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Review

Early embryonic development, assisted reproductive technologies, and pluripotent stem cell biology in domestic mammals

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ABSTRACT

Over many decades assisted reproductive technologies, including artificial insemination, embryo transfer, in vitro production (IVP) of embryos, cloning by somatic cell nuclear transfer (SCNT), and stem cell culture, have been developed with the aim of refining breeding strategies for improved production and health in animal husbandry. More recently, biomedical applications of these technologies, in particular, SCNT and stem cell culture, have been pursued in domestic mammals in order to create models for human disease and therapy. The following review focuses on presenting important aspects of pre-implantation development in cattle, pigs, horses, and dogs. Biological aspects and impact of assisted reproductive technologies including IVP, SCNT, and culture of pluripotent stem cells are also addressed.

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Introduction

Over the past decade, the landscape for veterinary research in embryo technology and stem cell biology has reshaped dramatically. The initial focus of embryo technology in the domestic animals was to optimize breeding for improvement of production and health. In some countries, such as Brazil and Argentina, embryo technologies have found extended practical application, and large numbers of bovine embryos are produced in vitro and transferred to recipients in these regions. In most parts of the world, however, the breeding-related use of such technologies is quantitatively limited. Investigations on pluripotent embryonic stem cells (ESCs) were initiated more than two decades ago with the aim of using the technology for the production of genetically-modified domestic animals. However, these initial efforts to establish ESCs in the domestic species were soon abandoned, due to the discouraging results and, more importantly, to the ground-breaking discovery that cultured embryonic or even somatic cells could be reprogrammed into totipotency by the egg cytoplasm, allowing for generation of genetically-modified animals by nuclear transfer.

Recently, however, renewed focus on domestic animal embryo technology and stem cell biology has emerged, due to the need for improved biomedical models for human diseases. This development has sparked in-depth research into fundamental aspects of

developmental and stem cell biology in the larger domestic mammals, and thus, the understanding of molecular and cellular aspects of initial embryology and phenomena such as pluripotency and cell differentiation in these species is exponentially evolving.

The understanding of pre-implantation embryonic development is a key to optimizing the use of domestic animals as models for human disease, e.g. via refinement by genetic modification and establishment of different stem cell tools, as well as for optimizing the use of embryo technologies for breeding and production. The present review is an attempt to analyse current knowledge of the molecular aspects of pre-implantation development in pigs, cattle, horses, and dogs as well as to discuss the significance of this knowledge for the practical refinement and utilization of in vitro production of embryos, cloning by somatic cell nuclear transfer, and pluripotent stem cell culture.

The anatomy of pre-implantation embryonic development in domestic mammals

Proper maturation of the oocyte to metaphase II is a prerequisite for fertilization and pre-implantation development. In the sow, cow, and mare maturation occurs in the pre-ovulatory follicle within approximately the last 42, 24, and 36 h before ovulation, respectively. Interestingly, in the dog the oocyte is ovulated with an intact germinal vesicle and completes maturation in the oviduct over a 2–4 day period.

Upon fertilization, major embryonic genome activation, which occurs at the 4-cell stage in pigs and around the 8-cell stage in cat-

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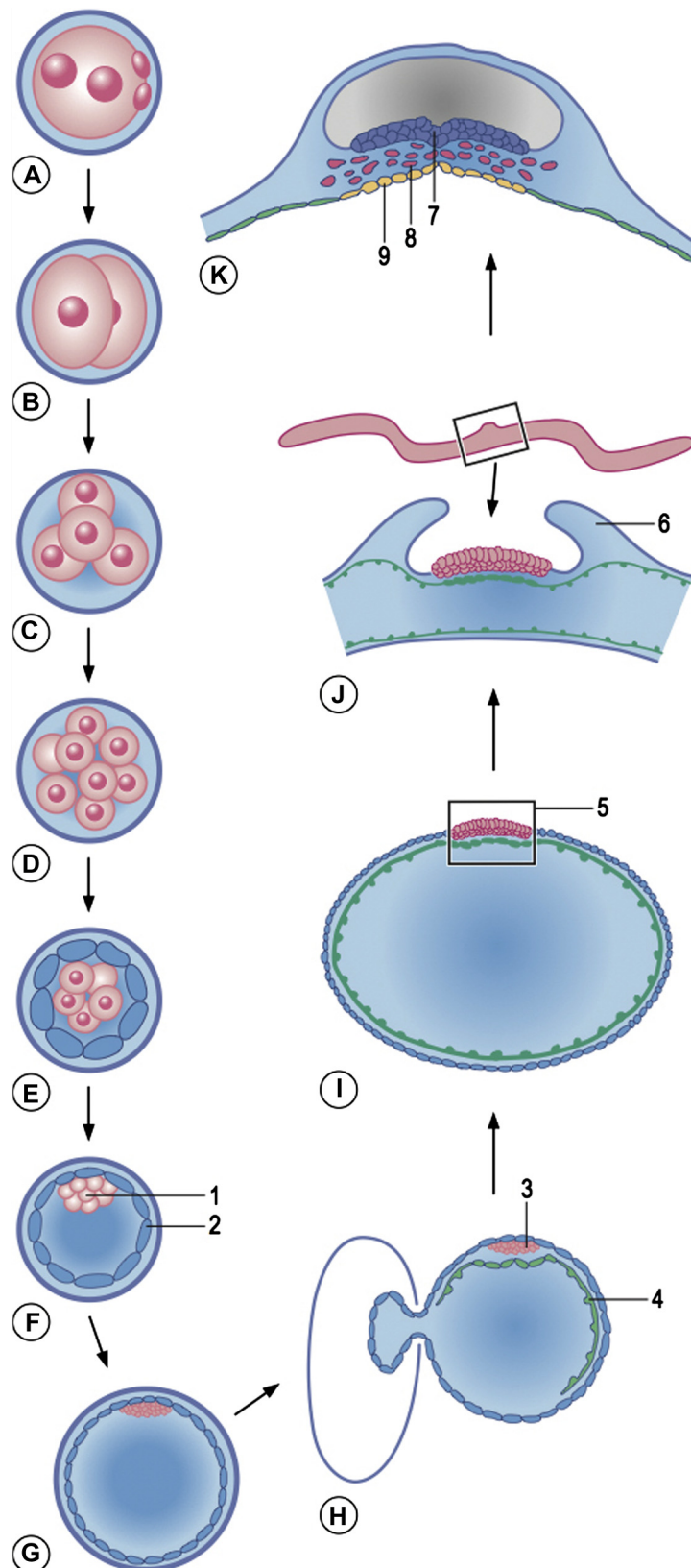


Fig. 1. Initial development of the bovine embryo. A: Zygote; B: 2-cell embryo; C: 4-cell embryo; D: Early morula; E: Compact morula; F: Blastocyst; G: Expanded blastocyst; H: Blastocyst in the process of hatching from the zona pellucida; I: Ovoid blastocyst with embryonic disc; J: Elongated blastocyst; K: Embryonic disc in the process of gastrulation. 1: Inner cell mass; 2: Trophoblast; 3: Epiblast; 4: Hypoblast; 5: Embryonic disc; 6: Amniotic folds; 7: Ectoderm; 8: Mesoderm; 9: Endoderm (from Hyttel et al., 2009).

tle, horses, and dogs, paves the way for the first lineage segregation into trophoblast and inner cell mass (ICM; Fig. 1). Around the time of hatching from the zona pellucida, the next lineage segregation of the ICM results in the formation of the pluripotent epiblast and the hypoblast, which develops into a flat layer epithelium that gradually covers the inside of the epiblast and trophoblast. The hypoblast is referred to as the primitive endoderm in the mouse; a structure which should not be confused with the definitive endoderm (see later). Along with this process, the trophoblast transforms from being a flattened cell layer to a more cuboidal cell architecture. Subsequently, the polar trophoblast, covering the epiblast, referred to as Rauber's layer, becomes increasingly thin, and, finally, the epiblast penetrates the trophoblast and establishes the embryonic disc, which thus becomes part of the outer lining of the conceptus exposed to the uterine environment.

