Nuclear transfer and reprogramming

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Nuclear transfer techniques for mammalian embryos have been developed in the last decade. Embryonic nuclei from advanced stages of preimplantation development can be fully reprogrammed and the totipotency is restored when nuclei are transferred into ooplasts. Transfer of nuclei after gene expression from the embryonic genome has started does not appear to restrict the reprogramming of these nuclei. The principles of nuclear transfer are outlined with respect to nuclear remodelling, nucleocytoplasmic interactions and effects of the cell cycle. However, the molecular mechanisms involved in reprogramming donor nuclei remain unknown. It is proposed that epigenetic DNA modification, such as DNA methylation that regulates gene expression, is related to the reprogramming of transplanted nuclei.

In mammals, oocytes are ovulated at metaphase II and remain arrested at this stage until fertilization. Once fertilized, the oocyte completes meiosis with the extrusion of the second polar body and the formation of male and female pronuclei. The embryos begin to develop by undergoing a series of mitotic divisions before differentiating into specific cells, resulting in the organization of tissues and organs. This developmental programme ensures the successful transition from oocyte to offspring. In general, once the programme is started, it cannot stop or be reversed, although cells of the early embryos remain totipotent (each capable of developing into a new individual) during the first one or two cleavage divisions. After this stage the individual cells cannot form a new individual presumably because changes have taken place in the DNA that alter gene expression during development. However, using nuclear transfer techniques to expose the chromatin (nuclei) to ooplasm, it is possible to reprogramme the DNA. The technique was originally developed in amphibians in the 1950s to answer questions of nuclear equivalence (for review see Gurden *et al.,* 1979). It was shown that the reprogrammed DNA could support complete embryonic development. Only relatively recently, owing to technical difficulties, have similar studies been conducted in mammals (McGrath and Solter, 1983; Willadsen, 1986; Kono *et al.,* 1991a), which showed that reprogramming of the nuclei from embryonic cells can occur after transfer to enucleated oocytes (ooplasts). This indicates that the oocyte cytoplasm can restore totipotency, or reprogramme the nucleus of embryonic cells to initiate and complete development. Thus, contrary to the original contention, it seems that the developmental programme can be re-initiated using suitable nuclear transfer techniques. These techniques provide a method for embryo cloning, as the nuclei of early (and perhaps advanced) stage embryos can be transferred to ooplasts to produce several new individuals of identical genotype.

Embryo cloning

The efficiency of embryo cloning by nuclear transfer differs between species and the procedures used. To clarify the potential for full reprogramming by nuclear transfer, we confirmed the studies that had produced live young by nuclear transfer. In mice, the stage of development of the donor nuclei that allows development to term after transfer to an ooplast is restricted to the two-cell stage (Tsunoda *et al.,* 1989; Kono *et al.,* 1991a, 1992). However, identical triplet mice were produced after the transfer of nuclei from a four-cell embryo into the enucleated blastomeres of embryos at the two-cell stage (Kono *et al.,* 1991b). The reason for restricted development of nuclei from more advanced embryos was thought originally to be related to the timing of embryonic genome activation, which in mice is about the middle of the two-cell stage (Flach *et al.,* 1982; Bolton *et al.,* 1984).

Now, by means of serial nuclear transfer, we have produced live young from four- and eight-cell nuclei synchronized at metaphase with nocodazole (an inhibitor of tubulin polymerization). Furthermore, these studies demonstrated that identical sextuplet mice can be produced from a four-cell embryo (see Fig. 1) (Kwon and Kono, 1996). In the first nuclear transfer, metaphase nuclei of four-cell embryos were fused with ooplasts using Sendai virus. After artificial activation, the reconstituted oocytes were cultured in a medium containing cytochalasin B, which inhibits microtubule polymerization, to inhibit the extrusion of an extra-polar body and achieve formation of two 'pronuclear-like nuclei'. Embryos with diploid sets of chromosomes were obtained by transferring the nuclei from each reconstituted embryo individually into separate enucleated fertilized one-cell embryos. Thus, the number of identical embryos is doubled by the second nuclear transfer. The results suggest that the second nuclear transfer to the fertilized zygote completes the reprogramming events that subsequently support development to term.

