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Reprogramming of mesenchymal stem cells and adult fibroblasts following nuclear transfer in rabbits

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ABBREVIATIONS

6-DMAP	6-dimethylaminopurine
ART	assisted reproduction technologies
Blast	blastocyst
BM	bone marrow
BMSCs	bone marrow stem cells
BSA	bovine serum albumin
°C	Celsius degree
CB	cytochalasin B
CHX	cycloheximide
Cm	centimeter
DAPI	4,6-diamidino-2-phenylindole
DC	direct current
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DMR	differentially methylated region
EDTA	ethylenediaminetetraacetic acid
EG cells	embryonic germ cells
EGFP	enhanced green fluorescent protein
ES cells	embryonic stem cells
ET	embryo transfer
FCS	fetal calf serum
FSH	follicle-stimulating hormone
g	gram
g	relative centrifugal force (RCF)
h	hour
H3-K4	histone H3 lysine 4
H3-K27	histone H3 lysine 27
hCG	human chorionic gonadotrophin
HDAC	histone deacetylase
ICM	inner cell mass
IU	international unit
IVF	in vitro fertilized
i.e.	that is
iPS cells	induced pluripotent stem cells
kV	kilovolt
LH	luteinizing hormone
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
µs	microsecond
M	molar
M199	Hepes buffered medium 199 supplemented with 10% FCS
mA	milliampere
MBD	methyl binding domain protein
MSC	mesenchymal stem cell

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)
NaBu	sodium butyrate
NEBD	nuclear envelope breakdown
ng	nanogram
nm	nanometer
NT	nuclear transfer
ntES	embryonic stem cell derived from nuclear transfer
PBS	phosphate buffered saline
PCC	premature chromosome condensation
PDs	population doublings
PGCs	primordial germ cells
PMSG	pregnant mare's serum gonadotrophin
POU5F1	POU domain, class 5, transcription factor 1
RFF	rabbit fetal fibroblast cells
RT	room temperature
SCNT	somatic cell nuclear transfer
sec	second
V	volt
vs.	versus

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1 INTRODUCTION

Rabbit is a good model for research in reproductive biology and is widely used to develop embryo micromanipulation technologies because of its reproductive and physiological characteristics, such as relatively short gestation period, reasonable amount of milk production, and its developmental biology closely related to large farm animals more than that of rodents (mouse, rat and hamster). Additionally, transgenic rabbits were supposed to produce large scale of therapeutic antibodies for human clinical application. Until now, transgenic rabbits can be produced only by pronuclear microinjection (Hammer et al. 1985; Massoud et al. 1991; Fan and Watanabe 2003) or by the novel technique of chimeric somatic cell cloning (Skrzyszowska et al. 2006). Pronuclear microinjection, however, has a number of disadvantages including a low proportion of transgenic founders, mosaic founders, a low transgenic transmission rate and uncontrolled expression. Nuclear transfer (NT) with transfected somatic cells overcomes all those limitations, and allows a more controlled introduction of transgenes and, in some circumstances, make it possible to preselect transgenic cell lines before the generation of cloned transgenic embryos by analyzing transgene integration sites (Schnieke et al. 1997; Bosze et al. 2003).

Although several research groups have made large amount of work on rabbit cloning using somatic cells (Mitalipov et al. 1999; Dinnyes et al. 2001; Yin et al. 2002a), no live births were obtained in these sudies. The first live somatic cell cloned rabbit, which were derived from freshly collected cumulus cells, were obtained as a result of improved oocyte activation and synchronization of the recipients (Chesne et al. 2002). Later, cloned rabbits from fresh follicular cells, cultured fetal and adult fibroblast cells were obtained by several groups (Challah-Jacques et al. 2003; Li et al. 2006a; Yang et al. 2007). Success of rabbit somatic cell nuclear transfer (SCNT) is expected to resolve some of the difficulities of producing transgenic rabbits by pronuclear microinjection and allow studies on gene knock-in and knock-out strategies with a major potential impact on pharmaceutical and medical applications.

The increasing amount of data on SCNT in different species suggests that cloning efficiency is closely related to incomplete or inappropriate epigenetic reprogramming of donor nuclei. The major players of epigenetic reprogramming are DNA

methylation and histone modifications including acetylation, methylation, phosphorylatioin, and ubiquitination of histone N-terminal tails (Bortvin et al. 2003; Shi et al. 2003). The delayed DNA demethylation, remethylation or absence of demethylation were observed in early bovine and rabbit embryos (Dean et al. 2001b; Shi and Haaf 2002). Aberrant histone methylation and acetylation associated with chromatin configuration (Nguyen et al. 2002) were found also in cloned bovine and rabbit embryos (Santos et al. 2003; Yang et al. 2007). As key chromatin modifications, di- and tri-methylated lysine 4 on histone H3 (H3K4m2/3), an epigenetic mark for transcriptionally competent euchromatin (Santos-Rosa et al. 2002; Schneider et al. 2004), and tri-methylated lysine 27 on histone H3 (H3K27m3), a mark for transcriptionally silent heterochromatin (Brinkman et al. 2006; Trojer and Reinberg 2007), might provide more insights into the relation between donor cell chromatin state and cloning efficiency. The latter modification is mainly found in so-called facultative heterochromatin, which can switch between an active/euchromatic and an inactive/heterochromatic state, as in the case of the inactive X chromosome in female mammals as one of the most prominent examples of facultative heterochromatin (Plath et al. 2003; Silva et al. 2003). Due to their involvement in transcriptional regulation and a dynamic behaviour (Bhaumik et al. 2007) both chromatin modifications appear to be well suited to assess reprogramming effects during NT using donor cell types with different developmental potential.

The aims of the present study were evaluation of the developmental potential of embryos cloned from different types of donor cells and their corresponding patterns of reprogramming hetero- and euchromatic histone modifications. For these purposes, we carried out NT with rabbit mesenchymal stem cells and adult fibroblasts.

2 REVIEW OF THE LITERATURE

2.1 History of nuclear transfer in mammals

The first concept of NT came from Spemann's book, titled <Embryonic Development and Induction> (Spemann 1938). He suggested a radical method for testing nuclear potentiality by isolating the nuclei from progressively older embryos and transferring them into enucleated eggs, and the possibility of NT from a somatic cell to an enucleated egg. But at that time there was no way to perform this kind of experiment due to the technical limitations.

2.1.1 Nuclear transfer using embryonic cells

Nuclear transfer (NT) in mammals began in 1983 with the report from McGrath and Solter regarding the pronucleus exchange experiments in mouse embryos (McGrath and Solter 1983). The NT technique developed by McGrath and Solter was most sophisticated with the use of micropipettes under a microscope. Two cytoskeletal inhibitors (cytochalasin B and colcemid) and a fusion procedure were used to treat embryos. Mouse NT experiments proved that the enucleated mouse one-cell embryos could only support the nuclei from one- or two-cell stage embryos to develop normally. However, transfer of nuclei from mouse eight-cell stage embryos into enucleated two-cell stage embryos resulted in normal blastocysts (Robl et al. 1986) and live mice (Tsunoda et al. 1987), supporting the suggestion that embryos reconstructed from mouse enucleated zygotes and nuclei of late stage embryos cannot develop to term. In 1986, Willadsen published a report on nuclear transplantation in sheep embryos, when donor cells from 8- and 16-cell embryos were fused with enucleated MII oocytes, and live cloned sheep were obtained for the first time (Willadsen 1986). Since then, many animals have been cloned from nuclei of embryonic cells including cattle (Prather et al. 1987; Sims and First 1994), pigs (Prather et al. 1989), goats (Zou et al. 1995), rabbits (Stice and Robl 1988), monkeys (Meng et al. 1997). Scientists at that period believed that somatic cell nuclei cannot give rise to an entire organism, especially in mice, and only nuclei of embryonic cells possess a mysterious power called totipotency and can be reprogrammed in oocyte cytoplasm.

2.1.2 Nnuclear transfer using somatic cells

The birth of sheep Dolly, the first mammal cloned from an adult somatic cell, has made a breakthrough in the field of cloning (Wilmut et al. 1997). This study not only confirmed that the genome may not undergo irreversible modifications, but also opened up a range of new ways to think about the questions that were of current interest, and provided the impetus to develop novel approaches for the genetic manipulation of mammals (Stewart 1997).

After Dolly, 16 other mammalian species have been produced by SCNT including cows (Butler 1998; Cibelli et al. 1998a; Cibelli et al. 1998b), mice (Wakayama et al. 1998), goats (Baguisi et al. 1999), pigs (Polejaeva et al. 2000), gaur (Lanza et al. 2000), mouflon (Loi et al. 2001), domestic cats (Shin et al. 2002), rabbits (Chesne et al. 2002), horses (Galli et al. 2003), mules (Woods et al. 2003), rats (Zhou et al. 2003), African wildcats (Gomez et al. 2004), dogs (Lee et al. 2005a), ferrets (Li et al. 2006c), wolves (Kim et al. 2007) and red deers (Berg et al. 2007). A large number of studies performed on different species tested various types of nuclear donors also with respect to their ability to be reprogrammed and to allow cloned embryos develop to term normally. It is generally accepted that relatively high efficiencies of somatic cell cloning in mammals can be achieved by using donor cells from the female reproductive system [e.g., cumulus/granulosa (Wakayama et al. 1998; Wells et al. 1999), oviduct (Kato et al. 1998) and mammary gland cells (Wilmut et al. 1997; Ogura et al. 2000)]. Cloned animals have also been derived from fetal or adult fibroblast cells (Schnieke et al. 1997; Wakayama and Yanagimachi 1999; Kubota et al. 2000; Onishi et al. 2000) and male Sertoli cells (Ogura et al. 2000). But with other terminally differentiated cells, such as mature B and T cells or neuronal cells, first cloned mice were produced only via a two-step NT involving embryonic stem (ES) cell generation and tetraploid complementation (Hochedlinger and Jaenisch 2002; Eggan et al. 2004; Li et al. 2004). Later, cloned mice from natural killer T cells were produced using direct NT procedure (Inoue et al. 2005). The majority of cell types found in animals have yet to be tested by NT for their developmental potential. In the near future, cloning researches will focus on improvements of the techniques and on selection of the best nuclear donors for NT and genetic modifications.

2.2 Donor cell sources

Developmental potential of a donor nucleus has a great impact on the efficiency of cloning by NT. So far, many undifferentiated and differentiated cells (or cell lines) have been proven to be the sources of donor cells for cloning. One aim for the near future is to establish cell lines, which can be cultured and genetically modified in vitro while their nuclei still maintain the ability to support development of cloned embryos to term.

2.2.1 Embryonic cells

Embryonic cells are isolated from early embryos, which are collected from the reproductive tract(s) of donor animals or obtained via in vitro maturation, fertilization, and culture. In mice, donor cells from 4- and 8-cell embryos were transferred into enucleated 2-cell embryos resulting in live offspring (Tsunoda et al. 1987). Live mice were also obtained when cells from 4- and 8-cell embryos were transferred into enucleated MII oocytes (Cheong et al. 1993). In livestock (cattle, sheep, pig and monkey), there were reports on production of live offspring from nuclei of the 16-cell to morula stage embryos as well as from the inner cell mass (ICM) of blastocysts (Smith and Wilmut 1989; Loi et al. 1997; Wolf et al. 1998; Li et al. 2000; Mitalipov et al. 2002). It is worth to note that transfer of rabbit trophectoderm cells into enucleated oocytes resulted in a striking drop of blastocyst formation compared with the use of cells from ICM (Collas and Robl 1991). However, it has been demonstrated later in mouse that not only ICM nuclei but also trophectoderm nuclei of blastocysts have a developmental totipotency (Tsunoda and Kato 1998).

2.2.2 Somatic cells and cell lines

As mentioned before cloning researches should focus on selection of the best nuclear donors for NT and genetic modifications. Cumulus/granulosa cells (Wakayama et al. 1998; Wells et al. 1999), oviduct cells (Kato et al. 1998), mammary gland cells (Wilmut et al. 1997; Ogura et al. 2000), fetal or adult fibroblast cells (Schnieke et al. 1997; Wakayama and Yanagimachi 1999; Kubota et al. 2000; Onishi et al. 2000), male Sertoli cells (Ogura et al. 2000), mature B and T cells or neuronal cells (Hochedlinger and Jaenisch 2002; Eggan et al. 2004; Li et al. 2004), and muscle cells (Green et al. 2007) have been successfully used as nuclear donors for SCNT.

Many strategies have been performed to modify donor cells for the purpose of improving the efficiency of NT, including using the fresh or in vitro cultured donor cells; using somatic cells from donors of different ages (Tian et al. 2000; Kasinathan et al. 2001a; Xue et al. 2002), tissue origins (Galli et al. 1999; Miyashita et al. 2002; Wells et al. 2003), passages (Arat et al. 2001; Liu et al. 2001b) and culture conditions (Zakhartchenko et al. 1999); synchronization of the cell cycle stage of donor cells and recipient oocytes (Kasinathan et al. 2001b; Gibbons et al. 2002; Wells et al. 2003); and modifying epigenetic status of donor cells with chemicals (Jones et al. 2001; Enright et al. 2003; Yang et al. 2007). Alothough the efficiency of SCNT has been dramatically improved from the initial success rate of one live clone born from 277 embryo transfers (Wilmut et al. 1997), none of the aforementioned efforts accomplished the common problems associated with this technique.

2.2.3 Stem cells

Stem cells are considered as the most promising cells today, because they have abilities to divide without limit to replenish other types of cells in the body as a backup for a sort of repair system. Stem cells include embryonic stem cells, embryonic germ cells, adult stem cells, and cancer stem cells. Two reports provided evidence that cloning efficiency using embryonic stem cells (ESCs) is five to ten times higher than that with somatic cells (Humpherys et al. 2001; Rideout et al. 2001). However, when hematopoietic adult stem cells at various differentiation states were used as donor cells for NT, terminally differentiated granulocytes were superior to less differentiated cells (Sung et al. 2006). It remains yet unclear whether somatic stem cells are better donors for NT than somatic cells or whether state of differentiation is a crucial factor affecting cloning efficiency.