The following process of gastrulation results in formation of the mesoderm, endoderm (i.e. the definitive endoderm) and ectoderm (Fig. 1): first, the primitive streak develops through cell migration, and cells continuously entering the streak undergo epithelial–mesenchymal transition and ingress to form mesoderm that spreads between the trophoblast/epiblast and hypoblast, and endoderm, which becomes inserted into the sub-epiblast portion of the hypoblast (i.e. definitive endoderm which becomes inserted into primitive endoderm). The fate of the ingressing cells depend on their site of ingress: those ingressing through the anterior streak and primitive node become prechordal plate mesoderm, notochord and endoderm; cells ingressing through 'mid' streak become paraxial mesoderm, and cells ingressing through the posterior streak become extra-embryonic and lateral plate mesoderm (Mikawa et al., 2004).

From the time when the trophoblast gradually becomes lined by extra-embryonic mesoderm on the inside, and becomes continuous with the ectoderm, through the transformation of the non-ingressing epiblast into ectoderm, the term trophoctoderm is applied to this cell compartment. The term trophoblast will, again, be used for those cells of the trophoctoderm, which engage in forming the placenta. At the time of gastrulation, chorio-amniotic folds consisting of trophoctoderm with an inner lining of extra-embryonic mesoderm develop into the amnion. A marked elongation of the conceptus occurs in pigs (to about a meter) and cattle at the time of gastrulation, whereas this phenomenon is not observed in horses and dogs.

Cattle (*Bos primigenius taurus* and *Bos primigenius indicus*)

Molecular regulation of pre-implantation development

In cattle the major activation of embryonic genome occurs at the 8-cell stage (Fig. 2; Kues et al., 2008) accompanied by changes in chromatin structure such as acetylation of core histones (Memili and First, 1999). At the 32–64-cell stage compaction occurs followed by blastulation at day 7–8, and hatching occurs at day 8–9 followed by elongation until implantation starts on day 20–21.

Around hatching, the ICM cells differentiate into an inner layer facing the blastocyst cavity, the hypoblast, while the remaining cells form the epiblast (Vejlsted et al., 2006). The polar trophoblast, Rauber's layer, soon degenerates and at the same time gene expression changes of key pluripotency transcription factors *POU5F1*, *SOX2*, and *NANOG* take place (Khan et al., 2012). Contrary to the mouse, this core triad is not confined to the ICM in the early blastocyst, but it is also expressed by the trophoblast together with trophoblast-specific genes *CDX2*, *HAND1*, *ETS2*, and *IFN-tau*. After formation of the epiblast and hypoblast, however, *POU5F1*, *SOX2*, and *NANOG* expression becomes restricted to the epiblast (Degrelle et al., 2005; Vejlsted et al., 2006).

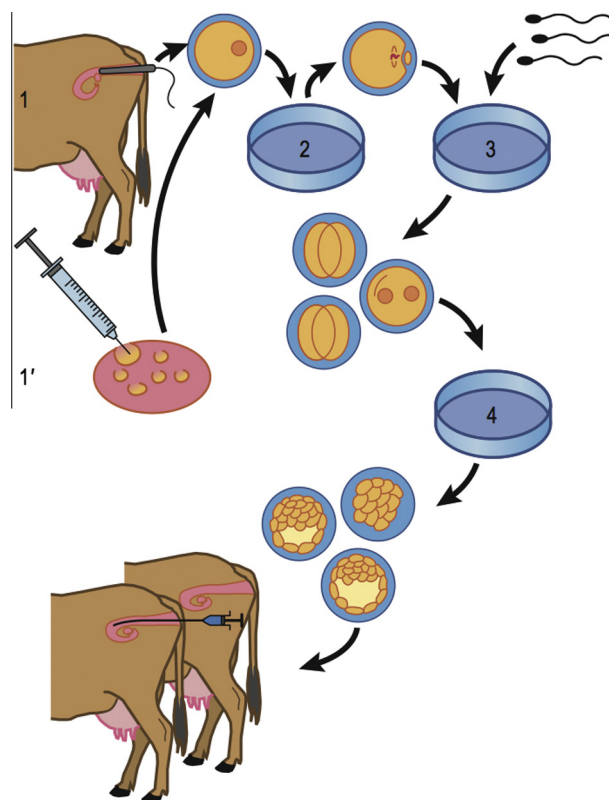


Fig. 2. In vitro production (IVP) of embryos in cattle. Immature oocytes are aspirated from live animals by ultrasound-guided ovum pick up (1) or from abattoir ovaries (1'). Immature oocytes at prophase I are submitted to in vitro maturation (IVM, 2) resulting in progression of meiosis to metaphase II, in vitro fertilization (IVF, 3) resulting in pronucleus formation and initial cleavages, and in vitro culture (IVC, 4) to the morula or blastocyst stage, at which time they can be transferred to recipients (from Hyttel et al., 2009).

In vitro production (IVP) of embryos

The birth of the first IVF calf derived from in vivo matured oocytes in 1982 (Brackett et al., 1982) and the discovery of heparin as capacitating agent for bull sperm 1986 (Parrish et al., 1986) were the two key events that started an era of intense research efforts for developing efficient bovine in vitro embryo production (IVP) procedures including in vitro maturation (IVM) of the oocyte to the metaphase II, in vitro fertilization (IVF), and subsequent in vitro culture (IVC) of embryos to the blastocyst stage (Fig. 3).

The initial lack of knowledge on embryo requirements was bypassed by temporary in vivo culture in the surrogate sheep oviduct (Galli et al., 2003a, 2003b). At the same time co-culture with oviduct cells, Vero cells, BRL cells, granulosa cells was developed followed by cell-free methods based on synthetic oviductal fluid formulations (SOF; Gardner et al., 1994). While over 30% blastocyst formation could be achieved in most culture systems, it soon became obvious that quantity did not always match quality (Loneragan et al., 2006) and that serum supplementation was detrimental to embryo/fetal development as the main causal factor of the so-called large offspring syndrome (LOS), characterised by abnormally advanced embryonic and fetal growth, altered gene expression patterns, and high perinatal losses (Young et al., 1998; Lazzari et al., 2002). A large field study demonstrated that the incidence of LOS was greatly reduced by in vitro culture in cell-free and serum-free SOF media (van Wagendonk-de Leeuw et al., 2000). At present the application of IVP combined with ovum pick up (OPU) from valuable donors is increasing due to developing breeding strategies based on genomics selection using SNP (single

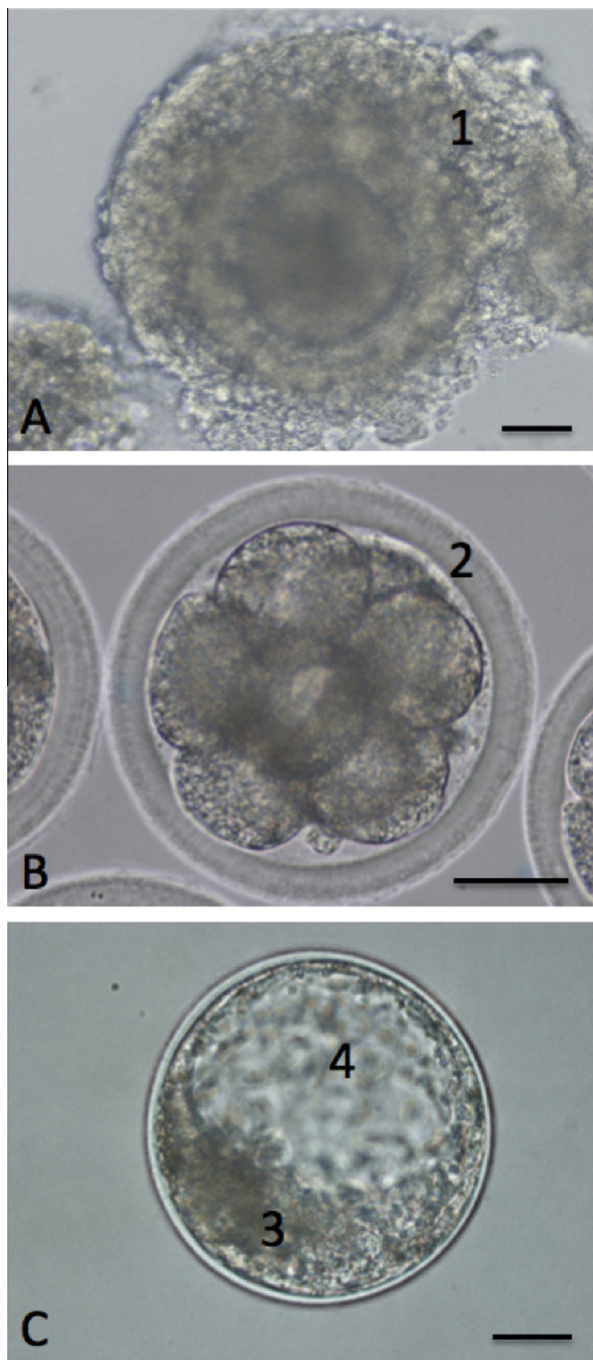


Fig. 3. Bovine in vitro production of embryos. Bovine immature oocyte (A), 8-cell embryo (B), and expanded blastocyst (C). 1: Cumulus cells; 2: Zona pellucida; 3: Inner cell mass; 4: Blastocyst cavity. Scale bars: 50 µm (A, B, and C).

