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Review article

The possible role of cell cycle stage in mammalian cloning

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Abstract. Progress in mammalian cloning started from cloning embryos (of mice, rats, rabbits, sheep, goats, pigs, cattle and rhesus monkeys) and culminated in obtaining clones of sheep, cattle, pigs and mice from adult somatic cells. Knowing the relationship between the cell cycles of the recipient and the donor of cell nucleus in embryonic cloning by nuclear transfer one can adjust the phases of the cell cycle properly. Metaphase II recipients accept G1 (in most species) or G2 donors (in the mouse). Interphase recipients can harbour nuclei in all stages of cell cycle. Relatively little is known about somatic cloning. Two attitudes are applied: either the donor is in the G0 phase or the recipient is in a prolonged MII phase.

Introduction

Clone (Greek *klōn* 'slip' or 'twig') is a group of at least two genetically identical cells or organisms derived from a single individual by asexual reproduction of various types. In many animals clones may occur naturally as a result of cell division (Protoza), gemmation (Coelenterata, Hydrozoa), strobilation (Coelenterata, Scyphozoa) and polyembryony (Insecta, Hymenoptera, Ichneumonoidea; Mammalia). In mammals, natural polyembryony is probably extremely rare although the incidence of genetically identical individuals (monozygotic twins and multiplets) is unknown in most species because they generally go unnoticed. Monozygotic multiplets occur, as a rule, in nine-banded (*Dasypus novemcinctus* L.) and in eleven-banded (*Dasypus hybridus* Desmarest) armadillos (Edentata). In humans the most common genetically identical multiplets are monozygotic twins, which also occur sporadically in sheep, cattle and horses.

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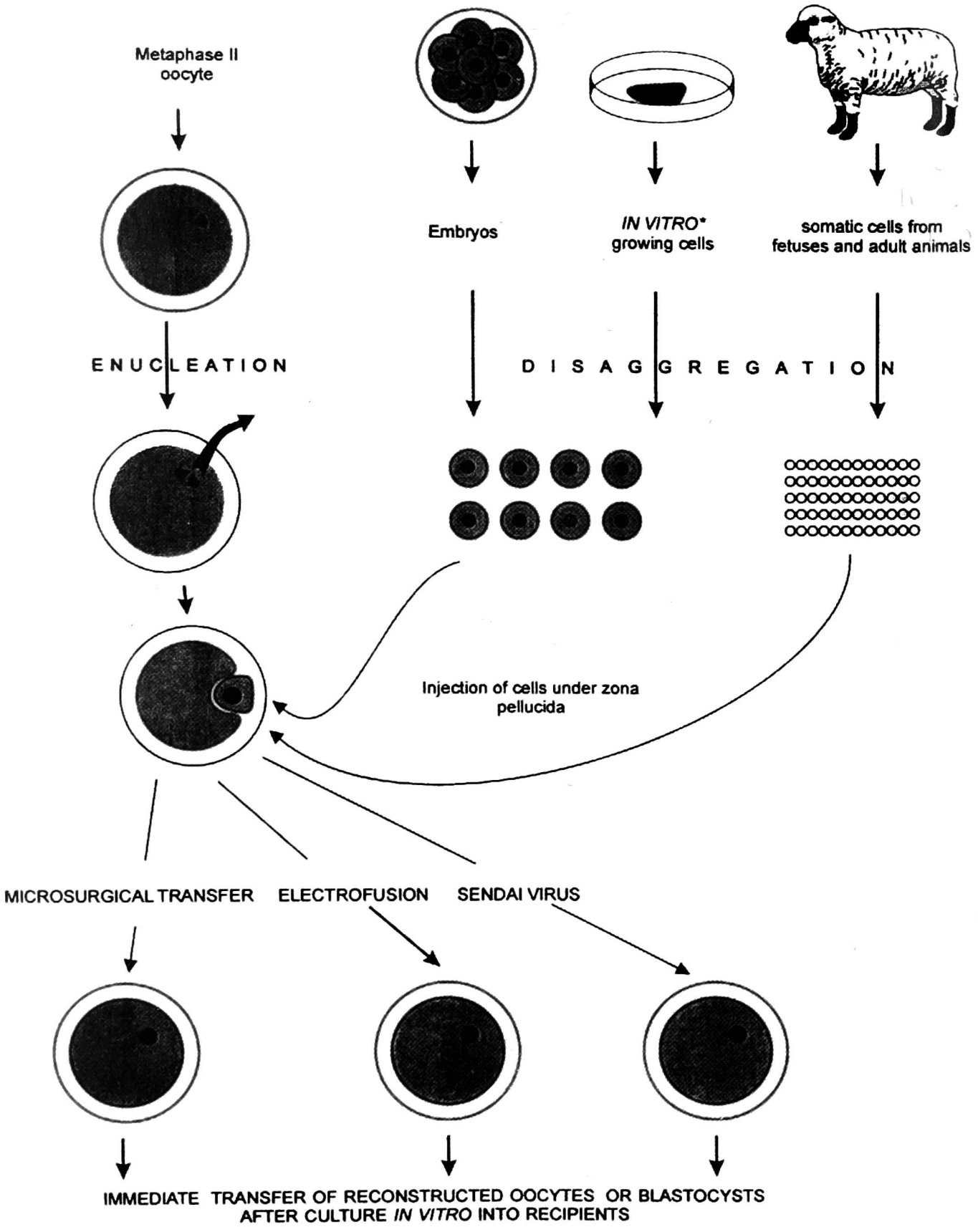

Figure 1. Cloning of mammals by nuclear transfer of embryonic and somatic cell nuclei (after MODLIŃSKI 1997, modified)

These monozygotic mammalian twins and multiplets are due to spontaneous self-cloning of preimplantation embryos. Monozygotic twinning usually begins at the blastocyst stage around the first 7-10 days of development and results in division of the inner cell mass (ICM), embryonic disc (ED) or embryoblast into two embryonic primordia. This type of cloning, which is termed embryonic cloning,

may be also performed experimentally by embryo splitting, blastomere isolation, recombination of isolated blastomeres (chimeric cloning) and nuclear transfer (see MODLIŃSKI 1997, MODLIŃSKI, KARASIEWICZ 1998 for review). In the first three cases all of the components of the nascent individuals, including genetic material and maternally inherited cytoplasmic factors, are derived from a single embryo, and thus they are true clones: "embryonic copies" or embryonic clones. On the other hand, the nuclear transfer technique that is in fact egg cell reconstruction involves the transfer of nuclear genetic material from a donor cell into a recipient cell (oocyte, zygote or blastomere) from which their own genetic material has been previously removed. The enucleated recipient cell is dubbed "cytoplast" or "ooplast" and the reconstructed cell is a nucleo-cytoplasmic cybrid cell. Since in the nuclear transfer technique the genetically identical nuclei are introduced into different cytoplasts, the resultant individuals are not true clones and may be more precisely described as "genomic copies" or genomic clones. However, it is the nuclear transfer technique which enables obtaining the most numerous flocks of genetically identical animals. Genomic cloning was originally limited to using cells from early cleaving embryos as nuclear donors (Figure 1) and have resulted in successful production of live animals including mice, rats, rabbits, sheep, goats, pigs, cattle and rhesus monkeys. However, a considerable progress has been made recently towards solving several technical and biological problems associated with nuclear transfer. This progress allows to demonstrate that successful development can be obtained using – as nuclear donors – the cells derived from late-stage embryos (mouse inner cell mass and trophectoderm cells: TSUNODA, KATO 1998), from *in vitro* cultured embryonic cells (sheep: CAMPBELL et. al. 1996, WELLS et al. 1997; mouse ES cells: MODLIŃSKI et al. 1996, WAKAYAMA et al. 1999) and finally, from foetal and adult tissues (Figure 1), culminating in the birth of Dolly (WILMUT et al. 1997) and Cumulina (WAKAYAMA et al. 1998), the first mammalian offspring derived from adult somatic cells.

