Cell cycle co-ordination in embryo cloning by nuclear transfer

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Exciting new opportunities in embryo cloning have been made possible by recent studies on the interaction of the donor nucleus with the recipient cytoplasm after embryo reconstruction. This article reviews information regarding the co-ordination of nuclear and cytoplasmic events during embryo reconstruction, in particular the direct and indirect effects of maturation/meiosis/mitosis-promoting factor (MPF), upon the transferred nucleus. This will be discussed in relation to DNA replication, the maintenance of correct ploidy, the occurrence of chromosomal abnormalities and development of reconstructed embryos. Although this review is primarily concerned with the reconstruction of mammalian embryos, specific examples from amphibians will also be cited.

Embryo reconstruction by the transfer of a donor nucleus to an enucleated one-cell egg was first proposed by Spemann (1938) to answer the question of nuclear equivalence or ‘Do nuclei change during development?’ By transferring nuclei from increasingly advanced embryonic stages, these experiments were designed to determine at which point the developmental potential of nuclei became restricted. Owing to technical limitations and the unfortunate death of Spemann, these studies were not completed until Briggs and King (1952) demonstrated that certain nuclei could direct development to a sexually mature adult. Their findings led to the current concept that equivalent, totipotent nuclei from a single individual could, when transferred to an enucleated egg, give rise to genetically identical individuals. In the true sense of the meaning, these individuals would not be clones, as unknown cytoplasmic contributions in each may vary and the absence of any chromosomal rearrangements would have to be demonstrated.

Since the demonstration of embryo cloning in amphibians, similar techniques have been applied to mammals (see Box 1). The ability to produce genetically identical clones has obvious advantages for research (i.e. as biological controls) and in commercial applications (i.e. uniformity of meat products, animal management).

After reconstruction, embryo development depends on many factors, including the ability of the nucleus to direct development, i.e. totipotency, nuclear reprogramming, developmental competence of the recipient cytoplast (i.e. oocyte maturation), oocyte activation and embryo culture (reviewed by Wilmut and Campbell, 1992; Campbell and Wilmut, 1994). Another group of factors can be described as cell cycle effects. Many reports on both amphibians and mammals have shown that the cell cycle stage of both the donor nucleus and the recipient cytoplasm, at the time of transfer, can have substantial effects upon the development of the reconstituted embryo.

Cytoplasmic states of different cytoplasm recipients

Most amphibian and mammalian oocytes become developmentally arrested at the germinal vesicle stage in prophase of the first meiotic division (for review see Masui and Clarke, 1979) (Fig. 1). Upon appropriate stimulation, meiosis resumes, the germinal vesicle breaks down, the first meiotic division is completed and the oocyte then becomes arrested at metaphase of the second meiosis. At this point, the mature oocyte (or unfertilized egg) can be fertilized. Upon fertilization, the second meiotic division is completed and the second polar body extruded; the male and female chromatin decondense and two pronuclei are formed. Shortly after their formation, DNA replication is initiated in the pronuclei. After DNA replication, equal segregation of the genetic material occurs by mitosis and the zygote cleaves to form two daughter blastomeres.

Of prime importance in these events is a cytoplasmic activity termed maturation/meiosis/mitosis-promoting factor (MPF) (Masui and Markert, 1971). MPF has been identified as a complex of two proteins, cyclin and p34cdc2, a protein kinase the kinase activity of which is regulated by changes in its phosphorylation state and by its association with cyclins. Throughout the cell division cycle, the concentration of p34cdc2 remains constant; however, the concentration of cyclins varies. The activation of p34cdc2 kinase triggers entry of the cell into mitosis or meiosis and results in breakdown of the nuclear envelope, chromosome condensation, reorganization of the cytoskeleton and changes in cell morphology (for reviews see Nurse, 1990; Maller, 1991; Masui, 1992).