nucleotide polymorphism) chips, whereby thousands of genetic markers can be screened even on a small embryo biopsy before embryo transfer, with enough precision to anticipate that the need for progeny testing will be dramatically reduced.

Somatic cell nuclear transfer (SCNT)

In 1986, Willadsen obtained the first cloned sheep using nuclei of embryonic blastomeres for nuclear transfer (Willadsen, 1986). The following year this result was reproduced in cattle (Prather et al., 1987). These achievements sparked a period of intense research in farm animals, which culminated with the birth of ‘Dolly’

(Wilmut et al., 1997), the first mammal to be cloned from a nucleus of a somatic cell. Following this event several reports on cattle cloned by SCNT emerged (Vignon et al., 1998; Galli et al., 1999). While cloning originally was applied for enhancement of breeding efficiency, including the rescue of a bovine breed close to extinction (Wells et al., 1998), it soon became a tool for obtaining genetically modified calves (Cibelli et al., 1998a).

The SCNT technology involves the enucleation of a mature oocyte creating a chromosome-free cytoplasm, which is typically electrofused with a diploid somatic cell (Fig. 4). This reconstructed embryo is subsequently activated and embarks on embryonic development. The subsequent steps that constitute the technology have been refined with the contribution of dozens of laboratories worldwide over several years. Mostly, enucleated metaphase II oocytes (Campbell et al., 1996; Oback and Wells, 2003) are used as recipient ooplasm, but also enucleated zygotes have been proven suitable to reprogram the transferred nucleus in a more physiological manner as compared to chemical activation of MII cytoplasm (Schurmann et al., 2006).

Quiescent G0 is the preferred cell cycle stage of the donor somatic cells, but also cycling cells (Cibelli et al., 1998a) and blood leukocytes (Galli et al., 1999) have been used as nuclear donors. Technical modifications such as the zona-free manipulation have improved the efficiency of enucleation and fusion (Oback et al., 2003) in several species beside cattle (Lagutina et al., 2007) although development to term is equal to conventional zona-enclosed methods. SCNT is characterised by high pregnancy losses occurring throughout gestation. A comparative embryo transfer study between IVP embryos and cloned embryos derived from embryonic, fetal, and adult cells provided evidence that while the initial pregnancy rate at 21 days is similar (from 55.6% to 62.7%), significant differences are evident at 70 days (49% vs. 37.3% vs. 22.5% vs. 14.3% for IVP, embryos and embryonic, fetal, and adult cell clones, respectively) and at calving (49% vs. 34.3% vs. 15% vs. 6.8%; Heyman et al., 2002a). For fetal and adult somatic cell cloning the efficiency is also influenced, for yet unclarified mechanisms, by the specific cell line used as source of nuclei (Powell et al., 2004).

Several studies have demonstrated that SCNT embryos present an altered gene expression (Smith et al., 2005) and epigenetic status (Dean et al., 2001), compared with IVP embryos, and the high rate of pregnancy loss has been clinically associated with hydroids and cotyledonary hyperplasia (Heyman et al., 2002b; Everts et al., 2008). These problems are, however, not observed in the offspring of clones, which are normal (Heyman et al., 2004). Because of their poor survival to term, cloned cattle have been subjected to intense studies to demonstrate that the composition of milk and meat from these animals is not different from controls (Heyman et al., 2007) and products from cloned animals or their progeny do not pose any health risk to the consumer.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)

ESCs were isolated for the first time from the murine ICM and cultured and characterized by Evans and Kaufman (1981) and Martin (1981). Recent evidence suggests that there are distinct states of pluripotency (naïve and primed) that differ both morphologically and functionally (De Los Angeles et al., 2012). Naïve murine ESCs (Evans and Kaufman, 1981; Martin, 1981) are derived from the ICM or early epiblast cells, proliferate in culture as packed dome-like colonies, are maintained in the undifferentiated state by LIF-JAK-STAT3 and BMP4 signalling, readily contribute to chimeric embryos, maintain two active X chromosomes (in female cells) and are relatively resistant to differentiation into primordial germ cells (PGCs) and extra-embryonic lineages (Kuijk et al., 2011).

In contrast, primed pluripotent stem cells have been derived from epiblasts of post-hatching murine blastocysts, are termed

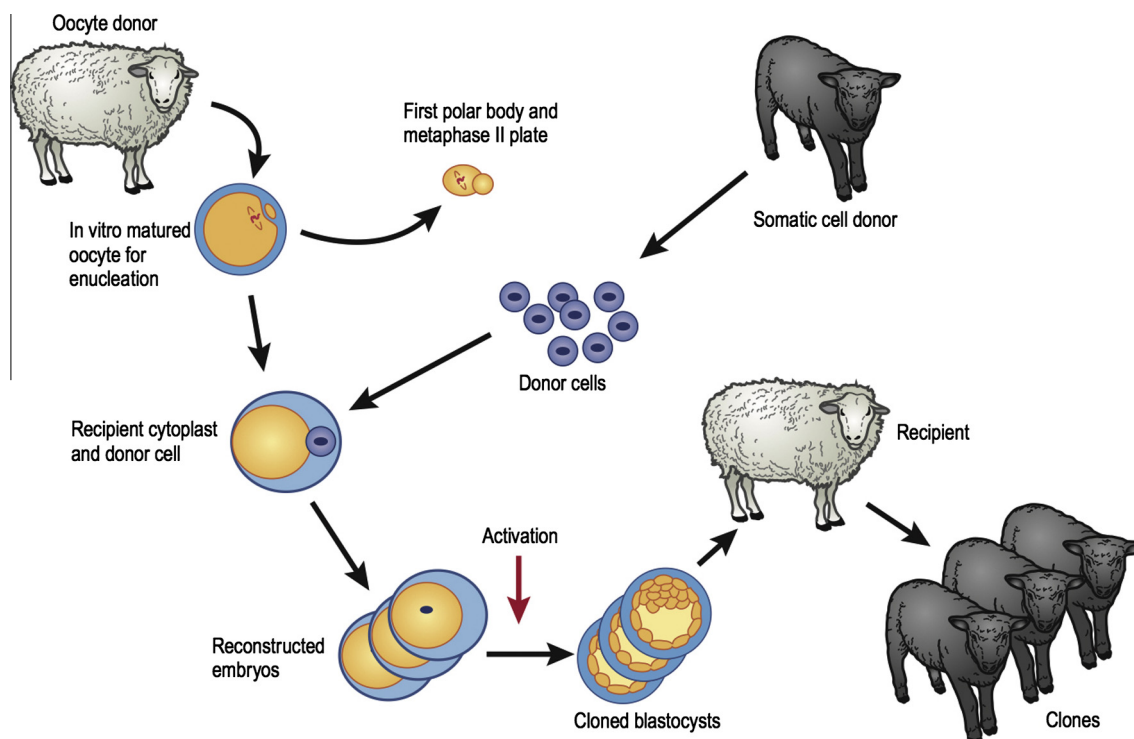


Fig. 4. Cloning of sheep by somatic cell nuclear transfer (SCNT). Oocytes are collected from a donor, matured in vitro to metaphase II, and enucleated (usually by micromanipulation) to remove the first polar body and the portion of the oocyte containing the metaphase plate. The resulting cytoplasm is combined with a donor cell (e.g. a cultured fibroblast cell) originating from the animal to be cloned. The cytoplasm and donor cell are submitted to electrofusion resulting in production of reconstructed embryos in which the oocyte cytoplasm is mixed with the donor cell cytoplasm and carries the donor cell nucleus. The reconstructed embryos are activated in order to initiate embryonic development and, after culture for about 1 week to the blastocyst stage, can be transferred to recipients for development into cloned offspring (from Hyttel et al., 2009).

