Inducing pluripotency in livestock somatic cells to enhance genome-editing opportunities

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Abstract

Although the manipulation of animal genomes has a history of more than three decades, there are only a few of the reported genetically modified (GM) animals that have passed safety regulations and found their way through to market. The production and safety regulations of GM livestock are hindered by a variety of issues, most importantly, lack of embryonic stem cells (ESC) in livestock species and concerns around the nature and biosafety of the genome manipulations, respectively.

As an alternative for ESC, induced pluripotent stem cells (iPSC) have reportedly been generated in livestock species. The initial attempts at generating iPSC were based on viral integration of specific factors into the genome of differentiated cells. However, attempts have been made in developing safer methods for generating virus-free iPSC. Although the possibility of inducing a pluripotency state in somatic cells raises hopes to overcome the lack of ESC in livestock, as yet there is no report on successful iPSC cell mediated transgenesis in large domestic animals.

Recent advances in genome editing exploiting site-specific endonucleases provide unprecedented potential for modifying the genome of livestock with reduction in off-target events, high efficiency and user-friendly approaches which are widely applicable across species. The high efficiencies achieved support the tantalizing prospect of achieving sophisticated genome-editing at the zygote stage even in livestock species.

Introduction

The term "*transgenesis*" coined by Gordon and Ruddle described a technical process enabling transfer of inheritable functional genes between organisms irrespective of species barriers (Gordon & Ruddle 1981). The introduction, expression and transmission of novel genes has been successfully demonstrated in a number of invertebrates, including *C. elegans* (Stinchcomb *et*

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al. 1985), *Drosophila* (Spradling & Rubin 1982), sea urchins (Mcmahon *et al*. 1984, Mcmahon *et al*. 1985) and lower vertebrates, frogs (Bendig 1981). Gene transfer was first reported in mammals by Jaenisch (1976), with a major advance occurring 5 years later when Palmiter *et al*. (1982) microinjected multiple copies of a gene construct, containing promoter elements to regulate the functioning of the introduced gene, into the pronucleus of fertilized one-cell mouse embryos, resulting in random integration into the host genome and subsequent passage *via* their germline to their offspring.

Following the success of transgenesis in mice the technology was transferred to a number of mammalian species (Hammer *et al*. 1985, Brem *et al*. 1986, Pursel *et al*. 1987, Vize *et al*. 1988, Murray *et al*. 1989, Rexroad *et al*. 1989). The potential for the application of this technology to livestock was huge, both from the scientific and commercial viewpoints. However technical limitations were identified with DNA injection in farm animals, including difficulty with visualizing the pronucleus, limited control over inserted copy number and random and low efficiency of integration resulting in mosiacism of the transgene presence in resulting animals (Nottle *et al*. 1997).

The cultivation of mouse ESC by Sir Martin Evans (1981), followed by the application of conventional HR techniques for targeting genes in mESC, resulted in the generation of the first knockout mouse in 1989 (Evans & Kaufman 1981, Capecchi 1989). However, this was not easily translated in livestock due the lack of true germ-line competent ESC in these species.

Induction of pluripotency in livestock

True germ-line embryonic stem cells have not yet been isolated from non-rodents, including livestock species, despite significant efforts. The unique characteristics of the ESC, if they could be generated from livestock, would make them an invaluable resource for both agricultural and biomedical applications. However, the goal of derivation of naïve (or ground state) ESC from livestock has remained elusive over the last two decades (Blomberg & Telugu 2012). For a comprehensive review on pluripotency in livestock species see review by Malaver-Ortega (Malaver-Ortega *et al*. 2012).

