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REVIEW

# Factors affecting early embryonic development in cattle: relevance for bovine cloning

**Yanna DANG, Kun ZHANG (✉)**

**Laboratory of Mammalian Molecular Embryology, Institute of Dairy Science, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China**

**Abstract** Female infertility represents a major challenge for improving the production efficiency in the dairy industry. Historically, fertility has declined whereas milk yield has increased tremendously due to intensive genetic selection. *In vivo* evidence reveals about 60% pregnancy loss takes place during the first month following fertilization. Meanwhile, early embryo development is significant for somatic cell nuclear transfer in cattle as a large proportion of cloned embryos fail to develop beyond peri-implantation stage. Oocyte quality is of utmost importance for the early embryo to develop to term for both fertilized and cloned embryos. Epigenetic reprogramming is a key process occurring after fertilization and critical roles of epigenetic modifiers during preimplantation development are now clear. Incomplete epigenetic reprogramming is believed to be a major limitation to cloning efficiency. Treatment of cloned embryos with epigenetic modifying drugs (e.g., Trichostatin A) could greatly improve cloning efficiency in both mice and cattle. Recently, the rapid progress in high-throughput sequencing technologies has enabled detailed deciphering of the molecular mechanisms underlying these events. The robust efficiency of genomic editing tools also presents an alternative approach to the functional annotation of genes critical to early development.

**Keywords** bovine cloning, embryo development, somatic cell nuclear transfer, X-inactive specific transcript

## 1 Bovine embryo development

A calf's life is initiated by the union of two highly differentiated gametes, an oocyte and a sperm (Fig. 1). During the first 2–3 weeks, the embryos are floating and migrating through the oviduct, entering the uterus, and elongating prior to attaching to the uterus endometrium for pregnancy establishment[2]. Briefly following fertilization, the zygote cleaves several times and then moves to the uterus on day 4 after feritilization. As a unique mechanism, the majority of mRNA, proteins and energy substrates consumed in early embryos are derived from the maternal source (i.e., the oocyte) since RNA synthesis is globally silenced in the cleaved embryos until the embryo genome is activated primarily at the eight-cell stage in cattle[3]. At the 16-cell stage, the embryo, usually called the morula, is polarized while blastomeres at this stage are difficult to distinguish under a regular stereo microscope[4]. Compaction takes place at the 32-cell stage, which is much later than in mice[5]. It has been believed that the outsides cells of a late morula would develop into the trophectoderm lineage and the inner cells migrate to one pole of the embryo to give rise to the inner cell mass lineage, which forms a special cavity-containing structure, namely the blastocyst[6]. The blastocyst is formed shortly after the entry of early embryos into the uterus followed by hatching from the zona pellucida by day 9[2].

Upon hatching, the spherical embryo grows rapidly in size and develops a tubular morphology before day 13. Considering the general similarity of development between fertilization and blastocyst stages among mammals, the early bovine embryo shows a characteristic of conceptus elongation[2,7]. This elongation generally occurs exclusively in the trophoblast and the recognition of maternal pregnancy is via the production of a critical molecule, interferon tau (IFNT). Maximum production and secretion of IFNT by the trophoblast usually occurs between day 13 and 17, which eventually triggers the attachment of the embryo to the uterus endometrium 18–22 d after ovulation[2,7].

Assisted reproduction technologies, including *in vitro* maturation (IVM) of oocytes and *in vitro* culture (IVC) of embryos, not only facilitate the study of fundamental developmental processes in early embryos but contribute extensively to infertility treatment, selective breeding and species conservation. Although the optimal requirements

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Correspondence: kzhang@zju.edu.cn

of oocytes and embryos *in vitro* have been intensively studied, the state of the art *in vitro* production system is still inferior to the normal *in vivo* environment of oocytes or embryos[8,9]. An important limitation of *in vitro* manipulation of embryos may be the lack of optimal bidirectional communication between the mother and the embryo[7]. In particular, improvements in *in vitro* culture conditions for bovine embryos have been limited given the higher efficiency that has been achieved for mouse and human IVC. Moreover, although *in vitro* development of fertilized embryos can be extended to day 14 in humans[10,11], it is usually only feasible to culture bovine embryos to blastocyst stage, and then only with a poor success rate (20%–50%)[12–14].

As described above, early embryos grow in a short developmental window, however, with a series of dynamic morphological, molecular and developmental changes. These changes are sensitive to environmental, physiological and nutritional challenges, which may lead to early embryo death. The goal of the present review is to highlight the factors and challenges affecting early embryo development, and to discuss the potential options for improving reproductive efficiency in cattle. Emphasis is placed on dairy cattle and relevance to bovine cloning as early embryo loss is relatively frequent in this context.

