Selected issues concerning biotechnology of farm animals breeding – a review*

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Achievements made in the last decades in biotechnology of farm animals breeding allowed to develop this branch of science, improve the animal breeding and also make progress in human and veterinary medicine. The most important biotechniques are: in vitro embryo production including maturation of oocytes, fertilization and embryos culture; cloning by somatic cell nuclear transfer and its modification as: interspecies cell nuclear transfer; nuclear transfer of embryonic stem cells and cloning by embryonic cell nuclear transfer. Also the important biotechniques are production of transgenic animals by microinjection or transgenic somatic cell nuclear transfer; xenotransplantation and production of chimaeras (using embryonic and somatic cells). Among all these biotechniques, the most beneficiary are those involved or used in biomedicine, as they form the link between farm animals and humans.

KEY WORDS: chimaeras / cloning / farm animals / in vitro embryos / transgenic animals / xenotransplantation

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Abbreviations

- ECNT – Embryonic Cell Nuclear Transfer
- EGCs – Embryonic Germ Cells
- ESCs – Embryonic Stem Cells
- faESCs – farm animal Embryonic Stem Cells
- ICSI – Intracytoplasmic Sperm Injection
- iSCNT – interspecies Somatic Cell Nuclear Transfer
- IVC – In Vitro Culture
- IVF – In Vitro Fertilization
- IVM – In Vitro Maturation
- IVP – In Vitro Production
- NTESC – Nuclear Transfer of Embryonic Stem Cell
- MOET – Multiple Ovulation and Embryo Transfer
- OPU – Ovum Pick Up
- SCNT – Somatic Cell Nuclear Transfer
- SMGT – Sperm Mediated Gene Transfer
- SSCc – Spermatogonia Stem Cells
- TSCNT – Transgenic Somatic Cell Nuclear Transfer

Biotechnology of farm animal breeding is one of the fields of science that has provided the most spectacular discoveries in the last two decades, from the sheep Dolly, created by the somatic cloning technique, to transgenic pigs that can be organ donors for humans, and to animal bioreactors producing human therapeutic proteins in milk [reviews: Niemann et al. 2005, Campbell et al. 2007, Robl et al. 2007].

Biotechnology of farm animal breeding includes many technologies, of which the most important are: in vitro embryos production, cloning, and production of transgenic animals and chimaeras.

In vitro production of farm animal embryos

Obtaining embryos in vitro is one of the most crucial techniques used in biotechnology of farm animal breeding, since it allows other processes to take place. It is multi-stage procedure involving in vitro maturation (IVM) of oocytes, in vitro fertilization (IVF) and in vitro culture (IVC) of embryos.

In the first stage, immature oocytes can be collected either from donor live animals (ovum pick up – OPU, laparoscopy, laparotomy) or from ovaries of slaughtered animals.
Isolated oocytes arrested at the prophase of the first meiotic division (MI) mature in vitro to reach the metaphase of the second meiotic division (MII). Timing of oocyte maturation differs depending on the species. Ovine, bovine and goat oocytes reach MII stage after 24 h, whereas horse oocytes within 24-30 h of culture. The longest maturing are the pig oocytes, reaching MII between 40 and 48 h of culture [reviews: Abeydera 2002, Cognie 2003, Hoshi 2003, Squires 2003].

Next stage, the IVF, requires incubation of mature oocytes with capacitated sperm. During this process, oocytes become fertilized. Timing of IVF is similar for most of the farm animals, taking between 18 and 20 h. There are, however, interspecies differences in timing of pronuclei formation and start of replication [reviews: Abeydera 2002, Cognie 2003, Hoshi 2003, Squires 2003]. In pigs, high frequency of polyspermy is observed both in vitro and in vivo leading to production of polyploid embryos [review: Abeydera 2002]. In horses, IVF proved to be very complicated and problems-bearing procedure. Thus, intra cytoplasmic sperm injection (ICSI) technique is preferably used for this species [Colleoni et al. 2007].

Third stage, IVC, involves in vitro culture of fertilized oocytes (zygotes), usually up until the blastocyst stage. Pig zygotes reach that stage in the shortest time, 144 h of culture. Ovine, bovine and goat blastocysts develop after 168 h. Horse embryos develop to the blastocyst the longest, between 168 and 192 h of culture [reviews: Abeydera 2002, Cognie 2003, Hoshi 2003, Squires 2003]. Once the embryos have reached the blastocyst stage, they can be transferred to the recipient animals or frozen/vitrified for using later. Freezing and vitrification allows creation of embryo and also oocyte banks [Gajda and Smorąg 2009].

