disadvantage of the embryo-mediated approach is the lack of control over the exact time and duration of nuclease and repair activities which can result in animals with variable degrees of mosaicism and one or several different mutated alleles (Table 1). Even with high HDR editing efficiencies, one could not completely rule out a low degree of mosaicism. Considering the large numbers of offspring that are produced from elite sires, any degree of mosaicism would pose a significant issue for breeding applications, as not all offspring would be of the desired genotype. However, this is not an unsurmountable problem and strategies to overcome mosaicism by embryonic cell transfer (ECT) are discussed in detail below.

3 Embryo-mediated editing

Genome edits have been introduced into eutherian zygotes, two-cell embryos or embryonic pluripotent stem cells (ePSCs), derived from high-value blastocysts. These embryo-mediated editing methods can be used indepen-

dently or in combination. Zygotic and two-cell editing are two approaches that were already successfully applied in mouse and cattle, while bovine ePSC-mediated editing is still under development.

3.1 Editing zygotes

Editing at the earliest point in embryogenesis means that, in theory, all cells of the developing embryo would acquire the precise modification. There are few reports of zygote-mediated genome editing in bovine embryos, differing in the type of nuclease used, as well as the timing, reagent and method of delivery (Table 1). A shared outcome from these diverse methods is the frequent production of mosaic embryos arising from several independent editing events from the one-cell stage onwards. These typically produce complex genetic mosaics, each with a unique spectrum of alleles and/or variable contribution of each allele to the whole animal, resulting in unpredictable and difficult-to-define phenotypes that necessitate further breeding of the founders[34].

Earlier publications carrying out zygote-mediated genome editing relied on ZFN and TALENs instead of Cas9, probably a reflection of the long generation time of cattle vs. the rapid development of new editing tools. In fact, the only embryo-mediated HDR-edited cattle offspring so far have come from TALENs producing lactoglobulin-ablated

Table 1 Publications of embryo-mediated genome editing in bovine zygotes

Nuclease[a]	Reagent[b]	Delivery method[c]	Delivery time (post IVF)/h[d]	Target locus	Intended editing pathway[e]	Edited embryos/%	Mosaic embryos/%[f]	Mosaic/total edited offspring	Reference
TALE	mRNA	CI	19	*ACAN* or *GDF8*	NHEJ	NHEJ: 2–50	~20	–	[25]
TALE	mRNA	CI	24	*GDF8*	NHEJ	NHEJ: 31–57	ND	1/3	[26]
ZF or TALE	Plasmid or mRNA	CI	8 or 18	*LGB*	NHEJ & HDR	NHEJ: 29–83 HDR: 11–46	~80	–	[27]
TALE	plasmid	CI	18	*LGB*	HDR	HDR: 21–32	ND	1/3[g]	[20]
Cas9	mRNA	CI	20–22	–	NHEJ	NHEJ: 83	ND	–	[28]
Cas9	Plasmid or mRNA	CI	5	*PRNP*	NHEJ & HDR KI	NHEJ: 25–45 KI: 12.5	~85	–	[29]
Cas9	RNP	E	8	*PMEL*	NHEJ & HDR	NHEJ: 57–100 HDR: 0–6	ND	–	[30]
Cas9	RNP	CI	10 (IVF), 1 (PG)	*POU5F1*	NHEJ	NHEJ: 86	~34	–	[31]
Cas9	RNP	E	18–22	*NANOS2*	NHEJ	NHEJ: ~63	ND	–	[32]
Cas9	RNP	E	10 or 15	*GDF8*	NHEJ	NHEJ: 20–60	~75	–	[33]

Note: [a] Transcription activator-like effector (TALE), zinc finger (ZF). [b] Nuclease delivered as mRNA, plasmid, or preformed ribonucleoprotein (RNP) complex. [c] Cytoplasmic injection (CI) or electroporation (E). [d] *In vitro* fertilized (IVF) or parthenogenetic (PG) embryos. [e] Non-homologous end joining (NHEJ), homology-directed repair (HDR), knockin (KI). [f] Normalized on the total number of edited embryos or not determined (ND). [g] Embryos were biopsied and screened for the presence of the HDR edit before transfer.