epiblast stem cells (EpiSCs), are molecularly and epigenetically different from murine ESCs (Brons et al., 2007; Tesar et al., 2007), have a more flattened colony morphology, depend on basic fibroblast growth factor (bFGF) or transforming growth factor alpha (TGF α)/activin signalling for self-renewal, exhibit a limited ability to contribute to chimeras, have undergone X-chromosome inactivation, and readily differentiate into PGC precursors in vitro (Brons et al., 2007). The optimal passaging procedure (single cells trypsinisation vs. disaggregation in clumps) and growth kinetics (14–16 h doubling time in murine ESCs vs. 36 h in murine EpiSCs) also differ. Moreover, the expression of some pluripotency markers is different: Pou5f1 (also known as Oct4), Nanog, and Sox2 are common, but Klf4, Dppa3, and Zfp42 are specific for murine ESCs. Surprisingly, ESCs derived from human blastocysts exhibit characteristics more like those of murine EpiSCs than their murine ESC counterparts (Thomson et al., 1998).

Most of the published studies on attempting bovine ESC derivation have applied the original mouse protocols (Stice et al., 1996; Cibelli et al., 1998b; Mitalipova et al., 2001; Saito et al., 2003; Keefer et al., 2007) starting from 2-cell embryos (Mitalipova et al., 2001) up to day-12 hatched blastocysts (Gjorret and Maddox-Hyttel, 2005). Colony formation ranges from 14% to 70% in the different studies. Some authors report the morula as the most suitable stage (Stice et al., 1996) and others the day-8 blastocyst (Talbot et al., 1995). There is some controversy with respect to expression of pluripotency markers: according to Saito et al. (2003), presumptive bovine ESCs express alkaline phosphatase (AP), FUT4 (also known as SSEA1), STAT-3, and POU5F1, but are negative for SSEA4, whereas other authors report that they are AP positive and stain for SSEA4, POU5F1, TRA-1-81, and TRA-1-60 (Wang et al., 2005; Munoz et al., 2008) and yet others consider AP staining negative while FUT4, SSEA3, and SSEA4 positive (Stice et al., 1996; Cibelli et al., 1998a, 1998b; Mitalipova et al., 2001).

All reported ESC-like bovine cells do not proliferate long-term, except for a few cases (Mitalipova et al., 2001), and are not capable of contributing significantly to chimeras following morula aggregation (Iwasaki et al., 2000). A recent study (Maruotti et al., 2012) has described the application of human ESC and mouse EpiSC culture protocols, based on bFGF and activin-nodal signalling, to post-hatching pre-implantation bovine blastocysts, but again undifferentiated proliferation could not be maintained.

Induced pluripotent stem cells (iPSCs) were first produced in the mouse in 2006, by inserting four transcription factors, including Pou5f1, Sox2, Klf4, and c-Myc into embryonic and adult cells, resulting in a reversion of these cells into a pluripotent state, similar to that observed in the ICM (Takahashi and Yamanaka, 2006). These pluripotent cells can now be created from a multitude of different factors, cell backgrounds, and methods in many different species (Hussein and Nagy, 2012). Derivation of bovine iPSCs was attempted (Huang et al., 2011) using transfection with a polycistronic plasmid containing the complete bovine cDNAs for POU5F1, SOX2, KLF4, and c-MYC, into bovine fibroblasts that were then cultured in presence of specific signalling inhibitors successfully used for mouse and rat ESC culture (Buehr et al., 2008; Ying et al., 2008). Reprogramming efficiency was 0.4% giving rise to non-proliferative dome-shaped colonies expressing markers of pluripotency, including endogenous iPSC factors, CDH1, DPPA3, NANOG, SOCS3, ZFP42, telomerase, Tra-1-60/81, and SSEA-3/4, but not SSEA-1.

In another study a lentiviral expression vector (pLentilox 3.7) for human POU5F1 and porcine SOX2, C-MYC, and KLF4 fused with EGFP was transduced into fetal fibroblasts obtaining a reprogramming efficiency of 0.0002–0.0007% in the presence of LIF and bFGF. The derived colonies resembled human ESCs rather than mouse ESCs, but the transgenes were only partially silenced, indicating incomplete reprogramming (Cao et al., 2012).

Pig (*Sus scrofa domestica*)*Molecular regulation of pre-implantation development*

The development of the porcine pre-implantation embryo is dependent on a number of key cell signalling events. Initial embryonic cleavage is controlled primarily by innate maternal components carried over from the oocyte in the form of RNA and proteins. However, recent reports suggest that RNA may be introduced via spermatozoa, which could contribute to initial development. Early cleavage events may also be steered or enhanced by external factors present in the oviduct such as oviduct-produced proteins or luminal secreted factors (Buhi et al., 1997). A number of key regulators have been found to act during the initial cleavages including the cell cycle controlling phosphatase cdc25 family (Kim et al., 1999; Anderson et al., 2001) and members of the Src family kinase (SFK) family (Levi et al., 2010). The major embryonic genome activation, which occurs at the 4-cell stage in the pig, paves the way for more complex developmental progression (Fig. 5; Jarrell et al., 1991).

This maternal to embryonic transition marks an important event in development: largely, overcoming transcriptional silencing of the embryo. This event is partly attributable to chromatin remodelling events. Chromatin remodelling genes, including *Smrca2*, have been shown to be important in porcine embryonic cleavage (Magnani and Cabot, 2007). A number of histone methyltransferases known to modulate H3K9 (which is associated with transcriptional silencing and cleavage control) have been found to be important in porcine embryonic cleavage (Park et al., 2011). Low expression of the H3K27me3 methylase *EZH2* and its co-factors *EED* and *SUZ12* at the 4-cell stage (Gao et al., 2010) suggests a reversal of H3K27me3-dependent transcriptional silencing occurs at this stage. Furthermore, histone H3K4me3 (implicated in gene activation) is thought to play a particular role in the maternal-to-embryonic transition in the pig (Gao et al., 2010).

The first differentiation event, occurring as the blastocyst forms, appears to be also regulated by key genes. Similar to the mouse, *ELF5* is expressed primarily in the porcine trophoblast and plays an important role in trophoblast specification (Gao et al., 2011b). *CDX2* is another key gene expressed in the porcine trophoblast (Gao et al., 2011a). Interestingly, *Eomes*, which is another important marker for lineage segregation in the mouse, is dependent on expression of *Cdx2* in the trophoblast (Ralston and Rossant, 2008), however, is only expressed in the porcine epiblast and not in the trophoblast (Wu et al., 2010).

Another important gene for ICM specification is probably *POU5F1*, which is expressed in these cells and becomes exclusively localized in the epiblast during later development (Hall et al., 2009; Gao et al., 2011b). It remains unclear whether the genes *NA-NOG* and *SOX2* play an important role in ICM specification in the pig. These genes are expressed in the murine ICM, but are only expressed in the pig epiblast (Hall et al., 2009; Wolf et al., 2011). Data on gene regulation during early development are more advanced in the well-studied mouse, but there are some reports which show that both similarities and differences exist between the mouse and the pig.

In vitro production (IVP) of embryos

The pig has been a particularly difficult species in which to obtain high rates of fertilization and subsequent blastocyst development in vitro. Problems in oocyte cytoplasmic maturation in vitro, high rates of polyspermy, and low embryonic development rates are the major obstacles that still need to be overcome (Gil et al., 2010). The rate of polyspermy has been reported to be over



Fig. 5. Porcine oocyte and embryos derived in vivo. Porcine mature oocyte (A), 8-cell embryo (B), and blastocyst (C). 1: Zona pellucida; 2: First polar body; 3: Inner cell mass; 4: Blastocyst cavity. Scale bars: 50 μ m (A, B, and C).