Recent experiments have demonstrated that full term development can be obtained from nuclei from beyond the two-cell stage of development (Tsunoda and Kato, in press). These experiments used nuclei from four-cell and late morula at the G1–S phase of the cell cycle, which were synchronized with nocodazole and aphidicolin (a DNA polymerase inhibitor), and retransferred nuclei of reconstituted embryos at the two-cell stage to enucleated fertilized two-cell embryos. The key manoeuvre in these studies was the transfer of nuclei from
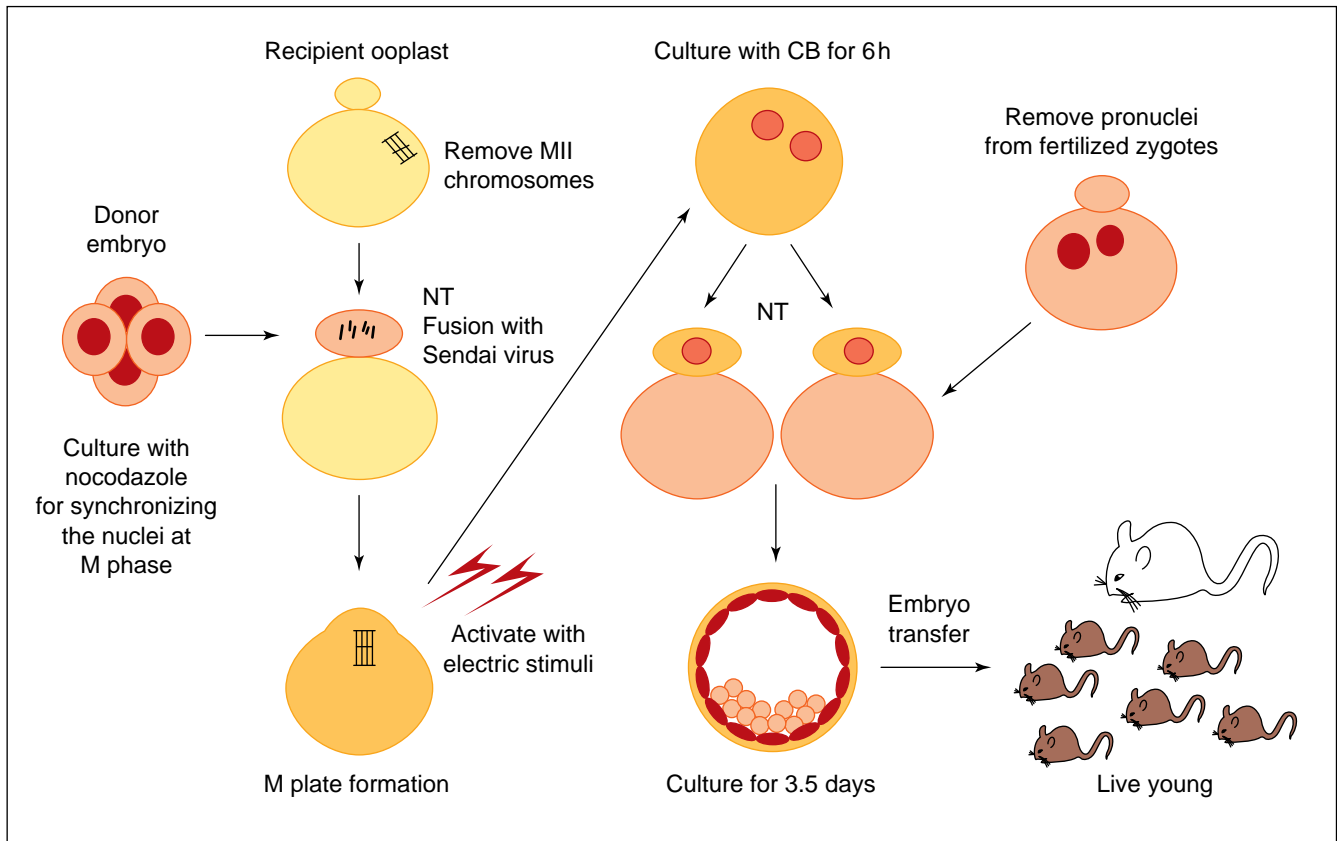
Fig. 1. Production of identical clone mice by serial nuclear transfer (NT) starting with the transfer of four-cell nuclei at metaphase into unfertilized ooplasts. The donor four-cell stage embryos were synchronized at metaphase with nocodazole. The oocytes receiving a donor nucleus at metaphase formed two nuclei after artificial activation and inhibition of cytokinesis with cytochalasin B (CB). Diploid embryos were obtained by transferring nuclei from each reconstituted embryo individually into separate enucleated fertilized one-cell embryos, thus doubling the number of identical embryos.

