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Genetic Manipulation of the Equine Oocyte and Embryo

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Abstract

As standard in vitro fertilization is not a viable technique in horses yet, many different techniques have been used to create equine embryos for research purposes. One such method is parthenogenesis in which an oocyte is induced to mature into an embryo-like state without the introduction of a spermatozoon, and thus they are not considered true embryos. Another method is somatic cell nuclear transfer (SCNT), in which a somatic cell nucleus from an extant horse is inserted into an enucleated oocyte, creating a genetic clone of the donor horse. Due to limited availability of equine oocytes in the United States, researchers have investigated the potential for combining equine somatic cell nuclei with oocytes from other species to make embryos for research purposes, which has not been successful to date. There has also been a rising interest in producing transgenic animals using sperm exposed to exogenous DNA. The successful creation of transgenic equine blastocysts shows the promise of sperm mediated gene transfer (SMGT), but this method is not ideal for other applications, like gene therapy, because it cannot be used to induce targeted mutations. That is why technologies like CRISPR/Cas9 are vital. In this review, we argue that parthenogenesis, SCNT, and interspecies SCNT can be considered genetic manipulation strategies as they create embryos that are genetically identical to their parent cell. Here, we describe how these methods are performed and their applications and we also describe the few methods that have been used to directly modify equine embryos: SMGT and CRISPR/Cas9.

1. Introduction

Compared to other domestic livestock species, the number of available equine oocytes for research purposes is low. Significant numbers of ruminant and porcine oocytes and ovaries can be recovered from animals sent to slaughter. As this is not an option for

horses in the United States, we are limited to oocytes collected from living horses via ovum pick up (OPU) or from euthanized horses. Additionally, the monovular nature of the mare's basic reproductive physiology as well as the inability to induce superovulation in mares, a technique which is feasible in other domestic species like cattle, limits the number of oocytes that can be obtained from a single mare. Thus, the relative shortage of equine oocytes that are available for study is further heightened by the relative difficulty in manipulating the mare's reproductive cyclicity.

Another limiting factor in equine developmental research is that standard in vitro fertilization (IVF) for horses is not possible yet. In standard IVF, oocyte maturation, introduction to capacitated spermatozoa, and fertilization by a single spermatozoon all occur in vitro. But, this inability to perform standard IVF has led to some innovative solutions, such as the use of intracytoplasmic sperm injection (ICSI), in which in vitro matured oocytes are fertilized through the microinjection of a single spermatozoon, to produce embryos for research purposes or to produce viable offspring.

Gene editing can further our knowledge about the function of individual genes and proteins in the early embryo. Gene knockouts are especially helpful in embryologic studies to determine protein function. For example, OCT4 (octamer binding transcription factor 4) is a transcription factor present in early mammalian embryos. Knockout studies in different species, such as mice [1], cattle [2], and humans [3], have been performed to better understand the regulatory role of this transcription factor in the early embryo and the species-specific differences in their activity. Gene editing experiments in the early embryo also pave the way for the use of embryonic gene therapy to treat genetic diseases at the organismal level, enabling the prevention of those diseases.

Gene therapy experiments have been performed in the embryos of several species including mice [4], zebrafish [5, 6], cynomolgus macaques [7], and humans [8]. Gene therapy is slowly beginning to be applied to livestock species as seen by the in utero gene therapy experiments performed in sheep [9,10]. But few, if any, of these gene therapy experiments have been done on the early embryos of livestock species. And, there have been no gene therapy experiments in equine embryos or fetuses in utero to date. However, there have been a few studies in which sperm mediated gene transfer (SMGT) or CRISPR/Cas9 were used to create genetically modified equine embryos. This review will focus on the methods, like parthenogenesis, somatic cell nuclear transfer (SCNT), and interspecies SCNT (iSCNT), that create clonal embryos and foals, along with the strategies that have been used to date to create genetically modified equine embryos: SMGT and CRISPR/Cas9.

2.   Parthenogenesis

Parthenogenesis is when an oocyte matures to an embryo-like state through chemical or electrical activation without the introduction of a spermatozoon or, in some cases, when the spermatozoon fails to correctly fuse with the oocyte nucleus. The resulting structure is called a parthenote [11]. As parthenogenesis occurs without the fertilization of an oocyte by a spermatozoon, the parthenote is essentially a mitotically derived clone of the original oocyte.

