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Nuclear Transfer in Rabbits with Different Types of Donor Cells

Thesis for the attainment of the title of Doctor in Veterinary Biology
from the Faculty of Veterinary Medicine, University of Munich

by
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Munich, 2005

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Lehrstuhl für Molekulare Tierzucht und Biotechnologie, Moorversuchsgut
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Kerntransfer beim Kaninchen mit unterschiedlichen Typen von Kernspenderzellen

Inaugural-Dissertation
zur Erlangung der Tiermedizinischen Doktorwürde
der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Von
Feikun Yang
aus Hunan, P. R. China
München, 2005

Gedruckt mit Genehmigung der Tierärztlichen Fakultät der
Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. R. Stolla
Referent:	Univ.-Prof. Dr. E. Wolf
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Tag der Promotion: 15. Juli 2005

Contents

Abbreviations

5-aza-dC	5-aza-deoxy-cytadine
6-DMAP	6-dimethylaminopurine
7-AAD	7 -aminoactinomycin D
Blast.	blastocyst
bPL	bovine placental lactogen
BSA	bovine serum albumin
°C	celsius degree
CB	cytochalasin B
CDKI	cyclin-dependent kinase inhibitor
CHX	cycloheximide
cm	centimeter
CSF	cytostatic factor
DAPI	4,6-diamidino-2-Phenylindole
DC	direct current
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyl-transferrase
dpc	days post coitum
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ES cells	embryonic stem cells
ET	embryo transfer
FCS	fetal calf serum
FSH	follicle-stimulating hormone
g	gram
g	relative centrifugal force (RCF)
GFP	green fluorescent protein
h	hour
H3-K9	histone H3 lysine 9
HAT	histone acetyltransferase
hCG	human chorionic gonadotrophin
HDAC	histone deacetylase
HMTase	histone N-lysine methyltransferase
ICM	inner cell mass
IGF	insulin-like growth factor
IU	international unit
IVF	in vitro fertilized
kb	kilobase
kDa	kilodalton
KV	kilovoltage
LH	luteinizing hormone
LOS	large offspring syndrome
µg	microgram
µl	microliter
µm	micrometer

µM	micromolar
µs	microsecond
M	molar
M199	hepes buffered- medium 199 supplemented with 10% FCS
mA	milliampere
MAP kinase	mitogen-activated protein kinase
MBD	Methyl binding domain protein
MeCP	methyl-CpG binding proteins
mg	milligram
MII	second meiotic division
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mPBS	modified PBS (PBS supplemented with BSA)
MPF	maturation promoting factor
MT	microtubule
NaBu	sodium butyrate
NEBD	nuclear envelope breakdown
ng	nanogram
nm	nanometer
NT	nuclear transfer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCC	premature chromosome condensation
PDs	population doublings
PEI	polyethylenimine
PGCs	primordial germ cells
PMSG	pregnant mare's serum gonadotrophin
RCC	rabbit cumulus cells
RFF	rabbit fetal fibroblast cells
RNA	ribonucleic acid
RT	room temperature
SA-ßgal	senescence-associated ß-galactosidase
SD	standard deviation
SDS	dodecyl sulphate sodium salt
sec	second
TAE	Tris-Acetate-EDTA buffer
TBS-T	Tris-buffered saline supplemented with tween 20
T-DMRs	tissue-dependent differentially methylated regions
TEMED	N,N,N',N'-Tetramethylethylenediamine
TSA	trichostatin A
UV	ultraviolet
V	voltage
vs	versus
X-gal	5-bromo-4-chloro-3-indoyl ß-D-galactopyranoside

1 INTRODUCTION

Due to their reproductive and physiological characteristics, rabbits have been one of the most popular animal models to study human physiological disorders (Adams 1970; Hahn 1984; Graur et al. 1996) and to produce valuable biomedical molecules as a potential bioreactor (Buhler et al. 1990; Hammer et al. 1985; Knight et al. 1988). Gene transfer is a technique for improving the performance and applications of rabbits in biomedical research and reproduction. However, to date transgenic rabbits can be produced only by pronuclear microinjection (Massoud et al. 1991), the approach which is associated with inevitable limitations, such as low efficiency (~5%), mosaicism, and position effects (Fan & Watanabe 2003). These limitations could be overcome by nuclear transfer with genetically modified donor cells (Bosze et al. 2003). As shown in several species (Cibelli et al. 1998; Schnieke et al. 1997), this approach reduces the number of animals required to produce a transgenic individual and moreover is so far the only technique allowing targeted mutations in livestock species by using homologous recombination in nuclear donor cells (McCreath et al. 2000).

Successful nuclear transfer in rabbits was first reported by Stice and Robl (1988). Using the procedure described by Robl et al. (1987), a modification of a procedure for nuclear transfer in the mouse (McGrath & Solter 1983), they produced six live young after transferring 8-16-cell embryonic nuclei into enucleated mature oocytes followed by electric pulse-induced activation and fusion. This study was repeated successfully and further improved by Collas and Robl (1990) who used multiple direct-current pulses to induce fusion and activation. With the same technique, later-stage embryos (32-64-cell stage) with an activated embryonic genome have been used successfully as donors of nuclei in several laboratories (Collas & Robl 1991; Yang 1991).

The birth of the sheep Dolly, the first animal cloned from a somatic cell, opened new way for wide scale application of nuclear transfer with diverse types of donor cells. There are now hundreds of animal clones around the world, including mice, cows, goats, pigs, rabbits, cat, horse, mule and rat (Wakayama et al.1998; Ogura et al. 2000; Kato et al. 1998; Cibelli et al. 1998; Kubota et al. 2000; Kasinathan et al. 2001; Baguisi et al.1999; Keefer et al. 2002; Ohkoshi et al. 2003; Polejaeva et al. 2000; Yin et al. 2002a; Park et al. 2002; Chesne et al. 2002; Shin et al. 2002; Galli et al. 2003; Woods et al. 2003; Zhou et al.

2003). However, to date, cloned rabbits from somatic nuclei could be obtained only from freshly collected cumulus or follicular cells (Chesne et al. 2002; Challah-Jacques et al. 2003). For the purpose of generating transgenic rabbits, donor cells must be cultured and then modified prior to use as nuclear donors. Although a relatively high proportion (13-35%) of enucleated rabbit oocytes receiving fibroblast or cultured cumulus cells developed to blastocysts (Mitalipov et al. 1999; Yin et al. 2000; Dinnyes et al. 2001; Inoue et al. 2002a), the implantation rates after embryo transfer were only 2-7% (Yin et al. 2000; Inoue et al. 2002), and no live cloned animals were produced.

The objectives of the present study were a) to evaluate the developmental potential of cloned embryos from different types of donor cells; and b) to produce cloned rabbits from cultured somatic cells. For these purposes, an effective protocol was established for nuclear transfer first with embryonic blastomeres. After some modifications of this protocol, we carried out several series of experiments with cultured cumulus and fetal fibroblast cells.

2 REVIEW OF THE LITERATURE

2.1 General review of nuclear transfer technique

Nuclear transfer is one tool for producing cloned animals, i.e. live mammals can be produced without fertilization events. But this does not mean that gametes are not necessary for cloning by nuclear transfer. Actually, successful cloning is in great part dependent on the female gamete – oocyte. Simply to say, nuclear transfer is the technique to replace oocyte chromatin with a foreign nucleus, thus oocyte cytoplasm adopts the new genetic material, then supports further development to term as those events occur as fertilization. The following elements are routinely considered in a nuclear transfer laboratory.

- Recipient oocytes
- Donor cells
- Removal of oocyte genetic materials (enucleation)
- Transfer of donor nucleus into recipient oocyte
- Artificial activation
- In vitro culture of reconstructed embryos
- Transfer of reconstructed embryos to recipient mothers

2.1.1 Recipient cytoplasm

To date, cloned mammals are usually produced using oocyte cytoplasm which can reset the transcriptional program of foreign nucleus. However successful development of reconstructed embryos can be obtained using not only mature oocytes (unfertilized eggs) (Willadsen 1986) but also zygotes, and early cleavage-stage embryos as recipient cytoplasts (Tsunoda et al. 1987).

2.1.1.1 Zygotic origin cytoplasm

The first claim to have created a cloned adult mammal was in 1981. Illmensee and Hoppe (1981) reported that they had obtained three cloned mice by injection of inner cell mass (ICM) cell nuclei into enucleated zygotes. But those results could not be repeated by others when using the same protocol for either ICM cells (McGrath & Solter 1984; Tsunoda et al. 1987), embryonic stem cells (Tsunoda & Kato 1993) or cumulus cells (Wakayama et al. 2000).

When using cytoplasm from 2-cell enucleated blastomeres, Robl et al. (1986) were able to obtain morulae and blastocysts after fusion with karyoplasts derived from 8-cell mouse blastomeres. However, they also reported that these apparently normal blastocysts were unable to support development beyond mid-gestation after transfer to synchronized females. With a slight modification of the technique for single 2-cell recipient cytoplasm, Tsunoda et al. (1987) were able to produce a small number of offspring from nuclei derived from 8-cell embryos.

Generally, the use of enucleated zygotes has been limited to the transfer of pronuclei or pseudopronuclei. This may reflect the removal, with the zygote pronuclei, of factors which are essential for early development, such as nuclear formation factors.

2.1.1.2 MII oocytes

Mature oocytes arrested at metaphase of the second meiotic division (MII phase) appear to be the optimal recipient cells for nuclear transfer since to date the majority of cloned mammalian offspring are originated from the use of enucleated MII oocytes. The cytoplasm of mature oocyte has the unique ability to promote nuclear envelope breakdown (NEBD, Szollosi et al. 1988), chromatin condensation/decondensation, nuclear reformation (Newport & Kirschner 1984; Fisher 1987), nuclear swelling (Gurdon 1964; Prather et al. 1990), translocation of cytoplasmic proteins into the nucleus (DiBerardino & Hoffner, 1975; Prather et al. 1989), and thus has the ability to incorporate the transferred nucleus and support development of a new embryo (Prather et al. 1989; Campbell et al. 1996a). Both developmental stage and morphology and the position in the cell cycle of the recipient cytoplasm have been proven to be important for the development of nuclear transfer embryos (Robl et al. 1986; Prather et al. 1987; Collas & Robl., 1990; Barnes et al. 1993; Yang et al. 1993).

In rabbits, in vivo matured MII oocytes can be easily obtained by superovulation. Considering the technical difficulties to collect a large number of rabbit oocytes soon after ovulation because the timing of ovulation varies from 10.5 h to 14.0 h after injection of LH (Harper 1963), Yin et al. (2002) tried to collect rabbit MII oocytes through in vitro maturation as for large domestic animals.

2.1.2 Nuclear donors

Various types of cells are successfully used as donors for nuclear transfer, but the efficiency varies among cell types.

2.1.2.1 Embryonic blastomeres

Results from early experiments in amphibian species demonstrated that differentiation causes irreversible modifications to the embryonic chromatin which render the nuclei less able to support development after nuclear transfer (King 1966; DiBerardino & Hoffner 1970). It was this assumption that guided the use of blastomeres from preimplantation embryos as nuclear donors in initial mammalian cloning studies.

In 1986, Willadsen (1986) used electrofusion or Sendai virus to fuse cells of 8- or 16-cell embryos into enucleated eggs of sheep, and obtained two healthy cloned animals. Nuclear transfer using embryonic donor cells was successfully performed in rabbits (Stice & Robl 1988), pigs (Prather et al. 1989a), mice (Cheong et al. 1993), cows (Sims et al. 1994) and monkeys (Meng et al. 1997).

2.1.2.2 Embryonic stem(-like) cells

To have both an unlimited source of donor cells and the possibility for the genetic modification of cells before nuclear transfer, an alternative to embryonic blastomeres for nuclear transfer is to use embryonic stem (ES) cells which are derived from the ICM cells of blastocysts and can be maintained in culture for unlimited time. Large source of nuclei for nuclear transfer provided by ES cells from domestic animals have yet to be established (Prelle et al. 1999), in contrast to mice where they have been obtained from a variety of strains (Robertson et al. 1987). After successful cloning of embryos using cells from ICM of bovine (Collas & Barnes 1994; Keefer et al. 1994; Zakhartchenko et al. 1996) and sheep embryos (Smith & Wilmut, 1989), isolated bovine and sheep ICM cells were cultured in vitro up to 13 passages, and live calves and lambs were obtained upon nuclear transfer of these cells (Sims & First 1994; Campbell et al. 1996a; Wells et al. 1997). Later, the successful cloning of mice from ES cells (Wakayama et al. 1999) and fetal neural stem cells (Yamazaki et al. 2001) has been achieved. Recently, Kato et al. (2004) demonstrated the developmental potency of adult bovine mesenchymal stem cells by nuclear transfer.

2.1.2.3 Primordial germ cells

Another choice of cell type suitable as nuclear donors is primordial germ cells (PGCs). Unlike other cell types, PGCs undergo the essential modification of imprinted genes that occurs during their differentiation, and might be the major limiting factor for the use of these cells for nuclear transfer. These cells have been isolated in a number of species from specific developmental stages (Leichthammer et al. 1990; Lavoir et al. 1994; Shim et al. 1997). In cattle, in early studies PGCs were shown to support the development of nuclear transfer embryos up to the blastocyst stage (Delhaise et al. 1995; Moens et al. 1996; Lavoir et al. 1997), and later, cloned calves were produced from cell lines of bovine PGCs (Strelchenko et al. 1998; Forsberg et al. 2002) and freshly isolated PGCs (Zakhartchenko et al. 1999b). In mice, when PGCs were used as donors for nuclear transfer, the resulting fetuses were unviable at day 10.5 because of the erasing and abnormal expression of imprinted genes that occurs during differentiation of PGCs into functional germ cells (Kato et al. 1999; Lee et al. 2002). Very recently, Miki et al. (2005) obtained four mouse kids from younger PGCs (embryonic day 10.5). After DNA methylation analyses, they found that only embryos exhibiting normal imprinting developed to term. Therefore, germ cell differentiation is not an insurmountable barrier to cloning, and imprinting status is more important than the origin of the nucleus donor cell per se as a determinant of developmental plasticity following nuclear transfer.

2.1.2.4 Somatic cells

The possibility that somatic nuclei could be used for nuclear transfer was first suggested by experiments in which differentiated fetal cells were used to produce clones of *Xenopus laevis* (Gurdon et al. 1979). In mammals, progress was retarded for a number of years by the mistaken belief that reconstituted embryos could develop to term only if the karyoplast was derived from a transcriptionally inactive blastomere (McGrath & Solter 1986; Howlett et al. 1987).

In 1996, Campbell et al. (1996b) performed nuclear transfer with the nuclei of an established cell line, originating from a day-9 embryo that had differentiated in vitro. They induced these cells to enter a quiescent state before electrofusing them with enucleated sheep eggs. These nuclear transfers resulted in two healthy cloned sheep. In 1997, they used the same technique with nuclei from cultured adult mammary cells and succeeded in producing a single cloned sheep “Dolly” (Wilmut et al. 1997).

Since the birth of Dolly, many other mammals have been successfully produced by nuclear transfer with somatic donor cells. These include mice (Wakayama et al. 1998), cows (Kato et al. 1998), goats (Baguisi et al. 1999), pigs (Polejaeva et al. 2000), rabbits (Chesne et al. 2002), a cat (Shin et al. 2002), a mule (Woods et al. 2003), a horse (Galli et al. 2003) and rats (Zhou et al. 2003).

2.1.3 Enucleation

Removal of genetic material from the recipient oocyte is the prerequisite for nuclear transfer because of the importance to maintain the correct ploidy. Aneuploidy leads to subsequent detrimental effects on development, genetic interference of the recipient cytoplasm, and possible parthenogenetic activation and embryo development without the participation of a newly introduced nucleus (Dominko et al. 2000). Enucleation may also affect the ultrastructure of the remaining cytoplast, thus resulting in a decline or destruction of its cellular compartments (Greising et al. 1999). To improve the enucleation procedure and thus cloning efficiency, several methods have been attempted.

2.1.3.1 Blind enucleation

Unlike mouse and rat whose nuclei can be easily observed and removed under an inverted-stage microscope, in some species including rabbit, sheep, goat, cattle, pig and horse, the metaphase zone is difficult to localize. Removing metaphase chromosomes in these species is generally done by aspirating the ooplasm adjacent to the first polar body without DNA staining. But one problem is that removal of large volume of cytoplasm is required to be sure of complete enucleation (Cheong et al. 1993). Another problem will be the fact that metaphase spindle is not always close to the first polar body. Removal of cumulus cells is required prior to oocyte manipulation, and the denudation process disrupts the connection between the first polar body and the metaphase spindle. Results with rabbit enucleation indicate more than 50% of metaphase spreads are located in a different region as compared to the first polar body location (Mitalipov et al. 1999). Therefore, the first polar body cannot be used as a reliable predictor for the location of the metaphase spindle.

2.1.3.2 Enucleation using DNA specific binding dyes

To define metaphase location, it is reasonable to stain the chromosomes with fluorescent dyes in combination with UV irradiation (Smith & Wilmut 1990; Heyman et al. 1994). Hoechst 33342, a short-wavelength, UV excitable fluorochrome, is routinely used to label

oocyte chromatin. Under a fluorescent microscope, metaphase spindle prestained with Hoechst 33342 can be easily removed together with very little surrounding cytoplasm, thereby preserving oocyte volume without compromising enucleation efficiency. Using this enucleation procedure, clones have been produced in several species (Forsberg et al. 2002; Kubota et al. 2000; Onishi et al. 2000; Loi et al. 2001). However, exposure to high energy UV light may damage maternal nuclear DNA and cytoplasmic organelles. For example, viability of rabbit oocytes decreased after being exposed to UV light for over half minute (Yang et al. 1990a).

2.1.3.3 Chemically assisted enucleation

Etoposide-cycloheximide treatment for enucleating metaphase I mouse oocytes was developed in 1993 (Fulka et al. 1993a,b). This noninvasive method yielded 96% enucleated mouse oocytes.

A combination of ethanol and demecolcine treatment to chemically enucleate metaphase II oocytes was used to produce mouse nuclear transfer embryos (Ibanez et al. 2003). Nuclear transfer mouse offspring were obtained using this procedure (Baguisi et al. 2000; Gasparrini et al. 2003).

Recently, Yin et al. (2002a,b,c) reported a partial chemically assisted enucleation procedure. Metaphase II oocytes were pre-treated by exposure to demecolcine which led to a membrane protrusion where the condensed maternal chromatin was located. These protrusions were easily identified and mechanically removed from the oocytes.

2.1.4 Nuclear transfer

There are different ways to introduce new genetic material from the donor cell into an oocyte which include fusion of the two cells by Sendai virus, direct current (DC) electric pulse (Willadsen 1986), injection of isolated nuclei directly into the oocyte cytoplasm (Collas & Barnes 1994) and by Piezo-assisted injection (Wakayama et al. 1998). Reconstructed embryos from the above methods have been proved able to develop to term. Compared to others, electrical fusion is easier to operate and by far the most widespread method.

An electric field is obtained when a voltage is applied between two electrodes. When submitting cells to electric pulses, due to the flexibility and mobility of the membrane components, the distribution of membrane proteins at the cell surface can be altered, thus membrane permeabilisation may occur (Zimmermann et al. 1974; Kinosita et al. 1977). Fusion takes place only when the two cell surfaces in contact are electropermeabilised. Cell fusion is a two-step phenomenon: membrane merging and then cytoskeleton reorganization (Blangero et al. 1989). While the first event is apparently very fast, the second one takes place only when the cell viability is preserved. It reflects a reorganization of the cytoskeleton. A key observation that the early events of embryonic fusion do not occur simultaneously with electric pulse suggests that fusion is not direct electric-field effect but results from the induced alteration of the membranes (Dimitrov & Sowers 1990). The delay in merging was indeed observed to be shorter when high field strengths were used to promote fusion.

The efficiency of fusion clearly depends on the electro-fusion system parameters which include direction of the fusion current, pulse duration, pulse length, number of pulses, fusion media and fusion chamber configuration. Moreover, beyond the electropulse system itself, there are other factors which are important to obtain high fusion rate. These include oocyte age, healthy cell membranes, degree of cell-to-cell contact and the size differential between cells to be fused. In rabbits, recently ovulated oocytes fuse at a higher rate than aged oocytes (Collas & Robl 1990).

It should also be mentioned that electropulse might induce adverse effects resulting from electropermeabilisation. These include blebbing of the electropermeabilized cell surface and induction of defense mechanism such as generation of reactive oxygen species (Escande-Geraud et al. 1988; Gabriel & Teissie 1994).

2.1.5 Artificical activation of oocytes

In mammals, the ovulated oocyte is arrested at the second metaphase. This arrest is maintained by the maturation promoting factor (MPF) and cytostatic factor (CSF). It has been firmly established that sperm penetration induces a series of calcium transients (calcium oscillation) in the oocyte (Miyazaki et al. 1993; Kline & Kline 1992; Carroll et al. 1996). These transient calcium spikes are propagated throughout the cortical granule exocytosis (Miyazaki 1990) and lead oocytes escape from metaphase II arrest (Whitaker &

Patel 1990), which is termed “oocyte activation”. As to nuclear transfer, activation of the recipient oocyte is a key step in the cloning procedure (Alberio et al. 2001a). Because of the lack of sperm-induced fertilization, an artificial activation must be applied to trigger further development of reconstructed oocytes. It has been reported that electropulse can induce calcium transient, and multiple electric pulses can result in calcium oscillation. Both the amplitude and frequency of calcium transients induced by electrostimulation affect the inactivation of MPF, the timing of pronuclear formation, and the rate of compaction and blastocyst formation in activated oocytes. Even though a series of calcium transients can be achieved by this physical way, its efficacy also varies among species, and usually fails to mimic completely the natural events. The common protocols used today are based on physical or chemical means to accomplish the objective of inducing calcium oscillation to degrade cell cycle proteins repeatedly and/or block new synthesis. These activation treatments affect a broad spectrum of cellular components, and, in addition to the activation effect, the physiology of the oocyte may be altered in various ways. For example, non-specific drugs such as cycloheximide (CHX) and 6-dimethylaminopurine (6-DMAP) affect several metabolic pathways in oocytes and consequently they can impair further normal embryonic development. A more specific, non-invasive method that could limit its actions to those needed for the activation is desirable. An attempt towards this goal is the use of specific cyclin-dependent kinase inhibitors (CDKIs), such as bohemine (Alberio et al. 2000a; Alberio et al. 2001b), butyrolactone I (Alberio et al. 2001a; Motlik et al. 2002) and roscovitine (Mitalipov et al. 2001).

Although activation of bovine oocytes with CDKIs could be achieved, no improvement in the pregnancy and survival rates after birth was observed in comparison with protocols using non-specific inhibitors of phosphorylation or protein synthesis. Activation by injection of sperm factor might trigger calcium oscillations similar to the naturally occurring ones (Wu et al. 2001). Injection of porcine sperm factor as activating agent has resulted in development of bovine nuclear transfer embryos, however, these results are still low compared to other methods (Knott et al. 2001).

The chronology of the events taking place during nuclear transfer and activation is relevant to the outcomes of these procedures. With embryonic nuclei, activation is initiated before or simultaneously with nuclear transfer; with somatic nuclei, activation might go on for a few hours after nuclear transfer and the optimal time seems to be 2-3 h with CHX and 2-6

h with 6-DMAP (Liu et al. 2001). Comparison of two chemical activation treatments, ionomycin/CHX and ionomycin/6-DMAP, did not reveal any significant differences in calving rates (Galli et al. 2002).

Since some chemicals target the cytoskeleton which is related to cytokinesis and karyokinesis, control of ploidy should also be a priority when activation follows nuclear transfer. If the donor nucleus is in G0/G1, extrusion of chromosomes should be prevented, either by the use of 6-DMAP or cytochalasin. In the case of donor cells in G2/M, the extrusion of half of the chromosomes is necessary to re-establish normal ploidy and therefore 6-DMAP can not be used.

2.1.6 Embryo culture and embryo transfer

For the aim of cloning, activated nuclear transfer embryos must enter into embryonic mitosis either under in vitro culture conditions or after transfer into recipients at a certain stage which is varying among species. In rabbits and pigs, the culture period of preimplantation embryos can be less than 24 h followed by oviductal transfer into recipients (Stice & Robl 1988; Prather et al. 1989b). A longer culture period has been the most practical procedure for sheep and cattle where nuclear transfer embryos can be cultured to the morula and blastocyst stage prior to transfer into recipient mothers (Smith & Wilmut 1989; Bondioli et al. 1990). But culture of embryos in vitro for long time may have some consequences on further post-implantation development. Increased embryonic and fetal mortality and some neonatal anomalies such as a large body weight and multiple deformities after transfer of cloned embryos have been reported both in cattle (Bondioli 1993; Garry et al. 1996; Shiga et al. 1999; Zakhartchenko et al. 2001) and in sheep (Campbell et al. 1996a; Wells et al. 1997).

Accumulating evidence indicates that experimental manipulations on the early stage embryos may result in abnormal development (Moore & Reik 1996). These manipulations can influence both the timing of transcription initiation and the expression of genes, which are known to be involved in early development (Reik et al. 1993; Wrenzycki et al. 1996).

Another important factor for successful recovery of nuclear transfer offspring is the selection of good recipient mothers which will receive cloned embryos. Although blastocyst rate is usually used as a parameter to evaluate the quality of cloned embryos, a

high blastocyst rate does not always result in a satisfying proportion of offspring. It has been suggested that the stage of estrous cycle of recipients should be synchronous with the transferred reconstructed embryos. But cloned embryos showed a significant delay in development compared to embryos originating from fertilization (Chesne et al. 2002). Therefore, in rabbits an extended asynchrony between cloned embryos and recipient females was applied.

2.2 Intrinsic factors contribute to successful nuclear transfer

Successful nuclear transfer is dependent not only on the technique itself, but mainly on the biological material: recipient oocytes and donor cells. The compatibility between these two elements is the internal determinant, which includes the maintenance of correct ploidy that is associated with cell cycle coordination, and the resetting of gene transcriptional activity that is related to nuclear reprogramming.

2.2.1 Cell cycle effects

2.2.1.1 Factors controlling the cell cycle

In mammals, the ovulated oocyte is arrested at the second metaphase. This arrest is maintained by MPF and CSF. MPF is composed of two sets of proteins: p34cdc2 and cyclins. Late in interphase during maturation, p34cdc2 becomes complexed with cyclin, and dephosphorylation of the complex provides p34cdc2 with H1 kinase activity, generating active MPF. The kinase activity of p34cdc2 leads to entry into the second meiotic arrest. On the other hand, the active component of CSF is MOS, the product of the c-mos proto-oncogene, pp39mos. It is a cytoplasmic serine/threonine kinase. Another component of CSF is mitogen-activated protein (MAP) kinase that mediates the activity of c-mos (Haccard et al. 1993) and the activity of which correlates with changes in microtubule organization and chromatin condensation during meiosis (Verlhac et al. 1994).
Mitosis of a somatic cell generates two identical daughter cells, each bearing a diploid complement of chromosomes. There are 4 distinct phases in mitotic cell cycle taking place successively: G1 phase (during which RNAs and proteins are synthesized), S phase (during which DNA replicates and total content of DNA increases one time), G2 phase (during this period, the cell has two complete diploid sets of chromosomes), and M phase (the period of actual division, corresponding to the visible mitosis). Under certain conditions, some cell phenotypes do not divide at all. This non-cycling state is called G0. Therefore, the cell cycle consists of transitions from one regulatory state to another. The change in regulatory

state is separated by a lag period from the subsequent changes in cell phenotype. The transitions take the form of activating or inactivating kinase(s), which modifies substrates that determine the physical state of the cell. Phosphorylation of MPF stimulates mitosis or meiosis. A prominent substrate is histone H1, and H1 kinase activity is now used as a routine assay for M phase kinase (MPF). Phosphorylation of H1 could be concerned with the need to condense chromatin at mitosis.

A general principle governing the above events is that the state of the substrates is controlled reversibly in response to phosphorylation, so that phosphorylated form of the protein is required for mitotic organization, while the dephosphorylated form is required for interphase organization (Lewin 1998).

2.2.1.2 Cell cycle synchronization

G0-phase: Common methods to obtain donor cells in G0 are starvation in low serum for several days or culture to full confluence. Both rely on the depletion of essential growth-promoting activities in the culture, but high confluence may be the more effective method. The first somatic nuclear transfer offspring were derived from cultures of ovine mammary epithelial cells synchronized at the G0 phase by serum starvation (Wilmut et al. 1997). However, nuclear damage is considered as one reason for the low offspring production of embryos reconstructed with cells that were synchronized at the G0 phase by serum starvation (Kues et al. 2002).

G1-phase: Early G1 cells can be obtained in sufficient numbers by picking mitotic cells on the microscope stage and allowing them to cleave. Resulting cell doublets are separated and used for nuclear transfer shortly after mitosis (Oback & Wells 2002a). It has been reported that nuclear transfer using cells in the early G1 phase as donor cells gave a high rate of offspring production (Urakawa et al. 2004).

S-Phase: Cells arrested at the late G1/early S-phase boundary can be obtained by variations of the double-thymidine block method involving reversible inhibitors such as thymidine, aphidicolin, mimosine or hydroxyurea (Tani 2001). Aphidicolin, which prevents DNA chain elongation by inhibiting DNA polymerase a, is the least cytotoxic drug and produces the highest synchrony efficiency (Oback & Wells 2002b).

G2-phase: Cell populations in G2 are most difficult to obtain. An efficient method involves the double thymidine block, followed by incubation with Hoechst 33342 which has been shown to be a topoisomerase II -inhibitor that can inhibit progression through the cell cycle. High concentration of butyrolactone I also arrests the cells at the G2/M boundary, which can be detected by cyclin B accumulation. For example, Kuhholzer and Prather (2001) pre-synchronized porcine fetal fibroblast cells by serum deprivation or aphidicoline-treatment, then incubated cells in medium containing 0.1 µg/ml Hoechst 33342. The resulting cells were suitable for nuclear transfer experiments.

M-phase: Mitotic phase cells are spherical, which makes it easier to dislodge them from the culture plate. Thus, M-phase cells can be preferentially recovered by shaking or treating with low dose of trypsin solution and subsequent replating. For example, mitotic phase bovine fibroblast cells were easily recovered by the combined treatments of 1 µM 2-methoxyestradiol, shaking, and selecting cells of the appropriate diameter (20 µm, Urakawa et al. 2004). Additionally, microtubule-depolymerising agents, such as nocodazole, colchicine or colcemid, reversibly arrest the cells in metaphase (Alberio et al. 2000b). Alternatively, neutral cystein protease inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) arrests cells without spindle damage by inhibiting cyclin B degradation and exit from M-phase (Uzbekov et al. 1998).

2.2.1.3 Cell cycle coordination and ploidy

During a single cell cycle, all genomic DNA must be replicated once and only once prior to mitosis. This is the prerequisite for the maintenance of correct ploidy in living cells. Immediately on fusion, the donor nuclear envelope breaks down and the chromosomes condense prematurely, and then DNA replication in the foreign nucleus is initiated (Collas et al. 1992a; Czolowska et al. 1992). These effects are regulated by cytoplasmic cell cycle regulators such as p34cdc2/cyclin B kinase, MPF, which induce the remodeling of nuclear structure (Fulka et al. 1996). Matured mammalian oocytes are arrested at metaphase II, and have high MPF activity (Campbell et al. 1996b). On fertilization or activation, MPF activity declines, the second meiotic division is completed, and the second polar body extruded, the chromatin decondenses and pronuclear formation takes place. In nuclear transfer, a prerequisite for the reconstruction of an embryo with normal karyotype is the correct cell cycle synchronization between the cytoplast and the karyoplast at the time when the transplanted nuclei are exposed to MPF (Campbell et al. 1996b). Chromosomal

abnormalities are observed when premature chromatin condesation (PCC) occurs at the S or G2 phase of the nuclear cell cycle because of a potential duplication of the genome (Collas et al. 1992b). During embryo reconstruction, correct ploidy can be maintained in one of two ways: first by transferring nuclei at a defined cell cycle stage, i.e. G1 diploid nuclei into metaphase II oocytes at the time of activation, or second, by activating the recipient oocyte and transferring the donor nucleus after the disappearance of MPF activity. In the second case the donor cell may be in G1, S, or G2 phases of the cell cycle (Campbell et al. 1993). On the other hand, if oocytes are aged for a period of time after the end of maturation, the MPF activity progressively decreases until reaching an interphase-like stage (Gall et al. 1996) in which the nucleus will continue its natural cell cycle progression (Collas & Robl 1991).

There are several combinations of the cell cycle stages between the recipient cytoplasm and the donor nucleus for maintaining correct ploidy in nuclear transfer experiments. To date, quiescent somatic donor cells arrested in presumptive G0/G1 phases of the cell cycle have been commonly used to produce cloned animals (Campbell et al. 1996b; Wilmut et al. 1997; Kato et al. 1998; Wakayama et al. 1998; Polejaeva et al. 2000; Betthauser et al. 2000; Chesne et al. 2002; Gibbons et a. 2002), and their nuclei are commonly introduced by fusion or injection into oocyte cytoplasm with a high level of MPF. Somatic donor cells, which are not in the G0/G1 phases, can also be used to clone offspring. Wakayama et al. (1999) produced cloned mice from ES cells synchronized in M phase by nocodazole treatment. This method was applied to produce a cloned calf from a cumulus cell (Tani et al. 2001) and cloned mice from fetal fibroblasts (Ono et al. 2001). A cloned piglet was obtained by using colchicine-treated somatic cell nuclei as donors, most of which are in the G2/M cell cycle stage (Lai et al. 2002). In contrast, when G2/M phase bovine blastomeres were synchronized by nocodazole or benomyl, donor nuclei were not properly remodelled and did not trigger development to blastocysts (Alberio et al. 2000b). This could be attributed to the specific combination of oocyte activation procedure and donor cell synchronization treatment.