parthenogenetically activated reconstituted embryos at the one- or two-cell stage to enucleated fertilized one- or two-cell embryos, respectively.

In recent studies (Kwon and Kono, 1996; Tsunoda and Kato, in press) an important contribution to success was the transfer of the donor nuclei first to a parthenogenetic and then to a fertilized recipient embryo. This raises the question as to why cytoplasm from fertilized one-cell embryos has greater potential for supporting the development of reconstituted embryos than that of parthenogenetic origin. One possibility is that new transcripts from the male genome support further development of the reconstituted embryos, as zygotic genome activation (Latham *et al.*, 1992) and exogenous gene expression (Ram and Schultz, 1993) occur as early as the late one-cell stage in mice. This contention is supported by the observation that using early fertilized one-cell embryos before the activation of transcription from the male genome as a recipient cytoplasm did not support development (T. Kono and O.Y. Kwon, unpublished). Alternatively, nucleocytoplasmic interaction (Reik *et al.*, 1993) in hybrid embryos, which were produced by transferring a female pronucleus into a recipient zygote from a different genotype, may be responsible for this phenomenon. Whatever the mechanism, it appears that the reconstitution of nuclei from

fertilized embryos with parthenogenetic cytoplasm is detrimental to development. These findings are not in agreement with the notion that embryonic genome activation is the main factor that restricts nuclear reprogramming.

Experiments in mice using primordial germ (PG) and embryonic stem (ES) cells as donor nuclei have also been performed by the single nuclear transfer method. The results show that the reconstituted embryos can complete preimplantation development but they do not survive beyond early implantation (Tsunoda *et al.*, 1989; Tsunoda and Kato, 1993; Kato and Tsunoda, 1995). When a type of stem cell is used as a nuclear donor, the reconstituted embryos either fail to implant or are absorbed soon after implantation. These results need to be confirmed using a serial nuclear transfer system.

Compared with embryonic nuclei from rodents, embryonic nuclei from domestic animals can be reprogrammed at much later stages of development. By standard nuclear transfer methods, nuclei from multiple cell embryos and even nuclei from inner cell mass cells can be reprogrammed to support development to term in cattle (Keefer *et al.*, 1994) and sheep (Smith and Wilmut, 1989). Recently, Campbell *et al.* (1996) described the production of lambs from cells derived from sheep embryos, which had been cultured for 6–13 passages. The early

passage cells with the features of embryo-derived epithelial cells, expressing differentiation markers such as cytokeratin and nuclear lamin A/C, were fully reprogrammed after transfer into an ooplast, as shown by their ability to develop to term. An important contribution to the success of this study may have been that the donor nuclei were from cells arrested in the G0 stage of the cell cycle. This was achieved by culture in a medium containing a low serum concentration. Thus, in cattle and sheep, ooplasts appear to have the ability to reprogramme donor nuclei from many stages of development and therefore provide an excellent opportunity for embryo cloning. However, the nuclear transfer technique for embryo cloning has not proved a reliable procedure. One problem, alluded to above, has been synchronizing the cell cycle of the donor nuclei with that of the recipient cytoplasm.

