Beyond the mouse: non-rodent animal models for study of early mammalian development and biomedical research

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ABSTRACT The preimplantation development of mammals generally follows the same plan. It starts with the formation of a totipotent zygote, and through consecutive cleavage divisions and differentiation events leads to blastocyst formation. However, the intervening events may differ between species. The regulation of these processes has been extensively studied in the mouse, which displays some unique features among eutherian mammals. Farm animals such as pigs, cattle, sheep and rabbits share several similarities with one another, and with the human developmental plan. These include the timing of epigenetic reprogramming, the moment of embryonic genome activation and the developmental time-frame. Recently, efficient techniques for genetic modification have been established for large domestic animals. Genome sequences and gene manipulation tools are now available for cattle, pigs, sheep and goats, and a larger number of genetically engineered livestock is now accessible for biomedical research. Yet, these animals still make up less than 0.5% of animals in research, mainly due to our inadequate knowledge of the processes responsible for pluripotency maintenance (to date no stable naïve embryonic stem cell lines have been established) and early development. In this review, we highlight our present knowledge of the key preimplantation events in the 3 non-rodent species which present the highest potential for biomedical research related to early embryonic development: cattle, which offer an excellent model to study human *in vitro* embryo development, pigs which emerge as models to study the long-term effects of gene-based therapies and rabbits, which in many aspects of embryology resemble the human.

KEY WORDS: *embryo, embryonic stem cell, cattle, pig, rabbit*

Introduction

The fundamental plan of mammalian preimplantation development is universal for all mammals. It starts with a formation of a totipotent zygote and through many rounds of cellular divisions and differentiation leads to the formation of a blastocyst. During this phase, the first lineage specification occurs resulting in the segregation of the outer, unicellular layer of epithelialized cells named the trophectoderm (TE). This is accompanied by a formation of the inner cell mass (ICM) which thereafter segregates into the pluripotent epiblast (EPI - the nascent embryo proper) and the differentiated primitive endoderm (PrE) or hypoblast (HP). The TE cells will subsequently give rise to the embryonic part of the placenta after blastocyst implantation in the uterus. While the starting point (zygote) and the endpoint (blastocyst) are conserved

among the species, the intervening events have diverged across mammals. The differentiation of the outer layer of cells is already underway by the morula stage, while the population of the early

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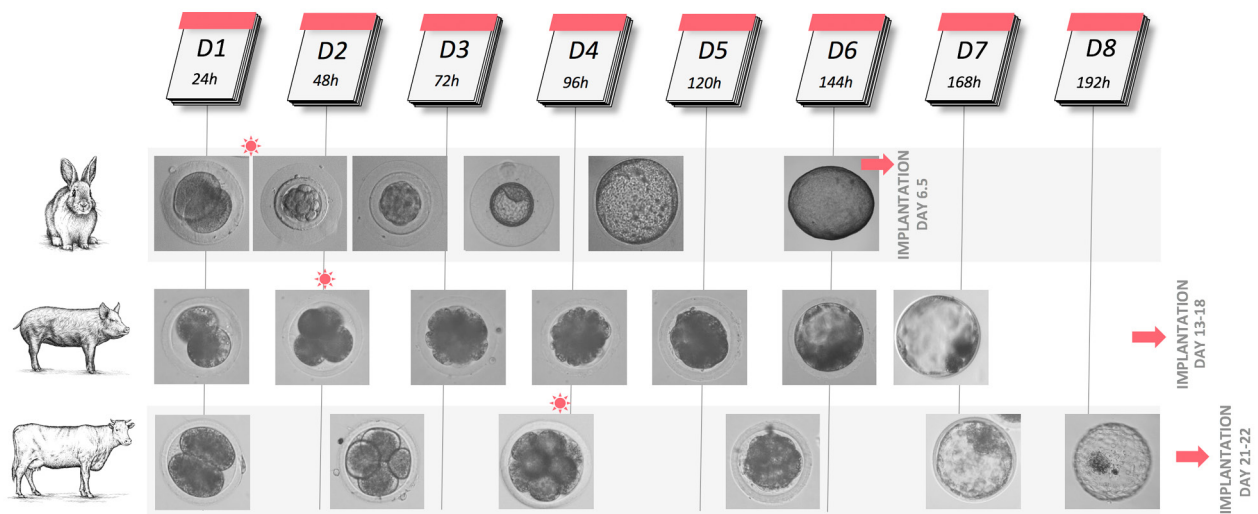
Fig. 1. Comparison of the morphology and timing of the preimplantation development of rabbit, pig and bovine embryos. *D, days of embryonic development; h, hours post insemination; asterisk, Embryonic Genome Activation (EGA).*

inner cells will retain its pluripotent state allowing the EPI formation. The genetic regulation of these processes has been extensively investigated in the mouse, yet the mechanisms involved are not always evolutionarily conserved. In comparison to other mammals such as ungulates, carnivores or primates, mouse preimplantation development is very short – the blastocyst is formed 3 days post coitum (dpc) and is quickly subjected to an invasive implantation at 4.5 dpc. This contrasts with blastocyst formation at 3-6 dpc in rabbit, 5-6 dpc in human, 6-7 days post insemination (dpi) in pig and horse, 7-8 dpi in sheep and 8-9 dpi in the cow (Fig. 1). In some species, the time gap between the blastocyst formation and implantation may be considerably extended in time: 6-11 days in pigs (Bazer and Johnson, 2014), 6-8 days in sheep (Bindon, 1971), 10-13 days in cattle (Peippo *et al.*, 2011) and 28 days in horse (Stout *et al.*, 2005). In most of the domestic mammals differentiation before implantation is significantly more advanced than in the mouse. This includes hypoblast migration, further differentiation of the trophoblast, and gastrulation of the EPI compartment. For a detailed review refer to our previous publication (Piliszek and Madeja, 2018).

The period from fertilisation to the blastocyst formation is the most vulnerable in mammalian development. For example, 50–70% of human embryos fail to reach the blastocyst stage and implant (Burruel *et al.*, 2014, Ellish *et al.*, 1996, Meseguer *et al.*, 2011, Vanneste *et al.*, 2009), similarly to some animal models. In cattle, the majority of embryo losses occur in the second week of gestation during which the blastocysts hatch (8-9 dpi) and the initial 3 lineages (EPI, HP and TE) are segregated (Berg *et al.*, 2010). The EPI and the HP lineages are particularly sensitive to developmental perturbations (van Leeuwen *et al.*, 2014). Studies have revealed that early cleavage events reflect the developmental potential of the embryo (Chavez *et al.*, 2012). Consequently, the processes of cell fate regulation, embryo morphokinetic development and the maintenance of genomic stability are critical elements of developmental competence acquisition. Therefore, in this review we will concentrate on similarities (and differences) in embryonic lineage commitment and pluripotency maintenance in mammalian embryos (non-rodent versus human). This is particularly important

in the context of establishing animal models to study human preimplantation development, as many fundamental aspects of the early stages of human development are found to be conserved in non-rodent mammals.

The ungulate model: cattle and pigs in biomedical research

The development of new therapies and diagnostic methods in human medicine requires animal models, as *in vitro* systems are incapable of modelling a whole organism pathophysiology. Non-rodent species may complement the preclinical studies conducted on mice, as many new therapies fail in the clinical trials because they do not allow to predict the safety and effectiveness in human patients. The lack of consent and the ethical issues arising from using primates in biomedical research place other large animals as a necessary element of preclinical trials. Due to their lifespan, anatomy and physiology farm animals may be used to test the long-term effects of gene therapy, stem cell and induced pluripotent stem cell (iPSC) treatments. Livestock may also indicate the obstacles arising from the application of medical advances in outbred populations.