One of the problems to be solved in cloning mammals by nuclear transfer is cell cycle co-ordination between the donor and recipient cells. The aim of this article is to describe and discuss the possible role of cell cycle stage of the recipient and donor cells in remodelling of introduced nuclear structures as well as in the maintenance of the correct ploidy in developing embryos .

Cell cycle progression

Cell cycle in somatic cells

In order to divide, the cell must replicate all its DNA and that is followed by a division when chromosomes are segregated into daughter cells. The cell cycle is a set of steps that enable the correct completion of these processes.

The cell cycle of differentiated somatic cells consists of four phases: most important is the S phase when DNA replication occurs and the M phase when chro-

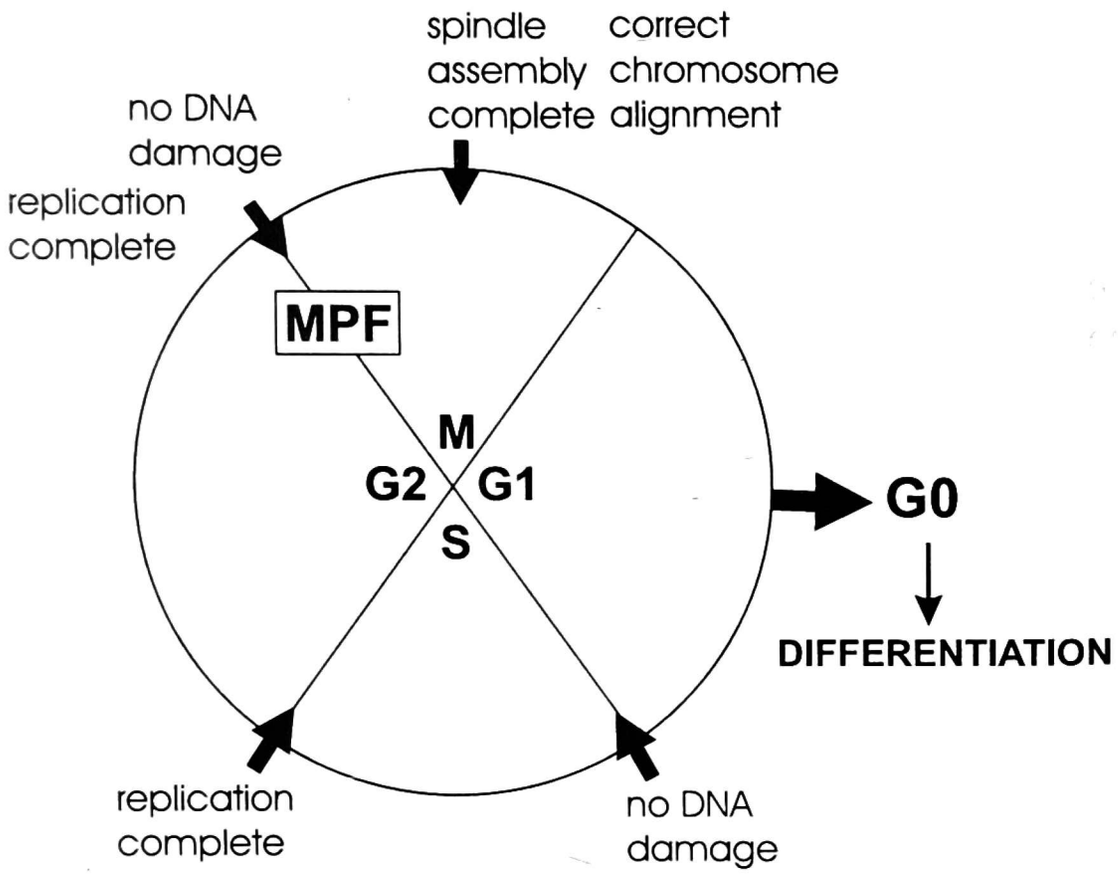

Figure 2. Schematic view of the cell cycle and the checkpoint controls of the cell cycle progression

mosomes are segregated into daughter cells. In majority of somatic cells, the S and M phases are separated by "gap" phases G1 and G2. There are several checkpoint controls that coordinate subsequent cell cycle events in space and time (Figure 2). They can break the cell cycle in response to some irregularities in the progress of particular phases of the cell cycle (see GRABAREK 1998 for review). It is well established now that a family of protein kinases termed cyclin-dependent kinases (CDKs) are key regulators of the cell cycle (see NIGG 1995 for review). The first discovered vertebrate CDK, p34cdc2 (CDK1), was shown to be a catalytic subunit of the maturation promoting factor (MPF), the universal regulator of the cell cycle in all eukarytic cells (MASUI, MARKERT 1971, GAUTHIER et al. 1988).

The G1 phase comes after mitosis. After the division, chromosomes decondense and form interphase nuclei in daughter cells. During the G1 phase the cell can grow and prepare for DNA replication before the next mitosis. It is also the time for the decision whether the cell will follow mitosis or exit the growth cycle and enter the quiescent state (G0), which is the first step for further differentiation of cells. Progression to the G0 stage is a reversible process, highly dependent on the presence of extracellular growth factors, mitogen antagonists and differentiation inducers or inhibitors. On exit from the cell cycle a number of substantial changes have been described to occur; these include: a reduction of transcription, changes in polyribosomes, destruction of the majority of mRNA species and condensation of chromatin. If the cell follows a mitotic cell cycle, it progresses through the G1 phase and prepares itself for DNA replication.

The mechanism controlling G1 phase progression is therefore thought to be responsive to damaged DNA present in the cell. When the S phase is initiated, cells start to replicate DNA. The mechanism monitoring this phase is sensitive to completion of DNA replication (NURSE 1994) and also to DNA damage. The S phase can be prolonged or even arrested until DNA damage is repaired. Once the DNA replication is completed, cells enter the G2 phase and prepare for the next division. The mechanism controlling the G2/M phase transition is also responsive to signals of DNA synthesis and repair completion.

The control of the M phase is one of the most conserved features of the cell cycle. The key regulator of this phase is MPF that is stabilised by a calcium-sensitive cytoplasmic factor termed cytostatic factor (CSF). CSF has been shown to be the product of c-mos proto-oncogene (HUNT 1992). MPF is not present in the cell during the G1 and S phases and is cumulated in an inactive form (pre-MPF) during the G2 phase. Once activated, MPF initiates a cascade of events that prepare cells for division. This activity causes nuclear envelope breakdown (NEBD), chromosome condensation, spindle assembly and reorganization of the whole microtubule network from interphase into the mitotic stage (ARION et al. 1988, DE PENNART et al. 1988, WARD, KIRSCHNER 1990, VERDE et al. 1990, GOTOH et al. 1991, SMYTHE, NEWPORT 1992, PINES 1994). Activation of MPF is essential for progress to the metaphase stage, but its inactivation is not required for the transition to anaphase (HOLLOWAY et al. 1993, IRNIGER et al. 1995). However, inactivation of MPF is necessary for the cell to complete cytokinesis and return to the interphase stage (KING et al. 1994). Mechanisms monitoring the M phase progression are sensitive to proper spindle formation and chromosome alignment completion during metaphase. If the spindle is not formed correctly or single unattached chromosomes are present in cell cytoplasm, division is inhibited (MCKIM, HAWLEY 1995).

Before the cell divides, it must double its DNA content from 2C (single chromatids) to 4C (double chromatids). At the onset of the M phase cells are diploid and have 4C DNA content. During mitotic division only chromatid segregation occurs. After division the cells remain diploid (2N) but with a 2C content of DNA.