2.2.3.1 Embryonic stem cells

Pluripotent stem cells include embryonic stem cells (ES) and embryonic germ cells (EG). Embryonic stem cells are isolated from morulae or the inner cell mass of blastocysts. The first mouse ES cell line were established by Evans and Kaufman in 1981 (Evans and Kaufman 1981), and the first human ES cell lines were derived by Thomson in 1998 (Thomson et al. 1998; Thomson and Marshall 1998). Pluripotent stem cells or stem-like cells have been reported in many species including mouse

(Matsui et al. 1991), human (Shamblott et al. 1998; Mai et al. 2007), pig (Piedrahita et al. 1990a; Piedrahita et al. 1990b), horse (Saito et al. 1992), cow (Saito et al. 1992; Cherny et al. 1994), sheep (Thuchiya et al., 1994), mink (Sukoyan et al. 1993), rhesus monkey (Thomson et al. 1995), chicken (Pain et al. 1996), common marmoset (Thomson et al. 1996; Sasaki et al. 2005), rabbit (Fang et al. 2006), monkey (Byrne et al. 2007), dog (Schneider et al. 2007) and cat (Yu et al. 2008). But the majority of ES cell lines from large animal hardly contributed to germ line. The reason might be that these the ES cell lines require a culture system which is different from the one used in the mouse, or there are factors or molecules which are yet unknown to control their differentiation. So they were named as embryonic stem-like cells in the most cases. The common essential characteristics agreed by many scientists of primate ES and EG cells should include derivation from the pre-implantation or post-implantation embryo, prolonged undifferentiated proliferation, and stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (Thomson et al. 1998).

Due to the characteristics of ES cells (long term culture in vitro, while maintaining pluripotentcy), their genetic modification is possible. ES cells are a very effective tool to study the expression and function of genes both in vivo and in vitro, which had never been possible before. Usually, the mouse model is used as an example to explain how the gene targeting can be performed since mouse ES cell lines are the only available over the world. Any gene in the mouse genome may be inactivated (knock-out) or inserted at a specific locus (knock-in) by homologous recombination, and gene targeting events are then identified by PCR and Southern blot. After analysis, positive ES cells are reintroduced back to mouse blastocysts to produce chimeras. Finally, transgenic individuals will be produced, if the chimeras have transgenic cells in the germ line.

Reports on the derivation of human ESC lines and EGSC lines have encouraged scientists to carry out more research on primate ES cells and to realise their potential applications in curing human diseases. If the cloning procedure is combined with human ES cell technology (called human therapeutic cloning), the immune compatibility and graft rejection in cell and/or tissue transplantation may be overcome. In this technology, the donor cells from patients themselves are transferred into enucleated human or animal oocytes to construct cloned embryos. The cloned

embryos are then cultured in vitro to produce blastocysts. ES cells are isolated from the ICM of these cloned blastocysts and then will be differentiated into different cell types and even tissues or organs for transplantation back to the patient when needed.

Very recently, after adding four genes – Oct4, Sox2, cMyc, and Klf4 - into mouse and human fibroblasts, cells showed morphology and proliferation similar to ES cells under exposing in careful culture conditions (Takahashi et al. 2007a; Takahashi et al. 2007b). When transplanted these cells into nude mice, teratomas containing various tissues of the three germ layers were produced. These "Induced pluripotent cells" (iPS cells) may help to resolve the difficulties of clinical applications of ES cells, including use of embryos and tissue/organ rejection after implantation. A quite new report showed that exogenous Oct4 together with either cMyc or Klf4 is sufficient to genetate iPS cells (Kim JB et al. 2008). However, this technique is at the beginning phase, still is so far to be perfect. For example, the exogenes are still necessary being added to permanently change the cell's genetic material; the process of the adding genes to be silenced during differentiation is still not entirely understood; at least cMyc is cancer-causing gene. All those defects have not been yet resolved. In new reports where more types of cells were induced into iPS cells or iPS cells were differentiated into other cell lines (Narazaki G et al. 2008; Park et al. 2008). Therefore, the question whether iPS cells are too dangerous to treat patients remains to be answered.

2.2.3.2 Adult stem cells

Adult stem cells, also know as somatic stem cells, are undifferentiated cells found throughout the body after embryonic development that divide to replenish dying cells and regenerate damaged tissues (Gardner 2002). As a main advantage, the use of adult stem cells in research and therapy is not controversial because adult stem cells are isolated from adult persons or children, that does not involve the destruction of an embryo (Zuk et al. 2002; Liu et al. 2005; Murrell et al. 2005). They have mainly been studied in humans and model organisms as mice and rats (Barrilleaux et al. 2006; Takahashi and Yamanaka 2006; Humphreys et al. 2008).

Meschymal stem cells (MSCs) from bone marrow (BM) are on the leading edge because they are easy to expand in culture while maintaining their multilineage potential. MSC is the first type of adult stem cells that has been used for NT. Kato et

al. (2004) produced one healthy calf from a MSC. Usually, mesenchymal precursor mononuclear cells can be separated by centrifugation of bone marrow (BM) aspirates through a solution of Ficoll or Percoll. Such mononuclear cells have been isolated from the BM of many mammals, including laboratory rodents (Friedenstein et al. 1976; Simmons et al. 1991), human (Pittenger et al. 1999), cats (Martin et al. 2002), dogs (Huss et al. 1995), pigs (Ringe et al. 2002) and rabbits (Wakitani et al. 1994). The MSCs from all species studied to date proliferate ex vivo as adherent fibroblast-like cells, a feature that has been exploited to enrich MSCs from hematopoietic stem cells (HSC) that normally remain in suspension. They are similar in that they are very rare, existing at an estimated frequency of about 1 in 100,000 bone marrow cells (Galotto et al. 1999). In cattle and pigs, even genetically modified MSCs retained the ability to differentiate as parental lines and supported preimplantation development of cloned embryos at the rates similar to those obtained with non-genetically manipulated MSCs (Colleoni et al. 2005; Bosch et al. 2006). All these findings show that MSCs are easy to isolate and to culture in vitro, can be genetically modified and used for cloning by NT.

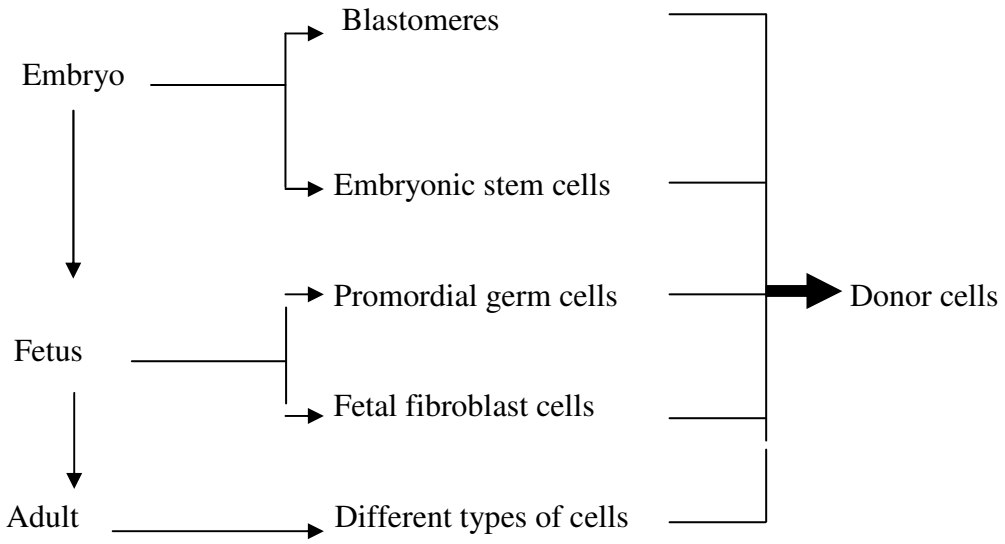

Fig. 1 Types of donor cells used for cloning

2.3 Nuclear reprogramming of cloned embryos

Despite the fact that the cloned animals derived from somatic cells have been successfully generated in 16 mammalian species, there are still so many unresolved problems with current cloning research, for example the low success rate of cloning

(less than 5%) (Tamada and Kikyo 2004), a high rate of abortion during early gestation and abnormalities in cloned animals. When and how these unexplained congenital malformations originate has been discussed in the progress of NT (Doherty et al. 2000; Dean et al. 2003). It is generally accepted that the defects are related to the reprogramming of specific nuclear activities in cloned animals. All these activities involve remodelling of the epigenetic features that overlay the gene sequences and find interpretation in new gene expression, but they do not relate to the change of the genetic sequences that comprise the genome (Surani 2001), that is epigenetic modification. Epigenetic modifications play vital roles in genome reprogramming during SCNT and include DNA methylation, histone modifications, telomere length regulation, X chromosome inactivation, genomic imprinting and other mechanisms.

2.3.1 DNA methylation during embryonic development of normal (in vivo derived) and cloned embryos

DNA methylation is the best characterized epigenetic mechanism and well known epigenetic regulator of gene expression. In mammals, between 60-70% of all CpGs (the 5'-cytosine residues at CpG dinucleotides) islands are methylated (Haines et al. 2001). DNA methylation may affect the transcription of genes by physically impeding the binding of transcriptional proteins to the gene, thus blocking transcription, or by binding with proteins known as Methyl-CpG-binding domain proteins (MBDs) to the gene.

The dynamics of DNA methylation in in vivo embryos must be understood if nuclear transfer is to succeed. It is widely agreed that, for example in mouse, normal fertilized embryos have global changes of DNA methylation (Dean et al. 2003). Fertilization initiates the decondensation of the sperm nucleus, resulting in the unwinding of the tightly packaged sperm DNA held in a unique, almost toroidal conformation by the sperm-specific protamines (Braun 2001). After fertilization, the paternal genome becomes actively demethylated in contrast to the maternal genome, which occurs to be passively demethylated during cleavage-stage divisions (Mayer et al. 2000). The global level of DNA methylation maintains at the lowest level in the morula and blastocyst stages, until sudden genome-wide de novo methylation (re-methylation) occurs by DNMT 3a and 3b (DNA methyltransferase) when embryos get implantation (Dean et al. 2001a).

Cloned embryos with methylation dynamics similar to normal embryos, have more chance to erase the tissue-specific DNA methylation pattern and to establish a new embryo-specific DNA methylation pattern on numerous genes successfully. A large proportion of cloned embryos showed abnormalities in the genome-wide DNA methylation levels and DNA methylation patterns on various repetitive sequences (Dean et al. 2001a), and high variability in DNA methylation levels among individual clones (Kang et al. 2001). One interesting observation was made in bovine cloned embryos when DNA methylation was undetectable in six out of nine spontaneously aborted bovine clones, but the methylation levels were normal in the clones that survived to adulthood (Cezar et al. 2003). In constract to this study, hypermethylation of DNA in fetal tissue, but not placenta, was linked to the over-growth phenotype of bovine SCNT and IVF fetuses (Hiendleder et al. 2004). A new interesting report showed that the methylation levels of IVF blastocysts were much more similar to NT and parthenogenetic blastocysts than to in vivo blastocysts (Bonk et al. 2008).

DNA methylation defects might be attributed to the specific features of the somatic chromatin structure and/or defective regulation of DNA methyltransferases (DNMTs) (Tamada and Kikyo 2004). For example in mouse, cloned embryos expressed not only the somatic form of DNMT1 at abnormally high level, but also showed defective nucleo-cytoplasmic translocation of the oocyte form of DNMT1 (Chung et al. 2003). The defects in DNA methylation can be also caused by culture conditions of cloned embryos as in vitro culture reduced the methylation level in the regulatory CpG site of the *H19* gene depending on the culture medium (Doherty et al. 2000).

2.3.2 Genomic imprinting

Genomic imprinting is an important mechanism in mammalian development by which some genes are activated depending upon their parental origin. In other words, it means that the activity of certain genes depends on whether the chromosome in which they are located is derived from mother or father. In several imprinted loci, imprinting control elements have been identified that are methylated on one of the parental copies of the gene. Imprinted genes frequently reside in clusters with genes expressed on opposite chromosomes being located adjacent to each other. Differential methylation of imprinted genes probably occurs during gametogenesis. For the gamete to acquire a sex-specific imprint, the previous genomic imprint must be

erased. It appears that methylation patterns are erased in the primordial germ cells of the midgestation mouse embryo.

As demonstrated in uniparental embryos (McGrath and Solter 1984) and NT embryos derived from male primordial germ cells (PGCs) (Kato et al. 1999) or nongrowing oocytes (Kono et al. 1996), genome-wide imbalance in imprinted gene expression or its disruption results in postimplantation lethality. Early mouse nuclear transplantation studies showed that both paternal and maternal DNA was essential for the development of embryos in mammals (McGrath and Solter 1984; Surani et al. 1984). Gynogenetic embryos (containing two female pronuclei) are normal but small, and their extraembryonic tissue is very poorly developed. Androgenetic embryos (containing two male pronuclei), on the other hand, are quite retarded, whereas their extraembryonic tissue is well developed. The two parental genomes apparently function in a complementary fashion during development. Regions of paternal chromosomes are apparently pre-reprogrammed to direct proliferation of extraembryonic tissues, whereas certain maternal chromosomal regions are pre-reprogrammed to direct development of the embryo body (Browder et al. 1991).

Recently, viable mice have been generated from engineered bi-maternal embryos produced by the construction of oocytes from fully grown and nongrowing oocytes that contain double deletions in the H19 differentially methylated region (DMR) and the Dlk1- Dio3 intergenic germline–derived DMR (Kawahara et al. 2007). These results demonstrated that imprinted genes regulated by these two paternally methylated imprinting-control regions are the only paternal barrier that prevents the normal development of bi-maternal mouse fetuses to term.

As demonstrated using conditional knockout technology (Kaneda et al. 2004), fetuses from *Dnmt3a* mutant females die in utero and lack methylation and allele-specific expression at all maternally imprinted loci examined; fetuses from *Dnmt3a* mutant males show impaired spermatogenesis and lack methylation at two of three paternally imprinted loci examined in spermatogonia. This provided the first evidence that DNMT3a is required for the establishment of both maternal and paternal imprints. However, both 102 *Dnmt3b* mutant males and 88 mutant females were found to be normal in the status of imprinting genes.

