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Animal Biotechnology III

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Preface

In the modern history of humans biology has an important role. Biology is one of the science area, which studies and discovers life and chemical processes, molecular interactions, physical structure and evolution of living organisms.

In 1960s, biologists had cracked the genetic code, but there was not a way to study gene functions. But in the 1970s everything changed. The term of *new biology* was coined with the introduction of recombinant DNA technology and biotechnology. Humankind has been using biotechnological methods for many years. Nowadays biotechnology is one of the key technologies of 21. century.

Biotechnology can be characterized as a set of unique techniques used for the benefit of humans and other animals. It includes traditional industries such as the production of beer, milk, wine as well as productions and biotransformation of vitamins, antibiotics etc.. This area of applied biology was the first step to develop special methods as a nuclear transfer technology, gene and stem cell therapy.

The potential of these techniques, methods and advances open up the prospect of generating new animal products (such as pharmaceuticals, organs, tissues) for example for the health sciences, molecular biology and reproductive medicine.

The book, entitled Animal Biotechnology III, is a compilation of nine chapters related to the pluripotent stem cells, somatic stem cells, animal genetic resources, gene banks, xenotransplantation and molecular approaches in animal breeding, sperm sexing technology, endoscopy in reproduction, ethics and future of animal biotechnology. These chapters consist from highly informative, practical and useful overviews of chosen areas of animal biotechnology.

A special thanks go to all contributors for their time and effort spent during the production of this modern and unique compendium of information about animal biotechnologies.

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1 Pluripotent Stem Cells

1.1 Introduction

This chapter focuses on the different types of pluripotent stem cells isolated from embryonic tissues, or produced from somatic cells by reprogramming with pluripotency transcription factors. The primary goal is to introduce the reader to the main types of stem cells and provide basic knowledge on the subject. Following a brief history, described are the main characteristics, methodologies, and potential uses of these cells in biotechnology or biomedicine. Another group of stem cells that are isolated from somatic tissues, termed somatic stem cells, are not included in the category of pluripotent cells and are therefore beyond the scope of this review.

1.2 Stem cells derived from pluripotent tissues

1.2.1 History and types of pluripotent stem cells

The establishment of embryonic stem cells is a major milestone in modern biology, with a potential to have an impact on human life in the future. The concepts of pluripotent stem cells were established in the 1970's during research in mouse testicular teratocarcinomas (TC), which are tumors from germ cell origin that arise spontaneously in the testes of certain inbred strains. Characteristic of these tumors is the extensive differentiation of the germ cells forming multiple layers of fully specified tissues, including even such formations as hair and teeth. The pluripotency potential of these cells was experimentally established when chimeric mice were produced by injecting cells directly from tumors into mouse blastocysts (Mintz and Illmensee, 1975). At the same time, Papaioannou et al. (1975) demonstrated the chimera potential of mouse teratocarcinoma cells cultured in vitro. Similarly, human TC cells have been grown in culture (Andrews et al., 1984, Thompson et al., 1984) and pluripotent cell lines with potential to form all three primary germ layers (endoderm, ectoderm, and mesoderm) have been derived in vitro (Pera et al., 1989). Since TC tumors not only occur naturally, but can also be artificially generated by the injection of early embryos into mouse extrauterine sites (such as kidney capsule), it did not take long for researchers to realize that the embryos themselves were a rich resource for pluripotent stem cells.

In 1981, two independent laboratories reported the derivation of the first pluripotent stem cells from inner cell masses (ICM) of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). These cells were referred to as embryonic stem cells (ESC) (Martin, 1981), and presently this term is

commonly used to denote pluripotent stem cells derived from ICM, although stem cells have been isolated also from other embryonic tissues. The mouse ESC established in these studies could proliferate indefinitely in culture and showed unlimited ability to differentiate into all of the cell types in the body, including the germ line. Moreover, they had the ability to maintain normal diploid karyotype over many cell divisions, unlike TC-derived stem cells, which usually contain chromosomal abnormalities. In addition to mouse ESC, the derivation of germ line-competent rat ESC was also achieved, but not until 27 years later (Li et al., 2008), despite the fact that this species is closely related to mouse.

The first primate ES cell lines were derived from rhesus monkey and marmoset blastocysts by James Thompson and his co-workers (Thompson et al., 1995; 1996). Subsequently, the same research group established human ESC using fertility clinic surplus blastocysts (Thompson et al., 1998). Compared with mouse ESC, their human counterparts had different morphology and required different culture conditions; however, they had the ability of unlimited self-renewal and were able to differentiate into all three germ layers in vitro and when injected into immunodefficient mice (teratoma formation).

During the time period between blastocyst hatching and gastrulation, the ICM forms the epiblast, which consists of epithelial-like cells and have either cylindrical (rodents) or discoid (other mammals) shape. It has been shown that at least some, if not all murine epiblast cells are pluripotent and can contribute to chimeras or to form teratomas. In 2007, mouse epiblast cells cultured in the presence of activin and bFGF gave rise to pluripotent stem cells (Brons et al., 2007; Tesar et al., 2007), called epiblast stem cells (EpiSC). In their morphology and culture requirements EpiSC seem to resemble human rather than mouse ESC. Although these cells demonstrate multilineage potential by forming teratomas, they have limited capacity to form chimeras.

Another type of pluripotent stem cells that has been established from fetuses are the so-called embryonic germ cells (EGC), which are derived from cultured primordial germ cells (PGC) isolated prior to their sexual differentiation (Matsui et al., 1992; Resnick et al., 1992). Primordial germ cells are the embryonic precursors of the gametes and (at least in the mouse) are considered nulipotent, i.e. not capable to form other cell types or contribute to chimera development. When injected into kidney capsule or testis of immunodefficient mice, they form pluripotent TC tumors. Additionally, in appropriate culture conditions they become reprogrammed into pluripotent state (EGC) during a process that is currently not well understood. Mouse EGC have shown to be equivalent to ESC in their ability for self-renewal and pluripotency, as demonstrated by their ability to form germ line chimeras (Labosky et al., 1994). Human EGC have also been established (Shamblott et al., 1998), however, they are not considered equivalent to ESC due to limited period of proliferation in vitro and

restricted pluripotency in vitro and in vivo, as evidenced by difficulties to produce teratomas (Shamblott et al., 2000; Turnpenny et al., 2005).

Lastly, pluripotent stem cells can be also derived from the male germ line in newborn or adult testis (termed germ stem cells (GSC), or spermatogonial stem cells (SSC)). Such SSC have been isolated from mouse (Kanatsu-Shinohara et al., 2004) and shown to possess some pluripotency characteristics. Human SSC from adult testis have also been derived (Kossak et al., 2009), but their pluripotent character is currently debated.

1.2.2. Generic definition of embryonic stem cells

All ES cell lines should possess certain characteristics that distinguish them from differentiated somatic cells. First, the cells should originate from pluripotent cells that are usually found in early embryos, such as the ICM, the epiblast, or the PGC from fetal genital ridges. Since the pluripotent state occurs only transiently in embryonic development, the cultured ESC derived from embryos can be considered a byproduct of development, because they have been arrested artificially in the pluripotent state.

The second and perhaps the most important characteristic of embryonic stem cells is pluripotency, which is the ability to differentiate into any cell of the body, including the germ line. This does not include the cells forming the placenta; in this respect only the zygote is considered capable of complete embryonic development and is designated as totipotent. Usually, all newly derived stem cell lines are tested for their ability to form different cell types that represent the three primary germ layers in culture. Although the extent of differentiation is somewhat limited by the culture conditions, true pluripotent ESC can form in suspension culture three dimensional spherical aggregates, called embryoid bodies, which may contain cells from the three germ layers and can be further expanded when allowed to attach to the culture dish. Differentiation into the germ line has also been achieved in vitro, although fully functional gametes have not been yet produced. Pluripotency is also tested by injection of the cells into immunodefficient mice, where they form teratomas containing tissues derived from the three germ layers. The ultimate proof of pluripotency is considered the ability to contribute to all the tissues in a live animal following injection of ESC into the blastocoel of a blastocyst. In this case, the resulting chimeric animals contain mix of cells coming from the original ICM and the injected pluripotent cells. In another in vivo test, the stem cells are aggregated with tetraploid 8-cell embryos (tetraploid complementation). Since the tetraploid embryonic cells can develop into placenta, but have poor ability to form ICM, the resulting chimera is almost entirely derived from the donor stem cells.

The third characteristic of pluripotent stem cells is the ability to proliferate indefinitely without differentiation (also refered to as immortality, or indefinite self-renewal). The division of the true stem cells is symmetric, and results in two pluripotent cells with identical properties, unlike the division of a somatic stem cell, where at least one of the resulting cells is more committed to particular somatic cell lineage (asymmetric division). During hundreds of cell divisions, the ESC can maintain stable karyotypes, showing generally uncompromised genomic integrity. This is in contrast with the differentiated somatic cells, which usually fail to maintain stable karyotypes and senesce after a limited number of divisions in culture (spontaneous immortalization has been observed in certain somatic cell types, but these cells have shown high degrees of aneuploidy).

It should be noted that so far only mouse ESC/EGC and rat ESC have satisfied all of the above criteria. Human ESC fulfill the requirements for pluripotency (demonstrated by in vitro differentiation and in teratoma formation) and self-renewal, but have not been tested for chimera formation for ethical reasons. However, most authors do not apply the above definitions too rigidly, and consider human ESC equivalent to their mouse counterparts. The ESC from other species often meet less than two of the criterias for true pluripotent stem cells, and should therefore be referred to as "ESC-like". For example, chicken ESC derived from blastoderm lose the ability to generate germ line chimeras when cultured for periods longer than 7 days (Pain et al., 1996) and therefore do not meet the requirement for indefinite self-renewal. Chicken EGC contribute to the germ line, but not to all somatic lineages when injected into chick embryos. Attempts to produce true ESC from other farm animals such as pig, sheep, and cattle have not been successful (for a review, see Keefer et al., 2007).

1.2.3. Morphology and molecular markers for pluripotent stem cells

The ESC lines grown in different labs are usually evaluated for "stemness" using a set of morphological and molecular markers. Mouse ESC normally form densely packed, dome-shaped colonies that are similar to ECC. In contrast, mouse EpiSC and human ESC form flatter, epithelial-like colonies with clear-cut and refractory borders. When the stem cell colonies differentiate, they lose their tightly packed structure and become flatter, with loosely associated cells that form clusters of differentiation.

Pluripotent stem cells can also be identified by their expression of variety of molecules, which is detected by various techniques such as RT-PCR, immunocytochemistry, and Western blot. Mouse ESC typically express tissue non-specific alkaline phosphatase (TNAP) and stage-specific embryonic antigen 1 (SSEA1). While human ESC also express TNAP, they do not have SSEA1 expression, but instead express surface antigens SSEA4, TRA-1-60, and TRA-1-81, which are absent in mouse ESC.

The maintenance of pluripotency in ESC is achieved by the regulated expression of multiple transcription factors, which form complex system of interaction with each other and with their target genes. An important stem cell marker is the octamer binding protein 4 (OCT4, also named Pou5f1), which is a member of the POU domain of transcription factors and is typically expressed in ICM, epiblast, SSC, and ESC. The expression levels of OCT4 are precisely regulated in ESC, and overexpression leads to differentiation similarly to downregulation. Another master pluripotency transcription factor is NANOG, which alone is able to maintain pluripotency of mouse ESC when overexpressed (Chambers et al., 2003). Although this gene is necessary for the formation of ICM in the mouse blastocyst, ESC can preserve pluripotency in absence of NANOG, provided that the other relevant transcription factors are expressed. OCT4 and NANOG interact with another transcription factor, SRY box protein 2 (SOX2) to form a core regulatory network. The three factors act together to regulate the expression of multiple pluripotency and lineage-specific genes, as evidenced by the co-occupation of the same promoters simultaneously by all three factors. In addition, these transcription factors may regulate each other as well as their own expression, in a manner of feedback loops. Other transcription factors that are highly expressed in ESC and serve as additional markers for pluripotency are C-Myc, KLF4, Stat3, LIN28, FoxD3 and many others. However, the expression of most of these genes, similarly to SOX2, is not restricted only to ESC, since they also play roles in various differentiation processes. The precise roles and interactions of these transcription factors in the pluripotency networks are subject of intense research.

1.2.4. Signaling pathways for regulating pluripotency and self-renewal of ESC

In order to be maintained in pluripotent state in vitro, the ESC need to be grown in certain culture conditions that activate signaling pathways responsible for cell proliferation and maintenance of pluripotency. There are significant differences between the different species in their signaling networks and therefore in their cell culture requirements. Mouse ESC depend mainly on leukemia inhibitory factor (LIF) and bone morphogenic protein 4 (BMP4) to maintain their pluripotent characteristics in culture. Binding of LIF to the heterodimeric LIF /g130 receptor protein initiates downstream signaling by Jak kinase and signal transducer and activator of transcription 3 (Stat3). Phosphorylated Stat3 translocates to the nucleus and initiated transcription of a number of pluripotency genes, one of which is believed to be c-myc. There is also a parallel signaling pathway induced by LIF that involves phosphatidylinositol 3 kinase (PI3K) and its downstream kinases. In this respect, inhibition of glycogen synthase kinase 3 beta (GSK3β), a downstream target of PI3K signaling, greatly enhances the derivation of mouse ESC. Of the two described signaling pathways, the LIF-Stat3 signaling is considered critical for mouse ESC culture; however, the cells also need

BMP4-Smad 1/5/8 signaling for transcription of genes responsible for inhibition of differentiation (Id genes). The BMP4 is present in fetal bovine serum, a standard additive to the cell culture media. When the medium is supplemented with LIF and BMP4, the mouse ESC can be maintained in serum-free conditions, which is a major advantage in achieving more defined culture environment for consistent results. Lastly, Activin A signaling has been shown to support proliferation of mouse ESC, but it is not necessary for preservation of pluripotency.

A new paradigm for maintenance of ESC pluripotency was established when Ying and co-workers (2008) reasoned that instead of using pluripotency-activating signals such as LIF, the cells can be kept from differentiation by inhibition of differentiation signals. It was found that mouse ESC could be propagated in undifferentiated state without LIF, but in medium supplemented with a cocktail of three inhibitors for GSK3β, mitogen-activated protein kinase (MEK), and fibroblast growth factor receptor tyrosine kinase. By using these inhibitors, it was possible to successfully establish rat ESC (Li et al., 2008) as well as ESC from strains of mice that have been previously considered non-permissive for stem cell derivation (Hanna et al., 2009).

Unlike their murine counterparts, human ESC cannot be maintained by LIF-Stat3 signaling, and differentiate in presence of BMP4. For proliferation and maintenance of pluripotency, they depend mainly on basic fibroblast grown factor (bFGF, or FGF2), Activin A, and PI3K agonists such as insulin-like growth factor (IGF) or insulin.

1.2.5. Stem cells exist in two distinct pluripotency states

Based on morphological and molecular characterization, researchers have found significant differences between mouse ESC and EpiSC. Typically, mouse ESC have dome-shaped, 3D colony structure, proliferate well as single sells following trypsin disaggregation, and have two active X chromosomes in all female lines. In contrast, the EpiSC grow as flat colonies that do not survive well disaggregation to single cells, and have one inactivated X chromosome, similar to somatic cells. These cells are considered pluripotent, although they have shown relatively low efficiency in generating chimeras. In addition, EpiSC require bFGF and Activin for their maintenance instead of LIF and BMP4, and express relatively lower levels of NANOG, but higher levels of some differentiation markers, such as major histocompatibility complex (MHC). For these reasons, EpiSC are considered to be in slightly advanced stage of differentiation, also termed “primed” state, while ESC have been considered to be in “naïve” state. Since human ESC are similar to EpiSC in their morphology and growth factor requirements, they are also categorized as “primed” cells. The two

states can be switched into one another under specific culture conditions, although the cells converted to “naïve” states have not been maintained for long periods of time (Hanna et al., 2010).

1.2.6. Use of ESC in transgenesis

Gene targeting is a powerful tool to study gene function, to produce transgenic animals with new genetic characteristics to be used in research as human disease models or for production of pharmaceuticals. In 1985, Smithies and co-workers first achieved directed genetic modification in mammalian cells by homologous recombination. In the following years, several groups demonstrated homologous recombination in mouse ESC (Gossler et al., 1986; Robertson et al., 1986). When injected into blastocysts, the genetically modified ESC produced germ line chimeras which in turn could generate transgenic offspring. The efficiency of the HR in mouse ESC is significantly higher compared with other cells types and for this reason they have been widely used in the production of “knock out” or “knock in” mice. Following the recent derivation of rat ESC, it was demonstrated that efficient gene targeting is also possible in this species (Meek et al., 2010). In the cells of other species, the efficiency of HR remains relatively lower, although in human ESC it has been significantly improved using different strategies, including the use of zinc finger endonucleases (reviewed by Nieminen et al., 2010).

1.2.7. Promises and problems for the use of ESC in regenerative therapies

Due to their ability to differentiate into any cell of the body, the ESC hold a significant potential for regenerative therapy, which generally involves replacement of dead or diseased tissues with newly generated tissues from differentiated stem cells. It is believed that many degenerative disorders such as cardiac disease, Alzheimer disease, Parkinson disease, diabetes, skin damage, bone damage, muscular dystrophy, and many others could be treated by grafting healthy ESC-derived cells. Over the years, the mass media has become responsible for creating unrealistic expectations among the general public regarding the fast development of stem cells-based cures for these diseases. However, many hurdles remain to be cleared before any ESC-based treatments can be applied in the clinic. Some of the main problems stem from the ethical implications of destroying embryos to isolate ESC. The debate regarding the ethics of human stem cell research has involved members of the religious, philosophical, and scientific community, and in many countries public funds have been withdrawn from research in this area.

Of the many human ESC lines that have been generated, relatively few have been expensively characterized. The results of the studies show that there are some very significant variations between

different lines regarding gene expression, making it difficult to standardize the material to be used in transplantation. Another problem is the oncogenic potential of ESC, due to their tendency to proliferate indefinitely. For this reason, the cells need to be differentiated into relevant somatic cell type before being used for treatments. Currently, most differentiation protocols give rise to mixed populations of cells, and the purification and expansion of the desired cells is difficult task. Moreover, problems with the generation of fully mature and functional differentiated cells additionally hamper the transition from the lab to the clinic. For example, differentiating human ESC selectively into cardiac cells from the left or right ventricle is necessary before cardiac treatment, and would require highly optimized culture protocols.

Another significant problem is overcoming immune rejection which is common for all transplantations that do not use allogenetic grafts. One way to deal with this problem is to use immunosuppressive drugs, which in turn increases the risks of other diseases. Another approach is to create patient-specific ESC by producing cloned blastocysts by nuclear transfer and derivation of ESC from them. This approach is even more controversial compared with the derivation of ESC from surplus blastocysts donated from fertility clinics, since it involves human cloning.

1.3. Induced pluripotent stem cells (iPSC)

1.3.1. Introduction to cellular reprogramming and history of iPSC

Embryonic development is considered a unidirectional process, where the totipotent zygote undergoes successive cell divisions that are characterized by an increased loss of pluripotency as the cells differentiate to form the different somatic tissues. In certain conditions, it is possible to reverse this process and to return the nucleus of a specialized somatic cell back to pluripotent or totipotent state. This was demonstrated by early experiments in nuclear transfer with amphibian cells (Gurdon et al., 1962) and later using mammalian somatic cells, resulting in the birth of the first cloned sheep, Dolly (Wilmut et al., 1997). Another method for reprogramming of somatic cells is by fusion with TC or ESC (Miller and Ruddle, 1976; Cowan et al., 2005). In 2006, Yamanaka and co-workers established a new milestone in cellular reprogramming by demonstrating that a somatic cell can be reprogrammed by artificially induced expression of pluripotency transcription factors (Takahashi and Yamanaka, 2006). After testing different combinations of 24 pluripotency-related genes, these researchers showed that exogenous expression of only 4 factors, namely, OCT4, SOX2, c-myc, and KLF4, can confer pluripotency to mouse fetal fibroblast cells. The reprogrammed cells, which were selected by the upregulation of endogenous Fbx15, had the ability to differentiate into all three germ layers and form teratomas, however, they could not form viable chimeras. The epigenetic and gene

expression profiles of these cells differed from ESC, suggesting only partial reprogramming. Germ line-competent mouse cells were generated a year later using the same methods, but selecting for the expression of NANOG (Okita et al., 2007). With their reprogramming experiments, these and other authors (Maherali et al., 2007, Wernig et al., 2007) demonstrated that the re-activation of endogenous expression of OCT4 and NANOG is crucial for complete reprogramming, further confirming the importance of these factors in pluripotency. The ability of the mouse iPSC to form germline chimeras upon injecting into blastocyst or to form nearly the entire mouse in tetraploid complementation experiments (Boland et al., 2009; Zhao et al., 2009) demonstrate beyond doubt their true pluripotent character.

Following the successful generation of iPSC in the mouse, two groups simultaneously reported the derivation of the first human iPSC lines. While the research group of Yamanaka reprogrammed adult fibroblasts using the same transcription factor combination as in mouse iPSC (Takahashi et al., 2007), the group of James Thompson achieved reprogramming of IMR90 human fetal fibroblasts by using a combination of OCT4, SOX2, NANOG, and LIN28. (Yu et al., 2007a). The obtained human iPSC closely resembled human ESC in their morphology and in the expression of pluripotency markers, and formed teratomas when injected into immunodeficient mice.

1.3.2. Understanding the reprogramming to iPSC state

During differentiation, the pluripotent stem cells undergo epigenetic changes characterized by specific changes in DNA methylation and histone modifications that are specific to the different types of somatic cells. The CpG islands in the promoters of pluripotency genes become hypermethylated as the expression of these genes is downregulated. Repressive chromatin modifications, such as H3K9 and H3K27 methylation are dominant over genes that are not characteristic for the particular somatic lineage. Reversing of the differentiation back into pluripotency requires removal or alteration of these epigenetic marks. It is therefore astonishing that this can be achieved by the exogenous expression of a few transcription factors. However, the iPSC generation is very inefficient process, since only a small percentage of the cells that initially express the transgenes become fully reprogrammed, while the rest remain in various intermediate states. The initial hypothesis that the iPSC originate from somatic stem cells that are present in many tissues was discarded after fully differentiated B-lymphocytes with terminal DNA rearrangements (Hanna et al., 2008) as well as terminally differentiated pancreatic β-cells (Stadtfeld et al., 2008a) were successfully reprogrammed to pluripotent state. Currently, it is generally believed that epigenetic reprogramming is the result of multiple stochastic epigenetic events. This was supported by experiments that showed that only some

subclones from the same parental cell that had identical transgene insertions could complete the reprogramming process, while the rest remained in intermediate state (Meissner et al., 2007).

Typically, the reprogramming of somatic cells into pluripotent iPSC takes 1 to 2 weeks and is characterized by morphological, epigenetic, and transcriptional changes in the starting cell population. One of the first visible signs in the reprogramming of fibroblast cells is the acceleration of cell proliferation and the transition from mesenchymal to epithelial phenotype. The increased proliferation is important for the epigenetic modification of the genome, since this process occurs mainly during DNA synthesis. Another event in reprogramming is the silencing of the somatic gene expression programme, as evidenced by the downregulation of THY1, a surface marker for fibroblast cells (Hochedlinger, 2008). Soon after induction, some pluripotency markers, such as AP and SSEA-1, are upregulated in mouse cells (Brambrink et al., 2008), followed in the end by the upregulation of the endogenous pluripotency factors such as OCT4, SOX2, NANOG, and many other stem cell-specific genes. In accord with these changes, analyses of the DNA methylation levels in iPSC have shown that the promoter regions of the reactivated pluripotency genes become hypomethylated (Okita et al., 2007). Upon completion of reprogramming, the transgenes are usually silenced by de novo methylation in iPSC, which become fully independent from exogenous transcription factor expression (Okita et al., 2007; Maherali et al., 2007). This process is more pronounced in mouse cells, while in human iPSC incomplete silencing of OCT4 has been reported (Yu et al., 2007a). Usually at the end of the reprogramming process, female mouse iPSC re-activate their inactive X-chromosome, similar to ESC.

The mechanisms of reprogramming to pluripotency by the exogenous transcription factors are at present incompletely understood. By mapping promoter binding in iPSC, it has been established that OCT4 and SOX2 co-occupy promoter regions that overlap with those in ESC (Sridharan et al., 2009). A part of these sites are also co-occupied by KLF4, which acts in synchrony to increase the transcription of the underlying genes. For these reasons, it is believed that these factors act mainly to induce the expression of the pluripotency gene network, including their own expression, as suggested by the finding that they also bind their own promoters. In addition, they may act to inhibit the expression of lineage-specific genes. The function of the fourth member of the reprogramming cocktail, c-myc, is uncertain, because this factor plays a role in many different cellular processes. For example, c-myc is involved in increase of proliferation by inhibition of p21, p15, and Cyclin E (Hooker and Hurlin, 2006; Vervoorts et al., 2003). In addition, it also promotes open chromatin structure by recruiting histone acetyl transferases (Knoepfler et al., 2006), which may help OCT4 and SOX2 to bind their targets. It is also known that c-myc causes increased DNA replication activity independently of its transcription factor properties (Domingues-Sola et al., 2007), which may also aid

the epigenetic reprogramming. Overexpression of c-myc induces p53-dependent cell apoptosis, an effect that is prevented by KLF4, which downregulates p53. In turn, c-myc prevents KLF4 inhibition of cell proliferation which is mediated by increase in p21 transcription. Reprogramming is also possible to achieve without c-myc; however, at much lower efficiency (Wernig et al., 2008).

Two other transcription factors, NANOG and LIN28, have been shown to improve the efficiency of reprogramming. Although the activation of NANOG expression is an important event in the reprogramming process, exogenous NANOG is dispensable for the induction of pluripotency in mouse cells, most likely because OCT4 and SOX2 activate its endogenous expression. In other species, such as marmoset, exogenous expression of NANOG may be necessary for complete reprogramming to pluripotency. The role of Lin28 is less clear, although it has been suggested that this factor enhances reprogramming by stabilizing mRNA transcripts of genes responsible for activating the pluripotency transcription networks (Welstead et al., 2008). Concurrent with its function in ESC, Lin28 may also help to prevent differentiation in iPSC by interfering with the processing of let-7 miRNAs, which are known to induce differentiation in cancer cell lines (Yu et al., 2007b).

1.3.3. Strategies for iPSC generation

The generation of iPSC is a slow and inefficient process that can be influenced by multiple factors and therefore it is amenable to a certain extent of optimization. The choice of the donor cell type has been shown to affect the kinetics and the quality of the reprogramming to pluripotency. To date, the most used cell type has been primary fetal fibroblasts that usually require expression of four transcription factors or some chemical substitutes and can take about 2 weeks to reach iPSC state. In comparison, human keratinocytes, which can be isolated from plucked hair, have been reprogrammed with 100-fold higher efficiency (Aasen et al., 2008). Some other cells that have high endogenous expression of one or more reprogramming transcription factors have been successfully reprogrammed with fewer transgenes. For example, neural stem cells (NSC) express high levels of SOX2 and can be reprogrammed using only OCT4 and KLF4 or OCT4 and c-myc (Kim et al., 2008), and even with OCT4 alone (Kim et al., 2009). The extent of differentiation of the starting cells also affects the efficiency of reprogramming, as evidenced by experiments where hematopoetic stem cells were reprogrammed 300 times more efficiently compared with terminally differentiated B and T cells (Eminli et al., 2009).

The method of delivery of the reprogramming transgenes is another factor in the process of iPSC production. Most popular so far have been lentiviral vectors due to their high transduction efficiency, although their use in the laboratory and in the clinic is a cause for significantly higher safety concerns

compared with other methods. Another approach that relies on integration of the transgenes in the genomes of the recipient cells is the use of transposon vectors, which are safe for use and can be removed from the iPSC genome after reprogramming. By using PiggyBac transposition, two groups have reported the production of transgene-free iPSC from mouse embryonic fibroblasts (Woltjen et al., 2009; Yusa et al., 2009). Non-integrating methods of reprogramming factor delivery have also been described in the literature, although these methods have shown in general lower efficiency of transfection, delayed reprogramming kinetics, and inconsistent reproducibility. By using integration-defective adenoviral particles, Stadtfeld et al. (2008b) were able to produce transgene-free iPSC from mouse hepatocytes. Another method that avoids the use of viruses is by transfection with episomal vectors. The advantages of this method are that the episomes can be introduced into the cells by transfection and therefore are safe for use, and subsequently are maintained at low copy number during many cell divisions without being inserted in the genome. After completing the reprogramming process, a small percentage of the iPSC that lose their episomes can be selected for expansion of transgene-free clones. The feasibility of this method was demonstrated by Yu et al. (2009), who generated iPSC from human fibroblasts using oriP/EBNA1 episomal system. In other particularly original approaches, transgene-free iPSC have been produced by using synthetic mRNAs (Warren et al., 2010), or recombinant proteins (Zhou et al., 2009).

Finally, to facilitate the conversion to pluripotency, some small molecule inhibitors that act as epigenetic modifiers or as inhibitors of particular differentiation signaling pathways have been used to complement or substitute some of the pluripotency transcription factors. Since DNA methylation is a major barrier to reprogramming, DNA demethylating reagents such as DNMT1 inhibitor 5-azacytidine can be used to accelerate the rate of epigenetic changes (Mikkelsen et al., 2008). Similarly, use of histone deacethylase inhibitors valproic acid (VPA) or trichostatin A (TSA) can induce changes in the repressive chromatin configurations and lead to increased number of iPSC colonies. For example, VPA has been shown in some cases to improve the efficiency of reprogramming up to 100 fold (Huangfu et al., 2008) and to act as substitute for c-myc and KLF4. In addition to epigenetic modifiers, some inhibitors of specific signaling pathways have proven active in the process of iPSC production. For instance, inhibitors targeting MEK and GSKβ have been shown to convert cells trapped in partially reprogrammed state into fully pluripotent iPSC (Silva et al., 2008).

1.3.4. Applications of iPSC in the biomedicine

Due to their pluripotent character, iPSC have the same potential as ESC to be used in regenerative therapies. Since these cells can be derived from any somatic cell type, they do not require the destruction of embryos, which makes their use relatively free of the ethical implications suffered by

the potential applications using ESC. Hypothetical ethical problems are seen mostly in cases where the cells could be reprogrammed to totipotency, however, this has not been yet achieved. Another advantage of using iPSC is the possibility to generate custom-made pluripotent cell lines from each patient, thereby avoiding the immune rejection when these cells are used for transplantation. The high efficiency of HR in iPSC makes them attractive for gene therapy, where the genetic defects are repaired before using the cells for transplantation. Additionally, the so-called "disease modeling" in vitro is also possible, where iPSC produced from particular patient can be differentiated into the affected tissues and analyzed for genetic disease markers, which allows for better understanding the genetic basis of the disease. Another area of the medicine that will benefit from the availability of iPSC is the in vitro testing of new drugs using iPSC cells derived from different patient backgrounds (drug screening). Using cells obtained from biopsies, iPSC have already been produced from different diseases, such as amyotropic lateral sclerosis, Parkinson's disease, heart disease, Down syndrome, Hutchinson-Gilford progeria syndrome, and many others, even from complex disorders such as autism.

Before the iPSC can be used in clinical applications, some hurdles need to be cleared so they are safe and ready for transplantation. A major concern is the oncogenic potential of the reprogrammed cells, as evidenced by the fact that mouse chimeras have relatively higher occurrence of tumor formation, mostly due to the reactivation of some of the reprogramming factors (Okita et al., 2007). For this reason, only iPSC that have been produced without viral or other integration could be considered for clinical use. To further decrease the possibilities for cancers, the cells need to be differentiated into the desired tissue type before transplantation. This requires that the current differentiation protocols be optimized for the generation of pure populations of mature, functional somatic cells in sufficient quantities, which still remains a challenge. Another question that still needs to be clarified is whether the number of genomic errors that iPSC have accumulated similarly to ESC (Louise et al., 2011) poses any danger to their use. Nevertheless, considering the quick expansion of the still young iPSC research field, and the involvement of multiple top scientific laboratories, there is no doubt that we can expect many new and exciting developments in the years to come.

2 Stem Cells in the Pig

2.1 Introduction

In 1981, Evans and Kaufman reported the establishment of pluripotent mouse embryonic stem cells (ESCs), i.e. stem cells, which had the capacity to differentiate into all cell types of the mammalian body (Fig. 2.1). This finding has had tremendous significance for the production of genetically modified mice. In 1998, this achievement was translated into man, when Thompson et al. reported the establishment of human ESCs with similar differentiation capacity. This finding sparked the research area of regenerative medicine, and over the past years, the potential of treating severe human diseases, including Alzheimer's, Parkinson's, diabetes, hepatitis, arthritis and consequences of stroke by cell replacement therapy, has been the focus of immense research activities.

In 2009, the first Phase I clinical trials based on human ESC-derived cells for treatment of spinal cord injuries received approval from the US Food and Drug Administration (FDA). Later, unfortunately, this trial was discontinued. It is clear from previous reports, that stem cell-based therapy imposes risks of tumor formation (Amariglio et al., 2009). Hence, in order to evaluate the potentials as well as the risks, proper animal models for stem cell-based therapy are urgently needed. As pigs are being refined as human disease models by e.g. introduction of human disease genes (Kragh et al., 2009), it would be ideal to develop stem cell tools, which can be critically evaluated in this model organism as well.

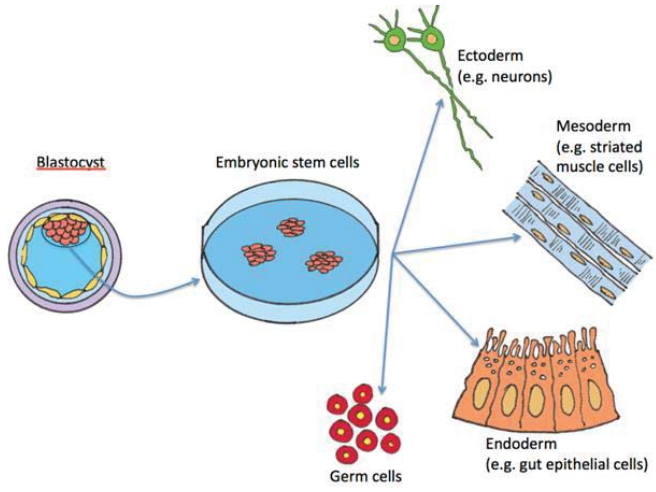

Fig. 2.1: Embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst and are pluripotent, i.e. they can be differentiated into all different cell types of the mammalian body

representing the three somatic germ layers, ectoderm, mesoderm and endoderm, ads well as germ cells.

2.2 General stem cell biology

Stem cells are defined by two characteristics: They have the capacity for indefinite cell division, i.e. they are able to self-renew, and they have the capability to differentiate into all or some of the about 230 cell types of the mammalian body.

Stem cells have the capacity for indefinite self-renewal. When a stem cell divides, it produces either two daughter stem cells (symmetrical division), as seen in e.g. ESC culture, or it divides into one stem cell and another cell destined for differentiation (asymmetrical division), as seen in e.g. haematopoetic stem cells, spermatogenic stem cells, and in keratinocyte stem cells in the skin (Fig. 2.2). The other feature of stem cells is that they are able to differentiate into all or some of the cell types of the mammalian body. Stem cells that have the capacity to differentiate into all of the about 230 cell types of the mammalian body are referred to as pluripotent. Stem cells that have a more limited differentiation capacity to a certain number of cell types (often restricted within a particular cell lineage) are referred to as multipotent, which stem cells that are restricted to differentiation into a singe cell type are considered as being unipotent. Stem cells may also be categorized according to their origin, and this categorization is, at least in mice to some degree, related to the potency of the stem cells: ESCs are derived from the inner cell mass of the blastocyst and are pluripotent. Another cell type, embryonic germ cells (EGCs), are derived from the primordial germ cells of the embryo at the time when the genital ridges are forming and are being populated by these cells, and are also considered pluripotent (Fig. 2.3). Somatic stem cells (SCCs), often referred to as adult stem cells, are derived from different components of the body of the fetus or the born individual and are, in general, considered as being multipotent or unipotent. Another type of pluripotent stem cell, which escapes the presented categorization of origin, is the induced pluripotent stem cell (iPSC). These cells are reprogrammed somatic differentiated cells, which have attained pluripotent characteristics. All of these stem cell types will be addressed in the following.

Characterization of stem cells is an important and versatile matter. Within the hematopoietic stem cell hierarchy, well-defined markers have been developed. Similarly, ESCs in mouse and human are also characterized by relatively well-defined markers. Some of these markers are transcription factors of importance for maintaining pluripotency and self-renewal, e.g. OCT4, NANOG, SOX2, KLF4, and C-Myc, while others are surface antigens, specific for certain stages of embryonic development, e.g. stage specific embryonic antigen (SSEA) 1, 2 and 4, or cell behavioral markers, e.g. tumor rejection antigens (TRA) 1-60 and 1-81. As will be presented, the marker characterization of porcine

stem cells (as well as stem cells from other domestic animals) has not been developed to the same degree.

Stem cells may also, as already mentioned, be evaluated and characterized by their ability to undergo differentiation. In the case of pluripotent cells, the differentiation capacity can be tested in vivo by the ability of cells to develop into teratomas when injected into immune deficient mice, or by their ability to contribute to formation of chimeric embryos and offspring. Stem cell-contribution to the germ-line of chimeric offspring is considered the golden standard of pluripotency. In vitro, their ability to undergo differentiation can be tested either by the formation of embryoid bodies, i.e. spherical embryo-like structures presenting derivatives of the three germ layers, ectoderm, mesoderm and endoderm, or by stimulation of growth factors or other signaling molecules to induce differentiation in monolayers. These different characterization approaches will all be addressed in the following. In the case of multipotent stem cells, these can also be characterized following injection in vivo into different tissues and later assessed for their differentiation capabilities. Multipotent cells may also be differentiated in vitro to assess the extent of their multipotency, by using chemicals and factors that signal differentiation events.

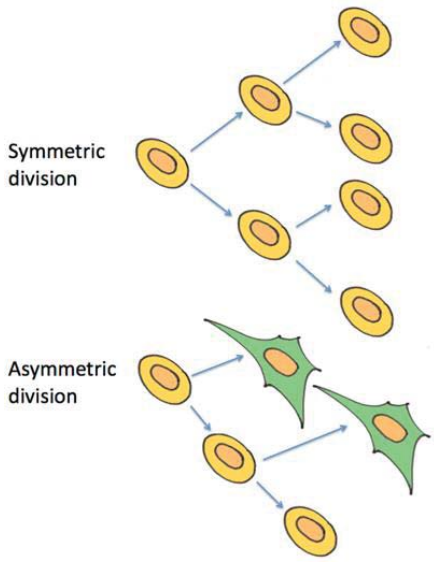

Fig. 2.2: Symmetric and asymmetric division of stem cells.

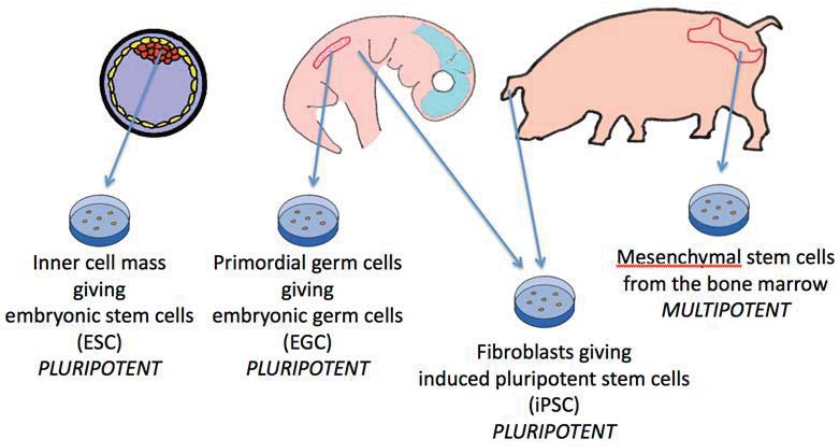

Fig. 2.3: Examples of stem cell types that can be retrieved from the pig. It should be emphasized that porcine ESC and EGC are not fully pluripotent as in the mouse.

2.3 Pluripotent stem cells

2.3.1 Embryonic stem cells (ESCs)

Researchers have since 1990 attempted to produce ESCs in the pig (Evans et al., 1990; Piedrahita et al., 1990a; Piedrahita et al., 1990b; Strojek et al., 1990). However, characterization of these cells hampered due to a lack of in vivo studies (e.g. chimera and teratoma production) and a lack of stable long-term culture of these cells, compared to mouse and man. Efforts have resulted in the production of a number of cell lines that can be cultured generally for the short-term and the cells exhibit some ESC characteristics, but not all. Thus, the porcine ESC-studies have to-date only resulted in the production of what at the best can be termed as putative ESCs or embryonic stem-like cells.

