Biotechnological bases of the development of cloned pig embryos

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The term 'clone' in animal biotechnology refers to an organism derived from non-sexual reproduction, which is both a direct offspring and a genetic copy of the parent organism. To date, the pig appears to be the most interesting object in cloning research. Somatic cell nuclear transfer in pigs has a wide range of potential applications in various fields of human scientific and economic activities. However, the efficiency of producing cloned embryos in swine is still lower than that of other livestock species, in particular horses and cattle. Somatic cell nuclear transfer is a technically complex multi-stage technology, at each stage of which the pig oocytes, which are more susceptible to changes of surrounding conditions, are affected by various factors (mechanical, physical, chemical). At the stage of oocyte maturation, changes in the cell ultrastructures of the ooplasm occur, which play an important role in the subsequent nuclear reprograming of the transferred donor cell. Before transfer to the oocyte donor somatic cells are synchronized in the G0/G1 stage of the cell cycle to ensure the normal ploidy of the cloned embryo. When removing the nucleus of pig oocytes matured *in vitro*, it is necessary to pay attention to the problem of preserving the viability of cells, which were devoid of their own nuclear material. To perform the reconstruction, a somatic cell is placed, using micro-tools, in the perivitelline space, where the first polar body was previously located, or in the cytoplasm of an enucleated oocyte. The method of manual cloning involves the removal of the oocyte nucleus with subsequent fusion with the donor cell without the use of micromanipulation techniques. The increased sensitivity of oocytes to the environmental conditions causes special requirements for the choice of the system for *in vitro* culture of cloned pig embryos. In this work, we have reviewed the modern methods used for the production of cloned embryos and identified the technological issues that prevent improving the efficiency of somatic cloning of pigs.

Key words: domestic pig; *Sus scrofa domestica*; oocytes; *in vitro*; somatic cell nuclear transfer; fusion; activation; cloned embryo.

Биотехнологические основы получения клонированных эмбрионов свиней

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Термин «клон» в биотехнологии животных обозначает организм, полученный в результате неполового размножения, который одновременно является прямым потомком и генетической копией родительского организма. На сегодняшний день домашняя свинья (*Sus scrofa domestica*) представляется наиболее интересным объектом в исследованиях по клонированию. Клонирование свиней имеет широкий спектр потенциальных возможностей использования в различных областях научной и хозяйственной деятельности человека. Тем не менее эффективность получения клонированных эмбрионов свиней все еще остается ниже, чем других видов сельскохозяйственных животных, в частности лошадей и крупного рогатого скота. Соматическое клонирование – сложная многоступенчатая технология, на каждом этапе которой более восприимчивые к изменениям окружающих условий ооциты свиней испытывают неблагоприятные воздействия различных по своей природе факторов (механические, физические, химические). На этапе созревания ооцитов происходят изменения клеточных ультраструктур ооплазмы, которые играют важную роль в последующем репрограммировании ядра пересаженной донорской клетки. Донорские соматические клетки перед переносом в ооцит синхронизируют в стадии G0/G1 клеточного цикла с целью обеспечения нормальной плоидности клонированного эмбриона. При удалении ядра у созревших *in vitro* ооцитов свиней следует обращать внимание на проблему сохранения жизнеспособности клеток после извлечения собственного ядерного материала. В ходе реконструирования соматическую клетку с помощью микроинструментов помещают в перивителлиновое пространство, где ранее находилось первое

полярное тельце, или в цитоплазму энуклеированного ооцита. Метод ручного клонирования (handmade cloning) предполагает удаление ядра ооцита с последующим слиянием с донорской клеткой без помощи микроманипуляционной техники. Повышенная чувствительность ооцитов к факторам окружающей среды обусловливает особые требования к выбору системы *in vitro* культивирования клонированных эмбрионов свиней. В рамках настоящего обзора проведен мониторинг современных методов, используемых при получении клонированных эмбрионов, выявлены технологические особенности, препятствующие повышению эффективности метода соматического клонирования свиней.

Ключевые слова: домашняя свинья; *Sus scrofa domestica*; ооциты; *in vitro*; соматическое клонирование; слияние; активация; клонированный эмбрион.