Pigs have proven to be an important model for cardiovascular diseases, colorectal cancer, osteosarcoma, diabetes, cystic fibrosis, Duchenne muscular dystrophy and Alzheimer's disease (reviewed in Perleberg, 2018). Pigs are also becoming a very attractive species to test stem cell therapies of a spinal cord injury (Kim, 2018). The advantage of using pigs arise from their relatively short time of pregnancy (115 days, in comparison to 283 in cattle), high fertility, and a possible direct translation of surgical techniques (such as endoscopy) which may be implemented in testing various medical diagnostic and therapeutic procedures. Similar organ size and the heart stroke volume places the domestic pig at the first place of the potential donors for xenotransplantation.

Due to the similarities in the reproductive cycles, cows become an important model to study human female fertility in the aspects of the ovarian function (Yapura *et al.*, 2011), the effects of ageing on fertility (Malhi *et al.*, 2005), and certain aspects of uterine infec-

tion and immunity (Herath *et al.,* 2006). The bovine model also facilitates studies on embryo-maternal communication and assisted reproduction techniques (reviewed in Bahr and Wolf, 2012). Other applications of cattle in biomedical research include studies of tuberculosis (and vaccine development) as *Mycobacterium* strains are similar in human and cattle (Waters *et al.*, 2011). Cattle also plays a minor role as biomechanical models for orthopedic surgery (Bisson *et al.*, 2008).

Genetic modifications of livestock became possible with the development of pronuclear DNA injections in the 1980s (Hammer, 1985), however, this method results in random transgene integration. The development of somatic cell nuclear transfer (SCNT), allowed for the generation of genetically engineered animals such as rabbits, sheep, pigs and cattle. In this method, somatic cells (typically foetal fibroblasts) are modified to introduce the intended genetic modification. Despite a steady improvement, SCNT still remains low in efficiency (Callesan, 2014). The more recent development of gene editing tools (such as TALENs and CRISPR/Cas9) has had the greatest impact on the development of genetic modifications in pigs and cattle.

An unquestionable advance of using cattle and pigs for the purpose of biomedical research also arise from numerous similarities that are shared at the preimplantation period of development with humans, such as the kinetics of cell division, the timing of the embryonic genome activation (EGA) or the moment of blastocyst formation. The majority of biomedical and agricultural applications rely on oocytes as the source of *in vitro* produced embryos, thus a detailed understanding of species-specific preimplantation development events is essential for the experimental success.

Cattle – early embryonic development

The early cell lineage specification in the mouse embryo has been broadly reviewed and described by numerous authors (Nichols *et al.*, 2009, Rossant *et al.*, 2003, Saiz and Plusa, 2013). Our understanding of these processes is quite complete on many levels - i.e., molecular, cellular and spatiotemporal. However, in species such as cattle (and other non-rodent mammals), the available information is still limited. Only in recent years, the developmental biologists took an interest in the farm animals and understood their importance for biomedical research and for unravelling the secrets of mammalian embryogenesis.

Mice display unique features, such as the cup-shaped EPI, the maintenance of polar trophoblast, early epiblast cavitation, precocious allantois formation and a complex set of specialised cells involved in invasive implantation that are not typical for eutherian mammals and other rodents (Chazaud and Yamanaka, 2016, Chazaud *et al.*, 2003, Kaufman, 1995). Recently a progress has been made in establishing rabbits and pigs as model systems for human development (reviewed in Piliszek *et al.*, 2016), yet cattle embryos, which are of high economic importance and represent over 250 distinct ruminant species, are still less described.

The moment of major EGA may be associated with the initiation of major differentiation events within the embryo. In rodents the major burst of EGA takes place at the 2-cell stage (Aoki *et al.*, 1997, Seshagiri *et al.*, 1992, Zernicka-Goetz, 1994), while in other mammalian species this process spans several cell cycles and occurs close to the first cell differentiation events, just before the morula formation (Table 1).

TABLE 1

TIMING OF THE MAJOR DEVELOPMENTAL EVENTS IN THE SELECTED MAMMALIAN SPECIES

Species	major EGA cell stage	Embryonic day of EG	morula stage embryonic days	blastocyst stage embryonic days
Human (*Homo sapiens*)	4-8	3	3	5-6
Cattle (*Bos taurus*)	8-16	3-4	5	8-9
Pig (*Sus scrofa*)	4-5	4-5	4-5	6-7
Sheep (*Ovis aries*)	8	3	4-5	7-8
Rabbit (*Oryctolagus cuniculus*)	4	1.5	2	3-6
Mouse (*Mus musculus*)	2	1.5	3	3-4

It has been generally accepted for the mouse model, that a shift from totipotent properties of a zygote to the first cellular differentiation events may start as early as the 2-cell stage. Single blastomere of a 2-cell stage mouse embryo, obtained after the destruction of its counterpart, is capable of full-term development and creating a morphologically normal adult animal (Tarkowski, 1959). Such full developmental potential is only attained when the separated 2-cell stage blastomere generates a sufficient number of the epiblast cells (reviewed in Chen *et al.*, 2018). Although viewed with caution, some studies show that independently developing 2- and 4-cell stage mouse blastomeres demonstrate inequality in totipotency. These authors showed that experimentally separated blastomeres are in some cases able to develop into two individual organisms, although several groups reported low efficiency of such procedure, suggesting that blastomeres at this stage may not possess equal developmental potential (Casser *et al.*, 2017, Katayama *et al.*, 2010, Tsunoda and McLaren, 1983). Other authors showed that a single cell of an 8-blastomere mouse embryo may still be competent enough to contribute to the ICM and/or the TE of a chimeric embryo, but this cell alone cannot develop into a viable offspring (Tabansky *et al.*, 2013). In case of farm animals, the ability of a single cell to retain a full developmental potential is maintained at least up to the 4-cell stage, and it was the first method used to clone cattle and sheep. It was demonstrated that some of the individual blastomeres from 8-cell sheep embryos can give rise to an entire conceptus and that the viability of embryos produced by cell separation depends rather on the degree of reduction in the cell number than on the stage of development at which the separation was performed (Willadsen, 1981). In sheep, the efficiency of live births from separated 4-cell embryos was 36% (Johnson *et al.*, 1995). In cattle, embryos split into blastomeres at the 4-cell stage also developed to term, giving rise to multiple monozygotic healthy calves (Willadsen, 1980). Bisected or biopsied early bovine embryos gave pregnancy rates similar to those obtained from intact control embryos. It is a very important observation, showing that the timing of cell lineage specification among the mammalian embryos of different species is crucial to understand species-specific mechanisms of pluripotency maintenance. This has a further impact on the application of various bio-techniques in animal breeding and in research. For example, the efficiency of cloning, defined as the proportion of transferred embryos that results in a viable offspring is approximately 2 to 3% for all species. However, in cattle, the average cloning efficiency is ranging from 5 to 20% (Kim *et al.*, 2012). Among the factors thought to contribute to the greater success in cattle cloning are the relatively

late EGA and the optimization of reproductive technologies, such as *in vitro* embryo production and embryo transfer, brought about by the cattle industry. Additionally, the efficiency of nuclear transfer technology may be enhanced by better understanding the nature of reprogramming using cow as a model animal, since approximately half of all SCNTs worldwide is performed in this species (Akagi *et al.*, 2013, Tian *et al.*, 2003).