cattle[20]. Across all three DSB-based editing platforms, nucleases have been delivered as plasmid DNA, mRNA or as RNA/protein complexes (Table 1). Delivering the editing tools as plasmid DNA is convenient but carries the risk of unwanted insertions of plasmid vectors as has been observed in cattle that were edited for the Celtic polled mutation[35]. Unwanted insertion can be avoided by delivering the editors in a different form and several studies have successfully demonstrated on-target mutagenesis in zygotes with preformed gRNA/Cas9 ribonucleoprotein (RNP) complexes[30–33]. Since designing gRNAs for Cas9 is far simpler than TALENs or ZFNs, delivering preformed gRNA/Cas9 RNP complexes is likely to be the preferred option for future accelerated breeding programs.

The standard method of delivery is via cytoplasmic, rather than pronuclear, microinjection (Fig. 2). This
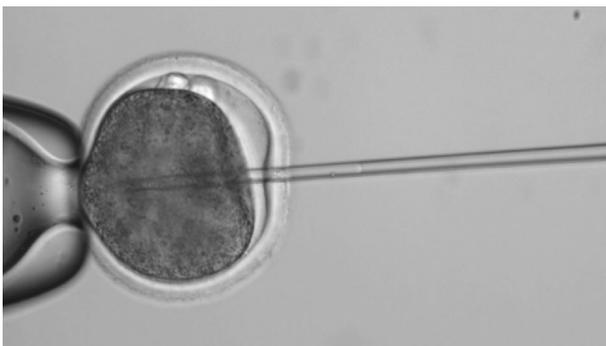
Fig. 2 Cytoplasmic injection in a bovine zygote. Image courtesy of Dr. Jingwei Wei (AgResearch, Ruakura Research Centre, New Zealand).

preference is due to the high lipid content in bovine oocytes and zygotes, which obscures visibility of the pronuclei by standard transmitted-light microscopy. Cytoplasmic injection is near 100% efficient and the associated plasma membrane damage and volume change is transient and generally well-tolerated by the zygote. Provided that the dimensions of the injection capillaries and injection parameters (e.g., injection pressure and duration) are standardized, this technique results in reproducible amounts of material being delivered. However, this method relies on high technical skills, introducing operator-dependent experimental variation as a confounding parameter in editing experiments. It also requires expensive micromanipulation, microscopy and pipette fabrication equipment and is relatively low-throughput.

By contrast, electroporation offers a higher throughput alternative, which is less labor- and cost-intensive. It can be simultaneously applied to many zygotes and only needs a stereomicroscope, electroporator and fusion chamber. This approach has been reported to deliver gRNA/Cas9 RNPs, Cas9 mRNA and gRNAs into rodent embryos, achieving high biallelic editing efficiencies[36,37]. This was followed by the validation of electroporation as a suitable method to edit bovine embryos[32,33]. In addition, the feasibility was demonstrated to HDR-edit bovine zygotes by electroporating gRNA/Cas9 RNPs and an exogenous repair template[30].

Multiple time points during the first bovine cell cycle have been evaluated for NHEJ or HDR editing efficiency. Most reports in cattle have focused on introducing NHEJ mutations for simple loss-of-function experiments, with nuclease delivery time ranging from 5 to 24 h after *in vitro*

fertilization (IVF) (Table 1). At each of these time points, moderate editing efficiencies have been achieved with usually at least half of the embryos carrying NHEJ generated indel mutations. However, fewer publications have assessed HDR-based editing in bovine embryos.

