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The Factors Affecting Cloning Bovine Embryo

-총설-

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소 수정란 핵 이식에 영향을 주는 요인

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적 요

핵 이식 기술을 이용한 cloning 송아지 생산이 처음 보고(Prather *et al.*, 1987) 된 후, 소 수정란 Cloning에 대한 많은 연구가 분자 생물학 등 여러 분야에서 꾸준히 계속되고 있다. 이 기술은 빈우의 번식 능력을 향상시켜 유전적 개량량을 증대할 수 있는 번식과 육종을 위한 도구로써 많은 잠재력을 가지고 있다. 최근 핵 이식 기술을 이용하여 유전적으로 우수한 빈우로부터 수천개의 수정란이 생산되어, 이들 수정란에서 생산된 송아지가 번식 축군으로 공시되어 있으므로, 그 결과가 주목되나 아직까지는 비용이 많이 들고 송아지 생산 효율이 저조하므로, 가까운 장래에 일반 양축가에 이용될 가능성은 낮다. 그러나 이 기술의 실용화를 위하여 선결되어야 할 많은 문제점들 중, 지난 몇 년 동안 많은 연구기관에서 수행된 활발한 연구의 결실로써, 난포란 제핵, cell fusion 과 oocyte activation의 방법등 주요 장애 요인들이 점차 극복되면서 실용화를 위한 접근이 예견되어지며, 구미의 일부 개량 기관에서는 이를 상업화 하기 위한 제반 여건을 다지고 있다. 그러므로 이 Review에서는 follicular dynamics, 난포란의 성숙, cell cycle, 난포란 제핵, cell fusion과 oocyte activation, 이식후 핵의 remodeling과 reprogramming에 대한 현재까지의 보고된 자료를 기초로 그 기본 원리를 재고하는데 초점을 맞추었다.

Key words : bovine, embryo cloning, nuclear transfer, oocyte activation, reprogramming

INTRODUCTION

Nuclear transfer in bovine which provides multiplication and production of identical embryos, offers the potential of producing an unlimited number of identical offspring of genetically superior animals. The production of cloned animals by nuclear transfer would be important for evaluation and selection of elite breeding cattle. The benefit of producing identical animals is that performance and production costs could be predicted more accurately by decreasing the large variability among animals. Accurate studies of interactions between genotype and environment are also possible with using fewer identical animals for experimentation. Maintenance of a clonal line in frozen storage while clones are tested would facilitate rapid advances in genetic improvement of livestock agriculture. This would benefit producers and processors who would be able to predict their product. With the increase in rate of genetic change possible, breeders could change

their product as consumer demand fluctuates. Use of a breeding program involving the production of an elite cattle group would result in greater genetic progress than is possible with conventional methods involving artificial insemination and embryo transfer (Robl and Stice, 1989).

Procedures used for nuclear transfer are too costly and relatively inefficient to justify large scale commercial use of the techniques. Thus, embryo cloning is still in the developmental phase. This review, therefore, will be focus on follicular dynamics, oocyte maturation, embryonic cell cycle, oocytes enucleation, cell fusion and oocyte activation, cytoplasm inheritance and nuclear remodeling and reprogramming.

FOLLICULAR DYNAMICS

Sexual differentiation, formed bovine fetal ovary, is achieved by d 39 of gestation (Shemesh, 1980). The first growing follicles that consisted mainly of primary and secondary follicles in fetal ovaries appeared around d 180 of gestation and few antral follicles were present before d 220 of gestation (Wandji *et al.*, 1992). A female is endowed with her entire life's complement of oocytes at birth, of the order of one million. Primordial follicles are present in cohorts of non-growing follicles in the ovaries of the heifer at birth (Roche and Boland, 1991). Between birth and puberty, this number decreases to around 100,000 to 400,000, constituting the pool of resting or dominant primordial follicles from which ovulatory follicles will develop in the process of folliculogenesis. This number remains stable until about the fourth year of life, after which it declines (Erickson, 1966).