Mechanisms of pluripotency maintenance in cattle

The first stable ESC lines were derived from mouse preimplantation embryos in 1981 (Evans and Kaufman, 1981, Martin, 1981), the first primate lines were published in 1995 (Thomson *et al.*, 1995), human in 1998 (Thomson, 1998) and rat ESC in 2008 (Buehr *et al.*, 2008). The 27-year long gap between mouse and rat ESCs shows the complexity of the system. The classical ESC derivation method relies on the activity of leukaemia inhibitory factor (LIF) in mouse and fibroblast growth factor (FGF) in human culture systems. Rat ESCs were derived only with the help of chemical inhibitors, which specifically eliminate differentiation inducing signalling. The classical ESC system is sufficient only for certain mouse strains and the initial lines were derived from the 129 strain that is consistently amenable to ESC derivation and genetic manipulation. Despite continuous efforts, the establishment of true/stable ESCs from farm animals remains elusive. Over the years, there have been numerous, sometimes contradictory, reports of successful ESC establishment from economically important ungulates, such as cattle and pigs, but the general consensus is that no stable, well-characterized ESC line from these species presently exists (Ezashi *et al.*, 2016). The existing ungulate ESC lines exhibit several deficiencies ranging from short live in culture, inability to contribute to chimaeras, to germline transmission failure. The basis of this phenomenon may arise from the genetic background of the species (or even strain in case of mice) and the differences in the preimplantation development. These include the time-frame of preimplantation development, gene expression patterns, the timing of EGA, morula formation, initiation of lineage specification and even the cell number at each developmental stage.

As it was already stated, all mammals share the same developmental plan, but the intervening events between the zygote formation, the emergence of the blastocyst and implantation exhibit species-specific differences. Bovine preimplantation development in one of the longest in mammals and takes about 3 weeks. The zygote is formed up to 24 hours post insemination (hpi) and the first cleavage may be observed between 22-48hpi. For comparison, human zygote undergoes the first cleavage division between 24-30hpi (Niakan *et al.*, 2012) and mouse up to 16hpi (Sozen *et al.*, 2014). Bovine morulae appear at day 5.0 and blastocysts at day 8-9, this is however only a mid-stage leading to the implantation at day 21-22. During this period the embryo further differentiates, finally obtaining the elongated morphology. The Rauber's layer (i.e. the polar TE overlying the ICM/EPI) disappears by day 11-12 and the EPI increases in size laterally (90-120μm) and in thickness. At day 12-13 the visceral hypoblast (VHP) may be noted underneath the EPI which is enlarged to a maximal diameter of 120-160μm. Cavities began to appear separating the EPI from the VHP. Unlike in mouse and human embryos, gastrulation in cattle (and other ungulates) begins prior to implantation and takes place around 16 dpi. Mural TE proliferation causes further embryo extension,

which finally reaches up to 20cm in length before the implantation (Degrelle *et al.*, 2005, van Leeuwen *et al.*, 2015). Due to the differences mentioned above, for a long time, it was believed that bovine/ungulate blastocyst may not be similar to the known mouse or human model of cell differentiation, lineage-specific signalling pathways activity and/or ICM/TE marker gene expression. The increasing body of evidence, however, shows that bovine blastocyst with its pluripotency status falls closely to the human and primate model.

The differentiation of the TE lineage may be regarded as the hallmark event in mammalian preimplantation development. The process of TE formation initiates with the emergence of the outer layer of cells around the morula stage. In the mouse, the embryo comprises 8 cells at the beginning of compaction and human and bovine embryos begin this process at 16 cell stage (Sozen *et al.*, 2014). A fully compact bovine or human morula comprises about 32 cells (Sozen *et al.*, 2014, Van Soom *et al.*, 1997). The molecular interactions underlying the ICM/TE specification in cattle and in other ungulates still remain to be fully unravelled. However, our and other authors results show that the distribution of lineage-specific transcription factors in bovine blastocysts at day 8-9 mostly resemble the known models, but the differences are noted at the earlier stages. In the blastomeres of 5-cell stage bovine embryos, CDX2 and OCT4 positive signals were detected predominantly in the cytoplasm (Madeja *et al.*, 2013). CDX2 was evenly distributed within the cytoplasm and absent in the nuclei and OCT4 was localised at the apical surface of the blastomeres. Changes in the sub-cellular and sub-nuclear distribution of pluripotency-related factors during the subsequent stages of bovine preimplantation development were noted. From 8–16 cell stage both proteins displayed a nuclear localisation. From the morula stage, the specific lineage segregation became visible, as OCT4 became ICM specific (with only a small proportion of TE cells being OCT4 positive) and CDX2 located solely in the TE layer. This observation may be explained by the work of Berg and colleagues (Berg *et al.*, 2011) who showed that OCT4 expression in bovine TE may be retained even until the PrE/EPI segregation, indicating that the TE cells may not become lineage committed at equivalent stages to the mouse, or that different signalling factors regulate the maintenance of TE in cattle. It was shown that at the early stages, CDX2 does not repress *OCT4* expression, as bovine *OCT4* gene locus does not contain the cis-acting regulatory region necessary for extinguishing its transcription in TE (Berg *et al.*, 2011). Furthermore, the TE cells of an early bovine blastocyst (7 dpi) retain the ability to contribute to the ICM derivatives in chimaeras. In support of this data, a recent study by Simmet and colleagues (Simmet *et al.*, 2018) reports that the deletion of the *OCT4* gene (by the CRISPR-Cas9 system) in bovine embryos did not affect the CDX2 expression in the TE. Control blastocysts showed a typical salt-and-pepper distribution of NANOG and GATA6 positive cells in the ICM, a pattern similar to that previously described in the mouse (Chazaud *et al.*, 2006, Plusa *et al.*, 2008). In contrast, NANOG was absent in the ICM of KO bovine blastocysts, and no cells expressing exclusively NANOG were visible (Simmet *et al.*, 2018). This mimics the findings in OCT4-deficient human blastocysts (Fogarty *et al.*, 2017), but remains in contrast to *OCT4*-null mouse blastocysts (Nichols *et al.*, 1998). Studies performed on *in vitro* produced bovine embryos showed that at the morula stage NANOG may be detected in the cytoplasm of certain cells, but at 8 dpi becomes ICM specific (Kuijk *et al.*, 2008, Madeja *et al.*, 2013).

The key regulators of lineage specification belong to the large group of transcription factors, which play a pivotal role in ESC pluripotency maintenance and its induction in the somatic cells (iPSCs) (reviewed in Chen *et al.*, 2017). Signal transducing pathways that were shown to be involved in embryogenesis include mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, Wingless (WNT)/β-catenin, bone morphogenetic protein (BMP)-SMAD, transforming growth factor (TGF)β, and Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to name just a few (reviewed in Zhao and Jin, 2017). MAPK family plays an important role in many cellular processes and at least three MAPK families have been described: extracellular signal-regulated kinase (ERK) crucial for proliferation, differentiation and development, C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) with a role in proliferation, differentiation and apoptosis, and p38 kinase, which, in addition to proliferation and differentiation also participates in apoptosis, stress response, inflammation and development (reviewed in Zhang *et al.*, 2007). MAPK/ERK signalling is activated by growth factors (such as Fibroblast Growth Factor 4, FGF4) and cytokines, consequently inducing lineage commitment and hypoblast formation. Antagonistically, active WNT pathway plays a key role in pluripotency maintenance. It acts as a regulator of embryonic cell patterning, differentiation, cell adhesion, survival and apoptosis, through a variety of signalling systems generally divided into canonical and non-canonical (reviewed in Logan and Nusse, 2004).