50% in some laboratories (Mugnier et al., 2009). Despite these difficulties, IVP blastocyst development rates tend to vary from 30% to 50% from monospermically-fertilized oocytes in most laboratories (Gil et al., 2010). Problems with mitochondria migration during IVM have been postulated to be one potential reason for lack of developmental competence (Sun et al., 2001).

The addition of particular components, such as porcine follicular fluid, into the IVM media has been shown to improve the qual-

ity of porcine IVM (Algriany et al., 2004), as has hormones at particular stages of maturation and insulin–transferrin–selenium (Hu et al., 2011). Due to a refinement of techniques, IVM rates now vary from 75% to 85% (Gil et al., 2010). One reason for the high rate of polyspermy seen in this species may relate to a delay in the zona reaction, which under normal conditions establishes a prompt barrier to the fertilization by supernumerary spermatozoa (Wang et al., 1998). Reducing the time of exposure of oocytes to the spermatozoa has led to an increase in monospermic fertilization (Gil et al., 2010). A synthetic attempt to harden the zona has also been attempted using an amine-reactive cross linker, which resulted in a 5-fold increase in monospermic fertilization and an increase in fertilization rate (Coy et al., 2008).

Embryo culture (IVC) has been developed extensively in the pig, and two particularly successful media compositions are used widely today, including NCSU23 and NCSU-37 (Petters and Wells, 1993). Two independent studies have shown that removal of glucose during the first 48–72 h can significantly improve blastocyst development (Abeydeera, 2002; Kikuchi et al., 2002). A chemically defined media has also been developed (PZM5) which appears to be very successful for porcine embryo culture (Yoshioka et al., 2008).

The rate of live offspring resulting from IVP is generally relatively low in the pig compared to the number of transferred IVP embryos, and to date, successful generation of offspring depends on transfer of large numbers of blastocysts or earlier embryos, to produce sufficient litter sizes. A live offspring rate of 11% to 16% has been reported from transferred blastocysts when using chemically defined culture media (Kikuchi et al., 2002). The need to transfer relatively large numbers of embryos to achieve even a comparatively low litter size, as well as the lack of stable non-surgical procedures for embryo transfer remain significant obstacles towards the practical implementation of IVP. In contrast, artificial insemination (AI) remains a mainstream method for ART in swine and is used across Europe, the USA, and many other countries worldwide (Day, 2000).

Somatic cell nuclear transfer (SCNT)

Over the past decade, the efficiency of SCNT has significantly improved due to refinements and simplifications of the cloning technology (Vajta and Callesen, 2012). Development of a zona-free methodology, based on removal of the zona pellucida of the oocyte, combined with the so-called hand-made-cloning, where the oocyte is enucleated by simple hand-held bisectioning, are aspects that have helped to improve blastocyst development of SCNT-produced embryos in the pig (Lagutina et al., 2007; Vajta and Callesen, 2012). Pre-treatment of porcine fibroblasts using *Xenopus* egg extract has also led to improved in vitro SCNT embryo development (Liu et al., 2011, 2012). As for IVP, vast numbers of SCNT embryos are transferred to produce pregnancies and even relatively-low litter sizes.

A recent report has shown that the average rate of offspring from porcine SCNT embryos using two different pig breeds and two different methods was approximately 7% of transferred embryos (Schmidt et al., 2010). Perinatal mortality and malformations are unfortunately still major issues that are reported in this species (Schmidt et al., 2010). This is considered to be caused by errors in epigenetic and genetic reprogramming, and has been overcome in some studies by performing an additional re-cloning step (Fujimura et al., 2008; Cao et al., 2012). Despite these considerable setbacks, a staggering number of genetically-modified pigs, serving as potential human disease models, have been produced using SCNT, including animals carrying gene modifications potentially resulting in skin inflammation related to psoriasis (Staunstrup et al., 2012), Alzheimer's disease (Kragh et al., 2009), cystic fibrosis (Welsh et al., 2009; Klymiuk et al., 2012), and diabetes (Renner et al., 2010). Transgenic pig models have also been developed that

will aid cancer research and xenotransplantation, such as the recently produced SCID pig (Suzuki et al., 2012). Thus the pig is fast paving the way for an alternative biomedical animal model for varying diseases.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)

Production of bona fide porcine ESCs remains elusive, which is likely due to inadequate culture conditions (Hall, 2013). Several research groups have attempted to produce porcine ESCs, however these cells only undergo a limited number of cell passages and differentiate spontaneously in culture (Hall, 2008). One research group has successfully been able to culture porcine ICM cells following transduction with the pluripotency genes *POU5F1* and *KLF4* (Telugu et al., 2011), but transgene-free porcine ESCs remain lacking.

In contrast, iPSCs have been produced in the pig by several different groups (Fig. 6; Ezashi et al., 2012; Kues et al., 2012). These cell lines demonstrate pluripotency and have been shown to contribute towards the formation of chimeras (West et al., 2010) and may even be transmitted through the germline, although this type of transmission was considered rare and combined with perinatal death potentially due to epigenetic aberrations (West et al., 2011). However, unlike their mouse and human counterparts, these cell lines do not silence their inserted transgenes, either during culture or during cell differentiation (Ezashi et al., 2012; Hall et al., 2012). Furthermore, the cells are unable to maintain pluripotency and self-renew when the transgenes are turned off (Wu et al., 2009), indicating that the cells are neither stable in vitro, nor fully reprogrammed.

Problems with both ESCs and iPSCs therefore remain and further research is required in order to determine the exact underlying causes for the continued problems of these cells in culture. Transcriptional profiling of the naïve pluripotent ICM or epiblast may provide some clues as to whether any differences exist in these cells compared to ESCs and iPSCs from mouse and human. Some initial studies have already shown that differences do exist, such as the absence of *NANOG* and *SOX2* in the porcine ICM (Hall et al., 2009). Thus, in vitro cell tools have been developed in the pig,

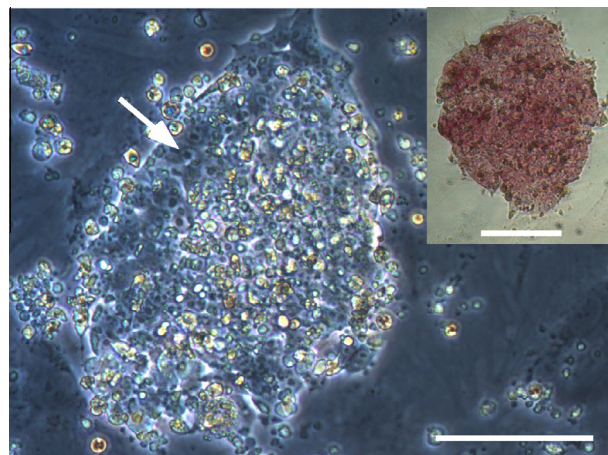


Fig. 6. Porcine induced pluripotent stem cell-like cells. Bright field image of a colony reveals small compact cells containing a large nuclear-to-cytoplasmic ratio with prominent single nucleoli (marked with arrow) and typical high lipid content (refractive yellow portions of colony). Colonies also express alkaline phosphatase (inset image). Colonies are at passage 4 and were grown on mitomycin C-treated mouse embryonic fibroblasts. Cell culture conditions used were KnockOut-DMEM containing 15% KnockOut Serum, 1× non-essential amino acids, 1× glutaMAX, 1× beta-mercaptoethanol, 1× penicillin–streptomycin and supplemented with MEK inhibitor PD0325901 and GSK3 inhibitor, CHIR99201. Scale bar: 100 µm.

however, further refinement of culture conditions is warranted in order to stabilize iPSC cells and enhance their reprogramming. Such advances may also allow for the development of porcine ESCs in the future.