Meiotic cell cycle in mammals

Meiotic division differs from mitosis. During the first meiotic division, reduction of chromosome number occurs. Thus, cells have a haploid (1N) number of double-chromatid chromosomes. Due to DNA amount reduction the cells have 2C DNA content. During the second meiotic division chromatid segregation occurs and the cells remain haploid with a 1C content of DNA. The meiotic cell cycle though limited to gametogenesis, is regulated by similar mechanisms.

Most fully grown mammalian oocytes are arrested in the prophase of the first meiotic division, at the germinal vesicle (GV) stage (Figure 3A). Upon appropri-

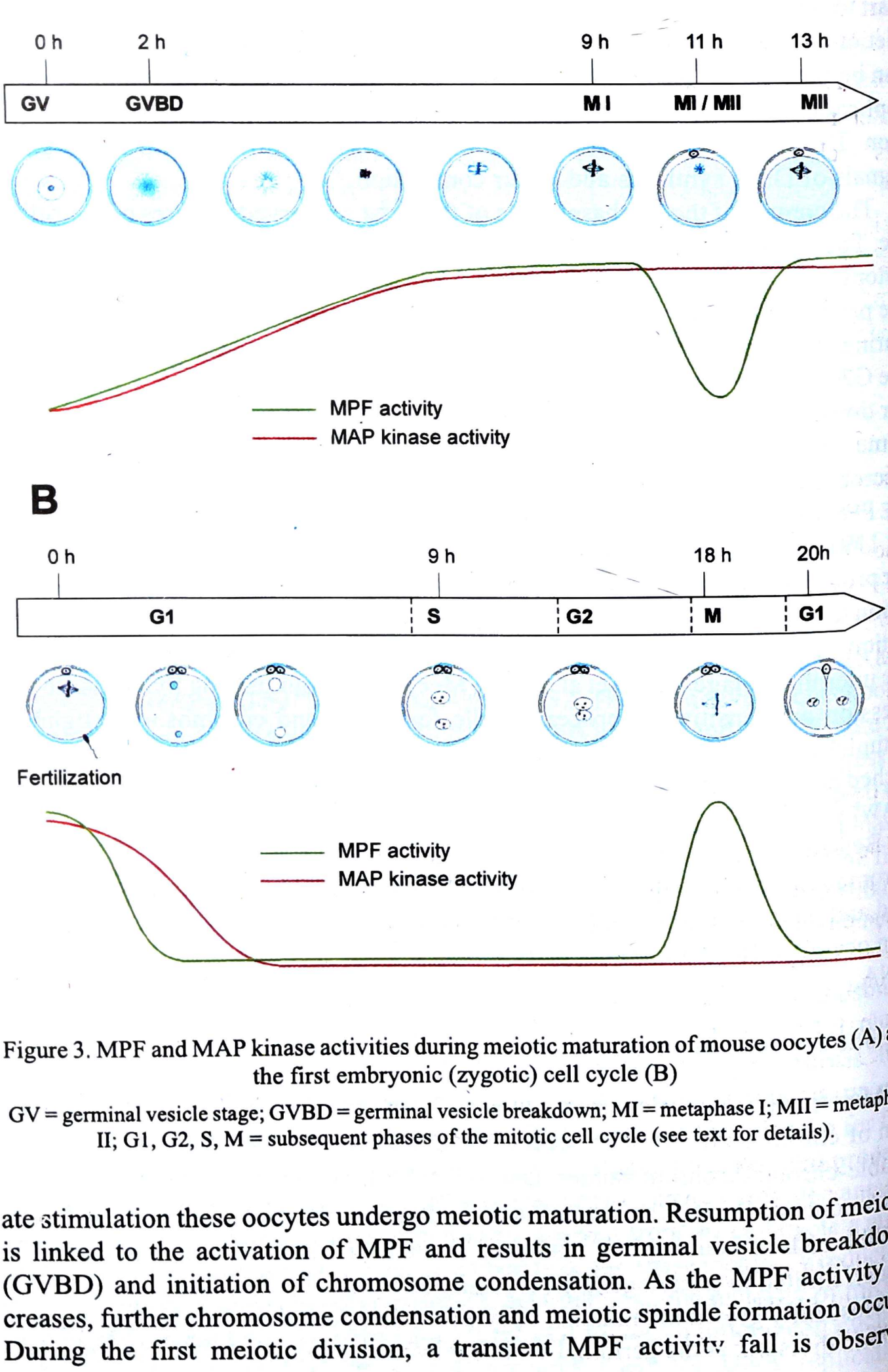

Figure 3. MPF and MAP kinase activities during meiotic maturation of mouse oocytes (A) and the first embryonic (zygotic) cell cycle (B)

GV = germinal vesicle stage; GVBD = germinal vesicle breakdown; MI = metaphase I; MII = metaphase II; G1, G2, S, M = subsequent phases of the mitotic cell cycle (see text for details).

ate stimulation these oocytes undergo meiotic maturation. Resumption of meiosis is linked to the activation of MPF and results in germinal vesicle breakdown (GVBD) and initiation of chromosome condensation. As the MPF activity increases, further chromosome condensation and meiotic spindle formation occurs. During the first meiotic division, a transient MPF activity fall is observed

(VERLHAC et al. 1994). After the extrusion of the 1st polar body, MPF is again activated and the metaphase II (MII) meiotic spindle is formed. Between the MI and MII stage chromosomes of an oocyte remain condensed and no replication occurs.

When oocytes reach the MII stage, they remain arrested until fertilization or parthenogenetic activation. Metaphase II arrest is maintained by the cytostatic factor – CSF (MASUI, MARKERT 1971). The nature of CSF and MPF interactions is unclear, although there is some evidence that CSF may stabilize the levels of the regulatory subunit of MPF and therefore prevent its inactivation (KUBIAK et al. 1993). The mos protein (SAGATA et al. 1989) and MAP kinase (HACCARD et al. 1993) have been shown to be involved in CSF activity. However, other factors may also be required to generate CSF. Cdk2 kinase has been proposed to be one possible candidate (GABRIELLI et al. 1993) but its role is still not definitely established.

Following activation, either natural or artificial, oocytes complete the second meiotic division and the 2nd polar body is extruded (Figure 3A). After the oocyte completes meiosis, maternal chromosomes rebuild the nuclear envelope and form a female pronucleus. Completion of meiosis is linked to the fall of MPF and CSF activities. While inactivation of MPF is rapid, inactivation of cytostatic activity seems to be gradual. In artificially activated mouse oocytes inactivation of MPF was observed within 15 min. (SZÖLLÖSI et al. 1993), whereas inactivation of MAP kinase began 2 hours after MPF inactivation (VERLHAC et al. 1994). Also, a high amount of Mos protein was detected in activated oocytes at that time (WEBER et al. 1991).

Zygotic cell cycle

The first mitotic cycle in mammalian embryos differs from subsequent divisions and is crucial for further development. The progression of the first embryonic cell cycle is maternally controlled (SCHULTZ 1993). During this cycle, gradual transition from meiotic to mitotic cell cycle controls takes place (KUBIAK, SZÖLLÖSI 1994, CIEMERYCH 1998). The first stage of this transition is inactivation of CSF. It has been shown that in mouse oocytes MAP kinase acts similarly to MPF. It causes delay in rebuilding of nuclear envelope and microtubullar reorganization from metaphase to the interphase state (VERDE et al. 1990, GOTOH et al. 1991). The probable effect of MAP kinase activity is a prolonged G1 phase in the first embryonic cell cycle (Figure 3B). In the mouse, G1 phase of the zygotic cell cycle lasts 4-9 hours (ABRAMCZUK, SAWICKI 1975, LUTHARD, DONAHUE 1973), while this phase in the second embryonic cell cycle lasts only 1 hour. The relatively long G1 phase of the first embryonic cell cycle allows proper transformation of oocyte and sperm chromatin into pronuclei. Subsequent embryonic cell cycles are typical mitoses and the G1 phase in these cycles is considerably shorter.