MPF activity during oocyte maturation is maximal at metaphase of both the first and second meiotic divisions. When the oocyte becomes arrested at metaphase II (MII), MPF activity remains high. Upon fertilization or activation, MPF activity declines rapidly (i.e. in cattle; Campbell et al., 1993a) (see Fig. 1). Thus it is immediately apparent that the cytoplasmic environments following nuclear transfer are different when MII oocytes or pronuclear zygotes are used as cytoplasts. When MII oocytes are used, MPF activity is high; in contrast, in pronuclear zygotes, MPF activity has declined. This reduction in MPF activity can...
Box 1. Techniques of nuclear transfer in mammals

A. Metaphase II Cytoplast

1. MII oocyte
2. Enucleation
3. Identification of polar body and meiotic spindle
4. Insertion of donor cell/karyoplast
5. Fusion

B. Zygotic cytoplast

1. Pronuclear zygote
2. Enucleation
3. Identification of pronuclei
4. Insertion of donor cell/karyoplast
5. Fusion

In mammals, there are two predominant methods of nuclear transfer which vary upon the point at which the recipient cell is enucleated. Both oocytes at metaphase II (MII) and one-cell zygotes have been used as recipients for nuclear transfer. Oocytes at MII that are to be enucleated are cultured in medium containing the microfilament inhibitor cytochalasin D and the DNA-specific fluorochrome Hoechst 3332. Disruption of the microfilaments imparts an elasticity to the cell membranes such that a portion of the oocyte enclosed within a membrane can be aspirated into a pipette. The metaphase plate is removed by aspirating a small amount of cytoplasm from directly beneath the first polar body. Enucleation is confirmed by examining the aspirated cytoplast under UV for the presence of both the polar body and the metaphase plate. Similarly, zygotes are also incubated in medium containing cytochalasin with the addition of the microtubule inhibitor colchicine. In mice, the pronuclei are visible under direct interference contrast (DIC) optics and can be removed by aspiration. However, in ungulates, the zygotes have to be centrifuged to visualize the pronuclei. Centrifugation has no detrimental effects upon the further development of either bovine or porcine zygotes. After enucleation, a donor cell (karyoplast) is aspirated into the enucleation pipette, the pipette is inserted through the hole that was created in the zona pellucida and the karyoplast expelled into the perivitelline space. The karyoplast is then placed in contact with the recipient cell or cytoplast. In most situations, cell fusion is induced by application of a DC electric pulse at 90° to the plane of contact between the two cells. Cells may be aligned in the fusion chamber either manually or by application of an AC current immediately before the fusion pulse. When using MII oocytes as cytoplasts, the same current that induces fusion also induces activation of the oocyte (for review see Wilmut and Campbell, 1992). The frequency of electrofusion is related to the area of contact between the cytoplast and karyoplast. The use of cultured cells as nuclear donors has resulted in a lower frequency of fusion; techniques have now been developed that allow direct injection of the donor cell into the cytoplast (Collas and Barnes, 1994; Ritchie and Campbell, 1995).
also be induced by parthenogenetic activation of enucleated MII oocytes; embryos can then be reconstructed after the decline of MPF activity (Barnes et al., 1993; Campbell et al., 1993b, 1994).

**Effects of MPF on the transferred nucleus**

All nuclei that are transferred into a cytoplast with high MPF activity undergo nuclear envelope breakdown and chromosome condensation. As chromosome condensation is induced prematurely in the donor nucleus by the recipient cytoplasm, it is referred to as premature chromosome condensation. The degree of premature chromosome condensation observed varies depending upon the MPF activity and the duration of exposure to MPF; in addition, the cell cycle stage of the transferred nucleus may have pronounced effects upon the degree of premature chromosome condensation observed. Observations in both somatic cell hybrids (Johnson et al., 1970) and nuclear transfer embryos (Collas and Robl, 1991) showed that the chromatin of S-phase nuclei induced to undergo premature chromosome condensation by exposure to MPF has a typical pulverized appearance (Schwartz et al., 1971); and chromosome analysis has shown a high incidence of abnormalities in such nuclei (Collas et al., 1992a). In contrast, when nuclei at G1 or G2 phases undergo premature chromosome condensation, the chromatin condenses to form elongated chromosomes with single- and double-stranded chromatids, respectively (Collas et al., 1992a).