Introduction

The ability of the somatic cell nucleus, which is transferred to the enucleated oocyte, to be reprogramed is one of the most important phenomena of biological science, the discovery of which made it possible to obtain reconstructed embryos and cloned animals. In practice, this was implemented in June 1996 by a group of Scottish researchers led by Ian Wilmut, who reported the birth of the first cloned mammal (Dolly the sheep) with hereditary material identical to another adult animal, which was produced using differentiated cells (breast epithelium) (Wilmut et al., 1997). The serious interest of the scientific community, caused by the revolutionary breakthrough in this field of reproductive technologies, has led to the cloning of more than 20 mammalian species (Singina et al., 2014).

The production of cloned piglets was conducted for the first time in 2000 simultaneously by two research groups from the United States and Japan (Onishi et al., 2000; Polejaeva et al., 2000). One of the main application fields of cloning technology is the use of genetically modified pigs as models for the study of human diseases and organ donors for xenotransplantation (Betthauser et al., 2000). Currently, such animals are used in preclinical testing of preventive or therapeutic medicines (Liu et al., 2008), testing the toxicity of drugs, studies of functional genomics (Wimmers et al., 2010). Production of genetically modified pigs is a potential tool for reducing physiological and immunological barriers to obtaining and transplantation of donor organs. Another equally important area of practical application of cloning is the production of animals with desired parameters of productivity by copying boars and sows with high breeding values.

At somatic cloning, instead of its own chromosomal material, the oocyte (cytoplast) acquires the nucleus of a somatic cell (karyoplast) from the animal, a genetic copy of which is to be obtained. The main stages of the cloning technology included the preparation (*in vitro* maturation) of the recipient oocyte and donor cells, removing nuclear material from the mature oocyte, reconstruction of the cytoplast obtained (fusion with karyoplasts), activation of the reconstructed oocyte and culture of the cloned embryo (Niemann et al., 2011; Simões, Santos, Jr., 2017).

Oocyte maturation

The ability of the oocyte to initiate successful development into the cloned, parthenogenetic embryo and the embryo after *in vitro* fertilization is largely determined by its maturation. Cytoplasmic maturation includes modifications of the cytoplasm, in particular, redistribution of organelles, changes in cytoskeletal dynamics, micro- and macromolecular alterations

(Ferreira et al., 2009). Nuclear maturation involves modifications of chromatin during the period from the destruction of the germinal vesicle to the metaphase of the second meiotic division (MII) (Marteil et al., 2009).

Unlike *in vitro* culture of other animal species, that of pig oocytes relies on a two-phase maturation protocol is used, which makes it possible to increase their competence to fertilization or to artificial activation. At the first stage, the proteins that are necessary for the early embryonic development are produced in the oocyte under the action of hormones. The second period of maturation takes place without external signals and includes the division of the nucleus and the structuring of cell organelles. The matured oocytes at the stage of metaphase II with the visualized first polar body are usually used as sources of cytoplasts for cloning (Hardarson et al., 2000). In pigs, the optimal duration of *in vitro* maturation of oocytes varies according to different studies in the range from 24 to 44 hours (Zhang et al., 2006; Sugimura et al., 2010).

Preparation of donor somatic cells

The efficiency of reprogramming after transfer of somatic cell nuclei depends on a number of factors, including the type, the number of passages and the stage of the cell cycle of the donor cell (Enright et al., 2003; Yang et al., 2007). Particular importance for the success of cloning has the level of differentiation of the donor cell (Jaenisch, 2012). To date, cloned swine embryos have been produced using different types of somatic cells, such as fetal fibroblasts, skin fibroblasts, neural stem cells, cumulus cells, granulosa cells and breast epithelial cells (Verma et al., 2000; Cervera et al., 2009; Zheng et al., 2009).

A necessary condition for nucleus reprogramming and for the successful development of the cloned embryo is the synchronization of the cell cycle of the cytoplast and karyoplast. With this aim, the donor cells of all types are normally subjected to artificial arrest in phase G0/G1 prior to micromanipulation procedures (Boquest et al., 1999). The transfer of somatic cell nuclei, of which DNA has not been replicated yet, to the oocyte at metaphase II reduces the risk of chromosomal abnormalities and ploidy disorders of cloned embryos (Campbell et al., 1996). Coordination between the cell cycles of oocytes and somatic cells of pigs is ensured by limitation of serum content or by contact inhibition. Despite the fact that the first cloned piglets were obtained after transfer of donor cell nuclei after the limitation of serum content (Polejaeva et al., 2000), this method is characterized by negative effects. One, for example, is the initiation of apoptotic phenomena along with increased DNA fragmentation at the culture of swine fibroblasts in a medium with a low serum content (Kues et al.,

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2002). The most commonly used method for synchronizing the cell cycle in a pig cloning procedure is contact inhibition of somatic cells in confluence (Park et al., 2010). In addition, chemical antimitotic agents (mimosine, rapamycin, roscovitine etc.) have become more broadly applied in the recent years (Vacková et al., 2003; Park et al., 2010; Hyun et al., 2016).