Direct reprogramming of somatic cells

An amazing discovery in stem cell research was the generation of iPSC. The significance of the work was recognized by the joint-award of the Nobel Prize for Physiology and Medicine in 2012 to Shinya Yamanaka for its discovery. Ectopic expression of just four transcription factors, namely *Oct4, Sox2, Klf4* and *c-Myc* in a variety of body cells resets the transcription profile and epigenetic state of the host cells to iPSC. These cell types are very much like ESC (Takahashi & Yamanaka 2006), can be germ line competent (Okita *et al*. 2007) and contribute to iPSC-mice by tetraploid complementation assay (Boland *et al*. 2009, Zhao *et al*. 2009, Kang *et al*. 2011). In this regard, establishment of iPSC from various farm animals holds considerable promise. The technology has since been applied to successfully generate iPSC from agriculturally important species, including cattle, sheep, and pig.

Cattle

Our laboratory published the first report on the establishment of iPSC from bovine fibroblasts (Han *et al*. 2011, Huang *et al*. 2011, Sumer *et al*. 2011). We delivered a combination of reprogramming genes identified by Yamanaka (*Oct4, Sox2, Klf4 and cMyc; designated as*

OSKM) plus *Nanog,* with human gene sequences, to bovine adult fibroblasts with a VSVG pantropic retroviral delivery system. The bovine iPSC generated expressed pluripotent markers, including AP, SSEA-1, REX1, Oct4 and Sox2, and stably transfected with CAG-GFP constructs as a reporter gene (Sumer *et al.* 2011). The bovine iPSC were also capable of forming teratomas in the immunodeficient mice, and also differentiated *in vitro* to the three germ layers (Sumer *et al.* 2011). Bovine iPSC were also generated using either a combination of *OSKM,* with *Lin28* and *Nanog* (Han *et al.* 2011), or using a combination of *OSKM* with dual kinase inhibitors (PD0325901 and ChIR9902) (Huang *et al.* 2011).

In our hands, it has proved difficult to reprogram bovine fibroblasts using only the Yamanaka reprogramming factors OSKM, with success achieved by including additional *Nanog* into the reprogramming cocktail (Sumer *et al.* 2011). It is suggested that species-specific requirements of reprogramming factors might exist regarding the transcription factors necessary for complete reprogramming in cattle (Malaver-Ortega *et al.* 2012).

Sheep

Induced pluripotent stem cells have been successfully generated from sheep fibroblasts (Bao *et al.* 2011, Li *et al.* 2011, Liu *et al.* 2012, Sartori *et al.* 2012). Interestingly, Nanog, which is a critical factor for reprogramming pluripotency in bovine fibroblasts, is not a required factor to establish completely reprogrammed sheep iPSC in our studies (Liu *et al.* 2012). The four Yamanaka factors (OSKM) were sufficient to reprogram sheep embryonic fibroblasts (SEF) to pluripotency. We routinely use amphotropic retrovirus to transduce human fibroblasts with OSKM to generate human iPSC (Liu *et al.* 2010, Liu *et al.* 2011). However, transduction of SEF with amphotropic retrovirus was inefficient; therefore, we examined the use of pantropic retrovirus (VSV-G) to deliver the factors, which significantly increased transduction rates from 19.5 to 83.5%, respectively. The sheep iPSC at passage 5 continued expression of all four transgenes. However, when the sheep iPSC were propagated to passage 12, significant down-regulation of the transgene Oct4 occurred. Further, the sheep iPSC maintained pluripotency with transcriptional silencing of the four transgenes even by passage 17. The sheep iPSC expressed endogenous Oct4, Nanog and Sox2, but did not express SSEA-1, SSEA3, SSEA4, Tra-1-60, or Tra-1-81 cell surface markers. The sheep iPSC typically grew in compact colonies, with a three-dimensional shape and distinct, glistening edge; morphology was similar to that of mouse ESC. The sheep iPSC were readily dissociated by trypsin-EDTA, which makes the cells more amenable to basic techniques desirable for creating GM sheep, such as FACS, efficient colony-cloning and blastocyst injection. Using these attributes, we successfully transfected the sheep iPSC with a CAG-GFP vector and enriched GFP expressing cells by FACS. Microinjection of GFP^{+}-iPSC into ovine 8- to 16-cell stage embryos demonstrated their integration in the ICM of the resulting sheep blastocysts. Two reports have used Dox-inducible lentivirus containing either using the four OSKM factors alone (Li *et al.* 2011) or OSKM combined with an additional four factors (NANOG, Lin28, SV40 and hTERT) (Bao *et al.* 2011) to transduce sheep fibroblasts. Maintenance of the putative sheep iPSC generated in both studies was dependent on addition of Dox to the culture medium. Withdrawal of Dox resulted in transgene silencing and an associated loss of pluripotency, thus confirming that the endogenous pluripotent genes were not fully reprogrammed in the putative sheep iPSC.