What must be mentioned is that maintenance of correct ploidy does not relate to the long-lasting effects of cloning. For instance, ES cell nuclei, synchronized in metaphase and injected into MII enucleated oocytes, provided high blastocyst formation and implantation rates, but the postimplantation development was as low as after nuclear transfer with

unsynchronized ES cells (Wakayama et al. 1999; Zhou et al. 2001). But the modification on donor cells to arrest them at a certain cell cycle stage might result in long-lasting effects. Both serum starvation and contact inhibition when cultured cells reach confluence are two methods used to synchronize cells at the G0/G1 cell cycle stage. However, it has been shown that about 25% of porcine fetal fibroblasts in serum poor medium have apoptotic nuclei containing fragmented genomic DNA compared to 0.2% of fibroblasts showing apoptosis in actively growing cells (Lee & Piedrahita 2002).

2.2.1.4 Cell cycle coordination and reprogramming

Early embryo development is controlled by maternally inherited RNA and proteins, and little or no transcription is detectable from the embryonic genome. At a particular stage of development, which is species-dependent, a switch to zygotic control occurs (Telford et al. 1990). As development proceeds, genetically identical cells gradually acquire different phenotypes and functions. The range of genes that are expressed within a cell type becomes specific; a process involving the acquisition of stable epigenetic changes (Jaenish & Bird 2003) that has long been considered unidirectional (Solter 2000). For successful development of nuclear transfer embryos, new embryonic transcriptional activities must be established in oocyte origin cytoplasm after receiving new genetic materials.

Nuclear reprogramming is a process of returning a differentiated somatic nucleus to a totipotent state. During nuclear reprogramming, genes inactivated during the process of cell differentiation are subjected to reactivation, allowing the reconstructed cloned embryos to develop and generate all tissue types. As mentioned above, quiescent somatic donor cells arrested in presumptive G0/G1 phases of the cell cycle have been commonly used to produce cloned animals. It has been reported that the chromatin of quiescent cells undergoes condensation, and these cells also show a reduction in transcription and changes occur in the polyribosomes (Campbell 1999). Quiescent cells reduce their metabolism to that which only required to maintain viability.

2.2.1.5 Cell cycle coordination and nuclear transfer efficiency

The cell cycle stages of both oocytes and donor cells have multiple effects on the reconstructed embryos, and the synchronization of their cell cycles has been traditionally thought to be one effective way to improve the efficiencies of nuclear transfer.

The benefit of using early cell cycle stage donor nuclei was confirmed by the enhanced rate of development of manipulated embryos to blastocysts with donor blastomeres in the G1 stage. G1 donor blastomeres showed that metaphase plates and spindles were intact in most cases of premature chromatin condensation (Collas et al. 1992a). However, their integrity was lost in most early S transplants, and gross abnormalities were detected in late S transplants. Embryos reconstructed with G1 donor blastomeres were more advanced in development than embryos reconstructed with late S phase blastomeres (Collas et al. 1992b). Bypassing the S phase was also considered important for effective nuclear transfer (Collas et al. 1992a,b). Embryos reconstructed from somatic cells except S phase developed further than embryos reconstructed with S phase somatic cells (Tani et al. 2001). Roscovitine-treated G0/G1 cells (Gibbons et al. 2002) or early G1 phase cells derived from mitotic cells (Kasinathan et al. 2001) enhanced fetal and/or calf survival. Furthermore, embryos reconstructed from transgenic fibroblast cells in the G1 phase were more likely to develop to calves at term and had higher post-natal survival to weaning than embryos reconstructed from transgenic fibroblast cells in the G0 phase (Wells et al. 2003).

It is generally believed that a diploid, G0/G1 stage of the cell cycle is required to initiate reprogramming following transfer of the donor nucleus into an inactivated, oocyte cytoplasm. This stage is also thought to ensure that the ploidy of the cloned embryo is normal. Cibelli et al. (1998) used cycling cells in presumptive G1 stage and also obtained offspring. As the majority of cumulus cells are to be in the G0/G1 stage (Liu et al. 2004), they have also been used as donor cells (Chesne et al. 2002).

The cell cycle stage of the donor cells could significantly interfere with in vitro development of stem cell generated nuclear transfer embryos in mouse (Zhou et al. 2001). However, the implantation rates at day 7 were quite similar among the three types of nuclei (G2, 23%; G1, 16%; and M-phase, 25%). The pup delivery rates at day 19 (M-phase, 2.0% vs. I-phase G1, 1.6% and G2, 1.9%) and the survival rates after one week (M-phase, 38% vs. I-phase G1, 33% and G2, 40%) were similar between metaphase and interphase groups. However, up to 85.1% of the cloned embryos developed to blastocysts when metaphase nuclei are injected, whereas this rate dropped to about 20% when interphase nuclei were used (G1 and G2). Evidence suggests that cell cycle synchronization change only the rate of blastocyst formation. Zhou et al. (2001) examined chromatin remodeling of the injected nuclei during activation. They found that metaphase donor nuclei reformed a metaphase

plate rapidly after transferring. Although 20% of the spindles were abnormal, with disordered chromosomal arrangement, 93.3% of metaphases formed one pseudo pronucleus and one polar body 6 h after activation. Interphase nuclei underwent PCC, after which only 50% of the G1 nuclei formed 2 pronuclei (PN) and 63% of the G2 nuclei formed 1 PN and 1 polar body (PB). In 20% of the cloned embryos derived from interphase donor nuclei, fragmented and condensed chromatin blocks were found. These data indicate that restoration of the nuclear totipotency depends more on the nature of the donor nucleus than its initial cell cycle stage.

2.2.2 Nuclear reprogramming

Although the maintenance of correct ploidy can be controlled, at present nuclear transfer efficacy is still low in any mammalian species. Increasing evidence suggests that successful nuclear transfer is mainly dependent on how and to what extent recipient cytoplasm set differentiated nucleus back to zygotic nuclear state (nuclear reprogramming). In other words, when a donor nucleus is transferred to the enucleated oocyte, the nucleus must be modified such that it behaves as though it was a zygote (pro)nucleus. This involves remodelling of the nucleus, which presumably results in its reprogramming. Nuclear remodelling is characterized by a variety of structural changes and it is thought that this process occurs completely and consistently only after nuclear envelope breakdown and chromosome condensation and is initiated by a high level of MPF (Fulka et al. 1996).

2.2.2.1 Epigenetics and differentiation

DNA and histones are the basic components of a chromosome, in which the DNA helix is wrapped around core histones to form the simple 'beads on a string' structure that is then folded into higher-order chromatin. Except DNA and histones, chromatin also contains various proteins required for its assembly and packaging, and for DNA replication, DNA and histone modification and transcription, and DNA repair and recombination. Among nuclei of embryonic and somatic cells, there are no differences in the basic components, but the modifications on them are quite different. Epigenetics refers to covalent modifications of DNA and core histones that regulate gene activity without altering DNA sequence and will have a significant impact on the development of the organism (Santos & Dean 2004). Epigenetic regulation is not only critical for generating diversity of cell types (i.e. differentiation) during mammalian development, but also important for maintaining

the stability and integrity of the expression profiles of different cell types. Most genes in differentiated cells are regulated through epigenetic modifications including DNA methylation and histone acetylation. Closely associated with DNA methylation is the acetylation of nucleosomal histone molecules at numerous lysine residues. Acetylated histone molecules in chromatin are associated with increased gene expression. Additionally, histone H1, a linker histone that associates with internucleosomal DNA, plays an important role in regulating chromatin structure and transcriptional activity. Interestingly, while these epigenetic changes are heritable and normally stably maintained, they are also potentially reversible, as evidenced by successful using nuclei of differentiated cells (Wilmut et al. 2002).

2.2.2.2 DNA methylation

2.2.2.2.1 DNA methylation and transcription activity

The most prominent form of epigenetic alteration in mammals is the symmetric methylation of cytosine in the 5' position in CpG dinucleotides. This alteration is heritable within mitosis and normally associated with transcription silencing by recruiting methyl-CpG binding proteins, such as MeCP2, MBD1, MBD2 and MBD3, as well as is associated with histone deacetylases, co-repressor proteins and chromatin remodeling machineries to the promoter of specific genes (Jaenisch & Bird 2003; Bird 2002). DNA methyltransferase enzymes transfer methyl groups donated from *s*-adenosyl methionine onto the C5 positions of over 70% of the cytosine residues in vertebrate DNA (Meehan 2003). Over 75% of the protein coding genes in the human genome contain long stretches of high density CpG dinucleotides known as CpG islands (Lander et al. 2001) and many of these are tissue-specific genes (Suzuki et al. 2001). In somatic tissues, multiple roles have been assigned to DNA methylation, including ensuring the heritability of transcriptional repression, involvement in X-chromosome inactivation, genomic imprinting and the inactivation of retroviral sequences (Bestor 2000; Robertson & Wolffe 2000; Bird 2002; Ehrlich 2003;). For example, gynogenetic embryos (diploid maternal) characteristically are growth restricted and fail to derive a functional placenta. In contrast, androgenetic embryos (diploid paternal) hyperproliferate extra-embryonic tissues instead of growth retardation. The essence of this difference was the understanding that during maturation of gametes there is marking of specific regions of the genome (genomic imprinting) for later differential expression (Surani et al. 1990). Moreover, DNA methylation has been implicated in ‘genome defense’ associated with the silencing of parasitic retrotransposons

(Yoder et al. 1997) and to a function in the maintenance of the structural integrity of chromosomes and prevention of chromosomal rearrangements (Chen et al. 1998). Functionally the above processes are linked by stable gene silencing, in which DNA methylation establishes or maintains a silent chromatin state in concert with the range of proteins that regulate nucleosomal structure (Jaenisch & Bird 2003).

2.2.2.2.2 DNA methylation during development

DNA methylation is subject to dynamic variations in preimplantation embryos. Prior to fertilization, the genomes of both sperm and metaphase II oocytes are transcriptionally inactive. However, asymmetry exists in the chromatin structure of both gametes that requires restructuring into a functional embryonic nucleus before embryonic transcription and the tightly regulated post-fertilization developmental program of gene expression can be initiated. At least three DNA methyltransferases are involved in the methylation of new CpG sites and maintenance of the already methylated CpG during DNA replication. Ubiquitously expressed DNMT1 functions primarily as a maintenance methylase that methylates CpG sites on the newly synthesized DNA strand copying the existing methylation pattern on the template DNA strand. Developmentally regulated DNMT3a and DNMT3b are responsible for methylation of new CpG sites to establish de novo CpG methylation patterns, especially in early development and germ cell development.

Global changes of DNA methylation patterns during preimplantation development seem to be conserved across species as observed in mouse, cow, rat, pig and human, although their timing with respect to developmental stages is slightly different (Dean et al. 2001; Tamada & Kikyo 2004; Young & Beaujean 2004). For example, in mice, upon fertilization a majority of the sperm-derived genomic DNA is rapidly demethylated before the onset of DNA replication by an uncharacterized active mechanism (Mayer et al. 2000; Santos et al. 2002a). In contrast, oocyte-derived DNA is passively demethylated only after DNA replication initiates, by the nuclear exclusion of DNMT1. The global level of DNA methylation decreases to about 15% in the blastocysts but returns to higher levels during implantation when genome-wide de novo methylation occurs by DNMT3a and DNMT3b (Reik et al. 2001).

Similar to mice, dramatic demethylation of one pronucleus in pigs, rats and also human beings embryo has been observed (Dean et al. 2001; Beaujean et al. 2004). However, the

lack of asymmetric pronuclear demethylation observed in the sheep zygote also occurs in the rabbit (Beaujean et al. 2004; Shi et al. 2004). Furthermore, while Dean et al. (2001) reported almost complete loss of methylation in one pronucleus in the bovine zygote, Beaujean et al. (2004) observed only a partial demethylation. These observations suggest an intermediate state between the pig/rat/mouse/human and the sheep/rabbit. Clearly, substantial pronuclear demethylation is not an obligate requirement for development in all mammalian species.

It is well documented that methylation patterns change as tissue-specific gene expression patterns are established during early lineage differentiation (Ehrlich, 1982; Riggs et al. 1998; Imamura et al. 2001; Jaenisch & Bird, 2003). A recent key demonstration in the mouse embryo is that the pluripotency gene *Oct4* is methylated as it is permanently silenced in the late mouse epiblast, concurrent with the loss of potency (Gidekel & Bergman 2002). Using the technique of Restriction Landmark Genome Scanning, Shiota et al. (2002) have also begun to analyze the genome-wide methylation changes that occur with early lineage differentiation. Sixteen percent of the CpG-island-associated methylated regions examined in differentiating mouse embryonic stem cells showed methylation changes, indicating the presence of a large number of tissue-dependent differentially methylated regions (T-DMRs) in the genome. This confirms the role of CpG-island methylation during developmental tissue formation.

2.2.2.2.3 DNA methylation and nuclear transfer

While cell-specific methylation patterns are relatively stable in somatic cells, successful nuclear transfer embryos have to follow the above methylation dynamics to erase the tissue-specific DNA methylation pattern and then to establish a new embryo-specific DNA methylation pattern on numerous genes.

Various types of cultured cells are influenced in their potential to differentiate in vitro by the degree of locus-specific methylation (Jones et al. 1990; Kawai et al. 1994; Ansel et al. 2003). Thus correct methylation reprogramming in the early stages of development, when new lineages are formed, is thought to be essential for regulating gene silencing at specific times and for prevention of damaging or lethal ectopic gene expression (Jaenisch & Bird 2003).

A majority of the cloned bovine embryos show a gross abnormality in the genome-wide DNA methylation level and DNA methylation pattern on various repetitive sequences when compared with in vitro fertilized (IVF) controls. The DNA methylation level in clones can be higher or lower than that in the control embryos depending on the donor cell types, target DNA sequences, examined embryonic stages and detection methods. Kang et al. (2001) demonstrated that bovine cloned embryos had genome-wide methylation changes compared to IVF embryos. Bisulfite sequencing was used to assess the methylation status of a repetitive element in the bovine genome, as a marker for genome-wide alterations. Abnormal methylation patterns were identified in cloned preimplantation embryos, where 75% of embryos had hypermethylation and 25% had hypomethylation at the target sequence, compared to IVF controls (Cezar et al. 2003). However, extraordinarily high cytosine methylation levels, even in samples from noncloned controls, were demonstrated in that study (Cezar et al. 2003). More recently, Hiendleder et al. (2004) reported that hypermethylation of bovine fetal DNA was associated with disproportionate overgrowth of somatic cell nuclear transfer fetuses and was, to a lesser extent, also observed in IVF fetuses.

Bovine somatic nuclei are resistant to the erasure of DNA methylation in early embryogenesis and the clones have a tendency to preserve the DNA methylation patterns inherited from the donor cells (Bourc'his et al. 2001; Dean et al. 2001). Re-establishment of DNA methylation was also potentially deregulated by precocious de novo methylation in clones (Dean et al. 2001). This abnormal methylation transition in cloned embryos could be due to the specific features of the somatic chromatin structure and/or defective regulation of DNMTs. For example, cloned mouse embryos expressed the somatic form of DNMT1 at abnormally high level and showed defective nucleo-cytoplasmic translocation of the oocyte form of DNMT1 (Chung et al. 2003). Culture conditions of the cloned embryos are also known to affect DNA methylation as shown by loss of methylation in the regulatory CpG site of the *H19* gene depending on the culture medium of the embryos (Doherty et al. 2000).

DNA methylation of imprinted genes is established during germ cell development and is protected from the genome-wide demethylation and re-methylation in early development by an unknown mechanism (Li 2002). It is intriguing to understand whether methylation imprinting in the donor somatic nuclei is protected from the global changes of DNA

methylation in the early embryos as effectively as that in the nuclei of embryonic cells. While Inoue et al. (2002b) found normal allele-specific expression of seven imprinted genes in mouse embryos obtained from Sertoli cells, two other groups reported grossly disrupted imprinting in cumulus cell clones (Humpherys et al. 2002; Mann et al. 2003). This abnormality in the imprinting status may suggest susceptibility of the methylation imprinting in the somatic nuclei to the global methylation changes during early embryogenesis. Epigenetic markers for the inactive X chromosome can also be erased and re-established on either X chromosome in cloning (Eggan et al. 2000) with the exception of some X-linked genes (Xue et al. 2002). ES cell-derived mouse clones show a striking variation in the DNA methylation pattern and imprinted gene expression, perhaps reflecting the instability of DNA methylation during the ES cell culture. In spite of this, some ES cell-derived clones developed to term implying that the epigenetic noise paused by aberrant DNA methylation and imprinting can be compensated by other mechanisms (Humpherys et al. 2001). This notion is consistent with the routine success in producing ES cell chimeras in transgenic experiments.

2.2.2.3 Histone modifications

2.2.2.3.1 Histone modifications and transcription activity

The organization of chromatin not only restricts physical access of nuclear factors to the underlying DNA, but also the post-translational modifications of histone proteins can alter chromatin conformation and play direct regulatory roles in gene expression (Felsenfeld & Groudine 2003). A variety of post-translational modifications such as acetylation, phosphorylation, methylation and ubiquitination lead to enormous diversity in the histone/nucleosome structures (Iizuka & Smith 2003; Zhang 2003). Some modifications, including acetylation and phosphorylation, are reversible and often associated with inducible expression of individual genes. But other modifications, such as methylation, are more stable and related to the long-term maintenance of the expression status of regions of the genome. These modifications occur on multiple but specific sites on the histones, and it has been suggested that histones can act as signaling platforms, integrating upstream signaling pathways to elicit appropriate nuclear responses such as transcription activation or repression (Cheung et al. 2000). In addition, with many possible modifications and their combinations that occur on a variety of sites on histones, it has been proposed that different combinations of histone modifications may result in distinct outcomes in terms of

chromatin-regulated functions. Based on this idea, the Histone Code Hypothesis has been formally proposed (Strahl & Allis 2000; Turner 2000; Jenuwein & Allis 2001).

Histone acetylation has been most extensively studied and has an established association with transcriptionally active or 'poised' chromatin (Waterborg 2002). This chromatin is enriched in histones H3 and H4 that contain three or more acetylations per histone, which are characterized by half-lives on the order of minutes (Davie & Hendzel 1994; Katan-Khaykovich & Struhl, 2002). The steady-state levels of histone acetylation are actively maintained through the action of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes that catalyze reactions on the euchromatin component of the genome with a much higher frequency than the remaining inactive chromatin (Davie & Hendzel 1994). More recently, histone methylation has gained attention as an epigenetic mark (Arney et al. 2002; Richards & Elgin; 2002; Wang et al. 2001) through discovery of the SET domain, which encodes a histone N-lysine methyltransferase (HMTase) activity (Rea et al. 2000). Numerous HMTases have recently been characterized and are thought to catalyze the addition of methyl groups onto the amino terminal tails of histones H3 (K4, K9, K27, K36) and H4 (K20) (Lachner et al. 2003). Interestingly, the apparent lack of a histone demethylase suggests that methylation may provide a lasting epigenetic mark whose removal requires histone turnover or replacement. In this regard, methyl-histone modifications may have the capacity to stabilize chromatin states that are required to maintain cell fate decisions during development.

Histone lysine methylation is the addition of mono-, di-, and trimethyl groups, each of which may confer a different functional state (Wang et al. 2001). For instance, while the trimethyl-lysine 4 derivative of histone H3 is exclusively associated with active genes, the dimethyl moiety is characterized by a broader distribution that does not always correlate with gene activity (Santos-Rosa et al. 2002). In addition, modifications of different lysine residues can also direct the recruitment of distinct regulatory molecules—methylation of histone H3 at K9 by the HMTase Suv39h provides a binding surface for heterochromatin protein 1 (HP1), which leads to propagation and stabilization of heterochromatin (Lachner et al. 2001). In contrast, Drosophila Polycomb recognizes histone H3 that contains a trimethyl modification at K27 and is excluded from chromosomal regions enriched in trimethyl-K9 (Fischle et al. 2003). Based on these molecular studies, it would stand to

reason that the overall distribution of methyl-histone moieties should provide insight into higher order organization of the genome and its relationship to function.

2.2.2.3.2 Histone modifications during development

From a developmental standpoint, histone methylation appears to play a central role, given that severe defects in embryogenesis are observed upon targeted loss of the Ezh2, G9a, NSD1, Suv39H1/Suv39H2 or Mll HMTases (Erhardt et al. 2003; Peters et al. 2001; Rayasam et al. 2003; Tachibana et al. 2002; Yu et al. 1995). Moreover, the differences in phenotype associated with each HMTase suggest that they regulate distinct developmental events. Consistent with such an idea, studies of mono-, di-, and trimethyl lysine 9 derivatives of histone H3 in Suv39H1/2 and G9a knockout cells reveal that these HMTases can contribute to spatially unique methylation events (Peters et al. 2003).

Histone methylation acts as an epigenetic regulator of chromatin activity through the modification of arginine and lysine residues on histones H3 and H4. In the case of lysine, this includes the formation of mono-, di-, or trimethyl groups, each of which is presumed to represent a distinct functional state at the cellular level. To examine the potential developmental roles of these modifications, Biron and colleagues (2004) observed the global patterns of lysine methylation involving K9 on histone H3 and K20 on histone H4 in midgestation mouse embryos. For each lysine target site, distinct subnuclear distributions of the mono- and trimethyl versions in 10T1/2 cells were conserved within primary cultures and within the 3D-tissue architecture of the embryo. Three of these modifications, histone H3 trimethyl K9, histone H4 monomethyl K20, and histone H4 trimethyl K20 exhibited marked differences in their distribution within the neuroepithelium. Specifically, both histone H3 trimethyl K9 and H4 monomethyl K20 were elevated in proliferating cells of the neural tube, which in the case of the K9 modification was limited to mitotic cells on the luminal surface. In contrast, histone H4 trimethyl K20 was progressively lost from these medial regions and became enriched in differentiating neurons in the ventrolateral neural tube. The inverse relationship of histone H4 K20 methyl derivatives is even more striking during skeletal and cardiac myogenesis where the accumulation of the trimethyl modification in pericentromeric heterochromatin suggests a role in gene silencing in postmitotic muscle cells.

2.2.2.3.3 Histone modifications and nuclear transfer

Global release and uptake of linker histone H1 is one challenge for the donor nuclei during the nuclear reprogramming. The histone H1 exists at a very low level in mature mouse oocytes and gradually becomes abundant around the 4-cell stage (Clarke et al. 1997; Adenot et al. 2000). Following this temporal profile, blastomere nuclei lose histone H1 upon injection into oocytes and reacquire histone H1 during the subsequent development (Bordignon et al. 2001). This DNA replication-independent transition of the histone H1 level was also observed in bovine clones (Bordignon et al. 1999). In *Xenopus* somatic nuclei incubated in egg extract, the molecular chaperone nucleoplasmin is responsible for the exchange of the somatic linker histone with the egg type linker histone B4 (Dimitrov & Wolffe 1996). It is likely that mammalian nucleoplasmin (Burns et al. 2003) is involved in the loss of histone H1 from the donor nuclei, although its physiological role is unknown.

Alteration of histone modifications is also an important aspect of chromatin remodeling in cloning. Bovine oocytes and early embryos express several histone acetylases and deacetylases with some variability in the transcript levels depending on the developmental stages (McGraw et al. 2003). In mouse oocytes, histone H3 and H4 are globally deacetylated on several lysines at the metaphase II of the second meiosis, which was reproduced in somatic nuclei transferred into the same stage of oocytes (Kim et al. 2003). This genome-wide decrease of histone acetylation may contribute to the erasure of the previous gene expression patterns specific to the donor cell differentiation.

Methylation on histone H3 lysine 9 (H3-K9) is usually associated with gene inactivation, and acetylation on H3-K9 is linked with gene activation (Fischle et al. 2003). Fertilized control mouse embryos become hypoacetylated on H3-K9 at the 4-cell stage and are gradually hyperacetylated after the 8-cell stage (Santos et al. 2003). In contrast, cloned embryos retain hyperacetylation on H3-K9 throughout these stages. At the blastocyst stage, the cloned embryos show hypermethylation on H3-K9 in the trophectoderm compared with the controls. The detailed enzymology responsible for these transitions of histone acetylation and methylation in early embryos is not yet available, but these aberrant histone modifications should almost certainly affect expression of a number of genes.

2.2.2.4 Artificial modification of epigenetic state in donor cells

As above stated, with nuclear transfer, the somatic donor nucleus carries the specific epigenetic modifications of its tissue type, which must be erased during nuclear reprogramming. Therefore, the levels of epigenetic modification existing in donor cells may affect their reprogrammability following nuclear transfer. The discrepancy in the donor cell's susceptibility to reprogramming has been observed between different cell types, resulting in differences in vitro and in vivo development of cloned embryos. Therefore, treating donor cells with pharmacological agents to remove some epigenetic marks prior to nuclear transfer may improve the ability of the donor cells to be fully reprogrammed by the recipient cytoplast.

Two kinds of reagents have been considered for the alteration of the levels of epigenetic modification of somatic cells. One is aimed to increase histone acetylation, and the other one is to decrease DNA methylation. For the first purpose, trichostatin A (TSA) and sodium butyrate (NaBu) are generally used, and 5-aza-deoxy-cytadine (5-aza-dC) is widely used for the second purpose. The resulting epigenetic changes have been associated with an increase of gene expression.

Enright et al. (2003a) demonstrated that the pre-existing global epigenetic marks in donor cells can be modified by treatment with TSA or 5-aza-dC. Unfortunately, treatment of donor cells with 5-aza-dC resulted in reduced blastocyst rate. Similar effect of 5-aza-dC, but with much higher doses, has been demonstrated after treatment of bovine fetal fibroblasts (Jones et al. 2001) and mouse stem cells (Zhou et al. 2002). Effect of TSA treatment to donor cells on development of cloned embryos varied from the detrimental for mouse stem cells (Zhou et al. 2002) to neutral (Shi et al. 2003), or positive (Enright et al. 2003a) for bovine fetal fibroblasts. The differences between these findings may be due to the variation in the concentration of TSA used. The detrimental effect of a higher dose of TSA on embryo development may be explained by the fact that treatment of cells with high concentrations of TSA causes chromatin breaks and apoptosis (Nakajima et al. 1998).

Shi et al. (2003) used an immortalized bovine mammary epithelial cell line for nuclear transfer. Treatments of these cells and fetal fibroblasts, as control, with TSA, 5-aza-dC, or combination of both, did not result in improvement of development of nuclear transfer embryos. In contrast, after treatment of immortal cells with NaBu some nuclear transfer embryos developed to morulae. In addition, strongly marked positive effect of NaBu

treatment of fetal fibroblasts was observed since a much higher proportion of nuclear transfer embryos developed to blastocysts compared with untreated cells.

Overall, nuclear reprogramming is dependent on the technique of nuclear transfer itself, and it is still unclear to what extent nuclear reprogramming can be reached by this technique.

2.3 In vivo and postnatal development of cloned embryos

At present, the only way to ensure the success of nuclear transfer is to transfer cloned embryos into pseudopregnant recipient, consequently to produce normal cloned offspring. Data from preimplantation analysis on normal and cloned embryos are clearly helpful to improve nuclear transfer procedure, but the improvement of preimplantation development fail to result in expected improvement on offspring rate. A high rate of abortion during gestation has been observed in cloning experiments performed on different species (Ogonuki et al. 2002; Schnieke et al. 1997; Vignon et al. 1998). These late miscarriages are frequently associated with abnormal development of the placenta (Hill et al. 1999). Cloned embryos and/or offspring also often show many abnormalities, including circulatory distress, placenta edema, hydrallantois, and chronic pulmonary hypertension. The surviving offspring also have large placentae and increased birth weights, and they suffer a high incidence of death (Tamashiro et al. 2002; Ogonuki et al. 2002). Therefore, more data on in vivo development after embryo transfer will from another side improve the efficacy of nuclear transfer.

2.3.1 Implantation defects

Early pregnancy losses from somatic nuclear transfer are commonly above 50% in sheep (Wells et al. 1997; Wilmut et al. 1997), cattle (Cibelli et al. 1998; Wells et al. 1999; Zakhartchenko et al. 2001), and goats (Baguisi et al. 1999). Upon implantation, those fetuses that fail to develop often show retarded development, although such a feature had previously been observed with concepti derived from embryonic nuclei (Stice et al. 1996). When compared with in vitro produced embryos, the frequency of early fetal mortality is about two times higher with somatic nuclei obtained from either fetuses or adult cultured fibroblasts than with embryonic nuclei (Heyman et al. 2002a). Most of the losses observed with somatic nuclei occur during the second month of pregnancy in cattle (Wells et al. 1999; Zakhartchenko et al. 2001; Heyman et al. 2002b). They are most frequently associated with functional deficiencies that occur at the onset of placentation as evidenced

by poor development of placentomes and abnormal vascularization of extraembryonic tissues (cattle: Wells et al. 1999; Hill et al. 2000; sheep: De Sousa et al. 2001).

2.3.2 Placenta abnormality

Appropriate growth, development, and function of the placenta are central to the success of nutrient partitioning between the mother, placenta, and fetus. In cloned pregnancies, placental deficiencies, including increased placentome size, reduced placentome number, and increased accumulation of allantoic fluid, and failure to initiate adequate blood supply have also been reported in ruminants following transfer of in vitro produced embryos (Walker et al. 1996; Young et al. 1998). Lack of some placentomes and abnormal vascularization of extraembryonic tissues could lead to fetal malnutrition that, in turn, would influence development long after birth (Barker & Clark. 1997). In sheep, within the first quarter of gestation, cloned fetuses are characterized by a high incidence of developmental retardation and placental insufficiency (De Sousa et al. 2001). Variations in the degree of placental alterations do exist, as animals derived from both somatic cloning and IVF have placental abnormalities during early or late gestation (Young et al. 1998; Hill et al. 1999).

Ravelich et al. (2004a) observed dysregulation of expression of placental lactogen (bPL) and leptin in nuclear transfer placentomes which could contribute to aberrations in cell migration and invasion and subsequently to alterations in placental metabolism and transfer of nutrients to the fetus, thus leading to increased placental and fetal macrosomia in nuclear transfer pregnancies. Moreover, endocrine and paracrine perturbations of the insulin-like growth factor (IGF) axis may contribute to modulate placental dysfunction in nuclear transfer pregnancies. Furthermore, increased cell numbers in nuclear transfer placentomes likely have significant implications for fetomaternal communication and may contribute to the placental overgrowth observed in the nuclear transfer placentomes (Ravelich et al. 2004b). In addition, aberrant hypermethylation at the Sall3 locus is associated with abnormal placental development caused by nuclear transfer of somatic cells in mice (Ohgane et al. 2004).

2.3.3 Postnatal development

Postnatal mortality has been reported for almost all species cloned until now. These losses are frequently associated with prolonged pregnancies and dystocia (Wilmut et al. 1997;

Kato et al. 1998; Renard et al. 1999). The majority of surviving clones are not without complications and they often suffer from a variety of defects such as respiratory distress, bacterial infections, kidney and liver defects (Cibelli et al. 2002). Body weight of these animals is generally higher than normal (Campbell et al. 1996a; Cibelli et al. 1998; Wells et al. 1999; Zakhartchenko et al. 1999a,b,c; 2001; Kato et al. 2000; Heyman et al. 2002a,b) and may occasionally be twice the mean value of the corresponding breed (Schnieke et al. 1997; Wells et al. 1999; Tamashiro et al. 2000). A syndrome called Large Offspring Syndrome (LOS, Young et al. 1998; Wakayama & Yanagimachi 1999; Hill et al. 2000; Rideout et al. 2001; Tanaka et al. 2001) may also arise after embryo culture (Behboodi et al. 1995; Sinclair et al. 1999; Young & Fairburn 2000) or pronuclear injection (Walker et al. 1992). This syndrome may involve epigenetic modifications of imprinted genes (Young & Fairburn 2000). Such epigenetic changes occurring in the early embryo could be propagated through subsequent cell cycles and could affect gene expression during fetal or postnatal development (Dean et al. 1998).

3 MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO)

3.1 Nuclear transfer with embryonic cells

3.1.1 Animals

Animal experiments were approved by the Regierung von Oberbayern (Az 209.1/211-2531-5/01).

Mature New Zealand White rabbits were bought from Charles River Deutschland.

3.1.2 Collection of recipient oocytes

Female rabbits were superovulated by injection of 100 IU pregnant mare's serum gonadotrophin (PMSG, Intervet Deutschland GmbH, Unterschleissheim) intramuscularly and 100 IU human chorionic gonadotrophin (hCG, Intervet Deutschland GmbH) intravenously 72 h later. Mature oocytes were flushed from the oviducts 15 –16 h post-hCG injection in warm (25°C) mPBS (PBS supplemented with 4 mg/ml BSA) and incubated in 0.5% hyaluronidase in M199 (Hepes buffered- medium 199 supplemented with 10% FCS) for 15 min in the culture conditions of 5% CO_2 in air at 39°C. Cumulus cells were removed by gentle pipetting with a flame-polished narrow-bore pipette.