Embryos produced using assisted reproduction technologies (ART) in human or by NT in several species showed widespread methylation defects in imprinted genes

(Humpherys et al. 2002; Mann et al. 2004). The abnormalities in the imprinted genes from the NT embryos may indicate susceptibility of the methylation imprinting in the somatic nuclei to the global methylation changes during early developemt (Tamada and Kikyo 2004).

2.3.3 Histone modifications

Histone modifications are associated with important biological processes such as cell proliferation, gene regulation, and DNA repair (Kothapalli et al. 2005).

The modifications of the the H3 and H4 histones are considered as the key chromatin modficiations (Santos-Rosa et al. 2002; Schneider et al. 2004). For example in mouse, histone H3 and H4 are widespread deacetylated on several lysines at the metaphase II of the second meiosis, and similar events occur in somatic nuclei transferred into oocytes of the same stage (Kim et al. 2003). One study on fertilized bovine embryos showed that H3-K9 becomes hypoacetylated at the 4-cell stage and gradually hyperacetylated after the 8-cell stage, while in cloned embryos H3-K9 was found to retain hyperacetylation at 4- and 8-cell stages (Santos et al. 2003).

Nearly 50% of cloned bovine and sheep blastocysts were aberrant in genome-wide patterns of both DNA methylation and histone acetylation and exhibited a high degree of methylation errors at specific loci (Shiota and Yanagimachi 2002; Han et al. 2003; Santos et al. 2003). Although more and more studies provide evidence for abnormal reprogramming of histone modifications following SCNT (Santos et al. 2003; Enright et al. 2005; Suteevun et al. 2006), the molecular mechanism(s) underlying the possible reasons of such failures are not yet well understood. However, the use of some chromatin modifying chemicals like the histone deacetylase (HDAC) inhibitors, trichostatin A (TSA) and sodium butyrate (NaBu), to treat donor cells can correct to some extent the process of nuclear reprogramming and increase cloning efficiency (Kishigami et al. 2006; Yang et al. 2007).

2.4 Steps of NT

Each step of NT affects the overall efficiency of this technique. Standardization of the steps is important to obtain consistent results, and improvements in technical steps will definitely affect both pre- and postimplantation development of the reconstructed embryos. However, due to a biological variation of the recipient oocytes and donor

cells, which is difficult to control, the efficiency of this technology is unpredictable and remains yet to be largely improved.

2.4.1 Embryo reconstruction

For embryo reconstruction by NT, enucleation (removal of the maternal DNA content) and introduction of a donor nucleus into a recipient cytoplasm must be performed using a variety of methods. The conventional method is, firstly, to enucleate the oocyte to generate a cytoplastst with a sharp pipette, and secondly, to transfer the donor nucleus under the zonae pellucida. Because mouse oocytes are too easy to be damaged by injection pipette, Piezo-driven enucleation device was used to improve the penetration of very flexible zonae pellucidae (Wakayama et al. 1998). The first cloned rats were produced only by using a Piezo-driven injection of somatic cell nuclei (Zou et al. 2003). In farm animals, Piezo-device has not been adopted as enucleation can be successfully achieved without it. Whatever, injection of a cell nucleus directly into the cytoplasm using Piezo device may improve nuclear reprogramming allowing better access of cytoplasmic factors to the nuclear DNA.

NT can be also performed by so-called hand-made cloning (HMC) without the use of a manipulator unit (Vajta et al. 2001). HMC comprises of bisecting oocytes, seclecting those half cytoplasts that do not contain the metaphase plate and adhering two half cytoplasts together with one donor cell by using phytohaemagglutinin (PHA), a cell-aggultinating lectin glycoprotein (Sharon and Lis 1989), followed by an electropulse. Birth of cloned calves, pigs and horse from HMC were reported by several groups (Vajta et al. 2004; Hall et al. 2006;Kragh et al. 2004; Lagutina et al. 2007).

Using chemicals to assist enucleation can be also one of the approaches to improve embryo reconstruction by NT. The successful chemical enucleation of mouse oocytes have been reported using demecolcine (colcemid) to induce a membrance protusion containing a condensed chromatin mass in oocytes, which was subsequently mechanically removed (Baguisi and Overstrom 2000). Cloned pigs and rabbits were produced using chemically assisted enucleation (Yin et al. 2002b; Yang et al. 2007).

Serial NT (comprising of two or more NT steps) was also designated to improve cloning efficiency. Briefly, the first NT is carried out according to conventional methods. In the second round, a blastomere (karyoplast) from 2- to 8-cell cloned

embryos was introduced into enucleated zygotes or 2-cell stage embryos (Kwon and Kono 1996). This second round of NT was considered to help the somatic cell–derived pronuclei of the cloned embryos reprogramming into a naturally fertilized pronuclear stage cytoplast, from which the pronuclei were previously removed. In pigs (Polejaeva et al. 2000), mice (Amano et al. 2001; Ono et al. 2001) and cattle (Hall et al. 2006), serial NT was successfully applied.

2.4.2 Cell cycle synchronization between donor cells and recipient oocytes

The mature metaphase II (MII) oocytes are considered to be the most appropriate recipients for cloning by NT. These oocytes are at the stage MII of meiosis. When a donor cell is fused with or directly injected into the enucleated oocyte, and the reconstituted oocyte is activated, the cloned embryo starts its first cell division. During very early events in the reconstructed embryo the co-ordination of the cell cycles of a donor nucleus and a recipient oocyte plays a crucial role.

A review by Campbell et al (1996) summarised the relation between the cell cycles stages of donor cell and recipient cytoplasm in nuclear transplantation. When nuclei, regardless of their cell cycle stage, were transferred into MII oocytes, they underwent nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC), and then started DNA synthesis after reformation of the nuclear envelope. Theoretically only nuclei in G1 phase could support the development of cloned embryos to term when transferred into enucleated MII oocytes, and nuclei in G2 or S phases would produce chromosome abnormalities in cloned embryos which most likely fail to develop (Campbell et al. 1996; Wilmut et al. 1997). The cell cycles of donor nucleus and recipient cytoplasm are important factors for the successful development.

Use of nuclei in M phase of cell cycle in mouse nuclear transplantation appeared to be very successful. A set of sextuplet mice from a single four-cell stage embryo was produced (Kwon and Kono 1996). When nuclei at M phase are transferred into MII oocytes, the cell cycles of both nucleus and cytoplasm are perfectly matched. After activation, the reconstituted embryos cultured in vitro in a medium without cytochalasin B (CB), would expell a polar body to become normal diploid embryos. The advantages of usage of M phase nuclei are: a) synchronisation is simple and successful by applying inhibitors of tublin polymerisation, such as nocodazole and/or

thymidine, which are not toxic to embryo development; b) it can avoid the delay of chromosome formation in cloned embryos after donor cell transfer. Two mice were cloned from metaphase embryonic stem cell nuclei (Zhou et al. 2001).

Wilmut and Campbell (1998) argued that serum depletion of a cell culture medium to achieve the quiescent phase of the donor cell cycle was a key factor to clone the first cloned sheep "Dolly". Quiescense is achieved by culturing the cells in vitro in a defined medium with 0.5% or less FCS for a few days (serum starvation). But another group headed by Robl and Stice (Cibelli et al. 1998b) produced three cloned calves from non-quiescent cycling foetal fibroblast cells. Since the birth of Dolly, thousands of cloned animals have been produced in different species with or without serum starvation thus suggesting that the quiescent state of a donor nucleus is not a critical prerequisite for successful cloning by NT.

2.4.3 Activation of reconstructed embryos

In normal development, a sperm activates an oocyte, i.e. triggers the release from the meiotic arrest and subsequent development. It is generally accepted that the sperm induces oocyte activation by generating temporary transient increases in the intracellular free Ca^{2+} concentration, which in mammals takes the form of a series of repetitive Ca^{2+} transients that last for several hours (Whitaker and Swann 1993).

The most commonly used method for activation in nuclear transplantation at the early days was applying the electric stimulation to the reconstructed embryos. The electric stimulation, which also induces the fusion between the donor cell and recipient cytoplasm, results in the formation of temporary pores in the plasma membrane allowing an exchange of extracellular and intracellular Ca^{2+} and macromolecules to produce only a single Ca^{2+} transient (Rickords and White, 1992). Clearly, this demonstrates that the intracellular Ca^{2+} flux associated with fertilisation has not been reproduced by simple electric stimulation procedure. This might partially explain why most of the cloned embryos fail to develop normally.

Another method of activating reconstructed embryos along with elevation of Ca^{2+} using electric pulse or some chemicals is subsequent treatment with compounds that affect protein kinase activity. 6-dimethylaminopurine (6-DMAP) is a protein kinase inhibitor (Susko-Parrish et al. 1994) and cycloheximide (CHX) is a protein synthesis inhibitor (Presicce and Yang 1994), both of them could reduce the synthesis of cyclin

B in the embryos. Cyclin B is a component of metaphase promoting factor (MPF). In order to maintain adequate levels of active MPF, it must be continually synthesized. With the decline of MPF activity, the reconstructed embryos enter the first inter-phase (Collas et al. 1992; Alexander et al. 2006). Experiments have proved that better activation and development of cloned embryos can be obtained if two or more activation methods are combined together (Alexander et al. 2006).

2. 5 Applications and prospects of cloning

2.5.1 Identical animals for research and clinic trials

In research and clinic trials, the biggest problem is that the animals used are not genetically identical, which often produces a range of different or in some cases false results. In 1997, the SPF (specific pathogen free) animals (especially mice) have been available (Jacoby and Lindsey 1997). Because of their SPF status, they produce better results in experiments, but the results are still not consistent. Although identical twins are produced naturally or can be created by embryo splitting, the number is limited for scientific research. Cloning mat pave the way to produce large numbers of genetically identical animals for scientific research and clinic trails. Nevertheless, epigenetic differences among clones from the same genotype may cause problem.

2.5.2 Production of a large flock of the same quality and genetic background farm animals (reproductive cloning)

High quality farm animals are rare and very important for consumers and farm industry. Cloning can reproduce the animals with unique genetic background at a large scale. Perceived benefits of the cloning of farm animals include a range of possibilities within basic research, biomedical research and agricultural production. Applications within the areas of improving animal welfare, the recreation of extinct species, and the conservation of endangered and threatened species are also worth mentioning (Vajta and Gjerris 2006).

2.5.3 Rescue of endangered species

Expanding populations of endangered species for which breeding programmes or the availability of oocytes are limiting, is one of the most exciting potential opportunities

provided by SCNT. Approximately 100 species become extinct a day around the world. Extinction threatens 11% of birds, 25% of mammals, and 34% of fish species (Porter 2000). Cloning may offer a strategy for rescuing those endangered species. Lanza et al. (2000) reported several pregnancies after transfer of somatic cells from a gaur bull (Bos gaurus), a large wild ox on the verge of extinction, into enucleated oocytes from domestic cows. The gaur nuclei were shown to direct normal fetal development, with differentiation into complex tissue and organs, even though the mitochondria DNA (mtDNA) within all the tissue types evaluated was derived exclusively from the recipient bovine oocytes. This study illustrates both the viability and the likely limits of such an approach. Successful production of cloned progeny has been achieved mainly in closely related species, for example Ovis genera and Bos (Loi et al. 2001; Meirelles et al. 2001; Arat et al. 2003). It appears development efficiency of intra-species cloning tended to be higher than that of inter-species cloning (Li et al. 2006b), and the more species, the donors of cells for NT and recipient oocytes, close to each other the more greater blastocyst development in vitro (Loi et al. 2007). NT using more divergent combinations of donor cells and recipient oocytes lead to early developmental arrest (Arat et al. 2003), as indicated by the facts that only 6.2% of cloned embryos developed beyond the 8-cell stage when mouse embryonic fibroblasts were transferred into bovine oocytes, and 3% and 2% blastocyst rates were reported when transferring donor cells from marbled cats or domatic cats into rabbit oocytes, respectly (Thongphakdee et al. 2006). It is possible that further refinements in culture systems and NT steps could facilitate the development of inter-species clones to term.

2.5.4 Genetically modified animals for bioreactor factories and cancer research

One of the major applications of SCNT is production of transgenic cloned animals from donor cells whose genome has been modified (Table 1). There are a number of methods for the generation of transgenic animals, including pronuclear microinjection (Brinster et al. 1985), retroviral vectors (Chan et al. 2001), electroporation (Zou et al. 1995) sperm carrier of introducing a foreign gene(s) into the genome of donor cells (Shemesh et al. 2000). Lentiviral vectors and small interfering ribonucleic acid (siRNA) technology are also becoming important tools for transgenesis (Clark and Whitelaw 2003). Genome modifed animals have numerous attractive applications in

biomedicine such as production of recombinant human proteins, nutriceutical production, blood replacement, xenotransplantation, and genetic models for human disease as well as for improving livestock production traits including disease and disease resistance, more efficient feed conversion, and milk composition for manufacturers.

The first transgenic mammalian clones expressing human factor IX were two lambs produced at the Roslin Institute by using transfected fetal fibroblast cells (Schnieke et al. 1997). The cultured cells were transfected with DNA constructs composed of the coding sequences for neomycin resistance and human clotting factor 1X placed downstream of the promoter sequence for ovine β-lactoglobin. The cells were then exposed to G418, killing all cells except those expressing neomycin resistance. The surviving cells having integrated both the gene for clotting factor 1X and the promoter sequence were used for NT. The promoter sequence was included to ensure that the clotting factor gene would function specifically in the mammary gland of the transgenic sheep to be produced. The surviving transgenic sheep, Polly, has indeed produced milk containing human clotting factor IX (Wilmut and Campbell 1998).

Since then, transgenic clones of calves, goats, pigs, mice and additional sheep were produced from transgenic fetal cells. Three cloned calves expressing the neomycin resistance transgene (Cibelli et al. 1998b) and three cloned goats containing the human antithrombin III gene (Baguisi et al. 1999) were generated in the studies when the respective genes were inserted at random into the genome of the donor cells. Later, a gene targeted sheep was created in Edinburgh (McCreath et al. 2000). Human a1-antitrypsin (AAT) under beta-lactoglobulin (BLG) promoter control was homologously integrated into the a1(I) procollagen (COL1A1) locus by cloning. One lamb has been induced to lactate a favorable quantity of the human protein in her milk. This report showed for the first time that gene targeting in farm animal can be achieved by transfecting fetal fibroblasts, selecting them for homologous recombination, and using the targeted cell clones for nuclear transfer (Fig. 2). As there were no reliable ES lines in large domestic animals, cultured fetal cells have been used for inserting the respective transgenes.