Remodelling of nuclear structures in oocytes reconstituted by nuclear transfer

For several biological and technical reasons, in an overwhelming majority of nuclear transfer experiments, the enucleated MII oocytes have become the cytoplasts (ooplasts) of choice. However, the behaviour of introduced nuclei strongly depends on the cytoplasmic state of the ooplast used (CZOŁOWSKA et al. 1984, 1992). In non-activated ooplasts (with a high MPF level), the transferred nuclei – regardless of the cell cycle at the time of transfer – undergo NEBD and premature chromosome condensation (PCC). The effects of NEBD and PCC on the transferred nucleus depend upon their cell cycle stage at the time of transfer. Nuclei in the G1 and G2 stages form single or double chromatids, respectively.

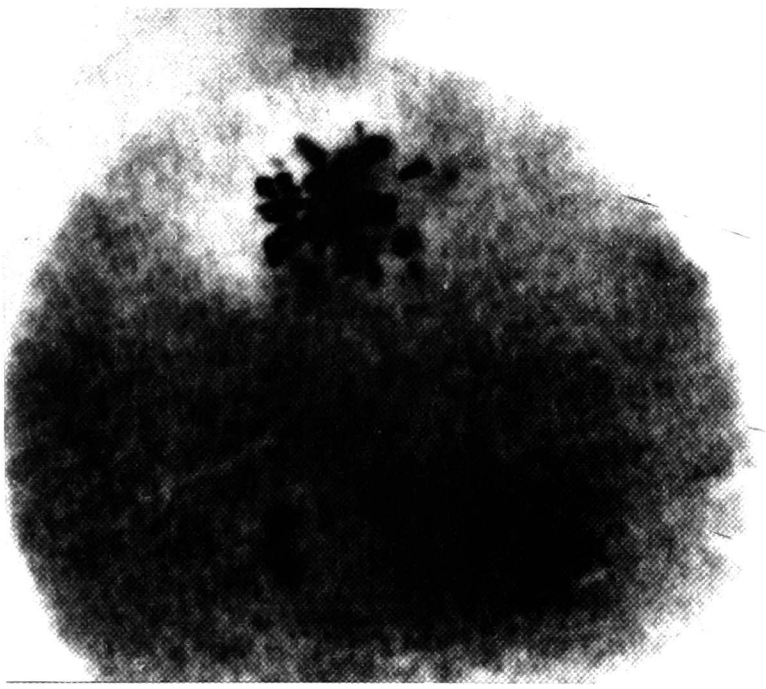

Figure 4. PCC of a mouse embryonic stem (ES) cell nucleus introduced into a MII oocyte. The pattern of chromatin condensation suggests that the donor nucleus was in the S phase (after MODLIŃSKI 1995).

In both cases full chromosome condensation is observed accompanied by the formation of the mature kinetochore (COLLAS et al. 1992a). Nuclei at the S stage attain a typical for that phase pulverised appearance of chromatin and even the most condensed fragments of chromatin do not approach the level of condensation of G1/G2 or meiotic chromosomes (Figure 4). Another effect of PCC on S-phase nuclei is the formation of an abnormal spindle and the absence of mature functional, trilaminar kinetochores on condensed chromosomes. As the cell cycle is restarted by activating reconstructed oocytes with an appropriate artificial stimulus, the nuclear envelope is rebuilt and interphase nuclei are reformed from the condensed

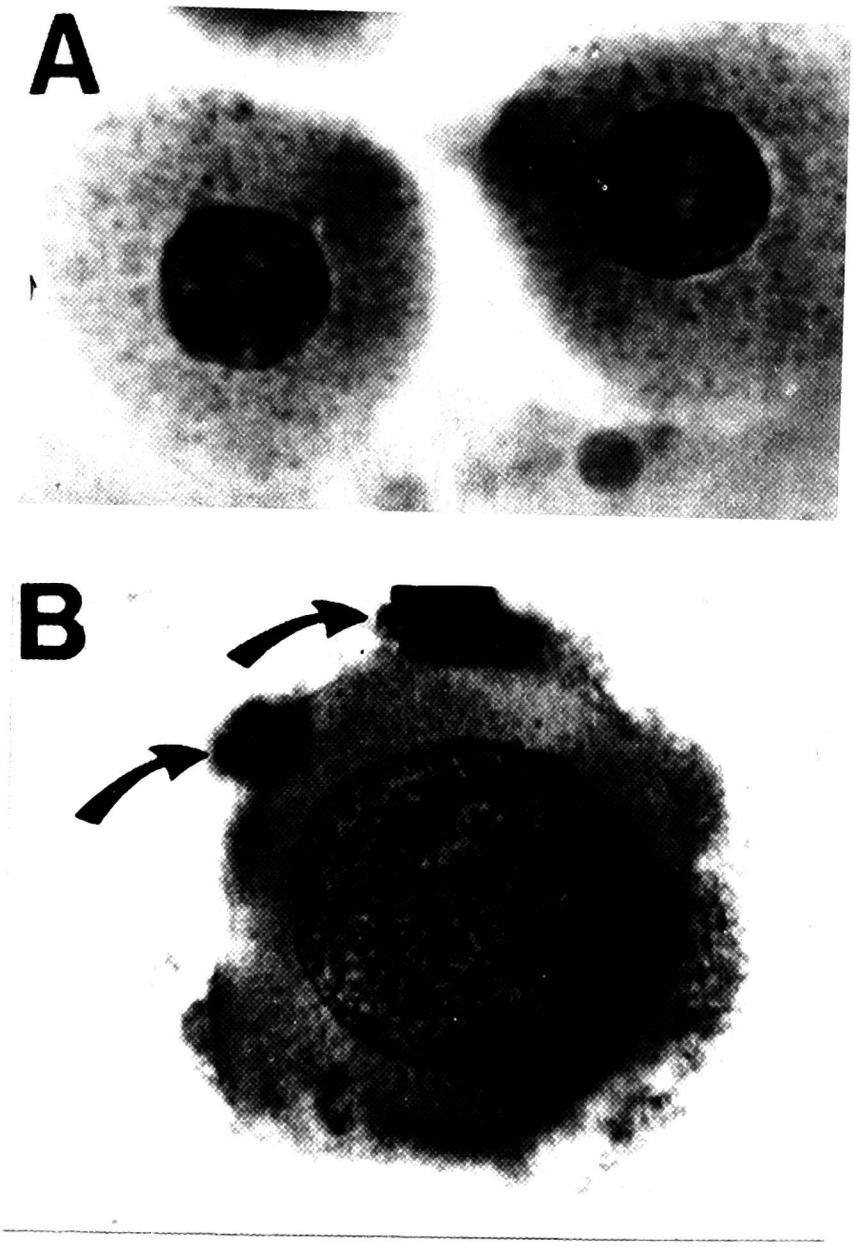

Figure 5. Mouse ES cell nuclei introduced into enucleated metaphase II oocytes and reformed after oocytes activation

A = formation of pronucleus-size nuclei, B = formation of overgrown nucleus indicating abnormal remodelling pattern. Compare the size of the reformed nucleus to the size of the nucleus of a nonfused ES cell (arrows) (after MODLIŃSKI 1995)

chromatin (Figure 5). Studies on reconstructed mouse oocytes receiving 1/8 blastomere nuclei (SZÖLLÖSI, SZÖLLÖSI 1988, CZOŁOWSKA et al. 1992) revealed that reformed nuclei initiated and continued "bleb" formation. "Blebbing" activity of nuclear envelope (SZÖLLÖSI, SZÖLLÖSI 1988) seems to be a phenomenon typical of early embryonic stages. It has been described in mouse, rat, rabbit, human and bovine pronuclei. It is thought that these membrane evaginations represented a specific nucleo-cytoplasmic communication system, serving for removal of certain excess materials (of unknown origin) from the nucleus at specific times of embryogenesis. Initiation of "blebbing" activity was also observed in bovine oocytes reconstructed from embryonic nuclei (KANKA et al. 1991) but not in pig oocytes receiving cultured embryonic ectodermal cell nuclei (OUHIBI et al. 1996).