Embryo transfer resulted in birth of living sheep [Gandolfi and Moor 1987], cattle [Lu et al. 1988], pig [Mattioli et al. 1989] and goat [Crozet et al. 1993]. The first horses were born after transfer embryos resulting from non in vitro matured oocytes - 1991 [Palmer et al. 1991].

Currently, in vitro embryo production is widely used both for scientific and commercial purposes.

This procedure helps to understand processes of oogenesis and spermatogenesis, fertilization, early embryonic development and formation of species-specific organs. It also helps to study mother-foetus interactions. Furthermore, it allows to analyse developmental potential of embryos, including the pattern of gene expression, epigenetic modifications and cytogenetic disorders during the development [reviews: Duszewska and Reklewski 2007, Galli and Lazzari 2008]. Early stages of bovine embryo development show many similarities with human embryos. Therefore, bovine embryos are used as a model organism [Niemann and Wrenzycki 2000].

In animal breeding, in vitro embryo production (IVP) is used for commercial purposes as an alternative to insemination or MOET (Multiple Ovulation and Embryo Transfer) technique, which comprises induction of multiple ovulation and embryo transfer to recipients. IVP of embryos allows using females only as donors.
of the oocytes without necessity of application further stages of MOET programme. Furthermore, pregnant or reproductively immature females can be used as donors in this technique. Recently, the IVP is used increasingly to deal with infertility issues in farm animals [reviews: van Wagendonk et al. 2000, Duszewska and Reklewski 2007, Galli and Lazzari 2008].

Finally, in vitro embryo production allows cloning and production of transgenic animals and chimaeras.

**Cloning**

Cloning technique involves transfer of somatic nuclei into enucleated oocytes (Somatic Cell Nuclear Transfer, SCNT) [review: Campbell et al. 2007]. The donor cells can be isolated during pre- or postnatal stage of animal development. Numerous types of somatic cells are used as donors in somatic cloning: foetal fibroblasts, adult fibroblasts, granulosa cells, hepatocytes, lymphocytes and many more [review: Campbell et al. 2007].

The recipient cell in somatic cloning is mature oocyte with removed metaphase II plate. It is worth to notice though, that enucleated zygotes or 2-cell stage embryos can also serve as the recipient in this technique [review: Campbell et al. 2007].

First animal obtained by somatic cloning was in 1997 sheep Dolly [Willmut et al. 1997]. Since then, SCNT was used successfully for cloning cattle [Cibelli et al. 1998], pig [Polejaeva et al. 2000], goat [Baguisi et al. 1999] and in 2000 a horse [Galli et al. 2003].

A very particular donor cells are embryonic stem cells (ESCs). They are pluripotent, which means they can differentiate in all three germ layers of the embryos (ectoderm, mesoderm and endoderm) and also in the germline. ESCs should exhibit molecules that support their pluripotency [Ralston and Rossant 2010]. Cloning procedure using ESC is called Nuclear Transfer-derived Embryonic Stem Cell (NTESC). However, although ESCs were derived for humans and some laboratory animals, derivation of farm animal Embryonic Stem Cells (faESCs) is still unsuccessful [Beyhan et al. 2007]. There were numerous attempts to produce such cells, and series of analyses indicate that faESCs were produced indeed. However, these cells occurred unable to produce germ-line chimaeras [Keefer et al. 2007, Brevini et al. 2008, Galli and Lazzari 2008]. The alternative to faESCs could be embryonic germ cells (EGCs) and spermatogonia stem cells (SSC) – Brevini et al. [2008].

SCNT is a procedure of cloning within the same species. However, there is a feasibility of interspecies cloning (interspecies Somatic Cell Nuclear Transfer -iSCNT) as well. The cloned animals have already been produced between closely related species like domestic cattle (Bos taurus) and wild ox (Bos gruniens) – Lanza et al. [2000], sheep (Ovis aries) and mouflon (Ovis orientalis musimon) – Loi et al. [2001] as well as between domestic cattle (Bos taurus) and yak (Poephagus gruniens) [Li et al. 2006a, 2006b].
Although no cloned animals were produced so far by iSCNT between non-related species, the work on this procedure is going on [review: Beyhan et al. 2007]. For example, iSCNT was attempted using somatic cells of domestic fowl as donors and bovine oocytes as recipients, resulting in embryos at blastocyst stage [Liu et al. 2004]. Interspecies human-bovine cloning studies were also attempted, however due to ethical reason the resulting blastocysts were not transferred to recipient mothers [Beyhan et al. 2007].