HDR occurs at higher frequency during late S and G_2 phase of the cell cycle[38]. So, timing the delivery of the editing tools may be important for achieving precise HDR editing. In bovine zygotes, G_1 phase lasts from about 6–14 h post-IVF, S-phase starts at 11–15 h and ends at 23–26 h, followed by a short G_2-phase at 26–31 h and the first cleavage divisions after 26 h[39]. For HDR editing in bovine embryos, the nuclease and repair template were either co-delivered before (5–10 h post-IVF) or during mid/late S phase (18–24 h post-IVF, Table 1). When delivered in G_1, cleavage and repair of the target sites will result in edits that are replicated in both DNA strands during subsequent S phase. By contrast, editing during S phase occurs while each chromosomal DNA strand is being replicated into two sister chromatids, doubling the number of target sites, but coinciding with the DSB repair machinery being most active[38]. Previous work by our group showed that there was no significant difference in the proportion of HDR-edited embryos between delivering the nuclease and repair template at G_1 (8 h) vs. S phase (18 h)[27]. These experiments were mostly performed with ZFNs delivered as a plasmid, with the DNA cleavage expected to be faster if the nuclease was delivered as a protein. Further experiments are required to optimize delivery time for HDR when using the more convenient gRNA/Cas9 RNPs. Since mosaic embryos with more than two alleles are commonly observed, the editing could be continuing throughout subsequent cleavage divisions[40].

HDR editing efficiency in zygotes is relatively low and was reported to be in the range of 11%–46%[27] and 21%–32%[20] of cytoplasmically injected zygotes for the introduction of a 9-bp deletion in the bovine *LGB* gene. By electroporation, the efficiencies were even lower with only up to 6% of embryos showing some degree of HDR editing[30]. For large transgene knockin experiments, Bevacqua et al.[29] reported that Cas9 improved targeted insertion with one of eight embryos correctly modified compared to zero of 175 when using just the repair template. This shows that direct HDR editing is possible in bovine zygotes, but the reported efficiencies would need to be greatly increased or correctly edited embryos identified and selectively multiplied in order to be useful for a breeding program.

3.2 Optimizing HDR outcomes

HDR efficiencies still remain low, especially for introducing large DNA sequences. A number of approaches have been devised to shift the balance between competing NHEJ and HDR pathways toward the latter. These include the use of chemical and biochemical compounds to interfere with the DNA repair machinery — either by blocking the NHEJ pathway, with DNA-dependent protein kinase inhibitors like M3814[41], or by stimulating the HDR pathway, with mRNA from HR effectors like RAD51[42] or with stimulating molecules like RS-1[43]. As pharmacological inhibitors can be cytotoxic and yield variable efficiency, HDR can also be enhanced by coupling Cas9 expression to cell cycle effectors at the late S to G2 phase[44,45] or by tethering the DNA template to the gRNA/Cas9 RNP complex, juxtaposing the template DNA to the DSB repair site[46–48]. In addition, HDR repair efficiency depends on how the DNA template is delivered: as a single-stranded DNA molecule[49], as recombinant adeno-associated viral vector[50], or in a cassette designed to trigger alternative DNA repair pathways, like microhomology-mediated end joining[51], homology-mediated end joining[52], or homology-independent targeted integration[53].

Direct delivery into embryos provides an additional opportunity to improve HDR outcomes. In contrast to zygotic injection, much higher HDR-mediated knockin efficiencies were achieved by cytoplasmic microinjection into two-cell mouse embryos, combined with a modified streptavidin-biotin Cas9 system[46]. This strategy resulted in a 10-fold increased knockin efficiency compared to standard methods, higher frequency of homozygous insertions, and increased germline contribution. Benefits were attributed to two factors. First, the two-cell stage marks the onset of major embryonic genome activation in the mouse, which is associated with increased open chromatin that may improve the accessibility for Cas9 to the target site. Second, mouse embryos display an exceptionally long G_2 cell cycle phase during the two-cell stage, extending the time for HDR-mediated repair to occur. The same approach is less likely to be successful in cultured bovine embryos, where embryonic genome activation occurs at the eight-cell stage[54], concomitant with the longest cell cycle duration at this stage[55]. Compared to zygote delivery, introducing editors into all blastomeres at the two- or eight-cell stage likely increases mosaicism, as there are now multiple independent genome editing events that need to resolve in the developing embryo. Also, transfecting multiple blastomeres, either by injection or electroporation, is technically more difficult, which makes its implementation less straightforward.