Pig

The first iPSC generated from a livestock species were from the pig in 2009 (Esteban *et al.* 2009, Ezashi *et al.* 2009, Wu *et al.* 2009), followed by several additional porcine iPSC lines generated in 2010 and 2011 (Telugu *et al.* 2010, West *et al.* 2010, Montserrat *et al.* 2011).

The porcine iPSC generated by all groups were pluripotent, as demonstrated by the potential to differentiate into tissues of the three germ layers in both *in vivo* and *in vitro* assays. Like the human ESC, the porcine iPSC were dependent upon the bFGF and activin/nodal signalling pathways, rather than LIF to maintain their pluripotency. The continued expression of the re-programming transgene is a concern for their potential applications.

Evolution of genome editing of mammals

Retroviruses and DNA microinjection

The very first attempts at introducing a gene of interest (GOI) into the genome were based on either a retroviral mediated gene delivery system (Jaenisch 1976) or direct microinjection of transgene cassette into the zygotes (Gordon & Ruddle 1981), with the latter approach mainly utilized for production of transgenic livestock (Hammer *et al*. 1985). Both methods have applicability to a wide variety of species; however, the main disadvantage with them is the random integration of the transgene into the genome and the limited ability to control the number of copies introduced into the genome. Both methods have limitations with the size of transgene that can be delivered and are unable to modify genes in their chromosomal contexts, in other words target the genome effectively. Therefore these methods are used only for inserting a gene randomly into the genome – commonly, referred to as 'gene addition', and not site-specific 'gene knock-in' for replacement of genes or disrupting specific targeted genes – commonly referred to as 'gene knock-out'. The fundamental limitation with these methods, which is the lack of the control of targeting the genomic site, was overcome by the discovery of HR techniques.

Conventional homologous recombination

In the 1980s, Mario Capecchi and Oliver Smithies showed HR could be used to specifically modify genes in mammalian cell types. Conventional HR techniques have numerous advantages compared with random integration-based methods including; (i) deletion of researcher-defined genomic sequences with concurrent insertion of an exogenous gene (knock-out and knock-in) in a site targeted manner, (ii) taking full advantage of molecular biology knowledge and publically available genome sequences for adding adequate gene regulation elements onto the targeting cassette to ensure the desirable expression of GOI in live born animals and (iii) precise genome editing by introducing single nucleotide polymorphisms.

Despite these limitations, the International Knock-Out Mouse Consortium (IKOM) have recently reported that more than 15,000 out of 20,000 protein coding genes in mouse have been targeted (Brown & Moore 2012a, Brown & Moore 2012b). Unfortunately in livestock, however, targeting genes using conventional HR is not as easy. Due to the lack of germ-line transmission capable pluripotent cells, the well-known and established procedure of injecting targeted mouse ESC into the blastocyst to generate a targeted offspring via the chimera approach is not applicable to the livestock species. Therefore gene-targeting needed to be carried out on somatic cells and an additional cloning step was required to produce a live sheep (McCreath *et al*. (2000). The long generation interval in livestock, compared with rodents, is an obstacle for generating either bi-allelic mutants or modifying multiple loci. The major disadvantage of conventional HR, low efficiency, has been overcome in recent times with the development of genome engineering nucleases, which exponentially enhanced HR success rate.

