The role of cell cycle co-ordination in the development of nuclear transfer reconstructed embryos

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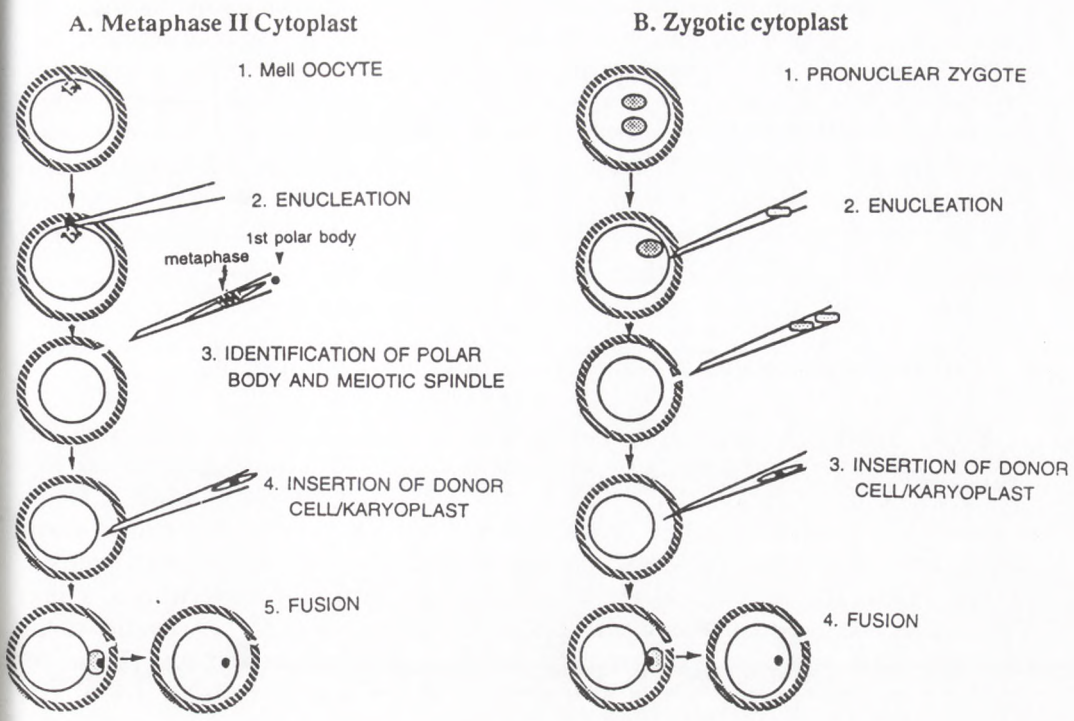
1. Introduction

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).



Box 1. Techniques of nuclear transfer in mammals.

In mammals there are two predominant methods of nuclear transfer which vary upon the point at which the recipient cell is enucleated. Both metaphase II (MII) oocytes and also one cell zygotes have been employed as recipients for nuclear transfer. MII oocytes that are to be enucleated are cultured in medium containing the microfilament inhibitor cytochalasin D and the DNA specific fluorochrome Hoescht 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 1st polar body. Enucleation is confirmed by examining the aspirated cytoplasm under U.V. 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 mouse the pronuclei are visible under direct interference contrast (DIC) optics and can be removed by aspiration. However, in ungulate species the zygotes have to be centrifuged in order to visualise 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 the majority of situations cell fusion is induced by application of a D.C. 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 A.C. current immediately prior to the fusion pulse. When using MII oocytes as cytoplasts the same current which induces fusion also induces activation of the oocyte (for review see Wilmut, 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 which allow direct injection of the donor cell into the cytoplast (Collas, Barnes, 1994; Ritchie, Campbell, 1995).

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, embryo culture (reviewed Wilmut, Campbell, 1992; Campbell, Wilmut, 1994). One other group of factors can be described as cell cycle effects. Many reports in 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.