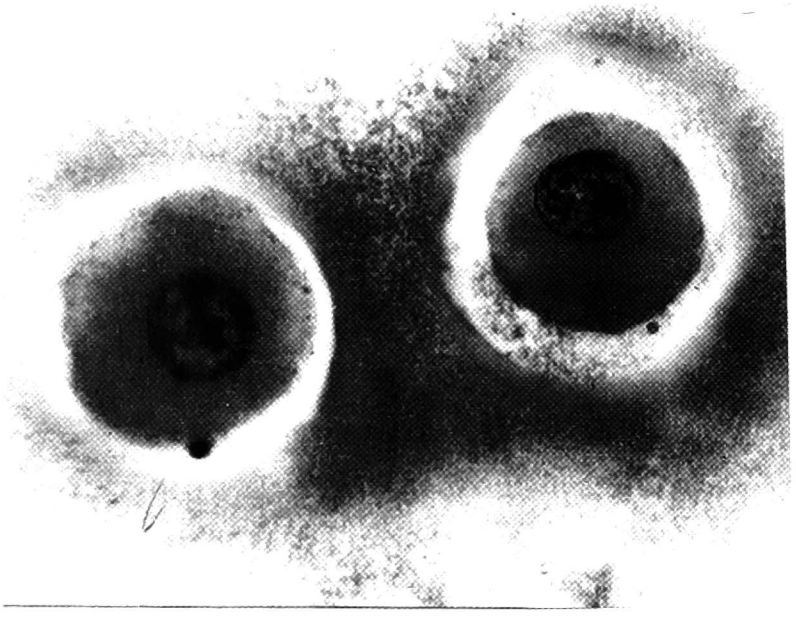

Figure 6. Swelling of an ES cell nucleus introduced into an activated mouse oocyte. The size of the nucleus is comparable to the size of the fully-grown pronucleus (after MODLIŃSKI 1995).

In the mouse, the remodelling process is most spectacular in nucleoli which revert from morphology characteristic for the transcriptionally active embryonic form (composed of compact fibrillar and fibrillo-granular parts and containing several fibrillar centers; SZÖLLÖSI 1971) to a structure typical for the zygote not engaged in rRNA transcription (the pronucleus – contained large electron dense, nucleolus-like body/bodies). The reconstructed embryonic nuclei contained, however, intranuclear annulate lamellae which are rarely observed in female pronuclei (CZOŁOWSKA et al. 1992).

Nuclei introduced into pre-activated interphase oocytes, after the cytoplasm has lost MPF activity, remain in the interphase state. The first overt event interpreted as morphological evidence of remodelling of the introduced nucleus is nuclear swelling (Figure 6). This may be the result of movement of some cytoplasmic materials into the nucleus. Specific examples of such movement of oocyte-made karyophillic materials are nuclear lamins (KUBIAK et al. 1991) and snRNAs and sRNPs (DEAN et al. 1989), which are exhausted in the cytoplasm shortly after oocyte activation. Some of proteins incorporated into the nucleus may be similar to the "early shifting proteins" which accumulate in *Xenopus* embryonic nuclei (DREYER 1987). As in reconstituted nuclei, the nuclear envelope "blebbing" is also observed in the introduced intact embryonic nuclei (CZOŁOWSKA et al. 1992), suggesting that this phenomenon is apparently independent of PCC. Mouse embryonic nuclei introduced into pre-activated oocytes retained features typical for blastomere nuclei: folded nuclear envelope, the presence of intracellular annulate lamellae and the structure of nucleoli (see above).

Cell cycle of donor nuclei and ploidy of reconstructed embryos

Coordination of cell cycle phases between ooplast and donor cells is essential for the maintenance of correct ploidy in the reconstituted embryos. In the majority of early studies the unsynchronized blastomeres were used as a source of donor nuclei. In preimplantation embryos the S phase is the longest phase of the cell cycle. In the mouse, for example, in the third and fourth cleavage cycle, which last 10-12 h, the S phase lasts 7 h, whereas the G1 and G2 phases last only about 1-2 h each (SMITH, JOHNSON 1986). Thus, it is reasonably certain that at any one time the majority of donor nuclei are in the S phase. As outlined above, nuclei in the G1 and G2 phases form in nonactivated MII oocytes single (2N; 2C) or double chromatids (4N; 4C), respectively, whereas the chromatin of S phase cell nuclei (2-4C) condenses in a pulverized manner. After activation and formation of a new nuclear envelope, the reformed nuclei undergo DNA synthesis. Studies on the development of nuclear transfer in bovine (CAMPBELL et al. 1993) and ovine embryos (CAMPBELL et al. 1994) suggest that the pattern of DNA synthesis in the reconstituted oocytes is dependent upon the cell cycle stage of the donor nuclei at the time of transfer and has an effect on the ploidy of daughter cells. Nuclei in the G1 phase or at the G1/S transition (before replication) undergo a normal round of replication which results in diploid descendant cells. Nuclei in the G2 phase (after replication) undergo an additional round of *de novo* replication resulting in formation of tetraploid daughter blastomeres. Nuclei in the S phase or at the S/G2 transition (during replication or just after replication) re-replicate a part or all of the DNA. A consequence of that is the formation of aneuploid or tetraploid cells. Furthermore, the partially condensed pulverized chromatin of S phase nuclei can sustain genetic damage resulting in abnormal development. This hypothesis was confirmed by experiments in both cattle and sheep (CAMPBELL et al. 1993, 1994). The frequency of development of reconstituted MII oocytes was significantly greater when the donor nuclei were at the early stage of the cell cycle (i.e. in G1) than when nuclei were in later stages.

In pre-activated oocytes no NEBD and PCC occur and the introduced nuclei remain in interphase. The DNA replication in these nuclei is coordinated with their cell cycle stage at the time of transfer. G1 nuclei undergo one round of replication, S phase nuclei continue replication and G2 nuclei do not replicate DNA. Thus, in all cases the resulting daughter cells are diploid. This protocol allows for using the unsynchronized blastomeres as nuclear donors. Since the pre-activated oocytes accept nuclei from all cell cycle phases (G0/G1, S and G2), they have been termed "universal recipients". The use of universal recipients has resulted in a significant increase in the frequency of full-term development in sheep (CAMPBELL et al. 1994) cattle (STICE et al. 1994). It is not clear, however, if that model of nuclear transfer (activation prior to fusion) can be successfully used in all mammals. In the mouse, for example, a similar increase in development was

not observed, probably due to differences in the cell cycle control mechanisms (ALI et al. unpublished; cited in: CAMPBELL 1999).

Polar body extrusion and ploidy of reconstituted embryos

The maintenance of the correct ploidy in the embryos reconstructed from metaphase II oocytes depends on the possible extrusion of the polar body. It means that in MII oocytes receiving G1 nuclei the extrusion of the polar body should not occur, otherwise haploid nuclei will be formed. The expulsion of the polar body following nuclear transfer has not been observed in the rabbit (COLLAS et al. 1992 a,b), sheep (SMITH, WILMUT 1989) and cattle (BONDIOLI et al. 1990, CAMPBELL et al. 1993). Thus, in these species transfer of G1 nuclei into MII oocytes will result in formation of diploid embryos, while introduction of the nuclei in later stages of the cell cycle will lead to production of aneuploid or tetraploid embryos.