Parthenotes can progress through metaphase II and undergo nuclear decondensation similar to fertilized oocytes, but they do not continue to develop [12]. This is believed to be caused by a lack of paternal genomic imprinting in these parthenotes [13]. Initial studies identified that in vitro activated equine parthenotes could only develop to the morula stage [14,15], but a subsequent study identified that a 40% blastocyst rate could be achieved in equine parthenotes [16] similar to cow [17] and rabbit [18] parthenotes. As parthenotes can cleave and begin to develop in culture, they have been used as positive controls for different activation treatments in experiments in equine embryo development. This is especially important for horses because standard IVF cannot be used as a positive control. For example, Choi and colleagues [19] used parthenotes as controls for their SCNT experiments. They concluded that the high rates of parthenogenetic cleavage they saw indicated that their activation protocol did not cause the low development rates they saw in their SCNT embryos [19].

The first reported methods to induce parthenogenesis in equine oocytes utilized ethanol, TCM-199, follicular fluid, cycloheximide, and/or calcium ionophore [12,20]. Later studies identified that stallion sperm extracts microinjected into equine oocytes could induce parthenogenesis in equine oocytes [21] and that culturing the cells with insulin-like growth factor I (IGF-1) or 6-dimethylaminopurine (6-DMAP) can increase the cleavage rates of equine parthenotes [14,22]. It has also been shown that how long the oocytes are allowed to mature prior to activation can affect their parthenogenic cleavage rate. Choi and coworkers identified a 58% parthenogenetic cleavage rate when oocytes were matured for 24 to 26 hours [22], whereas studies that allowed oocytes to mature for 48 hours prior to activation achieved parthenogenetic cleavage rates of 88% to 92% [23,24].

It has also been identified that horses have a high parthenogenic rate and therefore may be more prone to parthenogenesis after activation than other species. In one study, between 27% and 71% of equine oocytes that underwent ICSI with different activation treatments were actually parthenotes [25]. Additionally, several studies demonstrated that equine parthenotes had higher cleavage rates than embryos derived from SCNT or ICSI [15,16,19]. These findings all suggest that different activation treatments pose variable risks of inducing parthenogenesis in experiments on equine fertilization and equine embryo development.

A parthenote can also form when the spermatozoon fails to correctly fuse with the oocyte nucleus. In studies where sperm are introduced to an oocyte, it can be difficult to visually differentiate parthenotes from fertilized embryos. One method to differentiate the two is to stain them with a nuclear Hoechst stain [25]. In fertilized embryos, this stain shows a single nucleus in each blastomere of the two-cell embryo, but, in embryos that failed to fertilize after ICSI, the stain localizes to the condensed sperm head in the two-celled parthenote [25]. Heras and colleagues [26] determined that staining for epigenetic markers could be used to differentiate between parthenotes and fertilized oocytes. They found that the maternal and paternal pronuclei differ in their staining characteristics for the histone 3 lysine 9 trimethylation (H3K9me3) epigenetic marker. This technique can be used to differentiate parthenotes from fertilized embryos because parthenotes contained two strongly staining maternal pronuclei, whereas fertilized embryos contained one strongly staining maternal pronucleus and one weakly staining paternal pronucleus [26]. But, due to the fixation steps

required by this method, this technique cannot be used to distinguish parthenotes from fertilized oocytes prior to embryo transfer.

3. Somatic Cell Nuclear Transfer (SCNT)

Currently, the only method available to create a clone of an extant horse is through SCNT. To perform SCNT, equine oocytes must be collected and then matured in vitro. The equine somatic donor cells can be cultured as serum "starved," or grown in a medium that causes them to exit the cell cycle and enter the quiescent G_0 stage [27,28], or they can be grown in regular media until reconstruction with approximately the same success rate [23,24]. Next, the metaphase plate and first polar body are removed from the oocyte creating a cytoplast (enucleated oocyte) that is available for fusion with a somatic cell through electrofusion [28]. Then, the reconstructed oocyte is activated. Several methods have been utilized to activate reconstructed equine oocytes. Initial studies used 6-DMAP and cycloheximide, which had been successful at activating equine parthenotes, to activate the reconstructed oocytes [29]. More recent studies have used stallion sperm extract and ionomycin in addition to the initial methods [30]. Interestingly, the addition of IGF-1, which could be used to create equine parthenotes, did not increase activation rates after reconstruction [15] suggesting that further work is needed to investigate the role of IGF-1 in parthenogenetic activation.