Stage of cell cycle

The stage of cell cycle of the donor nucleus and recipient cytoplasm are important factors for successful development. A diploid set of chromatin and full nuclear reprogramming are required. The fusion of interphase nuclei with metaphase arrested ooplasts resulted in donor chromatin undergoing premature chromosome condensation (PCC) (Czolowska *et al.*, 1984; Szollosi *et al.*, 1988; Kono *et al.*, 1991a; Collas *et al.*, 1992). This is induced by maturation-promoting factor (MPF), a complex of p34^{cdc2} and cyclin (Gautier *et al.*, 1988; Choi, 1991; Kubiak *et al.*, 1993; Whitaker, 1996). The cell cycle is restarted by activating reconstructed oocytes with a suitable artificial stimulus, such as electric pulses or exposure to medium containing ethanol or strontium, to release them from metaphase arrest (Whittingham, 1980). Altering the timing of oocyte activation with respect to the fusion of the donor nucleus provides a number of possible approaches to synchronizing the respective cell cycles and to producing embryos with diploid genomes (Fig. 2).

When nuclei are fused into oocytes using Sendai virus, which does not cause oocyte activation (Kono *et al.*, 1995), nuclei at the G2 and M phases of the cell cycle are required to retain diploidy after fusion. The first production of live mice was obtained by transferring nuclei from two-cell embryos at the G2 phase to ooplasts (Kono *et al.*, 1991a, 1992). Synchronizing the donor nuclei of four-cell embryos at M phase of the cell cycle appears to be very successful, perhaps owing to the precision of synchrony, and has led to the production of identical sextuplet mice from a single four-cell stage embryo (Kwon and Kono, 1996). The main advantage of this method is that nuclei of preimplantation embryos can be arrested at metaphase of the cell cycle by inhibitors of tubulin polymerization, such as nocodazole and colcemid, which are not toxic to embryo development (Kato and Tsunoda, 1992).

When live young are produced from nuclei at the G0, G1 and S phases of the cell cycle, it is necessary to activate the ooplasts simultaneously (Fig. 2 b(i)) or before (Fig. 2. b(ii)) fusion to prevent the nuclei from forming a metaphase plate (a spindle apparatus). Mouse embryonic cells are synchronized in G1–S by treatment with nocodazole followed by aphidicolin, an inhibitor for DNA polymerase I (Tsunoda and Kato, in press). In this study, cell fusion was induced by Sendai virus and the oocytes were activated artificially before completion of fusion so that a MPF was destroyed, allowing the formation of a single

nucleus. The low rate of success was probably due to some asynchrony in the cell cycle or to variable timing with respect to cell fusion. Alternatively, DNA may not be so readily reprogrammed in the G1–S compared with the M phase.

In a different system, electric pulses are used for cell fusion; therefore, the oocyte is activated at the time of fusion (Fig. 2 b(i)) and the donor nuclei do not form metaphase plates. Although precise analysis is difficult, the results in rabbits suggest that the developmental potential of the reconstituted oocytes receiving G1–S phase nuclei is significantly greater than that of oocytes receiving nuclei at other stages of the cell cycle (Collas *et al.*, 1992a,b). An attempt using embryonic fibroblast cells synchronized in G0 phase resulted in the first successful production of offspring from differentiated cells in mammals (Campbell *et al.*, 1996). The fact that the cells were synchronized in G0 phase suggests that this stage of the cell cycle may be preferential for remodelling and reprogramming so that development to term is possible. In cattle, activation of recipient oocytes before fusion with unsynchronized donor nuclei (Fig. 2. b(ii)) significantly improved development of the reconstituted embryos. This may be because most of the asynchronous nuclei are in G1–S (the longest phase of the cell cycle), similar to that of the activated ooplast (Kono *et al.*, 1994). Together, these results suggest that it is important to synchronize the cell cycle of the recipient cytoplasm and donor nuclei. This synchronization can improve the efficiency of embryo cloning by nuclear transfer using donor nuclei at the M, G0 and G1–S phases of the cell cycle.