In mouse embryos, MEK/ERK signalling via the FGF receptor was shown to be involved in TE formation (Lanner and Rossant, 2010, Lu *et al.*, 2008). The inhibitor-mediated modulation of ERK signalling activity was shown to enhance the efficiency of rodent ESC derivation by supporting the retention of OCT4 positive cells during the initial outgrowth formation (Buehr, 2002). However, ERK pathway inhibition alone is insufficient to support undifferentiated ESC in culture, unless the cells are maintained at high densities or provided with LIF (Ying *et al.*, 2008). The maintenance of pluripotency in mouse and human ESCs is also possible through activation of WNT signalling via selected inhibition of glycogen synthase kinase 3 (GSK3) (Sato *et al.*, 2004). Ying and colleagues (Ying *et al.,* 2008) showed that the addition of a selective GSK3 inhibitor (GSK3i - CHIR99021) restored viability and allowed efficient expansion of undifferentiated mouse ESCs in the absence of ERK signalling. Based on these findings, Buehr and colleagues (Buehr *et al.,* 2008) equipped with two sets of chemically defined signalling pathway inhibitors were able to establish stable rat ESC lines. In the 3i system, the inhibitory effect on FGF signalling was supported by MEK/ERK pathway inhibition and WNT activity maintenance via the GSK3i. The 2i system relied only on the GSK3i and MEK/ERK pathway inhibition. Both systems were supplemented with LIF which is necessary to suppress differentiation, maintain viability and growth rate when FGF/MEK/ERK signalling is reduced. The 2i system utilised a more selective and potent MEK inhibitor than the 3i, thus eliminating the need to block the FGF receptor. Eventually, the 2i conditions proved optimal to derive rat ESC since it produced equivalent numbers of male and female cell lines and chromosomal abnormalities were not observed.

Still, a very limited amount of data exists with relation to the signalling requirement for the derivation of stable ESC lines from bovine embryos. The results of other authors (and our studies) show that WNT is active in bovine preimplantation embryos and that it affects lineage and pluripotency specific gene expression (Madeja *et al.*, 2015). *In vitro* culture of bovine embryos in the presence of the GSK3i (CHIR99021) throughout the whole period of *in vitro* preimplantation development resulted in the upregulation of *OCT4, NANOG, c-MYC* and *REX1* transcript expression in the ICM and *OCT4* expression in TE, accompanied by downregulation of *CDX2* expression in the TE (Madeja *et al.*, 2015). Our unpublished data also suggests that the 2i and 3i systems may exert an effect on pluripotency marker gene expression in bovine blastocysts, as in the case of both of these systems the number of OCT4 and NANOG positive cells significantly increases both within the ICM and the TE. Studies by Ozawa and colleagues (Ozawa *et al.*, 2012) showed that the exposure of bovine embryos to the 2i system at the time of morula to blastocyst transition facilitated the formation of self-renewing pluripotent cell lines from blastocysts and continuously maintained high *NANOG* and *SOX2* expression. The positive effect of the 3i system on bovine ESC lines survival and maintenance after thawing was also confirmed (Pashaiasl *et al.*, 2013). MEK inhibitor used in the 2i system (PD0325901) stimulated bovine blastocyst development and the ICM cell number (McLean *et al.*, 2014). Also, the 3i system improved blastocyst morphology, however the best results were noted in the 2i medium where *FGF4* and *NANOG* transcripts were upregulated and *PDGFRα* and *SOX17* downregulated. Thus it may be speculated, that at least at the mRNA level the inhibition of MAPK signalling prevents a natural shift from the EPI to HP state.

Pluripotent cell lines may exist in two states - naïve ESCs (equivalent to cells from the early epiblasts) and primed EpiSCs (developmentally more advanced epiblast stem cells). The genome of the naïve ESCs possesses a minimum of repressive epigenetic marks (Niwa, 2007), whereas EpiSCs have their epigenetic machinery active (Tesar *et al.*, 2007). The transition from the naïve to the primed state represents a pivotal event in the embryonic differentiation. Studies suggest that the pluripotent state of human ESCs corresponds to mouse EpiSCs. Mouse naïve ESCs efficiently contribute to chimaeras, maintain both X chromosomes active in female lines and rarely submit to primordial germ cell differentiation (Nichols and Smith, 2009). These cells may be cloned with high efficiency, grow as packed dome colonies, are stabilised by LIF/STAT3 and destabilised by basic Fibroblast Growth Factor (bFGF) and TGFβ/Activin signalling. In contrast, primed mouse EpiSCs exhibit flattened morphology, intolerance to passaging as single cells and rely on bFGF and TGFβ/Activin signals. Human ESCs share several important features with primed mouse EpiSCs. These include flattened morphology, intolerance to passaging as single cells, dependence on TGFβ/Activin signalling (Xu *et al.*, 2008), inactivation of X-chromosome in most of the derived female cell lines (Silva *et al.*, 2008) and higher susceptibility to differentiate into PGCs in the presence of BMP4. The results of our studies led us to speculate that the derived bovine ES-like cells may share features of both these stem cell types (Madeja *et al.*, 2015). The primary ESC lines from bovine embryos expressed transcripts for several pluripotency marker genes (*OCT4, NANOG, REX1, SOX2, c-MYC* and *KLF4*). It was shown that overexpression of KLF4 may revert mouse EpiSCs back to an ES-like state (Guo *et al.*, 2009). EpiSCs virtually do not express KLF4 but bovine cell lines derived in the presence of LIF and the GSK3i culture conditions maintained *KLF4* transcripts over several passages. JAK/STAT signalling was

active at the initial stages of bovine ESC derivation - LIF had beneficial effects at the initial passages, but alone was inefficient to support prolonged self-renewal in primary bovine ESC culture. Similarly to mouse ESCs, WNT activity supported by LIF had a positive effect on cell culture propagation, survival after passage, morphology and pluripotency-related marker gene expression. The derived bovine ES-like colonies shared features of mouse EpiSC and human ESC morphology and grew as flat, mono-layer colonies intolerant to passaging as single cells (Madeja *et al.*, 2015).

However, the issue of signalling pathway interactions in cattle may be much more complex. It was shown recently that employing a culture system containing FGF2 and an inhibitor of the canonical WNT signalling pathway Rho kinase (ROCK, Y-27632), allows for derivation of potentially pluripotent bovine ESCs with stable morphology, transcriptome, karyotype and population-doubling (Bogliotti *et al.*, 2018). These cells were also used as donors for nuclear transfer and produced blastocyst at standard rate. Yet these results must be taken with caution, as other authors show that WNT signalling regulates development of bovine embryos to the blastocyst stage (Denicol *et al.*, 2013). It has been postulated that the roles of different WNTs are variable according to developmental stage and factors such as cell environment, presence and abundance of different receptors and effector molecules (van Amerongen and Nusse, 2009). It is very likely that regulation of embryonic development by WNTs involves numerous receptors and signalling molecules participating in fine regulation of canonical and non-canonical pathways to balance maintenance of pluripotency, self-renewal and cell-fate commitment.