The first successfully activated equine reconstructed embryos were reported in 2001, but these embryos did not progress past the 10-cell stage in vitro [28,31]. Additional work in this field led to the birth of the first cloned equid, a mule, in 2003 (see Section 3.1 for more details) [32]. The first cloned horse was born later that same year [33]. Interestingly, this foal was the clone of her dam as the embryo happened to be derived from the mare's own fibroblast [33]. The most important outcome of this study was that a normal, healthy and fertile foal was born. Since then, 75 healthy equine SCNT foals have been reported in the scientific literature and hundreds more have been born from commercial cloning companies [34], suggesting that SCNT is a viable method to create equine clones.

The successes seen with equine SCNT are important because fetal and placental abnormalities are commonly seen in calves derived from SCNT [35,36]. The first equine SCNT foal was healthy and later studies identified that approximately 50% of foals born from SCNT are normal, healthy foals [37,38]. The other 50% of live SCNT foals tend to present with neonatal maladjustment syndrome, enlarged umbilical remnants, and angular limb deformities, all of which are treatable conditions. Interestingly, SCNT foals do not present with large offspring syndrome, which is commonly seen in SCNT calves [37,38]. These studies suggest that if the SCNT foal is gestated to term, it has a high likelihood of being born healthy or being born with treatable conditions.

Another study investigated the placental changes that occurred in equine SCNT derived pregnancies from a single donor showing that all placentas had edema, engorged allantoic vessels and enlarged umbilical vessels, including pregnancies that led to the birth of a foal [39]. This study also demonstrated that equine SCNT pregnancies may be more likely to develop placentitis than SCNT pregnancies in other species. In three of the eight mares

studied, there was ultrasonographic evidence of placentitis, which was treated aggressively and successfully resolved in two of the mares, allowing for those foals to be carried to term. Additionally, an ascending bacterial placentitis was histopathologically identified in all the placentas from the aborted pregnancies (n = 5) [39]. These findings suggest that mares carrying SCNT derived foals should be monitored closely for signs of placentitis as treatment can help improve the likelihood of a viable foal being born.

It has been proposed that the low blastocyst rates seen with equine SCNT embryos is due to differences in equine oocyte morphology. Equine oocytes tend to be fragile with lower rates of survival after enucleation than the oocytes of other species [31]. They also have a very pliable zona pellucida (ZP), making penetration with a pipette more difficult and necessitating a larger pipette or piezo drill be used for the enucleation procedure. The metaphase plate and the first polar body of the equine oocyte also tend to be far apart, often necessitating that the ZP be punctured twice to remove all the nuclear DNA from the oocyte. Additionally, the polar body tends to be firmly attached to the ZP making it more likely for the oocyte to be damaged when the polar body is removed [31]. One way around this is by using zona-free oocytes. The removal of the ZP makes oocyte enucleation quicker and less complex [40]. Additionally, the use of a zona-free oocyte allows for better fusion between the donor cell and the cytoplast due to their direct contact [40]. These zona-free embryos are capable of establishing a pregnancy as three live foals were born after blastocysts were transferred into 23 mares [40]. However, this rate is lower than the foaling rates seen with transfer of blastocysts produced from traditional SCNT (50%) [30,41].

In addition to the use of zona-free oocytes, embryo aggregation can also be used to improve SCNT embryo quality [42,43]. In embryo aggregation, two or three zona-free SCNT embryos that shared a nuclear donor (but not necessarily a cytoplasmic donor) are cultured together in a single well. Embryo aggregation increases blastocyst rates, early pregnancy rates, and improves embryo quality in vitro [42]. Another modification that can increase blastocyst rates is to decrease the number of passages the donor cells undergo in culture as high passage numbers negatively impact the ability of the nucleus to be reprogrammed in the oocyte [44].