There are a number of standard techniques used to help researchers characterize and prove that the cells they are cultivating are indeed true ESCs. Generally, a combination of techniques is required to prove that the cells are true ESCs. A number of in vitro techniques are generally performed to determine the gene expression profile of the ESCs, and include gene expression analyses (i.e. semi-quantitative or quantitative polymerase chain reaction), and protein analyses (i.e. immunocytochemistry and western blotting). The most well recognized transcription factors expressed in both mouse and human ESCs include OCT4, NANOG and SOX2. However, the list of ESC markers is quite long, and can be species-specific. For e.g. stage-specific embryonic antigen 1 (SSEA1) has been found to be expressed in mouse ESCs and not human ESCs, whereas, both SSEA3 and SSEA4 are detected in human ESCs and not their mouse counterparts (Henderson et al., 2002). Bona fide ESCs must also be able to be cultured for long time periods. Generally, if a line can be

maintained for at least one year in the lab, or above 30 passages, this suggests that the cells are indeed capable of self-renewal and long-term culture. Furthermore, ESCs must also be able to differentiate into cells of the three embryonic germ layers, i.e. ectoderm, mesoderm and endoderm. In vitro differentiation of ESCs is often performed, either using spontaneous or direct differentiation methods, and the resulting cell types are analyzed for their characteristics of differentiation using a combination of gene and protein analyses. Pluripotent stem cells are able to form spherical aggregates containing differentiated cells, called embryoid bodies (EBs), when cultured in suspension. When such EBs are plated, they give rise to a variety of differentiated cell types. Finally, it is required that ESCs can form teratomas after injection into immuno-compromised mice. Teratomas are tumors, which contain cells derived from the three embryonic germ layers, and which are observed only following the injection of pluripotent stem cells. The most ultimate verification of pluripotency is that the ESCs can contribute to the germline in chimeric animals. Chimeric animals can be produced by injection of a number of ESCs into a blastocyst. These cells may contribute into the inner cell mass and, in turn, to the development of the fetus and the resulting offspring. The ultimate verification that ESCs have contributed to the embryonic germline, is to mate chimeric offspring. If offspring with the genetic background of the ESCs results, then germline transmission from ESCs has been demonstrated. Germ-line transmission of genetically modified ESCs in the mouse has become a powerful technology for production of genetically modified mice (Fig. 2.4).

Attempts to establish porcine ESCs have been well reviewed previously in scientific journals (Hall, 2008; Oestrup et al., 2009). It is, however, fair to claim that true porcine ESCs have not yet been produced. One study has suggested that porcine epiblast stem cells (EpiSC) have been produced (Alberio et al., 2010). These cells are considered to be derived from the epiblast, rather than the inner cell mass, of the developing embryo. A recent insight has shown that mouse ESCs may be of inner cell mass origin and that human ESCs are of epiblast origin (Pera et al., 2009). This has largely come into acceptance following the derivation of another pluripotent cell line from mice, namely the EpiSC (Brons et al., 2007; Tesar et al., 2007). These cells have characteristics very similar to human ESCs. They are maintained in culture using the same factors used in human ESC culture and share a similar gene expression profile to human ESCs (Tesar et al., 2007). In the recently described porcine paper, the epiblast stem cells were able to be cultured for 22 passages and could differentiate in vitro into cell types representative of all three embryonic germ lineages as well as germ precursor cells and trophectoderm. However, characterization of the epiblast stem cells was performed by use of PCR only (i.e. gene expression) and not by immunostaining or western blotting. Furthermore, characterization of the differentiated cells was primarily performed by gene expression and no in vivo

experiments were performed, such as chimera production or teratoma formation. Thus, there are still uncertainties with respect to whether these cells are of similar nature as mouse epiblast stem cells.

There have only been two reports to date in production of chimeras from porcine ESC-like cells (Chen et al., 1999; Vassiliev et al., 2010). The former publication claimed somatic chimeric piglets could be produced, although clear analyses of these chimeric animals is lacking. The latter publication indicates that porcine ESC-like cells from an early passage could form chimeric piglets. Chimeric contribution was low, with the birth of 4 chimeric piglets from the transfer of apparently hundreds of embryos. Only 2 of the 4 chimeric piglets showed coat chimerism, and this contribution was low and restricted to a single spot near the tail. This chimeric contribution appears to be much lower than expected from mouse ESCs, which suggests that improvements to cell culture conditions may be required to improve the plasticity of these cells.

Lack of defined culture conditions required for this particular species may be one aspect hampering the production of porcine ESCs. Regulation of pluripotency appears to be controlled by more than one cell signaling pathway, and these pathways are different in mouse ESC-lines and human ESC-lines. This may also be, in part, due to the origin of the cells, i.e. the inner cell mass versus the epiblast, but it also appears to differ slightly between species. This has become clear with the recent publication showing even mouse EpiSCs regulate pluripotency slightly differently to human ESCs (Greber et al., 2010). The cell signaling that governs pluripotency in the pig remains largely unknown although new research is beginning to investigate this more thoroughly (Hall et al., 2009). This preliminary research indicates that the fibroblast growth factor (FGF) signaling may be active in the porcine epiblast, but that the JAK/STAT pathway is inactive. Furthermore, the Activin/Nodal pathway appears to be active in the porcine epiblast (Alberio et al., 2010). Culturing porcine epiblasts in medium containing basic FGF (bFGF), however, cannot prevent differentiation and other factors are apparently necessary to help maintain cellular pluripotency. Further studies revealing pathways important for both pluripotency and differentiation may help researchers refine the necessary culture conditions. Differences during early embryonic development in domestic ungulates, such as the pig, as compared with mouse and man could also account for the observed difficulties. The early development of the porcine embryo is longer and more attenuated prior to placentation. The inner cell mass differentiates into the hypoblast and epiblast at a later time point than in the mouse and man and the porcine epiblast expands and develops over a period of several days. Cell signaling controlling this development could differ significantly and needs to be investigated to ensure which stage of development may be most optimal for isolating the pluripotent cells. Perhaps later epiblast may already be pre-determined at the cell signaling level to undergo gastrulation, or the inner cell mass cells may not have acquired the necessary cell signaling to

maintain proliferation. These hypotheses still remain unsolved and further research in this area may provide further insights, which may aid in the future derivation of porcine ESCs.

Although research is continuing in this field, we are yet to observe bona fide ESCs produced from the pig. Coming research will certainly be of interest, as the pig is a particularly useful biomedical model for studying human disease.

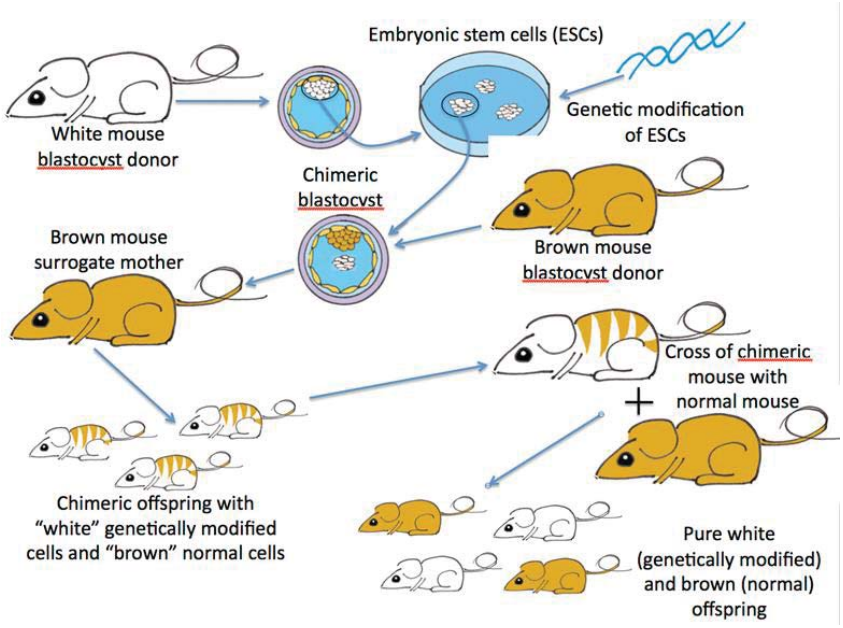

Fig. 2.4: Production of genetically modified mice by means of embryonic stem cells.

2.3.2 Embryonic germ cells (EGCs)

Embryonic germ cells (EGCs) are pluripotent cell derived in vitro from primordial germ cells. Mouse EGCs are morphologically similar to mouse ESC, and express similar pluripotency markers, such as alkaline phosphatase (AP), SSEA-1, OCT4, SOX2, and NANOG (Matsui et al., 1992; Resnick et al., 1992; McLaren, 2003; Surani et al. 2007; Shim et al., 2008). Their potential for differentiation is also similar to mouse ESC, as evidenced by their ability to differentiate in vitro into different tissues from the three germ layers (Rohwedel et al., 1996), and to re-enter the germ line when injected in blastocyst embryos (Laboski et al., 1994). Therefore, mouse EGCs may be considered equivalent to ESCs.

The conditions for derivation of mouse EGCs are similar to these used for ESCs, with two important growth factors being included in addition to leukemia inhibitory factor (LIF). The first is

stem cell factor (SCF), a ligand for the c-kit, which is important for the survival of PGCs in-vivo and in-vitro by suppressing apoptosis (Pesce et al., 1993). The second is basic fibroblast growth factor (bFGF), which plays a major role in the reprogramming of mouse PGCs into pluripotent cells in vitro (Durcova-Hills et al., 2006). The mechanism of this reprogramming is still poorly understood, but it has been found that bFGF downregulates the expression of basic lymphocyte maturation protein 1 (Blimp1) in vitro, which in turn causes upregulation of c-myc and KLF4 (Durcova-Hills et al., 2008). Together with OCT4 and SOX2, these two transcription factors have been shown to reprogram somatic cells to induced pluripotent stem cells (iPSC; Takashi and Yamanaka, 2006). Since mouse PGCs already express OCT4 and SOX2, iPSC-like mechanisms may be involved in their reprogramming to EGCs.

Porcine EGCs were first derived by Shim et al. (1997) who used embryos at day 25 of gestation in conditions similar to these used for culture of mouse EGCs. The established cell lines had ESC-like morphology, expressed strong AP, and showed ability to differentiate into different cell types in vitro and in vivo. Following this report, other groups have derived porcine EGC-lines using embryos at days 25-28 (Müller et al., 1999; Lee et al., 2000; Lee and Piedrahita, 2000; Durcova-Hills et al., 2008; Petkov and Anderson, 2009), and from the Chinese minipig (Tsung et al., 2003). Recently, we have established multiple porcine EGC-lines from migrating PGC at days 20-24 from Danish Landrace crosses and the Yucatan minipig (Petkov et al., 2011).

Porcine EGCs form flat, but very compact colonies with well-defined and refractory borders and consist of small cells with a low nuclear to cytoplasmic ratio. Characterization for pluripotency marker expression by immunocytochemistry has shown that in addition to strong AP activity, these cells express at various levels OCT4, SSEA-1, SSEA-3, and SSEA-4. With respect to gene expression, porcine EGCs seem to resemble human EGCs, and express AP, OCT4, SSEA-1, and SSEA-4 (Turpenny et al., 2003). Porcine EGC-lines have been shown to proliferate for many passages (in our hands, most EGC lines proliferate for 35 passages, and some lines can reach more than 55 passages and may proliferate for over a year), while maintaining stable karyotypes. This is far longer compared with fetal fibroblasts and other somatic cells, that typically senesce within 10-15 passages.

The tissue culture conditions used by all groups for derivation and propagation of porcine EGCs are similar to those used for human or mouse ESCs. Typically, the cells are grown on feeder layers of mitotically inactivated embryonic mouse fibroblasts.

Porcine EGCs can form simple EBs, consisting of large epithelial-like cells on the periphery surrounding mesenhymal-like cells in the center. Usually after a week in culture, the simple EBs cavitate and thus resemble embryos more fully. When allowed to attach on gelatin-treated plastic dishes, the EB-cells proliferate and spread around the structure, giving rise to several different types

of cells. In our experience, EB-outgrowths can contain epithelial-like, mesenchymal-like, fibroblast-like, neuronal-like, and endothelial-like cells (Petkov and Anderson, 2009).

So far only one group has reported teratomas resulting from porcine EGCs (Tsung et al., 2003). The authors reported that the teratomas contained cells from the three primary germ layers, such as epithelial, neuroepithelial, and adipose tissue. In our hands, porcine EGCs derived from migrating PGCs in serum-free conditions have failed to form teratomas, although they have shown the ability to differentiate in vitro (Petkov et al., 2011). This is similar to results reported for human EGCs, where injected cells have failed to form teratomas following injection into immune-deficient mice (Shamblott et al., 2000; Turnpenny et al., 2005), but in controversy with a more recent report on the formation of teratomas from human EGC cultured in serum-free conditions (Hua, 2009). Another way of testing the differentiation potential of pluripotent cells in vivo is by chimera formation, where the cells injected in early embryos contribute to the three germ layers and potentially to the germ line. Unlike mouse ESCs and EGCs that have been shown to re-enter the germ line in chimeras, pig EGCs have been able to form only chimeras with low percentage of donor-derived cells in the somatic tissues, and have not shown a proven germ line contribution (Shim et al., 1997; Müller et al., 1999, Piedrahita et al., 1998). However, similarly low chimeric contribution has been achieved by injection of somatic cells (fetal fibroblasts) in sheep blastocysts (Karasiewicz et al., 2008) and 8-cell mouse embryos (Piliszek et al., 2007). Therefore, caution is needed when interpreting chimera experiment results, and using proper controls (injection of somatic cells) is necessary to distinguish the "true" stem cells from somatic cells that can be integrated into the embryos after partial reprogramming by the surrounding embryonic cells.

2.3.3 Induced pluripotent stem cells (iPSCs)

In a revolutionary experiment, Takashi and Yamanaka (2006) discovered that the genome of a differentiated somatic cell could be epigenetically reprogrammed to pluripotency by induced expression of pluripotency transcription factors, resulting in the generation of iPSCs. The authors initially expressed 24 pluripotency genes in fetal fibroblasts in order to reprogram them into iPSC, but subsequently they found that expression of only 4 of these genes, namely, OCT4, SOX2, c-myc, and KLF4 was sufficient to achieve the same results. The reprogrammed cells were morphologically indistinguishable from mouse ESCs (Fig. 2.5). However, although these iPSCs had similar differentiation potential in vitro, they failed to re-enter the germ line in chimera formation assays. A year later, Okita et al. (2007) produced germ line competent mouse iPSC using the same growth factors, but by selecting the reprogrammed cells by NANOG expression.

The establishment of the first human iPSCs was reported by two groups simultaneously. The same group that produced the first mouse iPSCs successfully generated iPSCs from adult human fibroblasts using the same 4 transcription factors (Takashi et al., 2007). The other group, from the lab of human ESC pioneer James Thompson, was able to reprogram human fibroblasts by expression of OCT4, SOX2, NANOG, and LIN28 (Yu et al., 2007).

The quest for porcine iPSCs soon began (Fig. 2.6), and within another two years, three different groups reported the establishment of iPSC in the pig. One of these first groups generated porcine iPSC by lentiviral transduction of six human transcription factors (OCT4, SOX2, C-Myc, KLF4, Lin28, and NANOG) under the control of a doxycycline-inducible promoter (Wu et al., 2009). Almost at the same time, another Chinese group published report of iPSC derivation (Esteban et al., 2009), using constitutively expressed lentiviral vectors carrying the mouse cDNA sequences of OCT4, SOX2, c-myc, and KLF4. In the third report that followed shortly, Ezashi et al. (2009) also produced porcine iPSCs using 4 human transcription factors (OCT4, SOX2, C-Myc, and KLF4). Transcriptional profiling of the cell lines using Affymetrix microarray confirmed that the cells were indeed reprogrammed and had endogenous expression of a variety of ESC markers. However, continued expression of the exogenous transcription factors was detected in the iPSCs from all groups. This problem is not unique to the pig as it has been reported also in other species (Takashi et al., 2007). In any case, this continued expression of pluripotency genes did not pose any problems for the differentiation of the cells, and all three groups demonstrated that their cell lines were able to differentiate in-vitro (including EB formation) and in vivo by formation of teratomas containing all three germ layers. In a recent publication it was demonstrated that porcine iPSC could form chimeras with high efficiency (85.3%) and contribute to all three germ layers (West et al., 2010). Moreover, they can also contribute to the germ line, as germ line transmission was demonstrated at a low frequency of 2 out of 43 born offspring (West et al., 2011). One of the of the piglets, however, was stillborn and the other died 3 days after birth indicating that there may be underlying epigenetic problems in the process.

One of the strong advantages that may be provided by iPSCs in the future is the possibility to derive customized pluripotent cells from every patient that can be used for regenerative therapies without the risk of immune rejection. However, since the epigenetic reprogramming requires prolonged expression of the transgenes (2-3 weeks), most of the iPSCs produced to date have been generated with the use of lentiviral vectors that integrate known oncogenes (such as c-myc and KLF4) into the cell genome. The dangers of using these genes became evident in a study where mouse chimeras generated with iPSC-developed tumors following re-activation of the initially silenced

transgene c-myc (Okita et al., 2007). Another avenue opened by the iPSC-technology, is the establishment of patient-specific in vitro cell models for serious human diseases (Fig. 2.7).

To avoid problems associated with viral integration and the use of oncogenes, many groups have developed various strategies for iPSC-production where the disadvantages discussed above have been minimized. It was found that reprogramming can be achieved in both human and mouse cells without the use of c-myc, albeit at the expense of the efficiency (Nakagawa et al., 2008). The use of certain transcription factors can be omitted when using cell types that already express them, as in the case where neural progenitor cells that express endogenous SOX2 and c-myc have been reprogrammed using only induced expression of OCT4 and KLF4 (Kim et al., 2008; Shi et al., 2008).Viral integration, which also carries risks of insertional mutagenesis, can be avoided by using non-integrating adenoviral vectors (Okita et al., 2008; Stadtfeld et al., 2008), or by using episomal vectors, which are normally cleared from the cells after a certain number of divisions (Yu et al., 2007). Another attractive alternative to the use of viral vectors are the transposon vectors, which combine high transfection efficiency with enhanced safety, and can be removed from the cell genomes following reprogramming. PiggyBac is a DNA transposon, which following insertion can also be removed from the reprogrammed genome without leaving a trace, and it has already been used successfully in the production of iPSCs (Woltjen et al., 2009; Yusa et al., 2009). Lastly, in a particularly original approach, iPSCs have been produced by providing the transcription factors in the form of recombinant proteins (Zhou et al., 2009).

Regarding future research in porcine iPSC-production, the choice of cell type and strategy will likely play an important role in the reprogramming efficiency. It should be noted, however, that the same problems existing in porcine ESC-culture are likely to affect the long-term maintenance of the newly generated pig iPSC-lines. For example, it seems that the culture conditions that support pluripotency and self-renewal of the porcine iPSCs still need to be optimized. This suggestion comes from the fact that, in all published reports to date, the reprogrammed cell lines have been maintained with continuous expression of exogenous pluripotency genes. This is particularly evident in one of the studies, where instead of using growth factors or other supplements, the pig iPSCs were maintained with doxycycline-induced expression of the pluripotency transgenes, until the authors chose to differentiate the cells (Wu et al., 2009). There seems, however, to be underlying challenges with respect to establishment of bona fide iPSC lines in the pig. A recent investigation on partially reprogrammed porcine iPSCs revealed that the expressed transgenes seem to block for the endogenous expression of the same pluripotency genes (Hall et al., 2012). Hence, as for the ESCs the pig poses specific challenges to overcome.

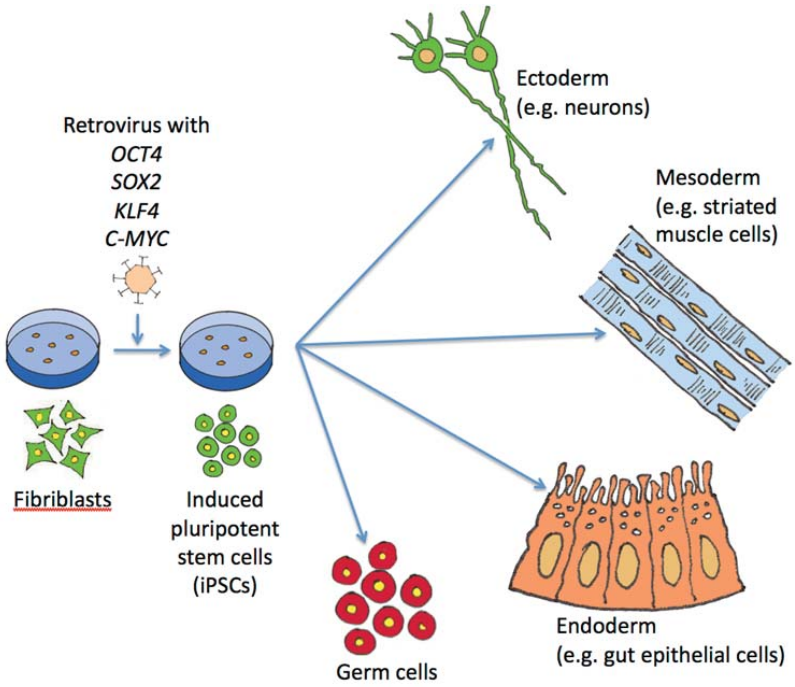

Fig. 2.5: Induced pluripotent stem cells (iPSCs) can be produced by reprogramming of somatic cells (e.g. fibroblasts) with genes of four important stem cell transcription factors. IPSCs are pluripotent and can be differentiated into all different cell types of the mammalian body representing the three somatic germ layers, ectoderm, mesoderm and endoderm, ads well as germ cells.

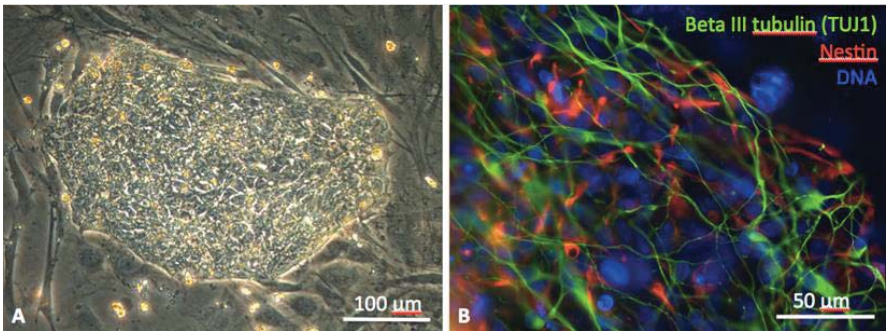

Fig. 2.6: Porcine induced pluripotent stem cells (iPSCs). (A) Porcine iPSC colony on top of mouse embryonic fibroblasts as feeder cells. The iPSCs were produced by reprogramming of porcine neural progenitor cells with a polycistronic lentiviral vector carrying a polycistronic construct including the porcine sequences for OCT4, C-Myc, SOX2, and KLF4. The genes were controlled by a doxycycline-inducible promoter. (B) Neural differentiation of the porcine iPSCs in A.

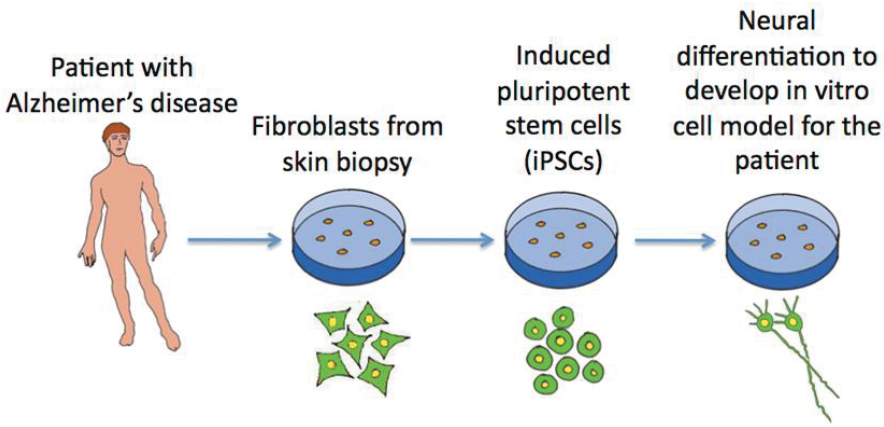

Fig. 2.7: Derivation of patient-specific neural in vitro cell model for Alzheimer's disease. The model can be used for unravelling of molecular disease patophysiology, for drug testing, and, hereby, for development of patient-specific treatment modalities.

2.4 Somatic stem cells

2.4.1 Mesenchymal Stem Cells

Perhaps the most promising clinical studies for treating disease and injury in the minipig come from studies of transplanted mesenchymal stem cells (MSCs) and their cell derivatives. MSCs are derived from varying tissues including the bone marrow, umbilical cord Wharton's jelly and cord blood, amniotic fluid and peripheral blood (Rho et al., 2009). These cells can be identified from both the presence and absence of cell surface marker expression. Cell surface antigens that have been detected from porcine MSCs include CD29, CD44 and CD90, whilst some surface markers lacking, include CD45 (Rho et al., 2009). Unfortunately, there is not one well-defined marker to characterize these cells from other cell types, which can make it difficult to trace MSCs in mixed cell populations. These cells are further defined by their ability to form osteoblasts, adipocytes and chondrocytes. There is some debate as whether such cells may also form cell types from other germ layers than the mesoderm. MSCs are considered to be a particularly favorable type of stem cell as they can be easily isolated from tissue and expanded in culture. There has also been some reports that they have low immunological rejection properties and are considered to be potentially useful for repair of myocardial tissues as well as bone and cartilage repair.

There have been a number of interesting studies reporting the beneficial effects of MSCs in repair of myocardial tissues in the pig with a small proportion of studies performed on the miniature pig. One study in pigs, investigated the effects of transplanted bone marrow stromal cells (which contain MSCs) treated with 5-aza-C into a myocardial infarct region (Tomita et al., 2002). The results

indicated that the cells induced angiogenesis, formed islands of myocardiac tissue, induced new capillary growth and improved cardiac function. In the case of the minipig, one recent and very promising study reported that male allogenic MSCs when injected into female minipigs suffering from myocardial infarction, could differentiate into cardiomyocytes, form vascular smooth muscle and endothelial cells (Quevedo et al., 2009). Similarly, in this study an improvement in heart contractility and improved blood flow was observed after a period of 12 weeks. Another more long-term study investigated the effects of isolated cells from the bone marrow of minipigs (these cells had some MSC-like properties) 6 months following transplantation into minipigs with acute myocardial infarction (Sheu et al., 2009). This report revealed autologous transplantation of these cells could improve the left ventricular ejection and reduce the mitral regurgitation. It is unclear from such studies the mechanisms for such improvements. Some proposed hypotheses include transdifferentiation (i.e. differentiating into cardiac cells rather than blood or bone), cell fusion or by release of cytokines or growth factors, such as vascular endothelial growth factor (VEGF) (Rho et al., 2009).

Minipig MSCs have also been demonstrated to be beneficial for bone and cartilage repair. Teeth regeneration studies have been especially promising. One recent report has shown that injection of minipig MSCs, platelet-rich-plasma and scaffolds, into damaged root sites within the mandible, resulted in the regeneration of new, vital bone between residual graft particles after a period of three months (Pieri et al., 2008). A study in Göttingen minipigs also shows that autologous MSCs can form cartilage in vivo (Jung et al., 2009). In this case, transplanted MSCs were injected into cartilage defects that were induced within the patellar groove. Cell distribution was more homogenous across the site and histomorphological scoring was higher compared with treatment using collagen membrane. Despite these good outcomes, poorer outcomes can be observed in older animals, repair on weight-bearing areas is still not optimal and new and improved scaffolds need to be developed (Rho et al., 2009).

Another promising field of study for MSCs is in trachea transplantations. A very recent article suggests that following partial trachea removal, a combination of autologous MSC-derived chondrocytes and epithelial cells together with de-cellularized matrix allowed the pigs to survive at least 60 days (Go et al., 2010). The matrices were completely covered by both cell types within 72 h of transplantation.

One drawback of MSCs is their inability to be cultured long-term in vitro. One research group has attempted to overcome this problem by overexpressing the human telomerase reverse transcriptase (TERT) gene in Chinese Guizhou minipig MSCs (Wei et al., 2008). This successfully results in extended culture of these cells, for up to 60 passages in vitro. Furthermore, despite some promising results in vitro suggesting that Banna miniature pig MSCs can suppress the proliferation

of peripheral blood lymphocytes and have low immunogenic profiles (Liu et al., 2004), emerging evidence also shows that complete immune responses can be observed in vivo in Massachusettes General Hospital-minipigs (Poncelet et al., 2007).

2.4.2 Neural Stem Cells

Neural stem cells (NSCs) can be derived from neurogenic zones in the developing fetus or adult animal brain. In the fetal brain, neuroepithelial cells can be obtained from the ventricular zone of the telencephalon, and other NSCs can be found in the rostral subventricular zone (Shin et al., 2007). In the adult brain, these cells have been isolated from the subventricular zone of the lateral ventricle and in the dentate gyrus located in the subgranular zone (Ma et al., 2009). NSCs may have multipotent or unipotent potential and can form neurons, glia and oligodendrocytes. Some researchers also may name such cells neural progenitor or precursor cells, however the underlying differences in these names can be difficult to delineate. Precursor or progenitor may be a more suitable name, if the cells have a finite lifespan.

There are currently no reports of production of NSCs from the minipig. However, there are some reports in other pig breeds. Neural precursor cells have recently been isolated from the porcine adult subventricular zone (Liard et al., 2009). These cells appear to have multipotent characteristics and apparently can form immature neurons, astrocytes and oligodendrocytes. NSCs may also be derived following differentiation of ESCs. This has been reported recently by one research group (Puy et al., 2010). In this study, ESC-outgrowths were allowed to differentiate in vitro. Isolated neural structures, called rosettes, were further cultured resulting in derivation of a NSC-line. These cells could form oligodendrocytes and astrocytes, but were not able to form neurons.

The minipig has also been considered a useful biomedical model for stem cell transplantation for treatment of neurodegenerative diseases, due to its large brain size and similar blood supply and immunogenic response compared to humans (Vodicka et al., 2005). One model of interest is the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) Göttingen minipig, which provides a useful model for studying Parkinson's disease (Bjarkam et al., 2008). However, there are no reports of stem cell transplantation into this porcine model. In contrast, more studies have investigated the effects of porcine cells transplanted into rodent neurodegenerative models. One example includes the transplantation of porcine neural precursor cells isolated from Day 22 fetal ventral mesencephalon into a rodent model of Parkinson's disease. In this study, survival of these cells and differentiation into neurons could be observed (Armstrong et al., 2003). However, two drawbacks were observed. Few of these cells could differentiate into dopaminergic neurons (a particular neuron which degenerates in Parkinson's disease progression) and an immune response was observed after 18

weeks. This study illustrated that immune-suppression is important and pre-differentiation of the cells in vitro and/or transplantation with differentiating factors should be considered. A later study revealed even more promising results following transplantation of porcine neural precursor cells derived from fetal ventral mesencephalon into an immune-suppressed rodent model of Parkinson's disease (Harrower et al., 2006). The grafts survived for 5 months and host vascularization and myelinated fibers could be detected with the graft. Hence, there remains the potential for using porcine neural stem cells as a cell source for modeling treatment of human neurodegenerative diseases such as Parkinson's disease.

2.4.3 Skin stem cells

The adult stem cell field is certainly large, considering there are so many different populations of stem cells within the fetal and adult body. Skin-derived stem cells have been isolated from fetal pig skin (Biernaskie et al., 2006; Zhao et al., 2009). The isolate stem cell population reportedly express pluripotent markers at the gene level, including OCT4, NANOG and SOX2 and appear to be multipotent and able to form immature neurons and glia in vitro (Zhao et al., 2009). There is also some evidence from this study that skin-derived stem cells can form smooth muscle cells in vitro (Zhao et al., 2009). Although this is relatively preliminary data, it does provide some early evidence of adult stem cell populations that could be useful for basic research studies on plasticity and cell differentiation.

There are many unexplored adult stem cell populations in the pig, which could be worth investigating further. It is likely that new reports will surface in the near future and further cell lines will be made available for both basic stem cell research and potential future cell therapies. Furthermore, the miniature pig provides a useful model for stem cell transplantation studies, as an alternative to rodent models for studying human disease.

2.5 Conclusions and perspectives

Stem cell biology in the pig is a rapidly developing research field. This is, for the most part, due to the increased availability of varying transgenic pigs being developed as models of human diseases. Although mouse and human stem cell research currently predominates over other species, the outcomes and findings from these species has provided a good setting and foundation for current and future porcine stem cell studies. In addition, the increasing popularity for developing alternate species' models, for studying human diseases and disease mechanisms has meant that porcine stem cell research is progressing at a new and heightened pace. With respect to the development of

pluripotent embryonic stem cells, the pig as a model has faced particular difficulties compared to mouse and man. Likewise, there are challenges with respect to the generation of fully reprogrammed iPSCs in this species. Despite this current limitation, the development of such pluripotent cell lines opens up the research field to assess the differentiation of many different cell types, which could ultimately be used for autologous or allogous cell transplantation studies, future cell therapy and treatment of disease. Regarding research in somatic stem cells, there is great promise from transplantation studies of mesenchymal stem cells into porcine heart, cartilage and bone.

3 Animal Genetic Resources and Gene Banks

3.1 Animal genetic resources

Animal genetic resources are a genetically unique population, together with their direct wild ancestors, created by processes of domestication within each animal species, which are used to production of food and agricultural purposes. The animal genetic resources have economic, scientific and cultural meaning. The economic meaning is connected with the fact, that animal husbandry production is important part of the economy. The scientific meaning of the animal genetic resources is based on the fact, that conserved populations are becoming control and they are used to monitor and to identify changes in genetic structure of the experimental populations. The animal genetic resources have their cultural meaning, because the breeding of some breeds is connected with the cultural and social development of nations.

By ratifying the UN (United Nations Organisation) convention on biodiversity was accepted the document of request for long-term management of biological resources in different countries of the world. In the 70's- 80's of the last century many activities were realized to the rescue of breeds in national character. Joint activities of FAO and EEAP have started program with general description of the breeds to be identified. These activities created an international databank of genetic resources at the Institute of Genetics and Animal Breeding in Hanover in 1987. For the animal resources is also important the global strategy FAO UN for the livestock genetic resources management, accepted in 1993. On the globe the existence of 30-50 million species of living organisms is presumed, about 15 000 birds and mammals species and about 30 of them breeds human for production of food and for the agricultural purposes. These 30 species have been domesticated over the past 10 000 years. During the domestication's processes, the genetically unique types or breeds allocated, acclimatized and adapted to conditions. According to FAO, about 10% of breeds of domestic animals were lost during the past century of and another 20% are in the risk of loss. The situation of breeds is especially adverse in Europe, where 68% of breeds are located at the different level of the risk of loss and 32% of breeds are out of the risk. The danger of disappear threatens 1 300 domesticated animal's breeds out of 6 300. In Europe 55% of mammalian breeds and 69% of avian breeds extinct or they are in the extreme danger. The worst situation in Europe is connected in an extent way with the fact, that the breeds on the other continents are not documented in detail. The livestock genetic resources are exploiting in Slovakia in the different range and with the different aims. The powerful pure blooded breeds are exploiting for the production and breading purposes. To a lesser extent, the animal breeds, which are part of world, or of the cultural heritage are breeding as well. About 40% of world's human population

is directly linked to the using of animals. The improvement and development of the animal husbandry production is dependent also on the existence of genetic variability within breeds.

3.1.1 The protection of animal genetic resources

The object of protection is a sample of population of genetic animal resource intended to isolated process of maintaining in the environment without such human activity that would cause genetic change. The protection is carried out by methods in situ and ex situ. The programmatic access to care for animal genetic resources is important for several reasons. The most often are those, which are connected with increased demand for products of animal origin and tendency to maintain all resources as food-stuffs for human and for agriculture. It cannot be ignored, that with the changing conditions less powerful breeds lose their competitiveness. The important reason for protection is the fact, that animal genetic resources are unique they are the part of the national heritage and integrating factor of heritage of nations. Last but not least, their main purpose is connected with an effort to prevent disease and to prevent their transfer to human population.

3.1.2 Conservation of animal genetic resources

Concept of conservation means managing human use of the biosphere in the way, which leads to systematic profit of current populations. The programs of animal genetic resources conservation are aimed on genes conservation or conservation of breeds or populations. New animal gene banks were created and they still keep forming. Resources of genetic information can be e.g.: nucleotides, chromosomes, male or female gametes, pronuclei, embryos, cells and adult animals. Preservation by steering is a system where by the sample or the whole population of animals is exposed to planned genetic change to maintain, use, recover and improve the quality and quantity of animal genetic resource and its products.

3.1.2.1 Methods for the conservation of animal genetic resources include:

1 a. Conservation of animal genetic material in the form of living ova, embryos or semen stored cryogenically in liquid nitrogen (ex situ).

b. Preservation of genetic information as DNA, stored in frozen samples of blood or other animal tissues or as DNA segments (ex situ).

2. Conservation of live populations in their adaptive environment or as close to it is practically possible (in situ).

In most developing countries, in situ conservation is the preferred conservation approach. In situ conservation has the benefit of allowing continued co-evolution of the genetic resources within the prevailing environment.

Ex situ conservation efforts generally for animal genetic resources, lag far behind similar efforts for plant genetic resources. The storage of genetic material for breeding purposes is common for some commercial breeds, but not in all species. Production and functional traits, and national capacity, should be taken into consideration in setting conservation priorities. The erosion of animal genetic resources has complex drivers and cannot be halted by one simple solution. A combination of in situ and ex situ conservation measures is necessary.

In such cases, it is important to support planned and targeted collecting of animal genetic resources, and to expand ex situ conservation activities :

1. To support populations conserved in vivo:
   a. as a back-up in case genetic problems occur in the living population (e.g. loss of allelic diversity, inbreeding, occurrence of deleterious genetic combinations);
   b. to increase effective population size of small populations and reduce genetic drift;
2. To reconstruct breeds, in case of extinction or loss of a substantial number of animals;
3. To create new lines (breeds), in case of breed extinction;
4. As a back-up, to quickly modify and/or reorient, the evolution / selection of populations;
5. For research.

Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands years. We know two methods :

1. In so-called „slow cooling“ methods, the biological material is cooled at a range of cooling rates that are fast enough to prevent „slow cooling damage“ but are slow enough to allow sufficient dehydration of the cells to prevent intracellular ice formation (IIF). The dehydrated cells in the „unfrozen fraction“ that remains between the masses of ice will ultimately reach a stable glassy state.
2. In so-called vitrification methods, the water content is lowered before cooling by adding high concentrations of cryoprotective agents (CPA).This allows fast cooling rates without risk of intracellular ice formation (IIF).

Fig. 3.1: Biological material stored in biological containers

Ex situ conservation takes place outside the native environment. It may or may not involve live animals, as there is the possibility of storing gametes, sperm or oocytes, or cells with the potential to develop new animals, e.g. embryos, using the scientific advances of cryopreservation.

Sperm - for many species the fertility of frozen semen is found to be lower than that of fresh semen. There may be considerable differences between breeds and between males, in the „freezability“ of the semen. As a consequence, frozen semen of some genetically interesting breeds or males may not be suitable as a gene bank resource, or can be used only with a poor efficiency.

Oocytes - the present efficiency and reliability of using frozen thawed oocytes for generating offspring is still much lower compared to cryopreserved embryos.

Further progress - more attention to fundamental aspects of cryobiology should enable further progress in cryopreservation methods. Recently a theoretical model was presented to predict the optimal cooling program for „slow cooling“ freezing methods. The model indicated that a nonlinear cooling profile could give better results than linear freezing programs. This and other models also demonstrate that the optimal cooling rate can be expected to be inversely related to the cryoprotective agents (CPA) concentration, and in fact this is found in empirical studies. It can also mean that a lower concentration of CPA would become feasible provided that a higher cooling rate is used. Improving technology (e.g. cryopreservation) is making long-term, ex situ, in vitro conservation of semen and embryos more feasible, affordable and applicable to a wider range of species.

The challenge is to decide :

- which animal genetic resources to conserve
- how to collect them
- where to store them
- when and how to characterize them
- and who can access, use and benefit from them in the future
- set and regularly review ex situ conservation priorities and goals

Action of ex situ collections

- Establish or strengthen national and regional facilities for ex situ conservation, in particular cryogenic storage. Support the efforts of countries within a region that have opted to establish a regional facility.
- Establish modalities to facilitate use of genetic material stored in ex situ gene banks under fair and equitable arrangements for storage, access and use of animal genetic resources.
- Develop and implement measures to secure ex situ collections from loss of genetic diversity resulting from disease outbreaks and other threats, in particular by establishing back-up samples.
- Identify and fill gaps in ex situ collections.
- Develop procedures for replenishment of genetic material taken from gene banks, by systematically developing links with live populations, or establishing in vivo populations of breeds at risk at off-farm locations, such as zoos and parks.

Ex situ, in vitro animal genetic resources conservation is a long-term insurance policy and an important first step in conserving animal genetic resources for future generations.

3.1.3 Endangered populations

The term endangered populations is used for description of population of an animal genetic resource, which is the subject of action of a force of change, affecting the maintaining of sufficient number of individuals. By the evaluation degree of threat of breed, the actual number of individuals is most often taken into account, which are actively used in breeding or the effective size of population. The both indexes are considered as basic. By the effective population range acts also the population age structure connected with steady number of offsprings in litters and longevity of animals. The evaluation degree of threat is dependent also on other indicators as are:

- the intensity of inbreeding in population and minimize the risks

associated with inbreeding

- gene drift
- genetic variability within population
- the need of time to satisfy a function of plan to survive the breeds
- increasing representation of individuals which has taken part in creation of the original population
- creation the subgroups in population, etc.