Pig - early embryonic development

The biomedical and agricultural applications of pigs as models for human diseases, stem cell research, transgenesis and cloning in many cases rely on the use of a large number of embryos obtained *in vitro*. Unfortunately, the efficiency of *in vitro* protocols including fertilisation, embryo culture and freezing is still unsatisfactory. Pig oocyte maturation procedures are based on well-established protocols that provide high (up to 90%) rate of oocytes reaching the metaphase II stage (Pawlak *et al.,* 2011). The subsequent stages, in particular gamete co-incubation, are still not reproducible between the laboratories and the results differ significantly within the published data. The attention in the field is mainly focused on culture media composition, semen selection, cytoplasmic maturation of oocytes, gamete co-incubation time, sperm concentration and embryo culture systems. Due to a high frequency of polyspermy, the gamete co-incubation time is drastically reduced (3-6h) and carried out with variable sperm concentrations. This approach allows to maintain an acceptable cleavage rate between 30 and 60% (Gil *et al.*, 2004). One of the most interesting aspects of porcine assisted reproductive technologies (ART) is oocyte *in vitro* maturation culture media composition, which requires supplementation with 10-20% v/v of the follicular fluid (FF), collected from ovaries upon oocyte aspiration. This procedure positively influences the process of cumulus-oocyte complex maturation, reduces the rate of polyspermy, improves pronuclei formation in zygotes and positively affects embryo development to the blastocyst stage, probably by enhancing the effectiveness of cytoplasmic maturation (Brevini *et al.,* 2005). The addition of the FF to the *in vitro* maturation media,

a procedure unique to this species, is intriguing, as the conditions prevailing in the ovarian follicles are to some extent transferred to the *in vitro* system. Therefore, FF compounds such as amino acids, fatty acids, hormones and growth factors may affect both the oocyte and the cumulus cells during the entire period of *in vitro* maturation, making the system suboptimal. The issue of data reproducibility arises due to the stimulatory or the inhibitory effect of FF on oocytes and embryos, depending on its quantitative and qualitative composition (Bagg *et al.*, 2007 Grupen *et al.*, 2010, Pawlak *et al.*, 2018). Moreover, working with an *in vivo* collected FF, demonstrating the action of a single factor is more difficult than in cattle, where IVM medium is supplemented with defined components such as fatty acids or amino acids. It is therefore difficult to compare porcine IVM to other species, also because the duration of this stage in the pig is 44h, which is considerably longer than in other mammals. On the other hand, the timing of porcine EGA (4-5 cells) and of blastocyst formation closely resembles human embryos and makes the pig a useful model for preimplantation development and ART research. Similar to cattle and other ungulates the differences arise after formation of the blastocyst, which further differentiates, to finally reach a very long filamentous shape (up to 60cm in length) (Anderson, 1978) before the implantation, which starts at 13-18 dpi.

Parthenogenetic embryos as an alternative for porcine IVF

Porcine embryos derived as a result of artificially induced parthenogenetic activation (PA) possess a great diagnostic potential and may be used as an alternative to embryos obtained in classical *in vitro* fertilization procedure. The first attempts to obtain embryos relying only on the uniparental genome date back to early 1940s and 1960s and the first studies were done on rabbit and mouse PA embryos (Chang, 1954, Graham, 1970, Kaufman and Gardner, 1974, Pincus and Shapiro, 1940, Tarkowski, 1971, Tarkowski *et al.*, 1970). With time, apart from a heat shock or hypoosmotic conditions, the use of chemical compounds to activate the oocyte and block the extrusion of the second polar body has been introduced. Today, the most effective activation stimuli rely on an electric impulse, what results in about 50% of porcine embryos developing to the blastocyst stage (Grupen *et al.*, 2002). Ionomycin is a commonly used chemical activator of porcine oocytes yielding up to 30-40% of diploid blastocyst under standard embryo culture conditions. In some species, the development of PA beyond the blastocyst stage is observed (9.5 dpc in mice) however, due to the phenomenon of genomic imprinting it is impossible to obtain a parthenogenetic mammalian offspring. On the other hand altering, by genome editing, just a few imprinted genes (of over 100 known) may result in circumvention of this barrier and enable the production of a viable animal from two maternal genomes (Moore *et al.*, 2015). Kono and colleagues proved that increasing the activity of the *Igf2* gene and monoallelic expression of the *H19* gene lead to the generation of live offspring from parthenogenetic mouse embryos (Kono *et al.*, 2004). In pigs, as well as in mice, rabbits and cattle, the aberrant expression of imprinted genes in parthenogenetic embryos has its origins in the epigenetic modifications, i.e. methylation patterns of DMR (differentially methylated regions) leading to failure in placenta formation and implantation (Han *et al.*, 2013, Park *et al.*, 2009, Park *et al.*, 2011, Wang *et al.*,

2017). Interestingly, maternally and paternally imprinted genes were shown to be expressed in uniparental porcine embryos with some distinct differences between haploid and diploid parthenotes (Tilghman, 1999). Mammals cannot develop only on the basis of a uniparental genome, but parthenogenetic-normal chimaeras are spontaneously found. In human, it is possible to detect this phenomenon only by chance, as it was in the case of a boy, whose parthenogenetic cells contributed to the hematopoietic cell line (Strain *et al.*, 1995). The proposed mechanism suggests that spontaneously activated oocyte was fertilized and a male pronucleus fused with one of haploid sets of oocyte, whereas the second underwent diploidization. Alternatively, activated oocyte underwent one or more divisions followed by fertilization of one of the resulting haploid blastomeres. Earlier studies on mice led to the birth of live chimaeras generated by the injection of ICM cells obtained from PA embryos to normal blastocysts (Surani *et al.*, 1977). The ongoing debate on parthenogenetic embryos attempts to create a classification based on their developmental abilities and pluripotency status, by drawing parallels to the SCNT embryos, the iPS cells and embryos derived from IVF or conceived *in vivo*. These issues, also of a philosophical nature, have been recently described by Schickl and colleagues (Schickl *et al.*, 2017). In 2014, the European Court of Justice, while debating on the proposal allowing to patent procedures of human embryonic stem cell derivation from parthenogenetic embryos, has judged that the PA embryos do not constitute a human embryo because of their incapacity to develop into a human being. Therefore, the studies on the derivation of ESCs from human PA embryos are ongoing and, interestingly, often generate better results than the embryos originating from the IVF procedures.

Parthenogenetic embryos and ESC derivation

In vitro culture of human embryos for the sole purpose of ESCs derivation is ethically controversial. Hence the idea of using parthenogenetic embryos enables the basic research and possible biomedical application bypassing the ethical barriers. The pluripotent ESCs retain the ability to differentiate into all embryonic lineages, survive multiple passaging, express pluripotency markers, form chimaeras after injection to blastocysts and contribute to the primordial germ cell (PGC) lineage. Ideally, they should also enable subsequent multiple gene targeting for the use in xenotransplantation. ESCs should also increase the efficiency of cloning, probably due to the fact that they are in a less differentiated state than fibroblasts and thus are more prone to reprogramming. The first attempts to produce putative porcine ESCs from *in vivo* obtained embryos date back to the 1990s (Chen *et al.*, 1999, Evans *et al.*, 1990, Piedrahita *et al.*, 1990). Although *in vivo* derived embryos exhibit superior quality, their derivation is laborious and costly. A notable problem often observed in porcine IVF and PA embryos is a non-obvious ICM or only a few cells contributing to the residual ICM. It was shown that under the same culture conditions, parthenogenetic ICMs generate more outgrowths and adhere better during the initial stages of ESC derivation than ICMs originating from the IVF embryos, probably due to the increased expression level of integrin-1 and vitronectin (Brevini *et al.*, 2010). This phenomenon was earlier described in human and murine parthenotes, giving hope for high-efficiency cell line establishment (Kim *et al.*, 2007, Mai *et al.*, 2007). The protocols to obtain human parthenogenetic embryos are also very efficient and result in 24-60% blastocyst rate, with ESC derivation efficiency of 50% (Kim *et al.*, 2007, Mai *et al.*, 2007, Revazova *et al.*, 2008, Revazova *et al.*, 2007). The human parthenogenetic stem cells (hpaESCs) exhibit morphological properties similar to hESCs, express the same pluripotency markers, have comparable telomerase activity and form teratomas when injected into mice. No clinical trials have been reported so far of hpaESC application, however studies on mice and monkeys (*Macaca fascicularis, Cyno-1 cells*) clearly indicate their great therapeutic potential, as it was shown that these cell lines may differentiate into hepatocytes or dopamine neurons restoring motor function in hemiparkinsonian rats (Cibelli *et al.*, 2002, Sanchez-Pernaute *et al.*, 2008, Vrana *et al.*, 2003). Porcine paESCs expressing green fluorescent protein (GFP) and differentiated into the neural lineage were used to cure a spinal cord injury in rats and an induced periodontitis in minipigs. Both models revealed the therapeutic potential of paESCs showing significant tissue recovery with no signs of teratoma formation (Yang *et al.*, 2013a, Yang *et al.*, 2013b).