**DNA replication in reconstructed embryos**

During a single cell cycle, all chromosomal DNA must be replicated once and only once. The mechanisms by which a cell co-ordinates DNA replication and prevents re-replication of previously replicated DNA are unclear. However, maintenance of an intact nuclear envelope appears to be central to this control (Blow, 1993). Experiments in somatic cell hybrids (Johnson and Rao, 1970), by injection of nuclei into *Xenopus* eggs (DeRoeper et al., 1977) and the *Xenopus* cell free system (Blow and Laskey, 1988), have shown that intact nuclei at G2 phase are not induced to re-replicate when transferred to an S-phase cytoplasm. However, if the nuclear membrane is permeabilized by treatment with detergent, these nuclei do undergo re-replication (Blow and Laskey, 1988). Similarly our experiments (Campbell et al., 1993b) have shown that in bovine embryos reconstructed by nuclear transfer into an MII cytoplast, all nuclei that undergo nuclear envelope breakdown, regardless of their cell cycle stage, undergo DNA synthesis after reformation of the nuclear envelope. However, if nuclei are transferred after the decline of MPF activity, when no nuclear envelope breakdown occurs, then replication depends on the cell cycle stage of the transferred nucleus. Nuclei that are in G1 or S phases initiate or continue replication, respectively, while those that are in G2 phase are not induced to re-replicate previously replicated DNA. From these results, we suggest that besides chromosomal damage induced by premature chromosome condensation, a further factor influencing the development of reconstructed embryos may be DNA content (summarized in Box 2). Furthermore, we hypothesize that when using oocytes at MII as cytoplasts, only nuclei that are in the G1 phase of the cell cycle should be transferred. In contrast, when nuclei are transferred after the decline of MPF activity, chromosomal damage induced by premature chromosome condensation is avoided and all nuclei, regardless of their cell cycle stage,
undergo co-ordinated DNA replication. We have termed such activated cytoplasts the 'Universal Recipient'. From this hypothesis, if the transferred nucleus can re-direct development (i.e. is totipotent), an increase in the frequency of development of reconstructed embryos should be observed. Our experiments in sheep support this. In a comparison of embryos that were reconstructed using unsynchronized donor nuclei obtained from 16-cell embryos at the time of activation, and in enucleated pre-activated oocytes after the decline of MPF activity ('Universal Recipient'), development to blastocyst was greatest in the latter group (21.3% versus 55.4%) (Campbell et al., 1994).

Besides the block to re-replication, sufficient time must be allowed for DNA replication of the transferred nucleus to be both initiated and completed before mitosis. During the early embryonic cell cycles of *Xenopus* embryos, DNA replication is completed within 30 min. When nuclei from other cell types, which typically require up to 12 h to complete DNA replication, are transferred into one-cell zygotes, although a high percentage of the transferred nuclei initiate replication, few complete replication before the onset of mitosis. It is postulated that this failure to complete replication is related to both the inability to develop and the occurrence of chromosomal abnormalities in such reconstituted embryos (see DiBerardino, 1979). In somatic cells there are a series of feedback mechanisms that monitor DNA replication (for review see Murray, 1991); however, these controls appear not to function during the early cell cycles of amphibian embryos. In contrast to amphibians, DNA replication during the first cell cycle of mammalian embryos typically occurs over a longer period (8 h in cattle: Barnes and Eyestone, 1990; 7 h in mice: Smith and Johnson, 1986). In mice, transfer of a nucleus from early in the second cell cycle to late in the first cell cycle of an enucleated zygote extends the duration of the first cell cycle in the reconstituted embryo (Smith et al., 1988). This finding suggests that the reconstructed zygote can respond to the replication state of the transferred nucleus. However, in these experiments, completion of replication is evidenced only by the high percentage of these embryos that develop to blastocysts. Similar
experiments have not been reported in other mammals and there is little evidence to support either the presence or absence of such control mechanisms.