In contrast to these experiments, the extrusion of the polar body has been observed in reconstructed murine MII oocytes receiving blastomere as well as embryonic stem cell nuclei (MODLIŃSKI 1995). Most of the enucleated telophase I oocytes (TI) reconstructed with 2-cell nuclei extruded a polar body within 2 h after activation and formed one pronucleus (KONO et al. 1992). However, the majority of the reconstructed oocytes that received nuclei from early (presumably G1 phase) and middle stages (presumable S phase) of the second cell cycle were arrested at the 2-cell stage. The reconstituted oocytes were able to develop to the blastocyst stage and then to term only when nuclei from late 1/2 blastomeres (presumably G2 phase) were transferred. These results are in contrast with those obtained by CHEONG et al. (1993), who transferred nuclei at early, middle and late cell cycle stage from 2,- 4,- and 8-cell embryos into enucleated MII oocytes. A high proportion of development to the blastocyst stage (77.8%) was obtained after transfer of nuclei from the early 2-cell stage embryos, as opposed to the nuclei from the middle (0%) and late (20.8%) stage. Also, after transfer of early-stage nuclei, high proportions of development to the blastocyst stage and of offspring were obtained from reconstructed oocytes receiving nuclei from 2-8-cell embryos. In these experiments, however, only oocytes reconstructed from middle-, and late-stage nuclei expelled a polar body (24.3% and 67.5%, respectively). The observed discrepancy is presumably due to differences either in the stage of recipient oocytes used (TI vs MII) or in activation treatment (DC pulses vs ethanol).

The experiments of CHEONG et al. (1993) were not confirmed by others (KWON, KONO 1996). The reason for this is not clear; one possible explanation is that it is extremely difficult to obtain embryonic nuclei in the G1 phase because it is so transitory. On the other hand, KWON, KONO (1996) showed that it is possible to obtain full-term development after transfer to MII oocytes of metaphase nuclei

derived from the mitotically arrested 4-cell mouse embryos. In this experiment the oocytes reconstructed with metaphase nuclei were activated in the presence of cytochalasin B, preventing polar body extrusion and resulting in the formation of two diploid pronucleus-like nuclei. Each "pronucleus" was then introduced into an enucleated zygote, which was transferred into a pseudopregnant recipient. This procedure, which mimics the pronuclear exchange, allowed the obtaining of six cloned mice from a single 4-cell embryo.

It means that the following conditions are required to maintain the correct ploidy in embryos developed from reconstructed MII oocytes: (1) in the mouse (and maybe in some other species), MII oocytes receiving G1 nuclei should be treated with cytochalasin B or D to prevent the extrusion of a "polar body" and formation of haploid nuclei, (2) in oocytes reconstructed by the transfer of G2 nuclei, the expulsion of the "polar body" is required to produce a normal diploid nucleus, (3) transfer of S phase nuclei is not recommended due to the resulting aneuploidy and possible damages of the genetic material.

The fact that the polar body and pronucleus-like formation type vary with species should not be disregarded, either. The lack of the "polar body" extrusion in reconstituted oocytes of domestic species may be related to a different mechanism of spindle formation that in the mouse. In the mouse the spindle is organized by self-replicating microtubule organizing centers (MARO et al. 1990) derived from maternal sources. Domestic species (and probably also some other mammalian species including marsupials and rhesus monkeys) follow the pattern similar to that observed in lower vertebrates and invertebrates. In bovine oocytes reconstructed with blastomere nuclei one or two asters were found in association with the introduced nuclei (NAVARA et al. 1994). This suggests that nuclear transfer involves also a transfer of the centrosome, and that the centrosome is not of oocyte origin. It is likely that the presence of one or two asters results from the cell cycle stage of the donor nuclei. If the nucleus is transferred before the time for centromere duplication (G1), one aster is formed; if after (G2), two asters may be formed.

Events associated with somatic cloning

In contrast to the studies on embryonic cloning, there are no reports comparing the effects of cell-cycle coordination between somatic donor nuclei and recipient ooplasts. So far, the only ways that are successful in yielding viable somatic clones is either using as nuclear donors the cells in a quiescent state (which is presumed to be the G0 phase; see above) or using as nuclear recipients the "metaphase-arrested" ooplasts (with high MPF activity) in which the introduced nuclei are exposed for prolonged periods to the cytoplasmic environment. The cells in the quiescent state can be obtained in three ways: (1) by culture in medium containing a drastically reduced serum concentration; (2) by using cells that

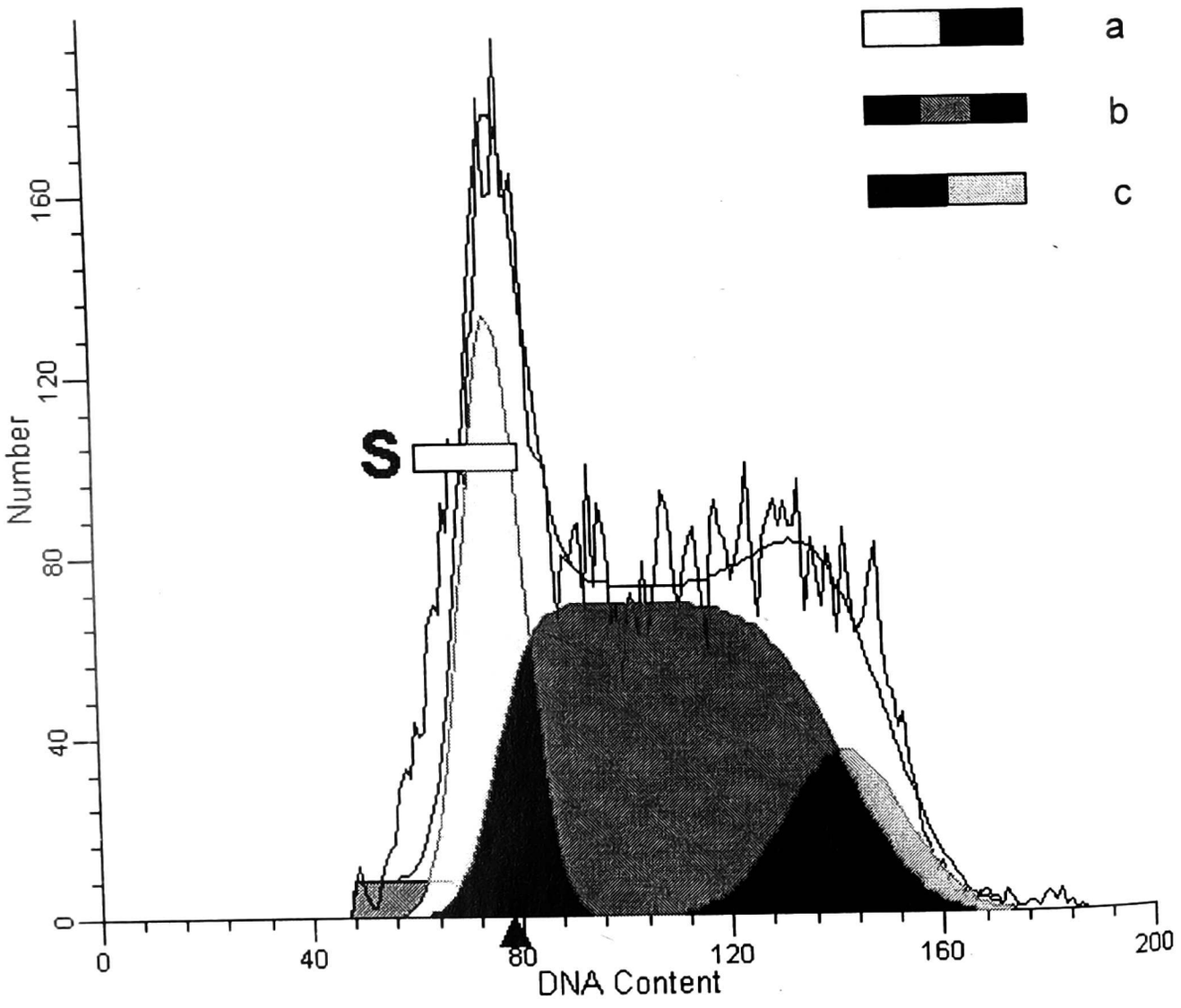

Figure 7. Distribution of cell cycle phases in mouse embryonic stem cells CGR8 population a = cells in G0/G1 phase (25.45%); b = cells in S phase (60.65%); c = cells in G2/M phase (13.90%); white rectangle – sorting gate (S) set on DNA histogram

are naturally arrested in this state (i.e. Sertoli cells, neurons, leukocytes); (3) by cell sorting using flow cytometry. The last method was successfully used for obtaining the G0/G1 fraction of mouse embryonic stem cells (MODLIŃSKI et. al. 1998; Figure 7).