In 2010 a new approach in porcine ESC derivation was proposed with regard to culture media (minimal essential medium (MEM) with 10% serum replacement, bFGF, LIF, Activin A and human recombinant EGF) and culture conditions (5% oxygen concentration) (Vassiliev *et al.*, 2010). Day 7 *in vitro* blastocysts and day 6 *in vivo* obtained blastocysts with removed zona pellucida cultured on mouse embryonic fibroblasts (MEFs) produced homogenous outgrowths and sustained passaging after vitrification. These cell lines expressed *OCT4, NANOG* and *SSEA-1,* were able to differentiate into three germ layers and contribute to ICMs, leading to the birth of chimeric piglets (Vassiliev *et al.*, 2010). Nevertheless, additional studies are needed to prove the germline transmission and chimaera generation from later passages, to state the pluripotent nature of these ESCs. The same group showed that ESC lines may be derived with the help of trichostatin A (which was shown to improve SCNT embryo development) from nuclear transfer porcine embryos at around 10% efficiency (Beebe *et al.*, 2009, Kishigami *et al.*, 2006, Vassiliev *et al.*, 2011). These lines expressed OCT4 and NANOG (but not the trophectoderm specific markers such as the CDX2*)*, maintained stability up to 15 passages and formed embryoid bodies, although no data on chimaera formation and other characteristics have been published (Vassiliev *et al.*, 2011). MEFs are still considered to provide the best feeder layer for the culture of porcine ESCs, but a debate exists regarding the influence of LIF and FGF signalling. There are no conclusive results showing the presence of LIFR in porcine ICM and EPI. Nevertheless, studies show that LIF improves cell attachment, self-renewal and maintenance of ESC culture in the undifferentiated state, suggesting its action via the alternative pathway involving PI3K, AKT and PTEN (Brevini *et al.*, 2012, Brevini *et al.*, 2010, Brevini *et al.*, 2008).

Compared to other species, pigs demonstrate differences in pluripotency and lineage-specific marker gene expression (Wolf *et al.*, 2011). In the mouse embryo, NANOG and OCT4 are found at the morula stage, and later become restricted solely to ICM cells, with OCT4 being quickly downregulated in the TE (Palmieri *et al.*,1994). Earlier studies showed no expression of *NANOG* in porcine blastocyst and detected *OCT4* both in the ICM and in the TE cells (Kuijk *et al.*, 2008). However, our preliminary study (Pawlak unpublished) indicate the expression of *NANOG* in PA

porcine blastocysts. Moreover, *OCT4* transcripts were detected throughout the entire period of preimplantation development, therefore it is of great importance to carefully analyse the distribution of pluripotency-related markers in different species. The low expression of *NANOG* may be sufficient to take an action as a pluripotency-related transcription factor at a particular stage of embryonic development. Brevini and colleagues showed a constant expression of NANOG in porcine ESC lines, contrary to OCT4 which is downregulated after few passages, suggesting its key regulatory function at earlier (embryonic) stages in the ICM or the epiblast (Brevini *et al.*, 2010). In support of these observations, Du Puy and colleagues detected high levels of *NANOG* transcripts in the ICM of the expanded porcine blastocyst, which was maintained also in the epiblast (du Puy *et al.*, 2011). In contrast to the mouse, in porcine embryos *CDX2* begins to be expressed later in the development and its expression is restricted to the trophectoderm at day 9.5 of development (du Puy *et al.*, 2011). Other pluripotency factors such as surface markers SSEA1, SSEA3, SSEA4 also exhibit species-specific expression patterns, therefore a global gene expression analysis of porcine ICM, TE and EPI is needed to characterize the real molecular picture of embryonic development and subsequently derived ESCs.

Since only cell lines from early passages have been positively verified in the chimera assay, a question has been raised, how long the ESCs would maintain the undifferentiated state *in vitro*.

In case of pluripotent stem cell derivation, many factors need to be considered, for example the source of embryos, embryo developmental stage, whether the isolated ICMs or the whole embryos are used, the frequency of passaging and the culture media used. Haraguchi and colleagues established self-renewing porcine ES-like colonies which underwent over 100 passages, under specific LIF dependent conditions using the inhibitors of MEK/ERK (PD184352) and GSKβ (CHIR99021) signalling (Haraguchi *et al.*, 2012). The embryoid body formation and the expression of the three germ layer specific factors (in the presence of LIF and the inhibitors of MEK and GSKβ) was also shown in porcine iPSCs, suggesting the importance of MEK/ERK and WNT signalling in the establishment of the naïve-like state (Rodríguez *et al.*, 2012). Recently, a few articles provided new insights into different porcine stem cell populations derived from the early embryos. A comparative study showed that porcine ESCs established from *in vitro* obtained embryos, parthenotes and nuclear transfer iPSCs (iPS-NT) (Kim *et al.*, 2016) expressed *OCT4, NANOG, SOX2, REX1, NROB2, KLF2* and factors involved in the signalling pathways activated in human primed ESCs such as *FGFR1* and *FGFR2*. These authors also showed that iPS-NT cells have some unique characteristics - they proliferate faster, upregulate *REX1* and *FGFR2* and contain a less mature form of mitochondria, but, in contrast to other cell lines, exhibit multiple aberrant patterns of nuclear H3K27me3 foci suggesting their epigenetic instability. Earlier, Hall and Hyttel showed that FGF and JAK/STAT signalling is critical for the maintenance and proliferation of porcine pluripotent cells *in vitro*. Porcine embryos express surface markers typical for both mouse (SSEA-1) and human ESCs and iPSCs (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81). Porcine ICM and epiblast express SSEA-1, while expression of human-specific surface markers was found solely in trophoblast which points to the similarities with the naïve state of mouse ESCs and iPSCs (Hall and Hyttel, 2014, Hall *et al.*, 2010). However, the germline transmission of porcine ESCs has not been proven up to date, therefore these cell lines do not fulfil the definition of true ESCs.