3.1.3.1 Some endangered species

Scientists are racing to set up a gene bank of the world's endangered animals, with thousands of species expected to become extinct within a generation due to climate change and habitat destruction. Giant panda - China will build its first genomic resource bank of endangered animal species to help save the giant pandas and other rare animals. Farming, development and clear cutting have destroyed the bamboo forests-pandas natural habitat. More than 50 per cent of these forests have been lost in the past few decades. The gene bank, to be built in the Giant Panda breeding and research base, will focus on keeping genetic materials of endangered animal species in cold storage, including sperm, eggs, cells, skin, hair and blood. The work will be done with the cooperation of chinese and american experts. The world's first use of frozen genes to successfully reproduce giant pandas in 1980. Since then, its aim has been to construct a genomic resource bank of endangered animal species, focusing on giant pandas.

Fig. 3.2: Gian panda

Saola - was discovered just 15 years ago by a team of scientists. It was the first large mammal discovered anywhere in the world since 1936. Scientists are still trying to figure out whether the saola is a goat, antelope or cow species. Only eleven have ever been recorded alive and fewer than 250 are estimated to exist today.

Fig. 3.3: Saola - pseudoryx nghetinhensis

Fig. 3.4: Some endangered species in the Slovak republic: 1. Pelegrine falcon; 2. Imperial eagle ; 3. Sea eagle; 4. Otther; 5. Falcon huntting; 6. Wigeon

3.2 Gene banks

Gene banks are defined as the systematic and organized collection, processing and storage of biological material for future use. These resources comprise samples of semen, testes, embryos, oocytes, ovaries, body tissue, fluid, cell lines and stem cells collected from both live and dead individuals. Gene banks are a means of preserving genetic material, be it plant or animal. In plants, this could be freezing cuts from the plant, or the seeds themselves. In animals, this is the freezing of sperm and eggs in zoological freezers until further need. This is one way scientists have prevented a gene family line from being wiped out. Genetic data bases represent the means to identify the relationship between genetic factors, lifestyle, or environmental exposures with disease susceptibility and treatment response. Depending on the type and structure of the bank, material from animals, patients, family members, healthy individuals, or population samples is preserved. One estimate suggests that a quarter of the Earth's species are at risk of extinction within the next thirty years. This

is primarily because of loss of natural habitat, though overhunting and pollution also play an important role.

- Natural selection can not operate unless there is genetic variability within a population.
- A population with a small gene pool would be unlikely to survive long periods of time because it lacks the ability to make evolutionary adjustments as conditions change.

One approach that may conserve animal genetic diversity by, for instance, helping to avoid inbreeding, is to compile a database of wild animal genetic resources. But animal genetic resources include all species, breeds and strains that are of economic, scientific and cultural interest to humankind for agriculture, now and in the future.

3.2.1. Historic development of gene banks

Historically, the revolution in domestic animal breeding technology over the last 50 years commenced with the collection, preservation and artificial use of semen collected from livestock, particularly in dairy cattle. The success of artificial breeding using frozen semen, embryo transfer procedures and now the advent of cloning in domestic species. A classic example of the development of these gene banks was the establishment of the first Australian Canine gene bank in 1976 with the collection and freezing of semen from domestic stud breeding dogs. This bank expanded over the years to include semen from selected and proven working Australian sheep and cattle dogs and guard dogs.

The final progression has been the extension of the bank to include species of wildlife with the collection and storing of semen from the Australian Dingo.

Fig. 3.5: Australian Dingo

For examle the Vietnam gene bank will keep stock of genes of all rare and precious animals including wild and native animals. For example, species under threat of extinction include the Saola, Peccary (Mexican hog) and Bos banteng.

3.2.2. Preparation of a DNA library

Gene bank is also term given to collection of cloned DNA fragments resulting from digestion of a genome by a restriction endonuclease. Each fragment is clonable by inserting it into an appropriate phage vector and introducing it into appropriate host cell (e.g. E.coli) for copying.

There are two types of DNA library:

1. The genomic library contains DNA fragments representing the entire genome of an organism.
2. The cDNA library contains only complementary DNA molecules synthesized from mRNA molecules in a cell.

- A cDNA of an eukaryotic organism (for example, a human) can be cloned into a prokaryotic organism (for example, E. coli) and expressed (translated into the appropriate protein) there (with limitations, for example post translational modification).
- A cDNA library is also important for analysis through bioinformatics. The complete cDNA library of an organism gives the total of the proteins it can possibly express.

Also, comparison of cDNA sequence between libraries constructed from cells derived from different organisms can provide insight into the genetic and evolutionary relationship between organisms through the similarity of their cDNA.

The gene banks secure the animal genetic resources, and in doing so provide new opportunities. One such opportunity is to enhance the exchange of animal genetic resources, and allow the benefits from animal genetic resources developed in one country to be shared elsewhere. Examples show that the importance of a breed can sometimes be more sustained in a country other than the one in which it is developed. Coordination of gene banks will be needed either through multilateral or bilateral agreements. In this context, there is a need to resolve how cryoconserved material can be stored in duplicate (or more) locations, to reduce the risk of catastrophic failure of one; how access and use can be made timely and traceable, with appropriate security to manage disease pathogens; and how replenishment of the gene bank can be achieved after access and use.

3.2.3 Modern day Noahs race to build wildlife gene bank

Scientists are racing to set up a gene bank of the world's endangered animals, with thousands of species expected to become extinct within a generation due to climate change and habitat destruction. While the biblical Noah collected live specimens to repopulate the world after the flood, the modern day Noahs will instead strive to preserve their biological details for posterity. The Arabian oryx and the Socorro dove are among the 10,000 species of animals listed by the IUCN world conservation union as being likely to vanish over the next 30 years Of these, some 33 species are already extinct in the wild, and nearly 1 000 are deemed to be critically endangered. Among the first species to enter the frozen ark will be the yellow seahorse, mountain chicken - which is actually a frog, the Seychelles fregate beetle and Polynesian tree snails. Frozen ark, which will hold samples of DNA and tissue at minus 80 Celsius, will save their genetic material for ever and give scientists an otherwise lost opportunity to study them. The project will collect DNA samples from mammals, birds, insects and reptiles, with priority being given to animals in danger within the next five years and those that are already extinct in the wild. Researchers will then focus on those expected to disappear within the next few decades.

3.2.4 The human gene bank

Since the early 1960's, people have seriously discussed the idea of freezing those who die in the hope that future technology would be able to revive them and restore them to health. This process is known as cryonics. Northwestern University has launched the Chicago area's first hospital gene bank and hopes to eventually recruit as many as 100 000 people willing to donate their DNA in the interest of science. The project, called NUgene, will use DNA samples and personal health information from volunteers, plus information from the human genetic code, to search out genes that play a role inmany diseases. Gene project may enable us to understand enough about the proteins produced by candidate genes that they can then be targeted as therapies. Genetic studies traditionally have been based on rare diseases afflicting smaller populations. Such studies will require access to thousands of DNA samples for genotyping and associated patient information from diverse populations. The first group of 2000 participants will be recruited among hospital patients who will be of varying age, sex, ethnicity and state of health and must be at least age 18 year. Similar project started up in Estonia. The aim of the Estonian Genome Project Foundation is to establish the gene bank, a database that contains health and gene data of the people of Estonia. The gene bank enables scientific and applied gene and health research to be carried out in order to find genes that influence the development of illnesses. Research carried out with the help of the gene bank shall not be limited to the present scientific level.

Legally dead - meaning the heart has stopped. This isn't the same as totally dead - because there's still some stuff going on inside the person's brain. The cryo-doctors and scientists will work to maintain that cellular brain function.

- The first thing they will do is connect your brain to a machine that supplies it with blood and oxygen. They are trying to keep your brain as "fresh" as possible.
- They will also inject a medicine called heparin into your blood stream so that the blood in your body does not coagulate.
- Then they will pack your body in ice to start cooling it down and ship you to the cryonics facility.
- The next step is to immerse the cells in your body in a cryoprotectant. The idea is to try to keep your cells from bursting when they freeze. Propanediol is one popular cryoprotectant. They pump this fluid through your blood stream so that it surrounds and permeates all of your cells.
- Now that your cells are protected, your body is placed in a bed of dry ice to cool it down to -120 degrees F. Then it is placed in a big vat of liquid nitrogen to give it a deep freeze at - 320 degrees F.
- The entire freezing process from legal death to liquid nitrogen temperature usually takes about three weeks.

Three things need to be worked out in order to bring cryonauts back to life :

1. Scientists will have to figure out how to thaw a full-size body so that there is no damage to the thawing cells.
2. They'll also have to figure out how to take a body that has technically "died" and reverse the whole dying process, plus fix any damage to the cells that "dying" caused.
3. They will have to figure out how to cure whatever.

One problem was that no one knew what sort of technology would be able to revive the frozen dead. The hope of cryonics was based on faith that new technologies would continue to develop so that, perhaps 200 years in the future, the frozen could be revived. Even so, there are currently about 180 people who have been frozen. And there are more than 600 people who have already paid to have their bodies frozen when they die. Who knows - maybe a hundred years from now these people will be walking and talking again! Gene banks are usually used for storing general genetic variability of endangered living populations but can be also used for storing alleles of a particular locus that are being eradicated through artificial selection programmes. In such scenarios gene banks would allow future re-introduction of one or more of the alleles being eradicated (and the associated diversity) into living populations. Frequencies within the bank for the locus of interest should have pre-determined

target values. An algorithm is derived to obtain the optimal contributions of all candidate donors to achieve the target frequencies of the removed alleles in the bank while maintaining at the same time genetic variability in other loci unlinked to those targeted in the eradication programme. The efficiency of the algorithm is tested using the case of gene banks storing prion protein alleles currently disfavoured in scrapie eradication programmes (the AHQ, ARH, ARQ and VRQ alleles). Results showed that the algorithm was able to find the combinations of candidate contributions fulfilling different objectives regarding target frequencies and restrictions on coancestry. The most important factors influencing the optimal contributions were the allelic frequencies and the levels of diversity (coancestry) of the living population. Heterozygotes were favoured over homozygous individuals as, for a given number of animals contributing to the bank, the use of heterozygotes leads to lower levels of coancestry.

3.2.5 National Center for Biotechnology Information (NCBI)

The GenBank sequence database is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced at National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration, or INSDC. GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100 000 distinct organisms. GenBank continues to grow at an exponential rate, doubling every 10 months. Release 155, produced in august 2010, contained over 65 billion nucleotide bases in more than 61 million sequences. GenBank is built by direct submissions from individual laboratories, as well as from bulk submissions from large-scale sequencing centers. Direct submissions are made to GenBank using BankIt, which is a Web-based form, or the stand-alone submission program, Sequin. Upon receipt of a sequence submission, the GenBank staff assigns an accession number to the sequence and performs quality assurance checks. The submissions are then released to the public database, where the entries are retrievable by entrez or downloadable by FTP. Bulk submissions of Expressed Sequence Tag (EST), Sequence Tagged Site (STS), Genome Survey Sequence (GSS), and High-Throughput Genome Sequence (HTGS) data are most often submitted by large-scale sequencing centers. The GenBank direct submissions group also processes complete microbial genome sequences. Cryoconservation of gametes and embryos is a quick and rather inexpensive way to prevent the loss of the genetic potential of a breed. Properly structured stores of frozen semen and embryos can also be used to support live animal conservation programmes with a minimum increase of inbreeding.

3.2.6 The future of gene banks

Regional or species specific wildlife gene banks have been in existence for over 20 years. Australia initiated the establishment of the first national gene bank in 1995 and this has been followed by similar banks in Africa, America and now The Frozen Ark Project in the UK who's goal is to link wildlife banks around the world into a international network. Gene
banks are not only a substitute for captive breeding programs or to fulfill the need to preserve our wildlife in its natural habitat. They are also an insurance for the future and a valuable reserve of information to supplement or complement the breeding and survival of our wildlife whether in captivity or in mainland island populations in the wild. The future for gene banks has great prospects.

4 Xenotransplantation

4.1 Introduction

Following the successful cloning of „Dolly“, the sheep, from an adult somatic cell ~15 years ago (Wilmut et al., 1997), somatic cell cloning has made steady progress and has been achieved in 13 different species (at the timepoint of writing this chapter). Although the overall efficiency is still unsatisfactory, nuclear transfer has emerged as a major breakthrough in the field of transgenic farm animal production (Vajta etal., 2006) because it allows targeted genetic modifications to be made in species for which embryonic stem cells are not yet available and because it dramatically reduces the cost of transgenesis in large farm animals.

One of the most exciting applications of transgenic technology is the development of genetically engineered pigs for use in xenotransplantation. Today more than 250,000 people are alive only as the result of successful human organ transplantation (allotransplantation). The success of organ transplantation technology has led to an acute shortage of appropriate organs, because cadaveric and live organ donation falls far short of meeting the demand in western societies. In the US more than 110,000 people are currently waiting for a suitable organ, while 18 people die each day waiting for an organ (http://organdonor.gov, Figure 4.1). To close the growing gap between demand and availability of appropriate organs, transplant surgeons are now considering the use of xenografts from domesticated pigs. Prerequisites for successful xenotransplantation are: (i) overcoming the immunological hurdles, (ii) preventing the transmission of pathogens from the donor animal to the human recipient, and (iii) verifying the compatibility of donor organs with human physiology.

Xenotransplantation is classified as concordant or discordant based on the immunological response of the recipient. Concordant xenografts are between closely related species, such as primate-to-human. They evoke an immunological response that is similar to an allograft and can thus be controlled by appropriate immunosuppression regimen. The use of primates for xenotransplantation is not a realistic possibility because all of the great ape species are already on the endangered list and there will never be sufficient numbers to meet the growing demand for suitable organs. Baboons, although potentially available in larger numbers, do not grow large enough to provide size-matched organs for most adult humans. Primate-to-human transplantation also implies an increased risk of transmission and recombination of infectious agents. In addition to these practical issues, the prospect of using higher primate species raises ethical concerns and would be unlikely to gain widespread acceptance.

Discordant xenografts, between distantly related species, invoke a more severe and violent immune response, which can not be fully prevented simply by the administration of immunosuppressive agents. Nevertheless, there are several advantages using a discordant donor species, in particular the pig, which has important physiological similarities to the human and can produce size matched organs. Pig production has reduced costs compared to primates and there is a long history of maintenance of pigs under strict hygienic specific pathogen free conditions. Ethical concerns are minimal as the pig is already a major source of animal protein throughout the world (Turk etal., 2004). Another key factor is the high reproductive capacity of pigs which have been bred for short generation intervals and large litters. This is critical for the eventual expansion of suitable transgenic donor populations to numbers which can match the growing demand for organs. Significant progress in the genetic modification of pigs for xenotransplantation has already been demonstrated by several groups and some new technologies that further improve the generation of transgenic animals have come to market.

4.2 Cloning and the generation of transgenic animals

Somatic nuclear transfer (SCNT) involves five major steps: 1) enucleation, 2) donor cell transfer, 3) fusion, 4) activation and 5) culture of the reconstructed embryos (Figure 4.2). As mentioned above, viable clones have been generated in 13 species. The number is rapidly growing as the technique is applied to the conservation of endangered species. Despite great efforts to improve cloning efficiency, cloning in all species is characterized by low success rates and frequent abnormalities in the cloned offspring such as the “Large Offspring Syndrome, (LOS)” observed in the cloned ruminants cattle and sheep .

Somatic nuclear transfer is making significant improvements in the generation of transgenic livestock. It has been shown that donor cells can be successfully transfected with various types of genetic constructs and that viable cloned transgenic offspring with stable integration and expression can be obtained in sheep, cattle, goats and pigs (Schnieke et al., 1997; Niemann et al., 2005). The main advantage is the possibility to preselect donor cell lines which exhibit optimal integration and expression of transfected transgenic constructs or carry targeted genetic modifications and then to use these cells in SCNT to produce one or more identical founder animals carrying the desired alterations. SCNT avoids the limitation of long generation intervals typical of large animal transgenesis where a founder animal was typically allowed to achieve sexual maturity and to produce an F1 generation before any functional testing was possible. Generation of multiple identical individuals in the first generation permits immediate testing for desired traits. For this reason, most groups interested in the transgenesis of large animals have switched from microinjection to nuclear transfer. This is especially

advantageous for the production of transgenic pigs for xenotransplantation due to the fact, that several identical founder animals are frequently born in a single litter. Some can be used immediately for transplantation related studies; while others can be allowed to mature as founder animals for natural breeding.

A great problem is still the generation of transgenic animals that feature a predictable and specific expression of the desired transgene. Transposons have been developed as important tools for transgenesis in flies, fish, frogs, mice and rats. DNA-based, or Class II, transposons are mobile genetic elements that move in the host genome via a "cut-and-paste" mechanism. Most DNA transposons are simply organized; they encode a transposase protein flanked by inverted terminal repeats (ITRs), which carry transposase binding sites and it has been possible to separate the transposase coding sequence from the ITR sequences. Any DNA flanked by the ITRs will be recognised by the transposase and will become enzymatically integrated into nuclear DNA. Apparently, no cellular cofactors are required The size of the integrated foreign DNA can exceed 10 kb. In a two-component system, the transposon is integrated solely by the trans-supplementation activity of the transposase.

Transposons have been successfully used in invertebrates, including Caenorhapditis elegans and Drosophila, for transgenesis and insertional mutagenesis. The first transposon sufficiently active for use in vertebrates was the Sleeping Beauty (SB) transposon which was developed in 1997 (Ivics et al., 2007). Since then, several other transposons have been found to function in higher eukaryotes, e.g. Piggyback, Frog Prince, Tol2 and Passport. Many drawbacks of classical methods for transgenesis can be overcome by transposition-mediated gene delivery, which increases the efficiency of chromosomal integration and facilitates single-copy insertion events. However, the main advantage of transposon-mediated transgenesis is that the integration of foreign DNA is directed to accessible euchromatic regions so that the transgene silencing which occurs after random integration into heterochromatic regions is prevented.

Co-transfection or injection of in vitro synthesized mRNA coding for transposase can enhance the efficiency of this technique due to the rapid availability of the transposase and circumvents the danger of integrated transposase sequences. This method has been employed for producing transgenic zebrafish (Suster et al., 2009) and Xenopus (transposons Tol2 and SB) (Yergeau et al., 2007); transgenic chickens (transposon Tol2) (Takahashi et al., 2008); transgenic mice (transposons SB and piggyBac) (Rad et al., 2010) and pigs (Garrels et al., 2011) (reviewed in (Garrels et al., 2012)). New technologies as e.g. the transposon-mediated transgenesis will further improve this important step in the production of transgenic pigs suitable for xenotransplantation.

4.3 Rejection responses after pig-to-primate xenotransplantation and strategies to prevent them

4.3.1 Hyperacute rejection:

Xenograft rejection includes several distinct physiological modalities, of which the hyperacute rejection (HAR) is the first and most destructive. HAR rejection of unmodified pig-to-primate vascularised organ xenografts resembles the rejection that occurs in allotransplantation without ABO blood group matching (Millan et al., 1994). It is characterized by an almost immediate loss of graft function and dramatic changes to the gross appearance of the xenograft including significant discoloration and swelling which can be observed within minutes to hours of reperfusion. The histopathological picture is that of haemorrhage, thrombosis and infiltration of neutrophils (Platt et al., 2001).

Hyperacute rejection is a consequence of the binding of xenoreactive antibodies, predominantly of the IgM isotype, to the xenograft endothelium (Gambiez et al., 1992). This activates the classical complement cascade leading to formation of the so-called ”membrane attack complex (MAC)” as well as endothelial cell activation, cell damage, platelet aggregation, platelet adhesion, and intravascular graft thrombosis leading to loss of graft function (Platt et al., 1991).

Certain bacteria and all mammals with the exception of humans, primates and old world monkeys exhibit a specific Gal epitope (galactose alpha 1,3 galactose linkage) on the surface of most cells including those of the kidney and heart. The enzyme responsible for the Gal epitope is alpha 1,3-galactosyltransferase (approved gene symbol - GGTA1). Humans have pre-existing antibodies for the Gal epitope, most likely from exposure to the capsular polysaccharides of bacteria that have the same sugar linkage. Approximately 1% of circulating antibodies in the human blood are directed against Gal epitopes. Thus, when pig cells or organs are transferred to humans or non-human primates, the combination of Gal epitopes and preformed antibodies result in instantaneous activation of the complement cascade and hyperacute rejection of the pig cells within minutes.

Prevention of HAR by transgenic approaches:

The most promising approach to suppressing the hyperacute response has been transgenic modification of the donor animals. Pigs expressing various human-complement regulating proteins including hCD59, human decay accelerating factor (DAF,CD55) and membrane co-factor protein (MCP,CD46) have been generated (Niemann et al., 2001; Byrne et al., 1997; Adams et al., 2001) and tested.

While the initial results were encouraging and xenograft (pig-to-primate) survival was increased up to 139 days (Kuwaki et al., 2004), the immune response was only partially blocked by this strategy and the transplanted organs were all eventually destroyed. After identification of alpha 1,3Gal

epitopes as the major antigens involved in the hyperacute immune response, two approaches to lowering the expression of the alpha 1,3Gal epitopes were evaluated. One was based on over-expressing enzymes, such as H-transferase, which compete for the substrates needed by the alpha 1,3 Galactosyltransferase. This approach was tested in pigs and mice both in vitro and in vivo (Mckenzie et al., 1998; Mckenzie et al., 2000; Costa et al., 1999; Cowan et al., 1998) and was found to decrease the levels alpha 1,3 Gal epitopes significantly. Unfortunately, the residual levels of Gal epitopes were still sufficient to trigger an immune response. The second approach was direct targeting of the gene responsible for the generation of these Gal epitopes - alpha 1,3 Galactosyltransferase.

With improvements in the efficiency of cloning technology, it recently became possible to generate alpha 1,3 Galactosyltransferase knockout pigs by combination of homologous recombination in primary fibroblast cultures and other types of differentiated primary cells, with somatic cell nuclear transfer (Dai et al., 2002; Lai et al., 2002; Harrison et al., 2002; Phelps et al., 2003).

Xenotransplantation of GalKO porcine kidneys and hearts into baboons gave encouraging results, leading to a significant increase in organ survival - up to 179 days for the heart and up to 83 days for the kidney (Kuwaki et al, 2005; Yamada et al., 2005) without any signs of hyperacute rejection.

It is becoming increasingly clear that a multiple transgene, multiple knockout approach will be necessary to produce an optimized xenotransplant donor animal and in the case of alpha 1,3 Galactosyltransferase, the combination of knockout and the H-transferase transgene might be superior to either approach on its own.

New technologies for the generation of knockout animals, that uses zinc finger molecules conjugated to an unspecific endonuclease FokI, have recently become available. Zinc Finger molecules were firstly described in Xenopus laevis and are part of the transcription machinery, where they serve as transcription factors. Zinc fingers have a specificity for 3 base pairs. The specificity can be increased by linking three zinc finger molecules which increases the specificity to 9 bp. The conjugated endonuclease needs to dimerize to cut the DNA and produce a strand break. Therefore, it is necessary to transiently transfect cells with two plasmids coding for each zinc finger nuclease (ZFN). The DNA strand break will subsequently repaired by a mechanism called “non-homologous end joining” (NHEJ) which could lead to the loss or integration of bases causing a reading frame shift, finally leading to the inactivation of the selected gene. Recently, it could be shown that this technology is also applicable to the pig. In one case, the scientist were able to completely knockout an endogenous gene with only one transfection (Hauschild et al., 2011). Compared to the homologous recombination approach, zinc finger nucleases increased the efficiency 10.000 fold. GalKO pigs have

been useful experimentally to investigate deeper barriers to xenotransplant survival. For example, porcine GalKO hearts transplanted into baboons functioned for 2 to 6 months but eventually succumbed to thrombotic microangiopathy (Kuwaki et al., 2005; Tseng et al., 2005) which was histologically quite different from the acute humoral xenograft rejection described above (Houser et al., 2004). Thrombotic microangiopathy was also seen in transplanted hearts from pigs transgenic for human decay-accelerating factor (hDAF) (Cozzi et al., 1995) in combination with an immunosuppressive regimen and continuous intravenous infusion of a Gal-alpha1,3Gal- conjugate to adsorb and deplete anti-Gal antibodies (Kuwaki et al., 2004). Although the survival of the GalKO hearts was not much longer than that achieved with hDAF hearts, avoidance of the necessity of continuous administration of the Gal-conjugate was a significant advance in practical terms. Less immunosuppression of the host immune system will be reflected in less complications and genetically modified donor pigs play an important role in reducing the levels of immunosuppression required for organ survival.

4.3.2 Acute vascular rejection and transgenic approaches to prevent it:

The acute vascular rejection (AVR) remains the major barrier to long-term pig-to-primate xenograft survival because microvascular thrombosis is a critical element of the rejection process. Persistent endothelial cell activation and cellular damage resulting from low levels of non-Gal-anti-pig antibodies overwhelm basal cellular anticoagulant defences and promote the development of thrombotic microangiopathy.

Microvascular thrombosis is a hallmark of the long-term rejection of porcine solid organ xenografts, regardless of the immunosuppressive regimen or donor type. Fibrin microthrombi are observed even in the absence of other markers of acute vascular rejection. Indeed, thrombotic microangiopathy is the predominant histopathological feature of rejected porcine GalKO cardiac xenografts (Tseng et al., 2005). No effective treatments for this have yet been developed. While there is increasing evidence that antibody-mediated activation of the graft's endothelial cells is the root cause of thrombosis, the relative importance of specific molecular incompatibilities that promote coagulation in xenografts remains unclear. These incompatibilities include: the inability of porcine tissue factor pathway inhibitor to adequately neutralize human factor Xa; the activation of both human prothrombin and factor X by porcine endothelial cells; the enhanced association of porcine von Willebrand factor with human platelet GPIb; and the failure of porcine thrombomodulin to bind human thrombin and activate the natural anticoagulant human protein C adequately (Bach et al., 1994; Jurd et al., 1996; Bach et al., 1996; Jurd et al., 1996a; Ierno et al., 1998; Robson et al., 1999). During transplantation, the unavoidable ischemia and reperfusion generates the production of a range of

damaging molecules in the vascular environment, including reactive oxygen species, activated complement, proteolytic enzymes and extracellular nucleotides. Activation and injury of endothelial cells induces numerous prothrombotic changes, including shedding of the glycocalyx and glycocalyx-associated proteins, loss of glycosylphosphatidylinositol (GPI) anchored molecules, internalization of thrombomodulin (TM), exposure of the sub-endothelial matrix and the expression of Tissue Factor (TF) and the fgl2 prothrombinase/fibroleukin gene (Ghanekar et al., 2004; Banz et al., 2006). These early injuries are insufficient to trigger the hyperacute rejection of GalKO or complement-regulator transgenic xenografts but it seems that persistent endothelial cell activation promotes the development of thrombotic microangiopathy due to molecular incompatibilities. Thrombomodulin (TM) is a component of a critical anticoagulant and anti-inflammatory system known as the protein C pathway (Esmon, 2003). TM is a multidomain integral membrane protein expressed on endothelial cells and it functions by conversion of thrombin from a procoagulant to an anticoagulant by blocking a site that is essential for thrombin´s procoagulant activity and then presenting protein C for cleavage by thrombin´s active site. The activated protein C (apC), with its cofactor protein S, proteolytically inactivates Factors Va and VIIIa to break the clotting cascade. In living tissues, ApC generation is enhanced 20-fold when protein C is bound to the GPI-linked endothelial protein C receptor (ePCR). It was shown that porcine aortic endothelial cells are not very effective at activating human protein C due to the inability of porcine TM to efficiently bind human thrombin (Siegel et al., 1997; Kopp et al., 1998). Indirect evidence for this molecular incompatibility has been provided by ex vivo perfusion experiments with human blood. This work showed that supplementation of the perfusate with soluble human thrombomodulin dramatically improved hepatic microcirculation in porcine livers (Shiraishi et al., 2001). Systemic anticoagulation treatment of recipients in pig-to-primate models has produced mixed results. Many anticoagulants have been tested including recombinant human antithrombin, heparin, aspirin and warfarin (Cowan et al., 2002; Kuwaki et al., 2004, Tseng et al., 2005; Cozzi et al., 2005) in pig-to-primate xenotransplantation, but none of the reagents could generate the levels of anticoagulant action required at the endothelial surface. First ex vivo perfusion results of porcine kidneys transgenic for human thrombomodulin showed no beneficial effect oh human thrombomodulin expression which could be due to the low and unspecific expression of the transgene or to the necessity of the expression of an co-factor the human endothelial Protein C receptor (ePCR) to accomplish full effects of human thrombomodulin expression on pig organs (Petersen et al., 2009). Various features of AVR could be delayed or prevented by continued aggressive application of one of the protocols such as xenoantibody depletion, used to prevent HAR. However, successful anticoagulation requires repeated depletion treatments and strong immunosuppression to prevent the rebound of antibody titers.

Due to the significant risk of hemorrhage associated with systemic anticoagulant treatment (Byrne et al., 2005), a safer approach to inhibition of thrombosis in xenografts is to genetically modify the donor organ to express tissue factor pathway inhibitor, thrombomodulin, CD39 or other human antithrombotic factors on the organ's endothelial surfaces. This approach has been successfully tested in several small animal models. Expression of human CD39 in mice, for example, significantly increased the survival of cardiac allografts in a model of antibody-mediated rejection (Dwyer et al., 2006). Protection of the endothelial cells from activation is an important step toward prevention of AVR. The transgenic introduction of "protective genes" in donor organs to increase their resistance to injury and to develop accommodation seems to be the most practical approach to creating a suitable line of donor animals. This principle has proven effective in many rodent models using a variety of genes including HO-I, Bcl-xl, Bcl-2 and A20 (Cooper et al., 1996; Bach et al., 1997) and was successfully transferred to the pig where similar effects could be observed in pigs transgenic for human heme oxygenase-1 (Petersen et al., 2011). Aortal endothelial cells from these pigs were protected against TNF-α mediated apoptosis and expressed significant lower levels of adhesion molecules as ICAM-1, VCAM-1 and E-selectin. In an ex vivo kidney perfusion circuit in which porcine kidneys were perfused with human blood no hyperacute rejection occurred and organs showed no formation of microthrombosis. Comparable effects were observed in organs and endothelial cells obtained from human A20 transgenic pigs (Oropeza et al., 2009).

4.3.3 Cellular rejection

Cellular rejection is the major problem to be managed in allotransplantation. The CD4+ T cells are the prime initiators of this response and CD8+ T cells and macrophages are the ultimate effectors. In the context of xenograft rejection, it is expected that the cellular response will involve these and other additional cell types. CD4+ T cells and neutrophils produce a strong response to porcine endothelium in vitro and in pig-to-primate xenografts macrophages and NK cells characterize the cellular infiltrate (Anrather et al., 1997). Not only the recruitment of additional cell types but also the very nature of the interactions between ligands and receptors can differ in a xenograft context, limiting the effectiveness of existing protocols for immunosuppression.

Prevention of cellular rejection

In 1983, the introduction of cyclosporine A to clinical allotransplantation gave a dramatic improvement in graft survival, due to the prevention of cellular rejection. More recently, the combination of cyclosporine A with other immunosuppressive drugs was successfully used to prolong the survival of porcine kidneys after transplantation into primates (Cozzi et al, 2003). On the other

hand, groups working on the genetic modification of xenograft donor pigs expect that transgenic pigs expressing T cell modulating genes such as HLA-E and PD1-L will be able to prolong survival of porcine xenografts without the need for high-dose application of immunosuppressive drugs. A third route to suppression of the post-hyperacute rejection mechanisms mediated by cellular components of the immune system is based on transgenic expression of human tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) in donor organs. Human TRAIL expression by porcine lymphocytes was shown to induce apoptosis of immortalized T lymphocytes (Jurkat and Hut 78.2 cells) in co-culture experiments. Neutralization of this effect with anti-TRAIL antibodies demonstrated that this was a TRAIL-specific effect (Klose et al., 2005). Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is co-stimulatory molecule that inhibits T-cell activity and may be useful in prolonging graft rejection. CTLA4, a T-cell surface antigen that is upregulated following CD28/B7 interaction, shares affinity for the B7 ligands with CD28, and binds CD80 and CD86 with greater affinity and avidity than CD28. In this way, CTLA4 successfully competes with CD28 in binding B7 on antigen-presenting cells and downregulates T-cell response. This co-stimulation blockade by CTLA4 offers a strategy for downregulation of the CD4+ T-cell response occurring in xenotransplantation. Pigs transgenic for CTLA4-Ig, that expressed very high titres in the serum had a severely compromised humoral immune status (Phelps et al., 2009). Therefore, a tissue-specific or controlled expression of CTLA4-Ig is necessary in transgenic pigs that could force a reduced T-cell response after xenotransplantation.

4.4 Porcine endogenous retroviruses:

Pigs maintained in specific-pathogen-free (SPF) facilities do not carry the bacteria, fungi and parasites that are known to be pathogenic in humans. A greater risk is posed by porcine endogenous retrovirus (PERV) sequences, which are a part of the porcine genome and cannot be simply eliminated by pathogen-free housing. It has been shown that PERVs are capable of infecting human cells in vitro (Patience et al., 1997; Martin et al., 2000; Martin et al., 2000a; Specke et al., 2001), raising concern over the safety of using porcine xenografts in large numbers of immunosuppressed patients. The pathogenic potential of PERVs is largely unknown but retrospective studies of the first experimental and clinical uses of porcine tissue (mostly short term skin transplants) for human xenotransplantation revealed no evidence of PERV transmission or recombination with human endogenous retroviruses (Paradis et al., 1999; Tacke et al., 2001; Denner et al., 2001; Winkler et al., 2005) despite experimental evidence of the adaptation of PERVs to human cells in vitro (Scheef et al., 2001; Scheef et al., 2002).

Pigs with a knockout for α1,3-galactosyltransferase theoretically present an increased risk for xenotransplantation as any virus particles released from Gal-free cells would not be recognized by preformed anti-Gal antibodies (Takefmann et al., 2002; Quinn et al., 2004). The other risk is related to recombinant PERVs. Recombination between the human-tropic PERV-A and the ecotropic PERV-C has been observed in pigs (Wilson et al., 1998; Wilson et al., 2000; Bartosch et al., 2004; Harrison et al., 2004; Niebert et al., 2005) and in some cases the recombined sequences were integrated into the pig genome. Exogenous forms of human-tropic porcine endogenous retrovirus have been identified in miniature pigs (Wood et al., 2004). Although no transmission of PERVs into human cells has been observed in vivo, a practical approach to minimizing the risk of recombinant PERVs based on down regulation of PERV expression by RNA interference has already been developed and tested. PERV expression was shown to be significantly down regulated in primary porcine cells by RNAi carried into the cells by lentiviral vectors (Dieckhoff et al., 2007). In this study, the expression of PERV-mRNA was significantly reduced in all primary porcine cells and in the porcine kidney cell line PK-15. These experiments demonstrate that it is feasible to down regulate PERVs (and potentially other retroviral expression) and that it will be possible to generate transgenic pigs carrying siRNA against PERVs. The application of this approach in vivo is currently being tested. Minipig lines which are free of specific types of PERV (Oldmixon et al., 2002) have been identified and tests have been developed for PERV identification and mapping within the porcine genome. Deletion of PERVs sequences from the porcine genome with the cloning technology would, at least theoretically, be possible in pigs. However, the presence of multiple copies of PERV in most pig genome makes this an onerous task. There is general confidence in the field that sufficient tools are available to control the risks presented by PERVs.

4.5 Conclusions and perspectives

Pig-to-human xenotransplantation can insure an unlimited supply of donor organs for patients with end-stage cardiac, liver or kidney failure, and for the treatment of diabetes, if immunological responses are controlled and if other technical problems are solved. The hyperacute rejection response, which was the premier hurdle in pig-to-human xenotransplantation, can already be overcome in a clinically relevant manner by expression of human complement regulatory proteins in transgenic pigs and/or by knockout of enzymes responsible for gal epitopes (GalKO). However, even with strong immunosuppressive treatment, acute vascular rejection and cellular rejection remain as major obstacles for long term survival of a porcine xenograft. The techniques for introducing

beneficial genes or removing undesired genes are available, but genetic modification of pigs and testing of these modifications in nonhuman primates is a very expensive and time- and labour consuming interdisciplinary endeavour. National and trans-national consortia have been established to advance this field and established methods for transgenesis and somatic nuclear transfer afford the possibility of making the genetic modifications needed to produce porcine organs capable of survival in a human being (www.xenome.eu). For some applications such as treatment of diabetes, this work has approached a level where clinical trials have recently been initiated while with solid organs such as porcine heart, kidney and liver transplantation, there is much work to be done. Nevertheless, heterotopic pig heart survival in a primate for nearly eight months has been demonstrated and existing problems are being eliminated systematically. Previous advances in control of hyperacute rejection and control of the human reaction to porcine gal epitopes have lead to the discovery of new challenges to xenotransplantation such as non-Gal antibody mediated rejection. Widespread application of pig-to-human whole-organ xenotransplantation will depend on optimization of a set of genetic modifications which suppress coagulation and protect the graft from cellular rejection over the long term. The advantage of this approach is that an appropriately modified organ can minimize the need for strong immunosuppressive treatment of the transplant recipient while inducing T- and B- cell tolerance and promoting immunological accommodation. This will be further supported by the development of protocols that effectively induce long term tolerance of the xenograft by using embryonic stem cells (Fandrich et al., 2002). Prolonged survival of transplanted porcine islets up to 260 days (Hering et al., 2006; Cardona et al., 2006) demonstrates the potential of xenotransplantation as a successful treatment in the near future.

Although additional refinements will always be possible, it is expected that appropriate lines of transgenic pigs will be available as organ donors for human xenotransplantation within the next decade. Transplantation of pancreatic islets from transgenic pigs may take place even earlier. Guidelines for the clinical application of porcine xenotransplants already exist in the USA and have been developed in other countries. The worldwide consensus is, that the technology is ethically acceptable as long as the individual's well-being does not compromise public health (e.g. the risk of PERV recombination). The improvement in quality of life for patients receiving conventional allotransplants is dramatic. Xenotransplantation with transgenic organs can extend this benefit to a greater number of patients and is economically attractive because the long term costs of treating patients with severe kidney or chronic heart disease is greater than the cost of a successful organ transplant.

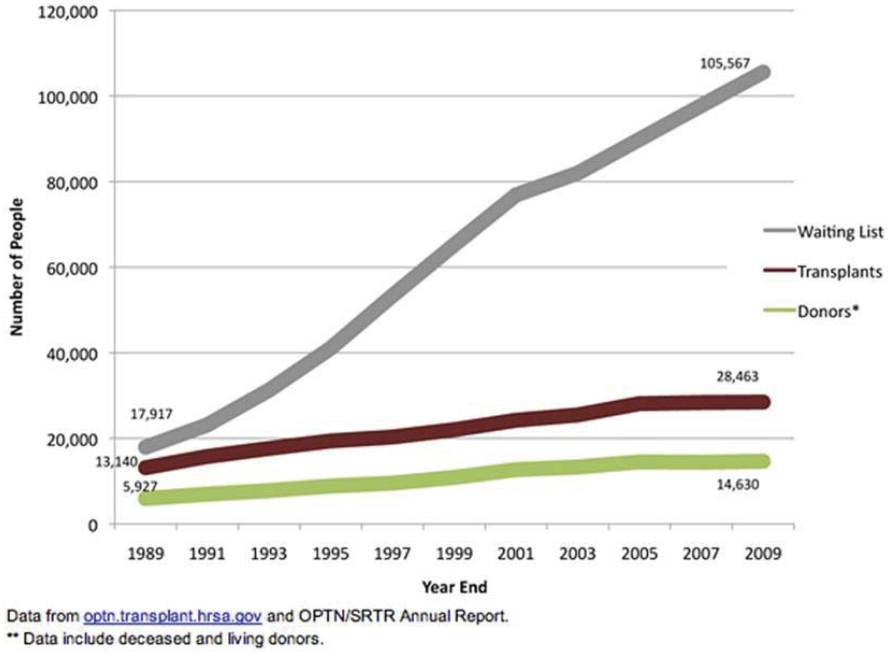

Fig. 4.1: Number of people on the waiting list and transplants in the United States.

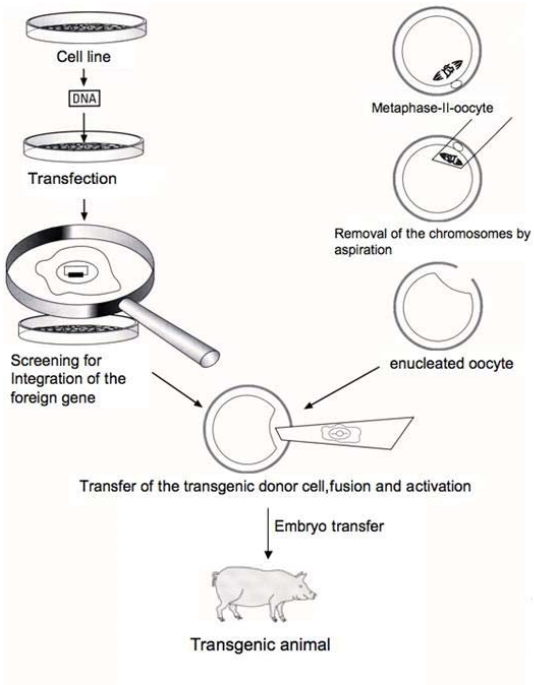

Fig. 4.2: Generation of transgenic pigs by somatic cell nuclear transfer (nach Niemann)

Author	Model	Maximum graft survival (days)
Mohiuddin et al., 2012	GalKO/hCD46 pig hearts to baboon. B-cell depletion	236
McGregor et al., 2004	hCD46 transgenic pig hearts heterotopic to baboon	113
Kuwaki et al., 2005	GalKO pig hearts heterotopic to baboon	179
Kuwaki et al., 2004	hDAF transgenic pig hearts heterotopic to baboon	139
Yamada et al., 2005	GalKO pig kidney to baboon	83
Chen et al., 2006	hDAF transgenic kidney to baboon	75
Chen et al., 2005	GalKO kidney to baboon	16
Baldan et al., 2004	hDAF transgenic kidney to monkey	90
Bhatti et al., 1999	hDAF transgenic pig hearts heterotopic to baboon	99
Vial et al., 2000	hDAF transgenic pig hearts heterotopic to baboon	39
McCurry et al., 1995	hCD59 transgenic pig hearts to baboon	30 hours

Table 4.1: Pig organ graft survival in nonhuman primates.