Another issue is using SCNT technique to produce transgenic animals (Transgenic Somatic Cell Nuclear Transfer – TSCNT). In this case, somatic donors’ cells are first transfected with appropriate constructs, resulting in transgenic cell lines [review: Niemann et al. 2005, Robl et al. 2007].

In all aforementioned examples of cloning techniques, somatic or embryonic stem cells were used as donors. However, embryonic cell from early stage embryo can also be used as a source of genetic material for cloning. The technique is called Embryonic Cell Nuclear Transfer (ECNT) and is the oldest method of cloning animals, initiated in 1960s. In 1980s, ECNT allowed to produce clones for most of the farm animals – sheep, pig and cattle [Kues et al. 2004].

Somatic cloning allows to broaden our knowledge about nuclear-cytoplasmic interactions in reconstructed embryos, epigenetic modification as well as the pre- and postnatal development of such clones [review: Campbell et al. 2007, Heyman et al. 2007, Galli et al. 2008, Loi et al. 2008, Palmieri et al. 2008].

Cloning enables multiplication of valuable genotypes. Hence, the technique is used to preserve individuals of outstanding properties, like high production of milk, or high quality of meat. In veterinary medicine, cloning is used to multiplication genotypes of individuals with high resistance to diseases. For example, thanks to this technique the mastitis-resistant cows were produced. Furthermore, somatic cloning can be helpful in assisted reproduction, remedying infertility. It is especially important in case of breeding highly efficient individuals, usually resulting from inbreeding and hence often showing low fertility [reviews: Kues and Niemann 2004, Niemann et al. 2005, Campbell et al. 2007].

Cloning offers not only preservation of valuable farm species, but also reconstruction of species endangered or extinct [Kues and Niemann 2004, Loi et al. 2007, 2008, Fulka et al. 2009].

Somatic cloning has enormous impact on biotechnology, due to the production of transgenic animals. In the future, cloning may be used in xenotransplantation, as it would allow multiplication of humanized pigs, the organs of which could be transplanted to humans [Kues and Niemann 2004, Duszewska and Reklewski 2007].

**Transgenic farm animals**

There are many ways to produce transgenic farm animals. One of the oldest methods known is the microinjection of exogenous DNA to the pronucleus of a
zygote. The technique allowed to produce transgenic sheep and pigs [Hammer et al. 1985] and also transgenic cattle [Krimpenfort et al. 1991]. Interesting in transgenic technology is using spermatozoa as carriers of foreign DNA – Sperm Mediated Gene Transfer (SMGT) – with the use of which, transgenic cow and pig were produced [Sperandio et al. 1996]. Using viral vectors to transfect or microinject oocytes or embryos allowed to obtain transgenic pig [Hofmann et al. 2003] and cow [Hofmann et al. 2004]. The highest hopes, however, are put in transgenic somatic cell nuclear transfer (TSCNT), which is a variant of SCNT technology. The method is more efficient than microinjection or SMGT [Robl et al. 2007]. Using TSCNT allowed production of transgenic sheep [Schneike et al. 1997], cow [Cibelli et al. 1998a], goat [Keefer et al. 2001] and pig [Park et al. 2002]. Entirely novel approach to transgenesis of farm animals was introduction of artificial chromosomes into nuclear donor cells and subsequently using these donors for cloning. This allowed to produce transgenic cow [Kuroiwa et al. 2002]. Undoubtedly interesting in transgenesis of farm animals is production of transgenic chimaeras [Cibelli et al. 1998b].

Transgenic farm animals can be used both in breeding and biomedicine [Robl et al. 2007, Wells 2010].

In breeding, transgenic individuals are produced to specifically improve quantitative and qualitative population features and to increase resistance to diseases. An example of this can be transgenic cows producing milk of increased β-caseine and κ-caseine content. In pigs, significant improvement in economically important traits, like body weight gain or fat to muscle tissue were obtained in transgenic individuals expressing human growth hormone. Thirty percent higher sirloin mass and 10% higher weight of fat are characteristic of transgenic pigs expressing human insulin-like growth factor I. Transgenic sheep with integrated keratin-IGF-I gene show 6.2% higher production of wool compared to normal individuals [Pursel et al. 1989, 1999, Damak et al. 1996a, 1996b, Brophy et al. 2003, Niemann and Kues 2003, Kues and Niemann 2004].