The rabbit as a model in biomedical studies

The rabbit (*Oryctolagus cuniculus*) is a eutherian mammal belonging to the order Lagomorpha, family Leporidae. It is considered a laboratory and a farm animal, as well as a pet. Lagomorphs are genetically close to primates, and some researchers even suggest their close evolutionary relation (Allard *et al.*, 1996, Graur *et al.*, 1996). Rabbit stands out among non-rodent mammals used in research: it is relatively small compared to other livestock species, and it has been of interest to breeders for many generations, resulting in a variety of inbred strains. Its relatively small size, short gestation period (29-31 days), the possibility of setting up timed matings and superovulation, and resilience of the embryos poses the rabbit as an attractive candidate for early embryonic studies. Rabbit embryology resembles that of human in many aspects, notably in the general morphology of peri-gastrulation embryo, which appears in the form of a flat disc, rather than acquiring cup-shaped cylinder typical of rodents (Idkowiak *et al.*, 2004). Recent evidence shows that also X chromosome inactivation (XCI) timing and mechanism (linked to the early lineage differentiation) is similar in rabbits and humans. In both species, the *Xist* homologue is not subjected to imprinting and XCI begins later than in mice. *Xist* expression is also upregulated on both X chromosomes in rabbit and human embryo cells, even in the inner cell mass (Okamoto *et al.*, 2011). Basic embryological and reproductive techniques, such as *in vitro* culture (Jin *et al.*, 2000, Kane and Foote, 1971, Maurer, 1978, Menezo *et al.*, 1984), *in vitro* fertilization (Chang, 1959, Pincus and Enzmann, 1934) and cryopreservation (Kasai *et al.*, 1992, Liu *et al.*, 2004, Renard *et al.*, 1984, Tsunoda *et al.*, 1982) have also been established for the rabbit. In fact, the first successful *in vitro* fertilization in mammals was achieved in rabbit (Chang, 1959, Pincus and Enzmann, 1934), and the first successful embryo transfer was performed in rabbit as early as 1890 by Walter Heape (Biggers, 1991, Heape, 1890, Heape, 1897).

The use of mammals as model organisms in biomedical research depends strongly on the possibility of performing precise genetic engineering such as gene targeting (knock-in, knock-out) and creating reporter lines. Whole genome shotgun sequence at 7.48x coverage is available for the rabbit (OryCun2.0, accession number AAGW02000000). In mice, genetic modifications can be effectively introduced by homologous recombination into ESCs (Bronson and Smithies, 1994), however, germline competent rabbit ESC lines are not yet available. Previously, reporter lines such as Oct4-EGFP have been created by transfection of somatic cells and subsequent somatic cell nuclear transfer (Quan *et al.*, 2014, Yin *et al.*, 2013). Although effective, this method is very time- and labour- consuming. Zinc finger (ZFN) (Kim *et al.*, 1996) and TALEN (Boch, 2011) nucleases have been successfully used for generation of rabbit knockout lines (Flisikowska *et al.*, 2011; Song *et al.*, 2013). Recently, the CRISPR/Cas system has been emerging as an efficient gene-targeting technique with application not limited to cultured cells (Yang *et al.*, 2014). Because of its versatility and specificity, and possibility of precise genome editing *in ovo* (homologous recombination after injection of gene construct directly into one cell embryo, without the necessity of transfecting cells cultured in vitro), the CRISPR/Cas system opens new avenues for animal

transgenesis, including wider use of rabbit as model organism in biomedical research (Honda and Ogura, 2017). CRISPR/Cas system has been already used in rabbits to create gene knockouts (Honda *et al.*, 2015, Kawano and Honda, 2017, Xu *et al.*, 2018, Yang *et al.*, 2014) of single or multiple genes (Yan *et al.*, 2014), including creating rabbit models of several muscular dystrophies (Sui, Lau, *et al.*, 2018, Sui, Xu, *et al.,* 2018), as well as targeted rabbit Rosa26 knock-ins (Yang *et al.*, 2016).

Rabbit is also currently used in the studies of atherosclerosis, diabetes, lipoprotein metabolism and cardiovascular diseases (Bosze and Houdebine, 2010, Duranthon *et al.*, 2012, Fischer *et al.*, 2012, Niimi *et al.*, 2016, Püschel *et al.*, 2010). Because of the similarities between rabbit and human embryonic development and placentation, it is a popular model in pregnancy-related conditions, such as testing of developmental toxicity (Bourdon *et al.*, 2018, Martin *et al.*, 2010, Pentsuk and van der Laan, 2009) and maternal Diabetes mellitus (Navarrete Santos *et al.*, 2004 a,b, 2008, Ramin *et al.*, 2010, Thieme *et al.*, 2012). The advance of a novel gene editing techniques will enable the creation of new, more accurate rabbit models for use in biomedical studies.

Preimplantation development of the rabbit embryo and early lineage specification

Preimplantation period of rabbit embryonic development lasts for 6 days, and at the time of implantation the blastocyst reaches around 5mm in diameter and approximately 5000 cells. De novo RNA synthesis is initiated at 2-cell stage, but major genome activation occurs at 8-16 cell stage (Brunet-Simon *et al.*, 2001, Manes, 1973), coincident with degradation of maternal transcripts starting at 8-16 cell stage (Henrion *et al.*, 1997). Importantly, rabbit genome activation is achieved gradually, and maternal to embryonic transition is achieved over several cell cycles, in a manner similar to that observed in human embryos (Braude *et al.*, 1988). As in several other mammalian species, early rabbit embryonic cells exhibit developmental plasticity, and single blastomeres remain totipotent up to 8-cell stage (Moore *et al.*, 1968). Cell polarity is established in rabbit embryos just prior to cavitation, at 32–64-cell morula stage (Koyama *et al.*, 1994, Ziomek *et al.*, 1990), as shown by polarised localization of the microvilli at the surface of the outside cells.

A number of transcription factors that have been associated with pluripotency in the mouse, human, and livestock embryos, are also expressed in the rabbit. *OCT4* is expressed throughout the whole preimplantation period, from a zygote to an implanting blastocyst at 6 dpc, although the expression level decreases over time, as evidenced by mRNA expression analysis (Henderson *et al.*, 2014, Kobolak *et al.*, 2009, Mamo *et al.*, 2008, Táncos *et al.*, 2015), immunofluorescent labelling (Canon *et al.*, 2018, Henderson *et al.*, 2014, Yin *et al.*, 2013), and expression of GFP reporters (Quan *et al.*, 2014, Yin *et al.*, 2013). Recent evidence has shown that the decrease might be related to progressive methylation of the 5' regulatory region of *POU5F1* and its gradual repression in the rabbit TE (Canon *et al.*, 2018). Krüppel-like factor 4 (KLF4) is also expressed in rabbit preimplantation embryos (Henderson *et al.*, 2014, Táncos *et al.*, 2015) and embryo-derived stem cells (Táncos *et al.*, 2017). It has also been shown that overexpression of human *KLF2* and *KLF4* transcripts in rabbit iPSCs induces their reprogramming towards pluripotency (Tapponnier *et al.*, 2017). SOX2 is chiefly ICM- and EPI-specific in rabbit embryos, and is found in

nuclei only starting from mid-blastocyst stage (Henderson *et al.*, 2014, Piliszek *et al.*, 2017, Táncos *et al.*, 2015) similarly to porcine (Hall *et al.*, 2009, Liu *et al.*, 2015) and equine embryos (Iqbal *et al.*, 2014). Expression of a key pluripotency factor *NANOG* (Chambers *et al.*, 2003) is initiated in the rabbit embryo at 2-cell stage, and expressed throughout the preimplantation period (Henderson *et al.*, 2014, Táncos *et al.*, 2015), but becomes strictly EPI-specific only upon EPI compartment sorting around 4 dpc. Interestingly, although OCT4, SOX2, KLF4 and C-MYC epitope regions show high homology between rabbit and human, only low homology has been observed in case of NANOG (Táncos *et al.*, 2015).