In addition to the number of passages in culture, different nuclear donor cells can be used to increase the efficacy of equine SCNT. The most common nuclear donor for SCNT studies is the equine fibroblast as these cells are easy to collect, but cells such as cumulus cells, induced pluripotent stem cells (iPSCs), umbilical cord-derived mesenchymal stem cells (UC-MSCs), fetal fibroblasts, and bone marrow derived mesenchymal stem cells (BM-MSCs) have also been used as equine nuclear donors [45–47]. Yet, of these additional donor cells, foals have only been produced from fetal fibroblast cells and BM-MSCs [46,47].

As the goal of equine cloning tends to be to preserve and expand the genetic material of a high performing individual, it is important that the clones are truly a genetic match of their donor. However, most foals born from SCNT are not exact clonal copies of their somatic cell donor because the oocyte that the donor nucleus is fused with contains different mitochondrial DNA. Choi and colleagues [48] performed SCNT with oocytes from mares that were maternally related to the somatic cell donor, and thus had the same mitochondrial

DNA. This method yielded a foal that had identical genomic and mitochondrial DNA as the donor horse [48], showing that it is possible to create a true equine genetic clone. However, producing mitochondrial-identical horses is more challenging because the somatic cell and cytoplast donors need to be selected more rigorously than they normally are for SCNT.

Another interesting concern with equine SCNT is its potential to propagate diseases as some viruses can be identified in the oocyte. As oocytes for SCNT can come from any horse, there is a concern that oocytes from untested horses from other countries could lead to the infection of a foal with equine infectious anemia virus (EIAV), a lentivirus [49,50]. As there have been no direct studies on this phenomenon, Gregg and Polejaeva [49] reviewed the existing literature on EIAV and similar viruses to determine the risk for infection of the recipient mare or the SCNT derived foal. They found that, between the inability of the virus to penetrate the ZP and the wash steps included in the standard SCNT procedure, there was a very low risk of infection [49]. Asseged and coworkers [50] performed a risk analysis for the spread of EIAV from the importation of cloned embryos identifying that the risk of transmission was very low, suggesting that the use of imported SCNT embryos is safe from this perspective. As both these studies used data collected from other lentiviruses, there is a chance that these conclusions are not a true reflection of EIAV, but there is no contrary evidence for this in the literature.

3.1. Interspecies SCNT (iSCNT)

One potential way around the low numbers of equine oocytes available is to use interspecies SCNT (iSCNT) for research instead. Interspecies SCNT is achieved when the nucleus from one species is transferred into the cytoplast of a different species [43]. To create an iSCNT embryo, similar steps to the creation of an SCNT embryo are followed (Fig. 1). This involves the collection of oocytes, the in vitro maturation of those oocytes, and the removal of the first polar body and metaphase II plate from those cells [51]. Then, the cytoplasts are ready to be combined with the donor cell nucleus.

The timing of the first cleavage is similar between equine and bovine embryos [28], suggesting that there could be developmental similarities across those two species. Because of this proposed similarity and the increased availability of bovine cytoplasts, there have been several studies where equine somatic nuclei have been inserted into bovine cytoplasts. These reconstructed embryos have done quite well with studies showing between 63% and 86.1% reconstruction rates and 53% to 88% cleavage rates of the reconstructed oocytes [23,28,31,52]. There have been a few studies that reported equine-bovine reconstructed embryos reaching the blastocyst stage [52,53], but this result has not been achieved in all studies on equine-bovine reconstructed embryos.

Different studies have been performed that show that equine nuclei can be inserted into the cytoplasts of other species (Table 1). For example, Gambini and colleagues [43] used porcine and feline oocytes as the host cell for the equine nucleus. The porcine oocytes achieved the highest fusion rates at 83%. However, the feline cytoplasts were able to develop to the blastocyst stage with three embryos of 57 reconstructed embryos reaching that stage. This was not the case for the bovine or porcine cytoplasts used in this study, suggesting that the equine-feline iSCNT embryos should be the subject of further study. But, these embryos

were low quality as they had low cell numbers and were smaller in size than equine-equine SCNT embryos [43]. Murine oocytes have also been used as the cytoplast donor for equine iSCNT [52]. The reconstruction rates achieved using mouse cytoplasts were not as high as was achieved with bovine cytoplasts with a 52.2% fusion rate and no blastocysts were derived from these embryos [52]. Another interesting finding that has been noted is that iSCNT development tends to arrest at the stage of embryonic transcriptional activation of the cytoplast host species [43]. This suggests that there might be species specific differences in cytoplasmic regulators of embryonic development that must be overcome to increase the efficiency of this technique. A variety of species have been used as cytoplasts for the creation of equine iSCNT embryos, but none of them have been developed past the blastocyst stage to enable the creation of an interspecies clone.