Genome Engineering Nucleases

The efficiency of gene targeting techniques greatly improved with the development of genome engineering nucleases. In chronological order of development, Zinc Finger Nucleases (ZFNs), Transcription Activator Like Effector Nucleases (TALENs) and Clustered Regulatory Inter-Spaced Palindromic Repeats (CRISPRs) are three major types of engineering nucleases that have been proven to efficiently target genes in their chromosomal contexts (Fig. 1).

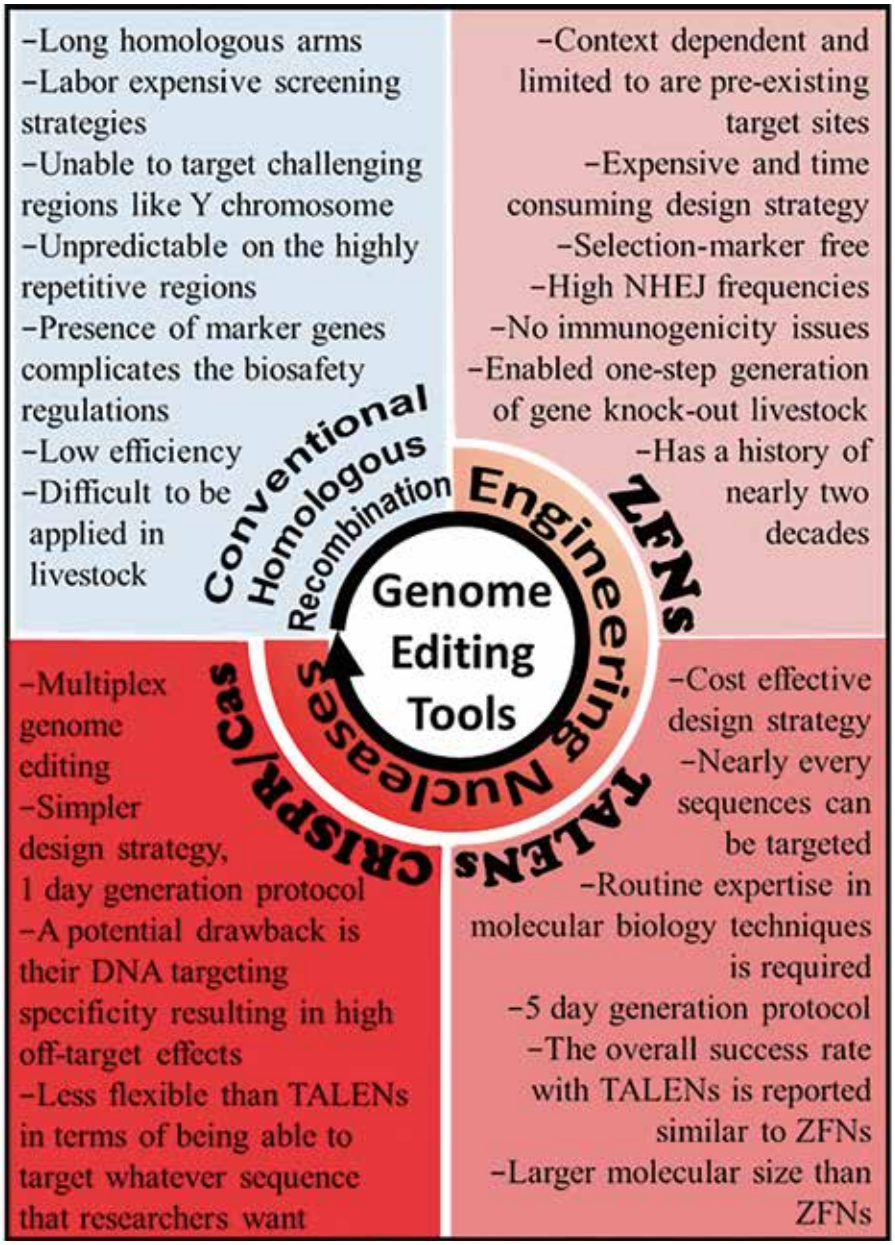

Fig. 1. Genome editing techniques demonstrating the pros and cons.

