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Double Sperm Cloning: Could Improve the Efficiency of Animal Cloning

Dharmendra Kumar*, Meeti Punetha, Sonu Bansal, Sheetal Saini, Pradeep Kumar, R K Sharma, P S Yadav*

Animal Physiology and Reproduction Division, ICAR-Central Institute for Research on Buffaloes, Hisar-125001, Haryana, India

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ABSTRACT

Somatic cell nuclear transfer (SCNT) is an assisted reproductive technology that produces an individual from a single somatic cell nucleus and an enucleated oocyte. SCNT has presented an extensive application in the multiplication of superior germplasm, production of transgenic animals, the rescue of endangered animals, and therapeutic cloning. Despite that, the competence of SCNT remains very low due to poor reprogramming of somatic cells and epigenetic modifications. The somatic cells used in SCNT as donor nuclei lack some important components which normally present in sperm cells that are transmitted during fertilization. Sperm-derived factors play an important role in nucleus reprogramming during SCNT and embryonic development. Loss of such factors in somatic cells is reflected as an imperative reason for the abnormal development of SCNT embryos. To improve the efficiency of somatic cells' nuclear reprogramming during SCNT, double sperm cloning (DSC) could be an alternative approach to produce clone embryos/animals. Here, we focus on the recent advances of SCNT and their bottleneck and explored the possibility of cloning efficiency using sperm as donor nuclei.

Introduction

25 years ago 'Dolly the Sheep' was born on 5th July 1996, the first cloned animal generated using an adult somatic cell as a nuclear donor known as somatic cell nuclear transfer (SCNT), marked a seminal moment in the arena of developmental biology (Wilmut et al., 1997). This cutting-edge experiment challenged a long-standing central dogma of irreversible cellular differentiation that triumphed for over a century. It demonstrated the re-establishment of the pluripotent state in differentiated cells which provided unambiguous evidence of genomic equivalence between embryonic and somatic cells. The success of animal cloning led quickly to a succession of birth of genetically modified livestock derived from genetically modified somatic cells (Schnieke et al., 1997; Cibelli et al., 1998). Later, the SCNT technique was successfully applied for the production of more than 20 species (reviewed by Klinger and Schnieke, 2021) including farm animals such as cattle (Cibelli et al., 1998), goat (Baguisi et al., 1999), pig (Onishi et al., 2000,

*Corresponding author.
E-mail address: Dharmendra.Kumar@icar.gov.in(Dharmendra Kumar); psycirb@gmail.com(P S Yadav)
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Polejaeva et al., 2000), horse (Galli et al., 2003) and buffalo (Shi et al., 2007) using a different type of somatic cell as donor nucleus (Table 1). Recently, a simplified methodology for animal cloning was demonstrated with equivalent efficiency to traditional SCNT cloning termed handmade cloning (HMC, Vajta et al., 2001). Now, HMC poses itself as an alternative method for the generation of cloned offspring in many species including buffalo (Table 1). Animal cloning has been successfully used for the preservation of endangered species (Kim et al., 2007), restoration of elite animals (Selokar et al., 2014), and production of genetically modified animals (Alberio and Wolf, 2021). Despite remarkable achievements made over the past two and half decades, cloning suffers low efficiency and high pregnancy losses associated with incomplete genome reprogramming, making it an expensive technique to reproduce animals (Simmet et al., 2020; Wang et al., 2020). Here, we focus on the recent advances of SCNT and their bottleneck and have explored the possibility of cloning efficiency using sperm as donor nuclei.

The bottleneck of animal cloning

Animal cloning is categorized as micromanipulator-based cloning known as classical cloning and other is micromanipulator-free cloning called HMC. Animal cloning is a multi-step process that involves the generation of cytoplast via enucleation of an oocyte by either aspiration or bisection, transfer of donor cell nuclei, in which a donor cell is attached to an enucleated oocyte and fused by either an electrical pulse or a donor cell can be injected directly into the cytoplasm of the enucleated oocyte followed by activation of the reconstructed embryos and culture them in CO_2 incubator at 38.5°C for 6-8 days to attain transferable stage blastocyst (Fig. 1). The method used for classical cloning requires expensive and sophisticated equipment namely micromanipulator for oocytes enucleation and transfer of somatic cells into enucleated oocytes. Further, it requires a specialized trained operator to perform SCNT and make manipulation tools. To simplify this technique, Peura and colleagues first time established the concept of HMC in