The process of folliculogenesis can be divided somewhat arbitrarily into several discrete steps including initiation of growth, recruitment into the pool of potential ovulatory follicles, and selection of dominant follicles, ending in ovulation (Armstrong, 1991). Gonadotrophins are of utmost importance in the control of recruitment, selection and dominance. Recruitment is triggered by FSH. The stimulatory effect of FSH on follicular differentiation will be more marked on the largest and weaker on the smaller follicles of the cohort of recruited follicles. Selection could be achieved in a way where the largest maturing follicle indirectly inhibits the growth of less mature follicles by reducing FSH concentrations below the threshold necessary to maintain other follicles. IGF-I, by amplifying the FSH action on aromatization and increase in number of LH receptors, plays a key role in the maintenance of dominance (Driancourt, 1991). Increased LH support appears to maintain a dominant follicle, whereas high progesterone concentrations decrease LH pulse frequency leading to turnover of the dominant follicle in cattle (Savio *et al.*, 1993). Dominant follicles apparently control development of their follicles through production of hormones, such as estradiol, inhibin, activin, follistatin, and other secretory products (growth promoting or inhibiting factors), which may act locally, systematically, or both locally and systematically (Lucy *et al.*, 1992). Final maturation and ovulation of a dominant follicle require an increase in LH pulse frequency, so that androgen and estrogen synthesis are stimulated sufficiently to give rise to a gonadotropin surge (Roche and Boland, 1991).

The number of follicles of the vesicular or graafian stage is in the range of 24 to 50 at a given time (Armstrong, 1991). The number of follicles with a diameter >70 um may vary between 53 and 881 in the bovine adult ovary (Monniaux *et al.*, 1983). The vast majority of

oocytes is destined to be lost through the process of atresia at various stages of follicular development (Armstrong, 1991). The process of follicular maturation takes four to five months, during which the oocyte increase about 30-fold in volume (Seidel and Elsden, 1989).

OOCYTE MATURATION

Meiosis begins around 75 of gestation (Shemesh, 1980) and by d 170, the majority of the oocytes are arrested at the pachytene stage (Russe, 1983). Oocytes become arrested at the diplotine (dictate germinal vesicle) stage of prophase I of meiosis during early postnatal life, until they are committed to ovulation or atresia. As the germ cells near completion of growth they are surrounded by a layer of granulosa cells, become competent to resume meiosis (Buccione *et al.*, 1990), and remain in meiotic arrest for many years. In cattle the oocytes are still growing when follicles reach the early antral stage (Motlik and Fulka, 1986). The follicular antrum is fully differentiated in follicles of about 0.5 mm in diameter and the presence of follicular fluid allows isolation of cummulus-oocyte complexes (Motlik, 1989).

Each oocyte at the end of its growth period contains a single large nucleus or germinal vesicle (GV). The interphase nucleus is referred to as a GV at this point. Changes in nuclear status of oocytes throughout maturation *in vitro* are identified by four different steps based on evaluation of chromosome status. Protein synthesis is required for germinal vesicle breakdown (GVBD). The GV was present from 0 to 6.6 h of culture, GVBD at 6.0 to 8.0 h, chromatin condensation at 8.0 to 10.3 h, metaphase I at 10.3 to 15.4 h, anaphase I at 15.4 to 16.6 h, telophase I at 16.6 to 18.0 h and metaphase II at 18.0 to 24 h (Sirard *et al.*, 1989).

The nuclear and cytoplasmic maturation of bovine oocytes is accompanied by a shift in follicular steroid production from being estradiol-dominanted before the LH to an increasing dominance of progesterone after the surge. Meiosis I is resumed after the preovulatory surge of LH. Oocyte maturation includes disruption of gap junctions between cummulus-cell projections and oocyte, and the breakdown of the envelope of the oocyte nucleus within 12 h after the LH peak. This junctional breakdown was paralleled by resumption of oocyte meiosis. At -15 h after GVBD metaphase of the first meiotic division occurs and spatial rearrangements of mitochondria and vesicles are seen in the ooplasm. At -19 h the first polar body is abstricted and the second metaphase appears. Subsequently, it degenerates and the polar body is generally not seen more than 7 h after ovulation (Hyttel *et al.*, 1988). At 21-22 h the cortical granules migrate to solitary positions along the oolemma, the golgi compartment decreases, and the smooth endoplasmic reticulum (SER) transforms. Ovulations occur in cattle at -24 h after the LH peak (Hyttel *et al.*, 1989). In cattle induced to superovulate by gonadotropin stimulation the ovulations start around 24 h and continue up to 33 h after the peak (Callesen *et al.*, 1986). In the period from the LH peak to ovulation the bovine oocyte completes a nuclear and cytoplasmic maturation (Kruip *et al.*, 1983; Hyttel *et al.*, 1986).