2. Cytoplasmic states of different cytoplast 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, Clarke, 1979) (Fig. 1). Upon appropriate stimulation, meiosis resumes, the germinal vesicle breaks down, the first meiotic division is completed, 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

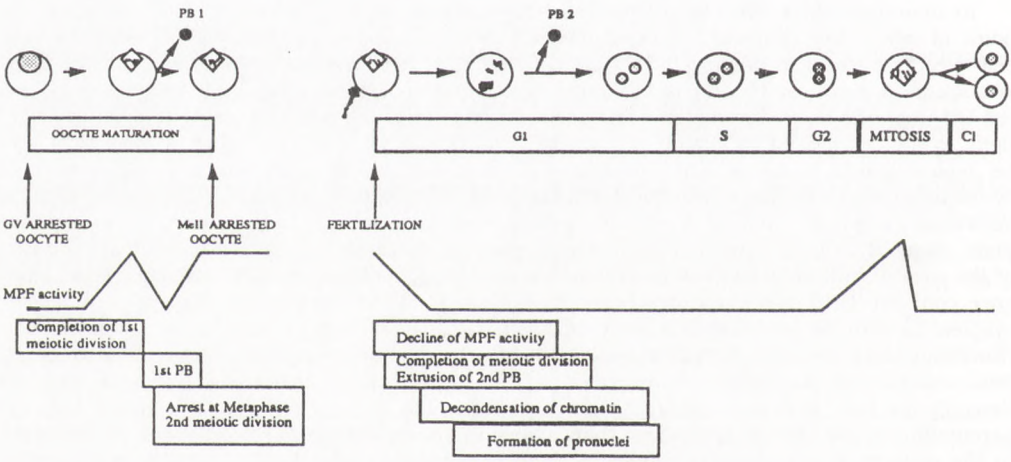


Fig. 1. Schematic representation of the major events during oocyte maturation and the first cell cycle of fertilised zygotes.
Cl = cleavage, PB = polar body, GV = germinal vesicle

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, Markert, 1971). MPF has been identified as a complex of two proteins, cyclins and $p34^{cdc2}$. $p34^{cdc2}$ is 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 $p34^{cdc2}$ remains constant; however, the concentration of cyclins varies. The activation of $p34^{cdc2}$ kinase triggers entry of the cell into mitosis/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).

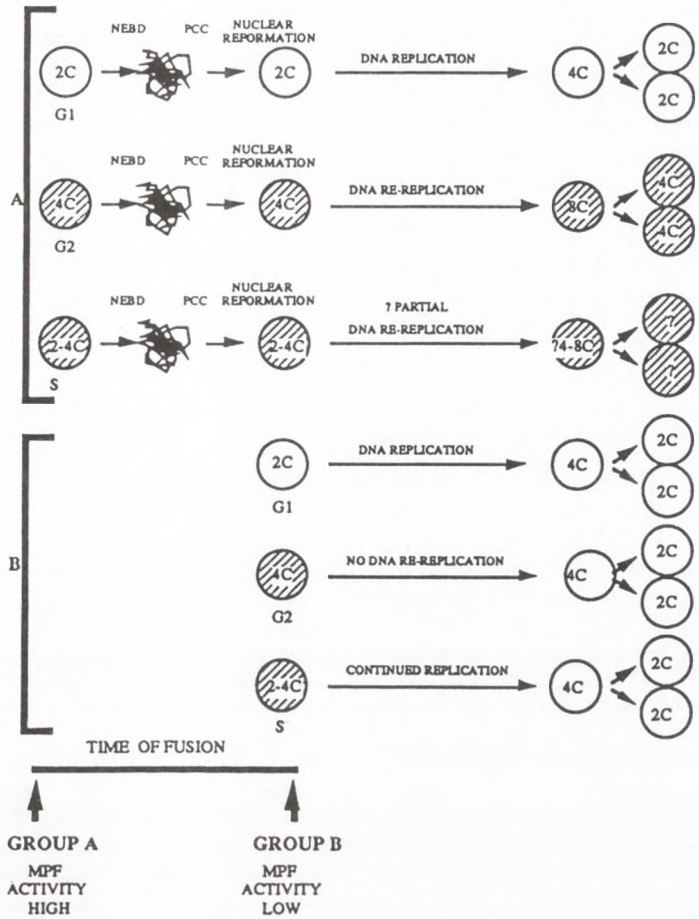
The MPF activity during oocyte maturation is maximal at metaphase of both the first and second meiotic divisions. When the oocyte becomes arrested at metaphase II (MII), MPF activity remains high. Upon fertilization or activation, MPF activity declines rapidly (i.e. bovine Campbell et al., 1993a) (see 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 also be induced by parthenogenetic activation of enucleated MII oocytes; embryos can then be reconstructed after the decline of MPF activity (Campbell et al., 1993b, 1994; Barnes et al., 1993).