3.1.3 Induction of MII metaphase protrusion and enucleation

Denuded oocytes were treated with 5 µM ionomycin in M199 for 3-5 min, followed by 0.6 µg/ml demecolcine in M199 between 40 min to 2 h (Yin et al. 2002a,b). The MII metaphase protrusions with a little under part were removed in M199 supplemented with 7.5 µg/ml cytochalasin B (CB) and 0.6 µg/ml demecolcine. The enucleated oocytes were used as recipient cytoplasts.

3.1.4 Activation

3.1.4.1 *Activation protocol I:* Activation of enucleated oocytes before/after nuclear transfer was initiated by 7% ethanol in M199 at 39°C for 5 min, then oocytes were treated with 10 µg/ml cycloheximide (CHX) and 5.0 µg/ml CB in Upgraded B2 INRA Medium (B2 medium, Laboratoir C.C.D, Paris, France) containing 10% FCS for 4-5 h under culture condition.

3.1.4.2 *Activation protocol II:* Activation of eucleated oocytes before/after nuclear transfer was initiated by double electric pulse (1.8 KV/cm, 40 µs×2, 1sec interval) using Eppendorf

Multiporator (Hamburg, Germany) in cell fusion medium (Eppendorf, Germany) with 1 h interval in M199, then followed by treatment with 1.9 mM 6-dimethylaminopurine (6-DMAP) and 10 µg/ml CHX in M199 for 1 h under culture condition.

3.1.5 *Preparation of donor blastomeres*

3.1.5.1 *Isolation of individual blastomeres:* Mature New Zealand White female rabbits were intramuscularly injected with 100 IU PMSG, then mated with fertile New Zealand White males 72 h later followed by intravenous injection with 100 IU hCG. Eight-12-cell in vivo fertilized embryos were collected by flushing oviducts with mPBS 42–44 h after hCG injection. Mucin coat and zona pellucida were removed by pipetting after 3–4 min treatment with 0.5% pronase in mPBS. Then naked embryos were incubated in 0.25% trypsin-0.01% EDTA for 2 min, and individual blastomeres were prepared by pipetting with a flame-polished narrow-bore pipette and kept in M199 till use.

3.1.5.2 *Synchronization of blastomeres:* Four-8-cell embryos were cultured in 0.6 µg/ml demecolcine in B2 medium for 10 h to arrest them in M phase. They were then extensively washed in M199 and immediately incubated in 0.1 µg/ml aphidicolin in M199. Cleavage once in aphidicolin was allowed, and daughter blastomeres remained arrested at the G1/S transition until the use for nuclear transfer.

3.1.6 *Nuclear transfer, fusion and embryo culture*

Transfer of donor karyoplasts was carried out essentially as described previously (Zakhartchenko et al. 1999a). Briefly, by 20-25 µm inner diameter injection pipette, a single individual blastomere was introduced under the zona pellucida of enucleated oocyte in M199 supplemented with 7.5 µg/ml CB. Reconstructed embryos were produced after fusion of karyoplast-cytoplast complexes by electric pulses (1.8 KV/cm, 40 µs×2, 1sec interval). After 15~20 min in M199, fused embryos were cultured in B2 medium containing 10% FCS at 39°C either overnight for embryo transfer or up to 4 days to assess the developmental potential to blastocysts.

In some cases, nuclear transfer was carried out before oocyte activation. Thus, enucleated oocytes were first fused with donor blastomeres, and then activated and cultivated as above.

3.1.7 *Embryo transfer*

Recipient females were given hCG 20-22 h after the oocyte donors (asynchronous recipients). Two-4-cell stage cloned embryos were transplanted non-surgically through the infundibulum into each oviduct of recipients (Besenfelder & Brem 1993). Pregnancy was determined by palpation 2 weeks after embryo transfer.

3.1.8 *DNA injection*

Foreign DNA constructs containing GFP-expressing sequence were prepared by the THP company. Using Eppendorf Microinjection Manipulator (Eppendorf, Hamburg, Germany), 1~2 ng DNA was injected either under the zona pellucida or directly into enucleated oocytes before or after activation. After overnight or 4 days in vitro culture, expression of GFP protein in reconstructed embryos was detected under UV light (Axiovert 200M Zeiss; Hallbergmoos Germany).

3.2 Nuclear transfer with cumulus cells

3.2.1 Animals

Mature ZIKA breed rabbits were obtained from Agrobiogen company.

Rabbits of *Ali/Bas* heterozygous were used as donors of fetal fibroblast cells. Alicia is a rabbit strain (*Ali*) which has a variant of the allotype allele a2 associated with the H chain of immunoglobin molecules. Basilea is another rabbit strain (*Bas*) with a mutant gene at the Ab allotypic locus controlling the synthesis of allotypic specificities (Ab4, Ab5, Ab6, Ab9, Ab4v, and Ab95) of K-immunoglobulin light chains (Garcia et al. 1982; Kelus & Weiss 1986). *Ali/Bas* origin female rabbits were donated by the THP Company.

3.2.2 Collection of recipient oocytes

Mature ZIKA breed rabbits were superovulated by injection of 100 IU PMSG intramuscularly and 100 IU hCG intravenously 72 h later. Mature oocytes were flushed from the oviducts 15 –16 h post-hCG injection in warm mPBS and incubated in 0.5% hyaluronidase in M199 for 15 min in the culture conditions of 5% CO_2 in air at 38.5°C. Cumulus cells were removed by gentle pipetting with a flame-polished narrow-bore pipette.

3.2.3 Induction of MII metaphase protrusion

Denuded oocytes were treated with 0.6 µg/ml demecolcine in M199 between 40 min to 2 h. The MII metaphase protrusion with a little under parts was removed in M199 supplemented with 7.5 µg/ml CB and 0.6 µg/ml demecolcine. Enucleated oocytes were kept in M199 and later used as recipient cytoplasts.

3.2.4 Preparation of rabbit cumulus cells (RCC)

Mature *Ali/Bas* origin female rabbits were superovulated by injection of 100 IU PMSG intramuscularly and 100 IU hCG intravenously 72 h later. Mature oocytes were flushed from the oviducts 15–16 h post-hCG injection in warm mPBS and incubated in 0.5% hyaluronidase in M199 for 15 min in the culture conditions of 5% CO_2 in air at 38.5°C. Following gentle pipetting, cumulus cells were collected and pelleted in M199 by centrifugation at 140 *g* for 5 min, then cultured in complete cell culture medium (Dulbecco Modified Eagle Medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 2 mM nonessential amino acids, 100 IU/ml penicillin, and 100 µg/ml streptomycin) in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

Confluent RCCs were washed twice with Ca^{2+}- and Mg^{2+}-free PBS, then treated with 0.25% trypsin-0.01% EDTA solution for 1-2 min. Cells were collected in complete cell culture medium and pelleted by centrifugation at 140 *g* for 5 min, then reseeded as 1:4 ratio. Cumulus cells at 1 - 5 passages were used as nuclear donors.

3.2.5 Nuclear transfer, fusion and activation

Individual cumulus cells were prepared by trypsinization, and then introduced under the zona pellucida of enucleated oocytes in M199. Reconstructed embryos were produced after fusion of karyoplast-cytoplast complexes by electric pulses (1.95 KV/cm, 25 µs×2, 1sec interval). After incubation in M199 for 20 ~ 40 min, cloned embryos were initiated by the same electropulse as for fusion, then immediately followed by treatment with 1 h 1.9 mM 6-DMAP and 5.0 µg/ml CB (in B2 medium containing 10% FCS) in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

3.2.6 Embryo culture and transfer

After activation, cloned embryos were cultured overnight in B2 medium containing 10% FCS at 39°C under 5% CO2 in air. Two-4-cell stage cloned embryos were transplanted non-surgically through the infundibulum into each oviduct of recipient females which had

been induced into pseudopregnancy state either by mating to vasectomized ZIKA breed males at 20-22 h after that of the females used as oocyte donors or at 20-22 h after for oocyte donors by injection of 80 IU PMSG intramuscularly and 80 IU hCG intravenously 72 h later. Pregnancy was determined by palpation 2 weeks after embryo transfer.
To assess the developmental potential to blastocyst stage, cloned embryos were cultured for up to 6 days.

3.2.7 Ali/bas genotyping

For the preparation of genomic DNA (ear and embryo samples) the QIAamp DNA Mini Kit (QIAGEN, Hilden) was used according to the manufacturer´s instructions. Due to the fact that the used wildtype rabbit strains belong to the e15 allotype (accession no. K00752) whereas *Ali/bas* rabbits belong to the e14 allotype (accession no. J00665), a differentiation between wildtype homozygous, *Ali/bas* homozygous and heterozygous animals is possible. For the determination of this 1 bp-difference, which maps in the CH2 domain of the IgH constant region, a specialized SNP genotyping kit (Genespector®) was developed by Variom, Berlin. The principle of this oligonucleotide ligation assay is based on the mismatch sensitivity of T4 DNA ligase. In brief, two oligonucleotides specific for the e14 or e15 allele were coated to different wells of a 96-well microtiter plate. A biotinylated signal probe together with a PCR product comprising the e14/15 mismatch were added to the wells. The signal probe was linked to the coated oligonucleotide by T4 DNA ligase if the corresponding allotype was present in the PCR product. A visualization of the results was possible by addition of a streptavidin-peroxidase conjugate detecting the linkage of the signal probe and catalyzing the processing of a substrate.

3.3 Nuclear transfer with fetal fibroblast cells

3.3.1 Animals

Mature ZIKA breed rabbits were obtained from the Agrobiogen company (Germany); and *Ali/Bas* female rabbits were donated by the THP Company.

3.3.2 Collection of recipient oocytes (the same as 3.2.2)

3.3.3 Induction of MII metaphase protrusion (the same as 3.2.3)

3.3.4 Preparation of Rabbit fetal fibroblast cells (RFF)

3.3.4.1 Establishment of primary RFFs

a) *Ali/Bas* origin 15 ~ 16 dpc fetuses were washed 3 times with Ca^{2+}- and Mg^{2+}-free PBS containing 100 IU/ml penicillin and 100 µg/ml streptomycin.

b) After removal of head and heart, remnant fetal tissues were minced into 1 mm^3 pieces in 0.25% trypsin-0.01% EDTA drop, and then collected into 15 ml centrifuge tube.

c) Additional trypsin-EDTA solution was added to cover all minced pieces, and mixed well by pipetting. The mixture was incubated at 38.5°C for 10-15 min.

d) Complete cell culture medium was added to stop trypsin digestion. Tissue pieces were thoroughly pipetted, and then sedimented by gravity for minutes.

e) Supernatant was transferred to new 15 ml centrifuge tube. Primary fetal fibroblast cells were pelleted by centrifugation at 140 *g* for 10 min.

f) Cell pellet were resuspended in complete cell culture medium, and then seeded into 35 mm cell culture dishes.

g) After 3-4 days culture in a humidified atmosphere of 5% CO_2 in air at 38.5°C, primary fetal fibroblast cells grew to confluence.

3.3.4.2 Cell passage, freezing and thawing

a) Cell passage: Confluent RFFs were washed twice with Ca^{2+}- and Mg^{2+}-free PBS, then treated with 0.25% trypsin-0.01% EDTA solution for 1-2 min. Cells were collected in complete cell culture medium and pelleted by centrifugation at 140 *g* for 5 min, then reseeded as 1:4 ratio.

b) Cell freezing: As cell passage, confluent cells were pelleted by centrifugation, then suspended in cold FCS in a density of 1×10^6 cells/ml. Dimethyl sulfoxide (DMSO) was added to 10% final concentration. After incubation for 10 min at 4°C, 20 min at –20°C, cells were finally stored at –80°C.

c) Cell thawing: Frozen cells were fast thawed by adding prewarmed (37°C) complete cell culture medium with gentle pipetting. Cells were pelleted by centrifugation at 140 *g* for 5 min, and then seeded into 35 mm cell culture dishes.

3.3.4.3 Preparation of cells for nuclear transfer

Confluent primary RFFs were either directly used as donor nuclei, or split as 1:4, then serum “starved” (FCS final concentration in cell culture medium decreased to 0.5%) for 4-

5 days prior to use as nuclear donors, or split as 1:4, then treated with 1 mM sodium butyrate (NaBu) in complete cell culture medium for 72 h prior to use as nuclear donors.

3.3.4.4 Characterization of NaBu-treated RFFs

3.3.4.4.1 In vitro life span of RFFs

Confluent freshly prepared primary RFFs (Passage 0) were pelleted and seeded into 35 mm dishes at a density of 5×10^4 cells/ml. After 3 days culture without medium change, cells grew to confluence, and total cell number were determined with a hemacytometer after trypan blue exclusion of dead cells before next generation was carried out as above. The same procedure was repeated until cells reached replicative senescence which was assessed by enlarged cell morphology and extremely slow proliferation rate.

3.3.4.4.2 Cell proliferation

To measure the effect of NaBu on RFF growth, confluent RFFs in 35 mm dish were split as 1:4, then in parallel either cultured in complete cell culture medium or treated with 1 mM NaBu in complete cell culture medium. Cells were harvested every 24 h up to 4 days. Cell number at the various time points was determined with a hemacytometer after trypan blue exclusion of dead cells.

3.3.4.4.3 Karyotype analysis

The ploidy of untreated and treated RFFs was examined using a standard preparation of metaphase spreads.

a) Eighty percent confluent RFFs on a 35 mm dish were split as 1:2, and then cultured for another 24 h.
b) Demecolcine (stock solution: 120 µg/ml in Ca^{2+}- and Mg^{2+}-free PBS) was added to the culture medium to a final concentration of 0.1 µg/ml. Cells were put back to culture condition for another 150-180 min.
c) Culture medium was removed to a 15 ml conical tube.
d) Adherent cells were washed once with Ca^{2+}- and Mg^{2+}-free PBS, and medium was collected into the same 15 ml conical tube as in step c).
e) 0.25% trypsin-0.01% EDTA solution was dropped to cover cell surface. Cells were treated for 1-2 min under cell culture condition.
f) Sloughed cells were collected in 2 ml complete cell culture medium, and then the cell suspension was transferred into the same 15 ml conical tube as in step c).

g) Cells were pelleted by centrifugation at 140 *g* for 10 min.

h) 0.5 ml of the supernatant was left in the tube, and the pellet was gently resuspended by flicking the tube with fingers. Ten ml of hypotonic solution (75 mM potassium chloride in bidistilled water) were slowly and gently added into the cell suspension.

i) The suspension was incubated in 37°C water bath for 10-12 min.

j) Three-5 drops of fresh fixative solution (3:1 methanol/acetic acid, Rothe) were added to the suspension to stop reaction.

k) After centrifugation as step g), 0.5 ml of the supernatant was left in the tube. Ten ml of freshly prepared fixative solution were slowly and gently added into the cell suspension.

l) Repeat step k) twice.

m) The cell pellet was resuspended in appropriate volume of fixative silution after removal of the supernatant.

n) Ten µl suspension was dropped onto clean glass slide.

o) After air-dry, the slide was placed in a Coplin jar containing Giemsa staining solution for 3 to 5 min.

p) The slide was rinsed with autoclaved distilled water until the stain no longer discoloured the water.

q) The slide was allowed to air dry. Fifty µl of 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml)/antifade medium was mixed well with 2 µl of 7-aminoactinomycin D (7-AAD, 1 mg/ml in PBS). The mixture was then dropped to the metaphase slide preparation. A coverslip was added.

r) Karyotype analysis was performed under fluorescent microscope (Axiovert 200M; Zeiss, Hallbergmoos Germany)

DAPI stock solution: An entire container of DAPI powder was diluted with sufficient bidistilled water to prepare a 100 µg/ml stock solution. Small aliquots were prepared and stored at -20°C for up to several months.

DAPI/antifade medium:

PBS	5 ml
DAPI stock solution	0.5 ml
p-phenylenediamine	0.05 g
Dissolve well	
Glycerol (Rothe)	45 ml

Mix well by continuous stirring (protecting from light!)

7-AAD stock solution: 7-AAD powder (1 mg) was first dissolved in 50 µl of absolute methanol (MERCK KGaA, Darmstadt Germany), then added with 950 µl of 1×PBS. Final concentration was 1 mg/ml. Small aliquots were prepared and stored at 4°C for up to 6 months.

3.3.4.4.4 Senescent analysis

Senescence-associated ß-galactosidase (SA-ßgal) activity was determined as described previously (Dimri et al. 1995). Confluent RFFs were split as 1:4 into 35 mm cell culture dishes with cover slides which had been treated with 0.4% gelatin in Ca^{2+}- and Mg^{2+}-free PBS for 1 day. After 3 days in culture, cells were washed in Ca^{2+}- and Mg^{2+}-free PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde in Ca^{2+}- and Mg^{2+}-free PBS for 3–5 min at room temperature, washed again in Ca^{2+}- and Mg^{2+}-free PBS, and incubated at 37°C in freshly prepared X-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indoyl ß-D-galactopyranoside), 40 mM citric acid/Na phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$ for 16 h. The proportion of blue-stained cells was counted in 10 fields at 200× magnification.

3.3.4.4.5 Global histone acetylation analysis

3.3.4.4.5.1 Histone extraction

Histones of cultured cells were extracted according to previous studies (Yoshida et al. 1990) with a little modification.

a) About 2×10^6 cells treated with(out) NaBu for 72 h were harvested using a rubber policeman, collected by centrifugation at 1000 *g* for 10 min, and washed once with Ca^{2+}- and Mg^{2+}-free PBS.

b) The washed cells were suspended in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$, 8.6% sucrose, pH6.5), then homogenized by passing through a 20-gauge needle 10 times.

c) The nuclei were collected by centrifugation at 1000 *g* for 10 min, washed three times with lysis buffer, and once with TE buffer (10 mM Tris-HCl, 13 mM EDTA, pH7.4) successively.

d) The pellet was suspended in 0.1 ml of ice-cold autoclaved H_2O using a Vortex mixer, and concentrated H_2SO_4 was added to the suspension to give a final concentration of 0.2 N, then incubated at 4°C overnight

e) The suspension was centrifuged for 5 min at the highest speed using a microcentrifuge

f) The supernatant were transferred to a new Eppendorf tube, mixed well with 1 ml of acetone, then incubated at –20°C overnight.

g) The coagulated materials were collected by centrifugation for 5 min at the highest speed using a microcentrifuge. The pellet was allowed to air dry.

h) The acid soluble histone fraction was dissolved in 50 µl of autoclaved H_2O

i) Protein was quantified using Bradford Reagent, and Varian UV-visible spectrometer (cary WinUV 50Bio) was used to read the absorbance at 595 nm.

3.3.4.4.5.2 SDS-PAGE

Proteins were separated through SDS-PAGE Metra electrophoresis system (Metra). The glass plates were assembled prior to gel preparation.

a) Polyacrylamide gel preparation:

Resolving gel: 10 ml of a 15% gel (the following reagents were mixed in sequence with continuous stirring)

bidistilled water	2.34 ml
30% Acrylamide/bisacrylamide (29:1 mix)	5.0 ml
Tris.HCl (1.5 M, pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% ammonium persulfate	50 µl
Ultrasonic for 3 min	
N,N,N',N'-Tetramethylethylenediamine (TEMED)	5 µl

The mix (i.e. the resolving gel) was immediately poured into the gap between two glass plates, and enough space was left for the stacking gel (about 3.5 cm). The resolving gel was overlaid with bidistilled water to ensure an even surface. The procedure could be stopped here, and left for overnight. After complete polymerisation, the water was discarded before preparing the stacking gel.

Stacking gel: 12.5 ml of a 4% gel (the following reagents were mixed well in sequence with continuous stirring)

bidistilled water	7.95 ml
30% Acrylamide/bisacrylamide (29:1 mix)	1.25 ml
Tris-HCl (0.5 M, pH 6.8)	3.15 ml
10% SDS	125 µl
10% ammonium persulfate	62.5 µl
Ultrasonic for 3 min	
TEMED	12.5 µl

The mix (i.e. the stacking gel) was immediately loaded on the top of the resolving gel. The comb was inserted with no air bubbles under the teeth. After complete polymerisation, the comb was removed, and the wells were washed three times with 1× running buffer, and then the plates were mounted in the electrophoresis system. The system was filled with 1× running buffer.

10 × running buffer:

Tris	30.3 g
glycine	144 g
SDS	10 g

Bidistilled water was added to final volume 1000 ml.

The buffer was stored at RT and diluted to 1× before use.

b) Sample loading and electrophoresis

Twenty micrograms of proteins were prepared in a final volume of 24 µl, then mixed with 12 µl Laemmli loading buffer following incubation at 95°C for 5 min. After short centrifugation, samples were chilled on ice.

Samples were loaded without migration to neighbour well(s), and the electrophoresis was performed initially at 95 V for about 50 min and then at 135 V until the bromophenol blue left the separating gel at the bottom (about 4 h). Molecular weight standards (Protein ladder, 10-200 kDa and Prestained Protein Ladder, ~10-180 kDa; Fermentas) were pipetted in the first and the last slot for estimation of the protein size.

3.3.4.4.5.3 Electrophoretic blotting

Before blotting, an Immobilon-p PVDF membrane (Millipore, Bedford, MA, USA) was soaked in methanol for 10 min, then immersed into 1× transfer buffer for about 1 h.

10 × transfer buffer: Tris 58.2 g
glycine 29.2 g
SDS 3.7 g
Bidistilled water was added to final volume 1000 ml.

The buffer was stored at RT. Before use, 1× transfer buffer was prepared: 1 volume of 10× transfer buffer was mixed with 2 volumes of methanol and 7 volumes of bidistilled water.
The gel was removed from the electrophoresis chamber and the separated proteins were transfer to the PVDF membrane by semidry electrophoretic blotting in the Milliblot-Graphite Electroblotter (MBBDGE001; Millipore) as following.

Six sheets of gel blotting paper (Schleicher & Schuell) cut to the same size as the gel (8.5 cm × 7 cm) were soaked in transfer buffer, stacked one on the top of the other on the bottom electrode and squeezed with a pipette to remove air bubbles. The PVDF membrane and then the gel were placed exactly over the paper stack and were covered with another six soaked gel blotting paper sheets. The upper electrode was placed and the system was connected to a power supply (POWER PAC 3000; BioRad). The transfer took place for 90 min at 60 mA for each gel (1 mA/cm^2).

3.3.4.4.5.4 Western immunoblotting

The membrane containing the separated proteins was washed with TBS-T at RT for 10 min, and then incubated at 4 °C overnight with blocking solution (TBS-T buffer with 3% BSA).

TBS buffer: Tris 2.42 g
NaCl 8.00 g
1 N HCl 3.80 ml
PH was adjusted to 7.6
Bidistilled water was added to final volume 1000 ml

TBS-T buffer TBS buffer 100 ml
Tween-20 50 µl

After blocking, incubation with the primary antibody (Anti-acetyl-lysine, clone 4G12, mouse monoclonal IgG, Upstate Biotechnology) diluted in blocking solution (1:350) was performed at RT for 90 min. The membrane was then washed 3 times for 5 min per time at RT with TBS-T. Incubation with the secondary antibody (Alkaline Phosphatase-Conjugates: Goat anti-mouse IgG, Santa Cruz Biotechnology Inc., Heidelberg, Germany) diluted in blocking buffer (1:1000) took place at RT for 1 h. Finally, the membrane was washed 3 - 5 times with TBS-T at RT for 5 min per time. After removal of the liquid, the membrane was covered with enough BCIP/NBT BLUE LIQUID SUBSTRATE FOR MEMBRANES detection solution in dark room for about 2 min. The developing reaction was stopped by rinsing membrane briefly with bidistilled water.

3.3.4.4.5.5 Quantitation of acetylated histone H3 and H4

Quantitation of immunoblotting bands was performed by Quantity One 1-D analysis software (Bio-Rad).

3.3.4.4.6 Global DNA methylation analysis

3.3.4.4.6.1 Extraction of genomic DNA from cultured cells

a) After two times wash with Ca^{2+}- and Mg^{2+}-free PBS, adherent cells grown on 35 mm dish were treated with trypsin/EDTA for 1-2 min, then cells were pelleted by centrifugation at 140 *g* for 5 min. The cell pellet was washed twice with Ca^{2+}- and Mg^{2+}-free PBS.

b) The cell pellet was suspended in 5 ml of DNA buffer (200mM Tris-HCl, pH 8.0; 0.1 M EDTA), and cells were pelleted again by centrifugation as above.

c) The pellet was suspended in 1.5 ml of DNA buffer, then 125 µl of Proteinase K (10 mg/ml, Carl Roth GmbH&Co. Karlsruhe, Germany) and 400 µl of 10% SDS were added. The suspension was incubated overnight at 45°C with gentle shake.

d) 1.8 ml of phenol was added to the suspension. After shake by hand for 10 min at RT, centrifugation at 1000 *g* for 10 min at 10°C was carried out.

e) The supernatant was transferred into a new tube. An eequal amount of phenol/chloroform/ isoamylalcohol (25:24:1, Carl Roth GmbH&Co. Karlsruhe, Germany) was added to the supernatant. After shake by hand for 10 min at RT, centrifugation at 1000 *g* for 10 min at 10°C was carried out.

f) The supernatant was transferred into a new tube. An equal amount of chloroform/sioamylalcohol (24:1) was added to the supernatant. After shake by hand for 10 min at RT, centrifugation at 1000 *g* for 10 min at 10°C was carried out.

g) The supernatant was transferred into a new tube. One-tenth volume 3 M sodium acetate (pH5.2) and 3 volume 100% isopropanol (2-propanol, Carl Roth GmbH&Co. Karlsruhe, Germany) were added. After gentle shake, the DNA was precipitated.

h) Using a sterile glass pipette, the precipitated DNA was transferred into a tube with 10ml of 70% ethanol. After thorough wash by inverting, DNA was transferred into a sterile Eppendorf tube.

i) After centrifugation for 20 min at the highest speed, the supernatant was removed, and the pellet was air-dried.

j) DNA pellet was dissolved in 200 µl of sterile water after incubation overnight at 37°C with gentle shake (Eppendorf thermomixer shaker).

k) The DNA concentration was measured using GeneQuant II RNA/DNA Calculator (Pharmacia Biotech).

3.3.4.4.6.2 Restricted enzyme digestion of genomic DNA

Two microgram of genomic DNA were digested with 10 U methyl-sensitive restriction enzymes *Aci*I (New England Biolabs GmbH, Frankfurt am Main, Germany) and *Sma*I (Fermentas GambH, St. Leon-Rot Germany) and 10 U methyl-nonsensitive restriction enzymes *Hind*III and *BamH*I (Fermentas GambH, St. Leon-Rot Germany) in the recommended buffer in a volume of 25 µl for at least 3 h. Five ul of 6 × loading buffer were added and the digested DNA was separated in a 0.8% TAE buffered agarose gel (Invitrogen Ltd. Paisley Scotland, UK) with DNA ladder (MBI), using 1 × TAE as running buffer. Ethidium bromide was added to permit visualization of nucleic acids under ultraviolet (UV) light (Gel Doc 2000, Bio-rad, Munich, Gemany).

50 × TAE buffer:	Tris	242 g
	glacial acetic acid	57.1 ml
	0.5 M EDTA, pH 8.0	100 ml
	Bidistilled water was added to final volume 1000 ml	
	The buffer was sterilized by autoclaving.	

3.3.5 Nuclear transfer, fusion and activation (the same as 3.2.5)

3.3.6 Embryo culture and transfer

After activation, cloned embryos were cultured overnight in B2 medium containing 10% FCS at 39°C under 5% CO_2 in air. Four-cell stage nuclear transfer embryos were transplanted endoscopically through the infundibulum into each oviduct of recipient females which had been induced into pseudopregnancy status 20-22 h after for oocyte donors by injection of 80 IU PMSG intramuscularly and 80 IU hCG intravenously 72 h later. Pregnancy was determined by palpation 2 weeks after embryo transfer.

To assess the developmental potential to blastocyst stage, cloned embryos were cultured for up to 6 days.

3.3.7 Preparation of aggregation embryos

3.3.7.1 Clone-fertilized embryo aggregation

ZIKA breed female rabbits were intramuscularly injected with 100 IU PMSG, then mated with fertile ZIKA breed males 72 h later followed by intravenous injection with 100 IU hCG. In vivo fertilized embryos were collected by flushing oviducts with mPBS 16 h after hCG injection, then cultured in B2 medium supplemented with 10% FCS. Six-12-cell embryos were treated with 0.5% pronase for seconds to remove the zona pellucida, and then incubated in 0.25% trypsin-0.01% EDTA for 2 min to isolate individual blastomeres.

One or 2 blastomeres were introduced into 4-10-cell stage cloned embryos in M199 containing 7.5 μg/ml CB. After thorough wash in M199 and culture medium sequentially, aggregation embryos were cultured in B2 medium containing 10% FCS either till embryo transfer or up to 6 days for the assessment of the developmental potential to blastocyst stage.

3.3.7.2 Clone-parthenote aggregation

Cumulus-free oocytes were parthenogenetically activated and cultured under the same conditions as for nuclear transfer embryos. Six-12-cell parthenogenetic embryos were treated with 0.5% pronase for seconds to remove zona pellucida, and then incubated in 0.25% trypsin-0.01% EDTA for 2 min to isolate individual blastomeres. One or 2 blastomeres were introduced into 4-10-cell stage cloned embryos in M199 containing 7.5 μg/ml CB. After thorough wash in M199 and embryo culture medium sequentially, aggregation embryos were cultured in B2 medium containing 10% FCS either till embryo

transfer or up to 6 days for the assessment of the developmental potential to blastocyst stage.

3.3.7.3 Culture of one or two blastomeres

After overnight culture, 1-2-cell cloned embryos which did not develop further were used as the source of zona pellucidae. Complete oocyte cytoplasm was removed by micromanipulation. One or 2 blastomeres from 6-12-cell in vivo fertilised or parthenogenetic embryos were introduced under the zona pellucida. If 2 blastomeres were used, they were intentionally put close to each other. Blastomeres were cultured 3 days for the assessment of their development to blastocyst.

3.3.8 Counting the cell numbers of blastocysts

Cell numbers of day-4 blastocysts from in vivo fertilized embryos, parthenogenetic embryos, NaBu-treated RFFs nuclear transfer embryos and aggregation embyos were counted after staining with Hoechst 33342.

a) Zona pellucidae were removed after treatment in prewarmed (39°C) 0.5% pronase for seconds.
b) After three times wash in mPBS at room temperature, naked blastocysts were incubated in mPBS containing 1.0 μg/ml Hoechst 33342 for 10 min under embryo culture condition.
c) After three times wash in mPBS, individual blastocysts were transferred onto Poly-L-lysine covered microscope slides with minimum volume.
d) Blastocysts were expanded by sucking away liquid with fine pipette, and air-dried for 2 min.
e) Slides were mounted by covering with antifade medium over the area where the embryos were fixed.
f) Cells were counted under the fluorescent microscope with a UV filter since nuclei appeared blue.

3.4 Statistic analysis

Results expressed as percentage were compared using chi-square analysis. Statistical differences for histone acetylation were determined using paired sample *t*-test. Student's *t*-test was used for determining the statistical difference among the cell numbers of blastocysts. $P<0.05$ was considered as level for a significant difference.

4 RESULTS

4.1 Nuclear transfer with embryonic cells (blastomeres)

4.1.1 Ionomycin/demecolcine induced condensation of MII metaphase

Under an inverted-stage microscope, it is difficult to observe nuclear material in rabbit oocytes. Therefore, chemically assisted enucleation was used in this study. Fig 1. shows that ionomycin/demecolcine induced extrusion of oocyte cytoplasm which contained all metaphase chromatin. Enucleation could be easily carried out by removal of the extruded cytoplasm, and a high enucleation rate was obtained. More than 90% of denuded oocytes showed cytoplasm extrusion after ionomycin/demecolcine treatment. Both the high extrusion rate and high enucleation rate were the prerequisite for the utilization of chemically assisted enucleation. Moreover, Fig. 1 also shows that the metaphase plate was not always adjacent to the first polar body which was the criterion for blind enucleation although MII oocytes were collected as early as at 15-16 h after hCG injection. This explains well why less than 50% enucleation rate could be obtained when blind enucleation procedure was conducted in some cases.

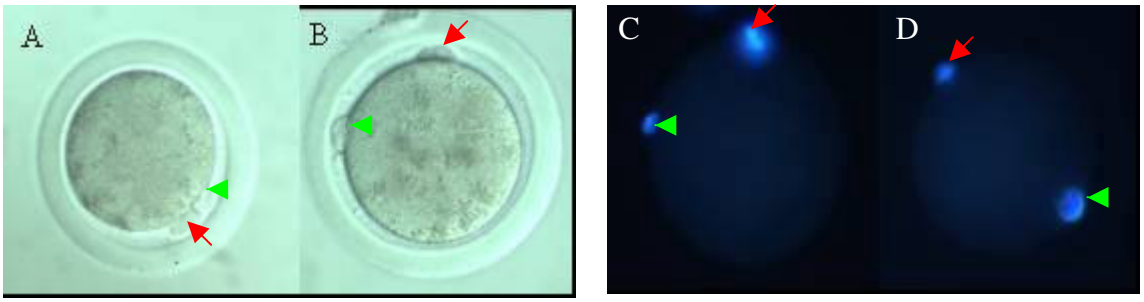


Fig. 1 Extrusion of maternal chromatin after ionomycin/demecolcine treatment (A: protruded part of the cytoplasm containing maternal chromatin (red arrow) is adjacent to the first polar body (green arrow head); B: protruded maternal chromatin is far from the first polar body; C, D: visualization of maternal chromatin by Hoechst 33342 shows that cytoplasm extrusion containing all maternal chromatin as well as maternal chromatin far from the first polar body).