All the above optimization strategies have so far been unable to sufficiently lift HDR efficiencies to make precision genome editing in embryos for breeding a realistic proposition. Without future improvements, multiplication of HDR-edited but rare embryos might offer another solution.

4 Multiplying edited embryonic genotypes

The probability of obtaining a viable calf from a single edited IVF embryo is about 45%[56]. To address this

biological limit, different strategies for multiplying edited genomic selection embryos are being developed. These also address low editing efficiency by identifying and selectively multiplying correctly targeted embryonic cells. For zygote-mediated editing, serial cloning from early cleavage-stage or morula blastomeres can multiply the desired genotype, resolve potential mosaicism and ensure the generation of live animals. For ePSC editing, robust *in vitro* proliferation enables genotype amplification and characterization before potentially generating a large number of animals.

4.1   Blastomeres

The zygote cleaves into progressively smaller nucleated cells, termed blastomeres, without increasing total embryo volume[57]. This process ends at the blastocyst stage when cells initiate growth and replicative proliferation for the first time during embryo development[57]. Until the eight-cell stage, bovine blastomeres are still totipotent, i.e., able to give rise to all cell types on their own, and genotype multiplication can be achieved by embryo splitting[58]. Following zygote-mediated editing, dissociating totipotent blastomeres at the two- to eight-cell stage could potentially resolve early embryonic mosaicism by segregating the different genotypes generated at the one-cell stage[59]. Multiplication of the edited genotype is maximally two to 8-fold with this approach, not accounting for some blastomeres that must be sacrificed for genomic selection and validation of edits. This limits the applicability of blastomere splitting for resolving mosaicism and genotype amplification.

The pre-compacting morula stage (32–64 cells) offers an intermediate starting point for embryo multiplication between early cleavage-stage stages, which are limited in numbers, and blastocyst-derived ePSCs, which can produce large numbers but have not been routinely established in cattle. Biopsy, genotyping and vitrification procedures, originally developed for genomic selection of blastocysts[22], can be applied to identify the best morula genotypes[60]. Modest genotype multiplication by ECT is

feasible with morula blastomeres and editing is already fixed because introduced editors are unlikely to still be active at this stage[40]. As each embryo derives from a single donor cell, ECT segregates edited from unedited donors and only generates non-mosaic embryos (Fig. 3). Hence, genomically selected morulae can be dissociated into single donor cells for ECT, resulting in genotype amplification and segregation. In principle, several rounds of iterative ECT can achieve further multiplication by serial embryo recycling and intermittent cryopreservation. Importantly, ECT multiplies both edited and unedited elite embryos.

4.2   Blastocysts and ePSCs

Blastocysts can be microsurgically bisected, but the biological multiplication factor to term is low (~1.2 times) due to the compromised *in vivo* survival of demi-embryos[61]. Instead, blastocyst-derived ePSCs offer a promising alternative for both multi-editing (see below) and multiplication. Under the right culture conditions, these cells remain capable, at the single cell level, to produce all adult cell types, including functional gametes[62,63]. This ability is retained even after extensive genetic modification by homologous recombination[64] or genome editing[65]. Only cells capable of germline transmission or tetraploid complementation, wherein ePSCs are introduced into a tetraploid host embryo and forced to form the entire animal[66], are considered fully pluripotent. Such cells have been referred to as naïve PSCs[67]. Naïve PSCs are capable of extended self-renewal and biological amplification of a selected genotype without genetic transformation. Their high clonogenicity from single cells allows maintaining stable diploid cultures over hundreds of population doublings[68]. This would be highly desirable for *in vitro* multiplication of rare high-value embryonic genotypes.