3. 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 level of 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, Robl, 1991) have shown that the chromatin of S-phase nuclei induced to undergo premature chromosome condensation by exposure to MPF have a typical pulverized appearance (Schwartz et al., 1971). Additionally chromosome analysis has shown a high incidence of abnormalities in such nuclei (Collas et al., 1992a). In contrast when G1 or G2 nuclei undergo premature chromosome condensation the chromatin condenses to form elongated chromosomes with single- and double-stranded chromatids respectively (Collas et. al., 1992a).

4. 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, Rao, 1970), by injection of nuclei into *Xenopus* eggs (De Roeper et al., 1977) and the *Xenopus* cell free system (Blow, Laskey, 1988) have shown that intact G2 nuclei 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, Laskey, 1988). Similarly our experiments (Campbell et al., 1993b) have shown that in bovine embryos reconstructed by nuclear transfer into a MII cytoplast, all nuclei that undergo nuclear envelope breakdown regardless of their cell cycle stage undergo DNA synthesis following 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, whilst those that are in G2 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). Further, we hypothesise that when using MII oocytes 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).



Box 2. Effects of nuclear transfer of karyoplasts at defined cell cycle stages into cytoplasts with either high (group A) or low (group B) maturation promoting factor activity upon DNA synthesis during the first cell cycle and potential effects upon the ploidy of the reconstructed embryo.

All nuclei transferred at the time of activation (group A) when MPF activity is high undergo nuclear envelope breakdown (NEBD) which is followed by premature chromosome condensation (PCC). The nuclear envelope is then reformed and DNA synthesis is observed in all nuclei. In this situation it is probable that unless the nucleus was in G1 at the time of transfer re-replication of previously replicated DNA will occur and that at the end of the 1st cell cycle the DNA content of the daughter nuclei will be incorrect. The increased amount of DNA present at the end of the 1st cycle may also adversely affect mitotis resulting in unequal segregation or possible chromosomal abnormalities. In contrast when nuclei are transferred after the disappearance of MPF activity no NEBD and no PCC are observed. Nuclei that are in G1 or S-phase initiate or continue DNA synthesis, however, no DNA synthesis is observed in nuclei that are in the G2-phase at the time of transfer.

In this diagramatic representation red circles represent nuclei which are 'out of phase' in terms of DNA content with the cell cycle stage of the recipient cytoplast, those which are green are 'in phase'.

TABLE 1

EFFECTS OF DIFFERENT CELL CYCLE PHASE COMBINATIONS ON DEVELOPMENT TO THE BLASTOCYST STAGE OF NUCLEAR TRANSFER RECONSTUCTED EMBRYOS IN A VARIETY OF MAMMALIAN SPECIES

Species	Cell cycle stage of cytoplast		MII	G1[a] S-phase early	S-phase MID	S-phase LATE	S-Phase LATE/G2
	Nuclear donor	Cell cycle stage of donor					
Sheep	16 cell [1]	S(93%)	21.3[b]	61.3	45.7	57.7	
Cow	16 cell[2]	S(90%)	1.25	16.3			
Rabbit	8 cell[3]	G1/S	15.0				
	8 cell[3]	G1	71.0				
Mouse	2 cell[4]	G1	77.8				
	2 cell[4]	S	0.0				
	2 cell[4]	G2	20.8				
	4 cell[5]	G1	43.0	60.0			0.0
	4 cell[5]	early S	0.0	14.0			0.0
	4 cell[5]	late S/G2	0.0	0.0			
	8 cell[6]	G1	27.0				
	8 cell[6]	S	0.0				

a) G1-post decline of MPF activity; b) 22.0% of oocytes activated spontaneously.