## 2 Challenges in early embryo development in cattle

### 2.1 Early embryos loss in dairy cattle

In practice, early embryo loss during the first month after insemination has been a significant challenge in dairy cattle as it accounts for approximately 60% of total embryo/conceptus loss during pregnancy (Fig. 1), which is a major contributing factor that results in poor fertility in dairy cows[15]. It has been observed that the calving rate is only 35%–40% after a single insemination in modern dairy cows and it is even lower in high yielding dairy cattle[15–17]. To make things worse, over the last half century, the fertility of dairy cows has decreased as evidenced by the dramatic decline in fertility of dairy cattle populations in the USA and globally[1,15,18,19]. In particular, daughter pregnancy rate, which refers to an estimate of the fertility of a bull's daughter to become pregnant in a 21-d period, has decreased from over 30% in 1950s to about 24% today, according to USDA statistics[2,19].

Accumulating evidence has indicated the time frame of early embryo loss. Fertilization seems normal for most cows as fertilization rates above 80% has been commonly observed in a range of independent studies[1,15]. Growing evidence demonstrates 30%–50% embryos are lost during the first week after insemination (Fig. 1), suggesting a critical role of development between fertilization and the blastocyst stage[1,15,20]. The second peak time for embryo loss occurs during peri-implantation development (Fig. 1), i.e, concurrent with trophoblast elongation and the secretion of IFNT[1,15].

### 2.2 Problems with early embryos produced by assisted reproduction technologies including somatic cell nuclear transfer

Numerous studies have shown that early embryos are vulnerable under *in vitro* culture conditions, which can lead to negative developmental outcomes, including early

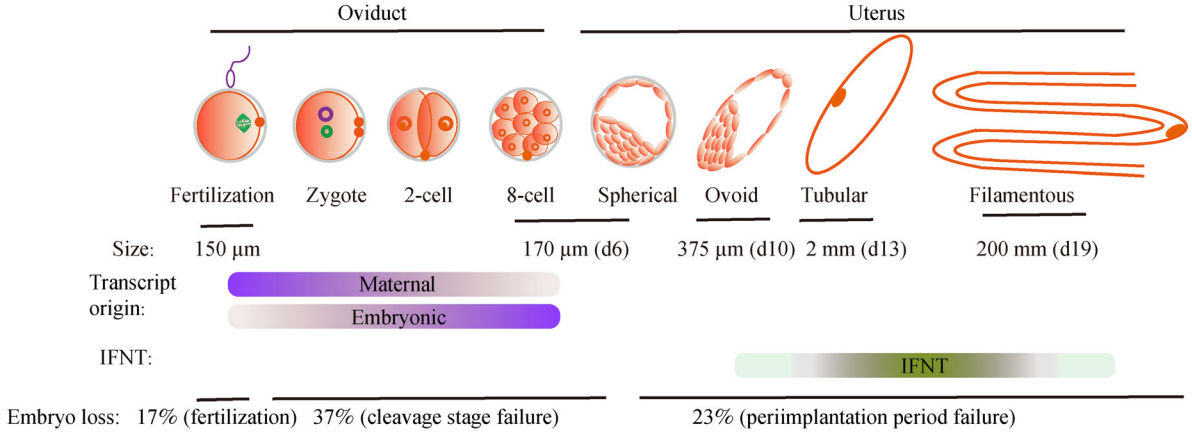


**Fig. 1** Overview of early embryonic development in cattle and critical developmental events. Upon fertilization, the embryo cleaves three times in the oviduct before entering the uterus. Once the blastocyst forms, the embryo's volume increases rapidly and the trophoblast elongates. Oocyte-derived molecules (e.g., mRNA and proteins) are the major drivers during initial development until the embryonic genome is activated at the eight-cell stage. The synthesis and secretion of IFNT from the elongated trophoblast is crucial for the maternal recognition of pregnancy. According to the statistics of Sartori et al.[1], the fertilization rate is high, however, the majority of pregnancy loss is observed during early embryonic development with 37% cleavage failure and 23% peri-implantation loss.

embryo death[16,21]. These environmental impacts include uncharacterized additives (e.g., serum), temperature, humidity, light and embryo density. To illustrate, embryos grown in media containing serum have reduced viability[22]. These embryos developed to day 14 after transfer and were 20% smaller than those produced from embryos cultured without serum or produced *in vivo*. Moreover, preimplantation embryos from ruminants are also vulnerable to environmental stressors.