Reprogramming of donor nuclei

Direct contact of chromatin with the ooplasm is probably required to induce nuclear reprogramming. Therefore, either complete and partial nuclear envelope breakdown, which is induced by high MPF activity, or nuclear swelling should occur after fusion into the ooplasm. The success of nuclear reprogramming can, to some extent, be evaluated by morphological changes during preimplantation development, such as the timing of cleavage, compaction and blastocoel formation. The series of morphological events that occurs during preimplantation development in nuclear transferred embryos is similar to that in fertilized embryos (Tsunoda *et al.*, 1989; Kono *et al.*, 1993). However, reprogramming is not necessarily complete, since embryos do not always develop to term after transfer into recipient females. For large scale embryo cloning, the efficiency of recloning, in which the nuclei from nuclear transferred embryos are transferred to ooplasts, has been examined. In mice, when embryos were reconstituted by serial nuclear transfer using four-cell nuclei synchronized at M phase, the proportion of the reconstituted embryos developing to blastocyst stage was reduced from 79% and 44%, to 17% and 4% in the first to the fourth generations, respectively (T. Kono and O. Y. Kwon, unpublished). Live young were obtained from only the first generation. Thus, recloning appears to reduce the effectiveness of reprogramming. In contrast, multiple nuclear transfer in cattle may be an effective procedure for large scale cloning since offspring have been obtained from the third generation embryos (Stice and Keefer, 1993).

Other evidence for the reprogramming of donor nuclei is the expression of stage-specific antigens and embryonic proteins in
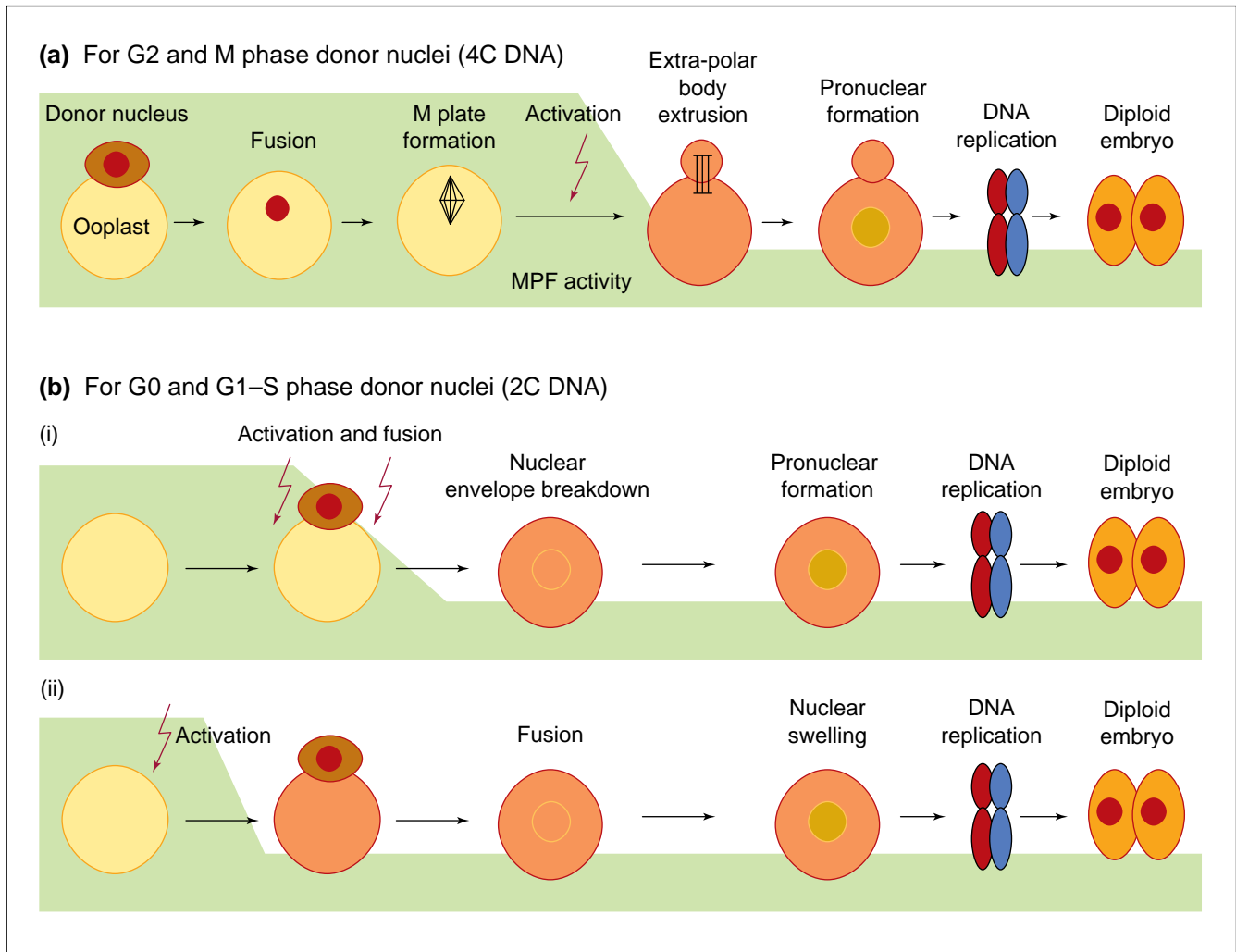
Fig. 2. Relationship between maturation promoting factor (MPF) concentrations and remodelling of donor nuclei at different stages of the cell cycle in nuclear transferred oocytes. (a) Nuclei at the G2 and M phases are fused with an ooplast using a Sendai virus to form a metaphase plate that is induced by the high activity of MPF. After artificial activation, the MPF concentrations decrease, a polar body is extruded and a single nucleus is formed. (b) When nuclei at the G0, G1 and S phases are used, the donor nuclei are fused with an ooplast that is activated at the time of fusion (i) or a preactivated ooplast (ii). The MPF concentrations are, therefore, low or decreasing rapidly at the time of fusion. The donor nucleus undergoes a normal S phase and the reconstituted embryos can develop to term.

