Chapter 7

Embryo Manipulation Techniques in the Rabbit

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Additional information is available at the end of the chapter

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Abstract

Rabbits are both productive and classic laboratory animals. Some particularities of female reproductive physiology make the rabbit an extraordinary model for the study of embryology and assisted reproductive techniques. For instance, as the ovulation is induced, the embryo development can be known with accuracy. Embryos are surrounded by a mucin coat which is crucial to prevent embryo mortality. Besides, the anatomy of the uterus does not allow embryo transmigration between both uterine horns, and so it is possible to test different reproductive techniques. Knowledge on early embryo development, and on influencing factors, has allowed to develop new insights into embryo manipulation, such as recovery, transfer, cryopreservation, *in vitro* fertilisation, cloning, or transgenesis. Also the rabbit may be used as a model for human reproductive health, because rabbit embryo and feto-placental development are similar to the human. This chapter reviews the aspects of the reproductive physiology in the female rabbit and discusses some embryo manipulation techniques available in the species.

Keywords: development, embryo, rabbit, reproductive techniques, survival

1. Introduction

Rabbit (*Oryctolagus cuniculus*) is a livestock species reared either for the production of hair, skin or meat [1] or as an experimental reference for other species, such as pigs or humans [2]. In rabbit meat production, litter size is an important economic trait [3, 4], and has been the objective in selection programs [5]. Litter size components are ovulation rate, fertilisation rate and prenatal survival, the latter component being defined by the embryo and foetal survival. Prenatal survival is around 30% [6], 15% corresponding to embryonic period. Therefore, knowledge of rabbit embryology is crucial for rabbit production.

Research in embryo biology using the rabbit as a model is associated with a number of well-known pioneer investigations on reproductive biology, e.g., fertilisation [7], oviduct physiology [8], embryo survival [9], prenatal mortality [10], experimental embryology [11], embryo *in vitro* culture [12], preimplantation embryo development *in vivo* and *in vitro* [13, 14] and embryo cryopreservation [15]. The reproductive characteristics of the rabbit have led to the development of embryo technologies with applications in genetic improvement, in the spread of genetic material or the study of diseases.

2. Reproductive particularities of the female

Female rabbit has certain physiological and anatomical characteristics, which make it especially important for the study of embryology and the application of embryo reproductive techniques.

The rabbit belongs to the few species in which ovulation is induced by mating [16, 17]. The coitus leads to the nervous stimulation of the vagina, triggering the production of gonadotropin-releasing hormone (GnRH) in the hypothalamus. Under the influence of GnRH, the anterior pituitary secretes the gonadotrophins and the follicle-stimulating hormone (FSH), which regulates follicular development, and the luteinizing hormone (LH), which leads to ovulation [16, 17]. In the absence of a mating stimulus, ovulation can be triggered by hormonal treatment, with either GnRH analogues [18–20] or human chorionic gonadotrophin hormone (hCG) [21]. As a consequence of induced ovulation, the age of the embryos [hours post coitum (hpc)] is precisely known.

Rabbit has a short reproductive cycle. Female sexual maturity occurs at about 17–20 weeks of age, depending on lines [5]. Gestation lasts for 31 days. Female shows oestrus early in post-partum, during lactation. Different reproductive systems can be used in rabbits' production, according to the production system: intensive (mating 4 days post-partum), semi-intensive (mating 11 days post-partum), semi-extensive (mating 18 days post-partum) or extensive (mating after weaning) [22]. Weaning takes place at 28 days of lactation. This implies that females sustain lactation and gestation simultaneously, except for the extensive system, but this overlap depresses sexual receptivity, ovulation, fertilisation, implantation and embryo survival due to hormonal antagonism between prolactin and gonadotropins [23, 24].

The morphology of the rabbit uterus represents a uterus duplex, i.e., constituted by two separated fully functional uterine horns and cervices opening into a sole vagina [2]. This morphology allows the transfer of two sets of embryos into the same recipient female without the occurrence of inter-horn migration. So, this species is particularly suitable to study the prenatal survival [25].