4.1.2 Optimisation of activation protocol

For blastomere (or somatic) cloning, two different types of recipient oocytes are generally used (Alberio et al. 2000; Piotrowska et al. 2000). A) Enucleated cytoplasts are activated before nuclear transfer. The resulting cytoplasts can adopt donor nucleus at any cell cycle stage due to low level of MPF activity. B) Enucleated cytoplasts are activated after nuclear

transfer. Hereby the donor nuclei should be synchronized at G1/ S transition because of the high level of MPF activity in the recipient cytoplasm. In this study we mainly focused on the first model (i.e. activation of recipient cytoplasm was carried out prior to nuclear transfer), and tested two activation protocols which are widely used in bovine and rabbit nuclear transfer (Shi et al. 2003; Chesne et al. 2002).

Enucleated oocytes were activated either by cycloheximide/cytochalasin B (CHX/CB) treatment following ethanol initiation or by 6-dimethylaminopurine/cycloheximide (6-DMAP/CHX) treatment following electric pulses. The resulting cytoplasts showed very high fusion rate (Table 1). Meanwhile, after receiving embryonic nuclei, activated cytoplasms from both activation protocols showed high cleavage and blastocyst rates. Regarding blastocyst rates, however, there were no significant differences between the treatments (Table 1).

Table 1 In vitro development of cloned embryos from embryonic cells

Activation	n	Fused (%)	Cleaved (%)	Blast. (%)
CHX/CB	3	128/131 (97.7)[a]	113/126 (89.7)[a]	64/126 (50.8)[a]
6-DMAP/CHX	4	124/126 (98.4)[a]	99/114 (86.8)[a]	67/114 (58.8)[a]

Blast.: blastocyst.

[a]: values within the same column with different superscripts were significantly different ($P<0.05$).

4.1.3 Production of cloned rabbits from embryonic cells

Total 220 nuclear transfer embryos (2-4-cell stage) from CHX/CB activated cytoplasts were transferred to 5 pseudopregnant recipients. Only one from 5 recipients was detected to be pregnant. This animal was euthanazed on day 16 after ET, and 8 resorbed conceptuses were found (Table 2; Fig 2a). A total of 284 nuclear transfer embryos (2-4-cell stage) from 6-DMAP/CHX activated cytoplasts were transferred to 5 pseudopregnant mothers. Again only one recipient became pregnant, but it gave birth 8 live pups (Table 2; Fig. 2b,c).

Table 2 Development of reconstructed embryos with different activation protocols

Activation	n	Fused (%)	Cleaved (%)	Embryos/ recipients	Pregnancies (%)	Offspring (%)
CHX/CB	5	282/283 (99.6)[a]	220/269 (82)[a]	220/5	1 (20)[a]	0* (0)[a]
6-DMAP/CHX	5	313/318 (98)[a]	284/310 (92)[b]	284/5	1 (20)[a]	8[#] (2.8)[b]

*: 8 conceptuses were obtained on day 16 after embryo transfer (ET).

[#]: 8 pups, including 1 died after birth, 1 died after 3 days, and others growing healthy, were produced on day 30 after ET.

[ab]: values within the same column with different superscripts were significantly different ($P<0.05$).

Fig. 2 Conceptuses in resorption and live rabbits obtained after ET of cloned embryonic cell embryos (a: 8 resorbed conceptuses on day 16 after ET; b: cloned rabbits after 1 month; c: cloned rabbits after 3 months).

4.1.4 Nuclear transfer of embryonic cells into recipient oocytes injected with DNA-GFP constructs

The birth of cloned pups (Photo 4.1.3b,c) proved that the protocol was suitable for nuclear transfer with embryonic cells. For the aim to produce cloned transgenic rabbits, injection of DNA directly into enucleated cytoplasts was performed in this part of experiments. To find the optimal "injection window" for foreign DNA integration, various procedures were tested with two kinds of DNA constructions.

4.1.4.1 Intracytoplasmic injection of naked DNA

We first attempted to use naked DNA for intra-cytoplast injection. Results in Table 4 show that with short time exposure to oocyte cytoplasm foreign DNA could have more chances to go into the transferred nucleus than that with long time exposure to empty cytoplast, although no GFP protein was detected in cloned blastocysts.

Table 4 In vitro development of cloned embryos after intra-cytoplast injection with naked-DNA

Procedure	n	Fused (%)	Cleaved (%)	Blast. (%)	GFP expression
I	3	98/105 (93.3)[a]	82/98 (83.7)[a]	59/98 (60.2)[a]	No
II	2	65/69 (94.2)[a]	43/65 (66.2)[b]	23/65 (35.4)[b]	3 uncleaved embryos

Procedure I: After intra-cytoplasmic injection with foreign DNA constructs, enucleated oocytes were first activated by 6-DMAP/CHX treatment following electric pulses, and then received donor nuclei.

Procedure II: After intra-cytoplasmic injection with foreign DNA constructs, enucleated oocytes were first received donor nuclei which were synchronized at G1/S transition stage, and then activated by 6-DMAP/CHX treatment following electric pulses.

Blast.: blastocyst.

[ab]: values within the same column with different superscripts were significantly different ($P<0.05$).

4.1.4.2 Subzonal injection of polyethylenimine(PEI)-DNA

The failure to produce GFP-positive embryos after injection of naked DNA prompted us to combine naked DNA construct with polyethylenimine (PEI) which could protect foreign DNA from digestion by intrinsic lysosomes of the oocyte cytoplasm (Godbey et al. 1999).

PEI-DNA constructs were injected under the zona pellucida of enucleated oocytes, but we failed to produce blastocysts expressing GFP protein when PEI-DNA was injected either prior to or after nuclear transfer (Table 5).

Table 5 In vitro development of cloned embryos after subzonal injection of PEI-DNA

Procedure	n	Fused (%)	Cleaved (%)	Blast. (%)	GFP expression
I	2	47/49 (95.9)[a]	18/47 (38.3)[a]	3/47 (6.4)[a]	No
II	1	75/75 (100)[a]	66/75 (88)[b]	46/75 (61.3)[b]	No

Procedure I: Enucleated oocytes were first activated by 6-DMAP/CHX treatment following electric pulses, then donor cells were introduced under zona pellucida followed by PEI-DNA subzonal injection, and then donor cells were fused with oocytes by electric pulses.

Procedure II: Enucleated oocytes were first activated by 6-DMAP/CHX treatment following electric pulses, then PEI-DNA solution was injected under zona pellucida followed by transfer of donor cells and membrane fusion.

Blast.: blastocyst

[ab]: values within the same column with different superscripts were significantly different ($P<0.05$).

4.1.4.3 Intra-cytoplasmic injection of PEI-DNA

Intra-cytoplasmic injection of DNA combined with PEI might be an alternative for transfer of foreign DNA into the cytoplast and then into the nucleus. But again this modification did not result in GFP protein expression in blastocysts (Table 6).

Table 6 In vitro development of cloned embryos after intra-cytoplast injection with PEI-DNA

Procedure	n	Fused (%)	Cleaved (%)	Blast. (%)	GFP expression
I	2	125/128 (97.7)[a]	123/125 (98.4)[a]	61/125 (48.8)[a]	No
II	1	24/24 (100)[a]	18/24 (75.0)[b]	8/24 (33.3)[a]	No
	1	46/48 (95.8)[a]	30/46 (65.2)[b]	ET	No pregnancy

Procedure I: After intra-cytoplasmic injection with foreign PEI-DNA construct, enucleated oocytes were first activated by 6-DMAP/CHX treatment following electric pulses, and then received donor nuclei.

Procedure II: After intra-cytoplasmic injection with foreign PEI-DNA construct, enucleated oocytes were first received donor nuclei which were synchronized at G1/S transition stage, and then activated by 6-DMAP/CHX treatment following electric pulses.

Blast.: blastocyst.

[ab]: values within the same column with different superscripts were significantly different ($P<0.05$).

4.2 Nuclear transfer with cumulus cells

Results from the above experiments proved that embryos produced by transfer of embryonic cells into recipient oocytes which were enucleated by using demecolcine treatment and were activated by DMAP/CHX could develop to term. However, it is obvious that the goal of producing transgenic cloned rabbits could be achieved only by using cultured somatic cells as donors for nuclear transfer.

4.2.1 Optimization of nuclear transfer procedure

Somatic cells were transferred into non-activated enucleated oocytes. Data from preliminary experiments showed that, following 6-DMAP/CHX treatment, 84% reconstructed embryos were with pseudo-polar body and none of these embryos could develop further although during culture the pseudo-polar body retracted back to the reconstructed oocyte (Table 7). This phenomenon was not observed after transfer of embryonic nuclei into recipient oocytes which were activated by 6-DMAP/CHX before nuclear transfer. Therefore, we modified the activation protocol by using CB to inhibit the extrusion of pseudo-polar body. Both 6-DMAP/CB and CHX/CB combination could completely inhibit pseudo-polar body formation when activation was carried out after nuclear transfer. Between these two activation protocols, 6-DMAP/CB treatment led to a higher cleavage rate ($p<0.05$) than CHX/CB treatment. Thereafter, activation of reconstructed embryos was initiated by 6-DMAP/CB treatment.

Table 7 In vitro development of cumulus cell cloned embryos activated with different procedures

Activation [#]	n	Fused (%)	Pseudo-polar body (%)	Cleaved (%)
6-DMAP/CHX[#1]	4	169/178 (94.9)[a]	98/116 (84.5)[a]	16/116 (13.8)[a]
6-DMAP/CB[#2] or CHX/CB[#3]	3	195/209 (93.3)[a]	0 (0)[b]	72/105 (68.6)[b]
			0 (0)[b]	45/90 (50.0)[c]

[#]: One hour interval after fusion, fused embryos were first initiated by the same electropulse as for fusion, then incubated in either 1.9 mM DMAP and 10 µg/ml CHX for 1 h ([#1]), or 1.9 mM 6-DMAP and 5µg/ml CB for 1 h ([#2]), or 10 µg/ml CHX and 5 µg/ml CB for 4~5 h ([#3]).

[abc]: values within the same column with different superscripts were significantly different ($P<0.05$).

4.2.2 In vitro and in vivo development of cumulus cell cloned embryos

Cloned rabbits from somatic nuclear donors were produced in 2002 (Chesne 2002). In that case the nuclear donors were freshly collected cumulus cells. In vitro culture of cells for nuclear transfer apparently increases the chance to apply the cloning technique for basic research and commercial interests. In this study, *Ali/Bas* originated cultured cumulus cells were used as nuclear donors for nuclear transfer, and 6-DMAP/CB treatment following electropulses were used for activation of reconstructed embryos. Ninety-three percent of fused embryos cleaved, and about 32% of embryos developed to blastocyst (Table 8).

Table 8 In vitro development of cloned embryos from cultured cumulus cells

n	Fused (%)	Cleaved (%)	Blast. (%)
3	88/91 (96.7)	78/84 (92.9)[a]	27/84 (32.3)[a]
3 (Parthenotes)	-	115/117 (98.2)[a]	113/117 (96.5)[b]

Blast.: Blastocyst.

[ab]: values within the same column with different superscripts were significantly different (P<0.05).

Total 172 cloned embryos (2-4-cell stage) were transferred into 5 pseudo-pregnant mothers (Table 9). By palpation, 2 animals were found to be pregnant. From these 2 animals, one was euthanized on day 16 after ET and 4 implantation sites were found. From the other recipient which was operated on day 27 after ET, one live pup and 2 conceptuses in resorption were found (Fig. 3a,b).

Table 9 In vivo development of cloned embryos from cultured cumulus cells

n	Fused (%)	Cleaved (%)	Embryos/ Recipients	Pregnancies (%)	Implantation sites (%)*	Offspring
5	291/321 (90.2)	177/246 (72.0)	172/5	2/5 (40)	>=7 (>=3.2)	1

*: Data from 2 pregnant females: one was euthanized on day 16 after ET, and another one was operated on day 27 after ET.

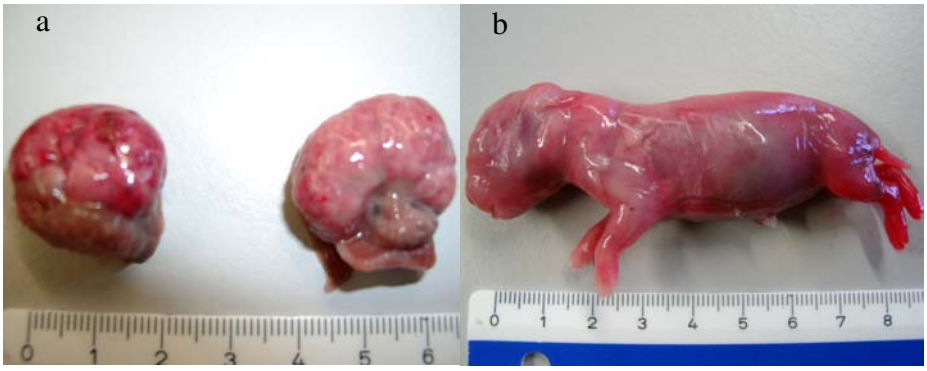


Fig. 3 Conceptuses in resorption and live rabbits obtained after ET of cultured cumulus cells (a: 2 resorbed conceptuses; b: 1 live pup was delivered by operation on day 27 after ET).

4.2.3 Genetic background analysis

Genomic DNA was extracted from donor cells and from tissue samples of recipient mother, the live pup and 2 resorbed conceptuses. ELISA analysis was carried out using GENESPECTOR rapid genotyping kit (see materials & methods 3.2.7). Results demonstrated that donor cells, tissue samples from the live pup and resorbed conceptuses were *Ali/Bas* origin, but samples from recipient mother were another genotype.

4.3 Nuclear transfer with cultured fibroblast cells

Although one cloned rabbit from a cultured cumulus cell was produced in our study, a relatively short lifespan of this type of somatic cells was observed under normal culture condition. Others also reported this phenomenon (Dinnyes et al. 2001). Due to their rapid growth and potential for many cellular divisions in culture (Schnieke et al. 1997; Cibelli et al. 1998; McCreath et al. 2000), fetal fibroblast cells were used as nuclear donors in further studies.

4.3.1 Nuclear transfer with confluent rabbit fetal fibroblast cells (RFFs)

In this study, confluent RFFs were used for nuclear transfer at passage 2 to 8. Reconstructed embryos developed to blastocyst at the rate (33%; Table 10) similar to that obtained with cumulus cells (Table 8). When 408 cloned embryos (2-4-cell stage) were transferred to 10 pseudopregnant recipients, only 2 females became pregnant, and no one gave birth. The low pregnancy rate prompted us to transfer cloned embryos together with in vivo fertilized 2-4-cell embryos (co-transfer). Two hundred and fourteen cloned embryos were co-transferred

with 37 in vivo embryos to 5 recipients. This modification resulted in 2 pregnant fosters including one that gave one live pup. Results of genetic analysis showed that this pup was not Ali/Bas origin.

Table 10 Development of cloned embryos from confluent fetal fibroblast donors

n	Fused (%)	In vitro development		In vivo development			
		Cleaved (%)	Blast. (%)	Embryo/ Recipient*	Embryo/ Recipient**	Pregnancies	Offspring
14	931/978 (95.2)	800/860 (93.0)	41/124 (33.1)	408/10	214+37#/5	2/10* 2/5**	0 1**

Blast.: blastocyst.

*: only cloned embryos were transferred.

**: co-transfer of cloned embryos and in vivo fertilized embryos.

#: in vivo fertilized embryos.

4.3.2 Nuclear transfer with serum starved RFFs

Serum starvation was routinely used to increase G0/G1 ratio in cultured cells. In our experiments, RFFs were serum-starved for at least 4 days after passage, and then used as nuclear donors. In this study, starvation of donor cells did not improve the development of reconstructed embryos (Table 11) compared to that obtained with confluent cells (Table 10). One hundred and sixty-four cloned embryos were co-transferred with 36 in vivo embryos to 4 recipients. Three of 4 recipients were pregnant, and total 8 pups were delivered from 2 mothers (each mother gave 3 live and 1 died pups). Genetic analysis showed all pups were not *Ali/Bas* origin. Additionally, 35 cloned embryos were transferred to one pseudopregnant mother without co-transfer, and this animal became pregnant but did not give birth.

Table 11 Development of cloned embryos from serum-starved fetal fibroblast donors

n	Fused (%)	In vitro development		In vivo development			
		Cleaved (%)	Blast. (%)	Embryo/ Recipient*	Embryo/ Recipient**	Pregnancies	Offspring
5	413/425 (97.2)	352/407 (86.5)	37/114 (32.5)	35/1	164+36#/4	1/1* 3/4**	0 6 (live) ** 2 (dead)**

Blast.: blastocysts.

*: only cloned embryos were transferred.

**: cotransfer of cloned embryos and in vivo fertilized embryos.

#: in vivo fertilized embryos.

4.3.3 Nuclear transfer with cultured fetal fibroblast cells treated with sodium butyrate

4.3.3.1 Life-span of cultured fetal fibroblast cells

Freshly isolated fetal fibroblast cells (Passage 0) from 3 individual fetuses were cultured independently until confluence, and then were passaged continuously until they reached replicative senescence. After 13 passages within 46 days, cells stopped proliferation and showed enlarged morphology. Total population doublings (PDs) was about 27 (Table 12).

Table 12 Life span of primary cultured RFFs

Cell source	Seeding density	Total passages	Days of culture	Total PDs
Fetus 1	4.5~5×10^{4}cells/ml	14	48	28.4
Fetus 2	4.5~5×10^{4}cells/ml	14	48	30.2
Fetus 3	4.5~5×10^{4}cells/ml	12	42	24.7
Average		13	46	27.4

4.3.3.2 Effect of NaBu treatment on RFFs growth

We analysed the effect of NaBu treatment on RFF growth. For untreated RFFs, the cell number increased by 5 fold after culture for 96 h. But when RFFs were treated with 1mM NaBu for 96 h, the cell number increased only 2 fold (Fig. 5). Meanwhile, after 72 h treatment with 1mM NaBu, RFFs also showed enlarged morphology (Fig. 4 a: untreated RFFs; b: NaBu-treated RFFs; both were from RFFs at passage 4).

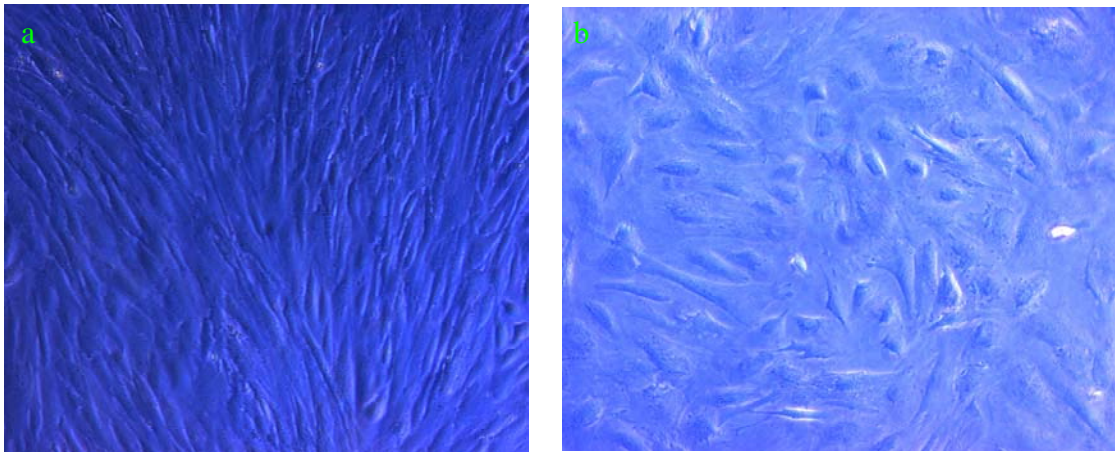
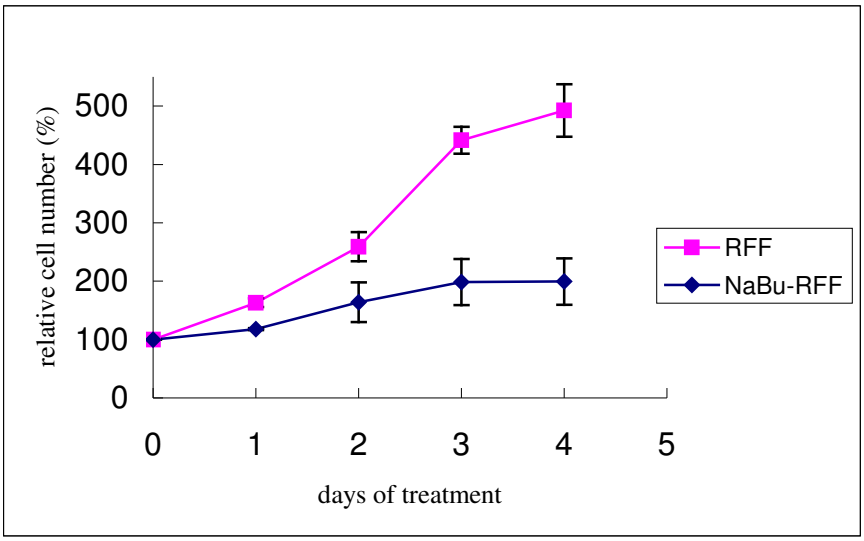


Fig. 4 Effects of NaBu treatment on RFFs morphology

Fig. 5 Growth curves of RFFs



4.3.3.3 Karyotype analysis

Since some chemical reagents could induce aneuploidy, which may affect embryonic development, chromosome content of RFFs before and after NaBu treatment was determined. From 3 replicates, total 63 metaphase spreads from untreated RFFs were counted, all these spreads had normal chromosome number 44 (Table 13). For NaBu-treated RFFs, total 72 metaphase spreads from 3 replicates were counted, and all these spreads had also normal chromosome number 44 (Table 13). Interestingly, after the same procedure for preparation of metaphase cells, NaBu-treated RFFs showed higher ratio of metaphase cells than the same origin untreated RFFs. Fig. 6 shows the normal karyotype in untreated RFFs (passage 6).

Table 13 Chromosome number analysis of RFFs treated with(out) NaBu

Cell type	Chromosome number		Spreads counted
	44 (%)	Abnormal (%)	
RFF	63 (100)	0 (0)	63
NaBu-RFF	72 (100)	0 (0)	72

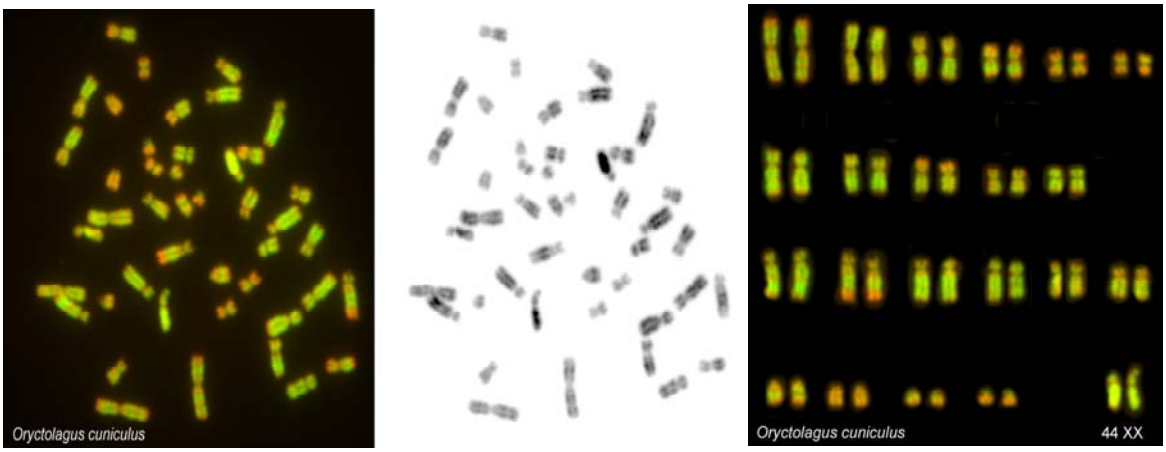


Fig. 6 Karyotype analysis of cultured rabbit fetal fibroblast cells

4.3.3.4 Senescent analysis

Senescence is associated with altered cellular morphology and size (Bayreuther et al. 1988). After treatment with NaBu, morphological changes of primary cultured RFFs were observed. Cells had an increased cell size and a more flattened appearance (Fig. 7, a: untreated RFFs; b: NaBu-treated RFFs; both were from RFFs at passage 6). SA-βgal staining was used to investigate whether the treated cells underwent biochemical senescence. Senescent cells have increased endogenous SA-ßgal activity, which is less detectable in terminally differentiated or quiescent cells (Dimri et al. 1995). In this study, compared with untreated RFFs, a significant increase of SA-βgal activity was observed in NaBu treated RFFs (Fig. 8, untreated RFFs 13.1% vs. NaBu-treated RFFs 55.8%, $P<0.001$).

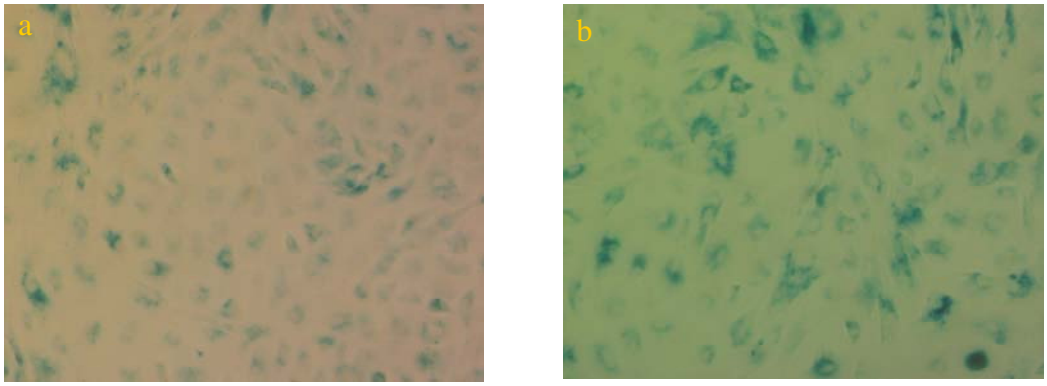
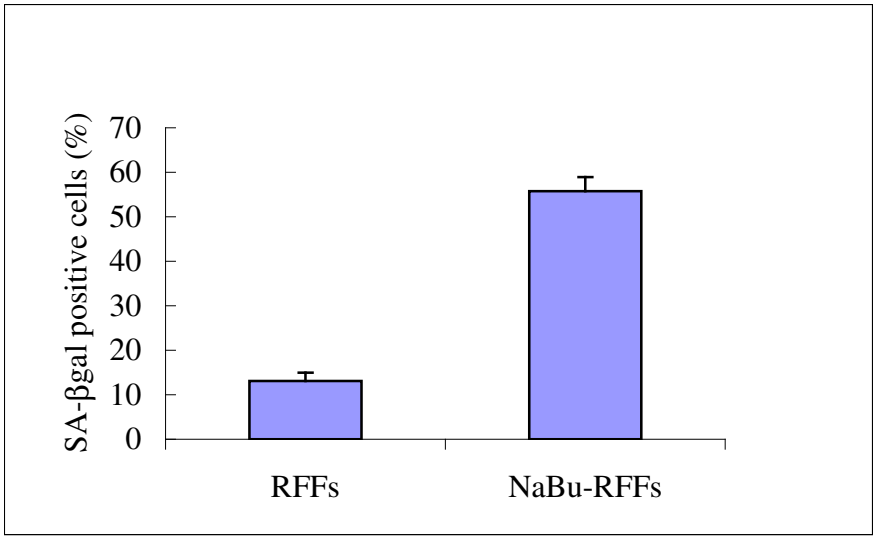


Fig. 7 Effects of NaBu treatment on SA-βgal activity of RFFs

Fig. 8 SA-βgal activity analysis



4.3.3.5 Histone acetylation

Among the core histones, H3 and H4 are the principal targets of regulatory posttranscriptional modifications. By Western blot, we compared the effects of NaBu on global histone H3 and H4 acetylation status of RFFs. After NaBu treatment, a significant increase of histone H3 acetylation was observed (Fig. 9, 10; $P<0.05$); but the increase of histone H4 acetylation was not significant ($P<0.05$). Meanwhile, among passage times, there were no differences in both H3 and H4 acetylation status (Fig. 9).

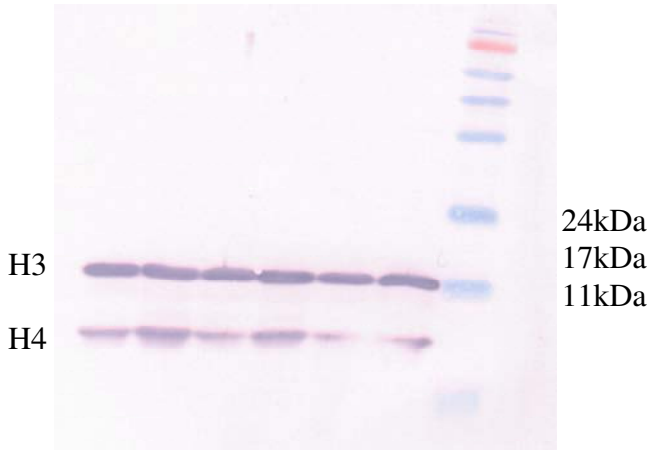
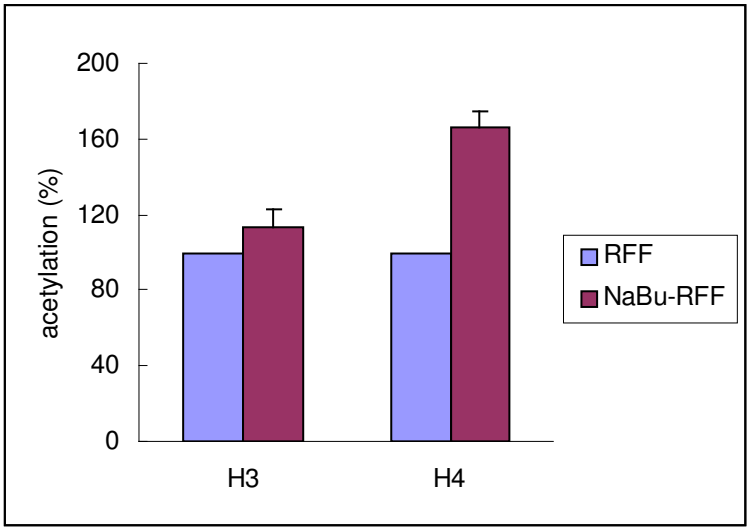


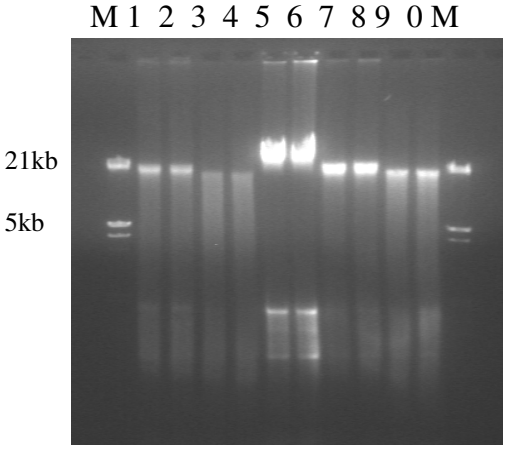
Fig. 9 Global histone H3 and H4 acetylation status in RFFs
(Lane 1, 3, 5: non-treated RFFs at passage 4, 10, 6; Lane 2, 4, 6: NaBu treated RFFs at passage 4, 10, 6; M: Prestained protein ladder)

Fig. 10 Global histone H3 and H4 acetylation status in RFFs



4.3.3.6 DNA methylation

Since histone acetylation is to some extent associated with DNA methylation, we investigated the effects of NaBu on methylation status of global genomic DNA in RFFs. Fig. 11 shows that NaBu treatment did not change the DNA methylation status of RFFs.



M Fig. 11 Global DNA methylation status analysis

4.3.3.7 Nuclear transfer with NaBu-treated cultured RFFs

Treatment of RFFs with NaBu resulted in a significantly higher blastocyst rate (49%; table 14) compared to that with non-treated RFFs (33%; table 10). Sixty-nine cloned embryos were co-transferred with 20 in vivo embryos to 2 recipients. One from them became pregnant, but did not maintain pregnancy after 19 days. One hundred and fifty-four cloned embryos were transferred to 5 recipients without co-transfer, and none of them became pregnant.

Table 14 Development of cloned embryos from NaBu-treated fetal fibroblast donors

n	Fused (%)	In vitro development		In vivo development			
		Cleaved (%)	Blast. (%)	Embryos/ Recipients*	Embryos/ Recipients**	Pregnancies	Offspring
9	464/476 (97.4)	409/431 (94.9)	86/175 (49.1)	154/5	69+20#/2	1/2**	0

Blast.: blastocyst.

*: only cloned embryos were transferred.

**: co-transfer of cloned embryos and in vivo fertilized embryos.

#: in vivo fertilized embryos.

4.3.4 Embryo aggregation

4.3.4.1 Clone-in vivo fertilized embryo aggregation

Although treatment of RFFs with NaBu increased the development of the resultant cloned embryos to blastocyst stage, in vivo development of reconstructed embryos was not

improved. Survival of manipulated embryos could be increased by their aggregation with normal, in vivo fertilized embryos or their cells. To improve in vivo development, 4-10-cell cloned embryos from NaBu-treated RFFs were aggregated with one or two blastomeres of in vivo fertilized 6-12-cell embryos.