Removal of the nuclear material of oocytes

Complete removal of nuclear material from the mature oocyte eliminates the possibility of aneuploidy, reduces the risk of spontaneous parthenogenetic activation and abnormal development of the cloned embryo. However, due to the presence of fat inclusions in the cytoplasm, the nuclei of swine oocytes are not visualized under an inverted microscope without prior staining of DNA with fluorochrome dyes in combination with ultraviolet visualization (Tatham et al., 1995). The disadvantage of the classical technique is the damage of mitochondrial DNA and ooplasmic organelles by ultraviolet light. Therefore, an alternative method of blind enucleation is used, which is based on the assumption that metaphase chromosomes in mature oocytes are attached to the mitotic spindle, and their position is determined by an indirect sign, the localization of the first polar body (FPB). Thus, after removal of the FPB and part of the cytoplasm, the mature oocyte also loses the metaphase plate (McGrath, Solter, 1983). The disadvantage of this approach is the migration of the FPB relative to the metaphase plate (Hardarson et al., 2000; Miao et al., 2004) due to aging of mature oocytes (Miao et al., 2009).

Another method of enucleation is based on the cutting of the zona pellucida close to the FBP with subsequent compression of the oocyte by a glass needle to remove a small volume of the ooplasm. The removed ooplast remains intact and is therefore convenient for DNA staining to confirm removal of the mitotic spindle without exposing the oocyte to the harmful effects of ultraviolet light. The disadvantage of the procedure is its complexity and difficulty to control the volume of the removed cytoplasm. The compression method, which is associated with removal of the metaphase plate by releasing part of the cytoplasm of the mature swine oocyte *in vitro*, is a time-consuming procedure, which is characterized by a higher rate of degeneration compared to the classical method (Lee et al., 2008).

In 2002, a group of researchers reported on the production of cloned piglets after chemical enucleation (Yin et al., 2002). Chemical enucleation is based on the use of topoisomerase II inhibitors blocking the onset of telophase II, resulting in the expelling of mitotic spindle to the cell boundary (Fulka, Mur, 1993; Savard et al., 2004). A short exposition of pig oocytes at metaphase II in 0.4 mg/ml demecolcine – a chemical agent causing the depolymerization of microtubules – in the presence of 0.05 M solution of sucrose causes membrane protrusion containing a condensed chromosome mass, which can be easily removed through aspiration (Kawakami et al., 2003).

The reconstruction of the oocytes with the aim of producing cloned embryos

The traditional method of reconstruction involves the transplantation of a donor cell into the perivitelline space of the recipient oocyte. After fixation of the oocyte with a holding pipette, the somatic cell is transferred to the oocyte through a hole or incision formed during enucleation using a transfer pipette (Popova et al., 2009). At intracytoplasmic injection, the karyoplast is introduced directly into the cytoplasm of the enucleated oocyte, bypassing the perivitelline space (Onishi et al., 2000; Lee et al., 2003; Kong et al., 2008). The factor limiting the use of this method of producing cloned embryos is the incomprehensible mechanism of destruction of the donor cell membrane in the cytoplasm of the recipient oocyte. In the case of maintaining the integrity of the membrane of the transferred cell, and thus, not passing the reprogramming of its nucleus during intracytoplasmic injection, embryos do not develop further (Lee et al., 2003).

Cloned embryos can be produced without the use of micromanipulation techniques (Vajta et al., 2005). In the framework of the method of manual cloning (Hand Made Cloning, HMC), oocytes at metaphase II are released from the zona pellucida with the help of the enzyme pronase, cut by a microscalpel into two equal parts, which are stained with the vital fluorescent dye Hoechst 33342 in order to accurately detect the location of the metaphase plate. Two halves of oocytes that do not contain chromatin are selected for fusion with the somatic cell (Vajta et al., 2001). The successful use of this method to produce cloned pigs has been reported (Kragh et al., 2004). The limiting factor in ensuring the reproducibility of the HMC technology is the need to set up adequate conditions for culture of zona pellucida free embryos. The use of two mature oocytes to produce one reconstructed embryo by the HMC method results in the loss of 50 % of the initial material. The presence in the HMC embryos of three genotypes of mitochondrial DNA potentially increases the level of mitochondrial heteroplasmy (Oback et al., 2003).