An important achievement was production of transgenic cows resistant to mastitis. Epithelial cells of their mammary glands excrete antibacterial agent lisostafin that acts specifically against Staphylococcus aureus [Wall et al. 2005].

There is a high interest in using transgenic farm animals as bioreactors producing human recombinant proteins in mammary gland [Kues 2004, Redwan 2009]. So far, a few dozen such proteins were obtained, for example antitrombin III and α-antitripsin from mammary gland of goat and sheep [Kues and Niemann 2004], and lactoferrin [Van Berkel et al. 2002], albumin [Echelardi et al. 2002] and growth hormon from a cow [Roschlau et al. 1989].

Mammary glands of transgenic goat produce also a number of monoclonal antibodies. Recombinant human bispecific antibodies were also obtained in blood of transgenic cow. The newest direction in transgenesis is production of transgenic farm animals bearing artificial chromosomes with complete sequences of human antibodies. Transgenic cow producing human immunoglobulins in blood was already obtained. Also, production of transgenic pig expressing human haemoglobin, which

Transgenic domestic pigs are used in studies on xenotransplants, i.e. transplantation of animal body parts into humans. Major concern in such procedure is related mainly to immunological background, i.e. rejection of transplant. Thanks to switching off the expression of pig 1,3-galactosyltransferase gene, responsible for acute transplant rejection, science is closer to being able to use animal body parts in human transplantation [Lai et al. 2002, Niemann et al. 2005]. However, one cannot exclude another possibility, which relates with using for xenotransplantation the organs origin from human embryonic or somatic stem cells [Behringer 2007].

Interesting is gene therapy, commonly used in human medicine [Laible 2009, Carlson 2010]. Although applying the method to farm animals seems very unlikely, using these animals as model organisms in studying how to prevent or remove genetic defect in humans is now very realistic. Transgenic pigs were successfully produced to study disorders of secretion of human growth hormone-releasing hormone (GHRH) [Forsberg 2005].

Only a few ways of practical application of transgenic farm animals were presented in this article, omitting production of environment-friendly transgenic individuals or using such animals in basic studies as a model to understand various physiological processes in farm animals and humans [Kues 2004, Niemann et al. 2005].

**Chimaeras**

Interesting area of biotechnology is production of chimaeras, which are individuals originating from more than one zygotes. Basic method of chimaera production is aggregation of two early-stage embryos or microinjection of embryonic stem cells (ESCs) into such embryos. Production of chimaeras allows to verify pluripotency of blastomeres and ESCs and to follow the differentiation and de-differentiation processes in prenatal development [Behringer 2007, Glover 2010, Ralston and Rossant 2010]. Several lines of ES cells were obtained from (1) inner cell mass of blastocysts, (2) single blastomeres isolated from embryos at earlier stages of development, or even from (3) one-cell stage embryos [Hwang et al. 2004, Chung et al. 2006, Klimanskaya et al. 2006].

It should be emphasised however, that majority of aforementioned achievements concerns humans and laboratory, not farm animals, as ES cells were not yet isolated from the latter. So far, chimaeric sheep – [Pighills 1968] – and cow – [Brem et al. 1984] were obtained by aggregation method, and pig – [Onishi et al. 2000] by injection of primary cultured inner cell mass cells. Furthermore, interspecies chimaeras were produced, for example sheep-goat chimaera [Polzin et al. 1968].

The most promising is production of transgenic chimaeras from farm animals. First, such individuals were transgenic chimaeric cows producing lacZ-neo, [Cibelli
et al. 1998b]. Entirely new challenge is the production of animal-human chimaeras, which could serve as a model for testing different types of therapy dedicated for use in humans [Niemann et al. 2005, Behringer 2007, Glover et al. 2010.]

Biotechnology of farm animal breeding is one of the fields of science that links academic and practical approach. On one hand it meets the needs of research problems, striving to answer different questions of basic science (oocyte maturation, fertilization, embryonic and postnatal development, etc). On the other hand it benefits biotechnology itself, together with animal breeding science and also veterinary and human medicine (in vitro embryo production, bioreactors, disease-resistant individuals, clones of rare animals etc). Among many biotechniques, production of transgenic farm animals as bioreactors seems to be of the highest importance, being a driving force behind general progress of biotechnology.

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