EMBRYONIC CELL CYCLE

The meiotic maturation of oocytes, fertilization and embryo development are all events influenced by cell cycle regulation. Success of embryo development is dependent on the correct functioning cell cycle control. The cell cycle consists of essentially two stage, mitosis and

interphase. The M phase refers to the period of mitosis and entails karyokinesis and cytokinesis of a parent cell resulting into daughter cells. Interphase is divided into three periods; G1 - the gap between the end of mitosis and the beginning of DNA replication, S - the period during which DNA replication occurs, and G2 - the gap between the end of S and the beginning of mitosis. During early cleavage of the embryo, there is no stage of no division (G0). The genetic information is duplicated by replicating the chromosomes during interphase and then partitioned by segregating the replicated sister chromosomes from each other in mitosis. The faithful transmission of genetic information depends not only on the accuracy with which the chromosomes are replicated and segregated, but also upon the coordination of these events. The cell cycle is regulated by a series of complex internal clocks and check points where events do not proceed until completion of the previous event (Barnes and Eyestone, 1990).

The first cell cycle may be appropriately timed from the point of second polar body extrusion. The total length of the first cell is 28 h. DNA synthesis begins at 16 h post insemination (hpi) and lasts for 8 h, until 24 hpi. S phase is followed by a 4~6 h G2 phase. Earliest chromosomal condensation is observed at 26~28 hpi, with the first cleavages appearing at 28 hpi. In cattle the first cell is long relative to other cell cycles under maternal control (Barnes, 1988). Cell cycle 2 is considerably shorter than the first with an overall duration of 12 h. The most striking feature of this cycle is the absence of G1. DNA synthesis begins immediately after mitosis. S phase is followed by a rather brief G2 (maximum of 2 h) and M phase of 2 h. The third cycle lacks a detectable G1 phase. S phase lasts for 8 h and in some blastomeres is followed almost immediately by chromosome condensation (M), while in other, a prominent G2 phase appears, lasting up to 6 h (Barnes and Eyestone, 1990). The variation in cell cycle length are due solely to variation in the length of G2 (Eyestone and First, 1986).

Synchronous cleavage of the bovine embryo occurs to the 4-cell stage. The presence of a G2 phase allows for new embryonic transcription (Barnes and Eyestone, 1990). The onset of transcription is temporally related to increase cell cycle length (Frei *et al.*, 1989). Major embryonic transcription started during the late 8-cell stage (Kopency and Niemann, 1993). Ribosomal RNA synthesis is active late in the fourth cell cycle (8-cell stage) (Frei *et al.*, 1989).

Cell block in bovine embryos is reported to occur at the 8- to 16-cell stage (Eyestone and First, 1986). This may in part be due to stress imposed by suboptimal environmental conditions as the embryo moves from the oviduct to the uterus (Smith and Johnson, 1986).

OOCYTE ENUCLEATION

Many problems remain to be solved before widespread use of nuclear transfer technology is possible. In cattle, a major obstacle is oocyte enucleation (removal or destruction of oocyte's genetic material), as it is impossible to visualize metaphase chromatin with standard light microscopy. One important aspect of the methodology to be improved relates to the removal of the genetic material contained within the cytoplasm of the recipient oocyte. This is necessary to completely eliminate the genetic contribution of the host cytoplasm and thus assure maximal similarity between cloned animals and avoid ploidy abnormalities with subsequent consequences to normal development (Smith, 1992). This has led to the development of four difleremt approaches for enucleation step. First, to

bisect the oocyte into two equal portions and use both halves for cloning (Westhusin *et al.*, 1991). In this procedure, both the nucleated and the enucleated halves are used as nuclear recipients. A disadvantage of the technique is that half of the nuclear transfers are likely to be polyploid, and therefore, unable to develop normally to term.