5 Molecular Approaches in Animal Breeding to Improve Commercially Important Traits

5.1 Introduction

Animals have always served for human as a source of food or valuable commodities (such as wool, cotton, or silk), for help with various types of work (such as transportation, protection, and warfare), scientific research, or simply to enjoy as companions. The process of selection for different environments (domestication) might have resulted in the generation of a wide variety of domestic breeds with divergent phenotypes.

Early animal breeders practised selective breeding by identifying what they considered worthwhile characteristics and sought a means of increasing the frequency of such desirable qualities in future generations. This has resulted to the present day specialised breeds of commercially used animals like the Belgian Blue Cattle well known for its lean meat, the Holstein-Friesian cattle noted for its milk-production, Superfine Merino Sheep for good quality wool or the Pietrain Pigs for very high yield of lean meat. For many farm animals, conventional breeding has already achieved high producing animals, but it seems increases in productivity by this means have peaked and are at the sedentary plateau phase. World population on the other hand is on the increase and so is the demand for animal products. Selective breeding can not keep up with the pace of population growth because it is a painfully slow process and can take many years (especially in cattle with long gestation periods and generation intervals) to establish the desired phenotypic changes.

5.2 Genetic markers

During the past few decades, advances in molecular genetics have led to the identification of multiple genes or genetic markers associated with genes that affect traits of interest in farm animals, including genes for single-gene traits and QTL or genomic regions that affect quantitative traits. The term “genetic marker for a trait” is related to known DNA variability which is associated with a trait and segregates in a predictable pattern, as the trait. Genetic markers facilitate the "tagging" of individual genes or small chromosome segments containing genes, which influence the trait of interest. Revealed genetic markers (and genes) provide opportunities to enhance response to selection, in particular for traits that are difficult to improve by conventional selection, for example due to low heritability or traits for which measurement of phenotype is difficult, expensive, only possible late in

life, or not possible on selection candidates. The process of selection for a particular trait using genetic markers is called “marker-assisted selection” (MAS). MAS can accelerate the rate of genetic progress by increasing accuracy of selection and by reducing the generation interval. About 50 % additional genetic gain can be obtained if the marker explains 20 % of the additive genetic variance and the economic trait has a heritability of 0.2.

5.3 Identification of genetic markers

The two complementary strategies used to identify genes or markers linked to quantitative traits are genome scans and the candidate gene approach.

In broad terms, the genome scan approach attempts to scan the entire genome for regions which are associated with variation in the traits of interest. In fact, a QTL region often spans 5–30 cM, and it is too large to find the target genes, so a fine mapping analysis needs to be performed. The fine mapping of a specific QTL is the goal of genome scan and it began in the relevant region by adding genetic markers and increasing the marker density to the linkage map. Genome scans usually use crosses of divergent breeds, and several-generation pedigrees are produced in which coinheritance of the trait and chromosomal markers can be followed. This approach has the potential to detect any loci with a large effect on a studied trait, whether or not their existence is known in advance. A drawback of the genome scan is that although it can locate of the genome containing a gene, it does not identify the gene itself. Another rate-limiting step is that the size of the observed effect for a given QTL may be too small to validate because of the costs and time constraints. Also, because of the use of divergent breeds in the crosses, identified QTL (for example in pigs) may not be relevant in commercial lines used by producers.

An alternative strategy is the candidate gene approach that focused on a limited number of known genes for which it is suspected or known that genetic variation within them could cause phenotypic variation in the traits of interest. Once a polymorphism has been identified in a candidate gene, association studies are completed to determine the relationship of the gene with traits of interest. This is a promising approach because genotyping is now increasingly easy and less expensive to perform. An advantage of this approach is that it limits the search to those genes that have been identified and characterised to some extent. Thus less genotyping may be required than is needed for the genome scan approach. Also, the studies do not require crosses of diverse breeds or lines, and they may be applied directly to commercial lines. This enables researches to use larger populations, and hence, there is a considerably more statistical power to detect smaller effects.

Recent technological advances have created new opportunities to study complex traits in animals. New genomic information is coming out from a number of projects on animal genomes. Instead of focusing only on the discovery of a single gene or DNA markers that co-segregate with a characteristics of a trait, in recent times the researchers focused their interests on elucidating complex traits by the detection of the large-scale molecular gene expression profiles, gene clusters and networks that are characteristics of a biological process or of a specific phenotype. The development of Next Generation Sequencing Techniques produces an increasing number of complete genome sequences as a source for a very high number of microsatellites and SNP markers. Morover, thanks to the high-throughput techniques such as DNA arrays and proteomic approaches allowing a global view of gene expression, it is now possible to add functional genomics to the range of approaches available for understanding the molecular basis of complex traits in farm animals. The application of these new genomic tools has the opportunity to identify pathways and interacting genes and to provide insight into epistatic effects of genes that can further improve the understanding of the genetic component of complex traits.

5.4 Types of genetic markers

O'Brien (1991) groups genetic markers into two types: Type I markers are associated with a gene of known function, and Type II markers are associated with anonymous gene segments of one sort or another (especially microsatellite sequences). According to Dekkers (2004), three types of observable polymorphic genetic loci can be distinguished:

1) direct markers: loci that code for the functional mutation;

2) LD markers: loci that are in population-wide linkage disequilibrium with the functional mutation;

3) LE markers: loci that are in population-wide linkage equilibrium with the functional mutation in outbred populations.

The LE markers can be detected on a genome-wide basis by using breed crosses or analysis of large half-sib families within the breed. Such genome scans require only sparse marker maps (15 to 50 cM spacing) to detect most QTL of moderate to large effects. The LD markers are close to the functional mutation for sufficient population-wide linkage disequilibrium between the marker and QTL (within 1 to 5 cM). The LD markers can be identified using candidate genes or fine-mapping approaches. Finally, direct markers are the most difficult to detect because causality is difficult to prove and, as a result, a limited number of examples are available, except for single-gene traits.

These three types of marker loci differ also in their application in selection programs. Whereas direct markers (to a lesser degree also LD markers) allow for selection on genotype across the

population because of the consistent association between genotype and phenotype, use of LE markers must allow for different linkage phases between markers and QTL from family to family. Thus, the ability to use markers in selection increases from direct markers to LD markers and LE markers. On the basis of this classification of genetic markers, MAS will be referred to as gene-assisted selection (GAS), LD markers-assisted selection (LD-MAS), and LE marker-assisted selection (LE-MAS).

5.5 Application of genetic markers in improvement of commercially important traits

The application of markers to enhance genetic improvement in livestock requires markers that trace within-breed variability. Direct and LD markers allow selection on markers across the population, which facilitates their use. Some of the earlier applications of MAS in livestock were prior to the era of molecular genetics (e.g., selection against the halothane gene in pigs using the halothane test). Subsequently, several genetic tests have been used to select against carriers of recessive genetic defects in livestock species. The first major genome scan in livestock was reported in 1994 from a cross between wild boar and Large White pigs. Since then, QTLs have been identified for a large number of traits segregating within numerous breeds of farm animals. For example, a study to identify quantitative trait loci (QTL) for reproductive traits in swine by using a genomic scan has identified regions with evidence for QTL or suggestive QTL on chromosomes 1, 3, 4, 6, 7, 8, 9, 10, 13 and 15 (Rohrer et al., 1999). Recently, a specific QTL database for pig, cattle, chicken, sheep and rainbow trout is available (www.animgenome.org/QTLdb/). With the candidate gene approach, many genes have been demonstrated to have allelic effects on different important traits in animals. For example, in pigs, major genes affecting meat quality include Ryanodine receptor (RYR1) gene that regulates Ca2+ transport across muscle cell membranes and Rendment Napole (RN) gene that affects glycogen content of muscle. Some other markers for meat and carcass quality with relevant effect include polymorphisms in the genes coding for calpastatin (CAST), insulin-like growth factor 2 (IGF2) or melanocortin receptor 4 (MC4R) gene. A non exhaustive summary of gene or marker tests that are available or used in commercial breeding programs is given in Table 1, with tests categorized by the type of trait and the type of marker.

5.6 Genomic selection and future perspectives

Over the past 2 decades, the rapid development of genomics has opened new paths to address the scientific basis of livestock biology and breeding, and has resulted in new production methods to

achieve increases in animal feed yields and long-term improvements in the efficiency of livestock production. This “genomics era” promises to enable the objective prediction of consequences based on direct access to the full DNA sequence of many individuals, and therefore more objective view of the genetic value of animals that is not limited to a few production traits.

With the production of whole-genome sequences for the major livestock species, the comparison of sequences from several individuals of different breeds with a reference sequence resulted in an almost inexhaustible source of genetic markers, primarily polymorphisms in the form of single nucleotide polymorphisms (SNP). With the release of the first draft of the chicken genome sequence (by International Chicken Genome Sequencing Consortium in 2004), the chicken community was the first animal community not only to have access to millions of SNP, but also to be organized in a freely accessible database. Similar sequencing and resequencing efforts in several other livestock species resulted in the discovery of hundreds of thousands of SNP covering the entire genome. These genetic markers were subsequently deposited in publicly accessible databases. Another major technological breakthrough has been the development of DNA array technology, which allows for the inexpensive measurement of SNP within a given sample. DNA arrays offer hundreds of thousands of SNP to be screened in parallel, allowing scientists to perform genome-wide association studies that simply would have been out of reach with traditional markers. With the use of whole-genome SNP panels, the traditional approach, e.g. positional cloning with a whole genome scan to map the region of interest, followed by a fine-mapping step, can be fully replaced with an efficient and cost-effective genotyping step using a whole-genome SNP array.

Trait category	Direct marker	Linkage disequilibrium marker	Linkage equilibrium marker
Congenital defects	BLAD (D) Citrulinaemia (D,B) DUMPS (D) CVM (D) Maple syrup urine (D,B) Mannosidosis (D,B) RYR (P)	RYR (P)	
Appearance	CKIT (P) MC1R/MSHR (P,B,D) MGF (B)		Polled (B)
Milk quality	κ-Casein (D) β-lactoglobulin (D) FMO3 (D)		
Meat quality	RYR (P) RN/PRKAG3 (P)	RYR (P) RN/PRKAG3 (P) A-FABP/FABP4 (P) H-FABP/FABP3 (P) CAST (P, B)	
Feed intake	MC4R (P)		
Disease	Prp (S) F18 (P)	B blood group (C) K88 (P)	
Reproduction	Booroola (S) Inverdale(S) Hanna (S)	Booroola (S) ESR (P) PRLR (P) RBP4 (P)	
Growth and composition	MC4R (P) IGF-2 (P) Myostatin (B) Callipyge (S)	CAST (P) IGF-2 (P) Carwell (S)	QTL (P) QTL (B)
Milk yield and composition	DGAT (D) GRH (D) κ-Casein (D)	PRL (D)	QTL (D)

Table 5.1: Examples of gene tests used in commercial breeding for different species by trait category and type of marker (according to Dekkers, 2004); D = dairy cattle, B = beef cattle, C = poultry, P = pigs, S = sheep

With sufficient genetic markers based on whole-genome SNP panels, one can follow the segregation of the entire genome and not merely a set of specific regions of interest, moving from MAS to genomic selection. Genomic selection is based on the principle that information from a large number of markers could be used to estimate breeding values without having a precise knowledge of

where specific genes are located on the genome. With tens of thousands of SNP, well chosen to be representative of the entire genome, it is expected that there will always be an SNP in close proximity to a particular gene or DNA fragment of interest; the existing linkage disequilibrium between one (or several) SNP and a causal mutation will be substantial and can then be used to explain a significant fraction of the variation of the observed trait. The first step in the genomic selection process is therefore access to a large group of animals, either a reference or training population with accurate phenotypes for the trait(s). This population should be genotyped using a whole-genome SNP array. The resulting data will then serve as a reference to develop a statistical model estimating the effect of each SNP with the trait(s) of interest. The result in this case is a predictive equation to calculate a genomic estimated breeding value. After a validation step, the genomic breeding value of new animals can be computed using the prediction equation, based on their genotypes from the SNP array. The accuracy of the genomic estimated breeding value depends of the size of the population and the heritability of the trait to be considered.

Genomic selection provides a new level of information that can be integrated into the breeding programs to identify and select the most promising animals. It can be implemented very early in life, is not sex limited, and can be extended to any traits that are recorded in a reference population. It also provides better selection accuracy while reducing the generation interval, thereby increasing the intensity of selection. In comparison with MAS it explains a much greater proportion of the genetic variance.

The ability to investigate the genome, the transcriptome, or the epigenome of any species by high-throughput sequencing methods is opening a new world of possibilities. Because of reduction in sequencing costs, all economically impactful agricultural species, subspecies, and their pathogens would be sequenced in the near future, offering important information for the implementation of genomic selection programs. Breeding programs will probably be driven primarily by array data because of superior economics and much higher throughput. The increasing value of genomics and the potential of genomics to increase the control of many economically important physiological functions is promising for further improvement of the important traits in farm animals.

6 Sperm Sexing Technology, a new Field in Applied Biotechnology

6.1 Introduction

The control of the sex ratio in farm animals permits faster genetic progress, higher productivity, and helps producing less environmental impact due to the elimination of the unwanted sex. The method of sex pre-selection must be effective and efficient, must result in fertility equal to or better than with unsexed semen, and must be reasonably inexpensive and convenient to be widely applied. Since the first reports of a successful and repeatable method to separate X-and Y- chromosome bearing sperm populations in mammals, the technology has been improved substantially and was introduced into commercial application for bovine semen ten years ago. Meanwhile sexed semen is available in several countries, especially in the USA, where the demand for replacement heifers is much higher than for instance in Europe. In other species such commercial standards could not yet be established either because the required sperm numbers /per AI are hardly to achieve within a reasonable time, or sexed sperm cannot be frozen with acceptable quality. An exception is the sheep, where a larger scaled commercial use is likely to be seen.

The only approved method for sperm sexing is the so called Beltsville Sperm Sexing Technology, which is based on individual sperm identification and separation by quantitative flow cytometry. Figure 6.1 shows the species specific DNA differences among x-and Y-chromosome bearing sperm. The production output is limited and requires special semen processing.

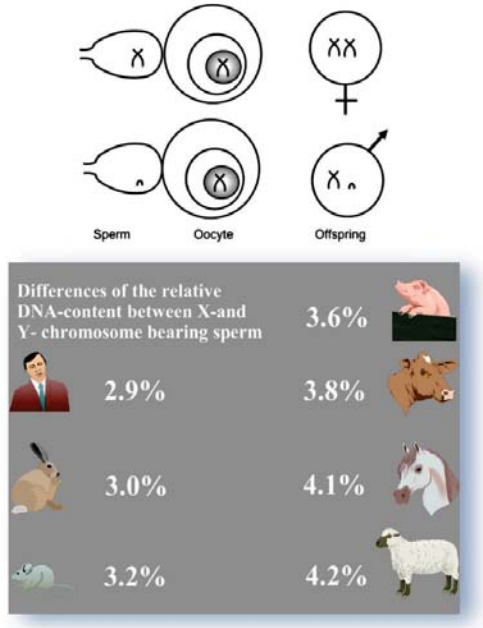

Fig. 6.1. Sex determination and the species specific differences in DNA content between x-and Y-chromosome bearing spermatozoa

6.2 Instrumentation

Sex selection by flow cytometry requires to understand the major steps of sorting. A flow cytometer with adjuncted sorting unit in shown in its principle in figure 6.2. The sample fluid, in this case containing diluted and DNA-labelled spermatozoa, is placed into the sample holder (5ml tube) and pressurized air is blown into the tube. The pressure pushes the semen into a capillary tube, which is connected to the nozzle assembly, a jet system, which is in principle very similar to those of an ink jet printer. The semen fluid is passed through the assembly and forms a core stream. The core stream is surrounded by a sheath stream that enters the nozzle assembly in parallel to the sample fluid. As the sheath stream has a higher pressure it focusses the core stream in a special way that controls the positioning of the sperm head relative to a Laser beam that hits the fluid stream directly at the orfice but outside the nozzle tip. The speed of the core stream with the sperm is accelerated by the design of the nozzle tip allowing a stable positioning of the cells after leaving the nozzle tip.

The stream is discontinous and forms droplets. Their size and breakoffpoint is related to the frequency that is generated by a piezo cristal, by which the stream is kept in vibration. This guarantees a highly stable positioning of the droplets, a prerequsite for the subsequent sorting steps.

The laser beam hits the droplet stream directly below the nozzle orfice. The fluorescence dye in the sperm head is excited and emits a fluorescence signal that is correlated to the amount of DNA in the sperm head. This signal is recognized and amplified in two photo multiplier tubes (PMT). As a speciallity to recognize not only the amount of DNA by the 0° PMT but to messure a fluorescene signal emitted by the small edge of the sperm head and to interpret this signal as degree of orientation of the sperm head relative to the position of the Laser beam, a second PMT (90°) recognizes the fluorescence signal, which bascially is an optical breaking effect against the surrounding medium.

The fluorescence signals are transformed in the PMTs to electric impulses, which are digitized and computed in the central sorter unit (CSU), which itself is controlled by a PC. Most of the setup functions, aquisition and sorting comands are controlled by the PC. Major controls are:

CSU settings, thresholds, gating of signals, setup of regions, sort decisions and sort directions. Depending on these setups, the computer recognizes the signals from the PMTs, which are DNA related and charges accordingly the last hanging droplet of the discontinous droplet stream (figure 6.3). The chared droplet disintegrates from the droplet stream and passes in a free flow the subsequent assembled eletrostatic field, which is generated between two capacitor plates. According to the electric charge, the droplet is deflected either to the left or the right side and collected in a collection tube, prefilled with collection medium. All empty droplet, droplets with non oriented sperm and those with more than one sperm are pushed into the waste.

The usual event rate is close to 30000 events/sec. The sort rate per sex is arround 3000 -6000 sperm/sec and in average 3-4 straws (250µl) can be produced per hour, containing 2 million intact sperm. In other farm animal species sort rates are very similar. However it takes about 5h to produce one AI dose (50 million sperm) from boar ejaculates and about the same time for one insemination straw for equine AI.

Once sperm are sorted an aliquot of about 1 million sperm is taken for re-analysis to evaluate sperm quantitavely. Sperm are relabelled with Hoechst 33342 and processed through the sorter again at very low speed (better orientation). This method is easy to perform and has been verified by FISH and PCR. Usually the purity is above 90% (figure 6.3).

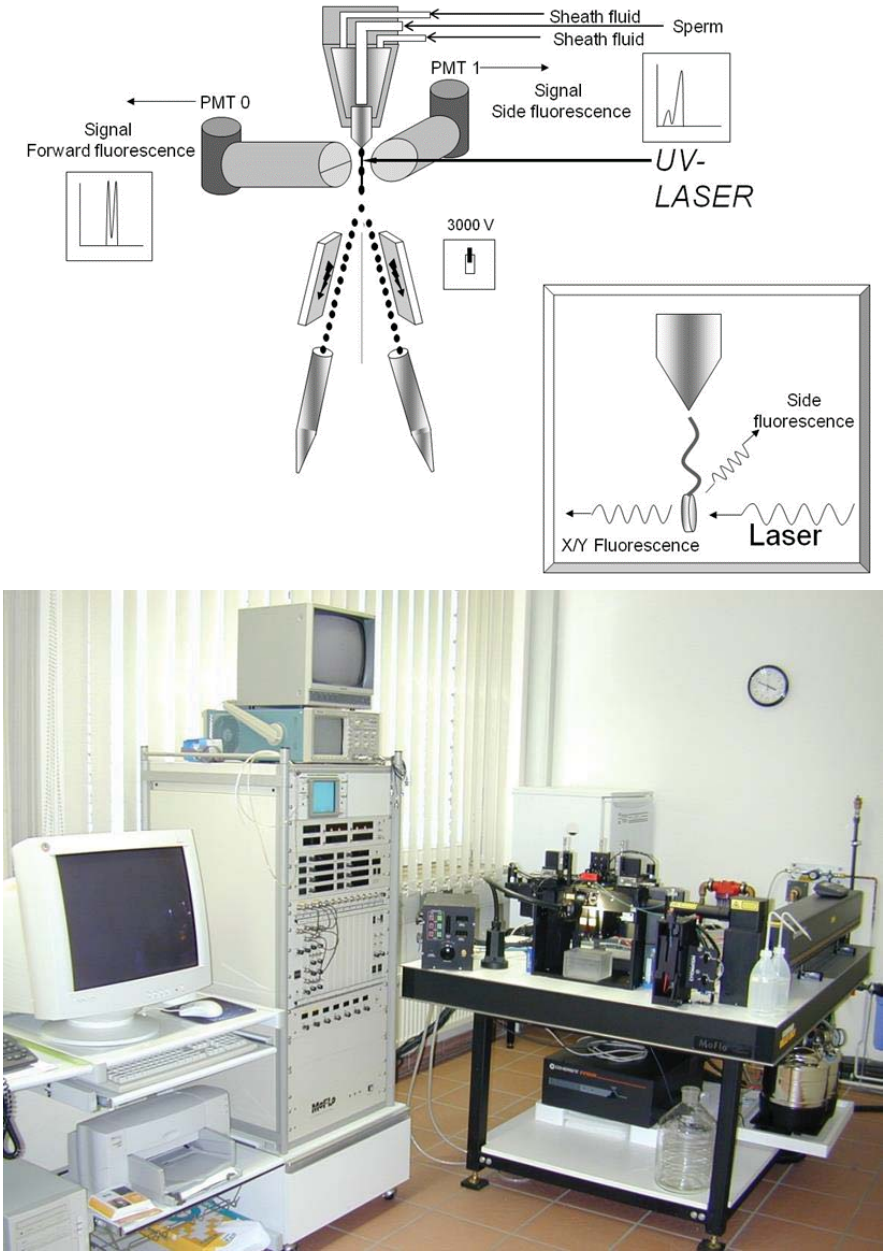

Fig. 6.2. Cell sorter (MoFlow; Beckman Coulter) and the principle of sperm sorting

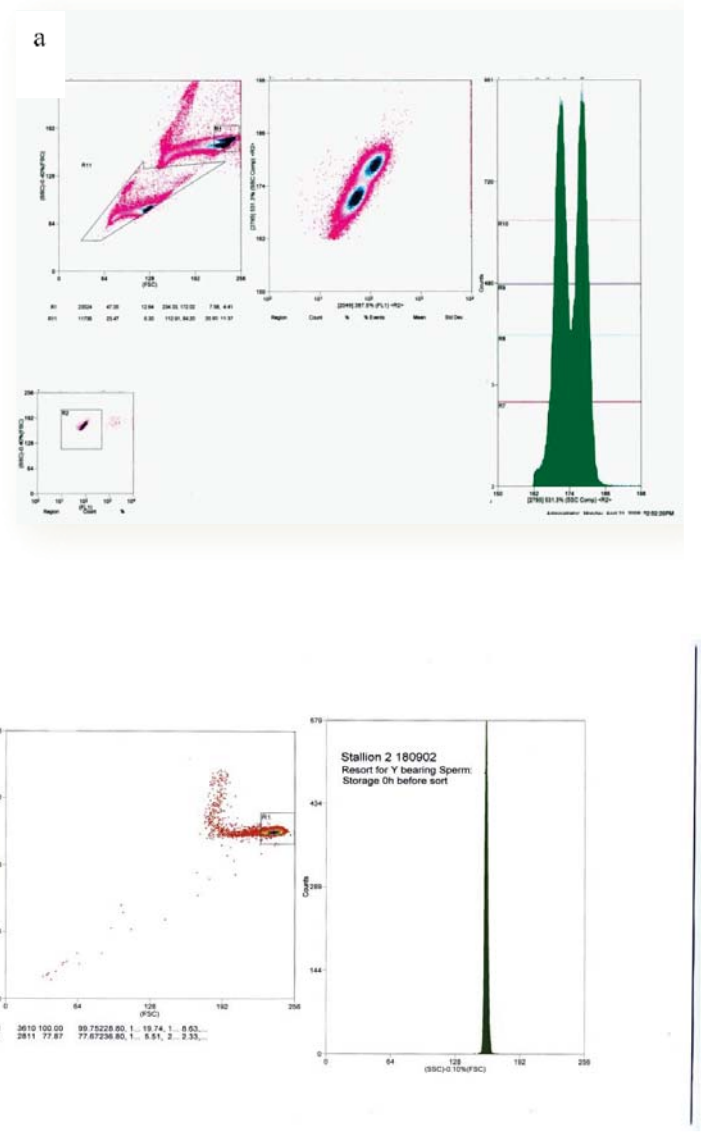

Fig. 6.3. Setup of sort decisions. Each red spot is a PMT recognized sperm. High sperm accumulations within the orientation/DNA content bivariate diagramm turn from red to blue. Fig 6.3a shows the sort screen setup, Fig. 6.3b displays the resort of a sample. Purity is estimated from the right histogram by curve fit statistics.

6.3 Semen processing

After semen collection and dilution sperm (100 million/ml) are labeled with a fluorescent dye (Hoechst 33342) in order to discriminate quantitatively between X- and Y- chromosome bearing spermatozoa. For maximal saturation sperm and dye are co-incubated for 75-90 minutes at 34°C

(bull) to 30°C (boar). Thereafter food dye FD40 is added. FD40 is made of relatively large molecules and enters only those sperm heads with defect membranes. FD40 bleaches the fluorescence signal of the DNA dye and such sperm can be eliminated from sorting (figure 6.3), making the sort process more efficient. The samples are filtered (50nm nylon mash) at room temperature and transferred into high pressure tubes (5ml). The tube is placed into the sample holder and the liquid is pushed with pressure (39 psi) into the sorting system.

Sorted samples are collected in tubes prefilled with collection medium. The composition of the collection medium varies among species but ever contains about 2% of seminal plasma. Components of the seminal plasma decapacitate those sperm that have undergone capacitation during flow sorting.

Most of the collected volume originates from sheath fluid, which basically is a physiological salt solution buffered with TRIS. The collection medium is therefore and the supernatant is discharged. The remaining sperm pellet is extended with an appropriate species specific extender for liquid preservation or freezing.

Even with the latest version of a high-speed flow cytometer less than 15 million spermatozoa can be sexed per hour. In the bovine this would be equal for one AI dosage of unsorted frozen semen. For AI in pigs about 100 hours are necessary to produce one regular AI dose containing 2x109 spermatozoa. Therefore, modified insemination protocols have been developed using 10 to 60 times diluted sperm dosages of the original insemination dose. During commercial sorting procedures about 75 % of sperm are lost due to different technical and biological reasons before they are inseminated. Recently developed flow technology combined with new filling systems for straws have reduced the sperm losses to 65% under experimentl conditions.

6.4 Offspring production by AI and other biotechniques

Offspring have been produced in many species with sex sorted sperm. Due to the reduced amount of these sperm, specific insemination schemes (AI) have to be performed like insemination close to the time of ovulation, deep intra uterine insemination and oviductal insemination. Besides in vitro fertilization (IVF) and intra cytoplasmatic sperm injection (ICSI) have been shown to efficiently produce offspring. The latter methods however, require advanced technical equipment like micro manipulators and incubation techniques. Therefore a combination of sex sorting and these methods are only a nichique for very specific applications. Animal production industry will only accept insemination strategies for the mass production of offspring with predetermined sex.

Take home message

- Sperm from domestic farm animals can be selected by flow cytometry according to their sex relevant DNA differences at high purity.

- High speed flow cytometers allow to produce 10-15 million sex sorted sperm per hour.
- Sexed sperm have reduced life span and need special processing before, during and after sorting.
- Sexed semen is available commercially from bulls. Sperm from other species are more difficult to sort.

7 Endoscopy in Reproduction

7.1 Introduction

Breeding of farm animals is inevitably linked to reproduction. The more reproduction is successfully managed the more it is possible to implement animal breeding strategies. Since 1960 worldwide animal breeding has undergone a crucial change especially in cattle to decreasing numbers of farms as well as increasing numbers of animals undoubtedly leading to a significant improvement of the genetic merit. In order to accelerate breeding progress of high genetic valued animals many additional efforts were put into the field of embryo production to increase selection intensity (Stroud, 2010). Consequently, milk production in the dairy cattle industry constantly increased, while a steady decline in fertility was noted (Lucy, 2001, Pryce et al., 2004). In this context it has to be emphasized that reproduction failure has reached an alarming dimension. A high proportion of fertilized oocytes does not result in the birth of calves (Diskin and Morris, 2008). The majority of reproductive wastage is due to early embryonic death (Humblot, 2001; Thatcher et al., 2001). It is well accepted that these dramatic changes in animal performance exert long lasting negative effects on reproductive physiology such as endocrine disorders, ovulation failure, and low conception rate or embryo/pregnancy losses (Wiltbank et al., 2006). Interestingly, this decline did not become evident when using embryo collection and embryo transfer (Hasler, 2006). Recently, scientific approaches clearly revealed a basic phenomenon of reproductive success as an import crosstalk between oocyte/embryo and the maternal environment. Environmental disturbances or disorders may lead to deviation in embryonic development. This communication with gametes and embryos plays a key role in early embryogenesis, implantation, progression of pregnancy until birth, the future health of offspring and animal performance (Fazeli, 2008).

In total there is a scientific as well as practical demand for an increased understanding of reproduction in order to meet ethic, legal and economic prerequisites for better performance of livestock animals.

Although significant improvements have been achieved, traditional fields in reproduction provide limited access to reproductive events. More detailed information was gained by means of assisted reproductive techniques such as ultrasonography and hormonal analyses. Formerly applied surgery in fact provided visual access either to the response of hormone pre-treatment and/or patho-physiological constitution of the anatomy of the reproductive organs and tissues. The inspection of the reproduction organs after slaughter only retrospectively accounts for fertility or problems during the life period. Although both, surgery and slaughter are very informative, none of these invasive

interventions have had an impact on practise. The idea of this chapter is to give a selective introduction into the use of endoscopy in reproduction. This review does not claim a unique position for the use of endoscopy but rather demonstrates that the application of this technique may have an additional benefit in the area of fertility and reproduction especially under the scope of:

-Collection of oocytes from antral follicles with regard to follicular dynamics

-Culture of in vitro derived embryos

-Application of semen of minor quality

-Collection of early stage tubal embryos with/without super stimulation, with regard to embryo kinetics

-Pregnancy diagnosis

This review will bypass traditional fields of endoscopic use in different species, and finally focus on our work we have recently done in cattle.

7.2 History & Endoscopy

The idea to evade surgery but to get optical access to inner organs dates back to more than two centuries. Bozzini (cited by Liess, 1936) used a bi-tubular instrument to perform laryngeal, vaginal and vesicular inspection. The light of a candle was transmitted by a concentrating reflector through the tubing system to the organs. More than 50 years later light was produced using gas and convex lenses and a concave mirror served for light bundling which significantly improved the light intensity in front of the endoscope. In the subsequent years many attempts were made in order to generate light more efficiently and to facilitate its handling: A platinum spiral was rectally placed to produce indirect light, or later on a so-called Mignon bulb fixed at the front of the tubing systems resulted in an optimal illumination at that time. During the last century enormous progress was made in the field of human as well veterinary medicine. The technique of endoscopy was applied to many disciplines and areas having natural entries such as laryngoscopy, rhinoscopy, pharyngoscopy, trachea- and bronchoscopy, oesophagoscopy, gastroscopy, rectoscopy, cystoscopy, hysteroscopy, or where an additional access had to be accomplished (endoscopy, thoracoscopy; cited by Liess, 1936).

Simultaneously, there were many demands which finally lead to revolutionary improvements: Harold HOPKINS created a fibroscope which consisted of a bundle of glass fibres for the transmittance of light and image. Shortly after this invention the rigid endoscope was technically improved by additionally embedding rod glass lenses into the optical axis. To date, rigid endoscopes provide a high resolution and high quality image which became very attractive for application in many medical disciplines. Among several manufacturers, Karl STORZ succeeded in designing a cold

light source, external light production, to generate very bright light. The combination of a rod lens system within an optical shaft with optical fibres to transmit light resulted in much brighter and clearer images having a more natural rendition of colours (Lhermette and Sobel, 2008). Thus, endoscopes can be manufactured of various sizes, length and diameter, flexible and rigid having either straight forward or different oblique angle optical axes. Moreover, combined techniques (ultrasound, laser, infra-red camera, forceps; Hunter et al., 2000; Wall et al., 2011), documentation (videos, pictures; Nezhat, 1994), and handling (disinfection etc.) of the endoscopes for different purposes have made this technique indispensable not only in the medical field (Wall et al., 2011).

7.3 Endoscopy in small ruminants

Endoscopy in sheep and goats has already passed through a long tradition. The industry of small ruminants aimed at speeding up breeding success with animals having a valuable genetic background. However, many limits such as accurate synchronisation protocols, semen processing including cryopreservation, and reliable AI techniques lowered the reproduction performance.

Endoscopy first served for the examination of ovarian function in sheep. The animals were fixed in a dorsal recumbent position. The preferred access was performed via midventral endoscopy. The time of ovulation was determined by repeated examinations and it was concluded that endoscopy could be done as often as every other day without adverse effects (Pukelov et al., 1971, Snyder and Dukelow, 1974; Whyman et al., 1979; Camp et al., 1983; Driancourt and Disenhaus, 1997). Synder and Dukelow (1974) already used this technique for pregnancy diagnosis based upon inspection of uteri and ovaries and recognized that the visualization through this endoscope also served for ovum pick-up.

Simões and co-workers (2005) performed a comparative study by means of ultrasonography and endoscopy. The number of ovulation sites was counted using both methods and finally the ovaries were removed and the total procedure was evaluated by slicing. Ultrasonography and endoscopy were found to be similarly reliable techniques for CL detection (Simões et al., 2005).

In reproduction most attention was paid to artificial insemination. To date, AI represents the most efficient applied reproduction biotechnology worldwide in livestock species. There are several technical steps which have been developed during the last decades in order to make this biotechnology feasible. On the male side many efforts have been made to improve semen collection, dilution, cryopreservation and transportation to deliver high quality semen for different industrial as well as scientific purposes. However, the anatomy and physiology of the female ovine reproduction organs turned out to be a barrier for artificial insemination. The ovine cervix is characterized by a very complex anatomical structure. This type of structure seems not to be uniformly existent. Different

breeds and even individuals show a great variability which hampers the technical standardisation of AI (Lightfoot and Restall, 1971). The introduction of an AI catheter is doomed to failure due to many folds varying in sizes, number and distance between folds which are misaligned and eccentrically placed. The passage of the cervix by a rigid catheter would need the skilful threading of the cervix over the catheter by rectal manipulation as is accomplished in cows (Kaabi et al., 2006, Halbert et al., 1990). For this reason, the utilization of endoscopy allows the deposition of semen in the lumen of the uterine horns.

Artificial insemination via endoscopy (Killeen and Caffery, 1982) in ovine insemination programmes is the only reliable methodology that results in satisfactory fertilization and lambing rates (Figure 7.1; Armstrong and Evens, 1984; Salamon and Maxwell, 1995; Kühholzer et al., 1997a; Anel et al., 2006). It is a relatively simple and convenient technique to additionally assess the reproductive organs regarding anatomic and endocrine disorders (adhesion, ovarian response to hormonal treatment etc.) simultaneously with intrauterine semen deposition. This information is not accessible when conventional transcervical insemination techniques are done.

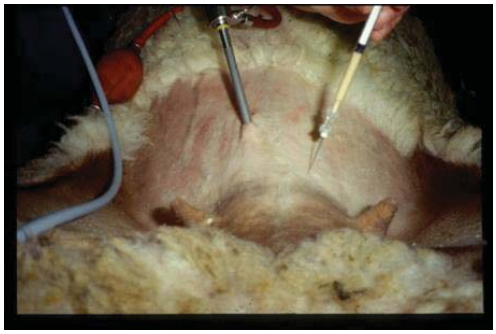

Fig. 7.1. AI in sheep: Endoscope and 1 ml syringe filled with semen.

Moreover, endoscopy becomes superior and beneficial when exceptional factors negatively affect success of fertilisation such as

-Insemination in super stimulated sheep

-Using of different ewe synchronisation protocols (gestagen sponges)

-AI using poor semen quality (individual rams, sorted semen)

-AI using ejaculates with low concentration of spermatozoa or a high dilution rate

-AI with cyropreserved semen

(Armstrong and G. Evans, 1984; Tervit 1996; Gourley and Riese, 1990; Kühholzer et al., 1997b; Baldassarre et al., 2002; Cognie et al., 2004)

AI of ewes with thawed semen via the vaginal or trans/intra-cervical routes is unfeasible or unreliable. Therefore, the application of endoscopy in commercial AI programmes plays a main part in breeding success.

In cases where fertility problems occurred or an extra number of embryos were required to maintain or accelerate animal breeding, hormonal super stimulated or non treated ewes were used to perform either ovum pick-up or embryo recovery.

In vitro production of ovine and caprine embryos has become an efficient technique which circumvents many critical steps being prerequisites for normal ovulation or superovulation. Many of the embryo donors do not adequately respond to hormonal treatment regarding number of ovulation sites and quality of corpora lutea and fertilization has to proceed under abnormal environmental conditions (concentration and duration of hormonal effects, sperm migration, consistence and amount of oviductal fluid, muscular activity of the oviduct, duration of ovulation interval; Killian et al., 1987; Baldassarre et al. 2002; Tervit 1996; Cognie et al. 2004). In 1996 Tervit proposed endoscopic puncture of follicles for oocyte retrieval and predicted one to three good quality blastocysts per aspiration session. He concluded that this reproduction performance is suitable for lamb production also from infertile ewes. But an improvement of the technology was recommended (Tervit, 1996). Kühholzer and her team (1996b) used endoscopy in hormonally untreated ewes to collect oocytes. Sedated ewes were fixed in a cradle in a 30 ° head down position. The endoscopic access to the peritoneal cavity was performed about 5 to 10 cm cranially to the mammary gland. Forceps and a 30 ° wide-angle endoscope (Hopkins, 6.5 mm in diameter) served for fixation and visualisation of the ovaries. A 20 G needle connected to a 5-ml syringe was introduced through the abdominal wall to individually puncture all visible follicles. Based upon the efficient collection of oocytes it was concluded that this technique is very simple and can be applied in ewes without hormonal super stimulation. Meanwhile the technique of endoscopic ovum pick-up has been permanently improved and systematically adapted to laboratory specific facilities and surgical expertise by the different teams, it is routinely used in ewes with/without super stimulation and promises to be an efficient method for the production of an extra number of offspring from small ruminants representing an exceptional breeding background (Tervit 1996; Stangl et al., 1999; Cognie et al. 2004; Baldassarre et al. 2007).

Alternatively an additional propagation of sheep and goats can be obtained by embryo collection of superovulation female animals followed by embryo transfer. In contrast to surgery, flushing and transfer of tubal stage and uterine stage embryos by means of endoscopy is recognised as less invasive and traumatic. Superovulation can be performed more frequently and accounts for appropriate

refinement, animal husbandry and animal welfare requirements (Besenfelder et al., 1994a,b; Kühholzer et al., 1996a,b; Baldassarre, 2002).

After synchronisation, stimulation of superovulation and endoscopic AI of the ewes and according to day of development, the desired embryonic stages are flushed using the standard dorsal recumbent position of sedated and anaesthetized animals. In total, three trocars serve for the transabdominal introduction of the endoscope, the forceps, and the flushing balloon catheter. The flushing balloon catheter is placed via uterine wall into the cranial part of the uterine horns and fixed by balloon inflating. At first the ovarian response to hormonal treatment is visualized (Figure 7.2).

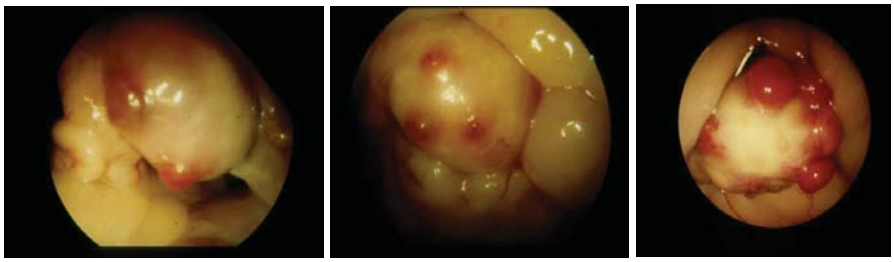

Fig. 7.2. Different ovaries showing (from left to right) an increasing number of ovulation sites.