Somatic cell nuclear transfer (SCNT) has been successfully used in a number of mammals for applications in animal breeding and therapeutic cloning. It appears that SCNT embryos have similar developmental potential to the blastocyst stage compared to that of *in vitro* fertilized (IVF) embryos[23]. It is believed most embryos losses occur during the peri-implantation stage leading to pregnancy failure, although, fetal losses are also observed until late gestation[23]. Consequently, the birth rate after embryo transfer is lower for SCNT than IVF. However, a plethora of aberrant phenotypes occur in the fetus and placenta for embryos generated by assisted reproduction, such as large offspring syndrome[24,25].

These abnormal phenotypes may be due to the abnormal epigenetic reprogramming of the transferred somatic donor cell from a differentiated status to a totipotent state[23,26,27]. Altered global DNA methylation has been observed in bovine SCNT embryos compared to IVF embryos and *in vivo* produced counterparts during preimplantation stages of development[26–28]. Developmental events that are heavily regulated by epigenetic mechanisms including X chromosome inactivation and genomic imprinting are also impaired in bovine SCNT embryos[29,30].

## 3 Factors influencing early embryo development

### 3.1 Genetic

Despite the low heritability for most fertility traits, accumulating evidence using state-of-the-art genome sequencing approaches has shown that reproductive functions are associated with various genomic loci or variations[31,32]. For example, mutations in genes, including progesterone receptor[33], FGF2[34] and STAT5A[35], are linked with embryonic developmental potential. According to data from the International Mouse Phenotyping Consortium, one quarter of all gene knockouts analyzed reveal themselves lethal and a large proportion of them are lethal to early embryos[36]. Thanks to the advent of the genome selection approach, it is feasible to select elite cows by avoiding homozygous mutations that may cause early embryonic loss in cattle.

*In vitro* production of embryos presents an excellent model for delineating fundamental genetic control in early bovine embryos. Both high-throughput sequencing and candidate approaches have been used to identify putative critical genes for early embryos. By using siRNA-mediated silencing tool, we have discovered and characterized multiple genes, including *Follistatin*[12], *JY-1*[37], *ZNFO*[38], *CHD1*[39], *H3F3A/H3F3B*[40], that are critical in early embryo development in cattle. Their special roles and potential mechanisms have been discussed in a recent review[41].

### 3.2 Follicle and oocyte quality

During preimplantation development, particularly for the first couple of days after insemination, the embryos are substantially dependent on maternally stored factors derived from the oocyte[41]. Oocyte quality, also termed oocyte competence, denotes the ability of an oocyte to complete meiosis, fertilization and cleavage, to proceed to early embryonic development, and eventually give rise to a healthy offspring[42]. Currently, poor oocyte quality is one leading factors associated with reproductive failures in dairy cattle[43]. It seems that oocyte quality could be disrupted by lactation in high yield dairy cattle, which may suggest there is a negative genetic correlation between fertility traits and milk yield traits[31].

The acquisition of oocyte competence is primarily dependent on the environment of the follicle in which the oocyte resides[44,45]. Indeed, a series of autocrine, paracrine (e.g., GDF9, BMP6 and FGFs) and endocrine (e.g., FSH, LH and estrogen) factors are pivotal in the growth and development of ovarian follicles, and thus impact oogenesis[45–47]. These extra and intrafollicular molecules have been demonstrated as crucial components of a signaling network controlling ovarian follicle development[48].

The role of progesterone in dairy cow fertility has received considerable attention[49–51]. It has been claimed that a reduction of blood progesterone could be responsible for reduced fertility in dairy cows[2]. The justification behind this hypothesis is that the metabolic activity is increased in the liver of high yielding dairy cattle and progesterone is subject to increased degradation. Administration of progesterone after insemination may improve fertility in dairy cattle[49,52,53]. However, the specific role of progesterone in oocyte quality and early embryonic development is still debatable[49].

Oocytes used as ooplasm recipients for SCNT are typically obtained from an abattoir, and the genetic background of these oocytes varies. Oocytes thus derived are matured *in vitro* under specific laboratory conditions. However, IVM conditions, including medium, air composition and PH, are still inferior relative to the *in vivo* conditions, which compromises the oocyte quality[21,23,54]. Thus, it is common that the rate of development to the blastocyst stage is lower in *in vitro* matured oocytes used

for SCNT than in *in vivo* matured oocytes[55].

### 3.3 Epigenetic

Epigenesis was coined and defined by Conrad Waddington as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being"[56]. More commonly, epigenetics is the study of heritable changes in gene function that are not the result of variation in DNA sequence. DNA methylation and histone modification are two the most intensively studied epigenetic modifications[57].