A second approach utilizes the position of the first polar body to locate the metaphase plate followed by aspiration and elimination of a quarter to half of the adjacent oocyte cytoplasm (Smith and Wilmut, 1989). However, possibly due to displacement or degeneration of the first polar body, a improper removal of chromosomes has been reported to occur in approximately one-third to one-quarter of the attempts in farm animals (Prather and First, 1990). Although an improvement from the previous technique, one is again left with an unknown percentage of nuclear transplanted embryos carrying a triploid genome.

A third approach, observation of demi-oocytes with ultraviolet microscopy following incubation in Hoechst stain provides a means of identifying and discarding those demi-oocytes containing chromosomes (Westhusin *et al.*, 1992). Despite this improvement, physical enucleation demands considerable technical skill, which limits the widespread use of nuclear transfer. In addition, the removal of the chromosomes by this procedure probably also inadvertently removes important cytoplasmic components which may reduce cytoplast viability.

Finally, a simple non-invasive method of enucleation would circumvent these problems and greatly simplify nuclear transfer. Fulka and Moor (1993) reported highly effective chemical technique for the complete enucleation of mouse oocyte. Strong chromosome to chromosome binding was induced by culturing early metaphase I oocytes in etoposide supplemented medium. Subsequent expulsion of the entire chromosome complex during polar body extrusion was facilitated by exposing the etoposide treated oocytes to a combination of cycloheximide and etoposide during anaphase and telophase. It is, however, important to note this enucleation procedure is successful only when applied to mouse oocytes at metaphase I. The same treatment applied to mouse oocytes at metaphase II gave highly inconsistent results. Likewise, the treatment of bovine oocytes at metaphase I has not yielded the same consistent enucleation rate as that achieved in the mouse. Although the reasons for this apparent species-specificity are not yet understood, the procedure will become suitable for a range of other animals. It will be useful as a practical technique in bovine nuclear transfer.

CELL FUSION AND OOCYTE ACTIVATION

Two key steps in the nuclear transplantation procedure are the fusion of the donor cell to the recipient oocyte and activation of the oocyte (Robl *et al.*, 1992). Electrofusion is now widely used for nuclear transfer in mammals, because of its simplicity and consistency. Important conditions for maximal cell fusion are electrical pulse voltage, duration, nonelectrolyte cell fusion medium which is designed to minimize heating in an alternating current field. Another factor that is important is the orientation of, and contact between, the karyoplast and the recipient cell. Fusion occurs as a result of membrane breakdown and pore formation between the opposing cells (Zimmerman and Vienken, 1982). Orientation of the cells such that the fusion membranes are perpendicular to the electrical fields is necessary because this is the point where the membrane breakdown voltage

will first be reached (Zimmerman and Veiken, 1982).

Alternating current (AC) wave is used to induce cell contact along with alignment and compression. Direct current (DC) pulse is applied to electroporation, and is performed causing transient pores in the cell membranes. The pores of adjacent (or touching) cells form interconnecting channels allowing for cytoplasmic mixing. The remaining pores close and an intact hybrid is formed. *In vitro*, the oocytes reach maximal activation potential by electrofusion at 30 h after the start of culture (Ware *et al.*, 1989). The ability of the oocyte plasma membrane to fuse after an electrical pulse is reduced slightly with aging of the oocyte and significantly after fertilization (Robl *et al.*, 1992). The low rate of development of activated aged oocytes has been partially attributed to an absence or abnormal cortical reaction and disorientation of microfilaments in the microvilli of the plasma membrane (Gulyas, 1976). The lack of fusion in pronuclear embryos correlated with a lack of pore formation, which may be related to cortical granule exocytosis and the vitelline block to polyspermy.

Repetitive transient elevations in intracellular Ca^{++} occur as a result of fertilization and are thought to be the key intracellular signal for oocyte activation (Robl *et al.*, 1992). Electrical pulses cause a single intracellular Ca^{++} elevation that rapidly returns to baseline. The Ca^{++} elevation is affected by pulse voltage and duration. Multiple pulses result in multiple Ca^{++} elevations. Electrical pulses which produce high rates of fusion give variable, very large (f-10 um) and prolonged (10~15 min) Ca^{++} elevations. The magnitude of the peaks ranged from approximately 400 to 900 nm and had a duration of 150 to 250 s (Robl *et al.*, 1992).