4.3.4.1.1 In vitro development of blastomere(s) from in vivo fertilized embryos

First, we assessed in vitro development of one or two blastomeres isolated from 6-12-cell in vivo fertilized embryos. One or two blastomeres were introduced into empty zonae pellucidae. Either one or two blastomeres could give rise to blastocysts or blastocyst-like structures (Table 15; Fig. 12). As it was expected, significantly better development was obtained with two blastomeres (81.8% vs. 45.7%, P<0.001).

Table 15 In vitro development of blastomeres from in vivo fertilized embryos

Embryo type*	n	Embryos	Cleaved (%)	Blast. (%)
1FB	3	46	44/46 (95.7)[a]	21/46 (45.7)[a]
2FB	3	33	33/33 (100)[a]	27/33 (81.8)[b]

*1FB: single blastomere from 6-12-cell in vivo fertilized embryos.

2FB: two blastomeres from 6-12-cell in vivo fertilized embryos.

Blast.: blastocyst.

[ab]: values within the same column with different superscripts were significantly different (P<0.05).

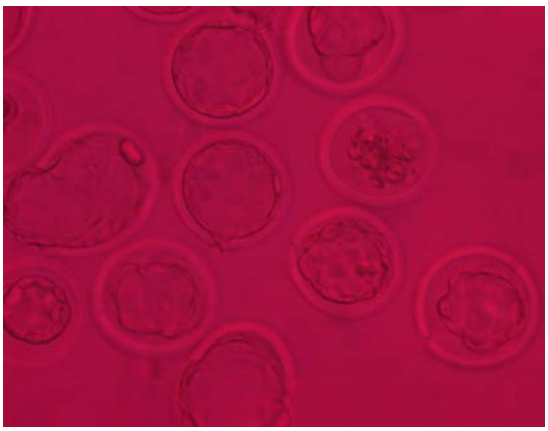


Fig. 12 Blatocyst-like structures from blastomeres of in vivo fertilized embryos

4.3.4.1.2 Cloned-in vivo fertilized embryo aggregation

Cloned embryos were aggregated with either one or two blastomeres of in vivo fertilized embryos. Compared to cloned, non-aggregated embryos, blastocyst rates tended to be higher or were significantly higher when using one or two blastomeres respectively (Table 16). One hundred and ninety-one aggregation embryos (with one blastomere) were transferred to 6 recipients, and one of them became pregnant, no offspring was obtained. When 201 aggregation embryos obtained with two blastomeres were transferred to 5 recipients, 2 of them became pregnant. To assess the origin of the fetuses, the pregnant recipients were euthanized on day 12 or day 13 after embryo transfer. Two and 3 fetuses in resorption were found in one and another recipient animals respectively. Results of genotypic analysis demonstrated that all fetuses were not *Ali/Bas* origin.

Table 16 Development of cloned embryos aggregated with blastomere(s) of in vivo fertilized embryos

Embryo type[#]	n	Fused (%)	In vitro development			In vivo development		
			Cleaved (%)	4-cell (%)	Blast. (%)*	Embryos/ Recipients	Pregnancies (%)	Offspring
Cloned	7	315/328 (96.0)^{a}	286/308 (92.9)^{a}	251/308 (81.5)^{a}	75/123 (61)^{a}	128/4	0/4 (0)^{a}	0
Cloned-1FB	6	317/331 (95.8)^{a}	269/306 (87.9)^{b}	242/306 (79.1)^{ab}	35/51 (68.6)^{a}	191/6	1/6 (17)^{a}	0
Cloned-2FB	6	354/361 (98.1)^{a}	331/343 (96.5)^{c}	252/343 (73.5)^{b}	44/51 (86.3)^{b}	201/5	2/5 (40)^{a}	0

[#] Cloned-1FB: cloned embryos aggregated with single blastomere.

Cloned-2FB: cloned embryos aggregated with two blastomeres.

Blast.: blastocyst; *: the blastocyst rate was calculated from 4-cell stage embryos.

^{abc}: values within the same column with different superscripts were significantly different ($P<0.05$).

4.3.4.1.3 Effects of aggregation on the cell numbers of blastocysts

The cell number of blastocysts is one of the parameters to evaluate their quality. Our results demonstrated that cloned non-aggregated blastocysts contained less cells than in vivo fertilized blastocysts (Table 17, Fig. 13). As compared to cloned non-aggregated embryos, aggregation of cloned embryos with one or two blastomeres resulted in slightly or significantly higher cell numbers of the resultant blastocysts, respectively. Blastocysts or blastocyst-like structures obtained from culture of one or two blastomeres contained

lower cell numbers than either type of blastocysts derived from cloned or in vivo fertilized embryos.

Table 17 Cell number analysis of blastocysts or blastocyst-like structures

Type of embryos*	Embryos	Aggregated embryos		Non-aggregated embryos[#]	
		Blast. (%)	Cell number	Blast. (%)	Cell number
Cloned-1FB	51	35/51 (68.6)^{b}	140 ± 39^{ae}	10/51 (19.6)^{a}	75 ± 11^{a}
Cloned-2FB	51	44/51 (86.3)^{b}	162 ± 52^{e}	6/51 (11.8)^{a}	96 ± 3^{b}
Cloned	34	20/34 (58.8)^{a}	125 ± 22^{a}		
1FB	46	21/46 (45.7)^{a}	35 ± 9^{c}		
2FB	33	27/33 (81.8)^{b}	72 ± 28^{d}		
In vivo fertilized	34	33/34 (97.6)^{b}	196 ± 21^{b}		

*1FB: single blastomere from 6-12-cell in vivo fertilized embryos.

2FB: double blastomeres from 6-12-cell in vivo fertilized embryos.

Cloned-1FB: cloned embryos aggregated with single blastomere.

Cloned-2FB: cloned embryos aggregated with two blastomeres.

Blast.: blastocyst.

[#]Non-aggregated embryos: some aggregation embryos developed to blastocysts or blastocyst-like structures, but independent cell aggregates and less cell numbers than that in cloned blastocysts were observed, suggesting that two types of blastomeres were not chimerized.

[abcde]: values within the same column with different superscripts are significantly different ($P<0.05$).

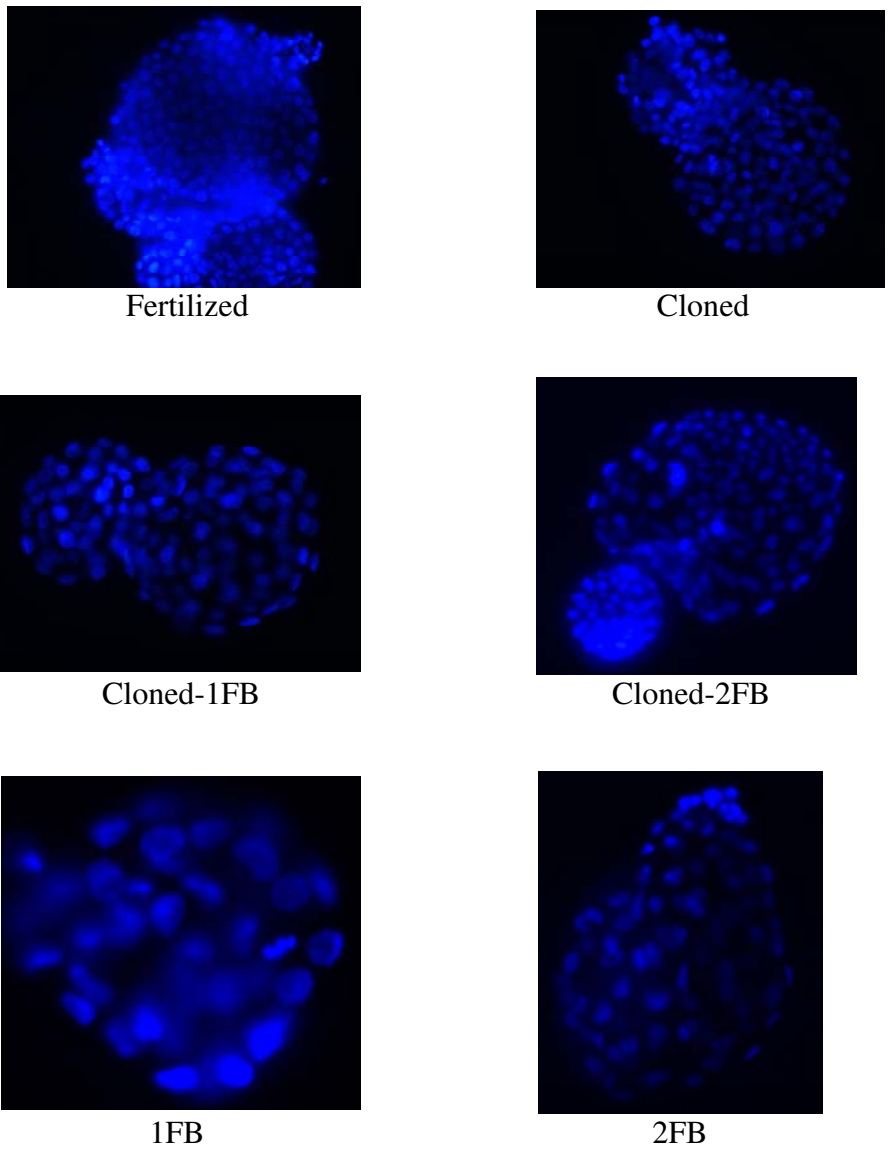


Fig. 13 Counting of the cell numbers of blastocysts after staining with Hoechst 33342

4.3.4.2 Clone-parthenote aggregation

Since individual blastomeres could develop to blastocyst, the reason for a failure to produce cloned fetuses from aggregation of nuclear transfer embryos with blastomere(s) of in vivo fertilized embryos might be that the in vivo blastomeres were more competitive than those of cloned embryos. Therefore, we aggregated cloned embryos with one or two blastomeres of 6-12-cell parthenogenetic embryos.

4.3.4.2.1 In vitro development of blastomere(s) from parthenogenetic embryos

We investigated in vitro development of one or two blastomeres isolated from 6-12-cell parthenogenetic embryos. Parthenogenetic blastomeres were introduced into empty zonae

pellucidae. The results shown in Table 18 indicate that single or two parthenogenetic blastomeres could develop to blastocysts or blastocyst-like structures, and the developmental potential of two blastomeres was significantly higher than that of single blastomeres.

Table 18 In vitro development of blastomeres from parthenogenetic embryos

Embryo type*	n	Embryos	Cleaved (%)	Blast. (%)
1PB	3	51	46/51 (90.2)[a]	24/51 (47.1)[a]
2PB	3	32	29/32 (90.6)[a]	23/32 (71.9)[b]

*:1PB: one parthenogenetic blastomere; 2PB: two parthenogenetic blastomeres
Blast.: blastocyst
[ab]: values within the same column with different superscripts are significantly different ($P<0.05$)

4.3.4.2.2 Clone-parthenote aggregation

4.3.4.2.2.1 Production of cloned rabbits from aggregation embryos

Cloned embryos were aggregated with one or two blastomeres of parthenogenetic embryos. Compared to the results with cloned embryos, there were no significant differences in the blastocyst rates when aggregation embryos were produced with one or 2 blastomeres. Between two kinds of aggregation embryos, the blastocyst rate of embryos obtained with 2 blastomeres was higher than that when using one blastomere, but the difference was not significant (Table 19). Ninety-five aggregation embryos from two blastomeres were transferred to 3 recipient mothers, and none of them became pregnant. When 128 aggregation embryos which obtained one blastomere were transferred to 3 recipient mothers, 2 of them were found to be pregnant on day 14 after embryo transfer. From these 2 recipients, one lost the pregnancy at the third week of gestation. Another recipient mother was operated on day 30, and 2 live pups as well as one resorbed conceptus were delivered (Fig. 14). The body weights of the 2 babies were 96 g and 54 g, respectively.

Table 19 Development of cloned embryos aggregated with blastomere(s) of parthenogenetic embryos

Embryo type*	n	Fused (%)	In vitro development			In vivo development		
			Cleaved (%)	4-cell (%)	Blast. (%)	Embryos/ Recipients	Pregnancies (%)	Offspring (%)
Cloned	7	315/328 (96.0)[a]	286/308 (92.9)[a]	251/308 (81.5)[a]	75/123 (61)[a]	128/4	0/4 (0)[a]	0 (0)[a]
Cloned-1PB	6	359/367 (97.8)[ab]	289/346 (83.5)[b]	216/346 (62.4)[b]	62/107 (57.9)[a]	109/3	2/3 (67)[a]	2 (2)[a]
Cloned-2PB	6	277/281 (98.6)[b]	237/275 (86.2)[b]	166/275 (60.4)[b]	50/71 (70.4)[a]	95/3	0/3 (0)[a]	0 (0)[a]

Cloned-1PB: cloned embryos aggregated with 1 parthenogenetic blastomere.

Cloned-2PB: cloned embryos aggregated with 2 parthenogenetic blastomeres.

Blast.: blastocyst.

[ab]: values within the same column with different superscripts are significantly different ($P<0.05$).

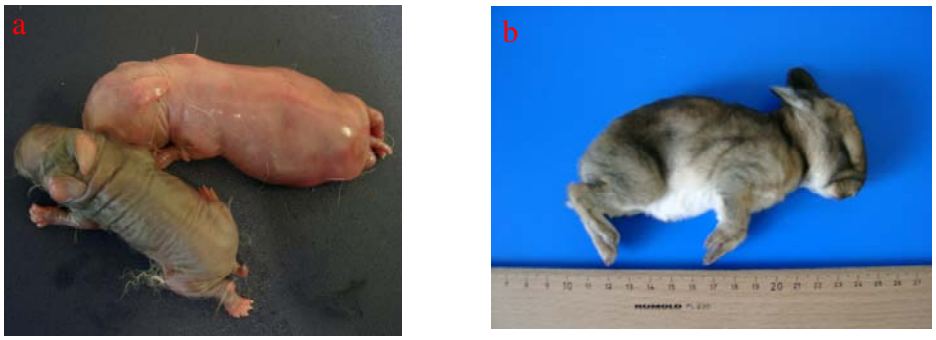


Fig. 14 Production of cloned offspring from aggregation embryos

a: 2 live pups were delivered by operation on day 30; b: The pup died accidentally 2 weeks after birth

4.3.4.2.2.2 Genotypic analysis

Genomic DNA was extracted from donor cells and from tissue samples of the recipient mother, the 2 pups and the corresponding placentae. ELISA analysis was carried out using GENESPECTOR rapid genotyping kit (see materials & methods 3.2.7). Results demonstrated that donor cells, tissue samples from placentae and pups were *Ali/Bas* origin, but samples from the recipient mother and the resorbed fetuses had another genotype.

4.3.4.2.3 Effects of aggregation on the cell numbers of blastocysts

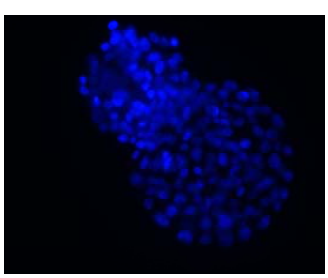
The cell numbers of blastocysts derived from one or two parthenogenetic blastomeres were significantly lower than those of cloned and parthenogenetic embryos. Aggregation of

cloned embryos with one or two parthenogenetic blastomeres, respectively, resulted in a slight or significant increase in cell numbers compare to cloned non-aggregated embryos (Table 20; Fig. 15).

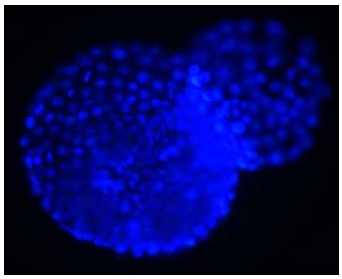
Table 20 Cell numbers of blastocysts produced after aggregation of cloned embryos with parthenogenetic blastomere(s)

Type of embryos*	Embryos	Aggregated blastocyts		Non-aggregated blastocysts#	
		Blast. (%)	Cell number	Blast. (%)	Cell number
Cloned-1PB	105	62/105 (59.0)	137 ± 35^{ed}	29/105 (27.6)^{a}	75 ± 11^{a}
Cloned-2PB	71	50/71 (70.4)	150 ± 34^{fb}	15/71 (21.1)^{a}	86 ± 3^{b}
1PB	51	24/51 (47.1)	31 ± 14^{ac}		
2PB	32	23/32 (71.9)	46 ± 19^{d}		
Cloned	34	20/34 (58.8)^{a}	125 ± 22^{ad}		
Intact parthenote	61	56/61 (91.8)^{b}	189 ± 57^{bf}		

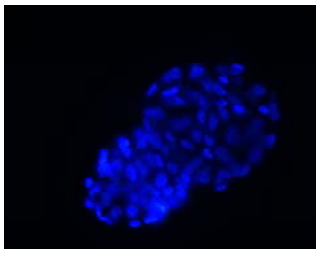
*1PB: single parthenogenetic blastomere; 2PB: two parthenogenetic blastomeres.
Cloned-1PB: cloned embryos aggregated with 1 parthenogenetic blastomere.
Cloned-2PB: cloned embryos aggregated with 2 parthenogenetic blastomeres.
Blast.: blastocyst.
#Non-aggregated embryos: some aggregation embryos developed to blastocysts or blastocyst-like structures, but independent cell aggregates and less cell numbers than that in cloned blastocysts were observed, suggesting that two types of blastomeres were not chimerized.
^{abcdef}: values within the same column with different superscripts are significantly different ($P<0.05$).



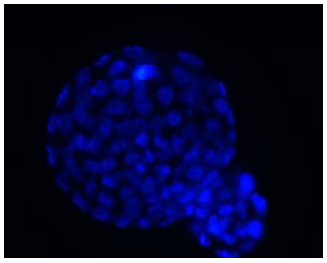
Cloned



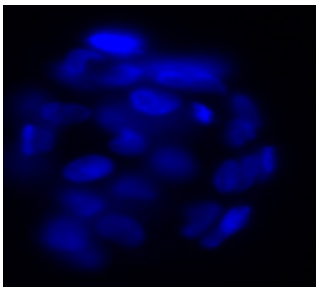
Parthenote



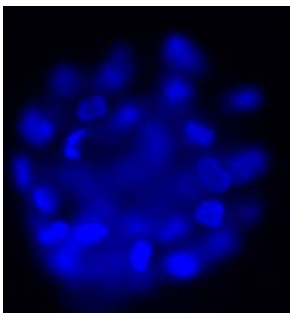
Cloned-1PB



Cloned-2PB



1PB



2PBFig. 15 Counting of the cell numbers of blastocysts after staining with Hoechst 33342

4.4 Effects of donor cell type on rabbit nuclear transfer

To assess the effect of donor cell type on rabbit nuclear transfer efficiency, we compared the results obtained with the following types of cells: cultured RCCs, confluent RFFs, serum-starved RFFs and NaBu-treated RFFs (Table 21). Among these four cell types, NaBu-treated RFFs were the best nuclear donors based on the development of cleavage and blastocyst rates. However, none of these cell types was superior to others when in vivo development of cloned embryos was evaluated.

Table 21 Effects of donor cell type on rabbit nuclear transfer

Cell type	n	Fused (%)	In vitro development		In vivo development		
			Cleaved (%)	Blast. (%)	Embryos/ Recipients	Pregnancy (%)	Offspring (%)
RCCs	8	412/447 (92.2)[a]	282/361 (78.1)[a]	27/84 (32.3)[a]	172/5	2/5 (40)[ac]	1 (0.5)[a]
RFFs	14	931/978 (95.2)[b]	800/860 (93.0)[b]	41/124 (33.1)[a]	408/10	2/10 (20)[ac]	0[a]
Starved-RFFs	5	413/425 (97.2)[bc]	352/407 (86.5)[c]	37/114 (32.5)[a]	35/1	1/1 (100)[ab]	0[a]
NaBu-RFFs	9	464/476 (97.4)[c]	409/431 (94.9)[b]	86/175 (49.1)[b]	154/5	0/5 (0)[c]	0[a]
Control*	3	117	115/117 (98.2)[b]	113/117 (96.6)[c]	-	-	-

Blast.: blastocyst.

*: parthenotes.

abc: values within the same column with different superscripts are significantly different ($P<0.05$).

5 DISCUSSION

The main result of the present study was the first production worldwide of cloned rabbits from cultured somatic cells. This achievement was reached by using a number of novel technical approaches.

5.1 Demecolcine-assisted enucleation

Enucleation of a recipient oocyte is a crucially important process for nuclear transfer. The optimal process should completely remove the oocyte chromatin material with a little loss of cytoplasm and associated organelles. The resultant enucleated oocyte should have no lasting detrimental effects on the remaining components within the cytoplast and their corresponding ability to support further development.

Unlike mouse and rat oocytes, chromosomes of rabbit oocytes are sometimes difficult to localize precisely in the cytoplasm under visible light because of the frequent presence of dark cytoplasmic granules. The first polar body is generally used to localize the metaphase chromosomes of MII oocyte since the chromosomes are presumably adjacent to the first polar body. However, results with rabbit enucleation indicate that more than 50% of metaphase spreads are located in different regions as compared to the first polar body location (Mitalipov et al. 1999). In our preliminary experiments, metaphase plate in denuded MII oocyte was located in different zones from the first polar body after visualization with Hoechst 33342.

To visualize the metaphase plate zone of rabbit oocytes, Inoue et al. (2002a) centrifuged cumulus-free oocytes in the presence of 5 µg/ml CB at 1250 g for 4 min, and the chromosomes could be clearly identified between the light-opaque and translucent layers under Nomarski optics. But the influence of centrifugation on the rearrangement of cytoplasm content is not clear. For instance, oocyte fragments were produced after centrifugation in bovine (Tatham et al. 1995). Another non-invasive method for enucleation of rabbit oocytes was developed by Yin et al (2002a,b,c). In vitro matured or superovulated oocytes were activated with 5 µM ionomycin for 3-5 min followed by 0.6 µg/ml demecolcine for 30 min to 2 h to induce extrusion of the pseudopolar body. The treated oocytes were incubated in manipulation medium containing 7.5 µg/ml CB and 0.6 µg/ml demecolcine for 15 min, and the maternal chromosomes were then eliminated by

removing the pseudopolar body with a small volume of the surrounding oocyte cytoplasm. Using this enucleation procedure, four fetuses, including two with beating hearts, were obtained on day 15 after embryo transfer. The same procedure was used in our study to prepare enucleated oocytes for nuclear transfer with embryonic blastomeres, and we obtained 8 live pups. This is the first time to report the production of live rabbit clones from oocytes which were enucleated by chemical assistance. In our later study, without pretreatment with ionomycin, denuded oocytes also showed cytoplasm extrusion containing condensed chromatin after demecolcine treatment, and cloned offspring from cumulus and fetal fibroblast cells were produced.

Demecolcine-induced membrane extrusion containing the condensed maternal chromatin can be easily identified and mechanically removed from the oocyte. In our study, more than 90% of rabbit oocytes had a membrane extrusion after demecolcine pretreatment, and enucleation rates were almost 100%. Production of cloned offspring in our and other studies (Yin et al. 2002a; Kawakami et al. 2003; Gasparrini et al. 2003) when using chemically assisted enucleation suggests that this procedure has no detrimental effect on the developmental competence of recipient oocytes.

The mechanism responsible for demecolcine induced chromatin condensation into membrane extrusion still remains unclear. It has been well known that demecolcine functions as microtubule (MT) destabilizing drug which binds tightly to tubulin dimers and prevents MT polymerisation, resulting in the loss of dynamic spindle MTs in mitotic and meiotic cells (Larkin & Danilchik 1999). Culture of activated mouse oocytes in the presence of demecolcine results in normal rates of oocyte activation and progressive cytoskeletal changes after activation. Disruption of spindle MTs by demecolcine impairs chromosome migration, suppresses spindle rotation and alters chromosome partitioning (Ibanez et al. 2003). In rabbits, after treatment with ionomycin or in vitro manipulation, denuded oocytes were somehow activated, thus the metaphase chromosomes started to move into anaphase, but the microtubules were disorganized by the following demecolcine treatment before complete separation occurred. The entire chromosome mass then moved into a second polar body and remained in a condensed form (Yin et al. 2002b).

5.2 Effects of donor cells on rabbit nuclear transfer

Appropriate choice of donor nuclei is the question of primary importance. Before Dolly was born, full-term development of cloned embryos could be obtained only after nuclear transfer of embryonic cells. Now somatic nuclear transfer is a routine procedure, and various types of differentiated cells have been used as sources of nuclei for cloning domestic and laboratory animals (Galli et al. 1999; Hochedlinger & Jaenisch 2002; Ogura et al. 2000; Wakayama & Yanagimachi 2001). Almost all cell types tested for nuclear transfer have resulted in live offspring, although with differences in efficacy.

5.2.1 Effects of donor cell type on nuclear transfer efficacy

5.2.1.1 Embryonic cells

Blastomeres were the first cells used in cloning amphibians (Briggs & King 1952) and all early successes in mammalian cloning were achieved with nuclei from embryonic blastomeres, also in rabbits (Stice & Robl 1988). Compared to somatic donor nuclei, when using the same serial nuclear transfer technique but different genetic backgrounds, both blastocyst development and number of live pups were dramatically improved with blastomere nuclei (Kwon & Kono 1996; Ono et al. 2001), Usually, the development of cloned embryos receiving low differentiated donor nuclei is more successful than that of nuclear transfer embryos originating from well-differentiated donor cells. Embryonic blastomeres show little or no differentiation, may retain factors which are necessary for early embryonic development and do not have to be reprogrammed. Furthermore, the DNA methylation status of blastomeres is more compatible with early development (Oback & Wells 2002a). In rabbit cloning, Stice and Robl (1988) produced the first six genetically verified nuclear transfer rabbits with donor nuclei from 8-16-cell embryos and enucleated mature recipient oocytes. Using the same type of nuclear donor embryos, Collas and Robl (1990) modified the procedures of nuclear transfer, and 21% of transferred embryos developed to offspring. These rates were similar to the development of nonmanipulated control embryos. Blastomeres from 8-cell embryos cultured for 20-24 h to the 32-64-cell stage were used as nuclear donor cells by Yang et al. (1992). A total of 243 embryos at 2-4-cell stage were transferred into 15 pseudopregnant recipients and 8 young (3%) were obtained. In our study, individual blastomeres were isolated from 8-16-cell stage embryos.

After modifying the enucleation and activation procedures, we transferred 284 cloned embryos at 2-4-cell stage to 5 recipient females, and 8 pups were produced. Although the cloning efficiency in our study was also low (3%), this was the first demonstration that oocytes enucleated by chemical assistance are suitable for rabbit nuclear transfer.

5.2.1.2 Cumulus cells

Among the somatic cell types tested, the consensus from numerous laboratories is that cumulus cells give the highest cloning efficiency with the least number of abnormalities in cloned animals (Tian et al. 2003). In mouse and bovine, cumulus cells were the first type of nuclear donors used for somatic cloning (Wakayama et al. 1998; Wells et al. 1999). In rabbits, both freshly collected and cultured cumulus cells were used as nuclear donors, however, to date, producing live cloned offspring could be possible only from freshly collected cumulus cells (Chesne et al. 2002). Higher blastocyst rates were obtained from cultured cumulus cells (27%) than that from fresh ones (16%; Cervera & Garcia-Ximenez 2003). In another study with cultured cumulus cells, 23% of cloned embryos developed to blastocysts, but no offspring was obtained (Yin et al. 2000). After modifying the activation protocol, Inoue et al. (2002) increased the blastocyst rate by 35%, but no live offspring were produced except one day 19 fetus. In our study, a high blastocyst rate (32%) was achieved from early passage cumulus cells after optimising the enucleation and activation procedure. When a total of 172 cloned embryos (2-4-cell stage) were transferred into 5 recipients, two of them were pregnant. From these two recipients, one lost pregnancy at the third week of gestation whereas the other one was operated on day 26 after ET and one live pup and two fetuses in resorption were recovered. To our knowledge, this is the first time to obtain live cloned pups from cultured somatic cells in rabbits.

5.2.1.3 Fibroblast cells

We and others (Dinnyes et al. 2001) observed that cumulus cells can not be cultured for long time under normal culture condition, which makes it difficult to establish stably transgenic colony. Due to their rapid growth and potential for many cellular divisions in culture (Schnieke et al. 1997; Cibelli et al. 1998; McCreath et al. 2000), fibroblast cells could be an alternative cell type to cumulus cells for nuclear transfer in mammals. In our study, fetal fibroblast cells were used as nuclear donors. The developmental potential to

blastocyst was as high as that from cumulus cells (RFF: 33.1% vs RCC: 32.3%, P>0.05). These data are not in agreement with the results from other studies where cumulus cells were better nuclear donors than fibroblast cells (Cervera & Garcia-Ximenez 2003) or vice versa (Liu et al. 2004a). These differences might be due to the various nuclear transfer protocols used in either study. For example, Mitalipov et al. (1999) also used adult fibroblast cells as nuclear donors, but they obtained a higher blastocyst rate (29.6%) than Liu et al. (18%, 2004a) when a different activation protocol was used.

When a total of 408 cloned embryos at the 2-4-cell stage were transferred into 10 recipient females, 2 from them were pregnant, but no offspring was produced. The contrast between high in vitro developmental potential and poor in vivo development suggests some implantation failures occurring after embryos transfer. To improve the in vivo developmental capacity of cloned embryos, we transferred them together with in vivo fertilized embryos into recipients. However, this approach did not result in increased pregnancy and offspring rates, suggesting that embryo transfer was not always successful.

In our study in vivo development of cumulus cell nuclear transfer embryos was slightly different from that of fetal fibroblast cell nuclear transfer embryos. In mice, however, embryos cloned from cumulus cells developed better both in vitro and in vivo than those cloned from fibroblast cells (Rideout et al 2000; Xue et al. 2002). These results imply that there must be some intrinsic differences (e.g. differences in epigenetic status) between these two cell types. Recently, Enright et al. (2003b) reported that in bovine more confluent cumulus cells were in the G0/G1 phase compared to confluent fibroblast cells which was consistent with, and perhaps results from, the fact that fewer cumulus cells were at the G2/M phase than fibroblast cells. In that study they also observed that cumulus and fibroblast cells were different in their levels of acetylated histones and amount of histone H1. Therefore, at least two aspects including cell cycle and epigenetic state differences are responsible for the above observations.

5.2.2 Effects of cell culture and treatment on nuclear transfer efficiency

In the present study we used donor cells which differed from each other either by culture conditions or treatments. Cumulus cells were cultured to confluence whereas fibroblast cells were either from confluent or serum-starved cultures. Additionally, confluent

fibroblast cells were treated with NaBu. The culture conditions and treatment of donor cells could have effects on their cell cycle, which plays a major role in the process of nuclear reprogramming and subsequent development of reconstructed embryos. In our study, however, cloning efficiency did not differ when using confluent or serum-starved donor cells irrespective of their origin. With fibroblast cells, NaBu treatment significantly increased developmental potential of cloned embryos indicating additional effects of this procedure. Indeed, as demonstrated in previous studies in several species (Wilmut et al. 1997; Cibelli et al. 1998; Liu et al. 2004b), under culture conditions used in our study, the majority of the cells were in G0/G1 phase of the cell cycle. Moreover, NaBu treatment also arrested cells at this cell cycle (Golzio et al. 2002; Shi et al. 2003; Huang et al. 2004).

We also used 8-cell blastomeres as nuclear donors, and obtained live cloned pups. Since the majority (about 80%) of embryonic cells are in the S phase of the cell cycle (Powell & Barnes 1992), recipient oocytes were activated prior to introducing donor nucleus to maintain correct ploidy (Campbell et al. 1993). Although activated oocytes are considered as universal recipient cytoplasm for nuclear transfer, it remains unclear whether activation of oocytes prior to nuclear transfer is necessary or not since the first rabbit clones were produced by activation of recipient cytoplasm following transfer of unsynchronised donor nuclei (Stice & Robl 1988).

5.2.3 Effects of NaBu-treatment on donor cells

With nuclear transfer, the somatic donor nucleus carries the specific epigenetic modifications of its tissue type, which must be erased during nuclear reprogramming. Therefore, the levels of epigenetic modification existing in donor cells may affect their ability for reprogramming following nuclear transfer. Treatment of donor cells with chemicals to remove some epigenetic marks prior to nuclear transfer may improve the ability of the donor cells to be fully reprogrammed by the recipient cytoplasm. In our study, treatment of RFFs with NaBu prior to nuclear transfer resulted in significantly better development of cloned embryos to blastocysts (49%) than that with non-treated RFFs (33%). When we investigated the effects of NaBu treatment on RFFs, cell proliferation activity was found to be significantly decreased and the majority of cells were SA-βgal-positive, which indicates that the cells reached a senescent or senescent-like state (Dimri et al. 1995). However, it is unknown whether the beneficial effect of NaBu-treatment was due to induction of a cell growth arrest. Senescent cells have been used to produce cloned cattle

(Lanza et al. 2000), but it seems that this type of cells is not superior to growing cells (Galat et al. 2002; Jang et al. 2004). It is more likely that the positive effect of NaBu treatment was due to epigenetic modifications of the donor cell chromatin. By Western blot analysis, we found increased levels of acetylated histone H3 and H4 in NaBu-treated RFFs. Hyperacetylation of these two histones is associated with an open chromatin structure and gene expression (Gorman et al. 1983). In bovine (Shi et al. 2003), NaBu-treatment of fibroblast cells had also positive effect on development of nuclear transfer embryos. But this effect was not related to the change of histone acetylation levels, suggesting species-specific differences in chromatin organization. In addition to histone acetylation analysis, we assessed the DNA methylation state of RFFs, and found no differences between NaBu-treated and non-treated RFFs. This finding was not unexpected since the major function of NaBu is inhibition of the activities of histone deacetylase, but not of DNA methyltransferases. The positive effect of NaBu-treatment on development of nuclear transfer embryos could be due to the alteration of histone acetylation levels.