3. Reproductive cycle, ovulation and fertilisation

Female rabbits do not have a typical oestrus cycle [26]. Their period of sexual receptivity is influenced by the development of follicular populations. The receptivity can be identified by the colour red or purple of the vulva [27]. The development of follicles occurs normally in waves, with 5–10 follicles in each ovary with an oocyte inside each. The follicles produce

oestrogen for 12–14 days. After this period, if there is no ovulation, the follicles degenerate, the oocytes are reabsorbed, and the oestrogen and sexual receptivity decrease. After about 4 days, a new wave of follicular growth begins. So, the reproductive cycle lasts 16–18 days, of which 12–14 days, the female is receptive and the remaining 4 days, it does not accept the male.

If ovulation takes place, the follicles become corpus luteum, starting a luteal phase characterised by high levels of progesterone that block new follicular waves. Whenever fertilisation, implantation and gestation occur, the corpus luteum is maintained throughout pregnancy. The oocytes are captured by the ovarian bursa of the infundibulum and are quickly transported through the ampulla, due to muscular contractions and ciliary activity. Fertilisation occurs shortly after ovulation in the ampulla by capacitated sperm that has already been present for 10–12 h in the female tract [28]. Fertilisation rate is generally high, about 95% [29, 30].

But if no fertilisation is achieved, or early embryonic losses are produced, the uterus produces prostaglandin-2α ($PGF_{2\alpha}$) 17 days after ovulation, which has luteolytic action. The progesterone levels decrease and a new wave of follicular growth is produced.

4. Embryo development

After fertilisation, the resulting zygote progresses through the next stage of development rather quickly in the isthmus and uterotubal junction. **Table 1** shows the timing of *in vivo* embryo development between 24 and 84 hpc. Overlap of different embryo developmental stages is commonly observed [30–33], and it could be related to the duration of ovulation or the oviductal and uterine fluid compositions [34, 35]. It is known that a high ovulation rate increases the overall duration of ovulation, and later ovulating follicles may be fertilised later [36].

Early embryo losses, defined from fertilisation to implantation, have been estimated at around 15% [6] and they may result of an inherited abnormal development, an asynchronism between the stage of embryo development and the uterine environment, or to an inadequate steroidogenic pattern [14, 37].

hpc	Zygote	2-cells	4-cells	8-cells	16-cells	Early morulae	Compacted morulae	Blastocyst	Expanded blastocyst
24	X	X							
28		X	X						
30		X	X	X					
48					X	X	X		
62						X	X		
72						X	X	X	
84								X	X

hpc: hours post coitum.

Table 1. Timing of *in vivo* embryo development in rabbits.

4.1. Embryo coats

The extracellular coverings of rabbit embryos are structurally more complex than in most mammalian embryos. The peculiarity of rabbit embryos is that they are surrounded not only by the zona pellucida but also by a mucin coat (**Figure 1**).

Zona pellucida is an extracellular embryonic coat that surrounds the early embryo (**Figure 1**). Zona pellucida proteins are synthesised by the oocyte and the granulosa cells to form concentric layers consisting of cross-linked zona proteins [38]. The steadiness of zona pellucida allows the intrazonal diameter of the embryo to remain constant (around 120 μm) up to 72 hpc [33, 39]. The zona pellucida thickness itself ranges between 15.9 and 19.2 μm [32, 40]. The zona pellucida selects morphologically normal spermatozoa [41], indices the acrosomal reaction [42] preventing polyspermy [43] and protects early embryo integrity during the transport through the oviduct [44]. It affects fertilisation; a thicker zona pellucida was found in oocytes with failed fertilisation collected from the oviducts compared with those of embryos [40]. Moreover, recent studies have shown that zona pellucida thickness is 8% lower in females under heat stress than in thermal comfort conditions, decreasing the number of normal embryos [45].

The mucin coat is a layer of acid mucopolysaccharides, which is deposited on the embryos during the passage through the oviduct [46]. The mucin coat thickness depends on the time spent in the oviduct [46], and it is half the thickness for embryos at 48 hpc (around 50 μm) than at 72 hpc (around 100 μm) [33]. The thickness of the mucin coat is essential for rabbit embryos to develop at term because it physically prevents the embryos from direct exposure to a deleterious uterine environment and allows them to expand until the appropriate time for implantation [46]. When *in vitro* embryos are transferred into recipients, the lack of a mucin coat predisposes to subsequent failure of pregnancy [47].

The embryo is covered by the zona pellucida and a mucin coat until 72–96 hpc. The zona pellucida disappears by then, and it is substituted by neozona (4.5 days) when the blastocysts enter into the uterus, while the mucin coat is covered by a new layer, named gloiolemma, around 6 days of gestation [38].