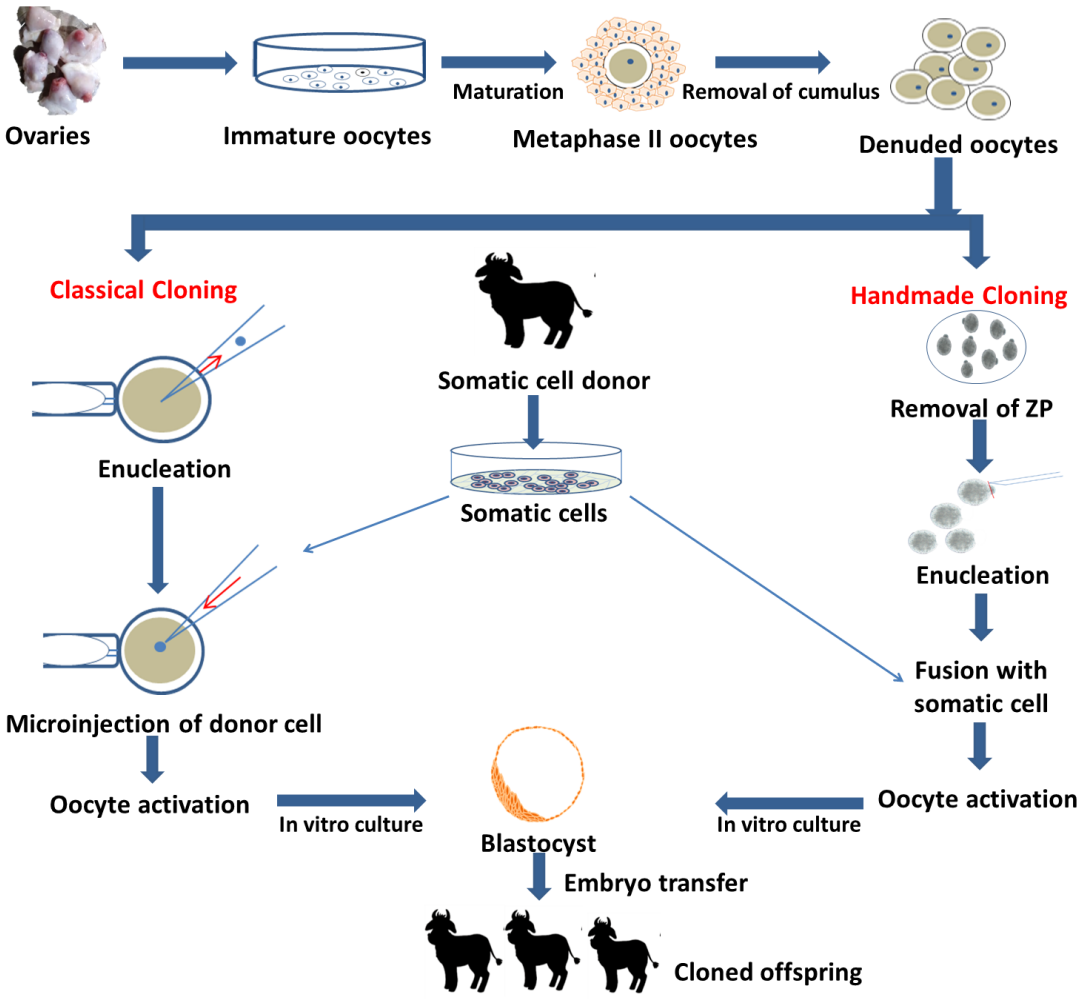

Fig. 1. Schematic representation of the procedure of classical and handmade cloning

which embryonic blastomeres were used as donors, and recipient oocytes were enucleated using microblade followed by fusion of two enucleated oocytes with one blastomere (Peura et al., 1998). Later, another group significantly refined and extensively used this approach of HMC due to simple and cost-effective (Vajta, 2007) and now it is widely used to produce cloned animals using differentiated somatic cells (Table 1).

Despite several significant technical improvements achieved in the selection of oocytes, donor cells, reconstruction of embryos, activation, and in vitro culture of cloned embryos, no major advancements have been made in nuclear transfer efficiency in terms of offspring development (Saini et al., 2018, Selokar et al., 2019, Loi et al., 2021). Mature oocytes present in metaphase II of the second meiotic division (MII) are the cytoplast donor of choice due to their highest embryonic developmental potential (Ozturk, 2020). Even then, developmental competence of cloned embryos was found lower, decrease pregnancy rate, abnormalities in fetal placental and large offspring syndrome are common concerns with cloned embryos transferred to animals (Keefer, 2015). The possible reasons for the common concerns of animal cloning are roughly classified into external factors: - such as removal of nucleus, electrofusion, and embryo culture, that may cause damage to the endoplasmic reticulum which subsequently induces cellular death (Rao et al., 2004). Further, toxic metabolites accumulated in the culture medium and changes in the osmotic pressure could trigger apoptosis of cloned embryos (Cordova et al., 2017), and, internal factors:- an abnormality in epigenetic and expression patterns in embryos during the cloning process, which are currently measured to be the main bottleneck for successful reprogramming during animal cloning (Matoba et al., 2018). Still many aspects of animal cloning remain unknown, and further in-depth studies are straightaway needed to investigate alternative approaches for somatic cell nuclear reprogramming.