1) Campbell et al., 1994; 2) Campbell et al., unpublished data; 3) Collas et al., 1992b; 4) Cheong et al., 1993; 5) Otaegui et al., 1994a; 6) Otaegui et al., 1994b.

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 (for discussion see Di Berardino, 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 (i.e. cattle 8 h (Barnes, Eyestone, 1990), mouse 7 h (Smith, 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 experi-

ment's 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.

5. 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 oocytes as cytoplasts. First, the transfer of G1 stage nuclei at the time of activation and second 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 Tab. 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.

TABLE 2
DEVELOPMENT OF OVINE EMBRYOS RECONSTRUCTED FROM CULTURED EMBRYONIC DISC CELLS

1993-94				1994-95			
Group	Number embryos recovered (%)	Number of morulae/ blastocysts (%)	Number transferred/ Number lambs	Group	Number embryos recovered (%)	Number of morulae/ blastocysts (%)	Number transferred/ Number lambs
16 cell A/F	34 (82.9)	2 (5.9)	2/1	16 cell	28 (96.6)	14 (50.0)	14/0
16 cell UR	22 (71.0)	6 (27.3)	6/2	ED P6	98 (84.5)	9 (9.2)	9/0
ED cell	15 (75.0)	1 (6.7)	1/0	ED P11	92 (87.6)	10 (10.9)	10/0
ED P1	19 (59.4)	4 (21.0)	4/1				
ED P2	11 (47.8)	1 (9.1)	1/1				
ED P3	36 (73.5)	2 (5.5)	2/2				

In vivo produced Day 9 ovine blastocysts were cultured on a feeder layer of mitotically inactivated 1^0 murine fibroblasts in DMEM 10% FCS. After attachment the trophectoderm was manually removed. ES like colonies were picked and passaged under the same culture conditions. For nuclear transfer cultured cells were fused to enucleated, pre-activated *in vivo* produced ovine MII oocytes.

If the frequency of development to blastocyst in ungulate species when using the 'Universal Recipient' cytoplast and unsynchronized blastomeres as nuclear donors is compared with the earlier reports, in which MII cytoplasts were used, it can see that the overall frequency of development has not increased significantly (i.e. sheep 48.3% (Willadsen, 1986), cattle 18.0% (Bondioli, 1993)). There are two possible explanations of this discrepancy: first the hypothesis would predict that only donor nuclei that are in G1 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 (i.e. sheep 16 cell 92% (Campbell et al., 1994), cattle 21-42 cell >80% (Barnes et al., 1993). Second, the recipient cytoplasts in these experiments were not at MII 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, Lu, 1990), recipient cytoplasts have commonly been used some 16 – 24 h later (Bondioli et al., 1990). Recent reports have shown that as the age of the oocyte increases they become 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, Barnes, 1992). We suggest that in many earlier studies, by the time of fusion the oocyte was no longer equivalent to one at second metaphase, but rather was similar to the 'Universal Recipient' advocated as a result of these analyses.

6. Nuclear Transfer in Sheep using cultured cells as a source of donor nuclei

The production of cloned offspring in mammalian species by the transfer of nuclei from an established cell line would provide many advantages in the fields of "Reproduction" and Biotechnology. One candidate for such a cell type is embryonic stem (ES) cells. ES cell lines have been established from murine blastocysts. These are defined as pluripotent, producing chimeric animals after injection into blastocysts, however, as yet there are no reports of development to term following nuclear transfer. At the present time, the isolation of ES *'like'* cells has been reported in the pig, cow and sheep, however, neither production of chimeric animals nor development to term following nuclear transfer has been shown.