5.3 Fusion rates between various types of donor cells

Fusion of the donor cell and recipient oocyte depends on a number of factors, some of which are related to the status of oocyte and donor cell. For example, in rabbits, recently ovulated oocytes fuse at a higher rate than aged oocytes (Collas & Robl 1990), and preactivated oocytes fuse better than nonactivated ones. Moreover, successful fusion of somatic cells with oocytes is more difficult to achieve as compared to embryonic cells (Dominko et al. 1999). In addition, time of oocyte maturation may influence the efficiency of fusion (Yin et al. 2002b). In our study, membrane fusion was initiated when the oocyte age was 18-19 h, and the fusion rates were higher than those reported by others regardless of the donor cell type (Table 22; Stice & Robl 1988; Collas & Robl 1990; Du et al. 1995; Dinnyes et al 2001; Yin et al. 2002b,c; Li et al. 2002; Chesne et al. 2002; Liu et al. 2004a). Among donor cell types used in our study, embryonic blastomeres showed the highest fusion rates while cumulus cells fused with the lowest efficiency. This difference might be due to the reduced area of contact between donor cell and cytoplast (Prather et al. 1987; Sims & First 1994; Wells et al. 1997).

Table 22 Fusion rates with different donor cells

Cell type	EB	RCC	RFF	Starved-RFF	NaBu-RFF
Fused (%)	1075/1099 (97.8)[a]	743/799 (93.0)[b]	931/978 (95.2)[c]	413/425 (97.2)[ac]	1771/1818 (97.5)[a]

[abc]: percentages with different superscripts are significantly different (P<0.05).

EB: embryonic blastomeres; RCC: rabbit cumulus cells; RFF: rabbit fetal fibroblast cells; Starved-RFF: RFF cultured in low serum medium; NaBu-RFF: RFF treated with sodium butyrate (NaBu).

In addition, electric pulse applied for fusion could to some extent initiate oocyte activation, which might have an adverse effect on further development of reconstructed embryos. Since calcium is the major factor related to electric pulse-induced activation, Yin et al. used calcium-free Zimmerman cell fusion medium (Yin et al. 2002b). This modification resulted in the production of cloned fetuses, but no offspring. Direct intracytoplasmic injection of donor nucleus into recipient cytoplasm is another way to avoid activation of oocytes by electric pulse-induced fusion (Inoue et al. 2002a), and one viable cloned fetus (day 19) with normal organogenesis was obtained. However, the production of first somatic cell nuclear transfer rabbits by Chesne et al. (2002) and also the birth of cloned rabbits either from cultured cumulus cells or fetal fibroblast cells in our study proved that the removal of calcium from the fusion medium is not necessary.

5.4 Activation

5.4.1 Time gap between fusion and activation

The exposure time of a donor nucleus into a recipient cytoplasm is related to the extent of chromatin condensation which affects further development of reconstructed embryos. Embryo development was significantly reduced when the donor chromosomes failed to undergo full condensation due to oocyte activation immediately after nuclear transfer (Wakayama & Yanagimachi 2001b). Moreover, it has been reported that reprogramming factors present in metaphase oocytes disappear 6 h after parthenogenetic activation (Tani et al. 2001). The reprogramming factors in oocytes are suggested to bind donor cell chromosomes soon after nuclear transfer (Surani 2001) and then induce the change of nuclei to a condition similar as after fertilisation. Therefore, the prolonged exposure of

donor nuclei to oocyte cytoplasm may increase the chances of reprogramming the nuclei. However, chromosomes of donor cells scatter into the cytoplasm as the exposure time in enucleated oocytes increases (Tsunoda & Kato 1997). Cevera and Garcia-Ximenez (2004) tested three different time intervals between fusion and activation (30 min, 60 min, 90 min). No differences were observed in blastocyst rates and hatching rates, but in the 60 min and 90 min groups, embryos reached the blastocyst stage earlier than in the 30 min group. Moreover, the quality of blastocysts was lower in the 30 min group than in the other 2 groups. In the present study, reconstructed oocytes from somatic donors were activated within 20-40 min after electric pulse induced fusion. This time gap was shorter than that in other studies (Yin et al. 2002a, b; Inoue et al. 2002; Chesne et al. 2002). But it is reasonable if considering the previous data that the rabbit zygotes enter S phase very early after fertilization (Oprescu et al. 1965) and that donor chromosomes in rabbits are fully condensed within 1 h after nuclear transfer (Yin et al. 2002b). The production of cloned offspring strongly supports the choice of the time gap between fusion and activation in our study.

Additionally, the oocyte age is another key factor related to the timing of activation, thus in turn determines the time gap between nuclear transfer and oocyte activation. In our study, electric pulse-induced fusion was initiated at about 18.5 h after hCG treatment, and oocyte activation was commenced at about 19 h after hCG injection. This time schedule is consistent with other studies (Chesne et al. 2002; Inoue et al. 2002a).

5.4.2 Optimisation of activation protocol

Optimisation of the activation regime greatly promotes the postimplantation development of parthenogenetic embryos (Ozil & Huneau 2001). Improvement of the oocyte activation protocol may be one of the key approaches to successful rabbit cloning.

Rabbit oocytes require long-lasting activation stimuli for complete escape from metaphase, because the activity of MPF is easily restored. Multiple electric pulses were carried out to mimic the calcium oscillation occurring during normal fertilization, and have resulted in greater activation success in rabbit nuclear transfer (Ozil 1990; Collas & Robl 1990; Berridge 1993). However, this protocol requires increased handling of the oocytes and is not well adapted for nuclear transfer manipulations. The treatments now most commonly

used to activate nuclear transfer embryos include the application of electric pulses in calcium-containing medium and incubation with chemical reagents that induce an increase in the concentration of intracellular-free calcium (Machaty & Prather 1998) or that function as kinase or/and protein synthesis inhibitors (Chesne et al. 2002). In our study, following electric pulses, 6-DMAP (a kinase inhibitor) together with cycloheximide (a protein synthesis inhibitor) were applied to activate enucleated MII oocytes when embryonic blastomeres were used as nuclear donors. This combination resulted in cloned offspring. Interestingly, the combination of 6-DMAP with CHX was not suitable for oocytes which had received somatic nuclei before activation, because the extrusion of pseudopolar body was formed within 1 h after the treatment and all resultant embryos failed to develop further. After staining with Hoechst 33342, donor nuclei were found being condensed in the extrusion. Chesne et al. (2002) did not report this phenomenon when using the same treatment for activating oocytes which were enucleated by Hoechst 33342 staining under UV light. To understand whether this phenomenon was due to the demecolcine-treatment for enucleation, we tried to use the same enucleation and activation protocols as those reported by Chesne et al. (2002). However, extrusions of pseudopolar bodies were still observed. These results indicate that demecolcine-treatment did not contribute to the formation of the pseudo-polar body.

It has been reported that CHX-treatment results in mouse egg activation, as assessed by emission of the second polar body and formation of a pronucleus (Siracusa et al. 1978; Clarke & Masui 1983; Fulka et al. 1994). 6-DMAP can induce the formation of a nuclear envelope after oocyte activation and subsequent normal postfertilization development (Verlhac et al. 1993; 1994). Incubation of porcine oocytes with 6-DMAP following electrical activation reduced the incidence of second polar body extrusion compared with electrical activation alone, but this phenomenon was not observed when electric pulsed oocytes were incubated with CHX (Grupen et al. 2002). Since the response of oocytes to 6-DMAP varies in a species-dependent manner (Ledda et al. 1996; Thibault et al. 1987), and CHX has effects on the microtubule rearrangement and the acquisition of competence for tubulin assembly (Rozinek et al. 1995), more studies are required to understand the dynamics of cytokinesis of nuclear transfer oocytes induced by 6-DMAP/CHX.

In the present study, to inhibit the extrusion of donor nuclei during treatment with 6-DMAP/CHX, reconstructed embryos from somatic cells were activated by 6-DMAP/CB

combination following electropulses. CB, which inhibits microfilament polymerization, is often used in current nuclear transfer procedures (Grupen et al. 2002; Yin et al 2002b). The combination of CB with 6-DMAP in our study completely inhibited the formation of pseudopolar body, and resulted in a higher developmental potential of cloned embryos in our experiments as compared to other studies.

Additionally, in the case of nuclear transfer, 6-DMAP-treatment of the electrofused oocyte-cell complexes may affect the reprogramming process of the donor nucleus. Prolonged exposure of bovine oocytes to 6-DMAP resulted in chromosomal abnormalities (De La Fuente & King 1998; Van de Velde et al. 1999), while shorter exposure did not (Liu et al. 1998a; Van de Velde et al. 1999). In our study, duration of 6-DMAP/CB-treatment was as short as 1 h, and with this modification we obtained the first cloned rabbits from cultured cumulus and fetal fibroblast cells worldwide.

5.5 Embryo culture

In addition to appropriate conditions that induce activation, in vitro culture of reconstructed embryos after micromanipulations is critical for successful cloning. In vitro culture may cause alterations in the expression of the zygotic genome (Stojanov et al. 1999; Blondin et al. 2000; Niemann & Wrenzycki 2000) and a great reduction in viability (Biggers & Papaioannou 1991; Holm et al. 1996; Sinclair et al. 1999). Several culture systems have been used for rabbit embryos, including defined temperature, air components, basic culture medium, concentration of FCS, co-culture and the use of biological fluids (Kane et al. 1987; Liu et al. 1996; Collas et al. 1991; Carney et al. 1990; Roh & Hwang 2002). When we compared M199 and B2 medium containing 10% FCS for culture of cloned embryos, the blastocyst rates of cloned embryos in M199 were lower than in B2 medium. Since rabbit clones have been produced when reconstructed embryos were cultured in B2 medium containing 2.5% FCS (Chesne et al. 2002), the same basic medium was used in our study but supplemented with 10% FCS. In addition, except CO2, the ratio of O2 in air also has effect on embryo development. For example, in vitro development to the morula-blastocyst stage was higher when nuclear transfer goat embryos were cultured at low oxygen tension (Apimeteetumrong et al. 2004). When we cultured nuclear transfer rabbit embryos in low O2 conditions (5%), the cleavage and further development rates showed no difference compared to normal air conditions (21%).

Overall, both the high developmental potential in vitro and the production of cloned offspring confirm that our culture system is suitable for rabbit cloning.

5.6 Embryo transfer

At present, the only way to ensure the success of nuclear transfer in mammalian cloning is to produce normal cloned offspring. Therefore, reconstructed embryos must be transferred into pseudopregnant recipients for further development. Numerous factors are related to the efficiency of this downstream event of nuclear transfer procedure. These include timing of embryo transfer, preparation of pseudopregnant recipients, methods for embryo transfer, and the number of transferred embryos.

5.6.1 Timing of embryo transfer

Rabbit embryos are unique in the formation of a mucin coat around their zonae pellucidae. This layer of glycoproteins is accumulated in the oviducts while embryos are transported to the uterus and is influenced by the ratio of progesterone/estrogen (Adams 1958; Greenwald 1969). The thickness of the mucin layer, which increases from nothing at the 1-cell stage to about 110 µm at 72 h (Adams 1958), appears to be an important factor for successful implantation of rabbit embryos (Murakami & Imai 1996). The potential of rabbit embryos to develop to young decreases markedly with the duration of in vitro culture (Maurer 1978). Murakami and Imai (1996) reported that the developmental potential of in vivo fertilized embryos was rapidly compromised after 1 day of in vitro culture, partly because of a lack of mucin coat covering the zona pellucida. Hence, in that study reconstructed embryos that developed beyond 3 cell cycles (4-6-cell stage) after only an overnight period of in vitro culture were transferred into recipients. Yang et al. (1990) also reported that the extended in vitro culture of rabbit embryos diminished their chance of developing to progeny. In our study, cloned embryos were transferred after an overnight in vitro culture. Since transportation of embryos from the oviduct to the uterus starts at approximately 60 h after fertilization (Ziomek et al. 1990), the influence of the overnight in vitro culture on the formation of the mucin coat is negligible. To test whether this is true, we collected transferred embryos on day 4 after embryo transfer, and a thick mucin coat was found around the zona pellucida. However, live offspring could be produced even when transfer of micromanipulated embryos was performed at 45-46 h after hCG treatment (Piotrowska

et al. 2000). Therefore, more investigations are required to understand the necessity of mucin coat for in vivo development of nuclear transfer embryos in rabbits.

In addition, we found that after an overnight in vitro culture (about 20 h after activation) cloned embryos were at different stages of development (from 2- to 10-cell stage). When 2-cell embryos were cultured further, poor in vitro development was observed, suggesting that these retarded embryos are developmentally deficient. Therefore, only at least 4-cell stage cloned embryos were transferred into recipients.

5.6.2 Synchronization of pseudopregnant mother

Mature females are induced into a pseudopregnant state either by mating with vasectomized males or by treatment with hCG. But it is still unclear whether the synchronization between the recipients and reconstructed embryos is necessary or not. In general, it is considered that the highest rates of survival are obtained after embryo transfer to recipients that are synchronized with donor embryos (Chang 1950). However, retarded rabbit embryos had a higher survival rate if they were transferred into recipients that ovulated later relative to the stage of the transferred embryos rather than to those that were synchronized to the same or to an earlier stage as compared to the transferred embryos (Tsunoda et al. 1982). The first cloned rabbits were produced as early as 1988 (Stice & Robl 1988). In that case, recipient females were mated to a vasectomized male concurrently with the hCG injection of mature oocyte donors. Two years later, the same group produced live offspring when cloned embryos were transferred into the recipient does which were mated to a vasectomized male and given 100 IU hCG 12 h after the time of hCG injection of the oocyte donors (Collas & Robl 1990). These data suggest that the "time window" for transfer of cloned embryos, at least derived from blastomeres, is relatively large.

However, in later studies on somatic nuclear transfer, cloned embryos failed to develop to term when they were transferred into recipients which were prepared the same as above (Yin et al. 2000; Liu et al. 2004a). Normally, transportation of embryos from the oviduct to the uterus starts at approximately 60 h; by 72 h, approximately 90% of them reach the uterus. Nevertheless, in some superovulated rabbits, the majority of embryos are still in the oviduct at this time. With nuclear transfer embryos, Chesne et al. (2002) reported that very

few cloned blastocysts could implant because their development was delayed. Therefore, they extended the asynchrony between donor and recipient females from 16 to 22 h, and obtained live offspring. These results indicate that such a marked asynchrony at early cleavage stage of development can be compatible with full-term development of fertilized eggs. Additionally, Joung et al. (2004) reported that the oviducts of asynchronous recipients secreted more mucin around the transferred embryos, causing higher rates of implantation of the in vitro-cultured blastocysts. Based on these data, in the present study, recipients were treated with hCG 20-22 h after the oocytes donors, and cloned offspring from all three different cell types were produced. Our results along with others demonstrate that synchronization between nuclear transfer embryos and recipient females is more important for somatic cell nuclear transfer embryos than for blastomere nuclear transfer embryos.

5.6.3 Methods for embryo transfer

Nonsurgical embryo transfer has been well developed in bovine (Rowe et al. 1980). But in rabbits, in most cases, transfer of rabbit embryos was conducted by surgical manipulation. Recipient females inevitably suffered from during and post the operation. Kidder et al. reported a nonsurgical method of embryo collection and transfer in domestic rabbits (Kidder et al. 1999). In that case, late stage embryos (78-89 h after hCG injection) were collected and transferred through cervices. This technique seems not suitable for nuclear transfer embryos since early stage embryos are transferred. In our study, specially designed catheters were used together with a fibre optic endoscope to visualize the ovary and infundibulum (Besenfelder & Brem 1993), and embryos were introduced through the infundibulum into each oviduct of recipients with minimum volume of transfer buffer. With this technique, recipient females suffered very little from manipulations, and live offspring was produced.

5.6.4 Numbers of nuclear transfer embryos per recipient

To date, no data are available on the optimal number of embryos that will give the highest offspring rates. Stice and Robl (1988) transferred 5-8 embryos into each oviduct and obtained first cloned rabbits from blastomeres (3.7%). Collas and Robl (1990) also obtained cloned pups when using the same number for embryo transfer (10%). But Yin et

al. (2000) could not repeat these results when transferring the same numbers of somatic nuclear transfer embryos. They increased the number by 45 per recipient, but except 6 fetuses, no offspring was produced (Yin 2002b,c). Dinnyes et al. (2001) used less numbers (16-22 per recipient) but failed to obtain offspring. Chesne et al. (2002) reported that increasing the number of reconstructed embryos transferred into one foster (up to 45) did not increase the rate of full-term development. They transferred 7 to 10 nuclear transfer embryos into each oviduct, and obtained cloned pups (1.6%). In addition, contrary to the mouse, a litter of only one pup can routinely be observed in rabbits, which is favourable for full-term development of nuclear transfer derived fetuses (Challah-jacques et al. 2003). One pup from follicular donor cells was produced after the transfer of 87 cloned embryos into five recipients (Challah-jacques et al. 2003). In our study, when a low number of cloned embryos was transferred, no pups were produced. All cloned pups from three types of cells were produced after transfer of high numbers of embryos (at least 45 per recipient). Therefore, further study should be carried out to understand the optimal number for transfer of cloned embryos into recipients.

5.6.5 Reasons for a low ratio of offspring after embryo transfer

The fate of cloned embryos after transfer into recipient females is mainly dependent on the quality of the embryos. Rabbit blastocysts greatly increase in size prior to implantation, and their rapid and significant expansion stretches the surrounding walls of the uterus (Denker 1981). To investigate the in vivo development of cloned embryos from the time of embryo transfer to implantation, we re-collected the embryos on day 5 after transfer into a recipient. In total 11 from 30 transferred embryos (37%) were collected, suggesting loss of embryos during embryo transfer or early resorption. Out of these 11 embryos, 6 were at the blastocyst stage (Fig. 16). The size of 4 growing blastocysts ranged from 750 to 2000 µm. These observations show that in our study about 13% of the transferred embryos could develop further. Lower offspring rates (<2%) in the present study imply that an additional loss of embryos or fetuses may occur during implantation and/or in the postimplantation stage which can result from the defective reprogramming of the genes related to these events.

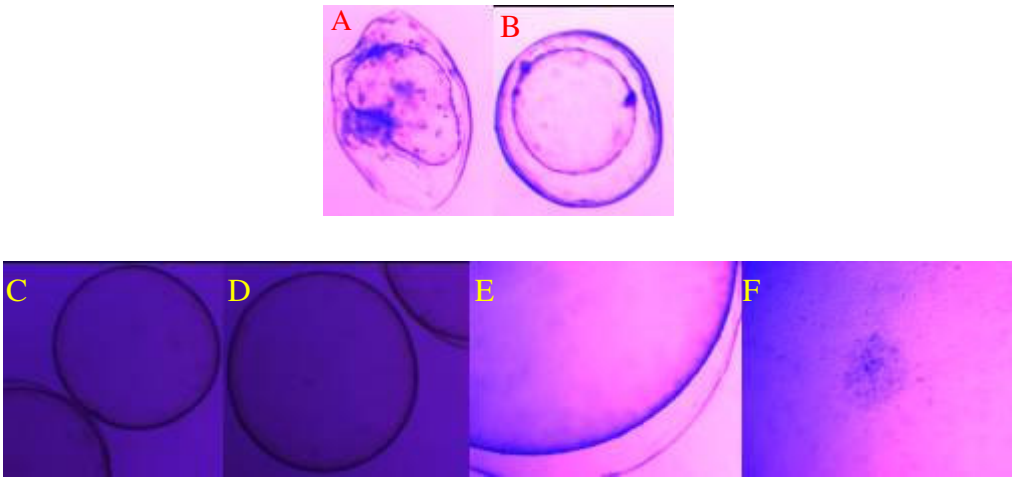


Fig. 16 Cloned embryos collected on day 4 after ET
(A-B: poor blastocysts (350 ~ 400 µm); C-D: good blastocysts (1300 ~ 1400 µm); E-F: excellent blastocyst (~2000 µm). Photos show the same blastocyst from different facet.)

5.7 Aggregation of cloned embryos with blastomeres of in vivo or parthenogenetic embryos

Aggregation of cloned embryos with other types of embryos or embryonic cells that could potentially improve their developmental competence may provide an alternative approach to producing cloned offspring. There is evidence for metabolic cooperation between genetically diverse cell types through permeable cell junctions that enable metabolically deficient cells to function in a normal manner (Pitts & Burk 1976). Developing intercellular junctions between blastomeres of different origin might play a role in communication and subsequently enhance development of the chimeric embryos (Ducibella & Anderson 1975). This approach proved to be useful for improving the efficiency of producing cloned transchromosomic calves (Kasinathan et al. 2004).

To improve the developmental competence of rabbit embryos cloned from fetal fibroblasts, we aggregated 4- to 10-cell stage cloned embryos with one or two blastomeres of 6-12-cell stage in vivo fertilized embryos. A greater proportion of aggregated embryos developed to blastocysts as compared with cloned, non-aggregated embryos (78% vs. 61%), but no pregnancies were established after transfer of 128 cloned embryos into 4 recipients. Transfer of 392 aggregated embryos into 11 recipients resulted in three pregnancies that were cancelled on day 12-13 to determine the origin of the conceptuses. Out of four conceptuses in resorption and one normal fetus, none appeared to be of the same genetic

background as donor cells indicating that blastomeres of cloned embryos may not be competitive with blastomeres of in vivo fertilized embryos.

We thought that the use of less competitive partners for aggregation might improve the survival of cloned embryos during postimplantation development. It is well documented that mammalian parthenogenetic embryos do not develop to term because of deficient expression of paternally derived imprinted genes and are characterized by poor development of their extraembryonic membranes (Surani et al. 1986; 1990). In rabbits, parthenogenetic embryos have the potential to implant but die near midgestation (Ozil 1990). However, in chimeric mice derived from aggregation of diploid parthenogenetic and normal fertilized embryos, parthenogenetic embryonic cells exhibit significant cellular contributions, including the germ line (Stevens 1978). Parthenotes were also used to assist the development to term of single blastomeres from 4-cell mouse embryos (Pinyopummin et al. 1994).

Considering that parthenogenetic blastomeres do not contribute to full-term development, we aggregated 4-10-cell stage cloned embryos with a single blastomere of 6-12-cell stage parthenogenetic embryos. The embryos produced by this approach appear to be superior to both cloned, non-aggregated and aggregated cloned-in vivo fertilized embryos. Although the development of cloned-parthenote embryos to blastocyst was similar to that of cloned embryos (58% vs. 61%), and even lower than that of cloned-in vivo fertilized embryos (78%), these embryos initiated more pregnancies than the latter ones (67% vs. 0% and 27%) and two of them developed to term. One of the two newborns, an overgrowth pup, died of respiratory failure within one hour while another one lived about two weeks until an accidental death. Genotypic analyses confirmed that both pups are clones derived from fetal fibroblasts.

Fetal fibroblast-derived live rabbits have been produced in our study after aggregation of cloned embryos with blastomeres of parthenogenetic embryos. However, we cannot conclude that our results are solely due to the direct effect of interactions between divergent types of cells after their aggregation. The prime aim of our aggregation approach was the improvement of cloning efficiency, and the contribution or mechanism by which pathenogenetic cells could assist the development of cloned embryos has not been determined yet. Blastocysts obtained after aggregation of cloned embryos with one

blastomere parthenogenetic blastomeres has similar cell numbers as compared to cloned non-aggregated blastocysts. This suggests that improved development of these aggregated embryos was related to another factors that may depend on the level of cell-to-cell communication. It might be that this situation is similar to that in mouse NT embryos when epigenetic complementation approach via clone-clone aggregation resulted in normalized Oct4 gene expression and higher rates of in vivo development (Boiani et al. 2003). However, in bovine, aggregation of cloned embryos did not improve somatic cell cloning efficiency (Oback et al. 2004). It is very unlikely that our results are related to the normality of the placenta since one of the major characteristics of parthenotes is poor development of extraembryonic tissues, and the development of one oversized pup was associated with placental overgrowth. Further studies on the contribution of parthenogenetic cells to both the inner cell mass and trophectoderm might resolve this enigma. Nevertheless, the major result of our study that cultured fetal fibroblast cells can be successfully used as donors for nuclear transfer opens a new way to more wide use of rabbit model for basic and applied research.

6 SUMMARY

Nuclear Transfer in Rabbits with Different Types of Donor Cells

Cloned rabbits were produced as early as 1988 by transferring embryonic nuclei into enucleated oocytes followed by electric pulse-induced activation and fusion. Transgenic rabbits were also obtained approximately two decades ago by pronuclear microinjection (Hammer et al. 1985; Brem et al. 1985). But production of cloned transgenic rabbits from somatic nuclei was hampered since the nuclear transfer technique had extremely low rate of success. The objectives of the present study were to evaluate the developmental potential of cloned embryos from different types of donor cells and to produce cloned rabbits from cultured somatic cells, thus to open a new way for transgenesis in this species.

In vivo matured MII oocytes were treated with 5 μM ionomycin following incubation in 0.6 μg/ml demecolcine for 30 min to 2 h. This treatment induced small extrusion from oocyte cytoplasm, and all maternal chromatin was condensed in the extrusion. Therefore, enucleation can be easily carried out by removal of the cytoplasmic extrusion. In our study, this chemically assisted enucleation protocol was successfully used with MII oocytes since high extrusion and enucleation rates were obtained.

The ooplasm prepared with the above enucleation procedure was first used to reprogram embryonic nuclei. To escape from meiotic arrest, enucleated oocytes were activated by electric pulses prior to nuclear transfer followed by treatment with protein kinase inhibitor (e.g. 6-DMAP) and protein synthesis inhibitor (e.g. CHX). High cleavage and blastocyst rates (87% and 59%, respectively) indicated the high activation efficiency from this protocol. The production of live cloned offspring proved that both chemically assisted enucleation and physical/chemical-combined activation are suitable for nuclear transfer in rabbits.

For the purpose of generating cloned transgenic rabbits, donor cells must be cultured for a relatively long time for genetic modification prior to use as nuclear donors. However, to date, somatic cell nuclear transfer rabbits could be obtained only from freshly collected cumulus cells. Therefore, we applied the above nuclear transfer procedure for cultured somatic cells. Interestingly, 6-DMAP/CHX treatment following electric pulses was not suitable for activation of enucleated oocytes after receiving donor nuclei, because after activation pseudo polar body containing donor nucleus formed. We produced reconstructed

embryos after using 6-DMAP/CB instead of 6-DMAP/CHX for activation. With this modification, high cleavage and blastocyst rates (RCCs: 93%; confluent RFFs: 93% and RCCs: 32%; confluent RFFs: 33%, respectively) were obtained when early passage RCCs and confluent RFFs were used as nuclear donors. Production of cloned offspring from RCC proved that activation by combination of 6-DMAP and CB following electric pulses together with chemically assisted enucleation was suitable for somatic nuclear transfer in rabbits. However, reconstructed embryos from either confluent or serum starved RFFs failed to develop to term. Sodium butyrate (NaBu) treatment led to higher in vitro development to blastocyst than that with confluent RFFs or serum starved RFFs (49% vs. 33% and 33%, $P<0.05$), but no full-term development was obtained.

Aggregation of cloned embryos with other types of embryos or embryonic cells could potentially improve their developmental competence, providing an alternative approach to producing cloned offspring. We aggregated 4-10-cell stage cloned embryos with one or two blastomeres of 6-12-cell stage in vivo fertilized embryos. Although a greater proportion of aggregated embryos developed to blastocysts as compared with cloned, non-aggregated embryos (78% vs. 61%, $P<0.05$), out of four conceptuses in resorption and one normal fetus, none had the same genetic background as donor cells. These results indicated that blastomeres of cloned embryos might not be competitive with blastomeres of in vivo fertilized embryos. Considering that parthenogenetic blastomeres do not contribute to full term development, we aggregated 4-10-cell stage cloned embryos with a single blastomere of 6-12-cell stage parthenogenetic embryos. Although the development of cloned-parthenogenote embryos to blastocyst was similar to that of cloned embryos (58% vs. 61%, $P>0.05$), and even lower than that of cloned-in vivo fertilized embryos (58% vs. 78%, $P<0.05$), these embryos initiated more pregnancies than the latter ones (67% vs. 0% and 27%; $P>0.05$) and two of them developed to term. One of the two newborns, an overgrowth pup, died of respiratory failure within one hour while another one lived about two weeks until an accidental death. Genotypic analyses confirmed that both pups are clones derived from fetal fibroblasts.

Overall, results from our study show that embryos cloned from embryonic cells had higher developmental potential than those cloned from somatic cells. Embryos cloned from somatic cells did not differ in their developmental capacity. After successive optimisation of all steps of nuclear transfer, we produced the first cloned rabbits from cultured somatic

cells including cumulus cell and fetal fibroblast cells. These achievements will open a new way to more wide use of rabbit model for basic and applied research.

7 ZUSAMMENFASSUNG

Kerntransfer beim Kaninchen mit unterschiedlichen Typen von Kernspenderzellen

Bereits 1988 wurden durch den Transfer embryonaler Zellkerne in enukleierte Eizellen, mit anschließender elektrischer Aktivierung und Fusion geklonte Kaninchen erstellt. Ebenso wurden bereits vor ca. zwei Jahrzehnten transgene Kaninchen durch Vorkern-Mikroinjektion erzeugt (Hammer et al. 1985; Brem et al. 1985). Die Erzeugung von geklonten transgenen Kaninchen durch Verwendung genetisch veränderter somatischer Zellkerne war jedoch bislang nicht möglich, da der Kerntransfer mit kultivierten Spenderzellen nicht etabliert ist.

Die vorliegende Arbeit hatte zum Ziel, das Entwicklungspotential klonierter Kaninchen-embryonen unter Verwendung verschiedener Zelltypen zu beurteilen und Klongruppen durch Verwendung somatischer Zellen zu erstellen und dadurch neue Möglichkeiten zur Erzeugung transgener Kaninchen zu schaffen.

In vivo maturierte M II Eizellen wurden mit 5 µM Ionomycin und einer darauf folgenden 30 bis 120 minütigen Inkubation mit 0,6 µg/ml Demecolcin behandelt. Diese Behandlung führte zu einer geringfügigen Extrusion des chromatinhaltigen Zytoplasmas der Eizelle.
Somit konnte die Enukleation durch das Entfernen des extrudierten Zytoplasmas problemlos durchgeführt werden. In der vorliegenden Arbeit führte diese chemisch unterstützte Enukleation an Oozyten in der MII Entwicklungsphase zu hohen Extrusions- und Enukleationsraten.

Die durch dieses Behandlungsverfahren erzeugten Zytoplasten wurden zunächst für die Reprogrammierung embryonaler Zellkerne verwendet. Um eine Blockade der Meiose aufzuheben, wurden die enukleierten Oozyten mit Hilfe eines elektrischen Impulses vor dem Kerntransfer aktiviert und anschließend mit einem Protein-Kinase-Hemmer (z.B. 6-DMAP) und einem Protein-Synthese-Hemmer (z.B. CHX) behandelt. Die Effizienz dieses Behandlungsprotokolls wurde durch die erzielten hohen Teilungs- und Blastozystenraten (87% bzw. 59%) nachgewiesen. Durch die Erzeugung von lebenden geklonten Nachkommen wurde gezeigt, dass beide Verfahren, sowohl die chemisch unterstützte

Enukleation, als auch die physikalisch-chemisch kombinierte Aktivierung für den Kerntransfer beim Kaninchen gut geeignet sind.

Für die Erzeugung geklonter transgener Kaninchen müssen Spenderzellen zur genetischen Modifikation relativ lange kultiviert werden. Es konnten jedoch bis heute ausschließlich mit frisch gewonnenen Kumuluszellen geklonte Kaninchen erzeugt werden. In der vorliegenden Untersuchung wurde deshalb das oben beschriebene Klonierungsverfahren mit kultivierten somatischen Zellen verwendet. Interessanterweise war die 6-DMAP/CHX Behandlung nach dem elektrischen Impuls nicht zur Aktivierung der enukleierten Eizellen nach dem Kerntransfer geeignet, da sich nach der Aktivierung ein Pseudo-Polkörper mit dem Spenderzellkern formte. In der vorliegenden Arbeit konnten durch Verwendung von 6-DMAP/CB anstelle von 6-DMAP/CHX zur Aktivierung klonierte Embryonen erzeugt werden. Dieses modifizierte Verfahren führte zu hohen Teilungs- und Blastozystenraten (RCCs: 93%, konfluente RFFs: 93% bzw. RCCs: 32%, konfluente RFFs: 33%), wenn frühe Passagen von RCCs und konfluenten RFFs verwendet wurden. Die erfolgreiche Erzeugung von geklonten Tieren mit RCC zeigte, dass die Aktivierung mit der Kombination 6-DMAP und CB nach dem elektrischen Impuls zusammen mit dem chemisch unterstützten Enukleieren für den Kerntransfer mit somatischen Zellen beim Kaninchen geeignet ist. Jedoch führte weder die Erzeugung von Embryonen mit konfluenten noch Serum reduziert kultivierten RFFs zu Nachkommen. Die Behandlung der Spenderzellen (RFF) mit Natriumbutyrat (NaBu) führte zu höheren in vitro Entwicklungsraten zu Blastozysten, als die Kultur zur Konfluenz oder in serumarmem Medium (49% vs. 33% und 33%, $P<0.05$), jedoch entwickelten sich keine Embryonen bis zur Geburt weiter.