The forceps serve for slightly lifting the infundibulum plus the adjacent part of the ampulla. Next to that position, a metal canula is introduced through the abdominal wall and inserted into the ampulla. A flexible catheter (we used x-ray labelled venous catheter) is pushed straight forward 3 to 5 cm deep into the ampulla. The position of the forceps is now used to hold together ampulla and the venous catheter. This procedure enables the flushing of embryos of different stages (from the zygote stage up to the blastocyst stage).

The transfers are done similarly. Either a glass capillary or a flexible catheter is loaded with embryos and pushed through a metal canula via infundibulum into the upper part of the ampulla (Figure 7.3).

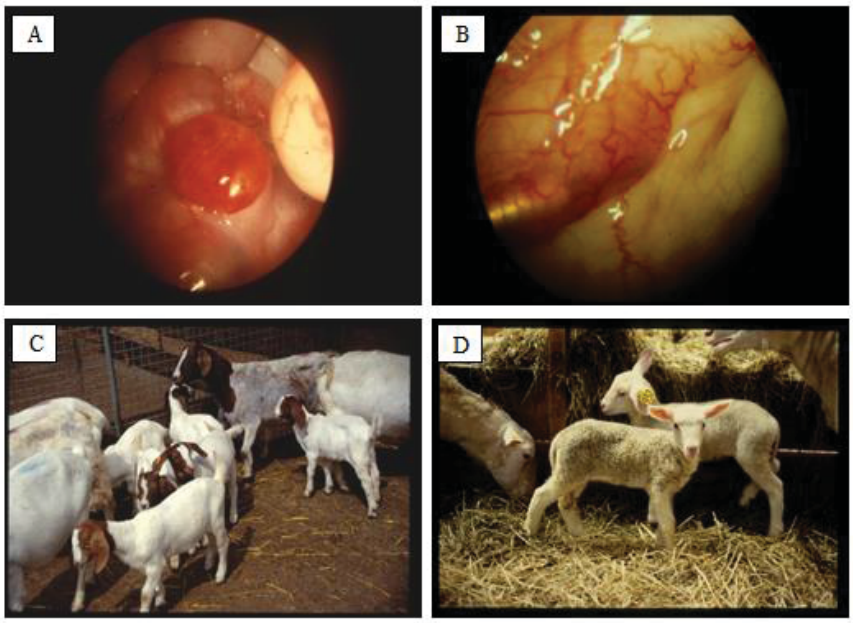

Fig. 7.3. A diagnosis of the presence of a day 2 CL, B metal canula bearing the flexible catheter load with embryos, and lambs born after endoscopic ET C , D.

Meanwhile the field of endoscopy has attracted many disciplines such as the utilisation of foetoscopy and endoscopic surgery during pregnancy for studying diseases (Calvano et al., 1998; O'Rourke and Kodali, 2006; Kohl et al., 2009) and the establishment of goats and sheep carrying in vitro reconstructed genes for numerous purposes (Kühholzer et al., 1998; Vainas et al., 1999; Ehling et al., 2003; Baldassarre et al., 2002).

7.4 Porcine Reproduction

At the same time as endoscopy in small ruminants has been developed many efforts have also been made in swine reproduction. In accordance to sheep and goats, studies first focused on ovulation characteristics. Gilts were frequently endoscopically visualized to evaluate factors and time influencing the onset of ovulation, duration and rate of ovulation. It was impressively demonstrated that ovulation rate increased with reproductive age and that hCG-treated gilts ovulated earlier compared to non treated ones (Faillace et al., 1994). Endoscopy also served for studies examining pathological influences such as the effect of tropical climatic conditions on ovulation rates followed by prenatal losses and reduced litter sizes in gilts (Tantasuparuk et al., 2005). Bolamba et al. (1991) endoscopically examined hCG-treated gilts and noted that the ovarian morphology described as

grape-type and honeycomb-type significantly reflects the ovulatory response to hCG injection. Hunter et al. (2000) successfully created an endoscope which was combined with an infra-red camera to record thermo-images of ovaries during the pre-ovulatory period. It was shown that within a closed abdomen, temperature gradients do exist in the ovarian tissues of mature animals, and these are generated at least in part as a consequence of endothermic reactions within Graafian follicles. It seems to be obvious that there are many more studies, which highlight the benefit from this instrument consisting of a thin optical axis to simply get access to the inner reproductive organs.

Brüssow and colleagues (1997) showed that aspiration of oocytes from preovulatory follicles can be successfully done using endoscopy. The stress for the animals was assessed as minimally invasive and the technique was reliable and adequate for studying intra-follicular development, oocyte maturation for examining IVM/IVF/IVC procedures.

In contrast to the inspection of the abdominal cavity many studies consistently express that the manipulation of the porcine reproductive organs for insemination, embryo collection and transfer requires somehow special expertise obtained by routine work.

Embryo collection in swine is preferentially done after slaughter (Blum-Reckow and Holtz, 1991), whereas surgical flushing (Springmann and Brem, 1989; Camaron et al., 1989, Rátky et al., 2001) and endoscopic flushing (Rátky and Brüssow 1995, Brüssow and Rátky 1996; Besenfelder et al., 1997, Rátky et al., 2001) is limited by the number of animals which justifies the exercise in individual valuable animals.

Rátky and Brüssow 1996 published a detailed description about endoscopic embryo flushing in gilts. A Veress needle, one trocar canula (10 mm in diameter) and four other small trocars (5.5 mm in diameter, Storz, Tuttlingen, Germany) served for the endoscope and for grasping forceps and flushing catheter (Rüsch-Gold balloon catheter, 12 Ch.).

Twenty-eight gilts were prepared and 80% could be flushed successfully which resulted in the collection of 208 ova (recovery rate: 53.6%). It was reported that routine application had a significant positive effect on the recovery rate. After the collection procedure the gilts were slaughtered. No adhesions were found but a prolapse of the endometrium remained from the introduction of the flushing catheter through the uterine walls (Rátky and Brüssow 1996).

Besenfelder et al. (1997) first started with unilateral flushing of oviducts in superovulated gilts. The medium was forwarded via the Fallopian tube to the tip of the uterine horn. Similar to the procedure of Rátky and Brüssow (1996) a balloon catheter served for collection of embryos. It is noteworthy that two animals were allowed to carry litters of 6 and 9 piglets to term, which assures the minimal invasive manipulation during flushing of the opposite horn. Both, the flushing of one

horn and both horns resulted in embryo collection rates which were comparable to those obtained by surgery (Besenfelder et al., 1997).

In swine embryo transfer has been done surgically (Springmann and Brem, 1989; Camaron et al., 1989; Blum-Reckow and Holtz, 1991; Rátky et al., 2001), nonsurgically via the cervical entry (Reichenbach et al., 1993; Hazeleger, 1999) or via endoscopy (Rátky et al., 2001; Besenfelder et al., 1997; Stein-Stefani and Holtz, 1987).

Stein-Stefani and Holtz (1987) introduced an endoscope abdominally, pierced a 15 cm needle through abdominal and uterine wall and deposited the embryos in the tip of one uterine horn. However, and for unknown reasons the endoscopic transfer did not result in satisfactory results.

In contrast, Besenfelder et al. (1997) also used a midventral laparoscopy to perform ET at Day 3 into the oviduct as well as at Day 6 into the uterine horns. The transfers were done unilaterally. The gilts were anaesthetized, fixed in a dorsal recumbent position and slightly turned to the lateral side. The access to the oviduct was provide using a Hopkins forward oblique telescope (30°, 6.5 mm), atraumatic forceps (7.0 mm) and glass capillary tubes (1.4 x 150 mm) or intravenous catheters (1.4 mm, 50 cm) loaded with 15 to 30 embryos. The capillary tube or the catheter was managed into the ampulla to a depth of 4 to 5 cm or 8 to 10 cm, respectively. The embryos were slowly d into the oviduct under visual control to guarantee that the medium flew straight forward. The uterus transfer was performed by means of a metal catheter (105 mm x 2.0 mm and 155 mm x 2.5 mm) which was inserted through the uterine wall. The embryos were positioned in front of a flexible tube (1 x 1.5 mm, 38 cm, with two lateral openings) which was introduced through the metal catheter into the uterine lumen. Embryos were transferred while the tube was slightly withdrawn. Both, oviduct transfers and uterus transfers resulted in pregnancies (Besenfelder et al., 1997). It was recommended that also both transfer techniques are suitable for embryo transfer programmes. In the following, routine transfer resulted in about 90 % of pregnancy. The use of flexible tubes for oviduct transfer was superior to the use of glass capillary due to the depth of tubal introduction. In some case we could observe that the number of embryos transferred via a flexible tube was identical to number of implantation sides (Besenfelder et al., 1998a).

Rátky et al. (2001) succeeded in doing ET endoscopically in 4 recipient gilts. A 17-gauge trocar served for introduction through the uterine wall 1.0 cm cranial to the uterotubal junction, and a 5 French PTFE transfer catheter loaded with 19 to 20 embryos was forwarded 2 cm into the uterine lumen. Half of the animals became pregnant and farrowed. It was concluded that an appropriate inter-breed ET program is a suitable tool to propagate the endangered breed such as Mangalica breed in Hungary (Rátky et al., 2001).

Artificial insemination guided by means of endoscopy turned out to be a subtle and efficient method with special regard to amount of semen and semen quality. It is well accepted that there is a significant semen gradient, which is found in the cervix, along the uterine horns, and in the oviduct which results in a manifold difference in the number of spermatozoa. Consequently, the efficacy of AI mainly depends on the deliberation about semen availability (amount and quality) and the chosen insemination method. In this context the intra-oviductal insemination has become a challenging position, because many factors which might have a negative influence on conception are compensated or/and circumvented (Roca et al., 2006; Garciá, 2007; Vazquez et al., 2008).

Three trocars are inserted bearing the endoscope, a pair of non-traumatic grasping forceps and the insemination needle. The grasping forceps serves for fixation of the oviduct while the insemination needle is injected through the tubal wall into the oviduct and a low dose of semen is injected. When tubal AI was performed after ovulation occurred the polyspermy was higher compared to AI done before ovulation (Vazquez et al., 2008). Tubal AI as it was described by Vazquez and his team also becomes relevant especially for using sex-sorted spermatozoa for the insemination (García et al., 2007).

7.5 Endoscopy in rabbits

In many ways the rabbit bridges the gap between laboratory animals and livestock species. In our group this was also the reason why endoscopy was developed and established, for the rabbit itself as well as for the rabbit serving as a model for other species.

The rabbit represents a historic model for embryo transfer due to the fact that even in 1891 Walter Heape was the first who flushed embryos of one breed and successfully transferred them into the reproductive tract of females of another breed which got pregnant (cited by Betteridge, 2003).

Meanwhile many attempts have been made to find a suitable route for transferring embryos. Embryos have been surgically transferred into oviducts and uterine horns, via the flanks or by midventral access (Chang, 1950; Hafez, 1962). In 1969 Testart performed the first nonsurgical transfer choosing the transvaginal route. Due to some problems this technique has never been adapted to practice. First approaches in rabbits also have been made to increase feasibility of endoscopy by evaluating standard reproduction parameters such as ovulation rate, embryo number and litter size (Fujimoto et al., 1974, Santacreu et al., 1990). Shortly after, the same team (Gracía-Ximenez et al., 1991) published a technique for transferring morulae into the recipient does. The rabbits were anaesthetised and placed in Trendelenburg position. The abdominal entry was performed 8 cm cranial of the navel region. The uterine horns were grasped with forceps fixed near the uterotubal junction allowing the trocar- canula needle to be inserted through the wall. Embryos were loaded in a

polyethylene tubing and transferred via the trocar canula needle at the tip of the uterine horn. All does (12/12) became pregnant (Gracía-Ximenez et al., 1991). Two years later, our team introduced an endoscopic technique for the transfer of embryos into the oviduct. Anaesthetised rabbits were fixed in a head down position. The endoscope was placed 1 cm cranial to the umbilicus which allowed the adspection of both ovaries and the adjacent part of the oviduct. A vein catheter (51 x 2.5/2.9 mm) was inserted through the abdominal wall close to the infundibulum and following the direction of the ampulla. A 5 µl glass capillary connected to a 1 ml syringe and filled with medium was loaded with embryos. This capillary was pushed through the vein catheter and inserted via infundibulum 2 to 3 cm deep into the ampulla. In this experiment 90 % of the recipient rabbits became pregnant (Besenfelder and Brem, 1993). Meanwhile this procedure has become our standard technique and, to date, we transferred some ten-thousands of embryos for different purposes (maintenance of lines and breeds, embryo banking, production of transgene rabbits). Moreover, we could successfully demonstrate within a European concerted action for "germplasm banking" (Bolet et al., 2000) that morulae and non-expanded blastocyst can be transferred into the oviduct leading to a high pregnancy rate.

In 1998, the same technique served for collection of rabbit embryos of different stages. The glass capillary was replaced by a flexible tube which was fixed in the oviduct during flushing. First the embryos were flushed through the oviducts into the uterine horns. An additional 20 to 40 ml was flushed through each uterine horn from a puncture side at the uterotubal junction. A cystoscope allowed the insertion of a collecting tube via each cervix into the uterine horns for medium collection. On average 13 ovulation sites were counted and 8 morulae/blastocysts were collected (Besenfelder et al., 1998b). The technique we currently use for routine flushing was refined and minimized in a way that we flush medium through both oviducts and uterine horns and collect this medium in the vagina. The embryos containing flushing medium is drained off via a balloon flushing catheter (CH18, for cattle) and collected in an embryo filter simply following the principle of pressure adjusting (Besenfelder et al., 2000). Mehaisen et al. (2004) also collected medium from the vagina by aspiration with a vacuum pump connected to a Foley catheter.

Based upon the success, animal welfare and feasibility it is recommended that endoscopic is superior to laparotomy in rabbit reproduction.

7.6 Endoscopy in cattle

As scored under the worldwide statistics of embryo transfer in domestic farm animals by the IETS Statistics and Data Retrieval Committee Report (Stroud, 2010) there is an enormous interest in

the cattle industry and in science to produce embryos in cattle. Besides relatively constant embryo collection data via the superovulation route there is a clear increase in embryo production based upon in vitro activities.

Although in vitro production of bovine embryos has significantly been improved over several decades, the complex function and intrinsic role of the oviduct has not been completely mimicked and replaced satisfactorily. Consequently, the suboptimal early embryo development in vitro is associated with alterations in survival of embryos, foetuses and calves (Farin and Farin, 1995; Wright and Ellington et al., 1995; Hansen and Block 2002; Wrenzycki et al., 2004; Farin et al., 2006; Farin et al., 2010). The architecture of the oviduct and its inductive as well as nutritive role provide an environmental milieu for oocytes, spermatozoa and early stage embryos which represents a failsafe system consisting of overlapping functions and components which are time-exactly orchestrated and adequately balanced (Killian 2004).

For this reason, many attempts aimed at replacing the tubal function by changing the medium composition (Wrenzycki et al., 2001; Rizos et al., 2003; De Oliveira et al., 2006), using conditioned media (Eystone and First, 1989) and co-culture systems (Eystone and First, 1989, Rief et al., 2002). Moreover, Knijn et al. (2002) used oocytes from different origin followed by in vitro production. Independently of oocyte source it was shown that alterations in blastocyst quality can be attributed to the post-fertilization culture period (Knijn et al., 2002). These observations were confirmed by Rizos et al. (2002), who performed studies which impressively demonstrated the necessity of tubal development especially for the embryo culture period.

Despite an increasing knowledge in the field of in vitro production of embryos many studies revealed a culture impact of oviducts for the improvement of embryo quality. A wide range of species served for the in vivo culture of bovine embryos followed by the re-collection and single transfer. In many cases the rabbit was an optimal model to firstly study the in vivo culture effect on bovine embryos (Lawson et al., 1972; Trounson et al., 1977; Fukui et al., 1983; Sirard et al., 1985; Sirard and Lambert, 1986; Sirard et al., 1988; Wall and Hawk, 1988; Ellington et al., 1990).

To date, the temporary culture of bovine embryos in the sheep oviduct has played the major practical part to achieve numerous high quality embryos mainly for research but as well for (Leibfried-Rutledge et al., 1987; Galli et al., 2001; Rexroad and Powel, 1999; Enright et al., 2000; Rizos et al., 2010). At least, several studies were also conducted to get access to the bovine oviducts (Jillalla et al., 1977; Trounson et al., 1977). In addition, Rizos et al. (2007, 2010) could demonstrate that the mouse oviduct in vitro could serve for development of bovine embryos from the zygote to the blastocyst stage. The pattern of expression of selected transcripts from bovine embryos cultured

in vitro in the mouse oviduct were assessed similar to that of in vivo derived embryos (Rizos et al., 2007).

All these studies point out a beneficial improvement of tubal embryo development, but the surgical access to the oviducts in different species, especially in cattle, finally and with some exceptions did not end up in routine application. This technical procedure was reserved for teams, which fulfilled necessary requirements such as specific surgical expertise and adequate facilities. The acceptance was also confronted by ethical issues and animal welfare concerns.

Initial work on endoscopic access to the bovine oviduct first has been described by Fayrer-Hosken and his colleagues (1989). In the year 1989 this team performed a paralumbar access to visualize both ovaries and oviducts using a bronchoscope. First, both ovaries were adspected for the presence of either a recent corpus hemorrhagicum (ovulation site) or a dominant, unovulated follicle. Then, the catheterization of the ampulla was accomplished with a polyethylene teflon tubing into which a tom cat catheter had been inserted. The complete transfer system was filled with medium. The embryos and medium were loaded into the tip of the catheter. The fimbrial part of the infundibulum was grasped with Semm´s atraumatic forceps to enable the catheterization of the oviduct lumen to a depth of 2.5 to 5.0 cm. Four endoscopies were done which resulted in one pregnancy. These results were assessed as encouraging for the application of in vitro maturation and in vitro fertilization for overcoming infertility in domestic and endangered species (Fayer-Hosken et al., 1989).

In our first studies we reported the transfer of 2 to 4 cell stage embryos into the oviduct by means of transvaginal endoscopy. The animals were fixed in a crush and an epidural-anaesthesia was performed. A bitubular system bearing the endoscope and the transfer capillary connected to a tube was mid-dorsally placed via the fornix vaginae in the abdominal cavity. The ovary plus the adjacent part of the ampulla were rectally presented in that way allowing the capillary to be inserted. Nine of 24 recipients got pregnant (Besenfelder and Brem, 1998). Once having established the endoscopic access we stepwise developed oviductal flushing of superovulated heifers. For this purpose the transfer glass capillary was replaced by a metal canula of the same size for the introduction into the oviduct. Lateral wholes of the flushing catheter were covered by a silicon tube, which was blown up when the flushing pressure was increased. This technique guaranteed the hermetical sealing of the oviduct for flushing. The embryos were flushed via the uterotubal junction to the tip of the uterine horn and passed into an embryo flushing balloon catheter connected to an embryo filter. Forty to 60 ml of medium was flushed to each oviduct which resulted in a visible medium flow in the embryo filter. We first flushed superovulated animals unilaterally. Subsequently, the technique, duration and manipulation were successfully aligned and adjusted. In the following superovulated animals were

flushed bilaterally. In accordance to the number of ovulation sites it was shown that all oocytes/embryos up to the blastocyst stage can be collected. Having stepwise developed the oviductal transfer of embryos followed by the uni/bilaterally flushing of the oviducts, both technical steps were combined for the in vivo culture of embryos derived in vitro (Havlicek et al., 2005a). In our first experiments we transferred more than 2000 embryos at Day 1 to 3 of synchronised recipients. Re-collection was performed 4 to 6 days later. Altogether, more 38 % of the embryos were re-collected and nearly 20 % of the complexes have been in the blastocyst stage (Havlicek et al., 2005b).

The shown technique has become a standard technique at our Institute (Besenfelder et al., 2010). The animals are fixed only for a very short time. Neglecting the time necessary to move the animals from the barn to the animal treatment room including washing, disinfection and time for epidural anaesthesia, endoscopic manipulation e.g. embryo transfer lasts about 5 to 10 minutes. Meanwhile, many experiments have been done in order to transfer more than 4,200 in vitro derived embryos to study the in vivo culture characteristics including morphological properties, different embryonic stages and embryo migration (Wetscher et al., 2005a; Wetscher et al., 2005b). Moreover, different super stimulation protocols were used and flushing of more than 1,400 complexes was performed at a different time period post insemination to study tubal embryo kinetics (Besenfelder et al., 2008). In total it was shown, that especially oocytes and early stage embryos are very sensitive to any kind of manipulation. The circular and longitudinal muscular structures of the oviduct are hyper-activated leading to a very motile oviduct during the ovulation period and the following 2 days (Ruckebusch and Bayard, 1975), which is reflected by a decreased recovery rate so far oocytes/embryos have been transferred denuded. After superovulation or after embryo transfer for in vivo culture flushing of uterine horns plus the oviducts results in higher collection rates compared to simply flushing of uterine horns (Besenfelder et al., 2010).

Alternatively to superovulation ovum pick-up has also been performed in cattle using endoscopy. It has turned out that endoscopy could serve for single OPU in non-stimulated vs. super stimulated animals whereas a frequent use would be too invasive.

Fundamental experiments were done in the years 1983 to 1991. Lambert et al. (1983) introduced an endoscopic technique, where the paralumbar route served for the examination of ovaries and for follicle aspiration. The use of a 19-G needle and a suction device with a vacuum pressure of 250 m Hg lead to the best results. Shortly after, this team endoscopically collected in vivo matured oocytes (Lambert et al., 1986). It was shown that experienced endoscopists had higher recovery rates compared to beginners. Oocytes surrounded by expanded cumulus cells were fertilised and showed a better developmental rate than the denuded counterparts (Lambert et al., 1986). In the following the

flank endoscopy has been accepted as a valuable method for OPU aspiration (Laurincik et al., 1988; Schellander et al., 1989; Fayrer-Hosken and Caudle, 1991; Laurincik et al., 1991).

Santl et al. (1998) and Becker et al. (1996) performed comparative studies testing ultrasonography vs. transvaginal endoscopy for OPU in cows and stated that endoscopy leads to a higher rate of denuded COCs compared to ultrasound-guided follicle aspiration. It was assumed that the aspiration system adapted to OPU by means of ultrasound is not suitable for endoscopic use due to the turbulent current. This problem has been solved by modifying the aspiration system. When a valve is attached to the tubing line to individually regulate vacuum pressure for follicle aspiration the cumulus-oocyte-complexes show an intact morphology (Mösslacher, 1998).

7.7 Conclusion

In conclusion, endoscopy has exerted a major influence in the field of reproduction in different species. Animal synchronisation, follicle aspiration, insemination, embryo collection and transfer are the main topics where the use of endoscopy can have an efficient and beneficial effect on animal breeding strategies. Besides any commercial application, there is a growing scientific interest regarding differences in early embryogenesis not only amongst in vitro produced embryos but also amongst poor quality embryos resulting from superovulation programmes (Greve and Callesen, 2001) and embryos derived from spontaneously ovulating animals, which are recommended to act as the "gold standard" (Wrenzycki et al., 2004) and to meet basic requirements for further studies in the field of embryo-maternal communication (Wolf et al., 2003; Fazeli, 2008). Many studies have revealed that endoscopy compared to laparotomy is less invasive, causes less adhesion formation and opens a wide range of application (Gutt, 2004).

8 Ethics of Animal Breeding and Biotechnology

8.1 Introduction

The animals that we normally breed are domesticated animals. Domesticated animals are of course descended from wild animals. Their wild ancestors gradually started to live in close proximity to humans and were later, for generations, bred under human control. Thus these animals are not only tamed but have been adapted genetically over many generations.

The first animal to be domesticated was probably the dog, which originates from the wolf. There is some scientific uncertainty about when and how the domestication took place, but even on a conservative estimate the dog has been domesticated for at least 12,000 years. Some breeds of dog – the so-called "purebreds" – are now defined by systematic breeding practices based on internationally recognized standards. The pedigrees of these dogs must be registered in special breeding registries. Today there are approximately 400 breeds of purebred dogs worldwide.

The domestication of livestock species such as sheep, goats, pigs and cattle took place, mainly in Asia, between 9,000 and 6,000 years ago. Chickens became domesticated about 4,000 years ago. The domestication of other animals is very recent. For example, the domestication of mink and other fur animals started in the late nineteenth century, and the domestication of deer only started in the 1970s. The breeding of rats and mice for experimental purposes began around 1900.

Domestication leads to dramatic changes in the physical appearance of an animal, as can be observed in the differences between the wolf and the multitude of dog breeds found today! Domestication is also associated with behavioural changes, with most domestic animals being calmer and less fearful than their wild ancestors. Typically it also brings about changes in reproductive biology. Thus while the wild ancestors of domestic animals display strict seasonal reproduction rhythms, most domesticated species can reproduce all through the year. Within the main species of domesticated animals different breeds have evolved to serve specific purposes. For example, some breeds of cattle are mainly working animals; others are used primarily for meat production; others again are dairy cows.

Despite the dramatic effects of domestication animal breeding was, until the beginning of the twentieth century, a relatively uncontrolled activity based mainly on the animal's physical appearance. The early animal breeders did not really have the knowledge and tools to predict and control what they were doing. This changed in the first half of the twentieth century, when Mendelian genetics was applied in farm animal breeding. Later, the second half of the twentieth century saw the development of new forms of animal biotechnology such as the freezing of semen, embryo transfer,

in vitro fertilization, gene transfer and cloning – all of which allow scientists and breeders even greater control over future animals.

With greater control comes greater responsibility and, in modern democratic societies at any rate, a higher degree of accountability. Certainly the new breeding technologies prompt a range of ethical questions about the application of science-based breeding and other forms of modern biotechnology. Who are we to decide what kinds of animal are going to exist in the future? Are breeding-induced animal welfare problems acceptable? Should breeding aim at producing animals that cope better with production systems? How should we react to the possibility of crossing the species barrier? What general ethical limits are in play here, and how should they be elaborated given the plurality of ethical perspectives? Before addressing these questions it must be taken into account what modern breeding and biotechnology actually involves, and how animals are (potentially) affected.

8.2 New ways of changing animals

The main breakthrough in the development of more advanced breeding practices came at the beginning of the twentieth century with the re-discovery of Mendelian genetics. With this new basis for understanding the heritability of different traits it became possible to design future generations of domestic animals using measurements of the genetic potential of potential breeding animals. From the beginning and for a long time this approach was mainly used in the breeding of farm animals.

The genetic potential of animals came to be measured by looking at the performance of their ancestors, siblings and offspring rather than looking merely at the performance of parent animals. Furthermore, advanced biometric models were put to use to estimate the genetic potential of possible parent animals. Breeding animals were now selected by referring to breeding goals – that is, goals that define the relative weight of the various traits that the breeders are trying to enhance. The fact that it is possible, generation after generation, to achieve progress on a breeding goal is based on the biological insight that relevant genetic properties vary among offspring. By always breeding on from the best-performing animals it is possible, over a number of generations, gradually to improve average performance.

For many years the main focus in the breeding of farm animals has been on production traits. Examples of such traits include milk yield in dairy cows, the number of eggs laid by laying hens, and growth and feed conversion in meat animals. The results have been staggering. For example, between the early 1960s and the late 1990s the time needed to produce a slaughter-weight broiler fell from 80 to 40 days, and the required feed consumption halved (Havenstein et al. 2003). Over the same period,

milk yields in most dairy cow breeds have more than doubled (Christensen 1998). Admittedly, these achievements derive in part from improved management practices. However, to a large, and still increasing, extent they are the outcome of genetic changes brought about by systematic farm animal breeding.

More recently, modern biotechnologies have also been used by breeders in their work with some farm animals. The technologies in question belong to what is called reproductive biotechnology, which aims to control (and often accelerate) the process of breeding. The first technology of this kind to be developed was artificial insemination (AI) in cattle. This allowed reproduction to take place without natural mating. In the 1950s a technique for freezing semen ensured that AI would become even more significant, since frozen semen could now be stored over a longer time and transported to a geographically wider area and, if necessary, across national boundaries. Today's breeders are, therefore, no longer restricted by the locally available gene pool. With this technique it is possible for one bull to sire hundreds of thousand calves. At the turn of the millennium approximately 110 million artificial inseminations were being carried out in cattle each year worldwide (Thibier & Wagner 2002).

Similarly, technologies have been developed to enable female animals to produce more progeny than they would naturally. These include superovulation, which allows several embryos/eggs to be produced per selected donor, and embryo transfer, which enables the breeder to shuttle embryos to surrogate mothers. A technique has also been developed that makes it possible to remove immature eggs from female animals, mature and fertilise these in vitro, and then transfer the fertilised eggs to recipient animals which serve as surrogate mothers. These technologies have been of particular interest to cattle breeders, because in cattle there are long intervals between generations; each cow normally produces only one calf per year.

One of the more spectacular forms of biotechnology so far has been the kind of animal cloning that took off with the sheep Dolly, born in 1996. Dolly was in a radical sense fatherless. She originated from a cell taken from the udder of her biological mother. This cell was inserted into an unfertilised sheep egg from which the nucleus had been removed. It was manipulated so that it fused with the 'egg-mass' to form an embryo. The embryo was then inserted into a foster mother who went through a normal, albeit closely monitored, pregnancy, resulting in the birth of Dolly – the first mammal to be cloned from an adult animal.

So far cloning has not been used to any significant extent in the breeding of farm animals. The main interest in the technology has come from scientists involved in research. Here there is a particular interest in the potential of combining cloning with another form of modern biotechnology: genetic modification. Genetic modification (sometimes referred to as 'genetic engineering' or

transgenesis) involves the direct manipulation of an organism's genetic make-up to create genetically modified animals. New genes can be introduced into a fertilised egg, or a cell line, so that the organism that subsequently develops inherits genes of mixed origin; or a gene can be "knocked out" so that it no longer functions. Using transgenic techniques, scientists can also move genes across species barriers. For example, it is now possible, for research purposes, to introduce genes of human origin into a mouse or a rat. In this respect genetic modification goes well beyond what has so far been possible in conventional breeding.

Most cloned and genetically modified animals are used in basic research and as disease models. Genetically modified animals have been produced to investigate the function of genes and gene products, and to create animals that mimic human diseases such as cancer or Parkinson's Disease. The aim is to facilitate research into the diseases and test possible treatments. In this area of investigation cloning is used mainly as a tool to produce genetically modified animals and to study abnormalities in reproduction. Another potential use of genetic engineering is to create animals which serve as bioreactors that produce biological compounds not naturally occurring in them (so-called "pharm animals"). Typically a gene of human origin is introduced in the animal genome. This may enable the animal to produce a specific protein, often in its milk, that can then be used in the production of a particular medicine.

There is no doubt that modern breeding practices and the various reproductive technologies reviewed above have delivered significant benefits across a wide range of applications. Since the Second World War farm animal breeding has ensured that animal products are produced evermore efficiently, and this has contributed to the significant drop in the relative price of meat and other animal products. Whether this should be considered a positive development from an ethical perspective is a matter for debate.

Furthermore, the application of biotechnology to animals delivers an important set of tools for biomedical research. It is hoped that this kind of research will enable researchers to find new ways to prevent, cure and alleviate serious human diseases, although for the most part this remains to be seen. Here again there is room for ethical debate.

Although modern animal breeding and recent developments in biotechnology deliver benefits, they also introduce problems – not least for the animals involved. These problems will be considered in the next section.

8.3 Problems caused by animal breeding and biotechnology

The main goal of farm animal breeding has so far been to increase the productivity of farm animals. Pursuit of this goal may as a side-effect lead to a higher occurrence of health-related welfare problems in farm animals. The breeding of dairy cattle for higher milk yield and breeding of broiler chicken for faster growth serve to illustrate this.

Over the last hundred years milk yield in dairy cattle has increased substantially. In Denmark the average milk yield has risen from approximately 2,000 kg to more than 9,000 kg per cow per year, thanks partly to improved management and partly to intensive breeding. This development can be seen as positive from the point of view of human standards of living. It may also be viewed positively from a resource perspective, since the pollution per kg milk produced may be less when production involves a smaller number of animals. However, it has become evident that excessive focus on raising milk yields leads to animal health problems. Thus on average cows get more mastitis and digestive disorders, and there are increased problems with reduced fertility and calving performance.

As was mentioned above, the huge acceleration in the growth rate of broilers has been secured largely by modern selection techniques. The time required for broilers to attain commercially desirable weight has, as a result, been cut substantially. But as an unintended side-effect the birds now suffer from severe leg problems. In a Danish study conducted in 1999 (Sanotra 1999), it was reported that nearly one third of the birds had a significantly reduced ability to walk normally. There is every reason to believe that this impairment is painful for the birds. A number of other problems also seem to be connected, directly or indirectly, with accelerated growth. For example, the parent animals used to produce eggs from which broiler chickens are hatched endure strict food restrictions under which they are permitted to eat only about half of what their appetite motivates them to eat. The resulting feeling of hunger may reduce their welfare, but in the absence of this restriction the animals become obese, with dramatic negative effects on both animal welfare and production.

The genetic correlations between production and health traits are typically unfavourable in the sense that the genes that bring increases in productivity introduce dispositions to disease and other health problems. Even so, carefully designed breeding programmes might allow breeders to improve health and increase production at the same time. In Scandinavia dairy cattle breeding programs were initiated in the 1980s which promote both production and health traits. Recently this approach to cattle breeding has spread to other countries. In response to public concern, companies involved in broiler breeding have in recent decades invested considerable resources in breeding for leg-health, and data from 2005 from one of the world's two main broiler breeding companies seem to indicate that there has been a significant reduction in leg problems in the birds.

However, it is also important to be aware that, because of widespread negative correlations between health and productivity, no breeding goal will at the same time deliver maximum improvements in animal health and welfare and maximum increase in productivity. Thus in farm animal breeding it will always be necessary to balance human benefits and the costs to the animals involved.

Modern farm animal breeding – like most other tools – can be used for doing good and bad things. Doing without these tools is not necessarily a good thing, as can be seen from breeding of pets such as cats and dogs, where advanced breeding techniques have helped breeders to reduce serious health problems in some breeds of dogs and cats.

The breeding of dogs and cats is mainly a low-tech, small-scale activity. Breeding animals are selected primarily on the basis of how well they have done in dog or cat shows. As a consequence health problems may occur in the animals' offspring. Some of these problems are due to the breeding goals. If, for example, a variety of dog or cat is bred with the goal of having an ever shorter nose, as happens in some breeds, several sorts of health problems, including breathing difficulties (dyspnoea), may arise. These problems can, of course, be dealt with by changing the breeding goal.

Other problems found in the breeding of cats and dogs, however, have no direct connection with the breeding goal. For example, some breeds of dog and cat happen to be badly affected by skin allergies. Such breeding related health problems are sometimes caused by a high level of inbreeding. Advanced breeding schemes like those applied to farm animals are sometimes required to deal effectively with these problems. Furthermore, in some cases the genetic testing of parent animals may be applied to tackle heritable diseases.

Again, also the more recently introduced forms of biotechnology, such as genetic modification and cloning, can create welfare problems. Thus, obviously, a mouse carrying the human Huntington's disease gene will be prone to suffer welfare problems as it develops the disease, including rapid progressive loss of neural control leading to premature death (Naver et al. 2003).

Genetically modified animals have mainly been used so far in biological research and as disease models. Usually the goal of modification is to produce animals that either under- or over-express certain genes, or that express a mutated, disease-causing human gene. In all these cases body function in the organism is in some way disrupted. In principle, modifications can involve any part of the animal genome, and the effects on the animal's phenotype range from those that are lethal to those that have no detectable effect on the health of the animal. It is therefore difficult to generalise about the welfare effects of genetic modification.

However, in some cases genetic modifications have a real impact on welfare. These cases can be divided into two main categories: those involving intended, and those involving unintended, genetic

change. Welfare problems stemming from intended genetic change are hard to avoid, since the very point of inducing the change is to affect the animal. By contrast, unintended genetic changes are the upshot of two factors: the present inaccuracy of the technology and our insufficient understanding of the function of different genes in different organisms. Both of these factors operate to create the rather unpredictable nature of genetic modification at the phenotypic level.

It is likely that at least some unintended welfare problems will be reduced as the technology and our scientific understanding develop. And where the intended consequences of genetic modification (e.g. in creating a disease model) are concerned, it may be possible to predict welfare consequences using information about the effects of similar mutations in other species, including human disease symptoms.

Turning to cloned animals, the current success rates of animal cloning are very low (3-5%), and of the few individuals born, many suffer from impaired health. Here, a wide range of problems includes placental abnormalities, foetal overgrowth, prolonged gestation, stillbirth, hypoxia, respiratory failure and circulatory problems, malformations in the urogenital tract, malformations in the liver and brain, immune dysfunction, lymphoid hypoplasia, anaemia and bacterial and viral infections. It is not yet clear to what extent the welfare problems currently associated with animal cloning can be avoided through technological or methodological improvements (Vajta & Gjerris 2006).

Besides problems relating to the welfare of affected animals, breeding and biotechnology may also have a negative effect on biodiversity. In its nature, intensive selection tends to lead to losses in genetic diversity, since very often a limited number of genotypes of particularly high breeding value are concentrated upon and put to heavy use. This danger is particularly great in dairy cattle, where artificial insemination enables a few bulls to have offspring all over the world. Already, less productive local breeds are being replaced by high-yielding and thus more profitable breeds, and as a result genetic diversity is being lost. Interestingly, however, it is desirable to protect biodiversity from the point of view of production: since it is not known what genes will be needed for future breeding goals, the preservation of genotypes may serve as a sort of insurance for the future. Of course, local breeds also have value as part of our cultural heritage.

8.4 Ethical limits to breeding and biotechnology?

In one way breeding and biotechnology seem merely to add more of the same kinds of problem and concern that is already found in various forms of animal use. Production-related breeding and biotechnology make it possible for farmers to put more pressure on animals and produce ever more

efficiently. When it comes to laboratory animals, breeding and biotechnology enable researchers to develop new animal models – for example, animals born with dispositions to develop certain diseases. And when it comes to companion animals, breeding is used to develop animals that meet human desires, such as the desire to own a dog of charming or endearing appearance.

Looked at in this way, breeding and biotechnology appear to give rise to the very same kinds of ethical dilemma that relate to other forms of animal use. In general, the dilemma is one in which there is, on the one hand, a human need, interest or preference, and yet, on the other hand, pursuit of the relevant human aim comes at a cost – a cost carried principally by the animals.

However, there is an important difference here. In most other form of animal use it can be assumed that the issues concern animals that either already exist or would very probably exist. But in deciding to adopt a certain breeding scheme, or to engage in the genetic modification or cloning of animals, the question is, not so much how existing animals should be treated, but rather which animals or what kinds of animal, are going to exist. So animals are being changed, they will be qualitatively different depending on how breeding and biotech is applied. However, they will also be numerically different – they will be different individuals.

The significance of this last feature of breeding and biotechnology can be illustrated with a hypothetical case. Suppose you own a dog to which you feel very strongly attached. However, the dog suffers from a rather mild heritable disease, which means that once a year it must be taken to the vet for an injection. This disease can be eliminated by breeding, since through screening it is possible to select parents that do not pass the disease on to their offspring. Given all this, you may feel tempted to say something like: “It would have been great if my dog had been bred so as to avoid this disease. It would have been good for the dog (which would have avoided the symptoms of the disease) and for me (since I would not have had the inconvenience and financial burden of visits to the vet).”

But if the dog had been bred so as to avoid the disease at least one of the parents would have been different. However, this would mean that your present dog would never have been born: another, presumably healthier dog would have been born instead. So to wish that the dog had been bred differently is really to wish that the actual dog had not been born, and that another dog had been born instead. It is nearly equivalent to saying “I wish that I had bought another dog!” – a sentiment that may come across as cynical.

This kind of reaction may be even stronger when it comes to humans. In many countries screening tests are allowed for certain serious diseases early in pregnancy and it is legal to induce abortion late in pregnancy if the test comes out positive. Families carrying serious heritable diseases may be offered genetic counselling. However, this is the exception to the rule. Generally speaking, the idea of breeding perfect children will trigger strong moral objections in most people. It is widely

assumed that we should love children as they are. Only in exceptional circumstances is it considered acceptable to interfere with the normal course of things when it comes to having children.

New biotechnologies that can be applied to animals and humans have given rise to a great deal of debate, and to strong public reactions, over the last three decades. There seems to be a widespread concern about the amount of responsibility being placed on our shoulders. In this context it can be argued that the moral choices we face become too problematic and complicated when we are obliged to decide not just how we treat animals and our fellow humans, but what future animals and humans are going to be like. In view of this many people feel that limits should be placed on researchers' interference with nature. This feeling can be defended in two rather different ways. It can be claimed either that we should refrain from interfering with nature because we cannot accurately foresee the consequences of what we are doing and may therefore bring about some kind of disaster, or alternatively that we should leave nature as it is because untouched nature has a value of its own.

According to the first line of thought the problem with interfering with nature is that we cannot properly predict the long-term consequences of what we are doing. If we try to manipulate nature on the basis of 'grand plans' for the future, there is a real danger that unexpected and harmful consequences occur – as indeed it has sometimes happened when species of plants and animals have been introduced by humans in new territory.

According to the other line of thought the problem with interfering with nature is that we should respect what is seen as the integrity of nature. For example, some time ago poultry breeders in Israel managed to breed featherless broiler chickens for use in poultry production in countries with a very warm climate. From a human-centred perspective this may seem to be a very useful and ingenious step to take. The use of these chickens would allow savings to be made in feedstuff (that would otherwise be required for the growth of feathers) and reduce or remove the cost of plucking at slaughter. Even though this is disputed, it can also be argued that there is no problem for the welfare of the birds as long as they are only used in warm areas where they don't need feathers to keep their body temperature. However, many people will probably still object to the breeding of featherless chickens, because they see it as a wrong-doing to the animal, or as wrong in itself, to make such drastic changes to a natural feature of birds.