ZFNs

Zinc finger nucleases (ZFNs) consist of a fusion of a non-specific nuclease domain of *Fok*I restriction endonuclease with user-defined arrays of zinc finger modules. Targeted double strand breaks (DSBs) introduced onto the DNA by ZFN dimers stimulate the DNA repair pathways. There are two main routes of DNA damage response in cells, the dominant but error prone Non-Homologous End Joining (NHEJ) and the less frequent but more precise Homology Directed Repair (HDR) pathway (Helleday *et al.* 2007) (Fig. 2).

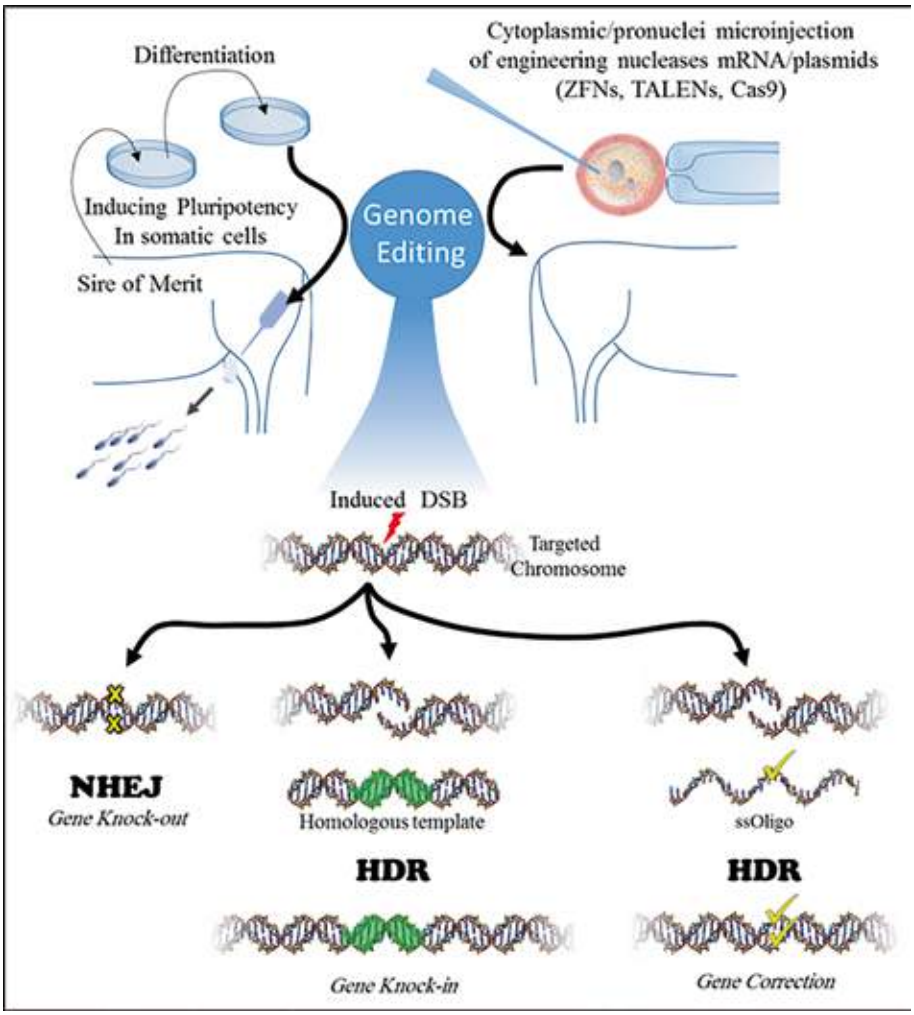

Fig. 2. Application of biotechnological state-of-the-art techniques in livestock. Genetic alteration has been facilitated by the evolution of genome editing tools.