Role of the cytoskeleton

Besides the effects of premature chromosome condensation and DNA replication on the development of reconstructed embryos, there is also the question of formation of a polar body in embryos reconstructed using MII oocytes as cytoplasts. In fertilized zygotes, the formation of a polar body removes half of the female genetic material so that the female pronucleus is haploid. What would be the effects of polar body formation from the transferred nucleus in reconstructed embryos? If the nucleus is in G2 at the time of transfer and if the meiotic or mitotic division induced by exposure to MPF is complete, the reconstructed embryo will maintain the correct ploidy after DNA replication. However, if the transferred nucleus is in G1 or S phase at the time of transfer or meiotic or mitotic segregation is unequal, chromosomal abnormalities or changes in ploidy may occur. Thus the effects of polar body formation combined with premature chromosome condensation and uncoordinated DNA replication may have pronounced effects on the ploidy, chromosomal constitution and the subsequent development of the reconstituted embryo (see Table 1). Cheong et al. (1993) reported the extrusion of a polar body when oocytes at MII were used as cytoplasts in mice. However, there are no reports of polar body formation in rabbits, cows, sheep or pigs.

Effects of cell cycle co-ordination on the development of reconstructed embryos

From the preceding discussion two distinct protocols emerge for embryo reconstruction by nuclear transfer when using MII

<table>
<thead>
<tr>
<th>Cell cycle stage of donor nucleus</th>
<th>Formation of a polar body</th>
<th>Hypothetical effect on the ploidy and chromosomal constitution (PCC) of daughter blastomeres derived from the reconstructed embryo after completion of the first cell cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>+</td>
<td>Unknown/aneuploid dependent upon two segregation events</td>
</tr>
<tr>
<td>G1</td>
<td>–</td>
<td>Normal ploidy</td>
</tr>
<tr>
<td>S</td>
<td>+</td>
<td>Probable chromosomal damage/ rearrangements due to premature chromosome condensation of the S-phase chromatin Probable partial reduplication of DNA Unknown/aneuploid dependent upon two segregation events</td>
</tr>
<tr>
<td>S</td>
<td>–</td>
<td>Probable chromosomal damage/ rearrangements due to PCC of the S-phase chromatin Probable partial reduplication of DNA Ploidy dependent upon segregation at first mitosis and the degree of reduplication</td>
</tr>
<tr>
<td>G2</td>
<td>+</td>
<td>Possible normal ploidy if PCC results in no chromosomal rearrangements and equal segregation occurs at the first mitotic/ meiotic division after reconstruction</td>
</tr>
<tr>
<td>G2</td>
<td>–</td>
<td>Tetraploid</td>
</tr>
</tbody>
</table>

Table 2. Effects of different cell cycle phase combinations on development to the blastocyst stage of nuclear transfer reconstructed embryos in a variety of mammals

<table>
<thead>
<tr>
<th>Species</th>
<th>Nuclear donor</th>
<th>Cell cycle stage of donor</th>
<th>MII</th>
<th>G1 / S phase early</th>
<th>S phase mid</th>
<th>S phase late</th>
<th>S phase late / G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>16-cell⁴</td>
<td>S(93%)</td>
<td>21.3⁷</td>
<td>61.3</td>
<td>45.7</td>
<td>57.7</td>
<td>-</td>
</tr>
<tr>
<td>Cow</td>
<td>16-cell⁶</td>
<td>S(90%)</td>
<td>1.25</td>
<td>16.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit</td>
<td>8-cell⁵</td>
<td>G1 / S</td>
<td>15.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8-cell⁶</td>
<td>G1</td>
<td>71.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td>2-cell⁴</td>
<td>G1</td>
<td>77.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2-cell⁴</td>
<td>S</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2-cell⁴</td>
<td>G2</td>
<td>20.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4-cell⁶</td>
<td>G1</td>
<td>43.0</td>
<td>60.0</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4-cell⁶</td>
<td>early S</td>
<td>0.0</td>
<td>14.0</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4-cell⁶ late S/G2</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8-cell⁶</td>
<td>G1</td>
<td>27.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8-cell⁶</td>
<td>S</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