nuclear transferred embryos. In bovine embryos, antigens defined by the TEC-03 and MPM-2 monoclonal antibodies appear at the two-cell stage (Van Stekelenburg-Hamers *et al.*, 1994). These antigens cannot be detected in reconstituted embryos that received a nucleus from morula stage embryos that express the antigen. The small nuclear ribonuclear protein (snRNP) and nuclear lamins are developmentally regulated during pig embryogenesis. Nuclei from blastomeres of 16-cell pig embryos react with antibodies against nuclear snRNP and not lamin A/C. After transfer to an enucleated oocyte, the expression changes to snRNP negative, lamin A/C positive, which reflects that of the oocyte (Prather *et al.*, 1989; Prather and Rickords, 1992). Embryonic RNA and protein synthesis have also been studied in nuclear transferred embryos. Transcriptional activity of bovine eight-cell nuclei arrests after transfer to an enucleated oocyte and resumes only when the embryo reaches the eight-cell stage

(Kanka *et al.*, 1991). In mice, data from SDS gel electrophoresis and fluorography show that the 68–70 kDa embryonic proteins expressed from the embryonic genome at the middle of the two-cell stage were detected in the two-cell embryos that received thymocytes (Kono *et al.*, 1993).

Molecular mechanism of reprogramming

Since reliable nuclear transfer procedures were established, there have been many studies attempting to produce identical domestic and laboratory animals. Nevertheless, how nuclei transferred into ooplasts are reprogrammed so that totipotency is restored remains unknown. Epigenetic DNA modification mechanisms, such as DNA methylation (Razin *et al.*, 1990) and changes in chromatin conformation (Croston and Kadonaga, 1993; Koide *et al.*, 1994), have been proposed as likely candidates