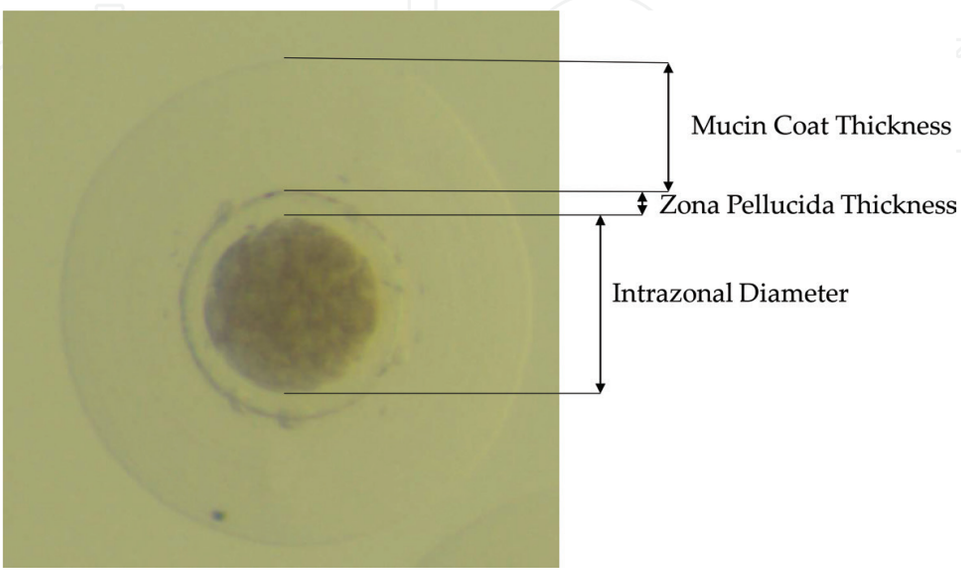

Figure 1. Rabbit compacted morulae. The image shows the measurements commonly used to assess early rabbit embryos.

5. Rabbit pregnancy

Gestation depends upon the early embryo's signalling its presence to the maternal system, a process termed maternal recognition of pregnancy [48]. The maternal recognition does not occur until the end of the first third of gestation [49], when the presence of the embryos is required to induce continued progesterone secretion by the corpus luteum.

Rabbits have a decidual and discoid haemochorial placenta [50]. The individual weight of the foetal placenta is higher than the individual weight of the maternal placenta, and both are higher for a live foetus than dead foetus [51]. Concerning the position in the uterus, the heaviest maternal and foetal placentae, and foetuses, are located near the oviduct [51, 52], probably due to the greater uterine space per foetus and higher blood flow in this region [53].

Abdominal palpation makes the simplest pregnancy diagnosis method, and it can perform by 12 dpc (days post coitum). To know not only the number of implanted embryos but also the ovulation rate, estimated from the number of corpora lutea in the ovary, it is necessary to perform a laparoscopy. Parturition takes place after 31 days of gestation.

6. Embryo cryopreservation

Since the 1970's, rabbit embryos can be cryopreserved and stored with optimal efficiency [54]. Embryo cryopreservation can be used as a tool in setting up genetic resources banks that preserve the genetic diversity and protect against loss through diseases or hazards [55, 56]. The establishment of control populations from cryopreserved embryos of genetically selected lines allows estimating the response to selection [6]. It further enables the mobility of genetic material in animal breeding, facilitating the diffusion of genetic improvement of animals with higher genetic value [57].

The first cryopreservation protocols were based on freezing. However, these have been replaced by the vitrification technique because it is an easier and cheaper technique. Vitrification permits the rapid cooling of the liquid medium avoiding ice crystal formation by the use of high levels of cryoprotectants. Several protocols have been developed for embryo vitrification, providing a survival rate *ca*. 60% after transfer [58–62]. Some vitrification procedures and solutions applied in rabbit [58, 61, 62] are similar to those used in human [63, 64]. An example of a protocol is one that is carried out in two steps, at 20°C. First, embryos are placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl sulphoxide (3.5 M) and 12.5% (v/v) ethylene glycol (4.4 M) in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.2% (w/v) of bovine serum albumin (BSA). In the second step, embryos are suspended for 1 min in a solution of 20% (v/v) dimethyl sulphoxide, and 20% (v/v) ethylene glycol in DPBS supplemented with 0.2% of BSA. Thereafter, the embryos suspended in the vitrification medium are loaded into 0.25 ml plastic straws, and two sections of DPBS are added at the beginning and the end of each straw, separated from the embryo containing medium by air bubbles. Finally, straws are sealed, identified and plunged into liquid nitrogen at −196°C [62].