Horse (*Equus ferus caballus*)

Molecular regulation of pre-implantation development

The time of major embryonic genome activation in the horse embryo appears to be around the 6-cell stage (Brinsko et al., 1995; Grondahl and Hyttel, 1996). The equine blastocyst cavity forms in a multicentric manner, resulting in a loose network of inner cells (Bruyas et al., 1993; Tremoleda et al., 2003; Hinrichs et al., 2007b). The segregation of these inner cells is strikingly different from that of other species: Enders et al. (1993) reported that some cells from this loose inner network form the ICM, and some migrate directly to individually seed the inside of the trophoblast, then spread to form a continuous endodermal layer, resulting in a bilaminar blastocyst.

An acellular capsule forms inside the zona pellucida after entry of the equine embryo into the uterus (Flood et al., 1982; Freeman et al., 1991). The equine capsule is composed of mucin-like glycoproteins produced by the trophoblast, containing a high proportion of sialic acid (Oriol et al., 1993a, 1993b). Sialic acid transporters and sialyltransferases are upregulated from days 8 to 14 (Klein and Troedsson, 2011).

Guest and Allen (2007) found that FUT4 (previously known as SSEA1), SSEA3, and SSEA4 proteins were expressed in both ICM and trophoblast in day-7 *in vivo*-recovered horse blastocysts, whereas POU5F1, TRA-1-60, TRA-1-81, and AP activity were localized to the ICM. The ICM cells of ~Day 10 IVP/transferred embryos expressed significantly higher levels of SOX2 and NANOG than did trophoblast; interestingly, CDX2 expression was present in both cell types (Choi et al., 2009a) and has been reported in the equine embryo proper at days 21–25 (de Mestre et al., 2009). Klein and Troedsson (2011) found that embryonic fibrinogen mRNA increased from day 8 to day 14, and that fibrinogen was present in the conceptus and environs.

Smits et al. (2011) found five genes upregulated in equine *in vivo*-derived vs. IVP blastocysts: *FABP3*, *HSP90AA1*, *ODC1* (previously known as *ODC*), *MOB3* (previously known as *MOBK3*), and *BEX2*. Heat-shock protein *HSPA1A* mRNA was higher in IVP vs. *in vivo*-derived embryos (Mortensen et al., 2010). Choi et al. (2009a) found that production of POU5F1 began at the compacted-morula stage in IVP embryos. POU5F1 protein was limited to the ICM in *in vivo*-derived embryos but not in IVP embryos, and transfer of IVP embryos to the uterus normalized expression. Similarly, GATA6 protein was present only in hypoblast of day-7.5 *in vivo*-derived embryos, but showed embryo-wide expression in IVP embryos (Desmarais et al., 2011).

At about day 37, specialized equine embryonic trophoblast cells (chorionic girdle cells) invade into the maternal endometrium, form nests (endometrial cups), and secrete equine chorionic gonadotropin (eCG). Expression of *GCM1*, a transcription factor found in human syncytiotrophoblast cells, was upregulated in chorionic girdle cells at day 34 (de Mestre et al., 2009). Chorionic girdle cells also showed high expression of the immunoregulatory cytokine, interleukin (IL) 22, which may modulate endometrial response to invasion (Brosnahan et al., 2012).

In vitro production (IVP) of embryos

Immature equine oocytes may be recovered post mortem, (Hinrichs and Williams, 1997; Hinrichs et al., 2005) or from live mares

via transvaginal oocyte aspiration (TVA; Brück et al., 1992; Colleoni et al., 2007; Jacobson et al., 2010). Mature oocytes may be collected by aspiration of the dominant pre-ovulatory follicle after gonadotropin stimulation, via TVA or puncture through the flank (Carnavale and Ginther, 1993; Hinrichs et al., 1998).

Immature oocytes may be held overnight in a modified M199 at room temperature before maturation, with no effect on development (Choi et al., 2006). Maturation is performed effectively in M199 with fetal bovine serum and FSH (Hinrichs et al., 2005; Choi et al., 2007; Ribeiro et al., 2008). The optimum duration of maturation is 24–30 h and 30–36 h for oocytes initially having expanded and compact cumuli, respectively. The maturation rate of compact oocytes is lower than that for expanded oocytes (~20% vs. ~65%), but there is no difference in blastocyst development after intracytoplasmic sperm injection (ICSI; Hinrichs et al., 2005).

Standard IVF has not been reliably successful in the horse. Treatment of sperm to induce hyperactivation has resulted in >60% fertilization in two studies (McPartlin et al., 2009; Ambruosi et al., 2013). Currently, fertilization in the horse is performed using ICSI. Good blastocyst rates (20% to 40% of injected oocytes) are achieved using the Piezo drill (Fig. 7; Hinrichs et al., 2005; Galli et al., 2007; Ribeiro et al., 2008). No exogenous activation is needed.

Low-glucose embryo culture media do not support equine blastocyst formation. Good rates of blastocyst development have been achieved using DMEM/F-12 with fetal bovine serum in a mixed gas atmosphere (5% O₂, 5% CO₂, 90% N₂), at 38.2 °C (Hinrichs et al., 2005; Choi et al., 2007; Ribeiro et al., 2008). Equine embryos develop to the blastocyst stage between days 7 and 10 after ICSI. Pregnancy rates after transfer of IVP blastocysts are 50–70% (Colleoni et al., 2007; Choi et al., 2011). IVP is currently used clinically in the horse, both in live mares (Colleoni et al., 2007) and post mortem (Hinrichs et al., 2012).

Somatic cell nuclear transfer (SCNT)

Woods et al. (2003) reported the birth of the first cloned equid, a mule. Viable foals from SCNT have been reported from the laboratory of Dr. Cesare Galli, in Italy (2 foals; Galli et al., 2003a, 2003b; Lagutina et al., 2005), from our laboratory at Texas A&M (13 foals; Choi et al., 2009b; Choi et al., 2013; Hinrichs et al., 2006, 2007a), and from the laboratory of Dr. Daniel Salamone, in Argentina (2 foals; Gambini et al., 2012). In addition, a company, ViaGen² has announced in the popular press the production of more than 160 viable cloned foals.

The reported blastocyst rate per reconstructed equine oocyte is typically less than 10%. Reconstruction was performed by fusion with zona-free oocytes in Italy (Galli et al., 2003a, 2003b; Lagutina et al., 2005), and this technique, accompanied by aggregation of multiple reconstructed oocytes, was used in Argentina (Gambini et al., 2012). Our laboratory in Texas synchronizes donor cells with roscovitine, injects the cells into enucleated oocytes, and injects sperm extract in addition to using chemical activation (Hinrichs et al., 2006, 2007a; Choi et al., 2009b, 2013). Live foal production per embryo transferred reaches 35% (Hinrichs et al., 2007a). We reported on the health of cloned foals after birth (Johnson et al., 2010). There was a 50% incidence of maladjustment, enlarged umbilical remnant, and/or front leg contracture. Two of 14 live-born foals in this series died within 2 weeks of birth; the other 12 were viable. One of three foals in Italy died within 2 days of birth (Lagutina et al., 2005). The two foals born in Argentina were healthy (Gambini et al., 2012). No reports on foal viability are available from ViaGen.

² See: www.ViaGen.com.