There have been several ways that equine nuclei have been introduced to cytoplasts of other species. Electrofusion is the most commonly used method to combine the equine nucleus with the bovine cytoplast with a 52% to 69% success rate [27,31]. This electrofusion technique can also be used to fuse cytoplast-donor cell-cytoplast triplets [52]. Choi and colleagues [23] describe the use of a piezo drill to efficiently inject the equine nucleus into the host bovine cytoplast, achieving an 81% reconstruction rate, suggesting that this method may be more effective than electrofusion for equine-bovine iSCNT embryo creation.

In addition, several methods have been used to activate iSCNT embryos. Initial studies used activation methods that were successful in inducing parthenogenesis in equine oocytes, such as ionophores or ionomycin with 6-DMAP [28,31,53]. Choi and coworkers [23] showed that when stallion sperm inhibited by treatment with Hoechst stain and UV light were injected into reconstructed embryos, 77% of bovine oocytes were activated. Stallion sperm extract was also shown to successfully activate interspecies reconstructed oocytes in several studies with cleavage rates as high as 97% [23, 24]. To produce the sperm extract, the ejaculate from one stallion is centrifuged and resuspended in Sperm-TALP then nuclear isolation medium [23]. Then, the suspension was subjected to four freeze-thaw cycles to induce cell lysis and release of cytoplasmic contents for use as a crude extract [23].

As equine oocytes are in limited supply, most of iSCNT studies involving horses have equine cells as the nuclear donor with the cytoplast coming from a different species. But, the first equid born from SCNT was actually from intragenus SCNT as a mule was cloned from the fusion of fetal fibroblasts with equine cytoplasts [32]. In this study, the mule donor nucleus was placed in the perivitelline space of the enucleated oocyte and electrofusion was utilized. Then, two to five SCNT embryos were surgically transferred into recipient mares. This procedure yielded a 6.9% pregnancy rate at 14 days following surgical transfer. At the time of publication, one SCNT mule foal had been born and two mares in late gestation were carrying two additional SCNT mule foals [32]. These findings support that this technique can be used to create clonal offspring with closely related equids, but the same success has not been seen with a more distantly related cytoplast donor.

4. Sperm Mediated Gene Transfer (SMGT)

The main limitations to creating transgenic large animals are the costs to introduce the transgene, the inefficiency of the methods that are available, the potential for lifelong quarantine by the Food and Drug Administration and the long gestation period and generation time [54]. This challenge is heightened in horses as standard IVF is not an option in this species yet. ICSI is an alternative to standard IVF that could be used to create embryos for modification, though. As the use of ICSI has been well established in horses, this technique has been paired with SMGT in the attempt to create modified equine embryos. This has the potential to be effective in equine embryo modification because the sperm itself acts as a vector to introduce exogenous DNA into the embryo [55]. In addition to its ability to create modified equine embryos in culture through the use of ICSI, SMGT can also be used in conjunction with artificial insemination (AI) of mares, unlike other available modification techniques.

Ball and coworkers [54] were the first to assess the ability of equine sperm to take up exogenous DNA and to use that sperm to fertilize equine oocytes in vivo. In this study, the authors evaluated the ability of equine sperm to take up a linearized plasmid with and without the addition of lipofection to the transfection cocktail, identifying that the presence of lipofection significantly increased the uptake of exogenous DNA to 63% uptake. To ensure that the transfected sperm were still viable, they were assessed morphologically and used to inseminate mares. After a single insemination, eight embryos were recovered from 11 mares with seven being viable embryos. Unfortunately, none of these embryos expressed the enhanced green fluorescent protein (EGFP) introduced by the plasmid, but PCR did confirm that two of the five embryos assessed contained the DNA for *EGFP* [54], suggesting that SMGT in horses had some promise.