The development of a cloned embryo is impossible without the fusion of the recipient oocyte with the transferred donor cell. In the practice of somatic cloning, there is a technique called 'electrofusion', which is fusion of the cytoplast and the karyoplast with the use of the phenomenon of electric breakdown of the membranes of the contacting pair in a pulsed electric field. The procedure of electrofusion involves the use of two types of electrical signals, inhomogeneous AC pulses and rectangular DC pulses. The electrical oscillations that occur during electrofusion cause excessive heating of the medium with high conductivity. For this reason, buffer solutions with low electrical conductivity, which can cause the formation of dielectric potentials within the cells to facilitate intercellular contact, are chosen for electrofusion. Mainly, the Zimmerman medium in various modifications is used for cell fusion (Robl et al., 1987; Nickoloff, 1995). The physical factors affecting the effectiveness of the fusion of the cytoplast and karyoplast include voltage, duration and repeatability of the electric field pulse. As a rule, the effect of dielectrophoretic forces on the cells is caused by the guidance of a high-frequency (1–3 MHz) sinusoidal AC field of low amplitude (~100–300 V/cm). Cells that come into contact with plasma membranes are fused by one or two rectangular pulses of high voltage DC (1–10 kV/cm) with a duration of 10–50 μs (Cao et al., 2008). The literature is rich in methods of cell electrofusion, which is due to both the technical characteristics of the devices used for these purposes, and the particularities of different types of cells.

Activation of reconstructed oocytes

In the process of cloning, the enucleated oocytes after fusion with the diploid donor cell are activated for further development (Campbell, 1999). For cloning of pig embryos, the activation signals used are DC pulses (Im et al., 2004; Hölker et al., 2005), the chemical agents ionomycin, ionophore Ca^{2+} A23187 (Yin et al., 2002; Hyun et al., 2003; Garcia-Mengual et al., 2008), and thimerosal in combination with dithiothreitol (Im et al., 2006; Whitworth et al., 2009). Among the existing methods, electrostimulation is the most commonly used for the production of cloned embryos. It is reported that the magnitude of the emission of Ca^{2+} ions is proportional to the number and magnitude of pores formed during electrical stimulation, and depends on the number and duration of electrical pulses and electric field strength (Fissore, Robl, 1992). One DC pulse leads to a single mobilization of intracellular calcium reserves. In contrast, the strategy of multiple electrical pulses (2–3) stimulates the generation of a long series of Ca^{2+} oscillator peaks, increasing many times the concentration of these cations in the ooplasm (Fissore et al., 1999).

In studies on somatic cloning of pigs (Verma et al., 2000; Zhu et al., 2002), the multiplication of electrical impulses positively correlated with the high level of development of reconstructed oocytes to the stage of morula and blastocysts. The specific feature of obtaining cloned pig zygotes is the simultaneous electroactivation and electrofusion of the enucleated oocyte and somatic donor cells (Hyun et al., 2003; Lee et al., 2003; Skrzyszowska et al., 2008). Transgenic cloned pig embryos produced from the oocytes reconstructed using fetal fibroblasts, which were activated by electric pulses and by subsequent incubation in a solution of ionomycin, were inferior in terms of development to the blastocyst stage to the oocytes fused with the somatic cell and activated simultaneously (Hyun et al., 2003). Simultaneous fusion and activation of pig oocytes led to the improvement of embryonal development of oocytes reconstructed using fetal fibroblasts compared to the use of shared electrical and chemical activation (Samiec, Skrzyszowska, 2010). At the same time, it should be noted that no clear and reproducible unified protocols of electrical activation – like those developed for other mammalian species – have yet been developed for pigs, and the parameters of the electric field (the number of electrical impulses, the duration of electrical stimulation and the interval between pulses) significantly vary (Koo et al., 2005; Cervera et al., 2010; Peng et al., 2013).