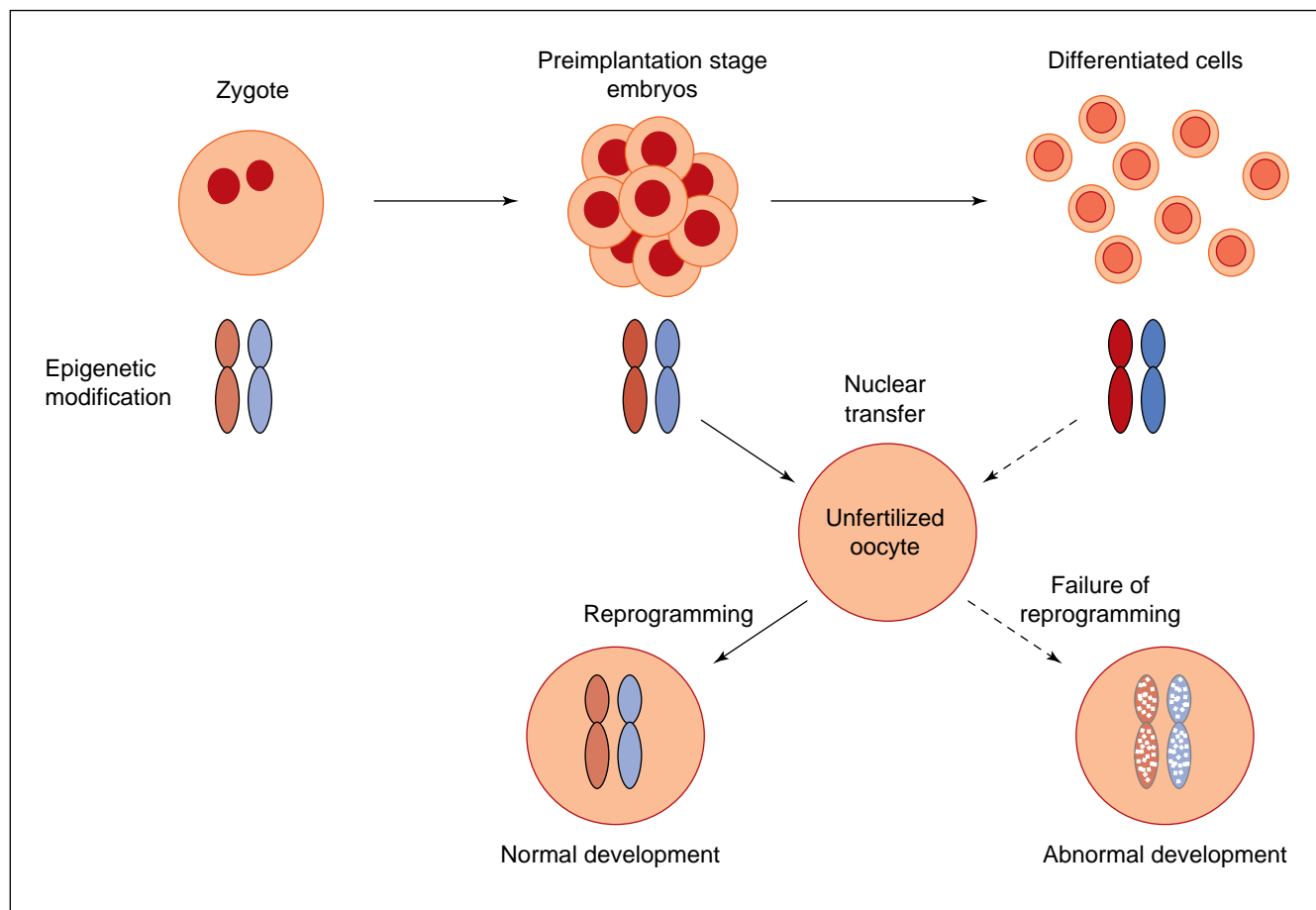
Fig. 3. Schematic diagram of the proposed mechanism for reprogramming after nuclear transfer. On transfer of nuclei from a preimplantation embryo to an activated or unfertilized oocyte, any epigenetic modification of the DNA, such as methylation, is reverted to that of the host cytoplasm DNA. The donor nucleus is now totipotent and the reconstituted embryo will develop to term. However, this becomes difficult as development and differentiation progress. The epigenetic modifications cannot be changed to be similar to the host cytoplasm; the nucleus is no longer totipotent and will not support development to term.

for controlling reprogramming after nuclear transplantation. Methylation of the pyrimidine base cytosine in DNA is thought to be one of the mechanisms underlying the epigenetic regulation of gene expression (Razin and Cedar, 1991). The methylation status in the site of newly replicated DNA is conserved by the action of DNA (cytosine-5)-methyltransferase (DNA MTase) for hemimethylated DNA. Therefore, methylation in the genome is stable. Undermethylation of CpG islands in DNA is closely correlated with gene expression and differentiation of cell lineage in mammalian development (Jaenisch *et al.*, 1995). Gene targeting experiments in mice show that a homozygous mutation of the DNA MTase gene results in severe stunting, developmental delay, and death at mid-gestation (Li *et al.*, 1992). The amount of cytosine-5 methylation in the DNA from day 10 of homozygous mutant embryos is 30% of that of wild-type embryo DNA, which suggests DNA methylation has an essential role in normal mammalian development.