TALENs

Despite having a short history, TALENs technology has been successfully used for targeting genes in rodents (Tong *et al.* 2012) and livestock (Carlson *et al.* 2012, Tan *et al.* 2013). Indeed, rapid achievements from TALENs, at least in part, are indebted to several years of pioneering research on ZFNs.

The off-target rate of TALENs, compared with ZFNs, is less explored, however, TALENs appear to be 10-fold more mutagenic than context-dependent assembled ZFNs in zebrafish (Chen *et al.* 2013). More than 80% of over one hundred generated TALENs in two large-scale studies, have shown an average mutagenicity rate of 20% (Reyon *et al.* 2012, Chen *et al.* 2013). Although TALENs and ZFNs contain the same *Fok*I nuclease domain, however they are associated with different mutation patterns at targeted loci. While ZFNs intend to induce both small insertions and deletions in nearly equal proportions, TALENs often induce deletions (Kim *et al.* 2013).

CRISPR/Cas system

The acquired immune system of *Streptococcus pyogenes* known as CRISPR/Cas system has been adopted to develop a powerful genome-editing tool, which makes use of guide RNA (gRNA) to direct Cas9 endonuclease to target sites for introducing sequence-specific DSBs (Cong *et al.* 2013, Mali *et al.* 2013b).

A potential drawback of the CRISPR/Cas system is their DNA targeting specificity (Fu *et al.* 2013, Hsu *et al.* 2013, Pattanayak *et al.* 2013). To reduce the off-target effects of CRISPR/Cas system different approaches, including targeting unique sequences in the genome, using modified gRNA and Cas9 have been suggested (Cho *et al.* 2013, Mali *et al.* 2013a, Ran *et al.* 2013). However, it is too early to predict the ultimate impact of CRISPR/Cas technology on different aspects of life science, although, results thus far are promising and present genome engineers with a new powerful tool that has huge potential.

Recent advances for enhancing gene addition experiments

Recent advances in the genome-editing area, in addition to enhancing HR, present additional approaches for increasing the efficiency of gene knock-in experiments.

Nickases

Engineering nucleases are highly efficient at producing knockouts by utilizing the NHEJ pathway. However, if the goal of genome editing is either gene addition or gene correction, NHEJ is not desirable because it competes with HDR and results in reduction of corrected clones. Since single-strand breaks (SSBs) or nicks cannot be repaired by the NHEJ pathway as stimulated DNA damage response by SSBs strongly bias repair toward HDR. Thus far all three types of engineering nucleases (ZFNs, TALENs and CRISPRs) that are reported to be functionally modified to introduce targeted SSBs. It has been demonstrated that ZFNickases show sufficient gene editing activity for the isolation of correct modified cells, although their gene editing activity remained lower than ZFNs (Kim *et al.* 2012, Ramirez *et al.* 2012, Wang *et al.* 2012). Cas9 nickase (Cas9n) also appear to efficiently target genes while having less off-target effects (Mali *et al.* 2013a, Ran *et al.* 2013). Thus, nickases-mediated gene addition approaches are much safer than use of engineering nucleases, which make targeted DSBs, due to the absence of adverse mutagenic effects.

Homology-independent knock-in

Obligation Ligation-Gated Recombination (ObLiGaRe) is described as an efficient approach for site specific gene insertion, which relies on NHEJ pathway (Maresca *et al.* 2013). A potential

drawback of the ObLiGaRe approach is the integration of vector backbone, though it can be removed either with Cre or Flp recombinases or be avoided by using minicircles. Most recently, a similar homology-independent approach has been implemented for gene tagging which relies on Cas9n-mediated double nicking of the target site to create defined overhangs (Ran *et al.* 2013). Thus double-stranded oligonucleotides, with complementary overhangs, can effectively integrate into the target site to encode small modifications. CRISPR/Cas9-mediated DSBs also can facilitate very efficient knock-in of transgenes in a homology-independent manner (Auer *et al.* 2014).