Devitrification is performed by immersing the central and the final sections of the straws in a water bath at 20°C for 10 s. The vitrification medium is eliminated in two steps. First, embryos

are expelled with the vitrification medium into a solution of DPBS with 0.33 M sucrose for 5 min; then, embryos are washed in a solution of DPBS for another 5 min [62]. The purpose is the elimination of the high concentration of cryoprotectants, which are toxic for embryos, as soon as possible.

The most recommended embryonic stage for cryopreservation is the compacted morulae. However, by different methodologies, it is also possible to cryopreserve cells from oocytes to blastocysts [65–67], bisected embryos [68] or cloned embryos [69].

A complete cryopreservation procedure involves several embryo manipulations such as *in vitro* handling, the exposure to toxic concentrations of cryoprotectants, or transfer into a female reproductive tract. The success of the vitrification-thawing procedures depends on the concentration and composition of the vitrification solution, the cooling and thawing conditions, the procedure used to dilute embryos from the vitrification solution, and the volume of the vitrification solution [70]. The genotype of the embryo and the genotype of the recipient female also affect the efficiency of the vitrification-thawing procedure [71].

Retarded embryonic development is produced in cryopreserved embryos, and it may be due to a slow and gradual restoration of normal metabolic and synthetic activities of the thawed embryo [72]. So, asynchrony between vitrified embryos and recipient females is applied to obtain higher rates of embryos developed to term [73]. Cryopreservation and transfer procedures affect the RNA expression [74], and consequently the placental transcriptome and proteome [75]. Vitrification also affects foetal survival and growth and modifies the placental development at term [60, 76] and growth from birth to adult age [77]. Nonetheless, positive long-term effects of cryopreservation and transfer procedures on female offspring reproduction have been reported, namely in the litter size and the number of newborns alive at birth [78].

7. Embryo reproductive techniques

Expanding knowledge on the embryology of the rabbit, the effective cryopreservation of embryos and the reproductive particularities of the rabbit allowed the development of embryo reproductive techniques. These techniques have been successfully applied in the rabbit industry and have been extrapolated to other species. Next, reproductive technologies related to the embryo are discussed, such as the embryo recovery and transfer, *in vitro* fertilisation, cloning and production of transgenic embryos.

7.1. Embryo recovery and transfer

The ability to recover and transfer preimplantation embryos has numerous associated applications which are inevitably linked to translational molecular genetics, cell biology and assisted reproductive technology [79]. In 1890, the first successful mammalian embryo transfer was performed in rabbits [80]. Nowadays, this technique has become a routine practice in human medicine [81].

Embryo transfer includes the generation of preimplantation embryos along with the development of those embryos until term in different recipient females. Production of preimplantation

embryos can be achieved *in vivo* or *in vitro*. In both cases, superovulation protocols can be applied to ensure the maximum number of embryos recovered per donor. The classical protocols have been performed with equine chorionic gonadotropin (eCG) or FSH (**Table 2**) [21, 82–84, 86–88]. But, both the number and quality of the oocytes obtained are highly variable when eCG is administrated [21, 83, 86]. The use of LH on super stimulation has been studied using porcine FSH (pFSH). The results are also variable due to the different LH concentration present, contamination from other hormones, inconsistencies within and among batches, and the possibility of the spread of diseases [88]. Nowadays, because of break-through in recombinant technology, it is possible to easily dispose of LH and FSH in an isolated manner [81], and the treatment with recombinant human FSH (rhFSH) alone or supplemented with LH is effective in stimulating superovulation without affecting the embryo quality [85].

Fertilisation of the oocytes can be achieved by natural mating or artificial insemination, if embryos are produced *in vivo*. Artificial insemination is usually performed with fresh or cooled semen stored for short periods of time (under 36 h) [89, 90], and obtains a high fertility rate and prolificacy. However, when fresh semen is used the sperm concentration per ml should be 4 million [91], while it should be increased to 15 million if the semen is refrigerated [89]. Fresh or cooled semen is used in the rabbit industry to improve breeding management [22]. However, frozen semen presents poor fertility after thawing [92, 93] and it is used mainly for conservation of banking resources, international exports and research [94].