In addition, a mechanism known as genomic imprinting, explained as the differential expression of genes depending on whether the allele is inherited via the spermatozoon or the oocyte, is thought to involve methylation (Surani *et al.*, 1990;

Gilligan and Solter, 1995). Analysis of imprinted genes suggests that DNA methylation is closely linked to genomic imprinting (Reik *et al.*, 1990; Surani *et al.*, 1990; Ueda *et al.*, 1992). The functional significance of DNA methylation in genomic imprinting is supported by the finding that CpG islands (or sites) in three imprinted genes, *H19* (Bartolomei *et al.*, 1993; Ferguson-Smith *et al.*, 1993), the insulin-like growth factor II gene (*Igf2*) (DeChiara *et al.*, 1991; Sasaki *et al.*, 1992), and the IGF-II receptor gene (*Igf2r*) (Barlow *et al.*, 1991; Stöger *et al.*, 1993), are differentially methylated depending on their parental origin. The gene expression study in MTase knockout mice showed that the normally silent paternal allele of the *H19* gene was activated, whereas the normally active paternal allele of the *Igf2* gene and the active maternal allele of the *Igf2r* gene were repressed (Li *et al.*, 1993). These genes have a definitive role in embryo development. Gene knockout studies showed that embryos inherit a nonfunctional *Igf2r* from their mothers, are 20–30% larger than wild type and die at birth (Lau *et al.*, 1993). However, when this gene is inherited paternally the embryos develop normally into adults. Conversely, mice lacking the functional paternal *Igf2* allele are 30–40% smaller than their wild type littermates (DeChiara *et al.*,

1991), but when the disrupted gene is inherited maternally the offspring are normal. The above may suggest that a normal amount of DNA methylation is required to control the differential expression of the paternal and maternal alleles of imprinted genes and for normal embryo development. It has been reported in sheep and cattle that the production of embryos by nuclear transfer and *in vitro* fertilization can influence fetal growth resulting in larger offspring (Walker *et al.,* 1996), although it is unknown whether the same mechanisms underlie both types of embryo production. This syndrome may be explained by over- or underexpression of imprinted genes as a result of alternations in methylation patterns by embryo manipulation (Fig. 3).

Furthermore, nuclear transfer studies have demonstrated that changes in the nucleocytoplasmic composition induce phenotypic effects through epigenetic modification. The methylation and expression of mouse urinary protein genes are influenced by transferring pronuclei to cytoplasts derived from mice of different genotypes (Reik *et al.,* 1993). Mice derived from nucleocytoplasmic hybrid embryos have a growth deficiency resulting in reduced adult body mass. Mature oocytes with maternal chromatin derived from non-growing oocytes have been produced by nuclear transfer techniques to explore the effects of maternal imprinting on the development of parthenogenetic embryos. The artificial activation of such oocytes leads to the development of normal fetuses with a well developed placenta on day 13.5 of gestation (Kono *et al.,* 1996). Analysis of the maternal methylation pattern expressed in the *Igf2r* and *H19* genes of the constructed oocytes and embryos showed that the pattern of methylation was indeed different from that of controls. The studies described above suggest that epigenetic changes occur in DNA during early development that have profound effects on gene expression later in development. The patterns of DNA methylation during preimplantation development appear to be quite labile (Shemer *et al.,* 1996). Around implantation the pattern of *de novo* methylation of the DNA becomes established (Gilligan and Solter, 1995) and this transition may govern the ability of donor nuclei to undergo complete reprogramming. Therefore, it is possible that nuclei from advanced stage embryos, including ES and PG cells, cannot be reprogrammed after nuclear transfer.

Conclusion

The fundamental technique for embryo cloning by nuclear transfer in mammals has been developed during the past decade. Recent progress has focused attention upon the totipotency of germ cell lines and the differential roles of male and female genomes. Precise nuclear transfer experiments and detailed molecular analysis will help to define the mechanism of reprogramming and accelerate the progression of studies of embryo cloning from a wide variety of embryonic cells.

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