DNA methylation: a type of post-replication covalent modification, which almost exclusively occurs in position 5 of cytosine, as well as CpG islands in mammals. By this modification, the chromatin is generally repressed, which therefore inhibits promoter activity[58]. Moreover, this process is catalyzed by DNA methyltransferases using S-adnosylmethionine as the methyl donor. It was in 1975, Holliday and Pugh[59] first proposed that DNA methylation might be involved in the maintenance of gene expression or chromatin structure. DNA methylation is now claimed to be vital for maintaining the stability of gene expression states, such as, X chromosome inactivation, gene imprinting and heterochromatin formation[60].

Genome-wide demethylation of parental chromatin occurs following fertilization, with methylation reinitiated from the 16-cell stage onward in early bovine embryos[61,62]. The mechanisms underlying the demethylation observed in early embryos have recently been identified[61,63]. A novel DNA methylation form, 5-hydroxymethylcytosine, and its modifier Tet3 have been extensively shown to be critical for this demethylation process[63,64]. Recent research has analyzed and mapped 5-methylcytosine and 5-hydroxymethylcytosine sites in early bovine embryonic stages[65]. Detail of the progress can be found in recent reviews[66–69].

Histone modification: chromatin in eukaryotes characteristically shows extensive compaction whereby 2 m of DNA is packed into cells of about 20–30 μm in diameter[70]. The compaction is used to protect genetic material and regulate DNA replication and RNA transcription. The basic unit of chromatin, the, nucleosome consists of 147 bp of DNA coiled around an octamer of histone proteins. The histone octamer consists of two copies of each histone protein (H2A, H2B, H3 and H4) with N-terminal tails protruding. Accumulating evidence indicates the core histones are subject to a growing list of more than 100 different post-translational modifications, including acetylation, methylation, ubiquitylation, phosphorylation and sumoylation[70,71].

Upon fertilization, protamines enriched in sperm chromatin are replaced by canonical histones when dramatic histone modifications occur[66,72]. Studies in somatic cell lines determined that multiple histone modifications, such as histone H3 lysine 4, histone H3 lysine 36 and histone H3 lysine 79 methylation, are positively associated with gene activation, while the methylation of histone H3 lysine 9, histone H3 lysine 27 (H3K27) and histone H4 lysine 20 are generally involved in silencing gene expression[70,73].

The specific roles of these histone modifications in early bovine embryonic development have only became apparent in recent years. H3K27 tri-methylation (H3K27me3) level appears dynamic in early bovine embryos with a sharp decline from the germinal vesicle to the eight-cell stage and an increase at the blastocyst stage, suggesting a critical role of H3K27me3 in early embryonic development[74]. Further analysis revealed Jumonji domain-containing 3, an active component of polycomb repressive complex 2, is responsible for the removal of H3K27me3 during early bovine development[75].

Aberrant epigenetic reprogramming, including DNA methylations and histone modifications, have been reported in SCNT embryos[23] (Fig. 2). During the last decade, two milestones have been passed that laid a foundation for the dramatic improvement in developmental potential of cloned embryos. First, the treatment of cloned embryos with trichostatin A (TSA), a histone deacetylases

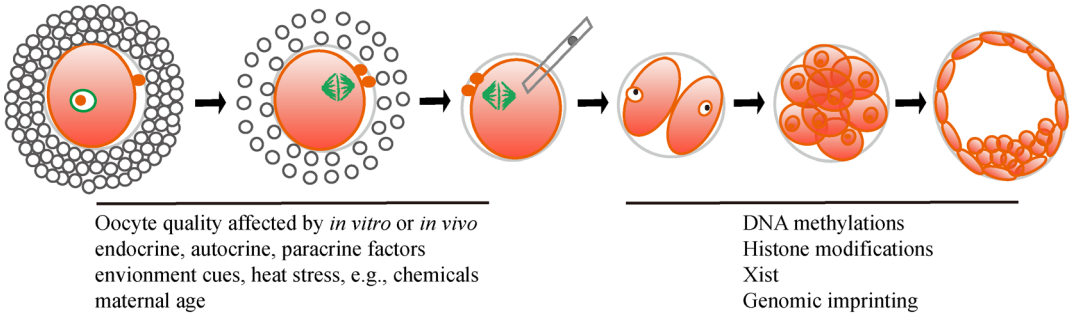


**Fig. 2** Critical factors influencing bovine cloning efficiency. The oocyte provides ooplasm to reprogram the differentiated somatic cell to a totipotent state, suggesting a crucial role for oocyte quality in the development of cloned embryos. Oocyte quality is influenced by a variety of endogenous (genetic) and exogenous factors, including hormone milieu and heat shock. Incomplete epigenetic reprogramming is frequent in cloned embryos with aberrant DNA methylations and histone modifications. Xist, X-inactive specific transcript.