A good understanding of the chronology of events that follows the ovulation is crucial to the recovery of preimplantation embryos [78]. At 24 hpc, all the embryos can be found in the isthmus [95]. At 78 hpc about one-third of the embryos are already found beyond the utero-tubal junction, whereas at 84 hpc more than 90% of the embryos have reached the uterus [95]. Embryo implantation occurs between 120 and 144 hpc [96]. Considering the chronology of embryo development some techniques have been developed to recovery embryos at any stage and location.

Embryos can be recovered by non-surgical, *post-mortem* or laparoscopy. In the non-surgical method, $PGF_{2\alpha}$ is administered 50–55 h after mating. It produces the expelling of embryos, located in the oviduct or the uterus, toward the vagina, where embryos can be recovered

Hormone	Dose	Ovulation induction	Ovulation rate	Recovered embryos	Reference
eCG	200 IU	—	19.2	8.8	[21]
	20 IU/kg BW	120 IU hCG	28.7	14.2	[82]
FSH	5 × 9 μg/ml /12 h	1.6 μg buserelin acetate	26.7	21.2	[83]
	6 × 0.5 mg /12 h	150 IU hCG	26.5	21.3	[84]
pFSH	3 × 18 μg/ml /24 h	2 μg buserelin acetate	34.4	—	[85]
rhFSH	3 × 0.6 μg /24 h	2 μg buserelin acetate	16.6	—	[85]

eCG: equine chorionic gonadotropin; FSH: follicle-stimulating hormone; pFSH: porcine FSH; rhFSH: recombinant human FSH; BW: Body weight; hCG: human chorionic gonadotrophin hormone.

Table 2. Examples of superovulation protocols.

without surgery [97, 98]. This procedure has an efficacy of 40% [97, 98]. When embryos are obtained *post-mortem*, the entire reproductive tract is removed after the female is euthanised. The embryos are recovered by perfusion of each oviduct and the first third of the uterus with 5 ml of phosphate-buffered saline containing 0.2% of BSA [33]. This method allows a retrieval rate of 64% [99]. Laparoscopy is a method that allows the recovery of both preimplantation embryos and also of oocytes [100]. If multiple cycles of embryo collection are required, this method is chosen because it guarantees minimal invasion through the use of a small entrance in the peritoneal cavity. However, this procedure has a lower efficacy in primiparous or multiparous females (around 50%), compared with nulliparous females (73%) [99].

Recovered embryos are subjected to morphological grading (**Table 3**, [101–103]); they are usually classified as having 'good quality' when they present homogenous cellular mass and intact zona pellucida and mucin coat [104]. Normal embryos can be maintained under *in vitro* conditions or cryopreserved until transferred. Zygotes have been successfully developed into blastocyst *in vitro* [10, 31, 104–108]. One example of culture media is TCM199 supplemented with 0.1% of BSA and culture is performed in 500 μl of medium layered under paraffin oil at 38.5°C, 5% CO_2 and saturated humidity [109]. However, these culture media do not mimic the uterine environment, and as a consequence, embryos developed *in vitro* have fewer cells and lower intrazonal diameter and mucin coat thickness than embryos developed *in vivo*, which reduces pregnancy rates after embryo transfer [40, 106].

Embryo transfer is performed by surgery (laparotomy) [110, 111] or by laparoscopy [112], into the oviducts or each one of the uterine horns. The use of laparoscopy aims at minimising the invasion site of the reproductive organs and *in situ* manipulation [79]. To guarantee the success of the transfer, a minimum number of embryos have to be transferred, and asynchrony between the embryo development and the recipient female has to occur [65, 70, 113]. On one hand, at least two foeto-placental units are required to maintain the gestation [114, 115], and on the other hand, when the survival rate is high, competition between foetuses can lead to a lower foetal survival [115]. So, the number of transferred embryos recommended per recipients ranges between 10 and 13 for both fresh and cryopreserved embryos [71, 116]. If the number of transferred embryos per donor female is lower than this range, it is possible

Embryo-grade categories[1]	Score grade[2]	Size blastomeres	Cytoplasmic fragments
Good quality	Grade 1	Equal	None
	Grade 2	Equal	Minor
Fair quality	Grade 3	Unequal	None or few
Poor quality	Grade 4	Unequal	Major
	Grade 5	Any	Severe or complete

[1]Embryos are clustered in embryo-grade categories following the Shulman criterion.
[2]Embryos are graded following the Veeck and Maloney criterion.