Foot-print free gene correction

Transposons are jumping genes that can change their positions within the genome. The mobilization ability of transposon systems, *Sleeping Beauty* and *piggyBac*, have been used for efficient transposition of genes between vectors and chromosomes for transgenesis (Carlson *et al.* 2011, Macdonald *et al.* 2012), generating transgene-free iPSC (Yusa *et al.* 2009). Most interestingly, the *piggyBac* transposon system can be used as a foot-print free tools specially applicable for gene correction (Yusa *et al.* 2011, Yusa 2013).

Genome editing methods

Unlike mouse, genome modified livestock are not only useful in biomedical research, but also they can be utilized in agriculture with potential commercial impact. Although livestock such as cattle, sheep, goat and pig are much closer genetically to people than rodents for mimicking human diseases, due to the numerous limitations with manipulating their genomes these species have been employed less than rodents for generating human disease models. Still GM livestock hold promise for providing tissues for xenotransplantation and producing therapeutics. So far, several GM livestock have been created that have potential impact on agriculture, the environment and animal welfare including: mastitis- and 'mad-cow- resistant' cattle (Wall *et al.* 2005, Richt *et al.* 2007), goats and cows that produce milk with altered composition (Maga *et al.* 2006, Kang *et al.* 2011, Jabed *et al.* 2012), fast growing salmon (Du *et al.* 1992), influenza resistant chickens (Lyall *et al.* 2011) and maybe in the near future double-muscled and Polled dairy cattle (Tan *et al.* 2013). Currently however, only transgenic salmon is close to passing the FDA approval process (Ledford 2013).

Genome editing zygotes (for random integration)

A number of approaches have been investigated to transfer transgenes into the genome of animals at the zygote stage, with varying degrees of success.

Pro-nuclear Microinjection

Microinjection of transgene constructs into the male pronucleus of a zygote (Palmiter *et al.* 1982) is still one of the most widely used techniques for delivering transgene constructs to produce transgenic animals. It has proven useful for both commercial and research purposes.

Sperm Mediate Gene Transfer (SMGT)

Another method that has promised much,but has yet to deliver was reported by Lavitrano *et al*. (Lavitrano *et al*. 1989). The use of sperm cells to transfer viral DNA into eggs was first described by Brackett *et al*. (Brackett *et al*. 1971). Despite the phenomenon being verified for a number of species including sea urchin (Arezzo 1989), pigs (Lavitrano *et al*. 1997), bulls, ram, goat, rooster, mouse and carp (Castro *et al*. 1990), blowfly and honey bee (Atkinson *et al*. 1991), no germline transgenic animals have resulted. Other variations of sperm mediated transfer that have been attempted is either the treatment of the sperm with liposomes (Bachiller *et al*. 1991) or electroporation of the sperm (Gagne *et al*. 1991, Muller *et al*. 1992) to facilitate uptake of DNA.

Targeted genome editing using pluripotent cells

Procedures of choice are based on ESC which allow manipulation (mutation) of resident genes *in situ* (Robertson 1986) and the insertion of transgenes into specific sites through homologous recombination (Capecchi 1989). Mouse iPSC resemble mouse ESC in morphology, gene expression profiles and epigenetic status (Stadtfeld *et al*. 2008, Wernig *et al*. 2008).

In the absence of robust ESC, the generation of iPSC might offer an alternative for livestock. The obvious motivating aims for the generation of iPSC, in lieu of ESC, are 1) gene targeting to perform locus-specific genetic modifications and 2) passing on the genetic alternations to the next generations through germ line transmission. However, with the advent of the TALENs and CRISPR/Cas9 system genome editing can be achieved biallelically in one round of gene targeting in zygotes of livestock, making the requirement of iPSC less critical. In addition to zygotes, somatic cells can be genetically altered similarly with site-specific nucleases and can be used as donor cells for SCNT (Carlson *et al*. 2012, Tan *et al*. 2013). Nevertheless, it remains to be determined whether using iPSC will make the same targeting events using TALEN and CRISPR/Cas9 more efficient in producing viable offspring by nuclear transfer. It has been reported that cloned piglets have been born using iPSC as donor cells, although this may not be critical because of the persistent expression of reprogramming transgenes (Fan *et al*. 2013).