CYTOPLASM INHERITANCE

Although there is likely little, if any, genetic variation in the nuclear genome between individuals within a clonal line, there may be significant variation within the cytoplasm. Since commercialization of nuclear transfer would require obtaining ovaries from slaughter house material followed by *in vitro* maturation of oocytes obtained from this material, it becomes important to know the genetic makeup of the those oocytes and the effects on traits of economic importance.

Two potential sources of genetic variation within the cytoplasm are the mitochondria and centrosome. Mitochondria contain a loop of double stranded DNA (mtDNA). Previously it had been thought that all mitochondria in mammals was inherited from the egg, although a recent paper suggests that the sperm also makes a contribution (Gyllensten *et al.*, 1991). Two interesting aspects of mtDNA is that each mitochondrion has its own mtDNA and significant changes can occur after a single generation (Brown *et al.*, 1989). It is thought that the mtDNA variation within a line could be accounted for by unequal amplification of the mtDNA during oogenesis (Michaels *et al.*, 1982).

In addition to the discussion of mtDNA, the centrioles may have their own DNA (Hall *et al.*, 1989). In the mouse they are maternally inherited (Schatten *et al.*, 1985) although they may be paternally inherited in domestic animals. Their mode of inheritance, either paternal or maternal, may have an effect on the developmental potential of the nuclear transfer embryo. In mammals, centrioles are absent during the first few cleavages (Szollosi *et al.*, 1972) and it would be interesting to determine the fate of centrioles that are transferred to oocytes. Thus,

the genetic uniformity of nuclear transfer clones remains to be determined.

REMODELING AND REPROGRAMMING

The nucleus must be modified such that it behaves as through it was a zygote (pro)nucleus, when a nucleus from developed embryo is transferred to the enucleated activated oocyte. The nuclei undergo a variety of structural changes during development. This involves remodeling of the nucleus, which presumably results in reprogramming of the nucleus, as well as synchronization of the cell cycles of the donor and recipient cells. Nuclear reprogramming is characterized by functional modification of the transferred nucleus to be ,able to direct normal early embryo developing, with potential to develop to term (Collas and Robl, 1991). The discussion is focused in mode of nuclear remodeling and reprogramming, and DNA synthesis.

The transfer of a nucleus into an enucleated, activated, mature oocyte leads to the remodeling of the nucleus, characterized by premature chromosome condensation (PCC), followed by pronuclear (PN) formation and growth of the nucleoli. Nuclear remodeling is a physical change in the structure of the nucleus that may be a result of the exchange of proteins between the nucleus and cytoplasm (Prather and First, 1990). This protein exchange is thought to result in a modification of both gene expression and DNA replication. Specific proteins associate with and disassociate with the nucleus after nuclear transfer. The exchange of proteins between the nucleus and cytoplasm appear to be limited by selective binding sites within the nucleus. There are in the distribution of nuclear antigens during early cleavage in cow (Stricker *et al.*, 1989). One class of proteins that changes is the nuclear lamins. Nuclear lamin A, B and C are present in fertilized egg and early cleavage blastomeres. A and C type nuclear lamins represent an example of the acquisition of a protein from the cytoplasm of the oocyte (Prather *et al.*, 1989).

Synthesis of lamin A was detected in eggs (Houliston *et al.*, 1988), lamin C becomes undetectable after the transition of zygotic control of development in cow (Prather *et al.*, 1989). The B core protein of the samll nuclear ribonuclear proteins (snRNPs) is an example of the loss of a protein from the nucleus. Synthesis of lamin B was detected in 8-cell stage, morula and blastocyst (Schatten *et al.*, 1985; Houliston *et al.*, 1988). This indicates that RNA processing has ceased, thus the donor nucleus has been remodeled to resemble a zygote (pro)nucleus.