⁴ G1-post decline of MPF activity; ⁷ 22.0% of oocytes activated spontaneously.
⁵ Campbell et al., 1994; ⁶ Campbell et al., unpublished; ⁷ Collas et al., 1992b; ⁸ Cheong et al., 1993; ⁹ Otaegui et al., 1994a; ¹⁰ Otaegui et al., 1994b.
oocytes as cytoplasts. The first is the transfer of nuclei in G1 phase at the time of activation and the second is the transfer of nuclei in G1, S or G2 phases into enucleated activated oocytes after the disappearance of MPF activity (The ‘Universal Recipient’) (Campbell et al., 1993b). Studies using both of these techniques have shown an increase in the frequency of development of reconstituted embryos to the blastocyst stage in different species (see Table 2). The synchronization of blastomeres to be used as nuclear donors is a limiting step in these studies. Although nocodazole has been used successfully in mice (Otaegui et al., 1994b), this and other procedures have proved to be unreliable in embryos of livestock species.

If the frequency of development to blastocyst in ungulates when using the ‘Universal Recipient’ cytoplast and unsynchronized blastomeres as nuclear donors is compared with the earlier reports, in which cytoplasts at MII were used, it can be seen that the overall frequency of development has not increased significantly (in sheep 48.3% (Willadsen, 1986); in cattle 18.0% (Bondioli, 1993)). There are two possible explanations for this discrepancy. First, the hypothesis would predict that only donor nuclei that are in G1 phase would promote development when using MII cytoplasts and, therefore, the percentage of development reflects the percentage of blastomeres in the G1 phase. However, recent reports have shown that at any time most nuclei in early embryos are in S phase (in sheep, 16 cell, 92% (Campbell et al., 1994); in cattle, 21–42 cell, >80% (Barnes et al., 1993)). Second, the recipient cytoplasts in these experiments were not at MII phase at the time of embryo reconstruction. When the methods for embryo reconstruction were first described (Willadsen, 1986), it was surprising that the recipient oocytes used routinely were far older than those used for in vitro fertilization. Whereas bovine oocytes are fertilized about 24 h after the onset of maturation (Gordon and Lu, 1990), recipient cytoplasts have commonly been used 16–24 h later (Bondioli et al., 1990). Recent reports have shown that as the age of the oocyte increases it becomes activated by minor changes in the environment, such as changes in temperature or exposure to the fusion medium. In addition, once activated, pronuclear formation occurs earlier than it does in younger oocytes (Powell and Barnes, 1992). We suggest that in many earlier studies, by the time of fusion, the oocyte was no longer equivalent to one at MII, but rather was similar to the ‘Universal Recipient’ advocated as a result of these analyses.

**Future perspectives in embryo cloning**

This review has discussed the co-ordination of cell cycle events in embryos reconstructed by nuclear transfer. The use of enucleated, activated MII oocytes (the ‘Universal Recipient’) as cytoplast recipients results in an increased frequency of development to blastocyst of embryos reconstructed from unsynchronized donor nuclei from totipotent cell types. This increase in development is due to a reduction in chromosomal damage or aneuploidy, which occurs as a result of premature chromosome condensation and unscheduled DNA synthesis in the transferred nucleus during the first cell cycle after reconstruction. A comparison of other cell cycle combinations is hampered (particularly in farm animal species) by the unreliability of methods for the synchronization of cell cycle stages of individual blastomeres from early embryos. The isolation and maintenance in culture of totipotent cells, which may lend themselves to such comparisons, would provide exciting opportunities not only for the fundamental understanding of nuclear cytoplasmic interactions during early embryo development but also for the production of genetically selected or modified offspring.

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