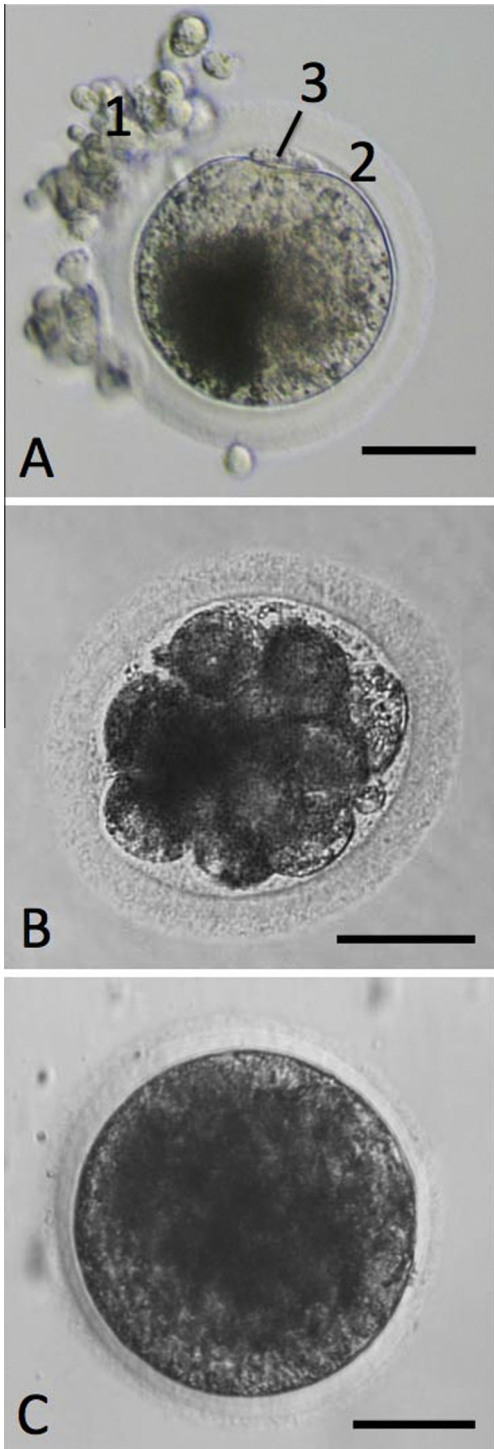


Fig. 7. Equine in vitro production of embryos. Equine in vitro matured oocyte (A), 8-cell embryo derived from intracytoplasmic sperm injection (B), and expanded blastocyst (C). Note the lack of a distinct inner cell mass in (C). 1: Cumulus cells; 2: Zona pellucida; 3: First polar body. Scale bars: 50 μm (A, B, and C).

Equine cloning presents an excellent tool for research on genetics vs. environment in a host of equine diseases, but has not yet been utilized for such studies. Commercial equine cloning is performed to preserve valuable genetics, but clones and their offspring are not eligible for registration in most breeds.

Embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC)

Saito et al. (2002) first described the production of equine ESC-like cells after microsurgical dissection of ICM from day-8 horse blastocysts. The cells expressed FUT4, STAT3, and POU5F1 and could be differentiated to neural precursor cells. Li et al. (2006) established ESC-like cells after immunosurgical dissection of ICM from day-7 to -8 blastocysts; cells proliferated for up to 28 passages were positive for AP activity, and for FUT4, TRA-1-60, TRA-1-81, and POU5F1 protein and mRNA. The cells differentiated into multiple lineages but did not form teratomas when injected into SCID mice. The gene pattern differed from those for mouse and human ES cells, but reflected the pattern of equine ICM cells (Guest and Allen, 2007). Desmarais et al. (2011) produced ESC-like cells from enzymatically-isolated ICM cells of in vivo-derived, parthenogenetic, and SCNT embryos. These authors concluded the cells most likely represented trophoblast stem cells rather than true ESCs.

Nagy et al. (2011) first reported the generation of equine iPSCs, after introducing human *POU5F1*, *SOX2*, *KLF4*, and *MYC* (also known as *c-MYC*) into equine fetal fibroblasts. The resulting iPSCs expressed AP activity, FUT4, SSEA4, TRA-1-60, TRA-1-81, and NANOG, as well as equine-specific mRNA for *POU5F1*, *NANOG*, and *KLF4*, and formed teratomas. Breton et al. (2013) and Khodadadi et al. (2012) reported production of iPSCs from adult equine fibroblasts; the latter study without use of *MYC*. Hackett et al. (2012) compared DNA methylation patterns of the *NANOG* and *SOX2* promoter regions and concurrent gene expression of *NANOG*, *SOX2*, and *POU5F1* in equine iPSCs (Nagy Lab) with those of mesenchymal progenitor cells (commercially marketed as 'stem cells' for treatment of orthopaedic injury in horses). All three pluripotency genes were highly expressed in iPSCs, whereas mesenchymal progenitor cells expressed *SOX2* at differentiated levels and did not express *NANOG* or *POU5F1*. ESCs and iPSCs have extensive clinical application in the treatment of orthopedic injury in horses, which presents an excellent model for their use in the human athlete; thus, this is an active and relatively well-funded area of research.

Dog (*Canis lupus familiaris*)

Molecular regulation of pre-implantation development

Several characteristics of canine female reproductive physiology and developmental biology, such as a pre-ovulatory follicular luteinization, ovulation of immature germinal vesicle (GV) stage oocytes, and prolonged pre-implantation development/transport in the oviduct are distinctly different from those seen in other mammalian species (Reynaud et al., 2006). These differences may account for the very poor efficiency of IVM, IVF, and IVC in canines (Chastant-Maillard et al., 2010). One unique feature of canine oocytes, zygotes, and embryos is their abundant lipid content that accumulates during follicle growth (Tesoriero, 1981). These lipids, mainly made up of triglycerides and phospholipids, are clearly visible by microscopy and can still be observed in the ICM and trophoctoderm of canine blastocysts (Fig. 8) and may be indicative of a unique metabolism that could contribute to their poor development in current in vitro regimes (see below).

Because of these hurdles very limited studies have examined the molecular regulation of canine pre-implantation development. In day-10 flushed canine embryos (morulae and early blastocysts) transcripts for key enzymes of prostaglandin synthesis (*COX2*), selected growth factors (*TGF- α* , *IGF-1*, -2), cytokines (*IL-1 α* , -6), immune cell receptors (*CD4*) and matrix-metalloproteinases (*MMP-2* and -9) are present (Schafer-Somi et al., 2008). We have detected early lineage markers of the epiblast (*POU5F1*), trophoctoderm



Fig. 8. Expanded canine blastocyst derived in vivo. At 11–15 days following detection of an LH surge canine blastocysts were retrieved from flushed reproductive tracts of inseminated bitches. Note the dark lipid-rich inner cell mass (1). 2: Zona pellucida; 3: Blastocyst cavity. Scale bar: 100 μ m.

(*CDX2*), and hypoblast (*GATA6*) in flushed canine blastocysts (Wilcox et al., 2009). Like other domestic species (Kirchhof et al., 2000), *POU5F1* is expressed at diminished levels in the trophectoderm (Wilcox et al., 2009). Clearly, further fundamental research is still required to understand the molecular mechanisms governing oocyte maturation and embryonic development in the dog.

In vitro production (IVP) of embryos

Although conventional assisted reproductive technologies such as AI and cryopreservation have been highly successful (Thomasen and Farstad, 2009), other advanced technologies have been exceptionally inadequate for obtaining high rates of in vitro embryo development in the dog (Chastant-Maillard et al., 2010). For starters, the IVM rates of canine oocytes are very low compared to results obtained in other domestic species (Otoi et al., 2000; Galli and Lazzari, 2008; Bukowska et al., 2012). Canine oocytes collected from anoestrous ovaries exhibit very low frequencies (10–20%) of maturation to the MII stage after 72–96 h of culture (Luvoni et al., 2005; Songsasen and Wildt, 2007), while the IVM rate of oocytes from pre-ovulatory follicles only reaches about 30% (Yamada et al., 1993).

Compounding this poor IVM is a reduced ability of canine spermatozoa to penetrate (10–50%) these oocytes in vitro, with only 4–10% of all oocytes forming two pronuclei after IVF (Mahi and Yanagimachi, 1976; De los Reyes et al., 2009). The poor fertilization is due, in part, to high rates of polyspermy (Saint-Dizier et al., 2001; Hatoya et al., 2006a). ICSI has equally been poor and has not overcome this dual problem of polyspermy and low fertilization ability (Fulton et al., 1998). Although we observed decent in vitro development of in vivo fertilized and flushed canine morulae to the blastocyst stages in SOF medium cultured under 5% oxygen tensions (Wilcox et al., 2009), a 4- to 8-cell block commonly occurs that contributes to their exceptionally poor in vitro development (Yamada et al., 1992; Otoi et al., 2000; Hori and Tsutsui, 2003; Hatoya et al., 2006a).