Fortunately, mouse ES cell lines were established in 1981 (Evans and Kaufman), and the production of transgenic mice through the use of ES cells has offered a powerful method to study gene expression and gene function (Pirity et al. 1998). Wakayama et

al. (Wakayama et al. 1999) obtained 26 cloned mice from late passages of an established ES cell line with a success rate of 8% based on the number of cloned embryos transferred to surrogate mothers. Subsequently, after integrating a tet-transactivator gene (rtTA2ASD) into the ROSA26 locus of ES cell lines via homologous recombination, a single cloned mouse was produced and developed into a fertile mouse expressing the transgene (Rideout et al. 2000). Although the proportion of transgenic newborns was low, the success was remarkable. A few founder clones would be that necessary minimum to start a breeding programm for transmitting and expressing the respective transgenes. Although, the production of large transgenic animals has been hampered by the lack of their respective ES cells, production of recombinant human proteins in the milk of transgenic dairy animals offers a safe, renewable source of commercially important proteins that cannot be produced as efficiently in adequate quantities by other methods. The first three transgenically produced recombinant proteins (Human factor VIII, Anti-thrombin III and Erythropoietin) are in the phase III of clinical trials (see table 2).

Table 1 Transgenic mammalian clones

Species	Transgene	Offspring/embryos transferred No.(%)	Reference
Sheep	human clotting Factor IXa	3 (7)	Schnieke et al. 1999
	human alpha 1-antitripsin [a,b]	14 (3)	McCreath et al. 2000
Cattle	neomycin[r]	3 (11)	Cibelli et al. 1998
Goats	human antithrombin IIIa	3 (3)	Baguisi et al. 1999
Mice	Tettransactivator [b,c]	1 (14)	Rideout et al. 2000
Pigs	human complement CD59 (hCD59)	- (-)	Imaizumi et al. 2000
Cattle	r28M	11(77)	Grosse-Hovest et al. 2004
Rabbits	Tg(Wap-GH1)	24(163)	Skrzyszowska et al. 2006

[a]: Integrated into fetal cells; [b]: embryonic stem cells; [c]: via gene targeting; [r]: resistance

Table 2. Partial list of medical-valuable proteins produced via SCNT

Proteins	Main functions	Application	Market value (Million dollar /year)
ATIII	Regulating blood clotting	Surgical operation and ATIII mutation disease	200
Human serum albumin	Artificial human blood	Transfusion	1500
Beta-Interferon	Increasing immune defence	Cancer and immune deficiency diseases	500
Calcitonin	Bone metabolism	Osteoporosis	1000
Insulin	Sugar metabolism	Diabetes	1500
Human growth hormone	Body height	Dwarfism	1000

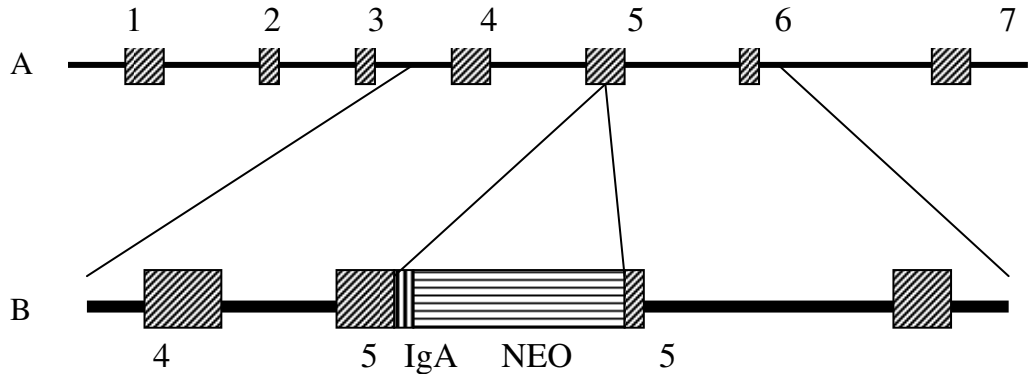

Fig. 2 Gene targeting of Beta-lactoglobulin

A. Genomic map of beta-lactoglobulin, ▨ indicated exons;

B. Gene target construct, NEO (neomycin) is a selective marker gene.

The deletion or mutation of any gene of interest in mammal is becoming into the most exciting field, which transforms the landscape of mammalian biology research. Knock-in or knock-out animal models provide valuable research tools for dissecting the biochemical pathways responsible for neoplasia and for testing new therapeutic agents. The first knock-out pigs were produced at 2002 (Lai et al. 2002). These authors disrupted the sequence of the α-1,3-galactosyltransferase gene (*GGTA1*) in fibroblasts using homologous recombination, then transferred these cells into enucleated oocytes, and finally obtained four knock-out pigs. The important practical significance of this research was to facilitate studies on xenotransplantation of pig organs to humans, as elimination of terminal α-1, 3-galactosyl epitopes from the pig was expected to resolve the problem of hyperacute and delayed vascular rejection. Another useful model is BHD (Birt-Hogg-Dube) knock-out mouse. Because renal cystadenocarcinoma nodular dermatofibrosis (RCND) in dogs (Lium and Moe 1985; Lingaas et al. 2003) and renal tumors in the Nihon rat (Okimoto et al. 2004) occur in animals that inherit a germline mutation in the corresponding BHD homolog, these naturally occurring animal models may harbour additional genetic changes that could confound studies of the functional consequences of BHD inactivation. A genetically engineered mouse model is necessary to provide a "clean" system to pursue functional studies of folliculin (FLCN). The targeted BHD knock-out mouse model was established by injection the BHD targeted embryonic stem cells into mouse blastocysts to produce chimeras (Baba et al. 2008). As the proof of the great scientific achievement in this field, three scientists, Mario Capecchi, Martin Evans, and Oliver Smithies, have been honoured by Nobel medicine prize at 2007 for significant contribution in establishment of the knock-out technology.

In summary, cloning with transgenic somatic cells offers similar advantages as with mouse ES cells in the production of transgenic animals because somatic cells can also be cultured, genetically modified and maintained in vitro over a long period and still are able to be reprogrammed to develop to term.

2.5.5 Cloned pigs for tissue and/or organ transplantation

The transplantation of organs from other species into human is considered to be a potential solution to the shortage of human donor organs. Organ transplantation from pig to human, however, results in hyperacute injection. Transgenic, gene targeting and

cloning technologies could be used to produce strains of pigs, which are labelled with human immune marker, or from which endogenous genes are deleted to avoid hyperacute rejection and acute vascular rejection.

The key enzyme responsible for the rejection is the *GGTA1* gene, which has opened up several novel therapeutic approaches to prevent hyperacute vascular rejection (Joziasse and Oriol 1999). As mentioned before, the first GGTA1 knock-out pigs were produced at 2002 (Lai et al. 2002). Recently, recloning method for production of *GGTA1* knockout pigs resulted in a higher pregnancy rate compared to that using direct NT (50 vs. 20%, respectively) (Fujimura et al. 2008). Molecular cloning, and expression in vitro of *GGTA1* cDNA, has allowed the development of strategies to induce immune tolerance, and deplete or neutralise the natural xenoreactive antibody.

2.5.6 Therapeutic cloning

The aim of therapeutic cloning is to produce human embryos not for generating cloned human beings, but rather to harvest stem cells that can be used to study human development and to treat disease. Using SCNT, "personalized" ES cell lines could be obtained from the cloned blastocysts (ntES). Such cell lines would offer the following advantages: a) minimize the risk of tissue rejection after autologous transplantation; b) allow for the correction of genetic defects; and c) serve as cell culture models to study pathomechanisms and disease progression. The ntES cells, which for the fist time were created in mice, had functions similar to those of fertilized embryo–derived ES cells (Wakayama et al. 2001). Later, when comparing the transcriptional profiles of multiple ntES cell lines to ES lines, Brambrink et al. found that the molecular characteristics of the lines were largely indistinguishable (Brambrink et al. 2006), suggesting that ntES cells are similar to ES cells, both in genetics and functions.

In 2001, scientists from Advanced Cell Technologies (ACT), a biotechnology company in Massachusetts, announced that human embryos had been cloned for the first time as the purpose of advancing therapeutic research. The research was carried out with eight woman oocytes, three began dividing, and only one was able to divide into six cells before stopping, finally no ntES cell lines have been derived. The results were limited in success. Still, derivation of ntES cell lines from humans has not yet been successfully performed. The field took a major step backward on the announcement that the Korean scientist Hwang and his group had fabricated data

outlining the derivation of ntES cell lines from humans (Lee et al. 2005a). Sometime later, their embryos were clearfied as parthenotes. Presently, there is a report on development of human nuclear transfer embryos to the blastocyst stage, but no generation of ntES cell lines (French et al. 2008). Two major limitations to the success in this research include the small numbers of human oocytes available and the low efficiency of successful nuclear reprogramming.

A recently described technique of isolating ES cells from the 8-cell embryos may improve the efficiency of ES and ntES cell derivation. For example, in both mouse and human studies, researchers at Advanced Cell Technologies generated ES cell lines from single-cell biopsies of the 8-cell embryos (Chung and Becker 2006; Chung et al. 2006; Klimanskaya et al. 2006). This technique may soon allow for more efficient ntES cell line isolation because nuclear transfer embryos increasingly fail to develop as culture time lengthens, and this technique also allows for the generation of ES or ntES cell lines without significantly damaging the remaining embryo from which the single cell originated.

There are technical and ethnic difficulties in obtaining human stem cells and using them for therapeutic purposes. In ten to twenty years' time, the technical difficulties might be overcome. But the ethical questions may hinder the development of this technology, and we have to weigh out between saving lives or having good lives and ethical concerns. Embryonic stem cells are not genetically matched to the patients and therefore not immuonologically competent for cell therapy. Only stem cells from the same patient are safe in the respect of avoiding the use of drugs to suppress immune response. Adult stem cells probably will the better choice in therapeutic purposes, but have a limited multilineage differentiation potential.

3 MATERIALS AND METHODS

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

3.1 Animals

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

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

Rabbits were maintained in 70 x 70 x 60 cm cages. They received deukanin energie pellets (Deuka, Etteldorf, Cat. No. 76320) and water daily ad libitum and hay.

3.2 Collection of recipient oocytes

Oocytes were collected from superovulated ZIKA® rabbits 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 using pre-warmed in 25°C mPBS (phosphate-buffered saline supplemented with 4 mg/ml BSA), then the oocyte-cumulus complexes were incubated in 0.5% hyaluronidase in M199 (Hepes buffered-medium 199 supplemented with 10% FCS) for 15 min in 5% CO_2 and 38.5°C incubator. The cumulus cells were removed by gentle pipetting.

3.3 Induction of MII metaphase protrusion and enucleation

Denuded oocytes were treated with 0.6 μg/ml demecolcine in M199 between 20 min to 2 h (Yin et al. 2002a; Yin et al. 2002b). The resulting MII metaphase protrusion with little underlying cytoplasm was removed in M199 supplemented with 7.5 μg/ml CB and 0.6 μg/ml demecolcine. Enucleated oocytes were washed and kept in M199 in an incubator for later use as recipient cytoplasts.

3.4 Preparation of donor cells: MSCs and RAFs

3.4.1 Establishment of primary culture of MSCs and RAFs

3.4.1.1 Isolation and culture of MSCs

a) Femurs of the rabbits (aged between 4~8 week old) were removed and cut under sterile conditions.
b) Bones were washed 3 times with Ca^{2+}- and Mg^{2+}- free PBS containing 100 IU/ml penicillin and 100 µg/ml streptomycin, and then muscles covering the bones were removed.
c) Bones were flushed with PBS containing 250UI/ml heparin to collect bone marrow (BM) (~20ml).
d) BM were put on the surface of Percoll (density, 1.077) in 2:1 and centrifuged at 800 g for 20 min at room temperature.
e) The mononuclear cells were recovered with pipette from the opaque interface and washed twice with culture medium, MesenCult Medium (Basal Medium for Mouse Mesenchymal Stem Cells (Cat: 05501) and Supplement Medium for Mouse Mesenchymal Stem Cells, StemCell Technologies, Vancouver, Canada) (MSC medium).
f) Cells were cultured in MSC medium in a humidified atmosphere of 5% CO_2 in air at 37°C.
g) After 24h culture, unattached cells were washed off during medium exchange. Adherent fibroblast-like cells were expanded for 7~10 days with medium replacement every third day. Cells were passaged after treatment with 0.05% (w /v) trypsin/EDTA and reseeded in 1:2.

3.4.1.2 Isolation and culture of RAFs

a) Small ear skin biopsies of young rabbits were cut under sterile conditions.
b) The tissues were washed three times with PBS and hairs were removed by sterile scissors.
c) Tissues were minced into 1x1 mm small pieces by sharp-tip sterile scissors and distributed on the bottom of 25-cm flasks filled with 1 ml DMEM/F12 supplemented with 10% FCS and penicillin/streptomycin (100 IU/ml penicillin and 50 µg/ml streptomycin). The flasks were turnovered and put in an incubator for 1-3 hours at 37°C with 5% CO_2 in air with saturated humidity.
d) Flasks were turned down and added 8 ml more culture medium.

e) The culture medium was changed when it is necessary. Growth of the cultures was monitored with an inverted optical microscope.

f) The cells were trypsinized when the cell monolayer was formed about 90%, and then diluted in complete culture medium, distributed into 35-mm dishes and incubated at 37ºC.

3.4.2 Cell freezing

a) Confluent MSC and RAF cells were trypsinized for 1-2 min.

b) Culture medium (1 ml) was added into dishes to stop enzyme treatment.

c) Cell suspension was transferred into 15-ml Falcon tube and centrifuged at 140 g for 5 min.

d) Supernatant was sucked off and pellet was resuspended in 2 ml culture medium.

e) Cell numbers were counted by haemocytometer using the following protocol:

- Place a drop (10 - 20 µl) of well-mixed cell suspension at each notch. Add drop all at once to ensure even distribution of cells.
- Count the number of cells in the 1 mm square (= 0.1 µl). Count eight 1mm squares. Select the 4 corner squares on the 2 grids on the haemocytometer. Calculate the average. Multiply by 1 X 10^4 to determine the number of cells per millilitre.

f) Medium was removed by pippet.

g) Cells were re-suspended in cold freezing media (10% DMSO, 90% FCS) in a density $1x10^6$ cells/ml. Cells were kept chilling while working.

h) Cell suspension (1 ml) was placed in a cryogenic tube, labelled and stored first for 30 min at 4°C, then for 2 h at –20°C, and finally overnight at -80°C.

i) Next day, cells were placed in liquid nitrogen or at -80°C.