inhibitor, which can significantly improve not only the blastocyst rate but also the full-term development success rate in mice[76,77]. Similar beneficial effects have been observed in cattle[78,79]. Second, transcriptomic analysis of cloned embryos has revealed that abnormal expression of certain histone modifiers is responsible for developmental failure of cloned embryos[80,81]. However, it remains unclear if this phenotype also occurs in cattle. Furthermore, complete rescue of all the cloned embryos is not currently feasible with a large proportion of cloned embryos still failing to develop to term[76,80,81].

### 3.4 Heat stress

In summer or after fever, a large reduction in female fertility in dairy cattle has been observed[82]. *In vitro* evidence indicates that elevated temperature can directly affect embryos by reducing their viability [83–85]. Early embryos gradually acquire the capability of thermotolerance via balancing free radical production and antioxidant protection and apoptosis[85]. Studies have shown that heat shock is detrimental to the developmental potential of zygotes and 2-cell embryos, however, 4- to 8-cell embryos are less sensitive and morulae are more resistant to heat shock.

The mechanisms by which embryos adjust to this stress have been studied, including altered hormone secretion, follicular development, steroid production and uterine blood flow[85,86]. Similar to other cell types, embryos after heat shock undergo a series of biochemical responses induced by the high temperature. For example, heat shock protein 70 family proteins are the most notable molecules contributing to induced thermotolerance[87]. In mice and cattle, the induced thermotolerance response has been observed in preimplantation embryos and is developmentally regulated[88–90]. Accumulating evidence indicates mitochondria are prone to damage following heat shock with their main functions, including apoptosis and oxidative stress, being disrupted[85].

### 3.5 Embryokines

Embryokines were first described by Hansen et al.[91] as molecules produced and secreted by maternal reproductive tracts that are critical for the proper development of early embryos. A few embryokines produced by oviducts or the uterus have been identified and characterized[92–94]. Thus, it is rational to believe that oocytes and embryos cultured *in vitro* are less competent than their *in vivo* counterparts due to the suboptimal conditions[95,96]. To illustrate, one such molecule, colony stimulating factor 2 (CSF2), is synthesized and secreted by the oviduct and endometrium. Exposure of the early embryos to CSF2 can improve the pregnancy rate and birth rate, potentially through modulation of apoptosis and cell proliferations in ICM[97,98]. The maternal age and metabolic status of the cow can affect the microenvironment of the reproductive tract. For example, the potential of embryos to develop to the blastocyst stage is reduced in postpartum lactating cows relative to nulliparous heifers[99] and non-lactating cows[100].

## 4 Conclusions and perspectives

### 4.1 Call for fundamental work in early bovine embryos

Understanding of the early embryonic development in mammals is far from complete, especially the molecular mechanisms governing critical developmental events, including epigenetic reprogramming, EGA, first lineage specification and implantation. Fundamental research in early mammalian embryos has usually been limited by scarcity of samples for molecular and biochemical assays. However, rapid progress in high-throughput analysis of genomic, transcriptomic and proteomic information with limited samples has greatly enhanced the capacity to dissect critical mechanisms of early embryonic development[101,102].

Most information on the mechanisms of early development is derived from rodent models. Functional annotation of critical genes in early embryos of large animals is usually dependent on the use of molecular inhibitors, recombinant growth factors or siRNAs delivered by microinjection, but all these approaches have limitations. Recent progress in genomic editing tools [e.g., clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)] will expand this toolkit and greatly enhance understanding of the fundamental principles of early bovine embryonic development.

### 4.2 Epigenetic solutions for improvement of SCNT efficiency

Certain chemicals, such as TSA, have dramatically improved SCNT efficiency, possibly by correction of epigenetic defects caused by SCNT[76]. Manipulation of X chromosome-linked gene expression can also increase cloning efficiency[30]. X-inactive specific transcript (*Xist*) knockdown in SCNT embryos can restore the genome-wide gene expression and increase cloning efficiency 10-fold[30]. *Xist* expression is also upregulated in SCNT embryos of cattle[29]. Further investigation of *Xist* knockdown in SCNT embryos might prove helpful for improving full-term development in cattle. Future study of the regulation of bovine epigenetic reprogramming in SCNT embryos is critical for providing novel insights required for developing drugs that may improve cloning efficiency.

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