Darüber hinaus wurde getestet, ob die Aggregation von geklonten Embryonen mit anderen Embryonen oder embryonalen Zellen ihre Entwicklungsfähigkeit verbessert, um die Chance der Erzeugung geklonter Nachkommen zu erhöhen. So wurden geklonte Embryonen im 4-10 Zellstadium mit ein oder zwei Blastomeren von in vivo erzeugten Embryonen im 6-12 Zellstadium aggregiert. Obwohl sich im Vergleich zu den nicht aggregierten Embryonen ein größerer Teil der aggregierten Embryonen zu Blastozysten weiterentwickelten (78% vs. 61%, $P<0.05$), waren weder die vier nach Embryotransfer erhaltenen, in Resorption befindlichen Föten noch der normal entwickelte Fötus mit den eingesetzten Kernspenderzellen genetisch identisch. Diese Ergebnisse deuteten darauf hin,

das Blastomeren von geklonten Embryonen möglicherweise nicht ausreichend konkurrenzfähig gegenüber Blastomeren von in vivo erzeugten Embryonen sind. Da sich parthenogenetische Blastomeren nicht zu Nachkommen weiterentwickeln können, wurden klonierte Embryonen im 4-10-Zellstadium mit einzelnen Blastomeren von parthenogenetischen Embryonen im 6-12-Zellstadium aggregiert. Die Entwicklungsrate von aggregierten Embryonen dieses Typs zu Blastozysten war vergleichbar der von geklonten Embryonen (58% vs. 61%, $P>0.05$) und lag sogar niedriger als nach der Aggregation mit in vivo Blastomeren (58% vs. 78%, $P<0.05$). Nach Transfer in Empfängertiere lag jedoch die Trächtigkeitsrate im Falle der mit parthenogenetischen Blastomeren aggregierten Kerntransferembryonen höher als in der anderen Gruppen (67% vs. 0% and 27%; $P>0.05$), und es wurden zwei Jungtiere geboren. Eines der zwei Neugeborenen litt an Überwuchs und starb innerhalb einer Stunde nach der Geburt an Lungenversagen, während das andere Junge phänotypisch normal war, jedoch nach ca. zwei Wochen auf Grund eines Unfalls starb. Durch genotypische Analysen konnte bestätigt werden, dass es sich bei den beiden Nachkommen tatsächlich um Klone aus den verwendeten fötalen Fibroblastzellen handelte.

8 REFERENCES

Adams CE 1958 Egg development in the rabbit: the influence of post-coital ligation of the uterine tube and of ovariectomy. *J. Endocrinol.* **16** 283-293.

Adams CE 1970 Ageing and reproduction in the female mammal with particular reference to the rabbit. *J. Reprod. Fertil. Suppl.* **12** 1-16.

Adenot PG, Campion E, Legouy E, Allis CD, Dimitrov S, Renard J and Thompson EM 2000 Somatic linker histone H1 is present throughout mouse embryogenesis and is not replaced by variant H1 degrees. *J. Cell. Sci.*. **113 (Pt 16)** 2897-2907.

Alberio R, Kubelka M, Zakhartchenko V, Hajduch M, Wolf E and Motlik J 2000a Activation of bovine oocytes by specific inhibition of cyclin-dependent kinases. *Mol. Reprod. Dev.* **55** 422-432.

Alberio R, Motlik J, Stojkovic M, Wolf E and Zakhartchenko V 2000b Behavior of M-phase synchronized blastomeres after nuclear transfer in cattle. *Mol. Reprod. Dev.* **57** 37-47.

Alberio R, Zakhartchenko V, Motlik J and Wolf E 2001a Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. *Int. J. Dev. Biol.* **45** 797-809.

Alberio R, Brero A, Motlik J, Cremer T, Wolf E and Zakhartchenko V 2001b Remodelling of donor nuclei, DNA-synthesis, and ploidy of bovine cumulus cell nuclear transfer embryos: effect of activation protocol. *Mol. Reprod. Dev.* **59** 371-379.

Ansel KM, Lee DU and Rao A 2003 An epigenetic view of helper T cell differentiation. *Nat. Immunol.* **4** 616–623.

Apimeteetumrong M, Thuangsanthia A, Leingcharoen N, Yiengvisavakul V, Harintharanon A, Kunavongkrit A, Sumretprasong J, Vignon X and Techakumphu M 2004 The effect of activation protocols on the development of cloned goat embryos. *J. Vet. Med. Sci.* **66** 1529-1534.

Arney KL, Bao S, Bannister AJ, Kouzarides T and Surani MA 2002 Histone methylation defines epigenetic asymmetry in the mouse zygote. *Int. J. Dev. Biol.* **46** 317-320.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM,

Godke RA, Gavin WG, Overstrom EW and Echelard Y 1999 Production of goats by somatic cell nuclear transfer. *Nat. Biotech.* **17** 456–461.

Baguisi A and Overstrom EW 2000 Induced enucleation in nuclear transfer procedures to produce cloned animals. *Theriogenol*. **54** 209 (abstract).

Barker D and Clark P 1997 Fetal undernutrition and disease in later life. *Rev*. *Reprod.* **2** 105-112.

Barnes F, Collas P, Powell R, King W, Westhusin M and Shepherd D 1993 Influence of recipient oocyte cell cycle stage on DNA synthesis nuclear envelope breakdown, chromosome constitution, and development in nuclear transfer bovine embryos. *Mol*. *Reprod*. *Dev*. **36** 33-41.

Bayreuther KH, Rodemann HP, Hommel R, Dittmann K, Albiez M and Francz PI 1988 Human skin fibroblasts in vitro differentiate along a terminal cell lineage. *Proc. Natl. Acad. Sci. U S A* **85** 5112-5116.

Beaujean N, Hartshorne G, Cavilla J, Taylor J, Gardner J, Wilmut I, Meehan R and Young L 2004 Non-conservation of mammalian preimplantation methylation dynamics. *Curr. Biol.* **14** R266–R267.
Behboodi E, Anderson G, Bondurant R, Cargill S, Kreuscher B, Medrano J and Murray J 1995 Birth of large calves that developed from in vitro-derived embryos. *Theriogenol*. **44** 227-232.

Berridge MJ 1993 Inositol trisphosphate and calcium signalling. *Nature* **361** 315-325.

Besenfelder U and Brem G 1993 Laparoscopic embryo transfer in rabbits. *J. Reprod. Fertil.* **99** 53-56.

Bestor TH 2000 The DNA methyltransferases of mammals. *Human Molecular Genetics* **9** 2395–2402.

Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S and Bishop M 2000 Production of cloned pigs from in vitro systems. *Nat. Biotechnol*. **18** 1055-1059.

Biggers JD and Papaioannou VE 1991 Postnatal compensatory growth of manipulated mouse embryos. *Hum. Reprod.* **6** 36-44.

Bird A 2002 DNA methylation patterns and epigenetic memory. *Genes Dev.* **16** 6–21.

Biron VL, McManus KJ, Hu N, Hendzel MJ and Alan D 2004 Underhill Distinct dynamics and distribution of histone methyl-lysine derivatives in mouse development. *Dev. Biol.* **276** 337-351.

Blangero C, Rols MP and Teissie J 1989 Cytoskeletal reorganization during electric-field-induced fusion of Chinese hamster ovary cells grown in monolayers. *Biochim. Biophys. Acta.* **981** 295-302.

Blondin P, Farin PW, Crosier AE, Alexander JE and Farin CE 2000 In vitro production of embryos alters levels of insulin-like growth factor-II messenger ribonucleic acid in bovine fetuses 63 days after transfer. *Biol. Reprod.*. **62** 384-389.

Boiani M, Eckardt S, Leu NA, Scholer HR and McLaughlin KJ 2003 Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? *EMBO. J.* **22** 5304-5312.

Bondioli K, Westhusin M and Looney C 1990 Production of identical bovine offspring by nuclear transfer. *Theriogenol*. **33** 165-174.

Bondioli K 1993 Nuclear transfer in cattle. *Mol. Reprod. Dev*. **36** 274-275.

Bordignon V, Clarke HJ and Smith LC 1999 Developmentally regulated loss and reappearance of immunoreactive somatic histone H1 on chromatin of bovine morula-stage nuclei following transplantation into oocytes. *Biol. Reprod.* **61** 22-30.

Bordignon V, Clarke HJ and Smith LC 2001 Factors controlling the loss of immunoreactive somatic histone H1 from blastomere nuclei in oocyte cytoplasm: a potential marker of nuclear reprogramming. *Dev. Biol.* **233** 192-203.

Bosze Z, Hiripi L, Carnwath JW and Niemann H 2003 The transgenic rabbit as model for human diseases and as a source of biologically active recombinant proteins. *Transgenic Res.* **12** 541-553.

Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP and Viegas-Pequignot E 2001 Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr. Biol.* **11** 1542–1546.

Brem G, Brenig B, Goodman HM, Selden RC, Graf F, Kruff B, Springman B, Hondele J, Meyer J, Winnacker EL and Kraublich H 1985 Production of transgenic mice, rabbits, and pigs by microinjection into pronuclei. *Zychthygience* **20** 251–252.

Briggs R and King 1952 Transplantation of living nuclei from blastula cells into enucleated frog eggs. *Proc. Natl. Acad. Sci. USA*. **38** 455-463.

Buhler TA, Bruyere T, Went DF, Stranzinger G and Burki K 1990 Rabbit beta-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. *Biotechnology (N Y)* **8** 140-143.

Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ and Matzuk MM 2003 Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science* **300** 633-636.

Campbell KH, Ritchie WA and Wilmut I 1993 Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. *Biol. Reprod.* **49** 933-942.

Campbell K, McWhir J, Ritchie W and Wilmut I 1996a Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380** 64–66.

Campbell K, Loi P, Otaegui P and Wilmut I 1996b Cell cycle coordination in embryo cloning by nuclear transfer. *Rev. Reprod.* **1** 40-46.

Campbell K 1999 Nuclear Equivalence, Nuclear Transfer and the Cell Cycle. *Cloning* **1** 3-15.

Carney EW, Tobback C, Ellington JE and Foote RH 1990 Co-culture of rabbit 2-cell embryos with rabbit oviduct epithelial cells and other somatic cells. *Mol. Reprod. Dev.* **27** 209-215.

Carroll J, Jones KT and Whittingham DG 1996 Ca2+ release and the development of Ca2+ release mechanisms during oocyte maturation: a prelude to fertilization. *Rev. Reprod.* **1** 137-143.

Cervera RP and Garcia-Ximenez F. 2003 Oocyte age and nuclear donor cell type affect the technical efficiency of somatic cloning in rabbits. *Zygote* **11** 151-158.

Cervera RP and Garcia-Ximenez F 2004 Effects of the time interval between fusion and activation on in vitro rabbit nuclear transfer efficiency when nuclear donor cells are derived from older adults. *Zygote* **12** 133-141.

Cezar GG, Bartolomei MS, Forsberg EJ, First NL, Bishop MD and Eilertsen KJ 2003 Genome-wide epigenetic alterations in cloned bovine fetuses. *Biol. Reprod.* **68** 1009-1014.

Challah-Jacques M, Chesne P and Renard JP 2003 Production of cloned rabbits by somatic nuclear transfer. *Cloning Stem Cells* **5** 295-299.

Chang MC 1950 Transplantation of rabbit blastocysts at late stage; probability of normal development and viability at low temperature. *Science*. **111** 544-545.

Chen RZ, Pettersson U, Beard C, Jackson-Grusby L and Jaenisch R 1998 DNA hypomethylation leads to elevated mutation rates. *Nature* **395** 89–93.

Cheong H, Takahashi Y and Kanagawa H 1993 Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. *Biol. Reprod.* **48** 958-963.

Chesne P, Adenot PG, Vigilietta C, Baratt M, Boulanger L and Renard J 2002 Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat. Biotech.* **20** 366-369.

Cheung P, Allis CD and Sassone-Corsi P 2000 Signaling to chromatin through histone modifications. *Cell* **103** 263-271.

Chung YG, Ratnam S, Chaillet JR and Latham KE 2003 Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol. Reprod.* **69** 146-153.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA and Robl JM 1998 Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280** 1256-1258.

Cibelli J, Campbell K, Seidel G, West M and Lanza R 2002 The health profile of cloned animals. *Nat. Biotech.* **20** 13–14.

Clarke HJ and Masui Y 1983 The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. *Dev. Bio.* **97** 291-301.

Clarke HJ, Bustin M and Oblin C 1997 Chromatin modifications during oogenesis in the mouse: removal of somatic subtypes of histone H1 from oocyte chromatin occurs post-natally through a post-transcriptional mechanism. *J. Cell. Sci.* **110 (Pt 4)** 477-487.

Collas P and Robl JM 1990 Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. *Biol. Reprod.* **43** 877-884.

Collas P and Robl JM 1991 Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol. Reprod.* **45** 455-465.

Collas P, Duby RT and Robl JM 1991 In vitro development of rabbit pronuclear embryos in rabbit peritoneal fluid. *Biol. Reprod.* **44** 1100-1107.

Collas P, Balise J and Robl J 1992a Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. *Biol. Reprod.* **46** 492-500.

Collas P, Pinto-Corriea C, Ponce de Leon F and Robl J 1992b Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. *Biol. Reprod.* **46** 501-511.

Collas P and Barnes F 1994 Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei. *Mol. Reprod. Dev.* **38** 264-267.

Czolowska R, Szollosi D and Szöllösi M 1992 Changes in embryonic 8-cell nuclei transferred by means of fusion to mouse eggs. *Int. J. Dev. Biol.* **36** 543-553.

Davie JR and Hendzel MJ 1994 Multiple functions of dynamic histone acetylation. *J. Cell. Biochem.* **55** 98-105.

De La Fuente R and King WA 1998 Developmental consequences of karyokinesis without cytokinesis during the first mitotic cell cycle of bovine parthenotes. *Biol. Reprod.* **58** 952-962.

De Sousa P, King T, Harkness L, Young L, Walker S and Wilmut I 2001 Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. *Biol. Reprod.* **65** 23-30.

Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses J, Reik W and Feil R 1998 Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: Association with aberrant phenotypes. *Development* **125** 2273-2282.

Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E and Reik W 2001 Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc. Natl. Acad. Sci. USA* **98** 13734–13738.

Delhaise F, Ectors FJ, De Roover R, Ectors F and Dessy F 1995 Nuclear transplantation using bovine primordial germ cells from male fetuses. *Reprod. Fertil. Dev.* **7** 1217-1219.

Denker HW 1981 Implantation: the role of proteinases and blockage of implantation by proteinase inhibitors. *Adv. Anat. Embryol. Cell Biol.* **53** 1-123.

DiBerardino M and Hoffner N 1970 Origin of chromosomal abnormalities in nuclear transplants - a reevaluation of nuclear differentiation and nuclear equivalence in amphibians. *Dev. Biol.* **23** 185-209.

DiBerardino M and Hoffner N 1975 Nucleocytoplasmic exchange of nonhistone proteins in amphibian embryos. *Exp. Cell Res.* **94** 235-252.

Dimitrov DS and Sowers AE 1990 A delay in membrane fusion: lag times observed by fluorescence microscopy of individual fusion events induced by an electric field pulse. *Biochemistry* **29** 8337-8344.

Dimitrov S and Wolffe AP 1996 Remodeling somatic nuclei in Xenopus laevis egg extracts: molecular mechanisms for the selective release of histones H1 and H1(0) from chromatin and the acquisition of transcriptional competence. *EMBO. J.* **15** 5897-5906.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith OM and Campisi J 1995 A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U S A* **92** 9363-9367.

Dinnyes A, Dai Y, Barber M, Liu L, Xu J, Zhou P and Yang X 2001 Development of cloned embryos from adult rabbit fibroblasts: effect of activation treatment and donor cell preparation. *Biol. Reprod.* **64** 257-263.

Doherty AS, Mann MR, Tremblay KD, Bartolomei MS and Schultz RM 2000 Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod.* **62** 1526-1535.

Dominko T, Mitalipova M, Haley B, Beyhan Z, Memili E, McKusick B and First NL 1999 Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. *Biol. Reprod.* **60** 1496-502.

Dominko T, Chan A, Simerly C, Luetjens CM, Hewitson L, Martinovich C and Schatten G 2000 Dynamic imaging of the metaphase II spindle and maternal chromosomesin bovine oocytes: implications for enucleation efficiency verification, avoidanceof parthenogenesis, and successful embryogenesis. *Biol.Reprod.* **62** 150-154.

Du F, Giles JR, Foote RH, Graves KH, Yang X and Moreadith RW 1995 Nuclear transfer of putative rabbit embryonic stem cells leads to normal blastocyst development. *J. Reprod. Fertil.* **104** 219-223.

Ducibella T and Anderson E 1975 Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Dev. Biol.* **47** 45-58.

Eggan K, Akutsu H, Hochedlinger K, Rideout W, Yanagimachi R and Jaenisch R 2000 X-chromosome inactivation in cloned mouse embryos. *Science* **290** 1578–1581.

Ehrlich M 1982 Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res.* **10** 2709–2721.

Ehrlich M. 2003 Expression of various genes is controlled by DNA methylation during mammalian development. *J. Cell Biochem.* **88** 899-910.

Enright BP, Kubota C, Yang X and Tian XC 2003a Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine. *Biol. Reprod.* **69** 896-901.

Enright B, Jeong B, Yang X and Tian X 2003b Epigenetic characteristics of bovine donor cells for nuclear transfer: levels of histone acetylation. *Biol. Reprod*. **69** 1525-1530.

Erhardt S, Su IH, Schneider R, Barton S, Bannister AJ, Perez-Burgos L, Jenuwein T, Kouzarides T, Tarakhovsky A and Surani MA 2003 Consequences of the depletion of zygotic and embryonic enhancer of zeste 2 during preimplantation mouse development. *Development* **130** 4235-4248.

Escande-Geraud ML, Rols MP, Dupont MA, Gas N and Teissie J 1988 Reversible plasma membrane ultrastructural changes correlated with electropermeabilization in Chinese hamster ovary cells. *Biochim. Biophys. Acta*. **939** 247-259.

Fan J and Watanabe T 2003 Transgenic rabbits as therapeutic protein bioreactors and human disease models. *Pharmacol. Ther.* **99** 261-282.

Felsenfeld G and Groudine M 2003 Controlling the double helix. *Nature* **421** 448-453.

Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD and Khorasanizadeh S 2003 Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev*. **17** 1870-1881.

Fisher P 1987 Disassembly and reassembly of nuclei in cell free systems. *Cell* **48** 175-176.

Forsberg E, Strelchenko N, Augenstein M, Betthauser J, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Koppang R, Lange G, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Voelker G, Watt S and Bishop M 2002 Production of cloned cattle from in vitro systems. *Biol. Reprod.* **67** 327-333.

Fulka J Jr and Moor RM 1993a. Noninvasive chemical enucleation of mouse oocytes. *Mol. Reprod. Dev.* **34** 427–430.

Fulka J Jr, Notarianni E, Passoni L and Moor RM 1993b. Early changes in embryonic nuclei fused to chemically enucleated mouse oocytes. *Int. J. Dev. Biol.* **37** 433–439.

Fulka J Jr, Moor RM and Fulka J 1994 Sister chromatid separation and the metaphase-anaphase transition in mouse oocytes. *Dev. Biol.* **165** 410-417.

Fulka J Jr, First NL and Moor RM 1996 Nuclear transplantation in mammals: remodelling of transplanted nuclei under the influence of maturation promoting factor. *Bioessays* **18** 835-840.

Gabriel B and Teissie J 1994 Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability. *Eur. J. Biochem.* **223** 25-33.

Garcia I, Brandt DC, Benammar A, Cazenave PA and Jaton JC 1982 BASILEA rabbits express two types of immunoglobulin light chains: lambda and kappa-like. *Proc. Natl. Acad. Sci. U S A.* **79** 4391-4394.

Galat V, Lagutina I, Mezina M, Prokofiev MI and Zakhartchenko V 2002 Effect of donor cell age on the efficiency of nuclear transfer in rabbits. *Reprod. Biomed. Online* **4** 32-37.

Gall L, Dedieu T, Chesne P, Ruffini S, Sevellec C, Peynot N, Renard J and Heyman Y 1996 Bovine embryo cloning: Characterization of the recipient cytoplasts by phosphorylation patterns and kinase activities. *Dev. Growth Differ*. **38** 517-525.

Galli C, Duchi R, Moor RM and Lazzari G 1999 Mammalian leukocytes contain all the genetic information necessary for the development of a new individual. *Cloning* **1** 161–170.

Galli C, Lagutina I, Vassiliev I, Duchi R and Lazzari G 2002 Comparison of microinjection (piezo-electric) and cell fusion for nuclear transfer success with different cell types in cattle. *Cloning Stem Cells*. **4** 189-196 .

Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Poderato N, Duchi R and Lazzari G 2003a A cloned horse born to its dam twin. *Nature* **424** 635.

Garry F, Adams R, McCann J and Odde K 1996 Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenol*. **45** 141–152 .

Gasparrini B, Gao S, Ainslie A, Fletcher J, McGarry M, Ritchie WA, Springbett AJ, Overstrom EW, Wilmut I and De Sousa PA 2003 Cloned mice derived from embryonic stem cell karyoplasts and activated cytoplasts prepared by induced enucleation. *Biol. Reprod*. **68** 1259–1266.

Gibbons J, Arat S, Rzucidlo J, Miyoshi K, Waltenburg R and Respess D 2002 Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. *Biol. Reprod* **66** 895–900.

Gidekel S and Bergman Y 2002 A unique developmental pattern of Oct-3/4 DNA methylation is controlled by a cis-demodification element. *J. Biol. Chem.* **277** 34521–34530.

Golzio M, Teissie J and Rols MP 2002 Cell synchronization effect on mammalian cell permeabilization and gene delivery by electric field. *Biochim. Biophys. Acta.* **1563** 23-28.

Gorman CM, Howard BH and Reeves R 1983 Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11** 7631-7648.

Graur D, Duret L and Gouy M 1996 Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). *Nature* **379** 333-335.

Greenwald GS 1967 Species differences in egg transport in response to exogenous estrogen. *Anat..Rec.* **157** 163-172.

Greising T and Jonas L 1999 The influence of enucleation on the ultrastructure of in vitro matured and enucleated cattle oocytes. *Theriogenol*. **52** 303-312.

Grupen CG, Mau JC, McIlfatrick SM, Maddocks S and Nottle MB 2002 Effect of 6-dimethylaminopurine on electrically activated in vitro matured porcine oocytes. *Mol. Reprod. Dev.* **62** 387-396.

Gurdon JB 1964 The transplantation of living cell nuclei. *Adv. Morphogenesis*. **4** 1-43.

Gurdon JB, Laskey RA, De Robertis EM and Partington GA 1979 Reprogramming of transplanted nuclei in amphibia. *Int. Rev. Cytol. Suppl.* **9** 161-178.

Haccard O, Sarcevic B, Lewellyn A, Hartley R, Roy L, Izumi T, Erikson E and Maller JL 1993 Induction of metaphase arrest in cleaving Xenopus embryos by MAP kinase. *Science* **262** 1262-1265.

Hahn J 1984 The value of laboratory animal models in embryo transfer research. *Theriogenol.* **21** 45–59.

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD and Brinster RL 1985 Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315** 680-683.

Harper MJ, 1963 Ovulation in the rabbit: the time of follicular rupture and expulsion of the eggs, in relation to injection of luteinizing hormone. *J. Endocrinol*. **26** 307-316.

Heyman Y, Chesne P, Lebourhis D, Peynot N and Renard JP 1994 Developmental ability of bovine embryos after nuclear transfer based on the nuclear source: in vivo versus in vitro. *Theriogenol*. **42** 695–702.

Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X and Renard JP 2002a Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol. Reprod.* **66** 6-13.

Heyman Y, Zhou Q, LeBourhis D, Chavatte-Palmer P, Renard J and Vignon X 2002b Novel approaches and hurdles to somatic cloning in cattle. *Cloning Stem Cells* **4** 47-55.

Hiendleder S, Mund C, Reichenbach HD, Wenigerkind H, Brem G, Zakhartchenko V, Lyko F and Wolf E 2004 Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques. *Biol. Reprod.* **71** 217-223.

Hill J, Rousel, A, Cibelli J, Edwards J, Hooper N, Miller M, Thompson J, Looney C, Westhusin M, Robl J and Stice S 1999 Clinical and pathological features of cloned transgenic calves and fetuses (13 case studies). *Theriogenol.* **51** 1451–1465.

Hill J, Burghardt R, Jones K, Long C, Looney C, Shin T, Spencer T, Thompson J, Winger Q and Westhusin M 2000 Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* **63** 1787–1794.

Hochedlinger K and Jaenisch R 2002 Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* **415** 1035-1038.

Holm P, Walker SK and Seamark RF 1996 Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. *J. Reprod. Fertil.* **107** 175-181.

Howlett SK, Barton SC and Surani MA 1987 Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. *Development* 101 915-923.

Huang YQ, Wang LW, Yan SN and Gong ZJ 2004 Effects of cell cycle on telomerase activity and on hepatitis B virus replication in HepG2 2.2.15 cells. *Hepatobiliary Pancreat. Dis. Int.* **3** 543-547.

Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout W, Biniszkiewicz D, Yanagimachi R and Jaenisch R 2001 Epigenetic instability in ES cells and cloned mice. *Science* **293** 95–97.

Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander E, Golub T and Jaenisch R 2002 Abnormal gene expression in cloned mice derived from ES cell and cumulus cell nuclei. *Proc. Natl. Acad. Sci. USA* **99** 12889-12894.

Ibanez E, Albertini DF and Overstrom EW 2003 Demecolcine-induced oocyte enucleation for somatic cell cloning: coordination between cell-cycle egress, kinetics of cortical cytoskeletal interactions, and second polar body extrusion. *Biol. Reprod.* **68** 1249–1258.

Iizuka M and Smith MM 2003 Functional consequences of histone modifications. *Curr. Opin. Genet. Dev.* **13** 154-160.

Illmensee K and Hoppe P Nuclear transplantation in *Mus musculus*: developmental potential of nuclei from preimplantation embryos. *Cell* **23** 9-18.

Imamura T, Ohgane J, Ito S, Ogawa T, Hattori N, Tanaka S and Shiota K 2001 CpG island of rat sphingosine kinase-1 gene: tissue-dependent DNA methylation status and multiple alternative first exons. *Genomics* **76** 117–125.

Inoue K, Ogonuki N, Yamamoto Y, Noguchi Y, Takeiri S, Nakata K, Miki H, Kurome M, Nagashima H and Ogura A 2002a Improved postimplantation development of rabbit nuclear transfer embryos by activation with inositol 1,4,5-trisphosphate. *Cloning Stem Cells* **4** 311-317.

Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F and Ogura A 2002b Faithful expression of imprinted genes in cloned mice. *Science* **295** 297.

Jaenisch R and Bird A 2003 Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33 Suppl.** 245-254.

Jang G, Park ES, Cho JK, Bhuiyan MM, Lee BC, Kang SK and Hwang WS 2004 Preimplantational embryo development and incidence of blastomere apoptosis in bovine somatic cell nuclear transfer embryos reconstructed with long-term cultured donor cells. *Theriogenol*. **62** 512-521.

Jenuwein T and Allis CD 2001 Translating the histone code. *Science* **293** 1074-1080.

Jones PA, Wolkowicz MJ, Rideout WM, Gonzales FA, Marziasz CM, Coetzee GA and Tapscott SJ 1990 De novo methylation of the MyoD1 CpG island during the establisment of immortal cell lines. *Proc. Natl. Acad. Sci. U.S.A.* **87** 6117–6121.

Jones K, Hill J, Shin T, Lui L and Westhusin M 2001 DNA hypomethylation of karyoplasts for bovine nuclear transplantation. *Mol. Reprod. Dev*. **60** 208-213.

Joung SY, Kim HJ, Choi WS, Im KS, Lee SH, Park CS and Jin DI 2004 Effects of transferring in vitro-cultured rabbit embryos to recipient oviducts on mucin coat deposition, implantation and development. *Zygote* **12** 215-219.

Kane MT 1987 Minimal nutrient requirements for culture of one-cell rabbit embryos. *Biol. Reprod.* **37** 775-778.

Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK and Han YM 2001 Aberrant methylation of donor genome in cloned bovine embryos. *Nat. Genet.* **28** 173–177.

Kasinathan P, Knott JG, Wang Z, Jerry DJ and Robl JM 2001 Production of calves from G1 fibroblasts. *Nat. Biotech* **19** 1176–1178.

Kasinathan P, Nichols MF, Griffin JE and Robl JM 2004 Production of chimeric transchromosomic claves *Reprod. Fertil. Dev.* **16** 144–145 (abstract).

Katan-Khaykovich Y and Struhl K Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes. Dev.* **16** 743-752.

Kato Y, Tani T, Sotomaru Y Kurokawa K, Kato J, Doguchi H, Yasue H and Tsunoda Y 1998 Eight calves cloned from somatic cells of a single adult. *Science* **282** 2095-2098.

Kato Y, Rideout W, Hilton K, Barton S, Tsunoda Y and Surani M 1999 Developmental potential of mouse primordial germ cells. *Development* **126** 1823-1832.

Kato Y, Tani T and Tsunoda Y 2000 Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J. Reprod. Fertil.* **120** 321-237.

Kato Y, Imabayashi H, Mori T, Tani T, Taniguchi M, Higashi M, Matsumoto M, Umezawa A and Tsunoda Y 2004 Nuclear transfer of adult bone marrow mesenchymal stem cells: developmental totipotency of tissue-specific stem cells from an adult mammal. *Biol.Reprod.* **70** 415-418.

Kawai J, Hirose K, Fushiki S, Hirotsune S, Ozawa N, Hara A, Hayashizaki Y and Watanabe S 1994 Comparison of DNA methylation patterns among mouse cell lines by restriction landmark genomic scanning. *Mol. Cell. Biol.* **14** 7421–7427.

Kawakami M, Tani T, Yabuuchi A, Kobayashi T, Murakami H, Fujimura T, Kato Y, Tsunoda Y 2003 Effect of demecolcine and nocodazole on the efficiency of chemically assisted removal of chromosomes and the developmental potential of nuclear transferred porcine oocytes. *Cloning Stem Cells* **5** 379-387.

Keefer CL, Stice S and Mattheus D 1994 Bovine inner cell mass as donor nuclei in the production of nuclear transfer embryos and calves. *Biol. Reprod.* **50** 935-939.

Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS, Zhou FJ, Kafidi N, Wang B, Baldassarre H and Karatzas CN 2002 Production of cloned goats after nuclear transfer using adult somatic cells. *Biol. Reprod.* **66** 199-203.

Kelus AS and Weiss S 1986 Mutation affecting the expression of immunoglobulin variable regions in the rabbit. *Proc. Natl. Acad. Sci. U S A.* **83** 4883-4886.

Kidder JD, Roberts PJ, Simkin ME, Foote RH and Richmond ME 1999 Nonsurgical collection and nonsurgical transfer of preimplantation embryos in the domestic rabbit (Oryctolagus cuniculus) and domestic ferret (Mustela putorius furo*). J. Reprod. Fertil.* **116** 235-242.

Kim JM, Liu H, Tazaki M, Nagata M and Aoki F 2003 Changes in histone acetylation during mouse oocyte meiosis. *J. Cell Biol.* **162** 37-46.

King T 1966 Nuclear transplantation in amphibia. *Meth. Cell Physiol*. **2** 1-36.

Kinosita K Jr and Tsong TT 1977 Hemolysis of human erythrocytes by transient electric field. *Proc. Natl. Acad. Sci. U S A.* **74** 1923-1927.

Kline D and Kline JT 1992 Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149** 80-89.

Knight KL, Spieker-Polet H, Kazdin DS and Oi VT 1988 Transgenic rabbits with lymphocytic leukemia induced by the c-myc oncogene fused with the immunoglobulin heavy chain enhancer. *Proc. Natl. Acad. Sci. U S A*. **85** 3130-3134.

Knott J, Kasinathan P, Wu H, Spell A, Fissore R and Robl J 2001 Effect of porcine sperm factor on intracellular calcium and activation of bovine oocytes and development of nuclear transfer embryos. *Theriogenol*. **55** 455 (abstract).

Kubota C, Yamakuchi H, Todoroki J, Mizoshita K, Tabara N, Barber M and Yang X 2000 Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc. Natl Acad. Sci. USA.* **97** 990–995.

Kues WA, Carnwath JW, Paul D and Niemann H 2002 Cell cycle synchronization of porcine fetal fibroblasts by serum deprivation initiates a nonconventional form of apoptosis. *Cloning Stem Cells* **4** 231-243.

Kuhholzer B and Prather RS 2001 Synchronization of porcine fetal fibroblast cells with topoisomerase-inhibitor hoechst 33342. *Anim. Reprod. Sci.* **66** 109-116.

Kwon OY and Kono T 1996 Production of identical sextuplet mice by transferring metaphase nuclei from four-cell embryos. *Proc. Natl. Acad. Sci. U S A.* **93** 13010-13013.

Lachner M, O'Carroll D, Rea S, Mechtler K and Jenuwein T 2001 Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410** 116-120.