3.4.3 Cell thawing

Frozen cells were fast thawed by adding prewarmed (37°C) complete cell culture medium with gentle pipetting. Then cells were pelleted by centrifugation at 140 g for 5 min in 5 ml volume, seeded into 35-mm cell culture dishes.

3.4.4 Preparation of cells for NT

Confluent MSCs and RAFs were washed with Ca^{2+}- and Mg^{2+}- free PBS twice, trypsinized for 1 min, and then collected by centrifugation at 140 g for 5 min in 5ml M199. Finally, the cells were resuspended in M199 for NT.

3.4.5 Characterization of MSCs and RAFs

3.4.5.1 In vitro life span of MSCs and RAFs

Primary isolated MSCs and RAFs were seeded into 35-mm dishes at a density of $1x10^4$ cells/ml in a medium for Mouse Mesenchymal Stem Cells or in DMEM/F12. The cells were passaged and counted with a haemocytometer as described before until they reached replication senescence, during which cell morphology considerably changed and proliferation capacity decreased. The number of population doublings were calculated by formula $PD = Log_{10}(N/N0) X 3.33$.

3.4.5.2 Karyotype analysis

a) 80-90% confluent RAFs and MSCs on a 35-mm dish were split as 1:2, and then cultured for another 24 h.
b) Demecolcine was used to synchronize cell cycle with a final concentration of 0.1 μg/ml in culture medium. Cells were cultured for another 150-180 min under treated with demecoline.
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) Cells were treated with 0.05% trypsin/EDTA solution for 1-2 min, and then 2 ml washed with culute medium.
f) Detached cells were collected into the same 15-ml conical tube as in step c).
g) Cells were pelleted by centrifugation at 140 g for 10 min.
h) Medium was carefully removed leaving about 0.5 ml supernatant in the tube, and the pellet was gently resuspended by flicking the tube with fingers.
i) The cells were resuspend in the remaining medium and approximately 2 ml prewarmed (37°C) 0.075 M KCl (potassium chloride in bidistilled water) was slowly added. Then an additional 8 ml KCL was added for a total of 10 ml and mixed well.
j) The suspension was incubated for 15 min at 37°C in the water bath.

k) A few drops of freshly prepared fixative solution (3:1 methanol/acetic acid, Rothe) were added to the suspension to stop reaction, re-cap the tube, and inverted to mix.

l) After centrifugation as step g), 0.5 ml of the supernatant was left in the tube. The cells were resuspended and fixed by adding 10 ml of fixative; the first 2 ml should be added dropwise while agitating gently.

m) The fixation procedure was repeated two more times.

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

o) 10 µl suspension was dropped onto a cleaned and cold microscope slide.

p) After air-dry, slide was covered with two drops of antifade medium (VECTASHIELD mounting medium for fluorescence with DAPI, Cat: H-1200, Vector labs, U.K). A coverslip was added and sealed.

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

3.4.5.3 Characterization of MSCs

3.4.5.3.1 Differentiation of MSCs into osteogenic lineage

Cells were seeded at 5000 cells per cm^2 in a 6-well plate, and maintained in culture medium (MesenCult Medium, DMEM or DMEM with 4ng/ml FGF (fibroblast growth factor) until they reached a confluence of 70-80% in a humidified 5% CO_2 incubator at 37°C. The cells were induced by osteogenic differentiation medium composed of high-glucose DMEM, 100 nM dexamethasone, 50 µM L-ascorbic acid, 10 µM β-glycerophosphate, 10% FCS (HyClone), and 1% antibiotic-antimycotic (Biological Industries) for 3 or 5 weeks. Half of the medium was replaced every 3 days. Calcium deposits were stained with the method of von Kossa. The differentiation assays were repeated 3 times for 3 weeks and one time for 5 weeks.

3.4.5.3.2 Differentiation of MSCs into adipogenic lineage

Cells were seeded at a density of 2 $x10^5$ cells/well in 6-well plate and cultured in culture medium until confluent. Then they were induced in high-glucose DMEM, 1 µM dexamethasone, 10 µg/ml insulin, 500 µM 3-methyl-isobutylxantine, 200 µM

indomethacine, 10% FCS, and 5% rabbit serum for three days, alternately cutured one day in maintenance medium, composed of high-glucose DMEM, 10 µg/ml insulin, 10% FBS, and 5% rabbit serum. After three cycles, adipogenic differentiation was visualized after staining with 0.3% Oil red O for 5 minutes at room temperature and rinseing with 60% isopropanol.

3.4.5.3.3 Differentiation of MSCs into chondrogenic lineage

Cells were seeded at a density of 5 x 10^5 cells/tub in high-glucose DMEM medium supplemented with 50 µg/ml ascorbate-2-phosphate, 40 µg/ml proline, ITS, 100 nM dexamethasone, 1% FCS, 10 ng/ml TGF-beta3, and 200 ng/ml BMP-2 . Medium was changed three times a week very carefully by aspirating using pipette. Glycosaminoglycans (GAG) were quantificated using chondroitin sulphate as standard curve, and DNA was quantificated by Quant-iT Pico Green dsDNA Assay Kit (Invitrogen, Cat. No. P7589).

3.4.6 Immunofluorescence staining of donor cells for detection of histone methylation modifications

An antibody that recognizes specifically di- and tri-methylated lysine 4 on histone H3 (H3K4m2/3) was used as an epigenetic marker for transcriptionally competent chromatin, i.e. euchromatin; and another antibody, binding specifically to tri-methylated lysine 27 on histone H3 (H3K27m3), was applied as a marker for transcriptionally silent chromatin, i.e. heterochromatin.

3.4.6.1 Immunofluorescence

a) MSCs and RAFs were grown and attached on glass coverslips until eighty percent confluent.
b) Specimens were briefly washed in PBS at 37°C and fixed in 3.7% formaldehyde/PBS for 10min at room temperature.
c) After three times washing in 0.02% Tween 20/PBS (PBST) specimens were permeabilized by incubation in 0.5% Triton X-100 in PBS for 30 min at room temperature.
d) Finally specimens were washed three times in PBST.

e) For an initial blocking, specimens were put in 4% BSA/PBST for 30 min at room temperature.

f) Specimens were incubated with primary antibodies diluted appropriately in 4% BSA/PBST for two hours at room temperature. The following primary antibodies were used separately: polyclonal rabbit anti-histone H3 tri-methylated at lysine 27 (Upstate 07-449; diluted1/100) and monoclonal mouse anti-histone H3 di-/tri-methylated at lysine 4 (Abcam ab6000; diluted 1/400).

g) After four times washing in PBST, the following secondary antibody dilutions in 4% BSA/PBST were applied for one hour at room temperature: goat anti-mouse IgG (H+L) Alexa 488 (Molecular Probes A-11029; diluted 1/400) and goat (Fab fragment) anti-rabbit IgG (H+L) Cy3 (Jackson ImmunoResearch 111-167-003; diluted 1/200).

h) Cells were washed 5 times in PBST, counterstained with the DNA dye TO-PRO-3 (Molecular Probes; 2 µM) for 5 minutes at room temperature and mounted on a glass slide using Vectashield (Vector Laboratories) antifade medium.

3.4.6.2 Confocal microscopy

Cells were imaged on a Leica TCS SP1 confocal laser scanning microscope using a 63x/1.32NA oil immersion objective lens. Fluorochromes were excited by a 488 nm Argon laser, 561 nm and 633 nm Helium-Neon lasers. An optimal detection range was adjusted by appropriate emission AOTF settings. To quantify fluorescence intensities mid-confocal sections of cell nuclei were collected using identical imaging parameter. Cultured cells from the various nuclear donor cell types used as a reference were fixed at a comparable passage number (±1).

3.4.6.3 Image analysis

Image analysis was done utilizing ImageJ 1.37v (http://rsb.info.nih.gov/ij/). To calculate mean fluorescence intensities within the limits of the nuclear area, all micrographs were synchronized to the image with the DNA counterstain where the nuclear border could be manually outlined. These margins were then applied to the micrographs of the antibody staining. The mean fluorescence intensity was normalized to the mean fluorescence intensity value averaged from 11 cellular nuclei of the respective donor cell type stained and imaged on the same day. This resulted in

a relative fluorescence intensity value representing the ratio of the signal intensity of an embryo nucleus to the signal intensity in the donor cell type. Statistics were analyzed by ANOVA using the MIXED procedure of SAS.

3.5 Nuclear transfer, fusion and activation

Nuclear transfer was done essentially as described by Yang et al. (2007). Briefly, individal RAFs or MSCs were inserted by micromanipulation under the zona pellucidae of the enucleated oocytes in M199. To induce membrane fusion, the reconstructed embryos were submitted to electro-stimulation (3DC pulses of 1.95 KV/cm, 25 μs×2, 1sec interval) and the fusion was checked in 20 min later. After fusion, the cloned embryos were immediately incubated for 30 min in B_2 medium (Laboratories CCD, Paris France) containing 1.9 mM 6-DMAP, 5.0 μg/ml CB and 10% FCS in a humidified atmosphere of 5% CO_2 in air at 38.5°C. Finally, embryos were applied one more time electro pulse and incubated in activation medium another 30min.

3.6 Embryo culture and transfer

After activation, cloned embryos were cultured overnight in 100μl microdrop of B_2 medium containing 10% FCS at 38.5°C under 5% CO_2 in air. Four-cell stage reconstructed embryos (about 15) were transplanted non-surgically through the infundibulum into each oviduct of recipients which had been induced into pseudopregnancy state at 20-22 h later in comparison to the oocyte donors. Pregnancy was checked by palpation around 14 days after embryo transfer. Blastocyst development rates were assessed after in vitro culture for up to 5 days.

3.7 Immunofluorescence staining of embryos

3.7.1 Embryo fixation

a) Embryos were washed 2 or 3 times in PBS to remove the culture medium.
b) 10-15 embryos were treated with acidic Tyrode's solution for 1-2 min, while pipetting them constantly in order to prevent them from sticking to the bottom. The process was controlled using a binocular microscope.
c) After zona removal, embryos were transferred into the fixation solution (3.7% paraformaldehyde/PBS) directly for 15 min at room temperature.

f) Embryos were washed three times in mPBS and one time in PBST, then stored in PBST at 4°C for no longer than 1 week before application of antibodies.

3.7.2 Immunofluorescence

a) Embryos were permeabilized by incubation in 0.5% Triton X-100 in PBS for 30min at room temperature.

b) For an initial blocking, specimens were put in 4% BSA/PBST for 30 min at room temperature.

c) Specimens were incubated with primary antibodies diluted appropriately in 4% BSA/PBST for two hours at room temperature. Following primary antibodies were used simultaneously: polyclonal rabbit anti-histone H3 tri-methylated at lysine 27 (Upstate 07-449; diluted1/100) and monoclonal mouse anti-histone H3 di-/tri-methylated at lysine 4 (Abcam ab6000; diluted 1/400).

d) After four times washing in PBST, following secondary antibody dilutions in 4% BSA/PBST were applied for one hour at room temperature: goat anti-mouse IgG (H+L) Alexa 488 (Molecular Probes A-11029; diluted 1/400) and goat (Fab fragment) anti-rabbit IgG (H+L) Cy3 (Jackson ImmunoResearch 111-167-003; diluted 1/200).

e) Embryos were washed and equilibrated in 20, 40 and 60% glycerol/PBST for 15 min each at room temperature before transferring them to poly-lysinated Lab-Tek 8-well chambers with a coverglass bottom (Nunc). Equilibration was performed to prevent morphological damaging of embryos due to the change of solution density from PBST to the glycerol based antifade medium. 8-well chambers were poly-lysinated by covering the glass surface with a Poly-L-lysine solution (5mg/ml) for 1h before removing it. Excess solution was removed from the transferred embryos and they were left in a small drop of 60% glycerol over night at 4°C to adhere them to the glass surface. The next day embryos were covered with Vectashield antifade medium supplemented with TO-PRO-3 (50 µM).

3.7.3 Confocal microscopy

It was done as the same as *3.1.4.6.2.* In order to compare fluorescence intensities of embryos stained on different days, signal intensities of embryonic nuclei were normalized to fluorescence intensities of cell nuclei stained on the same day. This was

done to correct for eventual differences in the efficiency of antibody staining in individual experiments done on different days.

3.7.4 Image analysis

It was done as *3.1.4.6.3.* In vivo fertilized embryos were normalized to RAF 95 nuclei. Between 7 and 19 embryos equivalent to 9-38 nuclei per stage were analyzed. Relative fluorescence intensity values of an individual stage were statistically compared to the respective previous stage as well as to the same stage of in vivo fertilized embryos by an ANOVA approach.

3.8 Culture of in vivo fertilized and cloned rabbit embryos

Cloned embryos were produced as above and in vivo fertilized zygotes were collected 16-18 h after mating. 15-20 embryos were cultured in 100-µl microdrop B_2 medium with 10% FCS and checked under microscope in every 2 hours.

4 RESULTS

Cloned rabbits have been produced from cumulus cells and cultured fibroblast cells in our previous work (Yang et al. 2007), but the cloning efficiency was very low (0.2-0.6%) and not considerably improved (1.8%) following aggregation of cloned embryos with blastomeres of in vivo fertilized or parthenogenetic embryos. In this study, MSCs were used for NT to test whether these cells which possess high degree of developmental plasticity would be better nuclear donors compared to other somatic cells.

4.1 Isolation and characterization of MSCs

4.1.1 Isolation of cell lines

In total, ten MSC lines were established from bone marrow using the same protocol and conditions. Most of non-adherent cells were removed during first medium change around 24 h. Discrete colonies of fibroblast-like cells attached to the plastic at 2-3 days after initial seeding. Most cell lines were composed of cells with a characteristic spindle shape, whereas others had cells with polygonal morphology. Cells reached 80-90% confluence on day 7-10 after seeding (Fig. 4).