In order to address this area at Roslin we have assessed the totipotency of embryonic disc (ED) cells taken directly from Day 9 ovine embryos and in preliminary experiments to establish whether totipotency is maintained when ED cells are cultured in conditions which promote the isolation of ES cell

lines in the mouse (Campbell et al., 1995a,b). The outline of the experimental procedure is described in Fig. 2 and the results summarised in Tab. 2. Live lambs were obtained up to and including passage 3. However, in subsequent experiments at passages 6 and 10, although development to the blastocyst stage was obtained, no live offspring were produced. These results show that cultured embryonic disc cells retain totipotency for at least 3 passages when placed into culture and offer exciting evidence that cultured cell lines may support development to term of nuclear transfer reconstructed embryos.

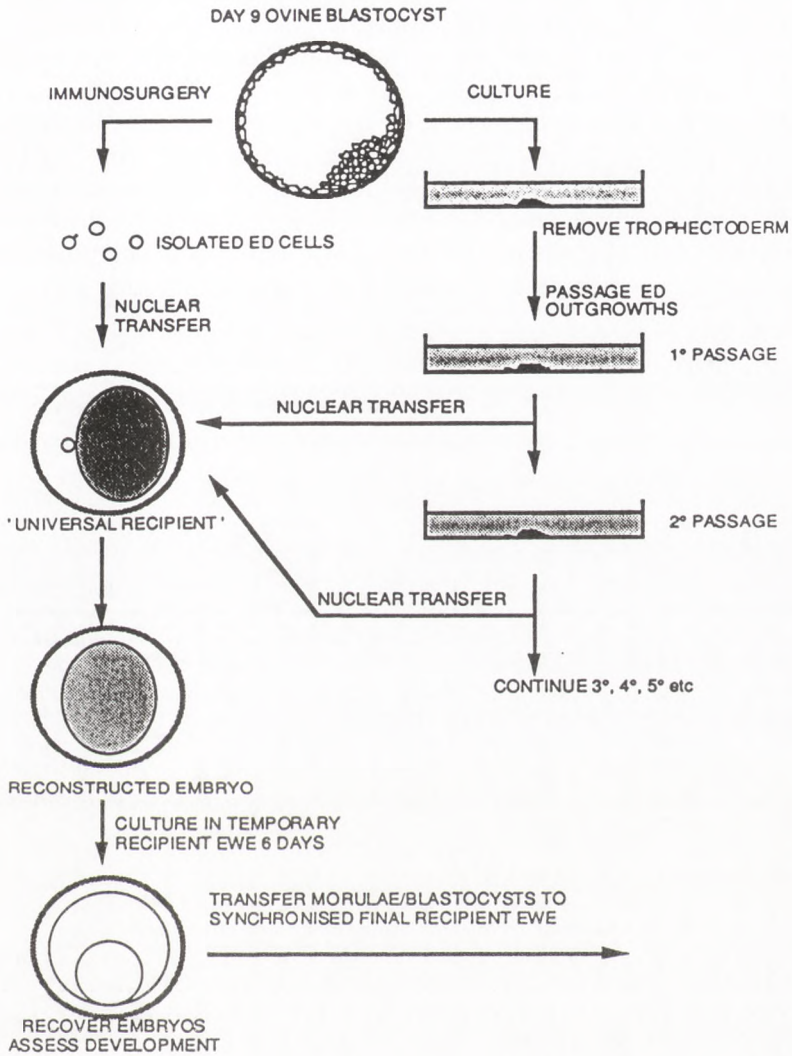


Fig. 2. Schematic representation of experimental protocol for nuclear transfer from cultured embryonic disc(ED) cells.

7. 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 occur as a result of premature chromosome condensation and unscheduled DNA synthesis in the transferred nucleus during the first cell cycle after reconstruction. At the present time a comparison of other cell cycle combinations is hampered (particularly in farm animal species) by the unreliability of methods for the synchronisation 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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Summary

Exciting new opportunities in embryo cloning have been made possible by recent studies on the interaction of the donor nucleus with the recipient cytoplasm following embryo reconstruction. The purpose of this paper is to review 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. These 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.

Key words:
embryo, cloning, cell cycle, DNA replication.

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