It is possible to see, then, why people are concerned about the idea of engineering future animals through breeding and use of modern biotechnology: (1) we are making choices which are not for us to make; (2) we should be very careful when trying to modify nature, because of potential negative consequences to the environment; and (3) it is wrong in itself to make gross changes to animals.

How do these thoughts fit into a more comprehensive account of our duties to animals? Is it possible to give a satisfying account of integrity of nature and the other concerns, and to explain why they matter? Answers to these questions will clearly depend on one's general ethical perspective.

8.5 Breeding and biotechnology viewed in light of four ethical views

Four ethical views will be discussed here, the first of which is the contractarian view. This view only considers human self-interest. Thus when making moral decisions one only has to consider what is in it for oneself (and for those fellow humans on whose collaboration one depends).

As was mentioned above, farm animal breeding has brought about huge gains in productivity and helped to reduce the price of animal products. Moreover, other forms of animal biotechnology hold out the promise of important human benefits – not least in the development of new ways to prevent, cure or alleviate serious human diseases.

From a contractarian perspective these potential and actual benefits all speak in favour of breeding and modern biotechnology. The only thing that may call for moderation from this perspective is the prospect of short-term benefits being achieved at the price of significant damage in the longer term. For example, from a contractarian perspective there might be good reasons to be cautious about breeding dogs that carry heritable diseases if these diseases will in the end have a negative effect on dog owners in terms of emotional, practical or financial burdens.

It may also matter, from a contractarian perspective, if a large proportion of the general public is concerned about the application of biotechnology to animals. For example, the poultry business may be well advised to abstain from using featherless broiler chickens – not out of concern for the animals, but because that use may alienate or disgust people who may then create problems for business.

The second ethical perspective to be discussed here is the utilitarian view. According to this view one needs to consider not just the interests of all affected humans, but of all affected sentient beings. For an adherent of the utilitarian view the strongest interest should prevail. More precisely, one should seek to produce the greatest total fulfilment of interests.

Thus from a utilitarian perspective the welfare of the affected animal enters the moral equation. However, from this perspective there is nothing inherently problematic about breeding or engineering animals as long as this is done with a view to maintaining or enhancing their welfare. For the utilitarian animals are replaceable. So just as it is, in this perspective, legitimate to kill an animal if this animal is replaced by another that will lead as least as good a life, it is also legitimate to change animals through breeding and biotechnology if the animals that come out of this process are at least

as well off as the animals that would have come into existence had breeding and biotechnology not been applied.

Indeed from the utilitarian perspective it is not only acceptable but our duty to breed animals, and use biotechnology in the process, if this is the best means of increasing welfare, including the welfare of humans and the affected animals. If it is possible to eliminate an inheritable dog disease that is painful to affected dogs and has a negative impact on owners, the breeders will have a duty to favour breeding practices aiming at reducing or eliminating that condition.

When it comes to agriculture, the utilitarian will be inclined to criticize cases in which animals have been bred in such a way that they suffer more seriously from production-related diseases. However, the utilitarian will not conclude from this that we should stop changing farm animals through breeding and biotechnology. Rather the tools should be used in pursuit of breeding goals that will improve the welfare of farm animals. Of course, some utilitarians will argue that it is better to stop farm animal production entirely, but while livestock farming persists breeding for better welfare can at least be seen as a step in the right direction.

On the issue of the alleged integrity of nature utilitarians will presumably support no single, practical policy. They will readily agree that there is nothing inherently problematic about interfering with nature. The only concern here may be about hubris – the worry, that is, that, mesmerized by technological optimism, we focus excessively on the potential benefits of breeding and biotechnology and fail to anticipate their more serious risks. Looking back at previous experience, the utilitarian may well note the negative side-effects of both breeding and biotechnology on animal health and adopt an attitude of caution.

According to the third view to be discussed here, the animal rights view, the interests of all affected beings count, but other things are to be considered as well. What matters is respectful treatment, including respect for life. On the animal rights view rights should be respected, and one should not allow interests to overrule rights.

Once animal rights are asserted the issues raised by breeding and biotechnology become more troublesome. Animals are no longer regarded as replaceable. The whole rationale of this perspective is to protect those animals (and humans) that happen to exist – they have rights. This means that there are certain things that we may not do to these individuals – even at the cost of securing a greater good. So it is not permissible to sacrifice an existing dog with a minor health problem for a future dog with a better health.

In truth, however, most of the choices connected with the application of breeding methods and biotechnology are not really between an existing and potential animal. They are between different

potential animals. More specifically, the choices are between different breeding schemes or strategies, or between different forms of biotechnology, or between the use of a biotechnology and its avoidance.

On the other hand, in some cases the defender of animal rights will have a clear, typically negative, view on breeding and the application of biotechnology to animals. This is because the rights view takes an abolitionist stance on some of the activities of which breeding and biotechnology are typically part and parcel. Thus according to the animal rights view animal production should be stopped. Everyone should adopt a vegan lifestyle. Similarly the use of animals in experimentation should be abandoned, and knowledge in biomedicine be gained in other ways. Obviously, given their belief that these activities ought to cease immediately and totally, advocates of animal rights need not enter into a specific discussion of the merits of using modern breeding techniques and biotechnology in animal production and animal experimentation.

Not all forms of animal use need be banned by the animal rights advocate, however. Some advocates of this view, for example, seem to have no objection to the human habit of keeping pets such as cats and dogs. The question then arises: to what extent is it morally acceptable, or even our duty, to use the tools of breeding and biotechnology to shape future generations of cats and dogs? Should limits be placed on the breeding of extreme phenotypes with a higher incidence of health problems? In fact, is there a positive duty to apply breeding and biotechnologies with the aim of enhancing the health of pets in the future? It is far from clear what answer the animal rights advocate would give to these questions.

None of these three views really seems to vocalize the concerns about not interfering with nature – about integrity of nature. At this point, then it may be necessary to introduce a further perspective of respect for nature. From this perspective what matters ethically is not only to look after the interests and rights of individuals. Larger entities such as species, breeds and ecosystems also matter. We should look after and protect original nature. We should certainly not try to shape everything around us according to our own interests and plans.

Those who sympathize with the claim that we should respect nature will tend to be critical of the view that, as long as the affected animals and humans are no worse off, there is really no problem about interfering with nature – a view eloquently formulated by the American philosopher Bernard E. Rollin:

Given an animal's telos, and the interests that are constitutive thereof, one should not violate those interests. If the animals could be made happier by changing their natures, I see no moral problem in doing so (unless, of course, the changes harm or endanger other animals, humans, or the environment). Telos is not sacred; what is sacred are the interests that follow from it. (Rollin 1995, p.172)

This view seems to be in line with both a utilitarian and an animal rights perspective, but from the point of view of respect for nature it is possible to disagree. From this point of view, in contrast to the view expressed by Rollin, it may be claimed that nature, as it happens to have evolved, is sacred and that it is always problematic to try to engineer nature.

More generally, respect for nature is bound to encourage a very cautious and restrictive policy when it comes to animal breeding and biotechnology. At least, this will certainly be the case with wild animals (and plants). Those who adopt respect for nature will, for example, worry about the breeding of, and application of biotechnology to, farmed fish. One worry may be that the fish could escape and mix with the gene pools of wild fish.

Clearly, however, the animals usually involved in breeding programmes, and usually subjected to biotechnological interventions, are not wild. They are domesticated. Should one care, from the perspective of respect for nature, about whether a dairy cow yields 5,000 or 10,000 kg of milk per year, or whether a bulldog or a Persian cat has a more or less flat nose? Are domestic animals and the human activities to which they relate part of nature or not? And are domestic animals natural in any sense relevant to those who advocate respect for nature? The answers to these questions are at best unclear. Whether or not respect for nature will recommend a restrictive view regarding breeding and biotechnology applied to domestic animals depends on how these animals are viewed.

8.6 Key points

Through modern breeding and the use of genetic modifications and other forms of modern biotechnology, it is possible to some extent to control what future animals will be like. This is an opportunity, but that opportunity comes with considerable ethical responsibility.

Farm animal breeding in particular has brought about dramatic changes. These changes have contributed to the huge growth of efficiency in animal production over the last 50 years, and hence to raised standards of living for humans, especially in the western world. However, the one-sided focus on production-related traits has had a negative impact on the health and welfare of many farm animals. To remedy this situation more balanced breeding goals are called for. When it comes to companion animals, advanced breeding methods might also be applied to help eradicate inherited diseases.

Genetic modification and the cloning of animals have provoked a strong negative reaction from the public. One widely felt concern seems to be that researchers cannot predict the consequences of what they are doing, and that more caution is therefore called for. Another concern seems to be that we should not interfere with nature – out of principle, and not only because of the consequences.

Four ethical perspectives deal with the issue of changing animals in different ways:

- From the contractarian perspective animals are just a resource, so there is not much of a problem.
- From a utilitarian perspective breeding and biotechnology should be used as tools to improve human as well as animal welfare; some degree of caution may be prudent.
- The animal rights view has little to say about choices regarding potential animals.
- From the perspective of respect for nature there may be moral reasons to abstain from animal breeding and biotechnology, but much depends on whether or not domesticated animals are seen as part of nature.

8.7 Further reading

Armstrong, S.J. & Botzler, R.G. (eds.) (2003). Part 6: Animals and biotechnology. The Animal Ethics Reader. Routledge, London and New York.

Comstock, G.L. (2001). Vexing Nature? On the Ethical Case Against Agricultural Biotechnology. Springer, New York.

Holland, A. & Johnson, A. (eds.) (1997). Animal Biotechnology and Ethics. Kluwer Academic Publishers, Dordrecht.

Lassen, J., Gjerris, M. & Sandøe, P. (2006). After Dolly -- ethical limits to the use of biotechnology on farm animals. Theriogenology, 65, 992–1004.

Olsson, A., Gamborg, C. & Sandøe, P. (2006). Taking ethics into account in farm animal breeding: what can the breeding companies achieve? Journal of Agricultural and Environmental Ethics, 19 (1), 37-46.

Reiss, M.J. & Straughan, R. (1996). Improving Nature? The science and ethics of genetic engineering. Cambridge University Press, Cambridge.

Rollin, B.E. (1995). The Frankenstein Syndrome. Ethical and Social Issues in the Genetic Engineering of Animals. Cambridge University Press, Cambridge.

Rollin, B.E. (2006). Science and Ethics. Cambridge University Press, Cambridge.

Thompson, P. (1997) Food Biotechnology in Ethical Perspective. Blackie Academic and Professional, London.

Vorstenbosch, J. (1993). The concept of integrity. Its significance for the ethical discussion on biotechnology and animals. Livestock Production Science, 36, 109-112.

9 The Future of Animal Biotechnology in Domestic Animal Production

9.1 Introduction

The breeding of domestic animals has a longstanding and successful history, starting with domestication several thousand years ago, by which men kept animals in his proximity and used products thereof (Niemann and Kues 2003). Using the technical options that were available in the respective time periods, humans have propagated those populations that deemed useful for their particular needs and purposes. Selection mostly occurred according to the Exterieur (morphology) and/or because of specific traits. A scientifically based animal breeding exists for no longer than ~50 years, starting mainly on the basis of population genetics and statistics. From early on, scientifically based animal breeding strategies involved biotechnological procedures, of which artificial insemination (AI) is the most prominent example. Today, AI is employed in more than 90% of all sexually mature female dairy cattle in countries with advanced breeding programs. Application of AI is also steadily increasing in pigs, where now on a global scale approximately 50% of sexually matured sows are fertilized by AI. AI allows to effectively propagate the genetic potential of valuable sires within a population. On average, 200 to 300 insemination doses can be produced from one bull ejaculate that can be successfully stored frozen; the corresponding figure for the boar is 10 to 20. In the second half of the 1980ies, embryo transfer (ET) technology has been transferred from an experimental stage into animal breeding. ET allowed for the first time a better exploitation of the genetic potential of female animals. On average, 5-10 viable embryos can be collected from a superovulated donor cow and approximately 600.000 embryos are transferred worldwide, of which 50% are used after freezing and thawing (Thibier 2006). In contrast to AI, embryo transfer is predominantly used in the top 1% of a breeding population.

The application of breeding strategies, based on population genetics, AI and ET technologies led to significant increases in the performances in domestic animals. However, three major disadvantages have to be taken into account:

1. The genetic progress is slow, with only 1-3% per year.
2. It is not possible to separate the desired traits from undesired ones.
3. A targeted transfer of genetic information between different species is not possible.

This situation is about to change. Reproductive biotechnologies are currently being complemented by new measures of molecular genetics. The genomes of several domestic animals

have recently been sequenced and annotated, including cattle, chicken, horse, dog and bee, and the pig genome is expected to be drafted shortly. Thus options to use the genetic information are being developed, mainly for diagnostic purposes and to alter the genetic make-up of farm animals. The technology for the production of transgenic animals, either by additive gene transfer or by selective deletion (knockout), has been advanced to the level practical application. A crucial step in this context was the development of somatic cell nuclear transfer (SCNT) cloning, which has replaced the previously used method of microinjecting the foreign DNA into zygotes for the production of transgenic animals. Thus, after approximately 7,000 years of domestic animal selection (Copley et al. 2003) based on the random mutations caused by radiation and oxidative injury to the genome, technology is now available to introduce or remove known genes with known functions.

9.2 Somatic cloning and transgenic animal production

The first transgenic livestock were produced 1985 via microinjection of foreign DNA into pronuclei of zygotes (Hammer et al. 1985). As microinjection has several significant shortcomings, incl. random integration into the host genome, low efficiency and frequent incidence of mosaicism, research has focused on alternate methodologies for improving the generation of transgenic livestock (Kues and Niemann 2004). These include

- sperm mediated DNA transfer (Gandolfi 1998; Squires 1999; Chang et al. 2002; Lavitrano et al. 2002),
- the intracytoplasmic injection (ICSI) of sperm heads carrying foreign DNA (Perry et al. 1999),
- injection or infection of oocytes or embryos by viral vectors (Haskell and Bowen 1995; Chan et al. 1998; Hofmann et al. 2003; Whitelaw et al. 2004) or
- the use of nuclear transfer (Schnieke et al. 1997; Cibelli et al. 1998; Baguisi et al. 1999).

Further qualitative improvements may be derived from technologies that allow targeted modifications of the genome (Dai et al. 2002; Lai et al. 2002), or conditional instead of a constitutive expression of transgenes (Kues et al. 2006). Emerging developments are the usage of large genomic constructs to optimize expression (Kuroiwa et al. 2002), the application of RNA interference to knock down specific genes (Clark and Whitelaw 2003) and the use of episomal vectors which might allow a more consistent expression due to the absence of position effects (Manzini et al. 2006).

To date, somatic nuclear transfer, which has been successful in eleven species – albeit at low efficiency (Kues and Niemann 2007) – holds the greatest promise for significant improvements in the production of transgenic livestock (Table 1). Cloning of mammals involves mainly somatic nuclear

transfer by which the haploid genetic material of an unfertilized ovum is replaced by the diploid genetic material of a somatic cell derived from fetal or adult tissue. However, the derived clones frequently differ in a number of characteristics, because the two cytoplasms involved contain different maternal proteins, RNAs and mitochondrial DNA. In addition, several epigenetic and environmental factors contribute to differences amongst groups of clones. The technical aspects of nuclear transfer in mammals have already been established in the 1980ties using embryonic donor cells (Willadsen 1986; Westhusin et al. 1991). However, attempts to multiply laboratory or farm animals by employing embryonic donor cells (embryonic cloning) have met with modest success. The assumption was that the closer the nuclear donor is developmentally to the early embryonic stage, the more successful nuclear transfer is likely to be. This was mainly based on earlier findings in frogs, in which cloning with embryonic cells was possible with the production of normal offspring whereas cloning with adult cells was only compatible with the development to tadpoles. This assumption dominated biology until the birth of "Dolly" in 1996 - the first cloned sheep generated from an adult mammary gland cell (Wilmut et al. 1997). Cloning of mammals by somatic nuclear transfer is still tainted by low success rate of live offspring, typically with a range of 1-5% of transferred embryos surviving to term (Kues and Niemann 2004, Niemann and Kues 2007). Recently, we have obtained significant improvement of porcine cloning success by better selection and optimized treatment of the recipients, specifically by providing a 24h asynchrony between the pre-ovulatory oviducts of the recipients and the reconstructed embryos. Presumably, this gave the embryos additional time to achieve the necessary level of nuclear reprogramming. This resulted in pregnancy rates of ~80% and only slightly reduced litter size (Petersen et al. 2007).

Despite current limitations, somatic cloning has already emerged as an essential novel tool in basic biological research and holds great application perspectives, incl. transgenic animal production. Further qualitative improvements may be derived from technologies that allow precise modifications of the genome, including targeted chromosomal integration by site-specific DNA recombinases, such as Cre or flippase (FLP), or methods that allow temporally and/or spatially controlled transgene expression (see Niemann and Kues 2003).

	Microinjection	Somatic nuclear transfer
Integration efficiency	+	+++
Integration site	random	random or targeted
Gene deletion	-	+++
Construction size	> 50 kb (artificial Chromosome)	~ 30 – 50 kb
Technical feasibility	technically demanding	technically demanding
Mosaicism	+++	-
Expression screen in vitro	+	+++
Expression pattern	variable	controlled, consistent
Multi-transgenics	+	+++
Required time until first characterized transgenic animals are available	long (F_1)	short (use of cloned transgenic offspring)

Table 9.1: Advantages and disadvantages of two gene transfer methodologies
- = not possible; + = weak advantage, ++ = moderate advantage, +++ = strong advantage (adapted from Niemann et al. 2005)

9.3 Agricultural applications of transgenic animals

Despite the inherent inefficiency of microinjection technology, a broad spectrum of genetically modified large animals has been produced for applications in agriculture and biomedicine (Niemann et al. 2005; Niemann and Kues 2007). Transgenic animals have been advanced to practical application in biomedicine, in particular for the production of pharmaceutical proteins (gene pharming) (dairy animals) and xenotransplantation (pigs), i.e. the production of porcine organs and tissue in human organ transplantation to cope with the shortage of human organs in terminally ill patients. The first recombinant pharmaceutical protein (human antithrombin III, ATryn®) isolated from the milk of transgenic goats has been approved in June 2006 by the European Medicine Agency (EMEA), clearly showing the great potential of this transgenic approach.

Agricultural applications of transgenic animals are lagging behind the biomedical area (Kues and Niemann 2004) but are now rapidly emerging. Table 2 shows an overview on the various attempts to produce animals transgenic for agricultural traits. An important step towards the production of healthier pork has been made by the first pigs transgenic for a desaturase gene derived either from spinach or Caenorhabditis elegans. These pigs produced a higher ratio of poly-unsaturated versus saturated fatty acids in skeletal muscle clearly indicating the potential of rendering more healthier pork in the near future (Saeki et al. 2004; Niemann 2004; Lai et al. 2006). A human diet rich in poly-

unsaturated fatty acids is correlated with a reduced risk of stroke and coronary diseases. In the pig, it has been shown that transgenic expression of a bovine lactalbumin construct in sow milk resulted in higher lactose contents and greater milk yields which correlated with a better survival and development of the piglets (Wheeler et al. 2001). The increased survival of piglets at weaning provides significant benefits to animal welfare and the producer. Phytase transgenic pigs have been developed to address the problem of manure-based environmental pollution. These pigs carry a bacterial phytase gene under the transcriptional control of a salivary gland specific promoter, which allows the pigs to digest plant phytate. Without the bacterial enzyme, the phytate phosphorus passes undigested into manure and pollutes the environment. With the bacterial enzyme, the fecal phosphorus output was reduced up to 75% (Golovan et al. 2001). These environmentally friendly pigs are expected to enter commercial production chains within the next few years.

The physicochemical properties of milk are mainly affected by the ratio of casein variants making them a prime target for the improvement of milk composition. The bovine casein ratio can be altered by over-expression of beta- and kappa-casein, clearly underpinning the potential for improvements in the functional properties of bovine milk (Brophy et al. 2003). Mastitis is a preeminent health problem in modern dairy cattle production associated with significant economic losses. Lysostaphin has been shown to confer specific resistance against mastitis caused by Staphylococcus aureus. Cows have been cloned from transgenic donor cells that express a lysostaphin gene construct in the mammary gland, rendering the animals mastitis-resistant (Wall et al. 2005). Recently, biologically active human butyrylcholinesterase has been produced in the milk of transgenic mice and goats that can protect against organophosphate poisoning. Large quantities of human butyrylcholinesterase are needed for effective prophylaxis and treatment of exposure to organophosphates, which are constituents of biological weapons (Huang et al. 2007).

Transgenic technology has already successfully been applied to create commercially available ornamental fish (Niemann and Kues 2007). Transgenic zebrafish expressing various fluorescent proteins are sold for ~5.00 US$/fish in the USA. These fishes are also sale in Taiwan, Malaysia and Hongkong; marketing is prohibited in Australia, Canada and the European Union (Niemann and Kues 2007). Transgenic application may become more widespread in farm animals with refinements of the genomic maps and more efficient gene transfer technologies allowing the targeting of specific genetic traits. Some of the emerging technologies are described below (Clark and Whitelaw 2003; Niemann et al. 2005; Niemann and Kues 2007).

Transgenic trait	Key molecule	Construct	Gene transfer method	Species	Reference
Increased growth rate, less body fat	Growth hormone (GH)	hMT-pGH	microinjection	pig	Nottle *et al.* 1999
Increased growth rate, less body fat	Insulin-like growth factor-1 (IGF-1)	mMT-hIGF-1	microinjection	pig	Pursel *et al.* 1999
Increased level of poly-un-saturated fatty acids in pork	Desaturase (from spinach)	maP_2-FAD_2	microinjection	pig	Saeki *et al.* 2004
Increased level of poly-un-saturated fatty acids in pork	Desaturase (from C. elegans)	CAGGS-hfat-1	somatic cloning	pig	Lai *et al.* 2006
Phosphate metabolism	Phytase	PSP-APPA	microinjection	pig	Golovan *et al.* 2001
Milk composition (lactose increase)	α-lactalbumin	genomic bovine α-lactalbumin	microinjection	pig	Wheeler *et al.* 2001
Influenza resistance	Mx protein	mMx1-Mx	microinjection	pig	Müller *et al.* 1992
Enhanced disease resistance	IgA	α,K-α,K	microinjection	pig, sheep	Lo *et al.* 1991
Wool growth	Insulin-like growth factor-1 (IGF-1)	Ker-IGF-1	microinjection	sheep	Damak *et al.* 1996*a,b*
Visna Virus resistance	Visna virus envelope	visna LTR-env	microinjection	sheep	Clements *et al.* 1994
Ovine prion locus	Prion protein (PrP)	targeting vector (homol. recomb.)	somatic cloning	sheep	Denning *et al.* 2001 (animals dead shortly after birth)
Milk fat composition	Stearoyl desaturase	β-lactogl-SCD	microinjection	goat	Reh *et al.* 2004
Milk composition (increase of whey proteins)	β-casein κ-casein	genomic CSN2 CSN-CSN-3	somatic cloning	cattle	Brophy *et al.* 2003
Milk composition (increase of lactoferrin)	human lactoferrin	α-s2cas-mLF	microinjection	cattle	Platenburg *et al.* 1994
Staph. aureus mastitis resistance	Lysostaphin	ovine β-lactogl-lysostaphin	somatic cloning	cattle	Wall *et al.* 2005

Table 2: Overview on successful transgenic livestock for agricultural production (from Niemann and Kues 2007)

9.4 Emerging transgenic technologies

9.4.1 Lentiviral transfection of oocytes and zygotes

Recent research has shown that lentiviruses can overcome previous limitations of viral-mediated gene transfer, which included the silencing of the transgenic locus and low expression levels (Wiznerowicz and Trono 2005). Injection of lentiviruses into the perivitelline space of porcine zygotes resulted in a very high proportion of piglets that carried and expressed the transgene. Stable transgenic lines have been established by this method (Hofmann et al. 2003). The generation of transgenic cattle by lentiviruses requires microinjection into the perivitelline space of oocytes and has a lower efficiency than that obtained in pigs (Hofmann et al. 2004). Lentiviral gene transfer in livestock promises unprecedented efficiency of transgenic animal production. It remains to be investigated whether the multiple integration of lentiviruses into the genome is associated with unwanted side-effects like oncogene activation or insertional mutagenesis.

9.4.2 Usage of pluripotent cells

Embryonic stem cells with pluripotent characteristics have the ability to participate in organ and germ cell development after injection into blastocysts or by aggregation with morulae (Rossant 2001). True ES cells (that is, those able to contribute to the germ line) are currently only available from inbred mouse strains (Kues et al. 2005b). In mouse genetics, ES cells have become an important tool for generating gene knockouts, and knockins and large chromosomal rearrangements (Downing and Battey 2004). Embryonic stem-like cells and primordial germ cell cultures have been reported for several farm animal species, and chimeric animals without germ line contribution have been reported in swine (Shim et al. 1997; Wheeler 1994; Anderson 1999) and cattle (Cibelli et al. 1998). Recent data indicate that somatic stem cells may have a much greater potency than previously assumed (Jiang et al. 2002; Kues et al. 2005a,b). Whether these cells will improve the efficiency of somatic nuclear transfer in farm animals has yet to be shown (Hornen et al. 2007).

9.4.3 Spermatogonial transgenesis

Transplantation of primordial germ cells into the testes is an alternative approach to generate transgenic animals. Initial experiments in mice showed that the depletion of endogenous spermatogenonial stem cells by treatment with the chemical busulfan prior to transplantation is effective and compatible with re-colonization by donor cells. Transmission of the donor haplotype to the next generation after germ-cell transplantation has been achieved in goats (Honaramooz et al. 2003). Current major obstacles of this strategy are the lack of efficient vitro culture methods for

primordial germ/prospermatogonial cells and the lack of efficient gene transfer techniques into these cells.

9.4.4 Conditional transgenesis in farm animals

Recently, we reported the first tetracycline-controlled transgene expression in a farm animal (Kues et al. 2006). An autoregulative tetracycline-responsive bicistronic expression cassette (NTA) was introduced into the pig genome via pronuclear DNA injection. The NTA cassette was designed to give ubiquitous expression of human RCAs independent of cellular transcription cofactors. Expression from this construct could be inhibited reversibly by feeding the animals doxycycline (tet-off system). In 10 transgenic pig lines carrying one NTA cassette, the transgene was silenced in all tissues with the exception of skeletal muscle, where the transgene was expressed in discrete skeletal muscle fibers (Niemann and Kues 2003). Interestingly, crossbreeding to produce animals with two NTA cassettes resulted in reactivation of the silenced NTA cassettes and a strong and broad tissue-independent, tetracycline-sensitive RCA expression pattern. The extent of reactivation in the double transgenic pigs correlated inversely with the methylation status of the NTA cassettes, and probably reflected effects of the different integration sites. This shows that selective crossbreeding of transgenic pig lines can overcome epigenetic silencing. This approach highlights the importance to study epigenetic (trans)-gene regulation in the pig.

9.4.5 Artificial chromosomes as transgene vectors

Artificial chromosomes have the potential to carry very large pieces of DNA that are maintained as episomal entities (Niemann and Kues 2003). A human artificial chromosome (HAC) containing the entire sequences of the human immunoglobulin heavy and light chain loci has been introduced into bovine fibroblasts, which were then used in nuclear transfer. Trans-chromosomal bovine offspring were obtained that expressed human immunoglobulin in their blood. This system could be a significant step forward in the production of human therapeutic polyclonal antibodies (Kuroiwa et al. 2002). Follow-up studies showed that the HAC was maintained over several years in the first generation cattle (Robl et al. 2007).

9.4.6 RNA interference mediated gene knock down

RNA interference is a conserved post-transcriptional gene regulatory process in most biological systems, incl. fungi, plants and animals. The common mechanistic elements are double stranded small interfering RNAs (siRNA) with 19-23 nucleotides, which specifically bind to complementary

sequences of their target mRNAs. The target mRNAs are then degraded by exonucleases and no protein is translated (Plasterk 2002). RNA interference is involved in gene regulation and specifically to control/suppress the translation of mRNAs from endogenous and exogenous viral elements and can be used for therapeutic purposes (Dallas and Vlassow 2006).

For a transient gene knock down, synthetic siRNAs are transfected in cells or early embryos (Clark and Whitelaw 2003; Iqbal et al. 2007). For stable gene repression the siRNA sequences must be incorporated into a gene construct. The combination of siRNA with the lentiviral vector technology is a highly effective tool in this respect. RNAi knockdown of PERV has been shown in porcine primary cells (Dieckhoff et al. 2006), as well as the knockdown of the prion protein gene (PRNP) in cattle embryos (Golding et al. 2006). Germline transmission of lentiviral siRNA has been shown in rats over 3 generations (Tenenhaus Dann et al. 2006). In contrast to knock out technologies, which require time consuming breeding strategies, RNAi can easily be integrated into existing breeding lines.

9.5 Health and welfare of transgenic farm animals

Due to integration and expression of transgenes, insertional mutagenesis and unwanted pathological side effects cannot be ruled out and concerns have been raised about the health of transgenic farm animals (Van Reenen et al. 2001). Transgenic farm animals have been produced since 1985 by pronuclear DNA injection, however only few systematic studies investigated the health status in cohorts of these animals. Over-expression of human growth hormone in pigs and sheep was associated with specific pathological phenotypes, which could be avoided by advanced constructs (Nottle et al. 1999). In pigs transgenic for human DAF, maintained at qualified pathogen free conditions, the haematology and blood chemistry, were normal compared to non-transgenic animals (Tucker et al. 2002). With the exception of a slightly exceeded growth rate, no abnormalities were found. A detailed pathomorphological examination of 9 lines of hemizygous pigs expressing human RCAs revealed no adverse effects correlating with transgene expression (Deppenmeier et al. 2006), providing clear evidence that transgenesis per se does not compromise animal health and welfare. Several of the investigated animals carried a bicistronic expression cassette, driving hCD59 and the tetracycline transactivator (Kues et al. 2006), suggesting that multi-transgenic animals are compatible with a normal health status. The hemizygous lines were fertile and produced normal litter sizes. However, attempts to breed these lines to homozygosity resulted in very small litter sizes in the first generation, likely reflecting inbreeding depression. In mice, several inbreeding rounds are necessary for extinction of a specific line (Taft et al. 2006).

High embryonic and fetal attrition rates of cloned animals are specifically observed in ruminants that seem to be particularly susceptible to epigenetic aberrations. Cardiopulmonary and placental lesions and lesions in the skeletal and muscle system have been observed in cloned cattle (Hill et al. 1999). The observed adverse effects seem to be related to the cloning procedure per se and not primarily to transgenic modification, as similar adverse effects are also seen in cloned cattle without genetic modification (Panarace et al. 2007). In contrast, transgenic cloned pigs which survived the neonatal period are apparently normal in all haematologial and biochemical parameters (Carter et al. 2002).

Studies with sufficient numbers of transgenic farm animals produced by emerging technologies, such as lentiviral and spermatogonial transgenesis, or expressing new constructs, like HAC and siRNA, will be necessary to assess the safety of these transgenic approaches.

9.6 Conclusions

Throughout history, animal husbandry has made significant contributions to human health and well-being. Recent advances in genomic technologies in farm animals present not only a major opportunity to address significant limitations in agricultural production, but also offer exciting prospects for medical research, significantly extending the important role of animals as models of human health and disease. Developments in animal genomics have broadly followed the route that the human genome field has led, both in terms of completeness of data and in developing tools and applications as demonstrated by the recent availability of advanced drafts of the maps of the bovine, chicken, dog and bee genomes. The possibility of extending knowledge through deliberately engineering change in the genome is unique to animals and is currently being developed through the integration of advanced molecular tools and breeding technologies. However, the full realization of this exciting potential is handicapped by gaps in our understanding of embryo genomics and the epigenetic changes which are critical in the production of healthy offspring.

The convergence of the recent advances in reproductive technologies with the tools of molecular biology opens a new dimension for animal breeding. Major prerequisites are the continuous refinement of reproductive biotechnologies and a rapid completion and refinement of livestock genomic maps. Embryonic stem cells (ES-cells) play a critical role in this context. Despite numerous efforts, no ES-cells with germ line contribution have been established from mammals other than mouse. ES-like cells, i.e. cells that share several parameters with true ES-cells, have been reported in several species. The time period over which these cells could be maintained in culture varied from 13 weeks to three years (see Gjorret and Maddox-Hyttel 2005). Derivation of true ES cells in farm

animals will permit to exploit the full power to recombinant DNA technology in animal breeding and will thus be critical to develop sustainable and diversified animal production systems.

Genetically modified animals will soon play a significant role in the biomedical field. Agricultural application might be further down the track given the complexity of some of the economically important traits. As the complete genomic sequences of all farm animals become available, it will be possible to refine targeted genetic modification in animal breeding and to develop strategies to cope with future challenges in global agricultural production.

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Niemann, H. and Kues, W.A. (2007). Transgenic farm animals – An update. Reprod. Fertil. Dev. 19, 762-770.

Niemann, H., Kues, W.A., and Carnwath, J.W. (2005). Transgenic farm animals: present and future. OIE-Report: Biotechnology applications in animal health and production, Revue scientifique et technique 24, 285-291.

Summary

Biotechnology is a well known and frequency term in the academic area, which have been coined in 1919 by Karoly Ereky. Biotechnology consists of eight disciplines- environmental, aquaculture, plant, food, industrial, molecular, medical studies and animal biotechnology. Animal biotechnology can be characterized as a set of special scientific techniques and engineering principles, which lead to production of the products derived from animals. Animal biotechnology has a long history, which began with domestification and artificial selection of animals. The advent of animal biotechnology has a potential to develop a variety of better or novel quality products for the treatment of a large number of diseases.

Knowledge gained through different disciplines of science as molecular biology, virology, transgenic DNA technology, lead to understand the mechanism of the biological processes and successful manipulations, which can find solutions for many prevailing and unresolved problems. For example the application of cloning techniques, genome manipulations and gene knockout strategies enhanced the production of transgenic animals, which represent source of human pharmaceuticals and proteins such as antibodies, clotting factor, enzymes etc.

This third edition of animal biotechnology was joint effort to produce a book to help students, teachers and instructors of animal biotechnology. The subject of this book includes practical application of animal genetic resources for isolation of pluripotent stem cell, xenotransplation, cloning and possible future of animal biotechnology. We are open to criticism, recommendations, suggestions that can help to improve the content and presentation of book and we hope that we will get support from readers of this book.

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References

Aasen, T. Raya, A. Barrero, M.J., Garreta,E., Consiglio, A., Gonzalez, F., Vassena, R., Bili, J., Pekarik, V., Tiscornia, G., Edel, M., Boué, S., Belmonte, J.C. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat.Biotechnol. 26, 1276–1284.

Adams DH, Kadner A, Chen RH, Farivar RS. Human membrane cofactor protein (MCP, CD 46) protects transgenic pig hearts from hyperacute rejection in primates. Xenotransplantation. 2001: 8: 36-40.

Alberio, R., Croxall, N., Allegrucci, C. 2010. Pig Epiblast Stem Cells Depend on Activin/Nodal Signaling for Pluripotency and Self Renewal. Stem Cells Dev. (ahead of print. doi:10.1089/scd.2010.0012).

Amariglio, N., Hirshberg, A., Scheithauer, B., Cohen, Y., Loewenthal, R., Trakhtenbrot, L., Paz, N., Koren-Michowitz, M., Waldman, D., Leider-Trejo, L., Toren, A., Constantini, S., Rechavi, G. 2009. Donor-derived brain tumor folowing neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med. 6(2):e1000029.

Anderson, G.B. (1999). Embryonic stem cells in agricultural species. In Transgenic Animals in Agriculture (J.D. Murray, G.B. Anderson, A.M. Oberbauer & M.M. McGloughlin eds), CABI Publ., New York, USA, pp 57-66.

Andrews, P. W., Damjanov, I., Simon, D., Banting, G. S., Carlin, C., Dracopoli, N. C., Fogh, J. (1984). Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Lab. Invest. 50, 147-162.

Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E, de Paz P. Improvement Strategies in Ovine Artificial Insemination. Reprod Dom Anim 2006,41(Suppl. 2),30–42.

Anrather J, Csizmadia V, Brostjan C, Soares MP, Bach FH, Winkler H. Inhibition of bovine endothelial cell activation in vitro by regulated expression of a transdominant inhibitor of NF-kappa B. The Journal of clinical investigation. 1997: 99: 763-772.

Armstrong DT, Evans G. Intrauterine insemination enhances fertility of frozen semen in superovulated ewes. J Reprod Fertil 1984,71,89-94.

Armstrong, R., Tyers, P., Jain, M., Richards, A., Dunnett, S., Rosser, A., Barker, R. 2003. Transplantation of expanded neural precursor cells from the developing pig ventral mesencephalon in a rat model of Parkinson's disease. Exp. Brain Res. 151:204-217.

Baguisi, A., Behboodi, E., Melican, D., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.A., Godke, R.A., Gavin, W.G., Overström, E.W., and Echelard, Y. (1999). Pro-duction of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17, 456-461.

Bach FH, Blakely ML, Van Der Werf WJ, Vanhove B, Stuhlmeier K, De Martin R, et al. Xenotransplantation: problems posed by endothelial cell activation. Transplant Proc. 1994: 26: 1029-1030.

Bach FH, Hancock WW, Ferran C. Protective genes expressed in endothelial cells: a regulatory response to injury. Immunology today. 1997: 18: 483-486.

Bach FH, Robson SC, Wrighton CJ, Stuhlmeier K, Ferran C, Winkler H. Genetic engineering of endothelial cells to ameliorate xenograft rejection. Clin Transplant. 1996: 10: 124-127.

Baldan N, Rigotti P, Calabrese F, Cadrobbi R, Dedja A, IACOPETTI I, et al. Ureteral stenosis in HDAF pig-to-primate renal xenotransplantation: a phenomenon related to immunological events? Am J Transplant. 2004: 4: 475-481.

Baldassarre H, Rao KM, Neveu N, Brochu E, Begin I, Behboodi E, Hockley DK. Laparoscopic ovum pick-up followed by in vitro embryo production for the reproductive rescue of aged goats of high genetic value. Reprod Fertil Dev 2007,19,612–616.

Baldassarre H, Wang B, Kafidi N, Keefer C, Lazaris A, Karatzas CN. Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and in vitro embryo production technologies. Theriogenology 2002,57,276-264.

Banz Y, Rieben R. Endothelial cell protection in xenotransplantation: looking after a key player in rejection. Xenotransplantation. 2006: 13: 19-30.

Bartosch B, Stefanidis D, Myers R, Weiss R, Patience C, Takeuchi Y. Evidence and consequence of porcine endogenous retrovirus recombination. J Virol. 2004: 78: 13880-13890.

Becker F, Kanitz W, Nurnberg G, Kurth J, Spitschak M. Comparison of repeated transvaginal ovum pick up in heifers by ultrasonographic and endoscopic instruments. Theriogenology 1996,46,999-1007.

Besenfelder U, Brem G. Laparoscopic embryo transfer in rabbits. J Reprod Fertil 1993,99,53–56.

Besenfelder U, Brem G. Tubal transfer of bovine embryos: a simple endoscopic method reducing long term exposure of in vitro produced embryos. Theriogenology 1998,50,739–45.

Besenfelder U, Haas C, Brem G. Reproduction technology and gene transfer in rabbits. J World Rabbit Sci Assoc 2000,8(Suppl. 1),37–59.

Besenfelder U, Havlicek V, Kuzmany A, Brem G. Endoscopic approaches to manage in vitro and in vivo embryo development: Use of the bovine oviduct. Theriogenology 2010,73,768–776.

Besenfelder U, Havlicek V, Moesslacher G, Gilles M, Tesfaye D, Griese J, et al. Endoscopic recovery of early preimplantation bovine embryos: effect of hormonal stimulation, embryo kinetics and repeated collection. Reprod Dom Anim 2008,43,566–72.

Besenfelder U, Házas G, Gyöker E, Gergátz E, Brem G. Laparoscopic embryo transfer into the fallopian tube in sheep. Theriogenology 1994a,41,162.

Besenfelder U, Mödl J, Müller M, Brem G. Endoscopic embryo collection and embryo transfer into the oviduct and the uterus of pigs. Theriogenology, 1997,47(5),1051-1060.

Besenfelder U, Müller M, Brem G. Transgenics and Modern Reproductive Technologies. In: GENETICS OF THE PIG (Eds.: Rothschild, M.F. and Ruvinsky, A.): Cab International, Wallingford, U.K, 1998a,345- 374.

Besenfelder U, Strouhal C, Brem G. A method for endoscopic embryo collection and transfer in the rabbit. J Vet Med A 1998b,45,577–579.

Besenfelder U, Zinovieva N, Dietrich E, Sohnrey B, Holtz W, Brem G. Tubal transfer of goat embryos using endoscopy. Vet Rec 1994b,135,480–481.

Betteridge KJ. A history of farm animal embryo transfer and some associated techniques. Anim Reprod Sci 2003,79,203–244.

Bhatti FN, Schmoeckel M, Zaidi A, Cozzi E, Chavez G, Goddard M, et al. Three-month survival of HDAFF transgenic pig hearts transplanted into primates. Transplant Proc. 1999: 31: 958.