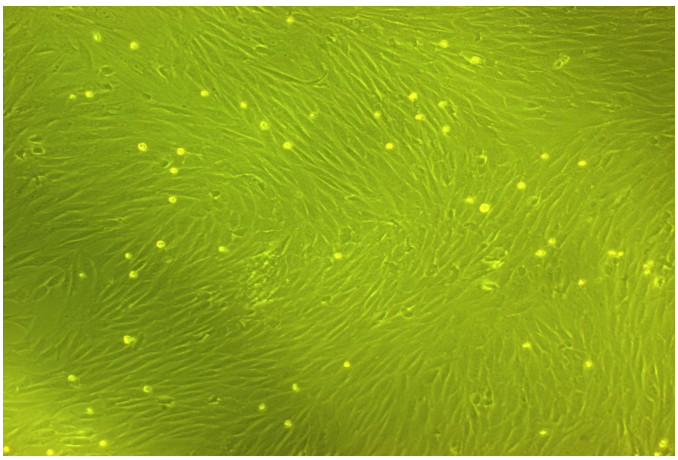

Fig. 4 Mesenchymal stem cells

4.1.2 Life span of cultured MSCs and RAFs

Freshly isolated MSC and RAF cells (passage 0) from 4 to 6-week-old rabbits were cultured independently until confluence, and were then passaged continuously until they reached replicative senescence. Passage one MSCs and RAFs were cultured either

in MesenCult Medium or in DMEM/F12 and the growth curves were recorded to determine their life span. In MesenCult Medium, MSC 93 and 95 were passaged 23 and 24 times until they reached 60 and 66 population doublings (PDs), respectively, whereas RAF 93 and 95 reached only 30 and 31 PDs, respectively, after 15 passages (Fig. 5). In DMEM/F12 medium, cells of both types, exhibited reduced proliferation capacity, however, MSC 93 and 95 reached more PDs (31 and 32, respectively) than RAF 93 and 95 (23 and 24, respectively), when they finally ceased to divide at passage 10-12 (Fig. 6). As passage and PD numbers increased, both MSCs and RAFs showed morphological changes, including flattened morphology and enlarged cell size.

Table 3 Proliferation capacities of MSCs and RAFs

Cell type	Culture medium	Total passages	Days of culture	Total PDs
MSC 95	MesenCult MSC medium	24	90	66
	DMEM/F-12 medium	12	63	32
RAF 95	MesenCult MSC medium	14	60	31
	DMEM/F-12 medium	11	51	24
MSC 93	MesenCult MSC medium	23	81	60
	DMEM/F-12 medium	12	59	30
RAF 93	MesenCult MSC medium	14	54	30
	DMEM/F-12 medium	11	44	23

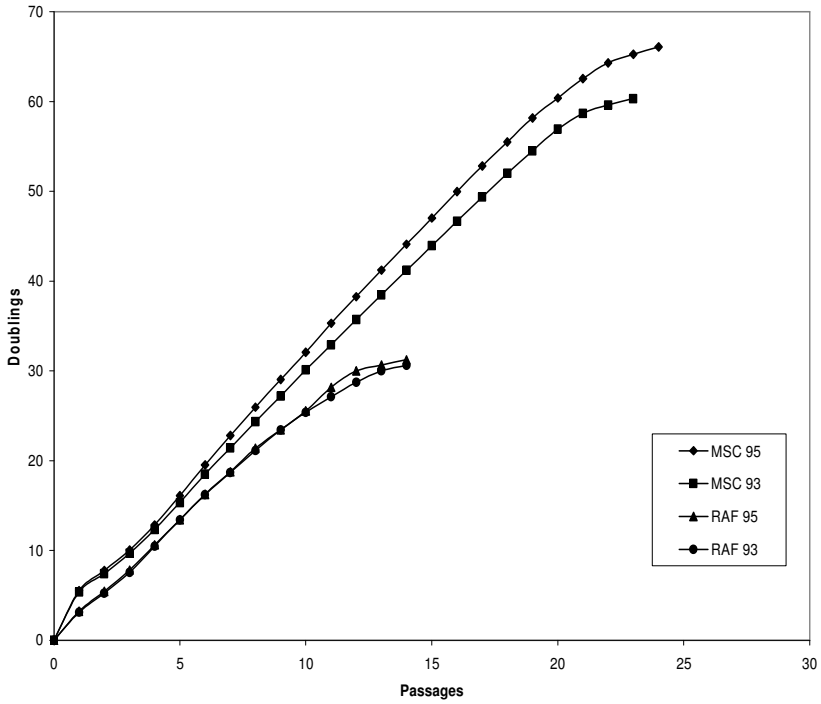

Fig. 5 Growth curves of rabbit MSCs and RAFs cultured in MesenCult MSC medium

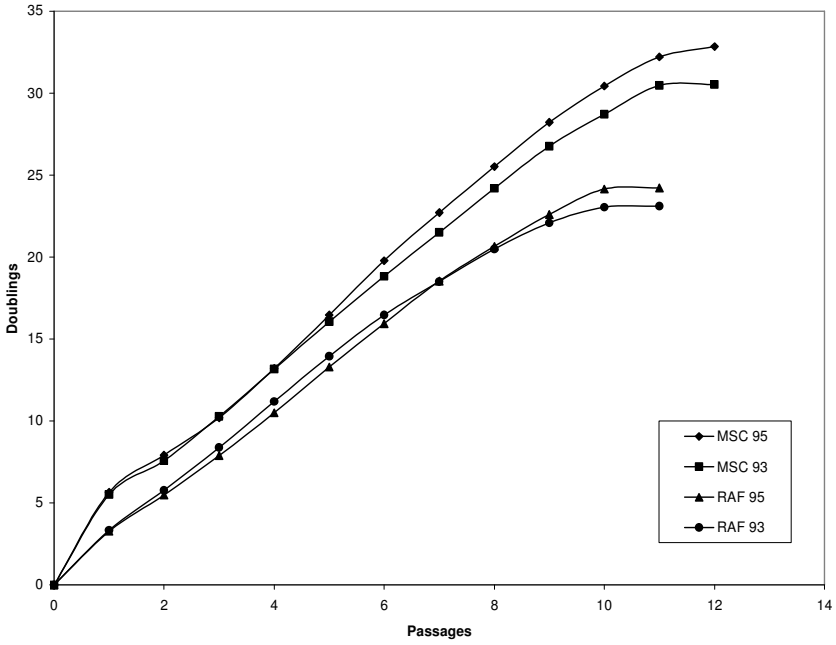

Fig. 6 Growth curves of rabbit MSCs and RAFs cultured in DMEM/F-12 medium

4.1.3 Karyotype analysis

In total about 60 metaphase spreads of RAFs and MSCs in 3 replicates were analysed at passage 12. All these spreads had normal chromosome number of 44 (Fig. 7).

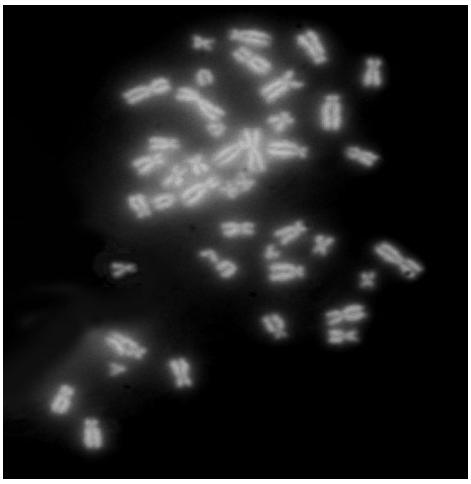

Fig. 7 Metaphase spread of a rabbit mesencymal stem cell

4.1.4 Differentation of MSCs into Osteogenic lineage

Cells cultured under osteogenic induction medium produced calcium deposits detected by von Kossa stain (Fig. 8).

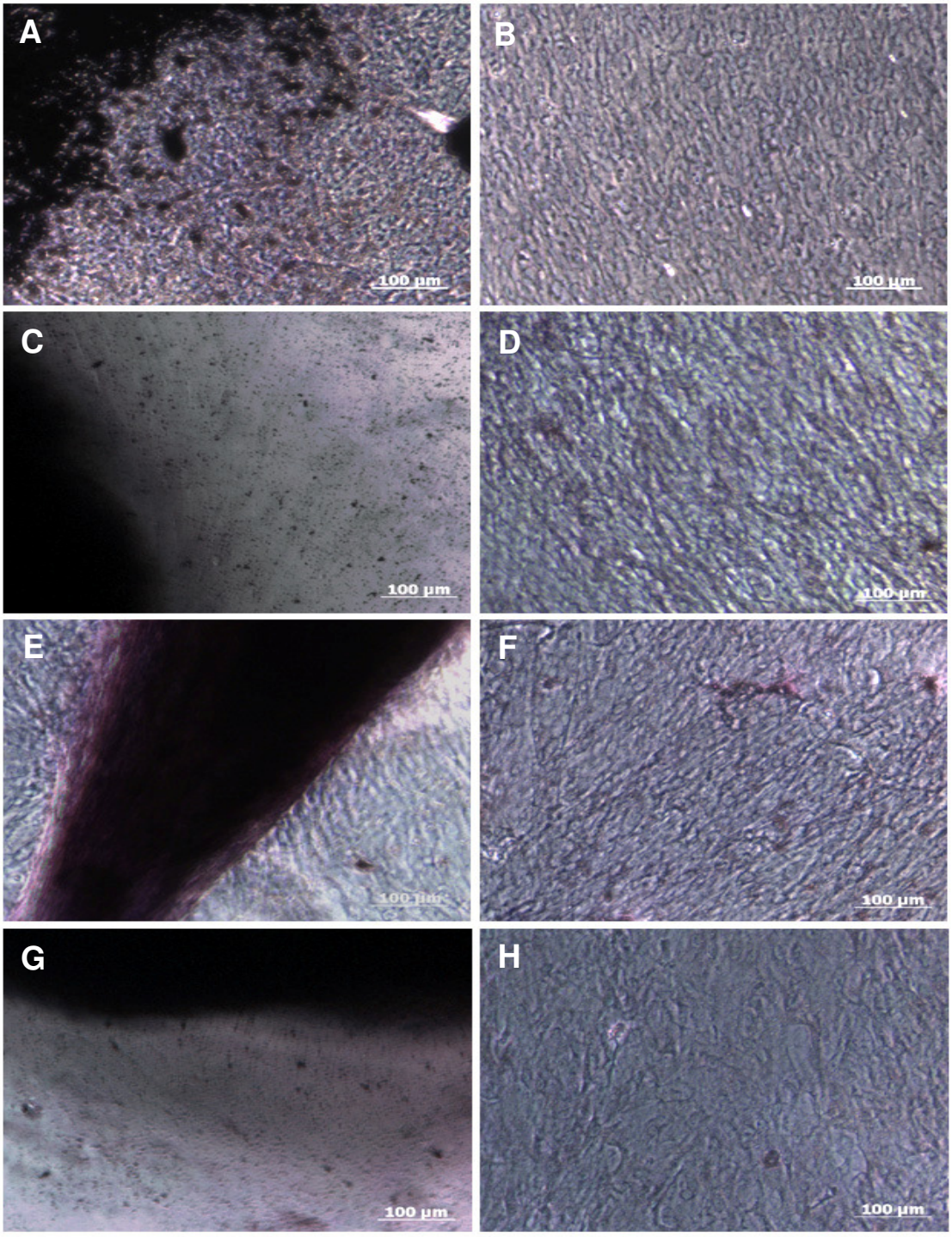

Fig. 8 Osteogenic differentiation of rabbit MSCs

A,C,E,G: stimulated MSCs; B,D,F,H: unstimulated controls MSCs; calcium deposits stained in black (von Kossa);

(A)+(B) human MSC; (C)+(D) rabbit MSCs expanded in MesebCult Medium; (E)+(F) rabbit MSCs expanded in DMEM; (G)+(H) rabbit MSCs expanded in DMEM with FGF

4.1.5 Differentation of MSCs into adipogenic lineage

Differentiation of MSCs into adipogenic lineage was identified by oil red staining of lipid vacuoles. In the adipogenic differentiated cells, red stained vacuoles were deteced (Fig. 9).

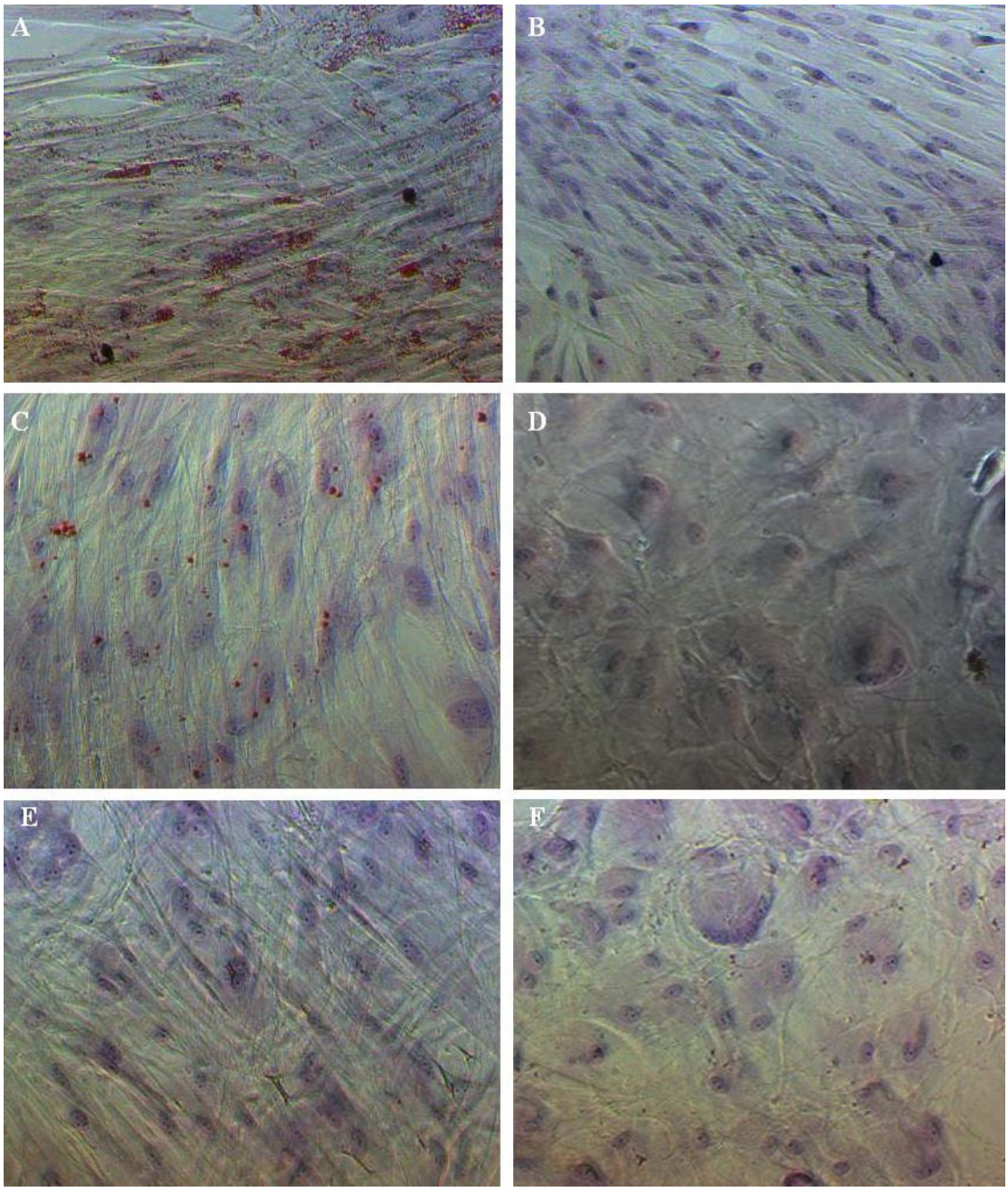

Fig. 9 Adipogenic differentiation of rabbit MSCs
A,C,E: stimulated MSCs; B,D,F: unstimulated controls MSCs; lipid deposits stained in red.