This technique has also been used in conjunction with ICSI to introduce a transgene into equine embryos. Pereyra-Bonnet and coworkers [56] were the first to induce exogenous gene expression in an equine embryo through the use of ICSI mediated gene transfer (ICSI-MGT) with the use of ionomycin and 6-DMAP as an additional activation step. However, this study reported a 0% blastocyst rate from their equine ICSI-MGT embryos [56]. In contrast to these findings, Zaniboni and colleagues [57] found that correct sperm manipulation, not chemical activation, was required for the success of ICSI-MGT as they had a blastocyst rate of 9.1% (2/22). Both blastocysts were PCR positive for *EGFP* and one of them expressed EGFP. Thus, this was the first study to create transgenic equine blastocysts using SMGT.

A limitation of SMGT is that it is challenging to identify if the exogenous DNA was incorporated into the genome of the spermatozoon or if gene is present in an extrachromosomal form. Smith and Spadafora [55] suggest that exogenous DNA integration could occur at "accessible" sites in the sperm chromatin where protamines were not placed during spermatogenesis. In addition, it is also unknown where the transgene will incorporate into the genome, if it does incorporate, or how many copies will incorporate into a single cell. For example, Webster and colleagues [58] used fluorescence in situ hybridization (FISH) to identify where the transgenes incorporated into the porcine genome after SMGT. They found that the transgenes incorporated in multiple sites and that different cells from

the same animal had different integration numbers with some cells showing no integration and some cells showing as many as three incorporations of the same transgene. This study assessed the incorporation ability of three different transgenes and interestingly, two of the transgenes integrated at the same site in different cell lineages [58], supporting the hypothesis proposed by Smith and Spadafora [55] that there are certain "accessible" sites for transgene incorporation.

One of the most important considerations when creating a transgenic animal is that animal's ability to pass the transgene onto its offspring. Transgenic offspring from a SMGT animal has been achieved in pigs [59], mice [60], and rabbits [61]. Lavitrano and coworkers [59] identified that transgenic pigs created with SMGT could transmit their transgene to their offspring at a rate of 20 to 50%, which is lower than expected if the gene was incorporated into the genome, suggesting that some of these pigs were mosaics. In their study on rabbits, Wang and colleagues [61] identified that one of six offspring from an SMGT induced transgenic animal expressed the transgene as well. Both of these studies put the economic viability of this technique into question. More research into why some species seem to be better or less able to pass on a transgene is needed to increase the efficacy of this method.

5. CRISPR/Cas9 Mediated Gene Editing

The use of clustered regularly interspaced short palindromic repeats (CRISPR) and the Cas9 protein to induce targeted mutations has rapidly emerged as a very effective gene editing technique [62]. The first use of the CRISPR/Cas9 system was in human cells, including embryonic kidney, chronic myelogenous leukemia, and iPSCs, and mouse cells showing that this method was an efficient way to edit mammalian genomes [63–65]. Since then, this system has been used to modify the embryos of several species including zebrafish [66], western clawed frogs [67], pigs [68], cattle [2], sheep [69] and cynomolgus macaques [70], showing that this technique is successful in a variety of species. But, application of this technique in an equine embryo has not yet been reported.

There have been five studies on the use of CRISPR/Cas9 in equine cells, three of which were performed on equine fibroblasts, one on equine fetal fibroblasts and one in adipose derived mesenchymal stem cells (aMSCs). Pinzon-Arteaga and coworkers [71] used CRISPR/Cas9 to remove the glycogen branching enzyme deficiency (GBED) mutation from equine fibroblasts and Hawkes [72] describes the use of CRISPR/Cas9 to edit the heritable equine regional dermal asthenia (HERDA) mutation from equine fibroblasts. These studies both show the potential gene therapy applications of this technology in the equine species as these mutations could be repaired in somatic cells, which, in turn, could be used to create embryos via SCNT, or for use in one-step gene editing directly in equine embryos to potentially prevent these diseases. Mançanares and colleagues [73] used the CRISPR/Cas9 system to edit the prostaglandin E2 and E4 receptors in cultured aMSCs with the goal of increasing their ability to self-renew, proliferate and survive in culture, showing another practical application of the CRISPR/Cas9 technology.