Somatic cell nuclear transfer (SCNT)

While poor rates of IVM and IVF have limited canine ART (Chastant-Maillard et al., 2010), SCNT could be used to improve canine reproduction and produce valuable disease models. Since the birth of ‘Snuppy’, the first cloned dog (Lee et al., 2005), several breeds of viable cloned puppies have now been produced by SCNT (Jang et al., 2008; Hossein et al., 2009; Kim et al., 2012). Due to the dog’s unique reproductive physiology, limitations of canine in vitro embryo technologies (see above) and poor ovulation induction, dog cloning has relied heavily on protocols to surgically collect in vivo matured oocytes by oviduct flushing after predicted natural ovulations (Johnston et al., 2001; Lee et al., 2005) and surgically transfer cloned embryos immediately after reconstruction to spontaneously synchronized recipients (Lee et al., 2005). Nevertheless, over 50 cloned dogs have been reported with an average pregnancy rate of almost 18% and an average live birth rate of 1.42% from total number of embryos transferred (Kim et al., 2012).

Cloning can propagate desired canine traits, restore the reproductive ability of old or neutered dogs, and even ‘resurrect’ a dead pet (Jang et al., 2008; Park et al., 2009). The concept of dog cloning was fostered in 1998 with the multi-million dollar Missyplicity project designed to clone a dog called ‘Missy’. Although initially unsuccessful, Missy’s clone was born in 2007 as the World’s first clone of a family dog. Currently, there are a few companies (e.g. RNL Bio; Perpetuate) that commercially offer canine cloning services and/or will cryobank cells for producing future dog clones when the technology becomes more efficient and cheaper.

Although phenotypic differences have been observed between clones due to stochastic epigenetic reprogramming events (Peat and Reik, 2012), SCNT allows elite characteristics related to nuclear genetic information to be passed on to the clones. Since re-cloned dogs have been recently derived from cells of cloned canines (Hong et al., 2011b; Oh et al., 2011), infinite propagation of these elite abilities, such as the unique scent sniffing capabilities of detection dogs (Park et al., 2009) or even transgenic dogs (Hong et al., 2009; Kim et al., 2011) are theoretically possible. Interspecies SCNT has even been used for preservation of endangered canine species. The grey wolf (*Canis lupus*), which is considered a threatened species in many countries, was successfully cloned using a wolf somatic cell and a dog oocyte and recipient (Kim et al., 2007; Oh et al., 2008).

Owing to a shared environment and to similarities in physiology, disease presentation, and clinical response at least half of canine diseases are known to have human equivalents making the dog an ideal model for human disorders (Starkey et al., 2005). Although controversial (Varner, 1999; Fiester, 2005), using SCNT to generate genetically modified disease models in dogs looks promising (Jeong et al., 2012; Oh et al., 2012). However, as with other domestic animal clones (Wells, 2005), there have also been some reports of abnormalities in cloned dogs that would currently limit this use (Kim et al., 2009; Hong et al., 2011a). These reports are contentious, however, since there are other published studies showing no adverse effects (Hong et al., 2010; Park et al., 2010). Nevertheless, the challenging reproductive physiological barriers combined with poor ART, low SCNT efficiency, and the high costs associated with these technologies are still limiting factors for achieving translational success with dog cloning.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)

One emerging research field that may contribute to a greater understanding of pre-implantation embryonic development in general and reveal the dog as a model system for developing therapeutic treatments is canine pluripotent stem cells. We were among five research groups to separately derive the first canine

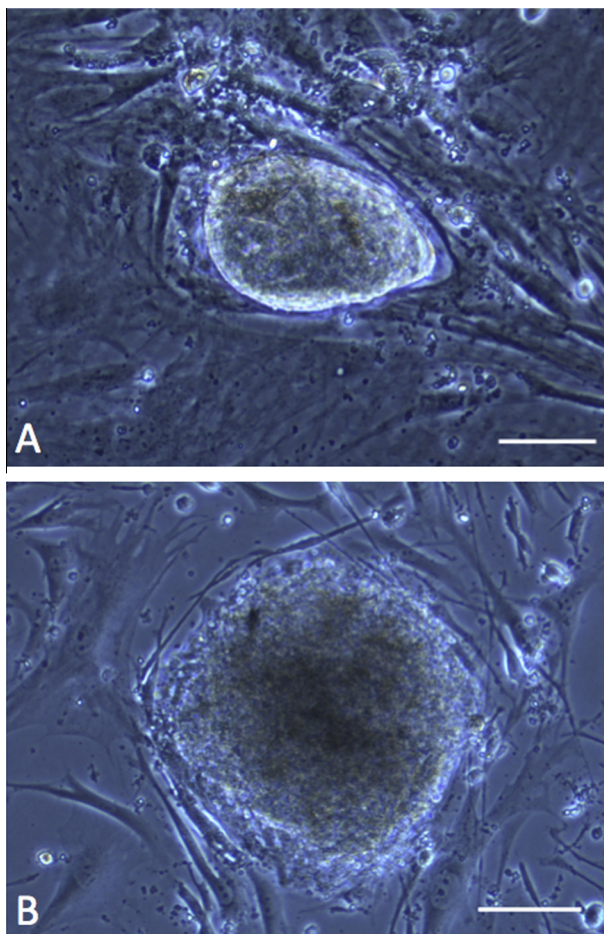


Fig. 9. Naïve (A) and primed (B) canine embryonic stem cell colonies. Canine ESC colonies appear in a primed (hESC-like) pluripotent state when cultured in media contain LIF and bFGF, but morphological changes associated with a more naïve (mESC-like) pluripotent state are observed upon propagation of explanted colonies in media containing just LIF + 2i (glycogen synthase kinase 3 β and mitogen-activated protein kinase inhibitors). Scale bars: 100 μ m (A and B).

ESC lines (Hatoya et al., 2006b; Schneider et al., 2007; Hayes et al., 2008; Vaags et al., 2009; Wilcox et al., 2009). All of our characterized lines exhibit prolonged propagation in the undifferentiated state by the expression of established pluripotency markers (e.g. POU5F1, SOX2, and SSEA3). Under culture conditions supporting differentiation, canine ESCs form embryoid bodies that give rise to ectodermal, mesodermal, and endodermal derivatives (Wilcox et al., 2009). Importantly, we have recently established neural progenitors and active neural cell types from our canine ESC lines (Wilcox et al., 2011). Although chimera formation and germ line contribution of canine ESCs have not yet been assessed, some ESC lines have displayed small overt teratoma formation after transplantation into immune compromised mice (Vaags et al., 2009; Wilcox et al., 2009).

Different pluripotent states (naïve vs. primed) likely arise from the species-specific developmental stage from which the cells are derived (Nichols and Smith, 2009) and stabilized by the *in vitro* conditions they are cultured in (Hanna et al., 2009). Although canine ESCs and canine iPSCs have been derived in LIF-only medium (Hayes et al., 2008; Whitworth et al., 2012), their long term propagation under these conditions have not been demonstrated. Interestingly, it appears that canine ESCs (Vaags et al., 2009; Wilcox et al., 2009) and iPSCs (Luo et al., 2011) require dual-factor culture (LIF and bFGF) to maintain proliferation in the undifferentiated state.

Canine pluripotent stem cells may exist in a stably distinct pluripotent state. However, preliminary studies in our laboratory have shown that canine ESCs are responsive to LIF and 2i (inhibitors of the Mek/Erk and GSK3 pathways) supplemented media with colonies displaying domed-like morphology typical of naïve pluripotency (Fig. 9). Further examination of these unique canine ESCs and iPSCs will allow us to understand to a greater extent the origin of different pluripotent states and will help define/optimize specific culture conditions for their unlimited self-renewal and differentiation into therapeutically relevant cell types to create animal models, stem cell transplantation treatments, and/or as drug screening modalities for human/canine diseases.

Conclusions

Over the coming years, because of new technological advancements and transcriptome- and proteome-based insight into cellular molecular signalling pathways, further progress in embryo and stem cell culture conditions will be forthcoming. This improved efficiency will increase the safety, efficacy and applicability of assisted reproductive technologies in animal production. However, the greatest impact is expected to be in the area of biomedicine. As with the human stem-cell field, advancements in domestic animal stem cells, and, in particular in iPSCs, will create cellular and animal models of disease that may also be used as drug-screening tools to treat various livestock and companion-animal ailments. In combination with newly-discovered genome editing tools, possible cell-based therapies to regenerate, repair, or even replace damaged or diseased tissue are envisioned in the near future.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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