Biernaskie, J., McKenzie, I., Toma, J., Miller, F. 2006. Isolation of skin-derived precursors (SKPs) and differentiation and enrichment of their Schwann cell progeny. Nat. Protoc. 1:2803-2812.

Bjarkam, C., Nielsen, M., Glud, A., Rosendal, F., Mogensen, P., Bender, D., Doudet, D., Moller, A., Sorensen, J. 2008. Neuromodulation in a minipig MPTP model of Parkinson disease. Br. J. Neurosurg. 22 Suppl 1:S9-12.

Blum-Reckow B, Holtz W. Transfer of porcine embryos after 3 days of in vitro culture. J Anim Sci 1991,69,3335-3342.

Bolamba D, Matton P, Sirard MA, Estrada R, Dufour JJ. Ovarian morphological conditions and the effect of injection of human chorionic gonadotropin on ovulation rates in prepuberal gilts with two morphologically different ovarian types. J Anim Sci 1991, 69,3774-3779.

Boland, M.J., Hazen, J.L., Nazor, K.L., Rodriguez, A.R., Gifford, W., Martin, G., Kupriyanov, S., Baldwin, K.K. (2009). Adult mice generated from induced pluripotent stem cells. Nature 461, 91–94.

Bolet G, Brun JM, Monnerot M, Abeni F, Arnal C, Arnold J, Bell D, Bergoglio G, Besenfelder U, Bosze S, Boucher S, Chanteloup N, Ducourouble MC, Durand-Tardif M, Esteves PJ, Ferrand N, Gautier A, Haas C, Hewitt G, Jehl N, Joly T, Koehl PF, Laube T, Lechevestrier S, Lopez M,

Masoero G, Menigoz JJ, Piccinin R, Queney G, Saleil G, Surridge A, Van Der Loo W, Vicente JS, Viudes De Castro MP, Virag JS, Zimmermann JM. Evaluation and conservation of european rabbit (oryctolaguscuniculus) genetic resources. First results and inferences. 7th World Rabbit Congress, Valencia. J Rabbit Sci Assoc 2000,8(Suppl. 1),281–315.

Brambrink, T., Foreman, R., Welstead, G.G., Lengner, C.J., Wernig, M., Suh, H., Jaenisch, R. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell 2, 151–159.

Brons, G.M., Smithers, L., Trotter, M.W.B., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448, 191-195.

Brons, I., Smithers, L., Trotter, M., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S., Howlett, S., Clarkson, A., Ahrlund-Richter, L., Pedersen, R., Vallier, L. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448:191-195.

Brophy, B., Smolenski, G., Wheeler, T., Wells, D., L'Huillier, P., and Laible, G. (2003). Cloned transgenic cattle produce milk with higher levels of b-casein and k-casein. Nat. Biotechnol. 21, 157-162.

Brüssow K-P, Ratky J. Endoscopic collection of porcine embryos. Reprod Dom Anim 1996,31,711–715.

Brüssow KP, Torner H, Rátky J, Hunter MG, Nürnberg G. Ovum pick up in swine: the influence of aspiration vacuum pressure on oocyte recovery from preovulatory follicles. Acta Vet Hung. 1997,45(2),189-196.

Byrne GW, Mccurry KR, Martin MJ, Mcclellan SM, Platt JL, Logan JS. Transgenic pigs expressing human CD59 and decay-accelerating factor produce an intrinsic barrier to complement-mediated damage. Transplantation. 1997: 63: 149-155.

Byrne GW, Schirmer JM, Fass DN, Teotia SS, Kremers WK, XU H, et al. Warfarin or low-molecular-weight heparin therapy does not prolong pig-to-primate cardiac xenograft function. Am J Transplant. 2005: 5: 1011-1020.

Calvano CJ, Moran ME, Mehlhaff BA, Reddy PP, Mandell J. Minimally Traumatic Techniques for In Utero Access and Fetal Surgery. JSLS 1998,2,227-233.

Cameron RD, Durack M, Fogarty R, Putra DK, McVeigh J. Practical experience with commercial embryo transfer in pigs. Aust Vet J 1989,66(10),314-318.

Camp JC, Wildt DE, Howard PK, Stuart LD, Chakraborty PK. Ovarian activity during normal and abnormal length estrous cycles in the goat. Biol Reprod 1983,28,673-681.

Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med. 2006: 12: 304-306.

Carter, B.D., Lai, L., Park, K.W., Samuel, M., Lattimer, J.C., Jordan, K.R., Estes, D.M., Besch-Williford, C., and Prather, R. (2002). Phenotyping of transgenic cloned pigs. Cloning Stem Cells 4, 131-145.

Cibelli, J.B., Stice, S.L., Golueke, P.L., Kane, J.J., Jerry, J., Blackwell, C., Ponce de León, F.A., and Robl, J.M. (1998): Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat. Biotechnol. 18, 642-646.

Clark, J., and Whitelaw, B. (2003). A future for transgenic livestock. Nat. Rev. Genet. 4, 825-833.

Clements, J.E., Wall, R.J., Narayan, O., Hauer, D., Schoborg, R., Sheffer, D., Powell, A., Carruth, L.M., Zink, M.C., and Rexroad, C.E. (1994). Development of transgenic sheep that express the visna virus envelope gene. Virology 200, 370-380.

Cognié Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. Theriogenology 2003,59(1),171-188.

Cooper JT, Stroka DM, Brostjan C, Palmetshofer A, Bach FH, Ferran C. A20 blocks endothelial cell activation through a NF-kappaB-dependent mechanism. J Biol Chem. 1996: 271: 18068-18073.

Copley M.S., Berstan R., Dudd S.N., Docherty G., Mukherjee A.J., Straker V., Payne S., and Evershed R.P. (2003). Direct chemical evidence for widespread dairying in prehistoric Britain. Proc. Natl. Acad. Sci. USA 100, 1524-1529.

Costa C, Zhao L, Burton WV, Bondioli KR, Williams BL, Hoagland TA, et al. Expression of the human alpha1,2-fucosyltransferase in transgenic pigs modifies the cell surface carbohydrate phenotype and confers resistance to human serum-mediated cytolysis. Faseb J. 1999: 13: 1762-1773.

Cowan PJ, Aminian A, Barlow H, Brown AA, Dwyer K, Filshie RJ, et al. Protective effects of recombinant human antithrombin III in pig-to-primate renal xenotransplantation. Am J Transplant. 2002: 2: 520-525.

Cowan PJ, Chen CG, Shinkel TA, Fisicaro N, Salvaris E, Aminian A, et al. Knock out of alpha1,3-galactosyltransferase or expression of alpha1,2-fucosyltransferase further protects CD55- and CD59-expressing mouse hearts in an ex vivo model of xenograft rejection. Transplantation. 1998: 65: 1599-1604.

Cowan, C. A., Atienza, J., Melton, D. A., Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309, 1369-1373.

Cozzi E, Simioni P, Boldrin M, Seveso M, Calabrese F, Baldan N, et al. Effects of long-term administration of high-dose recombinant human antithrombin in immunosuppressed primate recipients of porcine xenografts. Transplantation. 2005: 80: 1501-1510.

Cozzi E, Vial C, Ostlie D, Farah B, Chavez G, Smith KG, et al. Maintenance triple immunosuppression with cyclosporin A, mycophenolate sodium and steroids allows prolonged survival of primate recipients of hDAF porcine renal xenografts. Xenotransplantation. 2003: 10: 300-310.

Cozzi E, White DJ. The generation of transgenic pigs as potential organ donors for humans. Nat Med. 1995: 1: 964-966.

Dai, Y., Vaught, T.D., Boone, J., Chen, S.H., Phelps, C.J., Ball, S., Monahan, J.A., Jobst, P.M., McCreath, K.J., Lamborn, A.E., Cowell-Lucero, J.L., Wells, K.D., Colman, A., Polejaeva, I.A., and Ayares, D.L. (2002). Targeted disruption of the □1,3-galactosyltransferase gene in cloned pigs. Nat. Biotechnol. 20, 251-255.

Dallas, A., and Vlassow, A. (2006). RNAi: A novel antisense technology and its therapeutic potential. Med. Sci. Monit.12, RA67-74.

Damak, S., Jay, N.P., Barrell, G.K., and Bullock, D.W. (1996a). Targeting gene expression to the wool follicle in transgenic sheep. Biotechnology (NY) 14, 181-184.

Damak, S., Su, H., Jay, N.P., and Bullock, D.W. (1996b). Improved wool production in transgenic sheep expressing insulin-like growth factor 1. Biotechnology (NY) 14, 185-188.

Davoli R. - Braglia S.: Molecular approaches in pig breeding to improve meat quality. Brief. Funct. Genomic Proteomic. 6(4), 2007, p. 313-21.

De Oliveira ATD, Lopes RFF, Rodrigues JL. Gene expression and developmental competence of bovine embryos produced in vitro with different serum concentrations. Reprod Dom Anim 2006,41,129–136.

Dekkers J.C.: Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. J. Anim. Sci. 82, 2004, E313-E328.

Denner J, Specke V, Schwendemann J, Tacke SJ. Porcine endogenous retroviruses (PERVs): adaptation to human cells and attempts to infect small animals and non-human primates. Ann Transplant. 2001: 6: 25-33.

Denning, C., Burl, S., Ainslie, A., Bracken, J., Dinnyes, A., Fletcher, J., King, T., Ritchie, M., Ritchie, W.A., Rollo, M., de Sousa, P., Travers, A., Wilmut, I., and Clark, A.J. (2001). Deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat. Biotechnol. 19, 559-562.

Deppenmeier, S., Bock, O., Mengel, M., Niemann, H., Kues, W., Lemme, E., Wirth, D, Wonigeit, K., and Kreipe, H. (2006). Health status of transgenic pig lines expressing human complement regulator protein CD59. Xenotransplantation 13, 345-356.

Dieckhoff B, Karlas A, Hofmann A, Kues WA, Petersen B, Pfeifer A, et al. Inhibition of porcine endogenous retroviruses (PERVs) in primary porcine cells by RNA interference using lentiviral vectors. Archives of virology. 2007: 152: 629-634.

Dieckhoff, B., Karlas, A., Hofmann, A., Kues, W. A., Petersen, B., Pfeifer, A., Niemann, H., Kurth, R., and Denner, J. (2006). Inhibition of porcine endogenous retroviruses (PERV) in primary porcine cells by RNA interference using lentiviral vectors. Arch. Virol. 152, 629-634.

Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim 2008,43(Suppl 2),260–267.

Dominguez-Sola, D., Ying, C.Y. , Grandori, C., Ruggiero, L., Chen, B., Li, M., Galloway, D.A. , Gu, W., Gautier, J., Dalla-Favera, R. (2007). Non-transcriptional control of DNA replication by c-Myc. Nature, 448, 445–451.

Downing GJ, and Battey JF Jr. (2004). Technical assessment of the first 20 years of research using mouse embryonic stem cell lines. Stem Cells 22, 1168-1180.

Driancourt MA, Disenhaus C. Lack of effects of growth hormone administration on ovarian function of lactating goats. Animal Reproduction Science 1997,46,123-132.

Durcova-Hills G., Adams, I., Barton, S., Surani, A., McLarren, A. 2006. The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. Stem Cells 24:1441-1449.

Durcova-Hills, G., Tang, F., Doody, G., Tooze, R., Surani, A. 2008. Reprogramming Primordial Germ Cells into Pluripotent Stem Cells. PLoS ONE 3(10):e3531.

Dwyer KM, Mysore TB, Crikis S, Robson SC, Nandurkar H, Cowan PJ, et al. The transgenic expression of human CD39 on murine islets inhibits clotting of human blood. Transplantation. 2006: 82: 428-432.

Eggen A.: The development and application of genomic selection as a new breeding paradigm. Animal Frontiers 2(1), 2012, p. 10-15.

Ehling C,Wirth P, Schindler L, Hadeler KG, Döpke HH, Lemme E, Herrmann D, Niemann H. Laparoscopical intrauterine insemination with different doses of fresh, conserved, and frozen semen for the production of ovine zygotes. Theriogenology 2003,60,777–787.

Ellington JE, Farrell PB, Simkin ME, Foote RH, Goldman EE, McGrath AB. Development and survival after transfer of cow embryos cultured from 1-2-cells to morulae or blastocysts in rabbit

oviducts or in a simple medium with bovine oviduct epithelial cells. J Reprod Fertil 1990,89,293-299.

Eminli, S., Foudi, A., Stadtfeld, M., Maherali, N., Ahfeldt, T., Mostoslavsky, G., Hock, H., Hochedlinger, K. (2009). Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. Nat. Genet. 41, 968–976.

Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP. Culture of in vitro produced bovine zygotes in vitro vs in vivo: Implications for early embryo development- and quality. Theriogenology 2000,54,659-673.

Esmon CT. The protein C pathway. Chest. 2003: 124: 26S-32S.

Esteban, M., Xu, J., Yang, J., Peng, M., Qin, D., Li, W., Jiang, Z., Chen, J., Deng, K., Zhong, M., Cai, J., Lai, L., Pei, D. 2009. Generation of induced pluripotent stem cell lines from tibetan miniature pig. J. Biol. Chem. 284(26):17634–17640.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154-156.

Evans, M., Notarianni, E., Laurie, S., Moor, R. 1990. Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology 33:125-128.

Eyestone WH, First NL. Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. J Reprod Fertil 1989,85,715-720.

Ezashi, T., Telugu, B., Alexenko, A., Sachdev, S., Sinha, S., Roberts, M. 2009. Derivation of induced pluripotent stem cells from pig somatic cells. PNAS 106:10993–10998.

Faillace LS, Biggs C, Hunter MG. Factors affecting the age at onset of puberty, ovulation rate and time of ovulation in Chinese Meishan gilts.J Reprod Fertil 1994,100,353-357.

Fandrich F, Lin X, Chai GX, Schulze M, Ganten D, Bader M, et al. Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. Nat Med. 2002: 8: 171-178.

Farin CE, Farmer WT, Farin PW. Pregnancy recognition and abnormal offspring syndrome in cattle. J Reprod Fertil Dev, 2010,22,75–87.

Farin PW, Farin CE. Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 1995,52,676-682.

Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006,65,178–191.

Fayrer-Hosken RA, Caudle AB. The laparoscope in follicular oocyte collection and gamete intrafallopian transfer and fertilization (GIFT). Theriogenology 1991,36(5)709-725.

Fayrer-Hosken RA, Younls AI, Brackett BG, McBride CE, Harper KM, Keefer KL, Cabaniss DC. Laparoscopic oviductal transfer of in vitro matured and in vitro fertilized bovine oocytes. Theriogenology 1989,32(3),413-420.

Fazeli A. Maternal communication with gametes and embryos. Theriogenology 2008,70,1182–1187.

Fujimoto S, Rawson JMR, Dukelow WR. Hormonal influences on the time of ovulation in the rabbit as determined by laparoscopy. J Reprod Fertil 1974,38,97-103.

Fukui Y, Fukushima M, Ono H. Fertilization and cleavage of bovine follicular oocytes in rabbit reproductive tracts after maturation in vitro. J Exp Zool. 1983,226(1),137-142.

Galli C, Crotti G, Notari C,Turini P,Duchi R, Lazzari G. Embryo production by ovum pick up from live donors. Theriogenology 2001,55,1341-1357.

Gambiez L, Salame E, Chereau C, Calmus Y, Cardoso J, Ayani E, et al. The role of natural IgM in the hyperacute rejection of discordant heart xenografts. Transplantation. 1992: 54: 577-583.

Gandolfi, F. (1998). Spermatozoa, DNA binding and transgenic animals. Transgenic Res. 7, 147-155.

Garcia EM, Vazquez JM, Parrilla I, Calvete JJ, Sanz L, Caballero I, Roca J, Vazquez JL, Martinez EA. Improving the fertilizing ability of sex sorted boar spermatozoa. Theriogenology 2007,68,771–778.

Garcia-Ximenez F, Vicente JS, Santacreu MA. Embryo transfer in lactating rabbit does by laparoscopy. Anim Reprod Sci 1991,24,343-346.

Garrels W, Ivics Z, Kues WA. Precision genetic engineering in large mammals. Trends Biotechnol. 2012.

Garrels W, Mates L, Holler S, Dalda A, Taylor U, Petersen B, et al. Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLoS One. 2011: 6: e23573.

Ghanekar A, Mendicino M, Liu H, He W, Liu M, Zhong R, et al. Endothelial induction of fgl2 contributes to thrombosis during acute vascular xenograft rejection. J Immunol. 2004: 172: 5693-5701.

Gjorret, J.O. and Maddox-Hyttel, P. (2005). Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures. Reprod. Fert. Dev. 17, 113-124.

Go, T., Jungebluth, P., Baiguero, S., Asnaghi, A., Martorell, J., Ostertag, H., Mantero, S., Birchall, M., Bader, A., Macchiarini, P. 2010. Both epithelial cells and mesenchymal stem cell-derived chondrocytes contribute to the survival of tissue-engineered airway transplants in pigs. J. Thorac. Cardiovasc. Surg. 139:437-443.

Golding, M.C., Long, C.R., Carmell, M.A., Hannon, G.J., and Westhusin, M.E. (2006). Suppression of prion protein in livestock by RNA interference. Proc. Natl. Acad. Sci. USA 103, 5285-5290.

Golovan, S.P., Meidinger, R.G., Ajakaiye, A., Cottrill, M., Wiederkehr, M.Z., Barney, D.J., Plante, C., Pollard, J.W., Fan, M.Z., Hayes, M.A., Laursen, J., Hjorth, J.P., Hacker, R. R., Phillips, J.P., and Forsberg, C.W. (2001). Pigs expressing salivary phytase produce low-phosphorus manure. Nat. Biotechnol. 19, 741-745.

Gossler, A., Doetschman, T., Korn, R., Serfling, E., Kemler, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc. Natl. Acad. Sci. 83, 9065–9069.

Gourley DD, Riese RL. Laparoscopic artificial insemination in sheep. Vet Clin North Am Food Anim Pract. 1990,6(3),615-33.

Greber, B., Wu, G., Bernemann, C., Joo, J., Han, D., Ko, K., Tapia, N., Sabour, D., Sterneckert, J., Tesar, P., Scholer, H. 2010. Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. Cell Stem Cell 6:215-226.

Greve T, Callesen H. Rendez-vous in the oviduct: Implications for superovulation and embryo transfer. Biol Reprod 2002,66,629–34.

Gurdon, J. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J. Embryol. Exp. Morphol. 10, 622-640.

Gutt CN, Oniu T, Schemmer P, Mehrabi A, Büchler WM. Fewer adhesions induced by laparoscopic surgery? Surg Endosc 2004,18, 898–906.

Hafez ESE. Endocrine control of reception, transport, development and loss of rabbit ova. J Reprod Fertil 1962,108,237-353.

Halbert GW, Dobson H, Walton JS, Buckrell BC. The structure of the cervical canal of the ewe. Theriogenology 1990, 33(5),977-992.

Haley C. – Archibald A.: Progress in pig gene mapping. In: Wiseman J. – Varley M. A. – Chadwick J. P., Progress in pig science. Univ. Press, Nottingham 1998, p. 5-27.

Hall, V. 2008. Porcine embryonic stem cells: a possible source for cell replacement therapy. Stem Cell Rev. 4:275-282.

Hall, V., Christensen J, Gao Y, Schmidt MH, Hyttel P. 2009. Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. Dev. Dyn. 238:2014-2024.

Hall, V., Kristensen, M., Rasmussen, M., Ujhelly, O., Dinnyés, A. and Hyttel, P. (2012): Temporal repression of endogenous pluripotency genes during reprogramming of porcine induced pluripotent stem cells. Cell. Reprog. 3:1-13.

Hammer, R.E., Pursel, V.G., Rexroad, C.E. Jr., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D., and Brinster, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315, 680-683.

Hanna, J., Cheng, A.W., Saha, K., Kim, J., Lengner, C.J., Soldner, F., Cassady, J.P., Muffat, J., Carey, B.W., Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. 107, 9222-9227.

Hanna, J., Markoulaki, S., Mitalipova, M., Cheng, A.W., Cassady, J.P., Staerk, J., Carey, B.W., Lengner, C.J., Foreman, R., Love, J.,Gao, Q., Kim, J., Jaenisch, R. (2009). Metastable pluripotent states in NOD-mouse-derived ESCs. Cell Stem Cell 4, 513–524.

Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine,E.J., Cassady, J.P., Foreman, R., Lengner, C.J., Dausman, J.A., Jaenisch, R. (2008). Direct Reprogramming of Terminally Differentiated Mature B Lymphocytes to Pluripotency. Cell 133, 250–264.

Hansen PJ, Block J. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production.J Reprod Fertil Dev 2004,16,1-14.

Harrison I, Takeuchi Y, Bartosch B, Stoye JP. Determinants of high titer in recombinant porcine endogenous retroviruses. J Virol. 2004: 78: 13871-13879.

Harrison SJ, Guidolin A, Faast R, Crocker LA, GIANNAKIS C, D'APICE AJ, et al. Efficient generation of alpha(1,3) galactosyltransferase knockout porcine fetal fibroblasts for nuclear transfer. Transgenic Res. 2002: 11: 143-150.

Harrower, T., Tyers, P., Hooks, Y., Barker, R. 2006. Long-term survival and integration of porcine expanded neural precursor cell grafts in a rat model of Parkinson's disease. Exp. Neurol. 197:56-69.

Haskell, R.E., and Bowen, R.A. (1995). Efficient production of transgenic cattle by retroviral infection of early embryos. Mol. Reprod. Dev. 40, 386-390.

Hasler JF. The Holstein cow in embryo transfer today as compared to 20 years ago. Theriogenology 2006,65,4–16.

Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, Lucas-Hahn A, et al. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proc Natl Acad Sci U S A. 2011: 108: 12013-12017.

Havenstein, G. B., Ferket, P.R. & Qureshi, M.A. (2003). Carcass Composition and Yield of 1957 Versus 2001 Broilers When Fed Representative 1957 and 2001 Broiler Diets. Poultry Science, 82, 1509–1518.

Havlicek V, Lopatarova M, Cech S, Dolezel R, Huber T, Pavlok A, et al. In vivo culture of bovine embryos and quality assessment of in vivo vs. in vitro produced embryos. Vet Med–Czech 2005b,50,149–57.

Havlicek V, Wetscher F, Huber T, Brem G, Müller M, Besenfelder U. In vivo culture of IVM/IVF embryos in bovine oviducts by transvaginal endoscopy. J Vet Med A 2005a,52,94–98.

Hazeleger W. Non-surgical embryo transfer in pigs. PhD Thesis, Wageningen University, Department of Animal Sciences, The Netherlands, ISBN, 90-5808-072-2.

Henderson, J., Draper, J., Baillie, H., Fishel, S., Thomson, J., Moore, H., Andrews, P. 2002. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20:329-337.

Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, JIE T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med. 2006: 12: 301-303.

Hill, J.R., Roussel, A.J., Cibelli, J.B., Edwards, J.F., Hooper, N.L., Miller, M.W., Thompson, J.A., Looney, C.R., Westhusin, M.E., Robl, J.M., and Stice, S.L. (1999). Clinical and pathologic features of cloned transgenic calves and fetuses (13 case study). Theriogenology 51, 1451-1465.

Hofmann, A., Kessler, B., Ewerling, S., Weppert, M., Vogg, B., Ludwig, H., Stojkovic, M., Boelhauve, M., Brem, G., Wolf, E., and Pfeifer, A. (2003). Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep. 4, 1054-1060.

Hofmann, A., Zakhartchenko, V., Weppert, M., Sebald, H., Wenigerkind, H., Brem, G., Wolf, E., and Pfeifer, A. (2004). Generation of transgenic cattle by lentiviral gene transfer into oocytes. Biol. Reprod. 71, 405-409.

Hochedlinger, K. (2008). Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell 2, 230–240.

Honaramooz, A., Behboodi, E., Megee, S.O., Overton, S.A., Galantino-Homer, H., Echelard, Y., and Dobrinski, I. (2003). Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol. Reprod. 69, 1260-1264.

Hooker, C. W. and Hurlin, P. J. (2006). Of Myc and Mnt. J. Cell Sci. 119, 208–216.

Hornen, N., Kues, W.A., Carnwath, J.W., Lucas-Hahn, A., Petersen, B., Hassel, P., and Niemann, H. (2007). Production of viable pigs from fetal somatic stem cells. Cloning Stem Cells 9, 364-373.

Houser SL, Kuwaki K, Knosalla C, Dor FJ, Gollackner B, Cheng J, et al. Thrombotic microangiopathy and graft arteriopathy in pig hearts following transplantation into baboons. Xenotransplantation. 2004: 11: 416-425.

Hua, J. 2009. Derivation and characterization of human embryonic germ cells: serum-free culture and differentiation potential. Reprod. Biomed. Online 19:238-249.

Huang, Y.J., Huang, Y., Baldassarre, H., Wang, B., Lazaris, A., Leduc, M., Bilodeau, A.S., Bellemare, A., Cote, M., Herskovits, P., Touati, M., Turcotte, C., Valeanu, L., Lemee, N.,

Wilgus, H., Begin, I., Bhatia, B., Rao, K., Neveu, N., Brochu, E., Pierson, J., Hockley, D.K., Cerasoli, D.M., Lenz, D.E., Karatzas, C.N., and Langermann, S. (2007). Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning. Proc. Natl. Acad. Sci. U.S.A. 104, 13603-13608.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E., Melton, D.A. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat. Biotechnol. 26, 795–797.

Humblot P. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 2001,56,1417–1433.

Hunter RHF, Bogh IB, Einer-Jensen N, Müller S, Greve T. Pre-ovulatory Graafian follicles are cooler than neighbouring stroma in pig ovaries. Hum Reprod 2000,15(2)273-283.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., Smith, E. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113, 643–655.

Chan, A.W., Homan, E.J., Ballou, L.U., Burns, J.C., and Bremel, R.D. (1998). Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. Proc. Natl. Acad. Sci. USA 95, 14028-14033.

Chang MC. Development and fate of transferred rabbit ova or blastocysts in relation to the ovulation time of recipients. J Exp Zool 1950,114,197-216.

Chang, K., Qian, J., Jiang, M., Liu, Y.H., Wu, M.C., Chen, C.D., Lai, C.K., Lo, H.L., Hsiao, C.T., Brown, L., Bolen, J., Huang, H.I, Ho, P.Y., Shih, P.Y., Yao, C.W., Lin, W.J., Chen C.H., Wu, F.Y., Lin, Y.J., Xu, J.X. and Wang, K. (2002). Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. BMC Biotechnol 2, 5; http://www.biomedcentral.com/1472-6750/2/5.

Chen G, Qian H, Starzl T, Sun H, Garcia B, Wang X, et al. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. Nat Med. 2005: 11: 1295-1298.

Chen G, Sun H, Yang H, Kubelik D, Garcia B, Luo Y, et al. The role of anti-non-Gal antibodies in the development of acute humoral xenograft rejection of hDAF transgenic porcine kidneys in baboons receiving anti-Gal antibody neutralization therapy. Transplantation. 2006: 81: 273-283.

Chen, L., Shiue, Y., Bertolini, L., Medrano, J., BonDurant, R., Anderson, G. 1999. Establishment of pluripotent cell lines from porcine preimplantation embryos. Theriogenology 52:195-212.

Christensen, L.G. (1998). Future Market and Consumer-Orientated Breeding Goals. Acta Agriculturae Scandinavian, Section A, Animal Science, 28, 45–53.

Ierino FL, Kozlowski T, Siegel JB, Shimizu A, Colvin RB, Banerjee PT, et al. Disseminated intravascular coagulation in association with the delayed rejection of pig-to-baboon renal xenografts. Transplantation. 1998: 66: 1439-1450.

Iqbal, K., Kues, W.A., and Niemann, H. (2007). Parent-of-origin dependent gene-specific knock down in mouse embryos. Biochem. Biophys. Res. Commun. 358, 727-732.

Ivics Z, Katzer A, Stuwe EE, Fiedler D, Knespel S, Izsvak Z. Targeted Sleeping Beauty transposition in human cells. Mol Ther. 2007: 15: 1137-1144.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 447, 879-880.

Jillella D, Eaton RJ, Baker AA. Successful transfer of a bovine embryo through a cannulated fallopian tube. Vet Rec. 1977,100(18),385-386.

Johnson LA & Welch GR 1999 Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 52 1323-1341.

Johnson LA, Welch GR & Rens W 1999 The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci 77 Suppl 2 213-220.

Jung, M., Kaszap, B., Redohl, A., Steck, E., Breusch, S., Richter, W., Gotterbarm, T. 2009. Enhanced early tissue regeneration after matrix-assisted autologous mesenchymal stem cell transplantation in full thickness chondral defects in a minipig model. Cell Transplant. 18:923-932.

Jurd KM, Gibbs RV, Hunt BJ. Activation of human prothrombin by porcine aortic endothelial cells--a potential barrier to pig to human xenotransplantation. Blood Coagul Fibrinolysis. 1996: 7: 336-343.

Kaabi M, Alvarez M, Anel E, Chamorro CA, Boixo JC, de Paz P, Anel L. Influence of breed and age on morphometry and depth of inseminating catheter penetration in the ewe cervix: A postmortem study. Theriogenology 2006,66,1876–1883.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., Toyoshima, M., Niwa, O., Oshimura, M., Heike, T., Nakahata, T., Ishino, F., Ogura, A., Shinohara, T. (2004). Generation of pluripotent stem cells from neonatal mouse testis. Cell 119, 1001–1012.

Karasiewicz, J., Sacharczuk, M., Was, B., Guszkiewicz, A., Korwin-Kossakowski, M., Gorniewska, M., Szablisty, E., Modlinski, J. 2008. Experimental embryonic-somatic chimaerism in the sheep confirmed by random amplified polymorphyc DNA assay. Int. J. Dev. Biol. 52:315-322.

Keefer, C., Panta, D., Blomberg, L., Talbot, N. (2007). Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. Anim. Reprod. Sci. 98, 147-168.

Killen ID, Caffery GJ. Uterine insemination of ewes with the aid of a laparoscope. Aust Vet J 1982,59(3)95.

Killian GJ, Chapman DA, Kavanaugh JF, Deaver DR. Changes in the composition of oviductal fluid during the cow estrus cycle. Biol Reprod 1987,36(Suppl. 1),84.

Killian GJ. Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. Anim Reprod Sci 2004,82–83,141–153.

Kim, J., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M., Ruau, D., Han, D., Zenke, M., Schöler, H. 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454:646-651.

Kim, J.B., Sebastiano, V., Wu, G., Arauzo-Bravo, M.J., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrich, M., van den Boom, D., Meyer, J., Hübner, K., Bernemann, C., Ortmeier, C., Zenke, M., Fleischmann, B.K., Zaehres, H., Schöler, H.R. (2009). Oct4-induced pluripotency in adult neural stem cells. Cell 136, 411–419.

Kim, J.B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M., Scholer, H.R. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454, 646–650.

Klinc, P., D. Frese, et al. 2007 Insemination with sex sorted fresh bovine spermatozoa processed in the presence of antioxidative substances." Reprod Dom Anim 42 58-62.

Klose R, Kemter E, Bedke T, Bittmann I, Kelsser B, Endres R, et al. Expression of biologically active human TRAIL in transgenic pigs. Transplantation. 2005: 80: 222-230.

Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Herrmann D, van der Weijden GC, Niemann H, Dieleman SJ. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 2002,124,365–375.

Knoepfler, P.S., Zhang, X.Y., Cheng, P.F., Gafken, P.R., McMahon, S.B., Eisenman, R.N. (2006). Myc influences global chromatin structure, EMBO J 25, 2723–2734.

Kohl T, Tchatcheva K, Stressig R, Gembruch U, Kahl P. Is there a therapeutic role for fetoscopic surgery in the prenatal treatment of gastroschisis? A feasibility study in sheep. Surg Endosc 2009,23,1499–1505.

Kopp CW, Grey ST, Siegel JB, Mcshea A, Vetr H, Wrighton CJ, et al. Expression of human thrombomodulin cofactor activity in porcine endothelial cells. Transplantation. 1998: 66: 244-251.

Kossack, N., Meneses, J., Shefi, S., Nguyen, H.N., Chavez, S., Nicholas, C., Gromoll, J., Turek, P.J., Reijo-Pera, R.A. (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27, 138-49.

Kragh, P., Nielsen, A., Li, J., Du, Y., Lin, L., Schmidt, M., Bøgh, I., Holm, I., Jakobsen, J., Johansen, M., Purup, S., Bolund, L., Vajta, G., Jørgensen, L. 2009. Hemizygous minipigs produced by random insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res. 8:545-58.

Kues, W.A., and Niemann, H. (2004). The contribution of farm animals to human health. Trends Biotechnol. 22, 286-294.

Kues, W.A., Carnwath, J.W., and Niemann H. (2005b). From fibroblasts to stem cells: implications for cell therapies and somatic cloning. Reprod. Fertil. Dev. 17, 125-134.

Kues, W.A., Petersen, B., Mysegades, W., Carnwath, J.W., and Niemann, H. (2005a). Isolation of murine and porcine fetal stem cells from somatic tissue. Biol. Reprod. 72, 1020-1028.

Kues, W.A., Schwinzer, R., Wirth, D., Verhoeyen, E., Lemme, E., Herrmann, D., Barg-Kues, B., Hauser, H., Wonigeit, H., and Niemann, H. (2006). Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. FASEB J Express 20, E1-E10, doi: 10.1096/fj.05-5415fje.

Kühholzer B, Besenfelder U, Müller S, Reichenbach HD, Brem G.Laparoscopic insemination of seasonally anoestrous ewes by a simplified method under filed conditions. Reprod Dom Anim 1997a,32,309-312.

Kühholzer B, Mueller S, Prokofiev MI, Ernst LK, Besenfelder U, Brem G. Laparoscopic techniques for the recovery and transfer of microinjected goat zygotes. Theriogenology 1998, 49,245.

Kühholzer B, Müller S, Besenfelder U, Brem G. Endoscopic embryo transfer techniques in sheep 3. Part: intrauterine insemination and tuibal transfer of embryos. Wien Tierärztl Mschr 1997b,84,14-19.

Kühholzer B, Müller S, Besenfelder U, Brem G. Endoscopic embryo transfer techniques in sheep. 1. Part: Embryo flushing of uterine and tubal stage embryos. Wien Tierärztl Mschr 1996a,83,366-369.

Kühholzer B, Müller S, Besenfelder U, Brem G. Endoscopic embryo transfer techniques in sheep. 2. Part: Endocopic ovum pick. Wien Tierärztl Mschr 1996b,83,370-373.

Kuroiwa, Y., Kasinathan, P., Choi, Y.J., Naeem, R., Tomizuka, K., Sullivan, E.J., Knott, J.G., Duteau, A., Goldsby, R.A., Osborne, B.A., Ishida, I., and Robl, J.M. (2002). Cloned transchromosomic calves producing human immunoglobulin. Nat. Biotechnol. 20, 889-894.

Kuwaki K, Knosalla C, Dor FJ, Gollackner B, Tseng YL, Houser S, et al. Suppression of natural and elicited antibodies in pig-to-baboon heart transplantation using a human anti-human CD154 mAb-based regimen. Am J Transplant. 2004: 4: 363-372.

Kuwaki K, Tseng YL, Dor FJ, Shimizu A, Houser SL, Sanderson TM, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. Nat Med. 2005: 11: 29-31.

Labosky, P., Barlow, D., Hogan, B. (1994). Mouse embryonic germ (EG) cell lines: Transmission through the germ line and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development 120, 3197-3204.

Lai, L., Kang, J.X., Li, R., Wang, J., Witt, W.T., Yong, H.Y., Hao, Y., Wax, D.M., Murphy, C.N., Rieke, A., Samuel, M., Linville, M.L., Korte, S.W., Evans, R.W., Starzl, T.E., Prather, R.S., and Dai, Y. (2006). Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat. Biotechnol. 24, 435-436.

Lai, L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., Samuel, M., Bonk, A., Rieke, A., Day, B.N., Murphy, C.N., Carter, D.B., Hawley, R.J., and Prather, R.S. (2002). Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295, 1089-1092.

Lambert RD, Bernard C, Rioux JC, Beland R, D'amours 0, Montreuil A. Endoscopy in cattle by the paralumbar route technique for ovarian examination and follicular aspiration. Theriogenology 1983,20(2),149-161.

Lambert RD, Sirard MA, Bernard C, Béland R, Rioux JE, Leclerc P, Ménard DP, Bedoya M. In vitro fertilization of bovine oocytes matured in vivo and collected at laparoscopy. Theriogenology 1986, 25(1),117-133.

Laurent, L.C., Ulitsky, I., Slavin, I., Tran, H., Schork, A., Morey, R., Lynch, C., Harness, J.V., Lee, S., Barrero, M.J., Ku, S., Martynova, M., Semechkin, R., Galat, V., Gottesfeld, J., Belmonte, J.C., Murry, C., Keirstead, H.S., Park, H., Schmidt, U., Laslett, A.L., Muller, F., Nievergelt, C.M., Shamir, R., Loring, J.F. (2011). Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. Cell Stem Cell 8, 106-118.

Laurincik J, Bicha J, Pichova D, Oberfranc M. Timing of laparoscopic aspiration of preovulatory oocytes in heifers. Theriogenology 1991,3,415-423.

Laurincik J, Pivko J, Grafenau P, Oberfranc M. Oocyte recovery by aspiration of preovulatory follicles in heifers.Vet Med (Praha). 33,1988,727-734.

Lavitrano, M., Bacci, M.L., Forni, M., Lazzereschi, D., Di Stefano, C., Fioretti, D., Giancotti, P., Marfe, G., Pucci, L., Renzi, L., Wang, H., Stoppacciaro, A., Stassi, G., Sargiacomo, M., Sinibaldi, P., Turchi, V., Giovannoni, R., Della Casa, G., Seren, E., and Rossi, G. (2002). Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proc. Natl. Acad. Sci. USA 99, 14230-14235.

Lawson RA, Rowson LE, Adams CE. The development of cow eggs in the rabbit oviduct and their viability after re-transfer to heifers. J Reprod Fertil 1972,28,313–315.

Lee, C-K. and Piedrahita, J. 2000. Effects of growth factors and feeder cells on porcine primordial germ cells in vitro. Cloning 2(4): 197-205.

Lee, C-K., Weaks, R., Johnson, G., Bazer, F., Piedrahita, J. 2000. Effects of protease inhibitors and antioxidants on in vitro survival of porcine primordial germ cells. Biol. Reprod. 63(3):887-897.

Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL, First NL. Development potential of bovine oocytes matured in vitro or in vivo. Biol Reprod 1987,36,376–83.

Lhermette P, Sobel D. An introduction to endoscopy and endosurgery. IN: BSAVA Manual of Canine and Feline Endoscopy and Endosurgery. (Eds.: Philip Lhermette and David Sobel). British Small Animal Veterinary Association, Gloucester, UK, 2008, pp 1-10.

Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R.E., Schulze, E. N., Song, H., Hsieh, C., Pera, M. F., Ying, Q. (2008). Germline competent embryonic stem cells derived from rat blastocysts. Cell 135, 1299–1310.

Liard, O., Segura, S., Pascual, A., Gaudreau, P., Fusai, T., Moyse, E. 2009. In vitro isolation of neural precursor cells from the adult pig subventricular zone. J. Neurosci. Methods 182:172-179.

Liess J. Die Endoskopie beim Rinde. Habilitationsschrift an der Tierärztlichen Hochschule Hannover, M&H Schaper, Hannover, 1936.

Lightfoot RJ, Restall BJ. Effects of site of insemination, sperm motility and genital tract contractions on transport of spermatozoa in the ewe. J Reprod Fertil 1971,26,1-13.

Liu, J., Lu, X., Wan, L., Li, Y., Li, S., Zeng, L., Zeng, Y., Cheng, L., Lu, Y., Cheng, J. 2004. Suppression of human peripheral blood lymphocyte proliferation by immortalized mesenchymal stem cells derived from bone marrow of Banna Minipig inbred-line. Transplant. Proc. 36:3272-3275.

Lo, D., Pursel, V., Linton, P.J., Sandgren, E., Behringer, R., Rexroad, C., Palmiter, R.D., and Brinster, R.L. (1991). Expression of mouse IgA by transgenic mice, pigs and sheep. Eur. J. Immunol. 21, 1001-1006.

Lucy M C. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 2001,84(6),1277-1293.

Ma, D., Bonaguidi, M., Ming, G., Song, H. 2009. Adult neural stem cells in the mammalian central nervous system. Cell Res. 19:672-682.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R. (2007). Directly reprogrammed fibroblasts show global epigenetic reprogramming and widespread tissue contribution. Cell Stem Cell 1, 55-70.

Malau-Aduli A.E.O.: Use of Gene Marker Technology for Livestock Improvement. Invited paper of "The Genetic Future - More Than Just GMO's", Australian Institute of Agricultural Science and Technology (AIAST) Symposium, 30th September 2009, Launceston, Tasmania, Australia.

Manzini, S., Vargiolu, A., Stehle, I.M., Bacci, M.L., Cerrito, M.G., Giovannoni, R., Zannoni, A., Bianco, M. R., Forni, M., Donini, P., Papa, M., Lipps, H., and Lavitrano, M. (2006). Genetically modified pigs produced with a nonviral episomal vector. Proc. Natl. Acad. Sci. USA 103, 17672-17677.

Martin U, Winkler ME, Id M, Radecke H, Arseniev L, Groteluschen R, et al. Transmission of pig endogenous retrovirus to primary human cells. Transplant Proc. 2000: 32: 1157.