Ever since ePSCs were first described in mice, there has been an interest to derive analogous cells from farm animals, however, all attempts to isolate naïve bovine ePSCs have so far been unsuccessful[69]. Progress in
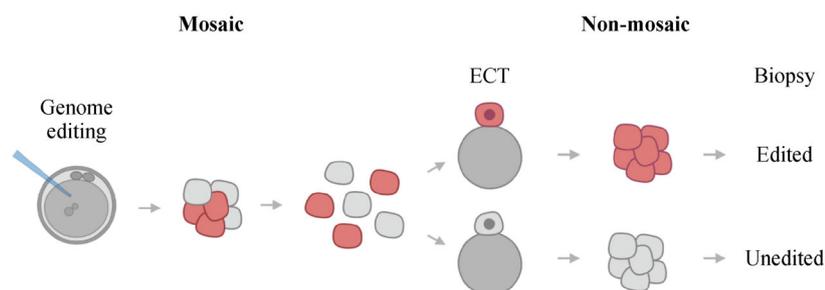
Fig. 3   Resolving mosaicism in edited embryos by embryonic cell transfer (ECT) blastomere cloning. After genome editing in the zygote, a mosaic morula can be dissociated and the blastomeres used for ECT. The resulting embryos derived from a single blastomere would then be non-mosaic, which can be confirmed with an embryo biopsy before transfer or cryopreservation.

understanding of pluripotency regulation in mice and humans, combined with extensive chemical screening, has led to the development of chemically defined culture conditions that yielded expanded potential stem cells in pigs[70]. These cells show molecular and functional features of naïve pluripotency, including expression of key pluripotency genes and permissiveness to genome editing. Initial plating and cell line derivation efficiency from individual blastocysts was only averaging ~30% but, once established, porcine ePSCs reached >40 passages without overt differentiation, while remaining karyotypically normal[70]. Similar results were also reported for bovine ePSC-like cells, achieving >50 passages with stable population-doubling times and normal karyotypes[71]. Importantly, porcine ePSCs also engendered all embryonic and extra-embryonic somatic cell lineages, but so far not the germline, in chimaeras. These advances suggest that the naïve pluripotent state may be a conserved feature of mammalian embryogenesis that could be effectively captured, without genetic intervention, in other livestock species.

4.3   Multiplex editing of ePSCs

The majority of important livestock traits are complex, quantitative and controlled by multiple genes. Their manipulation will require multiplex genomic modifications, which are likely to be challenging to achieve by zygotic injection alone. Long-term proliferating livestock ePSCs, once available, would present a practical alternative to zygote-mediated editing (Fig. 3).

In mouse and human ePSCs, the pluripotent cell state facilitates transgenesis, homologous recombination and genome editing with ZFNs and TALENs[72–74]. Likewise, Cas9 allows for fast and efficient multiplex mutagenesis at endogenous genomic loci in human[75] and mouse PSCs[65]. Even though biallelic HDR frequencies in human PSCs are generally 5–20-fold lower than in tumor cell lines, this can be overcome by positive selection strategies, efficient subcloning of cells with rare editing events or enhanced delivery methods for nuclease and template[75]. Similarly, transfected mouse PSCs can be enriched by either using drug selection or sorting for the expression of fluorescent reporters. Since ePSCs are highly

clonogenic under defined long-term culture conditions, single cells can be expanded into sufficient numbers of primary colonies to allow for thorough characterization of the introduced edits (Fig. 4). At the same time, ePSC clonal strains would resolve any potential mosaicism present in the founder population. Clonal strains derived from unedited cells that have undergone the same targeting process provide isogenic parental control cells.

While simultaneous editing is possible, its scope will be limited by the number of simultaneous DSBs in the genome that a cell can tolerate for survival. By contrast, the indefinite growth potential of ePSCs allows for essentially unlimited sequential editing and engineering of compound genotypes optimally suited to study complex biological processes. In livestock, this would facilitate stacking of beneficial causative variants into an elite genetic background in a single generation, short-cutting decades of traditional crossbreeding and backcrossing to generate enhanced phenotypes.