Table 3. Grading score used for rabbit embryos.

to transfer embryos from two donor females to each of the uterine horns of the recipient female, to ensure the survival of the embryos [71]. In case it is necessary to know the origin of the transferred embryos, it is recommended to perform a caesarean to the recipient female [71]. In general, asynchrony is obtained by artificially inducing ovulation in recipient female between 0 and 6 h before the donor females, for fresh embryos, or between 6 and 12 h before, for *in vitro* cultured or cryopreserved embryos [60, 65, 70, 113]. Besides, an association of the recipient genotype with implantation rate, foetal losses and offspring rate at birth has been documented [70, 71]. The recipient female usually belongs to a maternal line because recipient genotype is crucial in providing an adequate uterine environment to support adhesion, embryo-uterine cross-talk and placental and foetal development to term after vitrified and fresh embryo transfer [70, 111].

Embryo recovery and transfer influences mRNA expression of late blastocyst before implantation and may result in faulty embryonic implantation [117]. Transferred embryos have a lower transcript abundance of the transcription factor octamer binding 4 (OCT4) and higher epithelial membrane protein 1 (EMP1) [117]. OCT4 is a key regulator of the pluripotency maintenance system [118], and the main function of this transcriptional factor is to repress or activate several target genes involved in cell differentiation and early embryonic development [119]. The altered expression of OCT4 in the preimplantation embryo is associated with lower embryo quality [120]. EMP1 is involved in the regulation of cell cycle or cell-cell recognition, and high levels of EMP1 expression have been related to cell differentiation and arrest [121]. Signals involved in cell proliferation and differentiation during gastrulation and implantation events could be disturbed with embryo manipulation.

7.2. *In vitro* fertilisation and intracytoplasmic sperm injection

Oocyte collection and extracorporeal fertilisation represent an important embryo production in rabbits [73]. These embryos are of scientific interest for cloning and transgenesis [73].

Both *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) have been successfully developed [122, 123]. For IVF, oocytes are incubated with sperm for 5 h, and then moved to the embryo culture medium containing TCM199 medium suspended with 1.25 mM pyruvate, 0.1 mM EDTA, 10% FBS, and cultured at 37°C and 5% CO_2 [122, 123]. For ICSI, the micromanipulation is performed in the fertilisation medium on a warm microscope stage at 37°C. Cumulus cells of mature oocytes are removed by hyaluronidase treatment and gentle pipetting. The injection needle used for rabbit sperm is of 5.0 μm inner diameter. After injection, the oocytes were transferred to embryo culture medium [123].

7.3. Cloning and transgenesis

Cloning involves the transfer of a nucleus from a multicellular embryo, foetal or adult cell into an enucleated metaphase II (MII) oocyte [124]. This oocyte has the ability to incorporate the transferred nucleus and support development of a new embryo. Cloning of embryos by nuclear transfer technology (NT) has been developed in several species [125–127]; the rabbit was a pioneering species in NT by introducing embryonic cells into enucleated oocytes, at the