Lachner M, O'Sullivan RJ and Jenuwein T 2003 An epigenetic road map for histone lysine methylation. *J. Cell. Sci.* **116(Pt 11)** 2117-2124.

Lai L, Kolber-Simonds D, Park K, Cheong H, Greenstein J, Im G, Samuel M, Bonk A, Rieke A, Day B, Murphy C, Carter D, Hawley R and Prather S 2002 Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* **295** 1089-1092.

Lander ES, Linton LM, Birren B, et al. 2001 Initial sequencing and analysis of the human genome. *Nature* **409** 860–921.

Lanza RP, Cibelli JB, Blackwell C, Cristofalo VJ, Francis MK, Baerlocher GM, Mak J, Schertzer M, Chavez EA, Sawyer N, Lansdorp PM and West MD 2000 Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. *Science* **288** 665-669.

Larkin K and Danilchik MV 1999 Microtubules are required for completion of cytokinesis in sea urchin eggs. *Dev. Biol.* **214** 215-226.

Lavoir M, Basrur P and Betteridge K 1994 Isolation and identification of germ cells from fetal bovine ovaries. *Mol. Reprod. Dev.* **37** 413-424.

Lavoir M, Rumph N, Moens A, King W, Plante Y, Johnson W, Ding J and Betteridge K 1997 Development of bovine nuclear transfer embryos made with oogonia. *Biol. Reprod.* **57** 204-213.

Ledda S, Loi P, Bogliolo L, Moor RM and Fulka J Jr 1996 The effect of 6-dimethylaminopurine (6-DMAP) on DNA synthesis in activated mammalian oocytes. *Zygote.* **4** 7-9.

Lee C and Piedrahita J 2002 Inhibition of apoptosis in serum starved porcine embryonic fibroblasts. *Mol. Reprod. Dev.* **62** 106-112.

Lee J, Inoue K, Ono R, Ogonuki N, Kohda T, Kaneko-Ishino T, Ogura A and Ishino F 2002 Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development* **129** 1807-1817.

Leichthammer F, Baunack E and Brem G 1990 Behavior of living primordial germ cells of livestock in vitro. *Theriogenol.* **33** 1221-1230.

Lewin B 1998 Genes *Oxford University Press* **VI edition** 1089,1126-1127.

Li E 2002 Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.* **3** 662-673.

Li GP, Chen DY, Lian L, Han ZM, Zhu ZY and Seidel GE Jr 2002 Rabbit cloning: improved fusion rates using cytochalasin B in the fusion buffer. *Mol. Reprod. Dev.* **61** 187-191.

Liu Z, Foote RH and Simkin ME 1996 Effect of amino acids and alpha-amanitin on the development of rabbit embryos in modified protein-free KSOM with HEPES. *Mol. Reprod. Dev.* **45** 157-162.

Liu L, Shin T, Pryor J, Kraemer D and Westhusin M 2001 Regenerated bovine fetal fibroblasts support high blastocyst development following nuclear transfer. *Cloning* **3** 51-58 .

Liu JL, Sung LY, Du F, Julian M, Jiang S, Barber M, Xu J, Tian XC and Yang X 2004a Differential development of rabbit embryos derived from parthenogenesis and nuclear transfer. *Mol. Reprod. Dev.* **68** 58-64.

Liu CT, Yu KC and Ju JC 2004b Cell cycle stage analysis of rabbit foetal fibroblasts and cumulus cells. *Reprod. Domest. Anim.* **39** 385-390.

Loi P, Ptak G, Barboni B, Fulka J, Cappai P and Clinton M 2001 Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat. Biotech.* **19** 962-964.
Machaty Z and Prather RS 1998 Strategies for activating nuclear transfer oocytes. *Reprod. Fertil. Dev.* **10** 599-613.

Mann M, Chung Y, Nolen L, Verona R, Latham K and Bartolomei S 2003 Desruption of imprinted gene methylation and expression in cloned preimplatation stage mouse embryos. *Biol. Reprod.* **69** 902-914.

Massoud M, Bischoff R, Dalemans W, Pointu H, Attal J, Schultz H, Clesse D, Stinnakre MG, Pavirani A and Houdebine LM 1991 Expression of active recombinant human alpha 1-antitrypsin in transgenic rabbits. *J. Biotechnol.* **18** 193-203.

Mayer W, Niveleau A, Walter J, Fundele R and Haaf T 2000 Demethylation of the zygotic paternal genome. *Nature* **403** 501–502.

McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE and Kind AJ 2000 Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405** 1066-1069

McGrath J and Solter D 1983 Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science **220** 1300-1302

McGrath J and Solter D 1984 Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. *Science* **226** 1317–1319.

McGrath J and Solter D 1986 Nucleocytoplasmic interactions in the mouse embryo. *Embryol. Exp. Morphol.* **97 Suppl** 277-289.

McGraw S, Robert C, Massicotte L and Sirard MA 2003 Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. *Biol. Reprod.* **68** 383-389.

Meehan RR 2003 DNA methylation in animal development. *Semin. Cell Dev. Biol.* **14** 53–65.

Meng L, Ely JJ, Stouffer RL and Wolf DP 1997 Rhesus monkeys produced by nuclear transfer. *Biol. Reprod.* **57** 454-459.

Miki H, Inoue K, Kohda T, Honda A, Ogonuki N, Yuzuriha M, Mise N, Matsui Y, Baba T, Abe K, Ishino F and Ogura A 2005 Birth of mice produced by germ cell nuclear transfer. *Genesis* **41** 81-86.

Mitalipov SM, White KL, Farrar VR, Morrey J and Reed WA 1999 Development of nuclear transfer and parthenogenetic rabbit embryos activated with inositol 1,4,5-trisphosphate. *Biol. Reprod.* **60** 821-827.

Miyazaki S, Shirakawa H, Nakada K and Honda Y 1993 Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. *Dev. Biol.* **158** 62-78.

Moens A, Chastant S, Chesné P, Flechon J, Betteridge K and Renard J 1996 Differential ability of male and female rabbit fetal germ cell nuclei to be reprogrammed by nuclear transfer. *Differentiation* **60** 339-345.

Moore T and Reik W 1996 Genetic conflict in early development: parental imprinting in normal and abnormal growth. *Rev. Reprod.* **1** 73-77.

Motlik J, Alberio R, Zakhartchenko V, Stojkovic M, Kubelka M and Wolf E 2002 The effect of activation of mammalian oocytes on remodelling of donor nuclei after nuclear transfer. *Cloning Stem Cells* **4** 245-252.

Murakami H and Imai H 1996 Successful implantation of in vitro cultured rabbit embryos after uterine transfer: a role for mucin. *Mol Reprod Dev.* **43** 167-170.

Nakajima H, Kim Y, Terano H, Yoshida M and Horinouchi S 1998 FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Exp. Cell Res.* **241** 126-133.

Newport J and Kirschner M 1984 Regulation of the cell cycle during early *Xenopus* development. *Cell* **37** 731-742.

Niemann H and Wrenzycki C 2000 Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent developmMinimal nutrient requirements for culture of one-cell rabbit embryos.ent. *Theriogenol.* **53** 21-34.

Oback B and Wells D 2002a Donor cells for nuclear cloning: many are called, but few are chosen. *Cloning Stem Cells* **4** 147-168.

Oback B and Wells D 2002b Practical aspects of donor cell selection for nuclear cloning. *Cloning Stem Cells* **4** 169-174.

Oback B, Eichenlaub M, Laible G, Tucker F, Miller A, Oliver J, Troskie H, Forsyth J, Berg M, McMillan V and Wells D 2004 (Aug. 1-4) Aggregating nuclear transfer reconstructs increases inner cell mass-to-total cell ratio and does not improve somatic cattle cloning efficiency. *Biol. Reprod.* **Special issue** 239

Ogonuki N, Inoue K, Yamamoto Y, Noguchi Y, Tanemura K, Suzuki O, Nakayama H, Doi K, Ohtomo Y, Satoh M, Nishida A and Ogura A 2002 Early death of mice cloned from somatic cells. *Nat. Genet.* **30**, 253-254.

Ogura A, Inoue K, Ogonuki N, Noguchi A, Takano K, Nagano R, Suzuki O, Lee J, Ishino F and Matsuda J 2000 Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells. *Biol. Reprod.* **62** 1579-1584.

Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R and Shiota K 2004 The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. *Genes Cells* **9** 253-260.

Ohkoshi K, Takahashi S, Koyama S, Akagi S, Adachi N, Furusawa T, Fujimoto J, Takeda K, Kubo M, Izaike Y and Tokunaga T 2003 In vitro oocyte culture and somatic cell nuclear transfer used to produce a live-born cloned goat. *Cloning Stem Cells* **5** 109-115.

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H and Perry AC 2000 Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289** 1188–1190.

Ono Y, Shimozawa N, Ito M and Kono T 2001 Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biol. Reprod.* **64** 44-50.

Oprescu S and Thibault C 1965 Duplication de I'AND dans les oeufs de lapine après la fecondation. *Ann. Biol. Anim. Biochim. Biophys.* **5** 151-156.

Ozil JP 1990 The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* **109** 117-127.

Ozil JP and Huneau D 2001 Activation of rabbit oocytes: the impact of the Ca2+ signal regime on development. *Development* **128** 917-928.

Park KW, Lai L, Cheong HT, Cabot R, Sun QY, Wu G, Rucker EB, Durtschi D, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB and Prather RS 2002 Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. *Biol. Reprod.* **66** 1001-1005.

Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schofer C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A, Opravil S, Doyle M, Sibilia M and Jenuwein T 2001 Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107** 323-337.

Peters AH, Kubicek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, Kohlmaier A, Opravil S, Tachibana M, Shinkai Y, Martens JH and Jenuwein T 2003 Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol. Cell.* **12** 1577-1589.

Pinyopummin A, Takahashi Y, Hishinuma M and Kanagawa H 1994 Development of single blastomeres from 4-cell stage embryos after aggregation with parthenogenones in mice. *Jpn. J. Vet. Res.* **42** 119-126.

Piotrowska K, Modlinski JA, Korwin-Kossakowski M and Karasiewicz J 2000 Effects of preactivation of ooplasts or synchronization of blastomere nuclei in G1 on preimplantation development of rabbit serial nuclear transfer embryos. *Biol. Reprod.* **63** 677-682.

Pitts JD and Burk RR 1976 Specificity of junctional communication between animal cells. *Nature* **264** 762-764.

Polejaeva I, Chen S, Vaught T, Page R, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares D, Colman A and Campbell K 2000 Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407** 86-90.

Powell R and Barnes FL 1992 The kinetics of oocyte activation and polar body formation in bovine embryo clones. *Mol. Reprod. Dev*. **33** 53-58.

Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone W and First N 1987 Nuclear transfer in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol. Reprod*. **37** 859-866.

Prather RS, Sims MM and First NL 1989a Nuclear transplantation in early pig embryos. *Biol. Reprod.* **41** 414-418.

Prather R, Sims M, Maul G, First N and Schatten G 1989b Nuclear lamin antigens are developmentally regulated during porcine and bovine embryogenesis. *Biol. Reprod.* **41** 123-132.

Prather R, Sims M and First N 1990 Nuclear transplantation in the pig embryo: nuclear swelling. *J. Exp. Zool.* **255** 355-358.

Prelle K, Vassiliev I, Vassilieva S, Wolf E and Wobus A 1999 Establishment of pluripotent cell lines from vertebrate species – present status and future prospects. *Cells Tissues Organs.* **165** 220-236.

Ravelich SR, Breier BH, Reddy S, Keelan JA, Wells DN, Peterson AJ and Lee RS 2004a Insulin-like growth factor-I and binding proteins 1, 2, and 3 in bovine nuclear transfer pregnancies. *Biol. Reprod.* **70** 430-438.

Ravelich SR, Shelling AN, Ramachandran A, Reddy S, Keelan JA, Wells DN, Peterson AJ, Lee RS and Breier BH 2004b Altered placental lactogen and leptin expression in placentomes from bovine nuclear transfer pregnancies. *Biol. Reprod.* **71** 1862-1869.

Rayasam GV, Wendling O, Angrand PO, Mark M, Niederreither K, Song L, Lerouge T, Hager GL, Chambon P and Losson R 2003 NSD1 is essential for early post-implantation development and has a catalytically active SET domain. *EMBO. J.* **22** 3153-3163.

Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD and Jenuwein T 2000 Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406** 593-599.

Reik W, Romer I, Barton S, Surani M, Howlett S and Klose J 1993 Adult phenotype in the mouse can be affected by epigenetic events in the early embryo. *Development* **119** 933-942.

Reik W, Dean W and Walter J 2001 Epigenetic reprogramming in mammalian development. *Science* **293** 1089–1093.

Renard J, Chastant S, Chesné P, Richard C, Marchal J, Cordonnier N, Chavatte P and Vignon X 1999 Lymphoid hypoplasia and somatic cloning. *Lancet* **353** 1489-1491.

Richards EJ and Elgin SC 2002 Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108** 489-500.

Rideout WM 3rd, Wakayama T, Wutz A, Eggan K, Jackson-Grusby L, Dausman J, Yanagimachi R and Jaenisch R 2000 Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nat. Genet.* **24** 109-110.

Riggs AD, Xiong Z, Wang L and LeBon JM 1998 Methylation dynamics, epigenetic fidelity and X chromosome structure. *Novartis. Found. Symp.* **214** 214–225.

Robertson E 1987 Embryo-derived stem cell lines. *In*: Teratocarcinomas and embryonic stem cells: A practical approach (Robertson., E.J., Ed.). IRL Press, Oxford, 71-112.

Robertson KD and Wolffe AP 2000 DNA methylation in health and disease. *Nature Reviews. Genetics* **1** 11–19.

Robl J, Gilligan B, Critser E and First N 1986 Nuclear transplantation in mouse embryos: assessment of recipient cell stage. *Biol. Reprod.* **34** 733-739.

Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B and First NL 1987 Nuclear transplantation in bovine embryos. *J. Anim. Sci.* **64** 642-647.

Roh S and Hwang WS 2002 In vitro development of porcine parthenogenetic and cloned embryos: comparison of oocyte-activating techniques, various culture systems and nuclear transfer methods. *Reprod Fertil Dev* **14** 93-99.

Rowe RF, Del Campo MR, Critser JK and Ginther OJ 1980 Embryo transfer in cattle: nonsurgical collection techniques. *Am. J. Vet. Res.* **41** 106-108.

Rozinek J, Petr J, Grocholova R and Jilek F 1995 Microtubule rearrangement during in vitro maturation of pig oocytes. Effect of cycloheximide. *Reprod. Nutr. Dev.* **35** 685-694.

Santos F, Hendrich B, Reik W and Dean W 2002a Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev. Biol.* **41** 172–182.

Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, Emre NC, Schreiber SL, Mellor J and Kouzarides T 2002 Active genes are tri-methylated at K4 of histone H3. *Nature* **419** 407-411.

Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W and Dean W. 2003 Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr. Biol.* **13** 1116-1121.

Santos F and Dean W 2004 Epigenetic reprogramming during early development in mammals. *Reproduction* **127** 643-651.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A and Campbell KH 1997 Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278** 2130-2133.

Shi W and Haaf T 2002 Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Mol. Reprod. Dev.* **63** 329–334.

Shi W, Hoeflich A, Flaswinkel H, Stojkovic M, Wolf E and Zakhartchenko V 2003 Induction of a senescent-like phenotype does not confer the ability of bovine immortal cells to support the development of nuclear transfer embryos. *Biol. Reprod.* **69** 301-309.

Shi W, Dirim F, Wolf E, Zakhartchenko V and Haaf T 2004 Methylation reprogramming and chromosomal aneuploidy in in vivo fertilized and cloned rabbit preimplantation embryos. *Biol. Reprod.* **71** 340-347.

Shiga K, Fujita T, Hirosoe K, Sasae Y and Nagai T 1999 Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese Black bulls. *Theriogenol.* **52** 527-535.

Shim H, Gutierrez-Adán A, Chen L, BonDurant R, Behboodi E and Anderson G 1997 Isolation of pluripotent stem cells from cultured porcine primordial germ cells. *Biol. Reprod.* **57** 1089-1095.

Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L and Westhusin M 2002 A cat cloned by nuclear transplantation. *Nature* **415** 859.

Shiota K, Kogo Y, Ohgane J, Imamura T, Urano A, Nishino K, Tanaka S and Hattori N 2002 Epigenetic marks by DNA methylation specific to stem, germ and somatic cells in mice. *Genes Cells* **7** 961–969.

Sims M and First NL 1994 Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc. Natl. Acad. Sci. U S A.* **91** 6143-6147.

Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmut I, Broadbent PJ and Robinson JJ 1999 Aberrant fetal growth and development after in vitro culture of sheep zygotes. *J. Reprod. Fertil.* **116** 177-186.

Siracusa G, Whittingham DG, Molinaro M and Vivarelli E 1978 Parthenogenetic activation of mouse oocytes induced by inhibitors of protein synthesis. *J. Embryol. Exp. Morphol.* **43** 157-166.

Smith L and Wilmut I 1989 Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. *Biol. Reprod.* **40** 1027-1035.

Smith LC and Wilmut I 1990 Factors affecting the viability of nuclear transplantation embryos. *Theriogenol.* **33** 83–98.

Solter D 2000 Mammalian cloning: advances and limitations. *Nat. Rev. Genet.* **1** 199-207.

Stevens LC 1978 Totipotent cells of parthenogenetic origin in a chimaeric mouse. *Nature* **276** 266-267.

Stice SL and Robl JM 1988 Nuclear reprogramming in nuclear transplant rabbit embryos. *Biol Reprod.* **39** 657-664.

Stice SL and Robl JM 1990 Activation of mammalian oocytes by a factor obtained from rabbit sperm. *Mol. Reprod. Dev.* **25** 272-280.

Stice S, Strelchenko N, Keefer C and Matthews L 1996 Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biol. Reprod.* **54** 100-110.

Stojanov T, Alechna S and O'Neill C 1999 In-vitro fertilization and culture of mouse embryos in vitro significantly retards the onset of insulin-like growth factor-II expression from the zygotic genome. *Mol. Hum. Reprod.* **5** 116-124.

Strahl BD and Allis CD 2000 The language of covalent histone modifications. *Nature* **403** 41-45.

Strelchenko N, Betthauser J, Jurgella G, Forsberg E, Damiani P, Golueke P, Pace M and Bishop M 1998 Use of somatic cells in cloning. *Proc. Genet. Engeneering Cloning Anim.* June 21-23, Park City.

Surani MA, Barton SC and Norris ML 1986 Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* **45** 127-136.

Surani MA, Kothary R, Allen ND, Singh PB, Fundele R, Ferguson-Smith AC and Barton SC 1990 Genome imprinting and development in the mouse. *Dev. Suppl.* 89-98.

Surani MA 2001 Reprogramming of genome function through epigenetic inheritance. *Nature* **414** 122-128.

Suzuki Y, Tsunoda T, Sese J, Taira H, Mizushima-Sugano J, Hata H, Ota T, Isogai T, Tanaka T, Nakamura Y, Suyama, A, Sakaki, Y, Morishita, S, Okubo K and Sugano S 2001 Identification and characterization of the potential promoter regions of 1031 kinds of human genes. *Genome Res.* **11** 677–684.

Szollosi M and Szöllösi D 1988 Blebbing of the nuclear envelope of mouse zygotes, early embryos and hybrid cells. *J. Cell Sci*. **91** 257-267.

Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, Fukuda M, Takeda N, Niida H, Kato H and Shinkai Y 2002 G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev.* **16** 1779-1791.

Tamada H and Kikyo N 2004 Nuclear reprogramming in mammalian somatic cell nuclear cloning. *Cytogenet. Genome Res.* **105** 285-291.

Tamashiro K, Wakayama T, Akutsu H, Yamazaki Y, Lachey J, Wortman M, Seeley R, D'Alessio D, Woods S, Yanagimachi R and Sakai R 2002 Cloned mice have an obese phenotype not transmitted to their offspring. *Nat. Med.* **8** 262–267.

Tanaka S, Oda M, Toyoshima Y, Wakayama T, Tanaka M, Yoshida N, Hattori N, Ohgane J, Yanagimachi R and Shiota K 2001 Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biol. Reprod.* **65** 1813–1821.

Tani T, Kato Y and Tsunoda Y 2001 Direct exposure of chromosomes to non-activated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biol. Reprod.* **64** 324-330.

Tatham BG, Dowsing AT, Trounson AO 1995 Enucleation by centrifugation of in vitro-matured bovine oocytes for use in nuclear transfer. *Biol. Reprod.* **53** 1088-94.

Telford NA, Watson AJ and Schultz GA 1990 Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol. Reprod. Dev.* **26** 90-100.

Thibault C, Szollosi D and Gerard M 1987 Mammalian oocyte maturation. *Reprod. Nutr. Dev.* **27** 865-896.

Tian XC, Kubota C, Enright B and Yang X 2003 Cloning animals by somatic cell nuclear transfer--biological factors. *Reprod. Biol. Endocrinol.* **1** 98.

Tsunoda Y, Soma T and Sugie T 1982 Effect of post-ovulatory age of recipient on survival of frozen-thawed rabbit morulae. *J. Reprod. Fertil.* **65** 483-487.

Tsunoda Y, Yasui T, Shioda Y, Nakamura K, Uchida T and Sugie T 1987 Full term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. *J. Exp. Zool.* **242** 147-151.

Tsunoda Y and Kato Y 1993 Nuclear transplantation of embryonic stem cells in mice. *J. Reprod. Fertil.* **98** 537-540.

Tsunoda Y and Kato Y 1997 Full-term development after transfer of nuclei from 4-cell and compacted morula stage embryos to enucleated oocytes in the mouse. *J. Exp. Zool.* **278** 250-254.

Turner BM 2000 Histone acetylation and an epigenetic code. *Bioessays* **2** 836-845.

Urakawa M, Ideta A, Sawada T and Aoyagi Y 2004 Examination of a modified cell cycle synchronization method and bovine nuclear transfer using synchronized early G1 phase fibroblast cells. *Theriogenol.* **62** 714-728.

Uzbekov R, Chartrain I, Philippe M and Arlot-Bonnemains Y 1998 Cell cycle analysis and synchronization of the Xenopus cell line XL2. *Exp. Cell Res.* **242** 60-68.

Van De Velde A, Liu L, Bols PE, Ysebaert MT and Yang X 1999 Cell allocation and chromosomal complement of parthenogenetic and IVF bovine embryos. *Mol. Reprod. Dev.* **54** 57-62.

Verlhac MH, de Pennart H, Maro B, Cobb MH and Clarke HJ 1993 MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev. Biol.* **158** 330-340.

Verlhac MH, Kubiak JZ, Clarke HJ and Maro B 1994 Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes..*Development* **120** 1017-1025.

Vignon X, Chesne P, LeBourhis D, Flechon J, Heyman Y and Renard J 1998 Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. *C. R. Acad. Sci. Paris, Life Sci.* **321** 735-745.

Wakayama T, Perry ACG, Zuccotti M, Johnson KR and Yanagimachi R 1998 Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394** 369–374.

Wakayama T, Rodriguez I, Perry A, Yanagimachi R and Mombaerts P 1999 Mice cloned from embryonic stem cells. *Proc. Natl. Acad. Sci. U S A.* **96** 14984-14989.

Wakayama T, Taeno H, Mombaerts P and Yanagimachi R 2000 Nuclear transfer into mouse zygotes. *Nat. Genet.* **24** 108-109.

Wakayama T and Yanagimachi R 2001a Mouse cloning with nucleus donor cells of different age and type. *Mol. Reprod. Dev.* **58** 376-383.

Wakayama T and Yanagimachi R 2001b Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. *Reproduction* **122** 49-60.

Walker S, Heard T and Seamark R 1992 In vitro culture of sheep embryos without coculture: Successes and perspectives. *Theriogenol.* **37** 111-126.

Walker S, Hartwich K and Seamark R 1996 The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenol.* **45** 111-120.

Wang H, Huang ZQ, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, Briggs SD, Allis CD, Wong J, Tempst P and Zhang Y 2001 Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* **293** 853-857.

Waterborg JH 2002 Dynamics of histone acetylation in vivo. A function for acetylation turnover? *Biochem. Cell Biol.* **80** 363-378.

Wells D, Misica P, Day T and Tervit H 1997 Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro matured cytoplasts. *Biol. Reprod.* **57** 385-393.

Wells D, Musica P and Tervit H 1999 Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* **60** 996-1005.

Wells DN, Laible G, Tucker FC, Miller AL, Oliver JE, Xiang T, Forsyth JT, Berg MC, Cockrem K, L'Huillier PJ, Tervit HR and Oback B 2003 Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. *Theriogenol.* **59** 45–59.

Whitaker M and Patel R 1990 Calcium and cell cycle control. *Development* **108** 525-542.

Willadsen S 1986 Nuclear transplantation in sheep embryos. *Nature* **320** 63-65.

Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KHS 1997 Viable offspring derived from fetal and adult mammalian cells. *Nature* **385** 810–813.

Wilmut I, Beaujean N, De Sousa P, Dinnyes A, King T, Paterson L, Wells D and Young L 2002 Somatic cell nuclear transfer. *Nature* **419** 583-586.

Woods G, White K, Vanderwall D, Li G, Aston K, Bunch T, Meerdo L and Pate B 2003 A mule cloned from fetal cells by nuclear transfer. *Science* **301** 1063.

Wrenzycki C, Herrmann D, Carnwath J and Niemann H 1996 Expression of the gap junction gene conexin 43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo. *J. Reprod. Fertil.* **108** 17-24.

Wu H, Smyth J, Luzzi V, Fukami K, Takenawa T, Black S, Allbritton N and Fissore R 2001 Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod.* **64** 1338-1349.

Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H, Pereira LV and Yang X 2002 Aberrant patterns of X chromosome inactivation in bovine clones. *Nat. Genet.* **31** 216-220.

Yamazaki Y, Makino H, Hamaguchi-Hamada K, Hamada S, Sugino H, Kawase E, Miyata T, Ogawa M, Yanagimachi R and Yagi T 2001 Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer. *Proc. Natl. Acad. Sci. USA*. **98** 14022-14026.

Yang F, Kessler B, Ewerling S, Wolf E and Zakhartchenko V 2005 Rabbit nuclear transfer with cultured somatic cells. *Reprod. Fertil. Dev.* **17** 187 (abstract).

Yang X, Zhang L, Kovacs A, Tobback C and Foote RH 1990a Potential of hypertonic medium treatment for embryo micromanipulation: II. Assessment of nuclear transplantation methodology, isolation, subzona insertion, and electrofusion of blastomeres to intact or functionally enucleated oocytes in rabbits. *Mol. Reprod. Dev.* **27** 118-129.

Yang X, Chen Y, Chen J and Foote RH 1990 Potential of hypertonic medium treatment for embryo micromanipulation: I. Survival of rabbit embryos in vitro and in vivo following sucrose treatment. *Mol. Reprod. Dev.* **27** 110-117.

Yang X 1991 Embryo cloning by nuclear transfer in cattle and rabbits. *Embryo Transfer Newsletter* **9** 10-22.

Yang X, Jiang S, Kovacs A and Foote RH 1992 Nuclear totipotency of cultured rabbit morulae to support full-term development following nuclear transfer. *Biol Reprod.* **47** 636-643.

Yang X, Jiang S, Farrell P, Foote R and McGrath A 1993 Nuclear transfer in cattle: effect of nuclear donor cells, cytoplasm age, co-culture, and embryo transfer. *Mol. Reprod. Dev.* **35** 29-36.

Yin XJ, Tani T, Kato Y and Tsunoda Y 2000 Development of rabbit parthenogenetic oocytes and nuclear-transferred oocytes receiving cultured cumulus cells. *Theriogenology* **54** 1469-1476.

Yin XJ, Tani T, Yonemura I, Kawakami M, Miyamoto K, Hasegawa R, Kato Y and Tsunoda Y 2002a Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. *Biol. Reprod.* **67** 442-446.

Yin XJ, Kato Y and Tsunoda Y 2002b Effect of enucleation procedures and maturation conditions on the development of nuclear-transferred rabbit oocytes receiving male fibroblast cells. *Reproduction* **124** 41-47.

Yin XJ, Kato Y and Tsunoda Y 2002c Effect of delayed enucleation on the developmental potential of nuclear-transferred oocytes receiving adult and fetal fibroblast cells. *Zygote* **10** 217-222.

Yoder JA, Walsh CP and Bestor TH 1997 Cytosine methylation and the ecology of intragenomic parasites. *Trends in Genetics* **13** 335–340.

Yoshida M, Kijima M, Akita M and Beppu T 1990 Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* **265** 17174-17179.

Young L, Sinclair K and Wilmut I 1998 Large offspring syndrome in cattle and sheep. *Rev. Reprod.* **3** 155–163.

Young L and Fairburn H 2000 Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenol.* **53** 627-648.

Young LE and Beaujean N 2004 DNA methylation in the preimplantation embryo: the differing stories of the mouse and sheep. *Anim. Reprod. Sci.* **82-83** 61-78.

Yu BD, Hess JL, Horning SE, Brown GA and Korsmeyer SJ 1995 Altered Hox expression and segmental identity in Mll-mutant mice. *Nature* **378** 505–508.

Zakhartchenko V, Reichenbach HD, Riedl J, Palma G, Wolf E and Brem G 1996 Nuclear transfer in cattle using in vivo-derived vs. in vitro-produced donor embryos: effect of developmental stage. *Mol. Reprod. Dev.* **44** 493-498.

Zakhartchenko V, Alberio R, Stojkovic M, Prelle K, Schernthaner W, Stojkovic P, Wenigerkind H, Wanke R, Düchler M, Steinborn R, Mueller M, Brem G and Wolf E 1999a Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. *Mol. Reprod. Dev.* **54** 264-272.

Zakhartchenko V, Durcova-Hills G, Schernthaner W, Stojkovic M, Reichenbach HD, Mueller S, Prelle K, Steinborn R, Mueller M, Wolf E and Brem G 1999b Potential of fetal germ cell for nuclear transfer in cattle. *Mol. Reprod. Dev.* **52** 421-426.

Zakhartchenko V, Durcova-Hills G, Stojkovic M, Schernthaner W, Prelle K, Steinborn R, Mueller M, Brem G and Wolf E 1999c Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. *J. Reprod. Fertil.* **115** 325-331.

Zakhartchenko V, Mueller S, Alberio R, Schernthaner W, Stojkovic M, Wenigerkind H, Wanke R, Lassnig C, Mueller M, Wolf E and Brem G 2001 Nuclear transfer in cattle with non-transfected and transfected fetal or cloned transgenic fetal and postnatal fibroblasts. *Mol. Reprod. Dev.* **60** 362-369.

Zhang Y 2003 Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes Dev.* **17** 2733-2740.

Zhou Q, Jouneau A, Brochard V, Adenot P and Renard JP 2001 Development potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei. *Biol. Reprod.* **65** 412-419.

Zhou Q, Baquir S, Brochard V, Smith L and Renard J 2002 Donor nuclei are not well reprogrammed by nuclear transfer procedure. *Biol. Reprod.* **66(suppl.)** 237-238.

Zhou Q, Renard J, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A and Cozzi J 2003 Generation of fertile cloned rats by regulating oocyte activation. *Science* **302** 1179.

Zimmermann U, Pilwat G and Riemann F 1974 Dielectric breakdown of cell membranes. *Biophys. J.* **14** 881-899.

Ziomek CA, Chatot CL and Manes C 1990 Polarization of blastomeres in the cleaving rabbit embryo. *J. Exp. Zool.* **256** 84-91.

ACKNOWLEDGMENTS

This work was carried out in the Institute of Molecular Animal Breeding and Biotechnology, University of Munich.

First of all, I would like to thank with a deep sense of gratitude Prof. Eckhard Wolf for accepting me in his department, for the excellent working condition and atmosphere, for his consistent support and guidance throughout the research, and for the critical reading of this thesis.

I would like to express my special appreciation to Dr. Valeri Zakhartchenko for giving me the insights in animal cloning by nuclear transfer, for his consistent support and restrict guidance throughout the research, for the critical reading of this thesis, and for his care about my living conditions.

I am indebted to Dr. Barbara Kessler and Sonja Ewerling for the embryo transfer and the expert animal care. I am also greatly thankful to Myriam Weppert for her helpful comments, technical support and translation, to Dr. Regina Klose and Dr. Elisabeth Kemter for their helpful assistance in western blotting, to Dr. Hendrik Wenigerkind for his assistance with computer management, Dr. Felix Habermann for his helpful assistance with karyotype analysis, to Tuna Güngör, Wiebke Scholz and Sigrid Stärke for their assistance and technical support. Many heartfelt thanks go to Dr. Wolfgang Voss, Dr. Bernhard Aigner, Dr. Stephan Hiendleder, Dr. Sigrid Müller, Dr. Fabiola Lopes, Dr. Birgit Rathkolb, Dr. Marlon Schneider, Dr. Marc Boelhauve, Sylvia Jande, Franziska Rieger, Angelika Kammerer, Peter Rieblinger, Sigfried Elsner, Christian Erdle, Regine Rottmayer, Fernado Tenorio Filho, Partricia Gallindo Carazzoni, Ru Hao, Gabi Scholz for their helpful suggestion, discussion and assistance.

I am extremely grateful to my former Chinese supervisor, Prof. Yong-de Shi, who constantly encourages me to perform this work. For the financial support from University of Munich and the Therapeutic Human Polyclonals, Inc. I would like to express my gratitude.

Last but not least, I would like to thank my parents, my brother, my sister and all my friends, for their support and encouragement to finish this thesis.

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