5   Generating genome-edited animals from embryonic genotypes

5.1   Clones

The two main reproductive strategies for producing animals from either edited embryos or naïve ePSCs are (1) ECT cloning, and (2) germline chimaera formation (Fig. 5). In ECT cloning, the incidence and severity of abnormal phenotypes is greatly reduced with blastomere compared to somatic clones[76,77]. It remains to be shown that this also holds true for edited blastomeres, multiplied by serially cloning, but potentially this approach can substantially lessen animal welfare burdens. The situation is less clear with cloning from ePSCs. In mouse cloning experiments, ePSCs did not perform significantly better than genetically matched somatic cells[78]. However, mouse ePSC often contain non-reprogrammable karyotypic abnormalities[79] and are epigenetically unstable in long-term culture during targeting experiments[80], complicating assessment of their true reprogramming potential. This has prompted the notion that short-term cultures of primary ePSCs, which are more likely to be devoid of
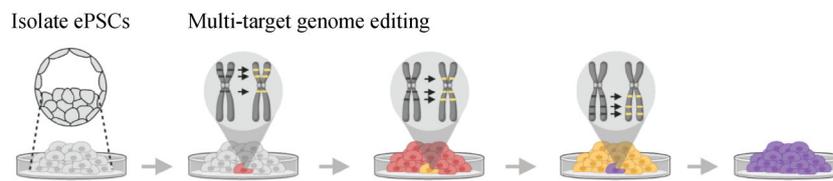
Fig. 4 Multiplex genome editing in embryonic pluripotent stem cells (ePSCs) isolated from a genomically selected elite blastocyst. With high clonogenicity and stable pluripotency, ePSCs would allow sequential multiplex genome editing of multiple loci (loci black to gold), followed by clonal expansion of edited strains.
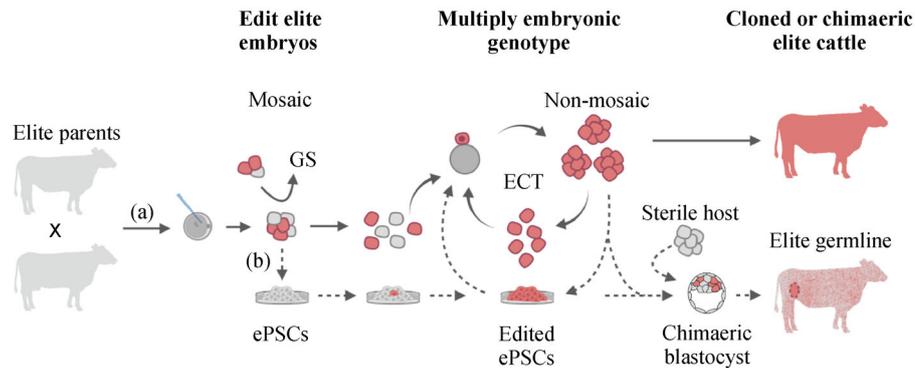
Fig. 5 Embryo-mediated accelerated breeding in cattle. (a) Using proven technologies (solid arrows), precise genomic edits can be introduced in zygotes from elite sires and the best embryos identified by genomic selection (GS). The desired embryonic genotype can then be multiplied by embryonic cell transfer (ECT) cloning and elite offspring generated. (b) A complementary pathway (dashed arrows) is centered around embryonic pluripotent stem cells (ePSCs). ePSCs would be isolated from an elite embryo and numerous genomic modifications introduced. Alternatively, ePSCs would be isolated from embryos multiplied by ECT for subsequent genome editing. Offspring could then be produced by cloning or as a chimaera with absolute transmission of the elite germline.

genetic and epigenetic aberrations, may result in higher cloning efficiency, similar to the results with embryonic blastomeres[78].