It is supposed that the events associated with the entrance of the donor cells into quiescent state may facilitate the remodelling of the introduced nuclei following exposure to cytoplasmic factors operating in MII oocytes. The lambs obtained after transfer of nuclei from quiescent cultured embryo-derived cells (CAMPBELL et al. 1996, WELLS et al. 1997), foetal fibroblasts and mammary gland cells (WILMUT et al. 1997) confirm that hypothesis. Furthermore, it seems that prolonged exposure (for 4-6 h) of somatic cell nuclei to the cytoplasmic *millieu* of unactivated MII ooplasts may increase and intensify the remodelling processes, enabling normal development to occur also after transfer of nuclei from non-quiescent cells (CIBELLI et al. 1998). The prolonged exposure of nuclei to the cytoplasmic factors operating in MII ooplasts is achieved by fusion of donor

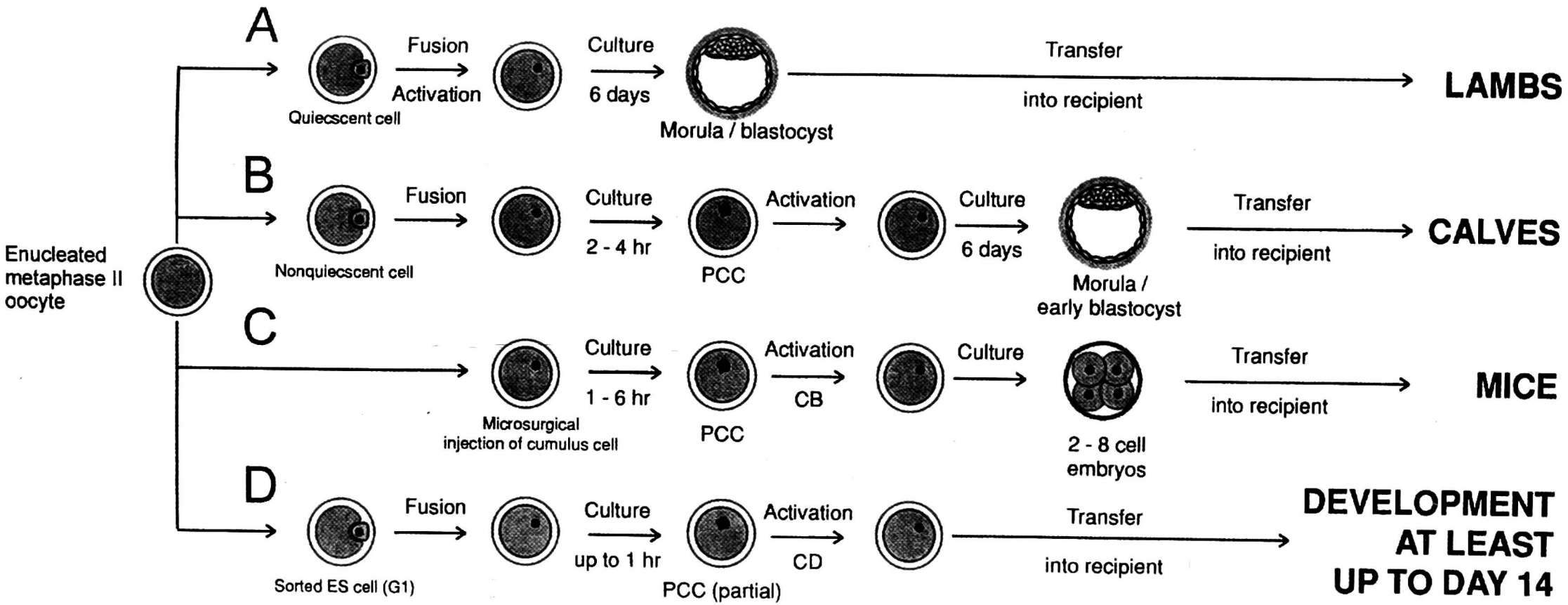

Figure 8. Nuclear transfer methods used in mammalian somatic cloningA = a quiescent cell nucleus is introduced into a MII sheep oocyte. Activation and fusion are performed simultaneously (as described by CAMPBELL et al. 1996). B = introduction of a nonquiescent cell nucleus into a non-activated bovine MII oocyte (fusion before activation) (as described by CIBELLI et al. 1998). C = microsurgical injection of G0/G1 cell nuclei into non-activated mouse oocytes (fusion before acivation; polar body extrusion is inhibited by cytochalasin B) (as described by WAKAYAMA et al. 1998). D = introduction of nuclei of sorted ES cells (G0/G1 phase) into post-activated mouse oocytes (after MODLIŃSKI et al. 1998).

Table 1. The use of somatic cells from newborn and adult animals in mammalian cloning

Species	Type of nuclear donor	Origin, sex, strain (breed)	Results References
Mouse	follicullar (granulosa) cells	cumulus oophorus surrounding ovulated oocytes, female, B6D2F1, B6C3F1	mice (females) WAKAYAMA et al.1998
	neural cells	brain, female, B6D2F1	7-day-old foetuses (females) WAKAYAMA et al.1998
	Sertoli cells	testes, male, B6C3F1	8,5-day-old foetuses (males) WAKAYAMA et al. 1998
	tail cells, phenotype unknown	tail tip, male, B6C3F1	mice (males) WAKAYAMA, YANAGIMACHI 1999
Sheep	mammary gland cells, phenotype unknown	mammary gland, pregnant 6-year-old female, Dorset	lamb (female) WILMUT et al. 1997
Cattle	granulosa cells	cumulus oophorus surrounding ovulated oocytes, female, Japanese Black	calves (females) KATO et al. (1998)
	epithelial cells	oviduct, female, Japanese Black	calves (females) KATO et al. 1998
	granulosa cells	mural granulosa, female, breed unknown	calves (females) WELLS et. al. 1999b
	muscle cells (fibroblasts ? fibrocytes ?)	musculus longissimus dorsi, male, Japanese Black	calves (males) SHIGA et. al. 1999
	leukocytes	peripheral blood, male, Brown Swiss	calf (male) GALLI et al. 1999
	fibroblasts	ear, male, Japanese Black ear, female, Japanese Black and Holstein	calves (males), calves (females), KATO et al. 2000
	skin cells (fibroblasts?)	skin, female, Japanese Black skin, newborn male, Holstein skin, newborn female, Holstein	calf (female) calf (male) calf (female), KATO et al. 2000
	liver cells	liver, newborn male, Holstein	calf (male), KATO et al. 2000
	mammary gland epithelial cells	colostrum, female, Holstein	calves (females), KISHI et al. 2000
Pig	granulosa cells	mural granulosa, female, Large White × Landrace × Duroc	piglets (females) POLEJAEVA et al. 2000

cells prior to activation of the reconstructed oocytes (post-activated oocytes). For the first time, the post-activated MII oocytes have been used as the recipients of mammalian somatic nuclei (thymocytes) by CZOŁOWSKA et al. (1984). This protocol (fusion before activation) is now commonly used for somatic cloning resulting in generating several viable murine, ovine, swine and bovine clones (Figure 8, Table 1).