4.1.6 Differentiation of MSCs into chondrogenic lineage

The glycosaminoglycans were quantified according to the chondroitin sulphate standard. Cell line MSC93 reached to 155.5 ng chondroitin/µg DNA, whereas other two cell lines didn't get that high concentration, 8.4 ng and 10.2 ng, respectively (Fig. 10).

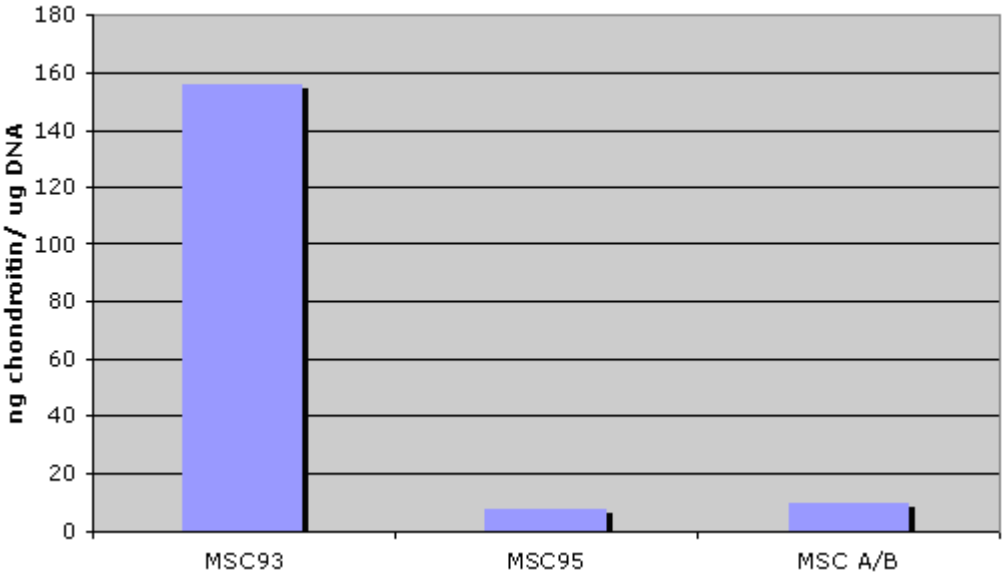

Fig. 10 Chondrogenic differentiation of rabbit MSCs

4.2 Development of embryos cloned from MSCs

In the first series of experiments, we established and tested as donors for NT two MSC lines. Rates of development to blastocysts varied from 50 to 76% and tended to be higher with the cells at early passages (Table 4). Transfer of 404 cloned 4 to 8-cell stage embryos into 10 recipients resulted in 5 pregnancies and the birth of one rabbit which lived only for a few hours. Neither transfer of cloned embryos at one-cell stage nor co-transfer with parthenogenic embryos improved pregnancy or offspring rates.

Table 4 Development of embryos cloned from MSC lines

Donor cell /passage	Fused (%)	Cleaved (%)	Blast (%)	Embryos/ Recipients	Pregnant recipients	Rabbits born
R3 p1-5	177/177 (100)[a]	135/151 (89)[a]	38/76 (50)[a,b]	68+8/2***	1*	0
R3 p8-12	340/340 (100)[a]	263/278 (94)[b]	26/48 (58)[a,b]	132/2 75+13/2***	0 0	0 0
R8 p3-7	206/207 (99)[a]	172/202 (85)[a]	26/38 (68)[a]	132/4	2(1*)	1
R8 p14-17	367/381 (96)[a]	190/235 (80)[a]	23/58 (40)[b]	140/4 106/2**	3* 0	0 0
Total	1081/1087 (99)	747/848 (88)	102/202 (50)	404/10 106/2** 143+21/4***	5(4*) 0 1*	1 0 0

*Recipients were slaughtered on day 13-17; **one-cell embryos were transferred; ***Co-transfer with parthenogenetic embryos; [a,b]: values within the same column with different superscripts were significantly different ($P<0.05$).

4.3 Development of embryos cloned from the cells of MSC A/B line

In the second series of experiments we used another MSC A/B line, either at early passage or after storage of donor cells in liquid nitrogen. Cleavage and blastocysts rates obtained with cells before their storage were significantly higher than that those obtained with cells used after one year storage in liquid nitrogen (Table 5). Transfer of 357 cloned embryos into 10 recipients resulted in four pregnancies and one cloned rabbit (Fig. 11) which lived for three days. When other three recipients were slauthered on day 13 after embryo transfer, nine implantations were found and 7 of those were in one recipient (Fig. 12).

Table 5 Development of embryos cloned from the cells of MSC A/B line

Donor cell/passage	Fused (%)	Cleaved (%)	Blastocysts (%)	Embryos/ Recipients	Pregnant recipients	Rabbit born
BMC A/B, 1-4	181/181 (100)	154/159 (97)[a]	77/102 (76)[a]	-	-	-
BMC A/B, 5-8*	261/269 (97)	212/246 (86) [b]	78/175 (45)[b]	37/1	1	1
BMC A/B, 9-13*	596/621 (96)	378/603 (63)[b,c]	23/68 (34) [b,c]	320/9	3	0

* Cells were used after one year storage in liquid nitrogen;

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

Fig. 11 One-day old live rabbit cloned from a MSC

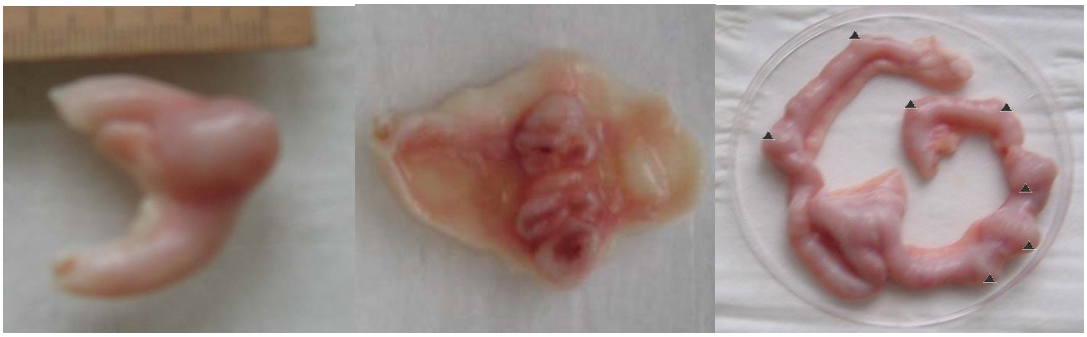

Fig. 12 Resorbed conceptuses on day 13 after embryo transfer

4.4 Development of embryos cloned from genetically matched MSCs and RAFs

To exclude possible effects of different genetic background of donor cells on the efficiency of NT, two MSC and two RAF lines from the same rabbits were established and tested as nuclear donors. The proportions of embryos that cleaved were not different between the MSC and RAF groups (Table 6). However, within the isogenic groups, significantly more embryos ($P<0.05$) cloned from RAF 93 cells developed to blastocyst than those derived from MSC 93 cells, whereas there was no significant difference in the proportions of cloned blastocysts derived from MSC 95 and RAF 95 cells.

Table 6 Development of embryos cloned from genetically matched MSCs and RAFs

Donor cells	Fused (%)	Cleaved (%)	Blastocysts (%)
BMC 93	204/207 (99)	188/203(93)[a]	53/182(29)[a]
RAF 93	172/175 (98)	151/171 (88)[a]	78/169 (46)[b]
BMC 95	209/223 (94)	164/200 (82)[b]	88/200 (44)[a,b]
RAF 95	173/189 (92)	132/161 (82)[b]	80/161 (50)[b]

[a,b]: Values within the same column with different superscripts were significantly different (P<0.05).

4.5 Immunofluorescence staining to detect histone methylation modifications

4.5.1 Histone methylation status of donor cells prior to nuclear transfer

Analysis of H3K27m3 modification revealed the expected staining pattern in nuclear donor cells, typically high lighting the inactive X-chromosome in female cell lines (MSC 93 and 95, and RAF 93 and 95) (Fig 13, 14, 15). For H3K4m2/3 modification, strong staining with small foci was detected in all donor cells. In female cells the inactive X chromosome (identified as Barr body via DNA staining) showed reduced levels of H3K4m2/3 (Fig. 13-15).

4.5.2 Histone methylation status of in vivo fertilized and cloned embryos

H3K27m3 modification was not detected in the zygote to morula stages of in vivo embryos (Fig. 13). Only in blastocysts a weak staining of a single domain in some

embryos in a few cells was visible, probably resembling the inactive X of female embryos. Since during simultaneous analysis of both modifications H3K4m2/3 signals were strong a staining, artifact can be excluded.

Irrespective of the type of donor cells, in all stages of NT embryos except for one-cell embryos and blastocysts, H3K27m3 was undetectable (again, as an internal control, signals for H3K4m2/3 were always observed) (Fig. 14,15). In the majority but not all one-cell embryos derived from MSC 95, RAF 93 and RAF 95 a very weak staining was observed, while essentially no signals were detected at the same stages of embryos cloned from MSC 93 and MSC A/B. In blastocysts derived from RAF 95 and MSC 95 a weak staining of one domain similar to in vivo embryos was seen in few cells of some embryos. No signals for H3K27m3 were detected in blastocysts derived from the other cell types. Generally, it seems that H3K27m3 is successfully reprogrammed in transferred nuclei of all donor cell types, with minor differences in embryos at the one-cell and blastocyst stages.

High levels of in H3K4m2/3 (euchromatin) observed in in vivo embryos at the one- and 2-cell stages decreased slightly at the 4-cell stage, followed by a more drastic drop at the 8-cell stage, where there was a minimum of measured fluorescence coinciding with the time point of major genome activation. At the 16-cell stage signals were slightly increased, while in morulae and blastocysts those reached similar levels as at the one- and 2-cell stages.

Fluorescence signals for H3K4m2/3 detected in NT embryos derived either type of donor cells differed from those of in vivo embryos (Fig. 14, 15, 16). A minimum was not reached at the 8-cell stage, but at the 4-cell stage, where in in vivo embryos the signals were still strong. Reprogramming of H3K4m2/3 modification occurred quite differently with either type of cells irrespective of the cell origin or type. However, all types of donor nuclei were reprogrammed to some degree, as the fluorescence increased already in one-cell stage and remained high or increased again at the 2-cell stage, except for RAF 95. It is worth noting that NT embryos derived from MSC A/B differed from all others in the way that fluorescence levels were generally lower and remained in the range of the donor cells, as if they would be more refractory to an elevation of global H3K4m2/3 levels. Similar to embryos generated from MSC A/B, those cloned from RAF 95 showed reduced H3K4m2/3 levels at the morula and blastocyst stages. Another noticeable aspect is that at the 8-cell stage, when in vivo

embryos reached their minimum, the H3K4m2/3 levels were still quite low for all MSC cell lines (MSC 93, 95 and A/B), while they were already elevated in embryos cloned from fibroblasts (RAF 93 and 95) (Fig. 14, 15, 16).