Possible solution: Double sperm cloning

To improve the efficiency of somatic cells' nuclear reprogramming during animal cloning, double sperm cloning (DSC) could be an alternative approach to produce clone embryos/animals. DSC is a micromanipulator-based SCNT technique in which two sex-sorted (either XX or XY) capacitated sperm are injected into an enucleated oocyte. Afterward, the fertilized embryos are cultured in a humidified CO_2 incubator at 38.5°C to form blastocysts which could be transferred to synchronized females and produce cloned animals having normal diploid karyotype (Fig. 2). This strategy mimics the fertilization process which possesses superiority over other reprogramming approaches and proposes a promising method for animal breeding and regenerative medicine (Zhang et al., 2020). The various studies revealed that the oocyte has the magical power to reprogram differentiated cells into a pluripotent state, but it is more efficiently reprogramming sperm cells during fertilization than somatic cells by SCNT (Matoba et al., 2018). It could be due to adequate epigenetic modification of sperm chromatin which assists in early embryonic development while somatic cell nuclei do not have such modifications (Teperek and Miyamoto, 2013). The epigenetic memories encrypted in sperm chromatin specify the unique roles of sperm in transgenerational inheritance (Teperek and Miyamoto, 2013).

Table 1. Details of cloned farm animals produced using different approaches of cloning

Method of cloning	Species	Donor cell type	Embryo transfer	Calf born	Reference
Micromanipulator based SCNT	Sheep	Adult mammary epithelium	29	1	Wilmut et al., 1997
	Cattle	Fetal fibroblast	28	3	Cibelli et al., 1998
	Goat	Fetal fibroblast	47	1	Baguisi et al., 1999
	Pig	Adult granulosa cell	72	5	Polejaeva et al., 2000
	Horse	Adult skin fibroblast	17	1	Galli et al., 2003
	Buffalo	Adult granulosa cell	42	3	Shi et al., 2007
	Camel	Adult cumulus cells	139	1	Wani et al., 2010
Handmade cloning	Cattle	Adult Fibroblast	16	1	Tecirlioglu et al., 2003
	Horse	Adult fibroblasts	71	2	Lagutina et al., 2005
	Pig	Fetal fibroblast	58	10	Du et al., 2007
	Buffalo	Fetal fibroblast	18	1	Shah et al., 2009; Saha et al., 2013
	Sheep	Adult skin fibroblast	53	3	Zhang et al., 2013

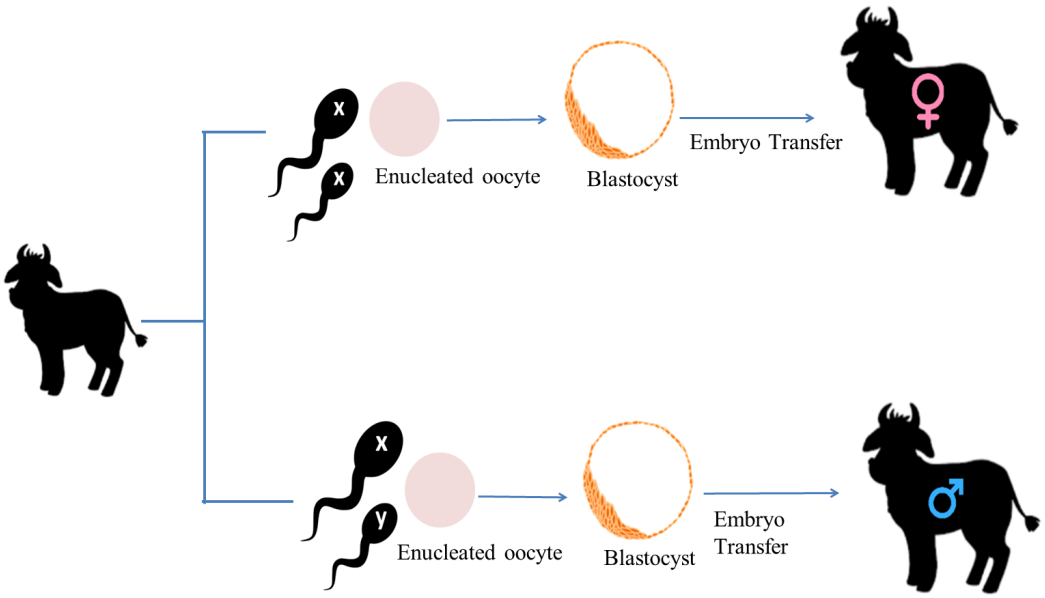

Fig. 2. Schematic representation of double sperm cloning by injection of two sperm containing either XX or XY into the enucleated oocytes to construct embryos, transfer to the synchronized recipient to produce the desired animal