Complete reprogramming seems possible only after remodeling of the donor nucleus (Collas and Robl, 1991). Nuclear reprogramming can be defined both morphologically and biochemically, based on the timing of the appearance of developmental stages and specific gene products, respectively (Prather and First, 1990). Morphological criteria have evaluated the development to term of nuclear transport embryo using embryonic donor nuclei (Prather *et al.*, 1987; Bondioli *et al.*, 1990). An indication of nuclear reprogramming is the time of blastocyst formation and blastocyst cell number. Moreover, nuclear transfer embryos form a blastocoele at a time corresponding to a zygote. During development the embryo undergoes differentiation events after nuclerar transfer. The bovine embryo begins to produce both mRNA and rRNA during the 8-cell stage.

Correlated with swelling and the exchange of proteins is the regulation of DNA synthesis. When random nuclei from cleavage stage embryos are transferred to the cytoplasm of an enucleated oocyte they must synchronize them-

selves with the recipient oocyte in terms of DNA synthetic phase. The nuclei that are transferred from a single embryo are likely in different phases of the cell cycle, i. e. G1, S or G2. Factors in the cytoplasm control mitosis (M), synthesis (S) and the gap stages (G1 and G2).

If a nucleus has already completed DNA synthesis and is in G2, then, for normal ploidy to result, the nucleus must remain in G2 and not undergo any additional DNA synthesis after nuclear transfer. Conversely, if a nucleus is in G1, i. e. before DNA synthesis, when transferred, then it must undergo a single round of DNA synthesis. Developmental studies show that nuclei in G1 result in significantly higher rates of development than nuclei in S or G2 (Collas *et al.*, 1992a). Thus, development to blastocysts was greatly affected by the stage of the cycle of the donor nucleus. After fusion of the donor blastomere, the morphology of prematurely condensely chromatin differs between G1, early S transplants, and late S transplants. Donor nuclei in G1 an early S typically condense into chromosomes within a spindle. In contrast, last S phase chromatin condenses into a non-uniform meshwork without individualized chromosomes, and spindle formation is dramatically impaired (Collas *et al.*, 1992b). Therefore, morphologically normal chromosomes are obtained in G1 and early S transplants, whereas a large proportion of blastomeres in late S transplants show chromosome abnormalities.

The embryo treated with aphidicolin, the DNA synthesis inhibitor, remained blocked at the G1 and S transition. This treatment was reversible, as assessed by the resumption of DNA synthesis, cleavage rate, and development to blastocysts of treated embryos (Collas *et al.*, 1992a). Nocodazole can synchronize cell division of mouse 2 cell embryos without reducing their ability to develop into live young (Kato and Tsunoda, 1992). Further investigation of the developmental ability and chromosomal constitution of young and aged oocytes receiving a synchronized nucleus at particular cell stages of cell cycle should be considered.

CONCLUSIONS

Bovine nuclear transfer has been accomplished to produce multiple offspring from one donor embryo. Cloning of cattle has brought about widespread interest in its commercialization because nuclear transfer is one of the most attractive biotechnologies for rapid genetic improvement of cattle. However, many problems remain to be solved before widespread use of this technology is possible. The answers to many of these questions are forthcoming as future work unfolds.

Critical events required for embryo development take place in the oocyte during the final stage of folliculogenesis. *In vitro* matured oocytes may be used for embryo cloning of cattle. The donor cell must be in G1 for high rates of development to result from the nuclear transfer. Some parent embryo may be better suited or more adaptable to the cloning procedures. A simple and novel method remains to be developed to improve the efficiency of oocyte en-ucleation. Very high fusion rates can be achieved in the condition of optimum pulse parameters. The most effective methods of achieving high activation are to use aged oocytes or to give multiple electrical pulses. The function of mtDNA and centriole DNA of cytoplasm in bovine remains to be explored to understand in the context of nuclear transfer clones. The nucleus is remodeled after nuclear transfer such that it resembles and behaves as pronucleus in a zygote. This reprogramming is accomplished by the exchange of proteins between

the nucleus and cytoplasm.

The efficiency of many steps in the procedure will be improved. Ultimately, biological limits to what can be achieved will be reached. More basic research on many steps in cloning is need to reach those limits and thus help the cattle breeding industry.

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