TE differentiation in the rabbit shows certain similarities to other non-rodent mammals. CDX2 is a transcription factor associated with TE, as in the mouse (Strumpf *et al.*, 2005) and other mammals (Berg *et al.*, 2011, Madeja *et al.*, 2013). However, in the rabbit (Piliszek, unpublished data), and pig embryos (Bou *et al.*, 2017, Liu *et al.*, 2015) *CDX2* is not expressed at morula and early blastocyst stage (the specific mRNA and protein are first detected only after the blastocyst cavity has been established), while human expanded blastocysts have no nuclear-localised CDX2 signal (Deglincerti *et al.*, 2016). Additionally, *OCT4* downregulation appears to be dispensable for TE specification in non-rodent mammals, because prolonged OCT4 expression in the TE and colocalization with CDX2 can be observed in rabbit (Kobolak *et al.*, 2009, Piliszek, unpublished data) as well as human (Kirchhof *et al.*, 2000), bovine (Berg *et al.*, 2011, Kuijk *et al.*, 2008, Madeja *et al.*, 2013), horse (Choi *et al.*, 2009, Iqbal *et al.*, 2014) and dog embryos (Wilcox *et al.*, 2009). A similar, prolonged expression of PrE-associated transcription factor GATA6 in TE is found in both rabbit (Piliszek *et al.*, 2017) and human (Deglincerti *et al.*, 2016) embryos up until day 6 of development. It should be noted that the expression (mRNA) of *OCT4*, *KLF4*, *NANOG* and *SOX2* is significantly higher in *in vitro*-cultured rabbit embryos than in their *in vivo* derived counterparts, potentially suggesting impaired differentiation towards extraembryonic lineages *in vitro* (Henderson *et al.*, 2014).

Mutual repression of *Gata6* and *Nanog* has been suggested by many authors as a mechanism driving the differentiation of PrE and EPI in the mouse (Bessonnard *et al.*, 2014, Frankenberg *et al.*, 2011, Singh *et al.*, 2007). In the rabbit embryos, GATA6 becomes downregulated in a subset of ICM cells around mid-blastocyst stage, but NANOG is found in all ICM cells at this stage, up until the final differentiation and segregation of PrE and EPI layers (Piliszek *et al.*, 2017), suggesting that the mechanism of PrE versus EPI specification might rely on additional factors. Other lineage-associated transcription factors, SOX2 (EPI) and SOX17 (PrE) show a much more restricted expression pattern in the rabbit embryo. Their expression is initiated at the mid-blastocyst stage, and from the onset is partially mutually exclusive, although a bi-positive cell population can be detected at this stage (Piliszek *et al.*, 2017).

In the mouse embryo, it has been extensively shown that PrE versus EPI specification depends on the activity of FGF/MEK/ERK signalling (Kang *et al.*, 2013, Nichols *et al.*, 2009, Yamanaka *et al.*, 2010). In other mammals, a modification of this pathway activity has varying effects. In human embryos, the specification of PrE is not affected by inactivation of FGF/MEK/ERK pathway (Kuijk *et al.*, 2012; Roode *et al.*, 2012). In the rabbit, similarly to the mouse, the activity of the MEK/ERK signalling pathway is necessary and sufficient to trigger cell differentiation towards the PrE (Piliszek *et al.*, 2017). Blocking of MEK/ERK signalling is also sufficient for

ablation of PrE in the rabbit. The remaining SOX2-positive EPI cells, unlike in mouse embryos, do not express the high NANOG levels characteristic for the naïve pluripotency (Nichols *et al.*, 2009; Piliszek *et al.*, 2017), which might potentially explain the differences in the derivation of pluripotent stem cell lines from mouse and rabbit embryos.

Derivation of rabbit pluripotent stem cell lines

Establishment of naïve pluripotent rabbit ESCs is of great interest because of their potential use in basic research, derivation of transgenic animals, and as a model in the establishment of stem-cell based therapies for human and animal medicine. Many research groups have reported derivation of rabbit ESCs or stem-like cells (Fang *et al.*, 2006, Graves and Moreadith, 1993, Honda *et al.*, 2008, Honda *et al.*, 2009, Honda, 2013, Intawicha *et al.*, 2009, Moreadith and Graves, 1992, Osteil *et al.*, 2016, Wang *et al.*, 2007, Zakhartchenko *et al.*, 2011). Currently available rabbit ESCs are morphologically and molecularly different from mouse ESCs, however, they share many characteristics with human, primate and other non-rodent ESCs (du Puy *et al.*, 2011, Tan *et al.*, 2011). Several features indicate that non-rodent ESCs exhibit primed- rather than the naïve state of pluripotency (Nichols and Smith, 2009), and are functional counterparts of mouse EpiSCs, rather than ESCs (Greber *et al.*, 2010). Indeed, they express pluripotency factors typical of mouse primed epiblast cells (Osteil *et al.*, 2013, Schmaltz-Panneau *et al.*, 2014). Primate and rabbit ESC lines also share the ability to differentiate into TE, while it is lost in mouse ESCs (Ginis *et al.*, 2004, Tan *et al.*, 2011). To maintain their characteristics, rabbit and human ESCs require FGF2 and Activin/Nodal, and not LIF (Ginis *et al.*, 2004, Honda *et al.*, 2009, Hsieh *et al.*, 2011, Wang *et al.*, 2007), while mouse ESC lines cultured without a feeder layer require LIF (Ginis *et al.*, 2004, Wang *et al.*, 2007). In a recent study, Osteil and colleagues meticulously analysed several rabbit ESC derivation conditions, including variations of dissociation methods and culture media, revealing a range of pluripotency states that cell lines acquire *in vitro* (Osteil *et al.*, 2016). Similar to human ESCs and mouse EpiSCs, rabbit ESCs are rarely able to colonize the ICM in chimaeras and do not contribute to the germline. The most successful rabbit ESC integration so far was shown by Zakharchenko and colleagues, who obtained one life born animal (out of 198 chimaeric embryos), of confirmed somatic chimaerism, which died 60 days after birth, and therefore germline transmission was never assessed (Zakhartchenko *et al.*, 2011). Other researchers reported a rapid loss of chimaerism in rabbit preimplantation embryos after the introduction of rabbit ESCs or iPSCs (Osteil *et al.*, 2013, Osteil *et al.*, 2016, Tapponnier *et al.*, 2017). Existing rabbit ESC lines are nonetheless capable of self-renewal over several passages, exhibit normal karyotype, and are well characterised by the gene expression profiling, teratoma formation and differentiation into derivatives of three germ layers *in vitro* (Honda *et al.*, 2009, Osteil *et al.*, 2013, Osteil *et al.*, 2016., Wang *et al.*, 2007). Several groups have also reported derivation of rabbit iPSCs, which share similar properties (Honda *et al.*, 2010, Táncos *et al.*, 2017, Tapponnier *et al.*, 2017).

Although mouse-like, naïve pluripotent embryonic or induced stem cells are not available in rabbit, the existing cell lines are more representative of other non-rodent mammalian cell lines, including human. They can be differentiated *in vitro* towards endoderm, epiblast and mesoderm derivative tissues, and in combination with an increasing number of rabbit disease model lines, present an attractive model for testing efficiency and safety of stem-cell based therapies.

Conclusions

To broaden our understanding of the basic mechanisms underlying mammalian development and differentiation it is necessary to access the data from phylogenetically different groups of mammals. The increasing interest in farm animal research (large animal models) not only expands our knowledge of the cellular and the molecular mechanisms of development through comparative studies but also equips us with better tools for biomedical research. Bearing in mind the complexity of mammalian preimplantation development and species-specific differences, it is advisory not to concentrate on a limited number of model species to study human (or other species) early embryonic development, but to divide these processes into specific stages/events, and for each find the appropriate counterpart.

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