It should be noted, however, that – for the reasons mentioned previously – in order to restore the correct ploidy in the reconstituted embryos and also to avoid chromatin damage only nuclei that have diploid DNA content (G0/G1 and M phases) can be successfully transferred to the post-activated MII oocytes. Since the reconstituted sheep and cattle oocytes do not extrude any polar body (see above) the correct ploidy is maintained. In addition, treatment of the reconstructed embryos with 6-dimethyloaminopurine (6-DMAP) following their activation by ionomycine probably increase the rate of embryo development. It is likely that 6-DMAP, which is the inhibitor of MAP kinase, prevents formation of micronuclei known to occur in post-activated nuclear transfer oocytes (CZOŁOWSKA et al. 1984). The situation is different with post-activated mouse oocytes receiving somatic nuclei. Since in this species a polar body is extruded after activation of reconstructed MII oocytes, the cytochalasin B should be added to the culture medium to prevent the expulsion of the polar body and thereby restore the diploid state in the oocyte nucleus (WAKAYAMA et al. 1998)

Recloning experiments

Although it is now clearly possible to produce cloned mammals after transfer of nuclei from somatic cells, the overall efficiency of that method is currently very low (0.3-2%). In order to increase the number of cloned embryos that could be transferred into recipients and also to allow a longer opportunity for nuclear remodelling to occur by passing the somatic nuclei through an additional exposure to oocyte cytoplasmic factors, the recloning procedures have been recently employed in somatic cloning. In multiple cloning, the nuclear transfer embryos are used as the donors of nuclei for the next round of cloning. In rabbit, caprine and bovine embryonic cloning this method permits to increase significantly the number of clones derived from the single embryo and to obtain viable offspring from the subsequent generations of the cloned embryos (see PIOTROWSKA, MODLIŃSKI 1998, MODLIŃSKI, PIOTROWSKA 2000 – for review). Both in the goat (YONG, YUQIANG 1998) and rabbit (PIOTROWSKA et al. 2000) the most numerous clones of embryos were obtained when the pre-activated ooplasts were used as recipients of unsynchronized blastomere nuclei in all rounds of cloning. In somatic cloning, however, the combination of post-activated and pre-activated ooplasts has to be applied. First, the somatic nuclei are introduced into post-activated ooplasts, then the reconstructed oocytes are cultured up to the morula stage. The somatic cell-derived nuclear transfer morulae are used as a source of nuclei for the second round of nuclear transfer (recloning). In this sec-

ond step, the unsynchronized morula cell nuclei are introduced into pre-activated ooplasts. The recloning procedures were used for production of cloned bovine embryos following transfer of nuclei from foetal fibroblasts (ZAKHARTCHENKO et al. 1999) and adult mural granulosa cells (WELLS et al. 1999b). However, the obtained results are inconsistent insofar as foetal fibroblasts and granulosa cells are used. When the morulae derived from fibroblasts were used for recloning, the proportion of blastocysts obtained with these embryonic nuclei was significantly higher (especially when non-starved fibroblasts were used) than it was with fibroblast nuclei used in the first round of cloning (55% vs 20%). In contrast, when adult mural granulosa cells were used, a significantly higher proportion of reconstituted oocytes of the first generation developed to the blastocyst stage than with recloning (27.5% vs 13%).

Recloning procedures were also used in one of the most spectacular experiments on somatic cloning, which was the production of a bull after transfer of leukocyte nucleus into an enucleated MII oocyte (GALLI et al. 1999) No significant difference was observed in the developmental rate to the blastocyst stage between the first and second generation of the cloned embryos (17% vs 19%) as well as in survival to day 35 of gestation (58% vs 53%). Of the 10 foetuses that survived to day 90 of gestation (16% from the 1st and 26% from the 2nd generation), 7 died before day 180, and 2 died thereafter. The foetus that survived to term came from a recloned embryo. The leukocytes used for nuclear transfer were not separated into B and T forms, and their cell cycle stage at the time of transfer was not determined. However, it is highly probable that the nuclei were in the G0/G1 phase since almost all circulating mononuclear blood cells are normally in quiescent or inactive state.

A certain type of recloning was also used for the production of cloned pigs (POLEJAEVA et al. 2000). Since in this species the artificial activation, although it stimulates pronuclear formation, results in very poor development, the technique of recloning and pronuclear exchange between zygotes has been applied (KWON, KONO 1996; see above). Granulosa cell nuclei were introduced into enucleated MII oocytes. The pronucleus-like nuclei formed in the reconstructed oocytes (first nuclear transfer generation) were then subsequently transplanted into enucleated zygotes (second nuclear transfer generation). Out of 401 double nuclear transfer embryos transferred into 7 recipient sows, five piglets were born. No piglets were obtained from 185 single nuclear transfer embryos.

Multiple somatic cloning was also applied to generational cloning of adult mice (WAKAYAMA et al. 2000). Founder cloned mice of two lines (A and B) were bred to six generations using cumulus cell nuclei. Although it was possible to obtain the 4th (A line) and 6th (B line) generation of the cloned mice, the success rate dropped in subsequent generations. In line A no single animal of the 5th generation was born from 670 reconstructed oocytes and in line B the only live-born pup was obtained from 724 reconstituted oocytes.

Conclusions and perspectives

Mammalian cloning is now most often achieved by nuclear transfer. In this technique nuclear material of an organism to be cloned is introduced into a recipient enucleated oocyte (ooplast, cytoplast). The recipient ooplast is either in metaphase (of the second meiotic division), that is in the M phase of the cell cycle, or in interphase (of the first cleavage – mitotic division), which covers the S or G2 phase.

Donor embryonic nuclei in the S phase should not be transferred to M phase recipients to avoid pulverisation of chromosomes, incomplete re-replication of DNA and the resulting incorrect ploidy of the embryos. Additionally, in those species in which a second polar body is extruded following nuclear transfer (e.g. mouse), introducing the G1 donor should be followed by cytochalasin treatment to prevent haploid embryo formation. On the contrary, in species not extruding the second polar body (rabbit, sheep, cattle) donors in G2 must be avoided to prevent tetraploid embryo development.

In interphase (preactivated) recipients ("universal recipients") embryonic nuclei in all phases (G0/G1, S, G2) can be accepted. However, this convenient procedure is not efficient in some species (mouse).

In somatic cloning the cell cycle coordination between recipients and donors has not been analysed. In practice effective are those procedures in which either the donor somatic nucleus is in quiescent state (G0 phase) or the recipient is in the M phase, which is maintained for the next few hours (postactivation).

The ability to produce animals by nuclear transfer cloning has a number of advantages and uses in both research and medicine. First of all, further research in this field will substantially contribute to the understanding of mechanisms underlying mammalian development and cell differentiation. Using modified somatic cells, this technique could soon become a very efficient way of large-scale production of transgenic animal-derived biopharmaceuticals that could be utilized to treat several human diseases. Advances in cloning offer also a way to preserve endangered species and even reincarnate some that are already extinct (WELLS et al. 1999a, CORLEY-SMITH, BRANDHORST 1999). The birth of Noah, the gaur (*Bos gaurus* H.Smith) obtained by interspecies somatic cloning is the world's first and the most sound example of progress in this area (LANZA et al. 2000, VOGEL 2001).

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Note added in proof: Recently, WAKAYAMA and YANAGIMACHI (2001) have shown that nuclei from murine foetal testicular and ovarian cells transferred to post-activated MII oocytes can support full term development. It is uncertain, however, whether the donor cells were germ or somatic cells. Oocytes receiving nuclei from adult spleen cells and macrophages stopped their development by day 6-7 p.c. The embryos reconstructed with nuclei of adult fibroblast sporadically were able to develop until day 9.5 [WAKAYAMA T., YANAGIMACHI R. (2001). Mouse cloning with nucleus donor cells of different age and type. Mol. Reprod. Dev. 58: 376-383].