The final two studies involving CRISPR in equine cells were performed in equine fibroblasts or in equine fetal fibroblasts. In these studies, the CRISPR/Cas9 system was used to

knockout the myostatin (*MSTN*) gene, which is a negative regulator of muscle growth and development [74,75]. After confirming with Sanger sequencing that those cell lines had the knockout, they were used to generate embryos via SCNT [74,75]. In the study by Vichera and colleagues [74], both of the modified cell lines could generate *MSTN* knockout blastocysts, but they both had a low blastocyst rate (2%) compared to their control SCNT experiments (6%–12%) [74]. Moro and coworkers [75] also identified a low blastocyst rate in their experiments with the modified fetal fibroblasts producing significantly fewer blastocysts than unmodified mesenchymal stem cells. These studies show the first step towards using the CRISPR/Cas9 system to genetically modify an equine embryo.

The decreased blastocyst rate seen in these studies was likely due to the need for clonal isolation and the increased number of passages the nuclear donors underwent in culture prior to SCNT to ensure that the knockout was incorporated [53,74]. The efficacy of gene editing in equine embryos could be increased if the mutation and fertilization were done all in one step. Navarro-Serna and coworkers [76] reviewed the use of one-step gene editing in livestock embryos. They found that off-target mutations, one of the main concerns with gene editing, rarely occurred with one-step editing. They also identified that mosaicism is one of the main problems associated with this method. Mosaicism occurs when the desired mutation is not incorporated into all chromosomes, likely due to incorporation of the mutation after the first round of DNA replication [76]. As this method does not require the clonal expansion of a donor cell and decreases embryo manipulation, the one-step method could be modified for use in equine embryos to increase the efficiency of edited blastocysts. Additionally, one-step editing also removes the need for a nuclear donor, thus allowing for the birth of genetically distinct offspring from these edited embryos.

There are many benefits to using the CRISPR/Cas9 gene editing technology in equine embryos. This method can be used to introduce site specific modifications that can be integrated into the genome of the individual before the first cleavage event ensuring that the mutation is present in all cells of the individual. Integration into all cells is especially important in the treatment of genetic diseases as a targeted edit can prevent the animal from ever developing the disease phenotype. In addition, this system can also be used for research purposes to learn more about the heritability of individual genes and their function in the embryo and in the adult organism.

The main limitations on the use of CRISPR/Cas9 in equine embryos is the low number of equine oocytes that are available for research. As these studies tend to require large numbers of oocytes to account for the anticipated losses after fertilization into blastocyst development and during gestation, the low numbers of available equine oocytes hinder our ability to perform these studies appropriately. Additionally, it is well documented that the blastocyst rates for equine embryos in culture are low and that they are even lower with additional manipulations [53,74].

Despite these limitations, studies using CRISPR/Cas9 in equine embryos are vital for the development of gene therapies for genetic diseases, such as GBED and HERDA. These studies can also help us gain more basic knowledge on the genetic mechanism of other genetic disorders seen in horses. To date, there have been no equine offspring born

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from CRISPR/Cas9 edited embryos, but this can and should be a working objective for researchers. We argue that the next step we need to take towards this goal is the creation of a one-step edited equine embryo.

6. Conclusions and Future Directions

This review described some research applications and the methods to clone equine embryos, including parthenogenesis, SCNT both within the equine species and between different species, and the genetic manipulation strategies that have been used in equine embryos to date, SMGT and CRISPR/Cas9. As parthenogenesis involves activation of an oocyte without the contribution of a spermatozoon, a mitotic clone is created, but these embryos do not continue to develop. SCNT has seen many successful cloned offspring born, but further work is needed to assess the ability to create a viable offspring from iSCNT with equine somatic donor cells. Equine sperm can take up exogenous DNA and remain viable, but no transgenic equids have been produced through SMGT yet. Finally, there are many potential uses for CRISPR/Cas9 in equine embryos, from basic genetics research to gene therapy, yet no attempts to use this technology directly in the equine embryo has been reported.