Martin U, Winkler ME, Id M, Radeke H, Arseniev L, Takeuchi Y, et al. Productive infection of primary human endothelial cells by pig endogenous retrovirus (PERV). Xenotransplantation. 2000: 7: 138-142.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. 78, 7634-7638.

Matsui, Y., Zsebo, K. and Hogan, B. L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70, 841-847.

Mccurry KR, Kooyman DL, Alvarado CG, Cotterell AH, Martin MJ, Logan JS, et al. Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. Nat Med. 1995: 1: 423-427.

McGregor CG, Teotia SS, Byrne GW, Michaels MG, Risdahl JM, Schirmer JM, et al. Cardiac xenotransplantation: progress toward the clinic. Transplantation. 2004: 78: 1569-1575.

McKenzie IF, Li YQ, Patton K, Sandrin MS. Fucosyl transferase (H) transgenic heart transplants to Gal-/- mice. Transplantation. 2000: 70: 1205-1209.

McKenzie IF, Li YQ, Patton K, Thall AD, SANDRIN MS. A murine model of antibody-mediated hyperacute rejection by galactose-alpha(1,3)galactose antibodies in Gal o/o mice. Transplantation. 1998: 66: 754-763.

McLarren, A. 2003. Primordial germ cells in the mouse. Dev. Biol. 262(1):1-15.

Meek, S., Buehr, M., Sutherland, L., Thomson, A., Mullins, J.J., Smith, A.J., Burdon, T. (2010). Efficient gene targeting by homologous recombination in rat embryonic stem cells. PLoS One 5(12): e14225.

Mehaisen GMK, Vicente JS, Lavara R. In vivo embryo recovery rate by laparoscopic technique from rabbit does selected for growth rate. Reprod Dom Anim 2004,39,347–351.

Meissner, A., Wernig, M., Jaenisch, R. (2007). Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat. Biotechnol. 25, 1177-1181.

Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature 454, 49–55.

Millan MT, Ferran C, Winkler H, Blakely ML, Van Der Werf WJ, Hancock WW, et al. Xenotransplantation: problems and approaches. Transplant Proc. 1994: 26: 3593-3596.

Miller, R. A. and Ruddle, F. H. (1976). Pluripotent teratocarcinoma-thymus somatic cell hybrids. Cell 9, 45-55.

Mintz, B. and Illmensee, K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cell. Proc. Natl. Acad. Sci. 72, 358-3589.

Mohiuddin MM, Corcoran PC, Singh AK, Azimzadeh A, Hoyt RF, JR., Thomas ML, et al. B-cell depletion extends the survival of GTKO.hCD46Tg pig heart xenografts in baboons for up to 8 months. Am J Transplant. 2012: 12: 763-771.

Mösslacher G. Transvaginale Endoskopie zur kontrollierten Aspiration von Kumulus-Oozyten-Komplexen (COC) beim Rind. Doctoral Thesis at the University for Veterinary Medicine, Vienna, 1998.

Müller, M., Brenig, B., Winnacker, E.L., and Brem, G. (1992). Transgenic pigs carrying cDNA copies encoding the murine Mx1 protein which confers resistance to influenza virus infection. Gene 121, 263-270.

Müller, S., Prelle, K., Rieger, N., Petznek, H., Lassnig, C., Luksch, U., Aigner, B., Baetscher, M., Wolf, E., Mueller, M., Brem, G. 1999. Chimeric pigs following blastocyst injection of transgenic porcine primordial germ cells. Mol. Reprod. Dev. 54:244-254.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., Yamanaka, S. 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol. 26:101-6.

Naver, B., Stub, C., Moller, M., Fenger, K., Hansen, A.K., Hasholt, L. & Sørensen, S.A. (2003). Molecular and behavioral analysis of the R6/1 Huntington's disease transgenic mouse. Neuroscience, 122, 1049-57.

Nezhat C, Nezhat F, Ceana Nezhat C. Operative Laparoscopy: Videolaparoscopy and Videolaseroscopy. Ann N Y Acad Sci. 1994,734,433-44.

Niebert M, Tonjes RR. Evolutionary spread and recombination of porcine endogenous retroviruses in the suiformes. J Virol. 2005: 79: 649-654.

Niemann H, Kues W, Carnwath JW. Transgenic farm animals: present and future. Rev Sci Tech. 2005: 24: 285-298.

Niemann H, Verhoeyen E, Wonigeit K, Lorenz R, Hecker J, Schwinzer R, et al. Cytomegalovirus early promoter induced expression of hCD59 in porcine organs provides protection against hyperacute rejection. Transplantation. 2001: 72: 1898-1906.

Niemann, H. (2004). Transgenic pigs expressing plant genes. Proc. Natl., Acad. Sci. USA 101, 7211-7212.

Niemann, H., and Kues, W.A. (2003). Application of transgenesis on livestock for agriculture and biomedicine. Anim. Reprod. Sci. 79, 291-317.

Niemann, H., and Kues, W.A. (2007). Transgenic farm animals – An update. Reprod. Fertil. Dev. 19, 762-770.

Niemann, H., Kues, W.A., and Carnwath, J.W. (2005). Transgenic farm animals: present and future. OIE-Report: Biotechnology applications in animal health and production, Revue scientifique et technique 24, 285-291.

Nieminen, M., Tuuri, T., Savilahti, H. (2010). Genetic recombination pathways and their application for genome modification of human embryonic stem cells. Exp. Cell Res. 316, 2578-2586.

Nottle, M.B., Nagashima, H., Verma, P.J., Du, Z.T., Grupen, C.G., McIlfatick, S.M., Ashman, R.J., Harding, M.P., Giannakis, C., Wigley, P.L., Lyons, I.G., Harrison, D.T., Luxford, B.G., Campbell, R.G., Crawford, R.J., and Robins, A.J. (1999). Production and analysis of transgenic pigs containing a metallothionein porcine growth hormon gene construct. In 'Transgenic Animals in Agriculture'. (Eds J.D. Murray, G.B. Anderson, A.M. Oberbauer, and M.M. McGloughlin) pp. 145-156. (CABI Publishing: New York, NY, USA).

O'Brien S.J.: Molecular genome mapping: lessons and prospects. Curr. Opin. Genet. Dev. 1, 1991, p. 105–111.

Oestrup, O., Hall, V., Petkov, S., Wolf, X., Hyldig, S., Hyttel, P. 2009. From zygote to implantation: morphological and molecular dynamics during embryo development in the pig. Reprod. Domest. Anim. 44 Suppl 3:39-49.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., Yamanaka, S. 2008. Generation of mouse induced pluripotent stem cells without viral vectors. Science 322:949-953.

Okita, K., Ichisaka, T. and Yamanaka, S. (2007). Generation of germline competent induced pluripotent stem cells. Nature 448, 313-317.

Oldmixon BA, Wood JC, Ericsson TA, Wilson CA, White-Scharf ME, Andersson G, et al. Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine. J Virol. 2002: 76: 3045-3048.

Omelka R. - Bauerová M. – Bulla J: Genetic markers for reproductive traits in pigs. Agric. 10, 2001, p. 731-740.

Oropeza M, Petersen B, Carnwath JW, Lucas-Hahn A, Lemme E, Hassel P, et al. Transgenic expression of the human A20 gene in cloned pigs provides protection against apoptotic and inflammatory stimuli. Xenotransplantation. 2009: 16: 522-534.

O'Rourke N, Kodali BS. Laparoscopic surgery during pregnancy. Curr Opin Anaesthesiol. 2006,19(3),254-9.

Pain, B., Clark, M.E., Shen, M., Nakazawa, H., Sakurai, M., Samarut, J., Etches, R.J. (1996). Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122, 2339–2348.

Pan, G. and Thomson, J.A. (2007). Nanog and transcriptional networks in embryonic stem cell Pluripotenc. Cell Res. 17, 42-49.

Panarace, M., Agüero, J.I., Garrote, M., Jauregui, G., Segovia, A., Cane, L., Gutierrez, J., Marfil, M., Rigali, F., Pugliese, M., Young, S., Lagionia, J., Garnil, C., Forte Pontes, J. E., Ereno Junio, J. C., Mower, S., and Medina. M. (2007). How healthy are clones and their progeny: 5 years of field experience. Theriogenology 67, 142-151.

Papaioannou, V.E., McBurney, M. W., Gardner, R. L., Evans, M. J. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. Nature 258, 70-73.

Paradis K, Langford G, Long Z, Heneine W, SANDSTROM P, SWITZER WM, et al. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. Science. 1999: 285: 1236-1241.

Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. Nat Med. 1997: 3: 282-286.

Pera, M. F., Cooper, S., Mills, J., Parrington, J. M. (1989). Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. Differentiation 42, 10-23.

Pera, R., DeJonge, C., Bossert, N., Yao, M., Hwa Yang, J., Asadi, N., Wong, W., Wong, C., Firpo, M. 2009. Gene expression profiles of human inner cell mass cells and embryonic stem cells. Differentiation 78:18-23.

Perry, A.C.F., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y., and Yanagimachi, R. (1999). Mammalian transgenesis by intracytoplasmic sperm injection. Science 284, 1180-1183.

Pesce, M., Farrace, M., Piacentini, M., Dolci, S., Felici, M. 1993. Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). Development 118(4):1089-1094.

Petersen B, Ramackers W, Lucas-Hahn A, Lemme E, Hassel P, Queisser AL, et al. Transgenic expression of human heme oxygenase-1 in pigs confers resistance against xenograft rejection during ex vivo perfusion of porcine kidneys. Xenotransplantation. 2011: 18: 355-368.

Petersen B, Ramackers W, Tiede A, Lucas-Hahn A, Herrmann D, Barg-Kues B, et al. Pigs transgenic for human thrombomodulin have elevated production of activated protein C. Xenotransplantation. 2009: 16: 486-495.

Petersen, B., Lucas-Hahn, A., Lemme, E., Herrmann, D., Barg-Kues, B., Carnwath, J.W., Ramackers, W., Schuettler, W., Tiede, A., Friedrich, L., Schwinzer, R., Winkler, M., and Niemann, H. (2007). Production and characterization of pigs transgenic for human thrombomodulin. Xenotransplantation 14, 371.

Petkov, S. and Anderson, G. 2008. Culture of porcine embryonic germ cells in serum-supplemented and serum-free conditions: The effects of serum and growth factors on primary and long-term culture. Cloning Stem Cells 10(2):263-276.

Petkov, S., Marks, H., Klein, T., Garcia, R., Gao, Y., Stunnenberg, H., and Hyttel, P. 2011. In vitro culture and characterization of putative porcine embryonic germ cells derived from domestic breeds and Yucatan mini pig embryos at Days 20-24 of gestation. Stem Cell Res 6(3):226-237.

Phelps CJ, Ball SF, Vaught TD, Vance AM, Mendicino M, Monahan JA, et al. Production and characterization of transgenic pigs expressing porcine CTLA4-Ig. Xenotransplantation. 2009: 16: 477-485.

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science. 2003: 299: 411-414.

Piedrahita, J., Anderson, G., BonDurant, R. 1990a. Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. Theriogenology 34:865-877.

Piedrahita, J., Anderson, G., BonDurant, R. 1990b. On the isolation of embryonic stem cells: Comparative behavior of murine, porcine and ovine embryos. Theriogenology 34:879-901.

Piedrahita, J., Moore, C., Oetama, B., Lee, C-K., Scales, N., Jagdeece, R., Bazer, F., Ott, T. 1998. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. Biol. Reprod. 58(5):1321-1329.

Pieri, F., Lucarelli, E., Corinaldesi, G., Iezzi, G., Piattelli, A., Giardino, R., Bassi, M., Donati, D., Marchetti, C. 2008. Mesenchymal stem cells and platelet-rich plasma enhance bone formation in sinus grafting: a histomorphometric study in minipigs. J. Clin. Periodontol. 35:539-546.

Piliszek, A., Modlinski, J., Pysniak, K., and Karasiewicz, J. 2007. Foetal fibroblasts introduced to cleaving mouse embryos contribute to full-term development. Reproduction 133:207-218.

Plasterk, R.H. (2002). RNA silencing: the genomes immune system. Science 296, 1263-1265.

Platenburg, G.J., Kootwijk, E.P., Kooiman, P.M., Woloshuk, S.L., Nuijens, J.H., Krimpenfort, P.J., Pieper, F.R., de Boer, H.A., and Strijker, R. (1994). Expression of human lactoferrin in milk of transgenic mice. Transgenic Res. 3, 99-108.

Platt JL, Fischel RJ, Matas AJ, Reif SA, Bolman RM, Bach FH. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation. 1991: 52: 214-220.

Platt JL. The immunological hurdles to cardiac xenotransplantation. Journal of cardiac surgery. 2001: 16: 439-447.

Poncelet, A., Vercruysse, J., Saliez, A., Gianello, P. 2007. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. Transplantation 83:783-790.

Pryce JE, Royal MD, Garnsworthy PC, Mao IL. Fertility in the high-producing dairy cow. Livestock Prod Sci 2004,86,125-135.

Pukelow WR, Jarosz SJ, Jewett DA, Harrison RM. Laparoscopic examination of the ovaries in goats and primates. Lab Anim Sci. 1971,21(4),594-7.

Pursel, V.G., Wall, R.J., Mitchell, A.D., Elsasser, T.H., Solomon, M.B., Coleman, M.E., Mayo, F., and Schwartz, R.J. (1999). Expression of insulin-like growth factor-1 in skeletal muscle of transgenic pigs. In Transgenic Animals in Agriculture (J.D. Murray, G.B. Anderson, A.M. Oberbauer & M.M. McGloughlin eds), CABI Publ., New York, USA, pp 131-144.

Puy, L., Chuva de Sousa Lopes, S., Haagsman, H., Roelen, B. 2010. Differentiation of porcine inner cell mass cells into proliferating neural cells. Stem Cells Dev. 19:61-70.

Quevedo, H., Hatzistergos, K., Oskouei, B., Feigenbaum, G., Rodriguez, J., Valdes, D., Pattany, P., Zambrano, J., Hu, Q., McNiece, I., Heldman, A., Hare, J. 2009. Allogeneic mesenchymal stem

cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. PNAS 106:14022-14027.

Quinn G, Wood JC, Ryan DJ, Suling KM, Moran KM, Kolber-Simonds DL, et al. Porcine endogenous retrovirus transmission characteristics of galactose alpha1-3 galactose-deficient pig cells. J Virol. 2004: 78: 5805-5811.

Rad R, Rad L, Wang W, Cadinanos J, Vassiliou G, Rice S, et al. PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. Science. 2010: 330: 1104-1107.

Rath D, Ruiz S & Sieg B 2003 Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen. Vet Rec 152 400-401.

Rath, D, Long CR, et al. (1999). "In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer." J Anim Sci 77 3346-3352.

Rátky J, Brüssow KP, Solti L, Torner H, Sarlos P. Ovarian response, embryo recovery and results of embryo transfer in a Hungarian native pig breed. Theriogenology 2001,56,969-978.

Rátky J, Brüssow KP. Successful embryo recovery from swine by a minimal invasive technique. Acta Vet Hung 1995,43(4),371-315.

Reh, W.A., Maga, E.A., Collette, N.M., Moyer, A., Conrad-Brink, J.S., Taylor, S.J., DePeters, E.J., Oppenheim, S., Rowe, J.D., BonDurant, R.H., Anderson, G.B., and Murray, J.D. (2004). Hot topic: using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition. J. Dairy Sci. 87, 3510-3514.

Reichenbach HD, Mödl J, Brem G. Piglets born after transcervical transfer of embryos into recipient gilts. Vet Rec. 1993,133(2),36-39.

Rens W, Welch GR & Johnson LA 1998 A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X and Y chromosome-bearing sperm. Cytometry 33 476-481.

Resnick, J. L., Bixler, L. S., Cheng, L. and Donovan, P. J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature 359, 550-551.

Resnick, J., Bixler, L., Cheng, L., Donovan, P. 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature 359(6395):550-551.

Rexroad CE Jr., Powell AM. The ovine uterus as a host for in vitrg-produced bovine embryos. Theriogenology 1999,52,351-364.

Rho, G., Kumar, B., Balasubramanian, S. 2009. Porcine mesenchymal stem cells--current technological status and future perspective. Front Biosci. 14:3942-3961.

Rief S, Sinowatz F, Stojkovic M, Einspanier R, Wolf E, Prelle K. Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced in vitro. Reproduction 2002,124,543–56.

Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De La Fuente J, Boland MP, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 2003,68,236–43.

Rizos D, Pintado B, de la Fuente J, Lonergan P, Gutiérrez-Adán A. Development and pattern of mRNA relative abundance of bovine embryos cultured in the isolated mouse oviduct in organ culture. Mol Reprod Dev 2007,74,716–723.

Rizos D, Ramirez MA, Pintado B, Lonergan P, Gutierrez-Adan A. Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. Theriogenology 2010,73,777–785.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002,61,234–48.

Robertson, E., Bradley, A., Kuehn, M., Evans, M. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323, 445–448.

Robl, J.M., Wang, Z., Kasinathan, P., and Kuroiwa, Y. (2007). Transgenic animal production and animal biotechnology. Theriogenology 67, 127-133.

Robson SC, Schulte Am Esch J, 2ND, Bach FH. Factors in xenograft rejection. Ann N Y Acad Sci. 1999: 875: 261-276.

Roca, J Vázquez JM, Gil MA, Cuello C, Parrilla I, Martínez EA. Challenges in Pig Artificial Insemination. Reprod Dom Anim 2006,41(Suppl. 2),43–53.

Rohrer G.A. - Ford J.J. - Wise T.H. - Vallet J.L. - Christenson R.K.: Identification of quantitative trait loci affecting female reproductive traits in a multigeneration Meishan-White composite swine population; J. Anim. Sci. 77(6), 1999, p. 1385-1391.

Rohwedel, J., Sehlmeyer, U., Shan, J., Meister, A., and Wobus, A. 1996. Primordial germ cell-derived mouse embryonic germ (EG) cells in vitro resemble undifferentiated stem cells with respect to differentiation capacity and cell cycle distribution. Cell Biol. Int. 20(8):579–587.

Rollin, B.E. (1995). The Frankenstein Syndrome. Ethical and Social Issues in the Genetic Engineering of Animals. Cambridge University Press, Cambridge.

Rossant, J. (2001). Stem cells from the mammalian blastocyst. Stem Cells 19, 477-482.

Ruckebusch Y, Bayard F. Motility of the oviduct and uterus of the cow during the oestrous cycle. J Reprod Fertil 1975,43,23–32.

Saeki, K., Matsumoto, K., Kinoshita, M., Suzuki, I., Tasaka, Y., Kano, K., Taguchi, Y., Mikami, K., Hirabayashi, M., Fashiwazaki, N., Hosoi, Y., Murata, N., and Iritani, A. (2004). Functional expression of a Delta12 fatty acid desaturase gene from spinach in transgenic pigs. Proc. Natl. Acad. Sci. USA 101, 6361-6366.

Salamon S, Maxwell WMC. Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. Anim Reprod Sci 1995, 38, 1-36.

Sanotra, G.S. (1999) Registrering af aktuel benstyrke hos slagtekyllinger. Dyrenes Beskyttelse, Copenhagen.

Santacreu MA, Vuides de Castro P, Blasco A. Évaluation par cœlioscopie des corps jaunes et des embryons. Influence sur la taille de portée chez la lapine. Reprod Nutr Dev 1990,30,583-588.

Santl B, Wenigerkind H, Schernthaner W, Mödl J, Stojkovic M, Prelle K, Holtz W, Brem G, Wolf E. Comparison of ultrasound-guided vs laparoscopic transvaginal ovum pick-up (OPU) in Simmental heifers. Theriogenology 1998,50,89-100.

Seidel GE, Jr., Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green RD & Cran DG 1999 Insemination of heifers with sexed sperm. Theriogenology 52 1407-1420.

Shamblott, M. J., Axelman, J., Wang, S. P., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R., Gearhart, J. D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc. Nat. Acad. Sci. 95, 13726-13731.

Shamblott, M., Axelman, J., Littlefield J., Blumenthal P., Huggins G., Cui Y., Cheng L., Gearhart J., (2000). Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. Proc. Nat. Acad. Sci. 98, 113–118.

Shamblott, M., Axelman, J., Littlefield, J., Blumenthal, P., Huggins, G., Cui, Y., Cheng, L., Gearhart, J. 2000. Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. PNAS 98:113–118.

Sharpe Neil F., Carter Roland F., (2006), Genetic testing Care, consent and lability, Wiley, 594 ., ISBN – 0 – 471 – 64987 – 2

Sheu, J., Yuen, C., Sun, C., Chang, L., Yen, C., Chiang, C., Ko, S., Pei, S., Chua, S., Bhasin, A., Wu, C., Yip, H. 2009. Six-month angiographic study of immediate autologous bone marrow mononuclear cell implantation on acute anterior wall myocardial infarction using a mini-pig model. Int. Heart. J. 50:221-234.

Shi, Y., Do, J.T., Desponts, C., Hahm, H.S., Scholer, H.R., and Ding, S. 2008. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. Cell Stem Cell 2:525–528.

Shim, H., Gutiérrez-Adán, A., Chen, L.R., BonDurant, R.H., Behboodi, E., and Anderson, G.B. (1997). Isolation of pluripotent stem cells from cultured porcine primordial germ cells. Biol. Reprod. 57, 1089-1095.

Shim, S., Han, D., Yang, J., Lee, B., Kim, S., Shim, H., Lee, H. 2008. Derivation of embryonic germ cells from post migratory primordial germ cells, and methylation analysis of their imprinted genes by bisulfite genomic sequencing. Mol. Cells 25(3):358-367.

Shin, S., Sun, Y., Liu, Y., Khaner, H., Svant, S., Cai, J., Xu, Q., Davidson, B., Stice, S., Smith, A., Goldman, S., Reubinoff, B., Zhan, M., Rao, M., Chesnut, J. 2007. Whole genome analysis of human neural stem cells derived from embryonic stem cells and stem and progenitor cells isolated from fetal tissue. Stem Cells 25:1298-1306.

Shiraishi M, Oshiro T, Taira K, Nozato E, Nagahama M, Nomura H, et al. Improved hepatic microcirculation by human soluble urinary thrombomodulin in the xeno-perfused porcine liver. Transplantation. 2001: 71: 1046-1050.

Scheef G, Fischer N, Flory E, Schmitt I, Tonjes RR. Transcriptional regulation of porcine endogenous retroviruses released from porcine and infected human cells by heterotrimeric protein complex NF-Y and impact of immunosuppressive drugs. J Virol. 2002: 76: 12553-12563.

Scheef G, Fischer N, Krach U, Tonjes RR. The number of a U3 repeat box acting as an enhancer in long terminal repeats of polytropic replication-competent porcine endogenous retroviruses dynamically fluctuates during serial virus passages in human cells. J Virol. 2001: 75: 6933-6940.

Schellander K, Fayrer-Hosken RA, Keefer CL, Brown LM, Malter H, M C Bride CE, Brackett BG. In vitro fertilization of bovine follicular oocytes recovered by laparoscopy. Theriogenology 1989,31(4),927-934.

Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K.H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278, 2130-2133.

Siegel JB, Grey ST, Lesnikoski BA, Kopp CW, Soares M, Schulte Am Esch J, 2ND, et al. Xenogeneic endothelial cells activate human prothrombin. Transplantation. 1997: 64: 888-896.

Silva, J., Barrandon, O., Nichols, J., Kawaguchi, J., Theunissen, T.W., Smith, A. (2008). Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol 6, e253.

Simões J, Potes J, Azevedo J, Almeida JC, Fontes P, Baril G, Mascarenhas R. Morphometry of ovarian structures by transrectal ultrasonography in Serrana goats. Anim Reprod Sci 2005,85,263–273.

Sirard M A, Lambert RD, Ménard DP, Bedoya M. Pregnancies after in-vitro fertilization of cow follicular oocytes, their incubation in rabbit oviduct and their transfer to the cow uterus. J Reprod Fertil 1985,75,551-556.

Sirard MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML, First NL. The culture of bovine oocytes to obtain developmentally competent embryos. Bio Reprod 1988,39,546-552.

Sirard MA, Lambert RD. Birth of calves after in vitro fertilisation using laparoscopy and rabbit oviduct incubation of zygotes. Vet Rec 1986,1191,167–169.

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., Kucherlapati, R. S. (1985). Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature 317, 230–234.

Snyder DA, Dukelow WR. Laparoscopic studies of ovulation, pregnancy diagnosis, and follicle aspiration in sheep. Theriogenology 1974,2,143–148.

Specke V, Rubant S, Denner J. Productive infection of human primary cells and cell lines with porcine endogenous retroviruses. Virology. 2001: 285: 177-180.

Springmann K, Brem G. Embryo transfer in swine in relation to a gene transfer program. Tierarztl Prax Suppl. 1989,4,21-25.

Squires, E.J. (1999). Status of sperm-mediated delivery methods for gene transfer. In 'Transgenic Animals in Agriculture' (Eds J. D. Murray, G. B. Anderson, A. M. Oberbauer, and M. M. McGloughlin.) pp. 87-96. (CABI Publishing: New York, NY, USA).

Sridharan, R., Tchieu, J., Mason, M.J., Yachechko, R., Kuoy, E., Horvath, S., Zhou, Q., Plath, K. (2009). Role of the murine reprogramming factors in the induction of pluripotency. Cell 136, 364–377.

Stadtfeld, M., Brennand, K., Hochedlinger, K. (2008a). Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr. Biol. 18, 890-894.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., Hochedlinger, K. (2008b). Induced pluripotent stem cells generated without viral integration. Science 322, 945–949.

Stangl M, Kühholzer B, Besenfelder U, Brem G. Repeated endoscopic ovum pick-up in sheep. Theriogenology 1999,52, 709–16.

Stein-Stefani J, Holtz W. Surgical and endoscopic transfer of porcine embryos to different uterine sites. Theriogenology 1987,27(1),278.

Strojek, R., Reed, M., Hoover, J., Wagner, T. 1990. A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts. Theriogenology 33:901-913.

Stroud B. IETS statistics and data retrieval committee report: The year 2009 worldwide statistics of embryo transfer in domestic farm animals. IETS Newsletter 2010, 28(4),11-21.

Surani, A., Hayashi, K., Hajkova, P. 2007. Genetic and epigenetic regulators of pluripotency. Cell 128(4):747-762.

Suster ML, Kikuta H, Urasaki A, Asakawa K, Kawakami K. Transgenesis in zebrafish with the tol2 transposon system. Methods in molecular biology (Clifton, NJ. 2009: 561: 41-63.

Tacke SJ, Bodusch K, Berg A, Denner J. Sensitive and specific immunological detection methods for porcine endogenous retroviruses applicable to experimental and clinical xenotransplantation. Xenotransplantation. 2001: 8: 125-135.

Taft, R.A., Davisson, M., and Wiles, M.V. (2006). Know thy mouse. Trends Genet. 12, 649-653.

Takahashi Y, Watanabe T, Nakagawa S, Kawakami K, Sato Y. Transposon-mediated stable integration and tetracycline-inducible expression of electroporated transgenes in chicken embryos. Methods Cell Biol. 2008: 87: 271-280.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676.

Takahashi, K., and Yamanaka, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-872.

Takefman DM, Spear GT, Saifuddin M, Wilson CA. Human CD59 incorporation into porcine endogenous retrovirus particles: implications for the use of transgenic pigs for xenotransplantation. J Virol. 2002: 76: 1999-2002.

Tantasuparuk W, Techakumphu M, Dornin S. Relationships between ovulation rate and litter size in purebred Landrace and Yorkshire gilts. Theriogenology 2005,63,1142–1148.

Tenenhaus Dann, C., Alvarado, A.L., Hammer, R.E., and Garbers, D.L. (2006). Heritable and stable gene knockdown in rats. Proc. Natl., Acad. Sci. USA 103, 11246-11251.

Tervit HR. Laparoscopy/laparotomy oocyte juvenile breeding. Anim Reprod Sci 1996,42,227-238.

Tesar, P., Chenoweth, J., Brook, F., Davies, T., Evans, E., Mack, D., Gardner, R., McKay, R. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196-199.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448, 196–199.

Testart J. Comparaison de différentes techniques de transplantation des blastocysts chez la lapine. Ann Biol Anim Biochem Biophys 1969,9,351-360.

Thatcher WW, Guzeloglu A, Mattos R, Binelli M, Hansen TR, Pru JK. Uterine-conceptus interactions and reproductive failure in cattle. Theriogenology 2001,56,1435–50.

Thibier, M. & Wagner, H.-G. (2002). World statistics for artificial insemination in cattle. Livestock Production Science, 74, 203-212.

Thibier, M. (2006). Transfers of both in vivo derived and in vitro produced embryos in cattle still on the rise and contrasted trends in other species in 2005. Embryo Transfer Newsletter 24, 12-18.

Thompson, S., Stern, P. L., Webb, M., Walsh, F. S., Engstrom, W., Evans, E. P., Shi, W. K., Hopkins, B.,Graham, C. F. (1984). Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic acid. J. Cell Sci. 72, 37-64.

Thomson, J. A. and Marshall, V. S. (1998). Primate embryonic stem cells. Curr. Topics Dev. Biol. 38, 133-165.

Thomson, J. A., Itskovitzeldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A., Hearn, J. P. (1995). Isolation of a primate embryonic stem-cell line. Proc. Nat. Acad. Sci. 92, 7844-7848.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A., Hearn, J. P. (1996). Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biol. Reprod. 55, 254-259.

Thomson, J., Itskovitz-Eldor, J., Shapiro, S., Waknitz, M., Swiergiel, J., Marshall, V., Jones, J. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–7.

Tomita, S., Mickle, D., Weisel, R., Jia, Z., Tumiati, L., Allidina, Y., Liu, P., Li, R. 2002. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. J. Thorac. Cardiovasc. Surg. 123:1132-1140.

Trounson AO, Willadsen SM, Rowson LEA. Fertilization and development capability of bovine follicular oocytes matured in vitro and in vivo and transferred to the oviducts of rabbits and cows. J Reprod Fertil 1977,51,321-327.

Tseng YL, Kuwaki K, Dor FJ, Shimizu A, Houser S, Hisashi Y, et al. alpha1,3-Galactosyltransferase gene-knockout pig heart transplantation in baboons with survival approaching 6 months. Transplantation. 2005: 80: 1493-1500.

Tsung, H., Du, Z., Rui, R., Li, X., Bao, L., Wu, J., Bao, S., Yao, Z. 2003. The culture and establishment of embryonic germ (EG) cell lines from Chinese mini swine. Cell Res. 13(3):195-202.

Tucker, A., Belcher, C., Moloo, B., Bell, J., Mazzulli, T., Humar, Y., Hughes, A., McArdle, P., and Talbot, A. (2002). The production of transgenic pigs for potential use in clinical xenotransplantation: baseline clinical pathology and organ size studies. Xenotransplantation 9, 203-208.

Turk JR, Laughlin MH. Physical activity and atherosclerosis: which animal model? Canadian journal of applied physiology = Revue canadienne de physiologie appliquee. 2004: 29: 657-683.

Turnpenny L., Cameron I., Spalluto C., Hanley K., Wilson D., Hanley N. (2005). Human embryonic germ cells for future neuronal replacement therapy, Brain Res. Bull. 68, 76–82.

Turnpenny, L., Cameron, I., Spalluto, C., Hanley, K., Wilson, D., Hanley, N. 2005. Human embryonic germ cells for future neuronal replacement therapy. Brain Res. Bull. 68:76–82.

Vainas E, Besenfelder U, Christodoulou V, Kuehholzer B, Amiridis GS, Belibasaki S, Brem G. Production of transgenic sheep: microinjection of the bovine P140 gene construct in Chios sheep breed zygotes. Bull Hellenic Vet Med Soc 1999,50,138–43.

Vajta, G. & Gjerris, M. (2006). Science and technology of farm animal cloning: State of the art. Animal Reproduction Science, 92, 210-30.

Van Reenen, C.G., Meuwissen, T.H.E., Hopster, H, Oldenbroek, K., Kruip, T.H., and Blokhuis, H.J. (2001). Transgenesis may affect farm animal welfare: a case for systemic risk assessment. J. Anim Sci 79, 1763-1769.

Vassiliev, I., Vassilieva, S., Beebe, L., Harrison, S., McIlfatrick, S., Nottle, M. 2010. In vitro and in vivo characterization of putative porcine embryonic stem cells. Cell. Reprogram. 12:223-230.

Vazquez JM, Roca J, Gil MA, Cuello C, Parrilla I, Caballero I, Vazquez JL, Martínez EA. Low-Dose Insemination in Pigs: Problems and Possibilities. Reprod Dom Anim 2008,43(Suppl. 2),347–354.

Vervoorts, J., Luscher-Firzlaff, J. M., Rottmann, S., Lilischkis, R., Walsemann, G., Dohmann, K., Austen, M., Lüscher, B. (2003). Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP. EMBO Reports 4, 484–490.

Vial CM, Ostlie DJ, Bhatti FN, Cozzi E, Goddard M, Chavez GP, et al. Life supporting function for over one month of a transgenic porcine heart in a baboon. J Heart Lung Transplant. 2000: 19: 224-229.

Visscher P. – Haley C.: Marker assisted selection in commercial pig breeding programmes. In: Wiseman J. – Varley M. A. – Chadwick J. P., Progress in pig science. Univ. Press, Nottingham 1998, p. 57-76.

Vodicka, P., Smetana, K., Dvorankova, B., Emerick, T., Xu, Y., Ourednik, J., Ourednik, V., Motlik, J. 2005. The miniature pig as an animal model in biomedical research. Ann. N. Y. Acad. Sci. 1049:161-171.

Wall RA, Bonnema GT, Barton JK. Novel focused OCT-LIF endoscope. Biomedical Optics Express 2011,2(3)421-430.

Wall RJ, Hawk HW. Development of centrifuged cow zygotes cultured in rabbit oviducts. J Reprod Fertil 1988,37,111–26.

Wall, R.J., Powell, A., Paape, M.J., Kerr, D.E., Bannermann, D.D., Pursel, V.G., Wells, K.D., Talbot, N., and Hawk, H. (2005). Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat. Biotechnol. 23, 445-451.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, G.Q., Brack, A.S, Collins, J.J., Cowan, C., Schlaeger, T.M., Rossi, D.J. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7, 618–630.

Watson James., D. Baker., A. Tanja, Bell Stepen P., Gann A. Mevine M., Losick R., (2004)Molecular Biology of the gene, 732 ., ISBN 0 – 321 – 22368 – 3

Wei. L., Gao, K., Liu, P., Lu, X., Li, S., Cheng, J., Li, Y., Lu, Y. 2008. Mesenchymal stem cells from Chinese Guizhou minipig by hTERT gene transfection. Transplant Proc. 40:547-550.

Welstead, G. G., Schorderet, P., Boyer, L. A. (2008). The reprogramming language of pluripotency. Curr. Opin. Gen. Dev. 18, 123–129.

Wernig, M., Meissner, A., Cassady, J. P., Jaenisch, R. (2008). c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell 2, 10-12.

West, F., Terlouw, S., Kwon, D., Mumaw, J., Dhara, S., Hasneen, K., Dobrinsky, J., Stice, S. 2010. Porcine induced pluripotent cells produce chimeric offspring. Stem Cells Dev. Apr 9. [Epub ahead of print].

West, F.D., Uhl, E.W., Liu, Y., Stowe, H., Lu, Y., Yu, P., Gallegos-Cardenas, A., Pratt, S.L., Stice, S.L. 2011. Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. Stem Cells 29:1640-3.

Westhusin, M.E., Pryor, J.H., and Bondioli, K.R. (1991). Nuclear transfer in the bovine embryo: a comparison of 5-day, 6-day, frozen-thawed, and nuclear transfer donor embryos. Mol. Reprod. Dev. 28, 119-123.

Wetscher F, Havlicek V, Huber T, Gilles M, Tesfaye D, Griese J, Wimmers K, Schellander K, Müller M, Brem G, Besenfelder U. Intrafallopian transfer of gametes and early stage embryos for in vivo culture in cattle. Theriogenology 2005a,64,30–40.

Wetscher F, Havlicek V, Huber T, Müller M, Brem G, Besenfelder U. Effect of morphological properties of transferred embryonic stages on tubal migration Implications for in vivo culture in the bovine oviduct. Theriogenology 2005b,64,41–48.

Wheeler, M.B. (1994). Development and validation of swine embryonic stem cells: a review. Reprod. Fertil. Dev. 6, 563-568.

Wheeler, M.B., Bleck, G.T., and Donovan, S.M. (2001). Transgenic alteration of sow milk to improve piglet growth and health. Reproduction 58 (Suppl.), 313-324.

Whitelaw, C.B., Radcliffe, P.A., Ritchie, W.A., Carlisle, A., Ellard, F.M., Pena, R.N., Rowe, J., Clark, A.J., King, T.J., and Mitrophanous, K.A. (2004). Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett. 571, 233-236.

Whyman D, Johnson DL, Knight TW, Moore RW. Intervals between multiple ovulations in PMSG-treated and untreated ewes and the relationship between ovulation and oestrus. J Reprod Fertil 1979,55,481-488.

Willadsen, S.M. (1986). Nuclear transplantation in sheep embryos. Nature 320, 63-65.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810-813.

Wilson CA, Wong S, Muller J, Davidson CE, Rose TM, Burd P. Type C retrovirus released from porcine primary peripheral blood mononuclear cells infects human cells. J Virol. 1998: 72: 3082-3087.

Wilson CA, Wong S, Vanbrocklin M, Federspiel MJ. Extended analysis of the in vitro tropism of porcine endogenous retrovirus. J Virol. 2000: 74: 49-56.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology 2006,65,17–29.

Winkler ME, Winkler M, Burian R, Hecker J, Loss M, Przemeck M, et al. Analysis of pig-to-human porcine endogenous retrovirus transmission in a triple-species kidney xenotransplantation model. Transpl Int. 2005: 17: 848-858.

Wisnerowicz, M., and Trono, D. (2005). Harnessing HIV for therapy, basic research and biotechnology. Trends Biotechnol. 23, 42-47.

Wolf E, Arnold GJ, Bauersachs S, Beier HM, Blum H, Einspanier R, Fröhlich T, Herrler A, Hiendleder S, Kölle S, Prelle K, Reichenbach HD, Stojkovic M, Wenigerkind H, Sinowatz F.

Embryo-maternal communication in bovine—strategies for deciphering a complex cross-talk. Reprod Dom Anim 2003,38,276–89.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hämäläinen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H., Nagy, A. (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458, 766-771.

Wood JC, Quinn G, Suling KM, Oldmixon BA, Van Tine BA, Cina R, et al. Identification of exogenous forms of human-tropic porcine endogenous retrovirus in miniature Swine. J Virol. 2004: 78: 2494-2501.

Wrenzycki C, Herrmann D, Keskintepe L, Martins Jr A, Sirisathien S, Brackett B, Niemann H. Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. Hum Reprod 2001,16, 893–901.

Wrenzycki C, Herrmann D, Lucas-Hahn A, Lemme E, Korsawe K, Niemann H. Gene expression patterns in in vitro-produced and somatic nuclear transfer-derived preimplantation bovine embryos: relationship to the large offspring syndrome? Anim Reprod Sci 2004,82–83,593–603.

Wright RW Jr., Ellington J. Morphological and physiological differences between in vivo- and in vitro-produced preimplantation embryos from livestock species. Theriogenology 1995,44,1167-1189.

Wu, Z., Chen, J., Ren, J., Bao, L., Liao, J., Cui, C., Rao, L., Li, H., Gu, Y., Dai H., Zhu, H., Teng, X., Cheng, L., Xiao, L. 2009. Generation of pig-induced pluripotent stem cells with a drug-inducible system. J. Mol. Cell Biol.:1–9.

www. aboutafricasafari.com/african-safari-parks/

www. Animal genetic resources information

www. gene banks

www. impex.uk..com/zoo_animal_transportation.php

Yamada K, Yazawa K, Shimizu A, Iwanaga T, Hisashi Y, Nuhn M, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. Nat Med. 2005: 11: 32-34.

Yergeau DA, Kuliyev E, Mead PE. Injection-mediated transposon transgenesis in Xenopus tropicalis and the identification of integration sites by modified extension primer tag selection (EPTS) linker-mediated PCR. Nat Protoc. 2007: 2: 2975-2986.

Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature 453, 519–523.

Yu, F., Yao, H., Zhu, P., Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E. (2007b). let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131, 1109–1123.

Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I., Thompson, J. A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. Science 324, 797–801.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., Thomson, J.A. (2007a). Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917-1920.

Yusa, K., Rad, R., Takeda, J., Bradley, A. (2009). Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nat. Methods 6, 363-369.

Zhao, M., Isom, S., Lin, H., Hao, Y., Zhang, Y., Zhao, J., Whyte, J., Dobbs, K., Prather, R. 2009. Tracing the stemness of porcine skin-derived progenitors (pSKP) back to specific marker gene expression. Cloning Stem Cells 11:111-122.

Zhao, X.Y., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C.L., Ma, Q.W., Wang, L., Zeng, F., Zhou, Q. (2009). iPS cells produce viable mice through tetraploid complementation. Nature 461, 86–90.

Zhou, H., Wu, S., Joo, J., Zhu, S., Han, D., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., Siuzdak, G., Schöler, H., Duan, L., Ding S. 2009. Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4:1-4.

Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., Siuzdak, G., Schöler, H.R., Duan, L., Ding, S. (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4, 381–384.

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