Direct targeted genome editing in embryos by DNA/RNA injection

Simultaneous generation of GM model organisms, such as mice (Wang *et al*. 2013, Wefers *et al*. 2013), rats (Geurts *et al*. 2009, Tesson *et al*. 2011, Qiu *et al*. 2013) and rabbits (Yang *et al*. 2012) as well as livestock (Hauschild *et al*. 2011, Carlson *et al*. 2012) carrying knock-out genes, have been achieved by direct microinjection of either DNA or mRNA of engineered nucleases (ZFNs, TALENs and CRISPR/Cas system) into pronuclear-stage embryos. The activity of custom designed TALENs and CRISPR/Cas system in embryo-based gene targeting experiments has approached 90% in mice (Wang *et al*. 2013) and nearly 100% in zebrafish (Dahlem *et al*. 2012, Bedell *et al*. 2013). Various factors can affect the success rate of experiments aimed at the direct modification of zygotic genomes. Cytoplasmic microinjection of custom designed nucleases has used mRNA as it has a higher success rate than pronucleus DNA microinjection for generating knock-out rats (Tesson *et al*. 2011, Mali *et al*. 2013a). It has been demonstrated that, in a dose dependent response, direct microinjection of different concentrations of TALENs into embryos varying mutagenesis frequencies are achieved and produce a different percentage of bi-allelic knock-outs, as well as variations in the blast formation rate (Carlson *et al*. 2012,

Wang *et al*. 2013). To enrich modified embryos, mRNA/DNA encoding fluorescent proteins, like EGFP, can be co-injected with engineering nucleases (Carlson *et al*. 2012). The mutations generated by all three types of custom designed nucleases in founder animals can be efficiently transmited to the offspring (Cui *et al*. 2011, Mali *et al*. 2013a, Qiu *et al*. 2013, Sung *et al*. 2013). One-step generated GM founders also can result in mosaic animals (Wang *et al*. 2013) and therefore offspring of the founder animals needs to be genotyped.

Conclusions

Concerns around the genetic manipulation of animal genomes and the safety of their products has been the subject of increasing discussion between opponents and proponents of animal transgenesis including researchers, policy makers and the public. Proponents argue that GM animals hold great promise for improved animal welfare, healthier food, less environmental impact and as the production of human drugs. However, concerns arise around the biosafety of techniques used for generating GM animals. In this review we have presented our perspective on the advances made for inducing pluripotency and progress in genome editing., The progress made to date should help to address both the technical and biosafety issues in generating GM livestock.

Site-specific nucleases are closest to presenting genome-engineering researchers with an ideal DNA microsurgery tool. The best potential features the use of engineering nucleases which can be summarized as having the least effects, highest efficiency, marker-free selection, nontoxic, cost efficient, user-friendly protocol and being widely applicable across different species. All of these properties would enable significant progress for achieving farm animals with improved genetic makeup, consistent with regulatory guidelines for GM livestock. In addition, being able to target genes in early stage embryos, by injecting the engineering nucleases into either the cytoplasmic or pronuclear space, is extremely important for livestock because as yet there is no germline transmission capable of producing pluripotent stem cells. Although direct genome modification of zygotes appears to be highly efficient in rodents, somatic cell genome editing, followed by cloning, would be extremely useful in large animals, due to small litter size of these animals and to avoid increased gestation costs in these species.

Although the recent advances in genome editing will rapidly increase the use of livestock biotechnology in a variety of applications, thus far, the majority of genes that have been targeted in livestock species are also genes with potential applications in human health (Bruce *et al*. 2013). Furthermore, as the efficiency of the process improves even further the application of these approaches for modulating and enhancing livestock for production and welfare gain is becoming exceedingly feasible, hence the increasing excitement in the field.

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