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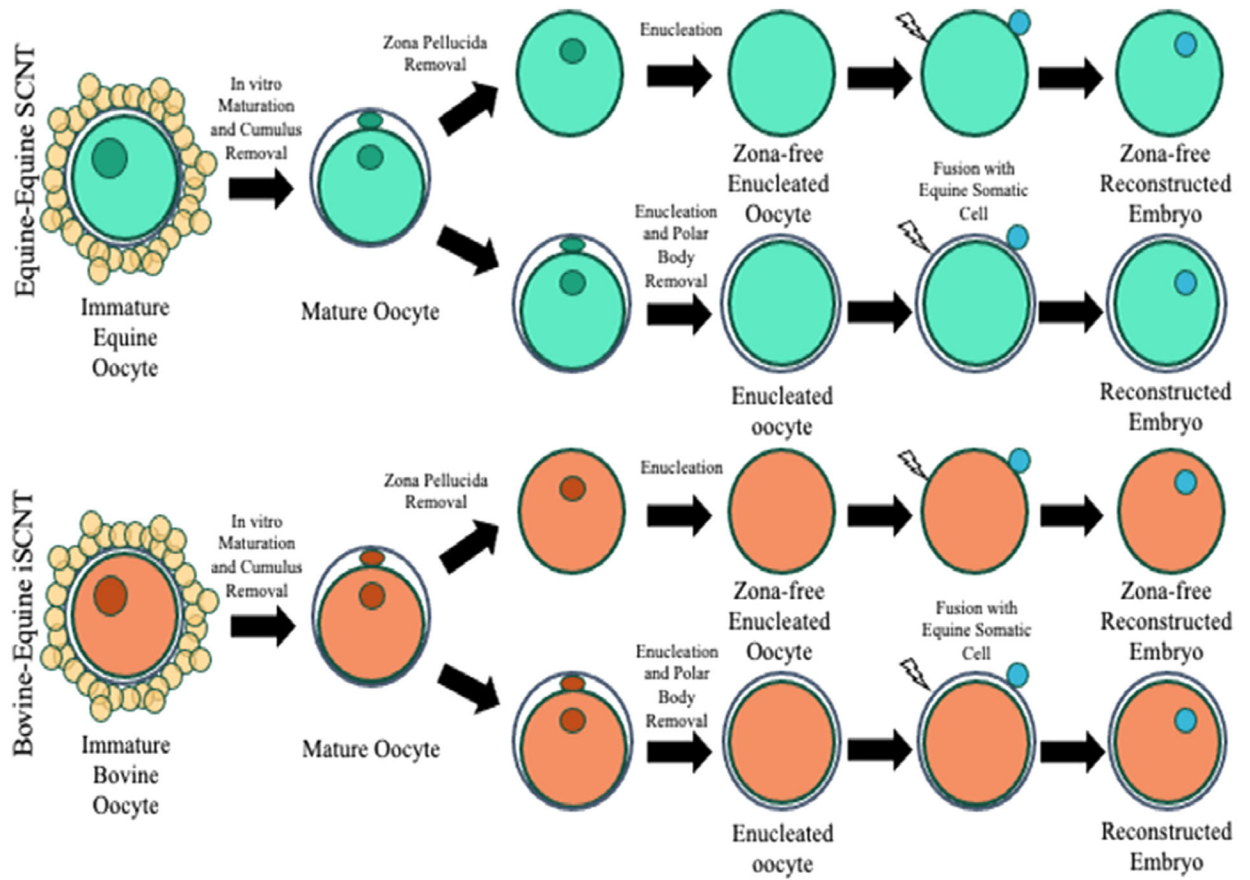
Fig. 1.
Schematic comparison of SCNT and iSCNT. A bovine oocyte was chosen as a representative example of iSCNT. Similar methods are employed for other species. Adapted from Gambini et al. [43].

Table 1

Comparing the ability of different cytoplast donors to create embryos.

Recipient Species	2-Cell Embryo	4-Cell Embryo	8-Cell Embryo	16-Cell Embryo	Blastocyst	Offspring	References
Equine	•	•	•	•	•	•	[33,34]
Bovine	•	•	•	•	•	•	[52,53]
Porcine	•	•	•	•	○	-	[43]
Feline	•	•	•	•	•	-	[43]
Murine	•	-	-	-	○	-	[52]

Closed circles represent that embryos reached this stage of development. Open circles represent that embryos failed to reach this stage. Dashes represent missing data.