One of the approaches for increasing the effectiveness of artificial activation is the by using stimuli that increase the concentration of Ca^{2+} ions in the cytoplasm, in combination with factors that inhibit the activity of the maturation promoting factor (Presicce, Yang, 1994; Cheng et al., 2007). Electrical stimulation in conjunction with postactivation culture in the presence of 6-dimethylaminopurine (6-DMAP) or cycloheximide resulted in an increase in the yield of blastocysts compared to conventional electroactivation (Kim et al., 2005; Im et al., 2006). Culture in the presence of 6-DMAP after electrical stimulation leads to an increase in the oscillations of intracellular Ca^{2+} ions, which is observed throughout the entire period of postactivation of activated pig oocytes (Im et al., 2006, 2007). The combination of the chemical agents 6-DMAP + cytochalasin B improved the parthenogenetic development of embryos to the blastocyst stage, but these blastocysts were characterized by a reduced number of cells. Postactivation of pig oocytes in 6-DMAP increased the yield of blastocysts on day 7 of culture in comparison with the incubation in the presence of cycloheximide or cytochalasin B (Grupen et al., 2002).

Culture of cloned embryos

Activated reconstructed oocytes, which have started their embryonic development, are cultured *in vitro* in special media until transplantation to the recipient animal. As is known, when comparing *in vivo* and *in vitro* produced embryos, the latter have a reduced potential to embryonic development (Uhm et al., 2009; Gil et al., 2017). On the other hand, it has been shown that cloned embryos are more sensitive to culture conditions compared to parthenogenetic embryos (Heindryckx et al., 2001). These observations, along with the increased susceptibility of embryos to environmental factors when they are maintained outside the sow's body, including temperature fluctuations, set special requirements to the systems of *in vitro* culture of reconstructed pig oocytes. Culture media should provide overcoming a developmental arrest of 4-cell pig embryos, activation of the own genome and *in vitro* development to advanced stages of embryogenesis (morula and blastocyst).

Different media such as NCSU-23 and NCSU-37, BECM-3, PZM-3, PZM-4 and PZM-5 are used for culture of pig embryos (Dobrinsky et al., 1996; Yoshioka et al., 2002; Im et al., 2004). NCSU-23 is a traditional and initially widely used medium for the development of *in vitro* fertilized and cloned swine embryos. On the other hand, PZM-3, of which the composition is similar to the environment of the pig oviduct, allows increasing the ratio of embryos developed to the blastocyst stage at the 7th day of culture, and the number of cells of inner cell mass compared to NCSU-23 media (Im et al., 2004). Cloned embryos are known to be more susceptible to apoptotic degeneration than embryos developed after *in vitro* fertilization of intact oocytes (Ju et al., 2010). It has been shown that culture in PZM-5 developed by Japanese researchers in 2004 (Suzuki et al., 2004) reduces the apoptotic index in the pig embryos, produced by nuclei transfer of somatic cells (Yamanaka et al., 2009).

However, it should be noted that, despite the local successes in the development and application of new culture media, currently the conditions for embryo culture of this animal species are still not optimal. That is why, according to scientists from Canada (Cordova et al., 2017), in most of the experiments on nuclei transfer of somatic cells, the reconstructed embryos are transplanted to the host animals prior to or at early stages of cleavage. For example, a more efficient transplantation of embryos to the recipient sow was shown at hours 4–6 following activation of reconstructed oocytes than at hours 20–24 (1–2 cell embryos), the increase in efficiency being seen as an increase in pregnancy rate and the overall effectiveness of cloning (Shi et al., 2015). On the other hand, *in vitro* incubation to the morula and blastocyst stage allows controlling each division-cleavage with the possibility to select high-quality embryos with the greatest potential for further development (Jin et al., 2018). As confirmation of this

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suggestion, the extension of the duration of *in vitro* culture of cloned embryos from 20 to 40 hours increased the number of pregnant recipients by 13 %, and from 22 to 120 hours by 61.8 % (Ju et al., 2010; Rim et al., 2013).

Conclusion

Analysis of literature data has shown that the cloning technology allows the researcher to create cloned pig embryos and to obtain viable offspring after transfer to the recipient animal. While some stages of cloning have become routine in many aspects (fusion, enucleation, reconstruction), others are still uncertain and require additional research (oocyte maturation, donor cells and embryo culture). It is also obvious that it will be necessary to pay special attention to studying the mechanisms of somatic cell reprogramming and regulation of oocyte quality – for using quality oocytes as sources of cytoplasts.

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