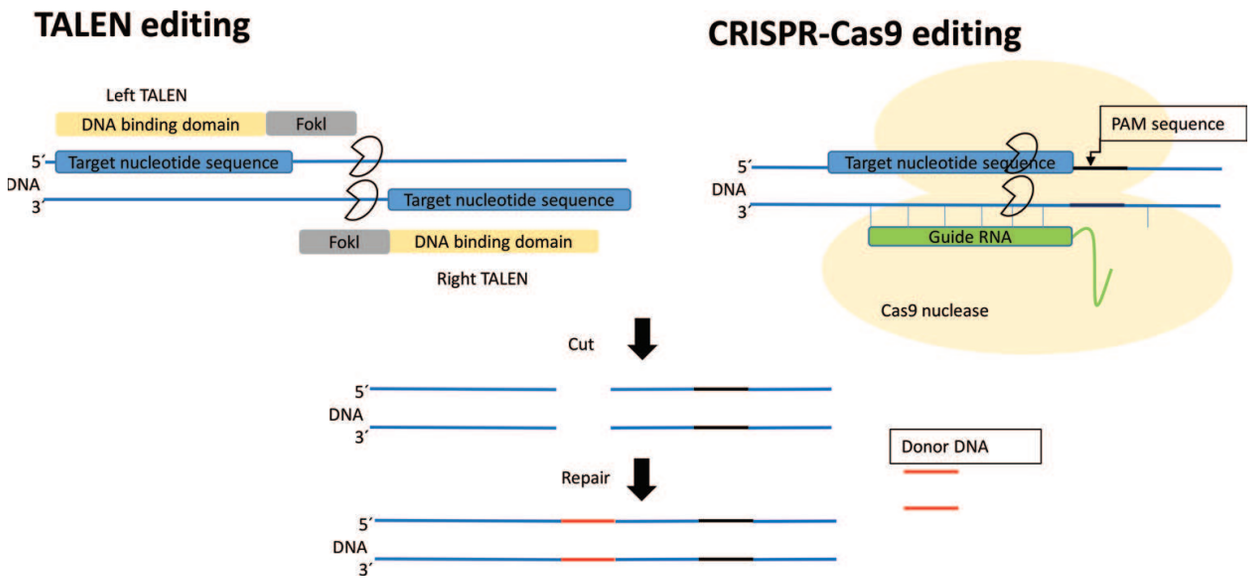

Figure 2. TALEN and CRISPR-Cas9 genome editing systems.

end of the 1980s [128, 129]. However, the use of highly differentiated somatic cells as nuclear donors remained a challenge. It was not until 2002 that live clones were generated from NT with freshly prepared adult rabbit cumulus cell [130]. And later, clones were produced from foetal [131] and adult fibroblast [132].

Overall, rabbit NT efficiency depends on the enucleation of the recipient oocyte, fusion of the transplanted nucleus to the enucleated oocyte, activation of the oocyte and reprogramming of the transferred nucleus. On enucleation, the visualisation of the MII is difficult, because of the presence of dark cytoplasmic granules. To overcome this problem, MII can be detected with low ultraviolet light, which allows the removal of MII and polar body under visible light using an enucleation pipette with a minimal volume of oocyte cytoplasm [133]. Enucleation rates vary from 60 to 90% [134, 135], and the age of the recipient oocyte plays an important role in successful NT [136, 137]. Briefly, for nuclear transplantation, single donor cell is introduced beneath the zona pellucida of the enucleated oocytes by micromanipulators. Electro-cell fusion (3.2 kV/cm, 20 μs and three pulses) is applied to fuse the donor cell with the cytoplasm of the reconstructed embryos [138].

Classically, gene transfer has been carried out by microinjection of DNA constructs into fertilised oocytes or by using viral factors. Notwithstanding, with the progress in molecular technology, embryos have been genetically modified using most recently developed tools including TALEN [14] and CRISPR-Cas9 [139, 140] (**Figure 2**).

Both techniques, cloning and transgenesis, have low applications in livestock production due to problems derived from detection of genetically superior animals and evaluation of the clones and the transgenic animals [141]. Some implications for the use of transgenic rabbits nowadays include to act as bioreactors [142] or model for detailed analysis of spermatogenesis [143], and more recently, to establish embryonic stem cell lines from blastocyst stage rabbit embryos cloned by somatic cell NT [138, 144].

8. Conclusion

There are clear advantages in the use of rabbit for embryo studies. The embryo development is similar to human, so rabbit is a suitable model for the application of embryo reproductive techniques. Important insights have been developed successfully in embryo recovery and transfer, cryopreservation, *in vitro* fertilisation, cloning or transgenesis. In addition, these findings have been applied to improve rabbit breeding.

Conflict of interest

The author declares that there is no conflict of interest.

Abbreviation list

BSA	bovine serum albumin
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats-associated nuclease Cas9
DPBS	Dulbecco's phosphate-buffered saline
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EMP1	higher epithelial membrane protein 1
FBS	foetal bovine serum
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotrophin
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilisation
LH	luteinizing hormone
MII	metaphase II
NT	nuclear transfer technology
OCT4	transcription factor octamer binding 4
$PGF_{2\alpha}$	prostaglandin F-2α
rhFSH	recombinant human FSH
TALEN	transcription activator-like effector nucleases

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