5.2   Germline chimaeras

Chimaera formation, on the other hand, involves introducing donor blastomeres or naïve ePSCs into a normal diploid host embryo of a different zygotic origin, resulting in widespread somatic and germline contribution of the donor cells. Since the donor germline contribution is seldom 100%, only some of the offspring originate from the donor genotype, necessitating time-consuming cross-breeding of the founder generation, which renders this approach impractical for livestock breeding. To overcome this problem, it is possible to genetically ablate the host germline[81–85], providing a suitable vehicle for exclusive transmission of the donor haplotype, either from transplanted spermatogonial stem cells[86] or embryonic cells developing into gametes. The latter approach relies on embryo complementation of germline-deficient hosts, a mechanism that has, in principle, been demonstrated as feasible in sheep[87], cattle[88,89] and pigs[90,91]. The generation of such absolute transmitters with exclusively embryo-derived elite donor germlines provides an exciting opportunity for accelerating genetic gain[22]. Absolute transmitters could functionally replace somatic clones, potentially reducing cloning-associated animal welfare problems.

5.3   *In vitro* gametogenesis

It may also be possible to differentiate edited diploid ePSCs into haploid male and female gametes, similar to what has been achieved in mice[92]. Depending on the time lines for cell differentiation, meiotic recombination and

proliferation, this approach, originally termed *whizzogenetics*, could theoretically shorten the generation interval, provided that the same selection accuracy can be maintained[93]. Even though the biological and practical hurdles for reconstituting gametogenesis in the dish are enormous, this new technology may provide an alternative source of gametes for *in vitro* breeding in the future.

6   Regulatory aspects

Genome editing is a relatively new technology that is associated with regulatory uncertainty. Regulations established for the control of transgenic technology appear no longer appropriate for the level of precision and control that is possible with the new editing technology and is a topic of intense international discussion[94]. While many countries are still developing their final position, economic heavyweights like the EU and the USA have opted to regulate genome-edited animals just like transgenic animals even though genome-edited animals can be indistinguishable from naturally-occurring animals[95,96]. However, there are also a few countries that have taken a different path. The regulations in Argentina, Australia and Brazil reflect the reduced risk profiles of genome-edited animals and regulation is not required if genome-edited animals do not contain any foreign DNA[97,98]. The simplest regulatory approach for new technologies has probably been implemented by Canada[99]. The trigger for regulation is not a new technology but rather a new animal trait, irrespective of what technology was used to create that trait. Realizing the great potential of genome-edited animals toward sustainable solutions for pressing environmental and food security issues will only be possible if more countries adopt risk-adjusted regulations that might allow the widespread use of such animals.

7   Conclusions

Almost two decades ago it was predicted that embryo-based genome selection, modification and multiplication would replace somatic cell-based approaches to accelerate livestock breeding[100]. With the advent of accurate marker- and sequence-based embryo selection, precise multi-editing and more sophisticated serial embryo multiplication, this prediction is now getting closer to reality (Fig. 5). Conceptually, some key advantages of embryo-mediated approaches for genetic improvement have not changed since then: (1) genomically-selected embryos capture the most recent genetic gains, compared to the genetic lag associated with adults; (2) embryonic cells are amenable to complex genomic modifications owing to their extensive proliferation potential; (3) embryonic donors are inherently easier to reprogram, resulting in greater cloning efficiency, reduced animal production costs and animal welfare issues; and (4) animal clones may even be replaced with chimaeras if complementation with embryonic donors can efficiently establish absolute germ-line transmitters. In addition, genome editing can selectively introduce naturally-occurring beneficial variants into various elite genetic backgrounds in a single generation without leaving foreign DNA in the targeted genome.

To use genome editing productively for livestock improvement, putative causative variants for desirable traits must first be identified, emphasizing the ongoing need for large-scale gene discovery programs. Genome editing can then determine causality of the most promising selection candidates before their embryo-meditated introgression for animal production. Stacking multiple edited variants in the most recent elite genetic background would rapidly generate fitter, healthier and more sustainable animals, required to reduce our vulnerabilities to diminishing natural resources and secure sustainable livestock production.

Compliance with ethics guidelines Zachariah McLean, Björn Oback, and Götz Laible declare that they have no conflicts of interest or financial conflicts to disclose.

This article is a review and does not contain any studies with human or animal subjects performed by any of the authors.

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