During fertilization, a sperm enters into the cytoplasm of matured oocyte where sperm-specific factor phospholipase C zeta 1 (PLCZ1) induces oocyte activation through calcium influx and regulates epigenetics via the demethylation process, which triggers oocytes to exit the M phase and initiate the developmental progress (Castillo et al., 2018; Qu et al., 2020). But, in classical or in HMC cloning, due to the absence of PLCZ1 in somatic cells, SCNT reconstructed oocytes need to be activated by using chemical or electric pulse after fusion of denucleated oocyte with somatic cell to initiate the developmental progress. But in DSC, oocytes are activated by sperm factors which are more natural for embryo development as compared to the SCNT approach. Besides these facts, the DNA of the somatic cell is enclosed around histones, whereas the DNA of sperm is tightly packed by protamines, which condenses sperm DNA to one-sixth of the size of the mitotic chromosomes which make them transcriptionally inert (Jullien et al., 2010). Upon fertilization, transcriptionally inert and highly condensed sperm chromatin is remodeled into the decondensed and transcriptionally proficient chromatin of the male pronucleus (McLay and Clarke, 2003). Recently, it was documented that sperm is not the only delivery boy for paternal DNA but also carries thousands of different RNAs to oocytes at fertilization, facilitating first cell division and early embryonic development (Ostermeier et al., 2004; Hosken and Hodgson, 2014). These features of sperm chromatin are likely to support embryonic development after fertilization. However, the chromatin of somatic cells does not have such kind of fine-tune features to support embryonic development and that could be a possible reason to show the reprogramming abnormality in embryos generated through SCNT compared to fertilized embryos (Teperek and Miyamoto, 2013; Long et al., 2014). Therefore, DSC could be considered as a promising alternative to produce cloned embryos that could have a similar reprogramming process as occurs during natural fertilization.

Future perspectives and challenges

Animal cloning has many potential applications such as faster multiplication of superior breeding germplasm, protecting the genetic resources of commercially important species, rescuing endangered species, establishing animal models to investigate the pathogenesis of human diseases, and producing genetically modified xenograft organs for patient transplantation, etc (Wang et al., 2020). Despite several clones produced from many species of mammals still this technology is facing technical hurdles which limit the practical utility. In the last decade's great effort have been made to improve cloning efficiency up to 8-10% of transferred embryos resulting in live offspring (Czernik et al., 2019; Shyam et al., 2020; Yadav et al., 2020). But, due to lack of understanding of somatic cell reprogramming and factors influencing reprogramming has hindered improvements in cloning efficiency.

Alternate, in DSC sperm cells, being used instead of the somatic cell which undergoes reprogramming process seems to be more natural as occurs during in vitro fertilization. Earlier studies support the notion of DSC in which

enucleated oocytes were successfully fertilized in vitro using male nuclear genetic material called an androgenetic embryo and this has been successfully demonstrated in the mouse (Kono et al., 1993). Later, bovine diploid androgenetic embryos were able to produce blastocysts, and following the transfer of these embryos to the surrogate, a pregnancy was established and maintained their pregnancy up to day 28 (Lagutina et al., 2004). Matsukawa et al. (2007) observed no significant difference in early cleavage and morula stage embryos generated through diploid androgenetic and IVF but the blastocyst formation rate was significantly lower in sheep. To date, no live animal had been born using DSC, perhaps due to epigenetic modifications. But, these studies showed that the enucleated oocytes are completely capable of reprogramming sperm and can be able to produce blastocysts. But, on the other hand, several challenges are also associated with this technique to implement it into reality. Most common, for normal embryonic development both maternal and paternal genomes are required which is lacking in DSC. Further, genome imprinting (means some imprinted genes are only expressed from their maternal allele, while others are only expressed from their paternal allele) occurs during embryonic development, and during this phenomenon certain genes to be expressed or not, depending on whether they are inherited from the mother or the father. In DSC, it could be a challenge to correctly reprogram two sperm from paternal genomes and two sets of Y chromosome-bearing embryos could be able to regulate embryonic development due to lack of the X chromosome.

If these challenges of DSC are successfully overcome, this would provide a great tool for increasing the efficiency of animal cloning. This technique could also be useful for animal breeding in which male animals can be able to produce male and female by either using two sperm (X and Y) or two X sperm from one male individual. Furthermore, it could help establish a new animal breeding system by assembling the sperm of different sexes from diverse breeds. Further in-depth studies are required to establish the fact of the influence of sperm and sperm factors in epigenetic inheritance and development of SCNT embryos and the possibility to establish a pregnancy.

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Conflict of interest

We have no conflict of interest.

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