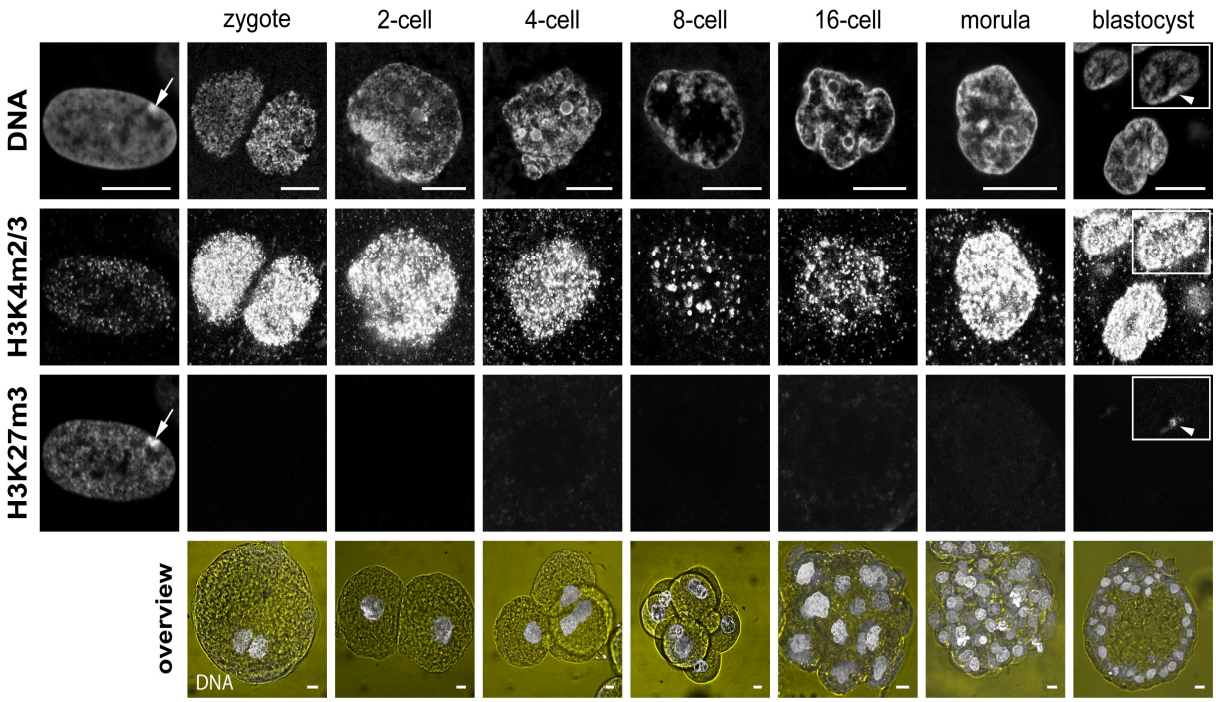

Fig. 13 Patterns of H3K4m2/3 and H3K27m3 in rabbit in vivo fertilized embryos and fibroblast cells 95 (left column)

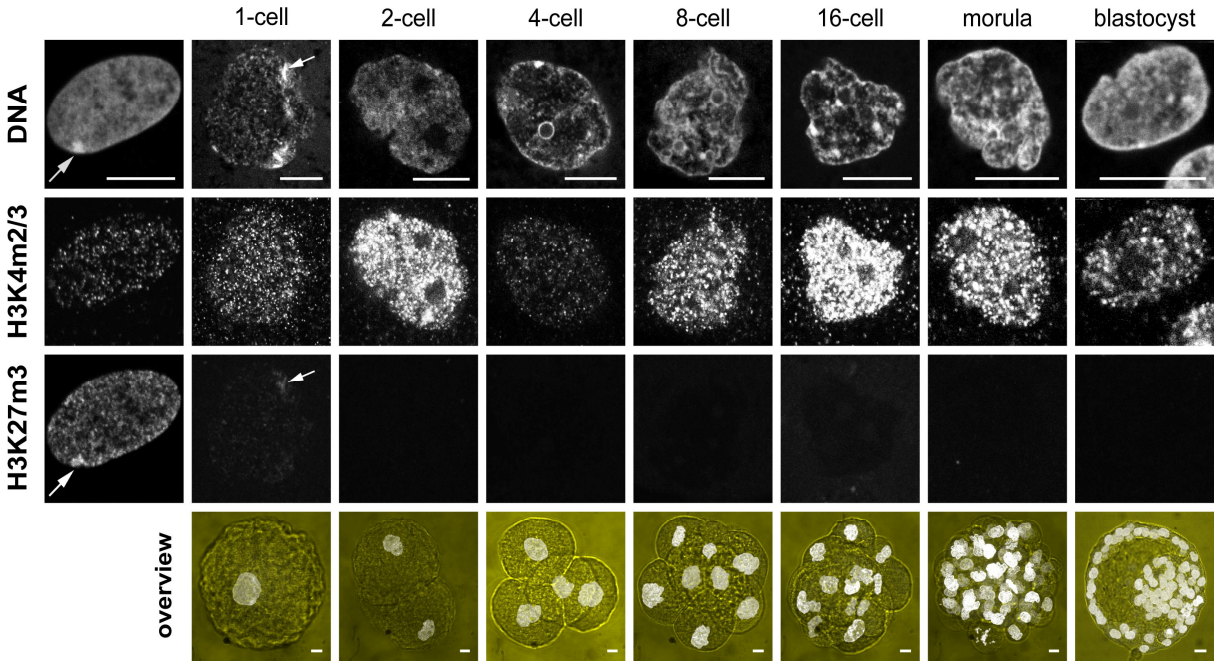

Fig. 14 Patterns of H3K4m2/3 and H3K27m3 in RAF 93 NT embryos and fibroblast cells 93 (left column)

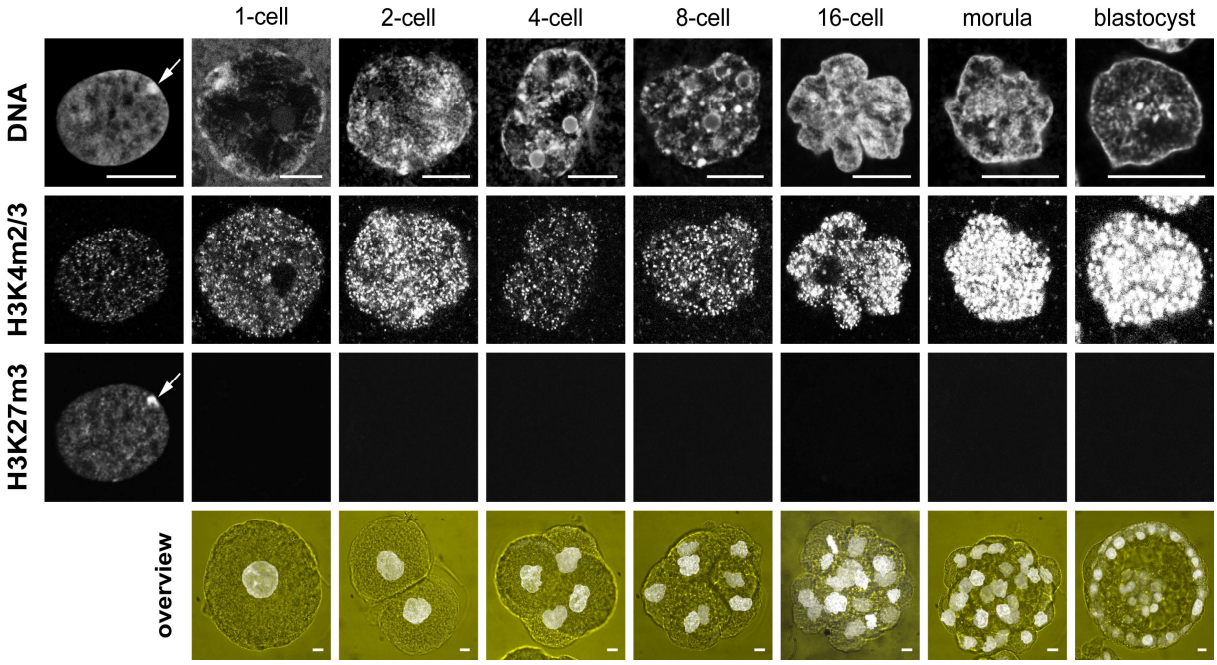

Fig. 15 Patterns of H3K4m2/3 and H3K27m3 in MSC 93 NT embryos and MSCs 93 (left column)

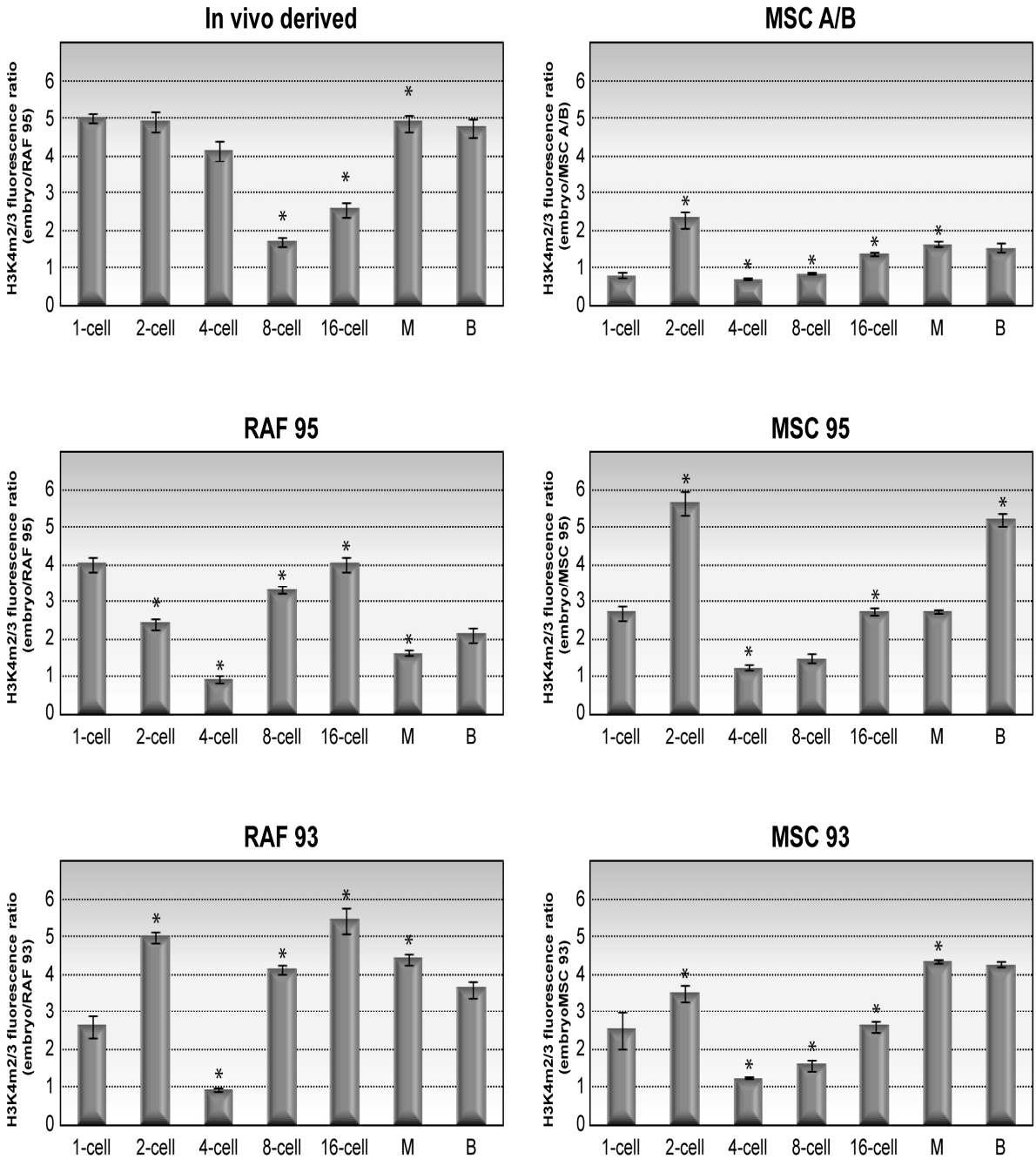

Fig. 16 Patterns of H3K4m2/3 rabbit in vivo embryos and embryos cloned from MSC (93, 95 and A/B), RAF (93 and 95)

4.6 Comparison of the time course of preimplantation development of in vivo fertilized and cloned rabbit embryos

We found that zygotes need about 24-26 h to cleave into 2-cell embryos, while cloned one-cell embryos undergo first cleavage division in 10-12 h after activation (Table 7). To reach futher stages of development cloned embryos need two hours more than in vivo fertilized embryos indicating some degree of developmental delay in the former.

Table 7 Timing of preimplantation development of in vivo fertilized and cloned embryos

Embryonic stage	Normal fertilized embryos, time post mating/doubling time (hours)	NT embryos, time post activating/doubling time (hours)
Oocyte	12-14/-	-
Zygote	18-20/-	-
2-cell	24-26/6	10-12/10-12
4-cell	30-32/6	18-20/8
8-cell	38-40/8	26-28/8
16-cell	46-48/8	36-38/10
Morula	54-56/8	46-48/10
Compact morula	64-66/10	58-60/12
Early blastocyst	76-78/12	72-74/14
Expanded blastocyst	84-86/8	82-84/10
Hatched blastocyst	94-96/8	92-94/10

5 DISCUSSION

Reprogramming of epigenetic modifications established on the chromatin of donor cells plays a key role following NT and is largely dependent on the cell origin and type. Whether various donor cells differ in their ability to successfully pass through the process of epigenetic reprogramming was the question addressed by numerous studies on SCNT (Kato et al. 2000; Santos et al. 2003; Tamada and Kikyo 2004; Armstrong et al. 2006). In our previous study in rabbit, we found that embryos cloned from cumulus cells were more similar to in vivo fertilized embryos in respect to reprogramming of acH3K9/14 than those derived from fetal fibroblasts (Yang et al. 2007). However, cloning efficiency estimated by blastocyst and offspring rates was not significantly different between these two groups. Here, we tested two types of donor cells, mesenchymal stem cells and adult fibroblasts, which are considered to be different in the state of differentiation. The main results of the present study include cloning for the first time rabbits from MSCs and demonstration of reprogramming dynamics of hetero- and euchromatic histone modifications.

5.1 Effects of donor cell type on nuclear transfer efficiency

5.1.1 Mesenchymal stem cells

MSCs have already been used for SCNT in different species, but the results do not shed light on the question whether these cells are better nuclear donors than other somatic cells. In the first report on the use of bovine MSCs for SCNT it has been suggested that cloned embryos do not have a higher developmental potential, both in vitro and in vivo, than those produced from the cells of non-stem cell origin (Kato et al. 2004). However, their findings were based on a relatively small data set, which lacked a comparison of NT with other cell types as a control. Subsequent studies provided less certainty but rather contradictory results. While Colleoni et al. (2005) in cattle and pigs, and Bosch et al. (2006) in pigs reported similar blastocyst rates reconstructed with MSCs and adult fibroblasts, Faast et al. (2006) showed that the percentage of blastocysts cloned from porcine MSCs was almost two-fold higher than those derived from adult fibroblasts. The latter study compared two different cell types originating from the same animal that excludes likely effect of genetic background, however, when porcine MSCs were isolated from blood but not from

bone marrow they were not superior to fibroblasts in generating cloned blastocysts. The reasons for such apparent difference between MSCs isolated from bone marrow and blood are unknown but a likely explanation could be that these cells were obtained from different animals and the experiments performed at different times. Similar to the findings of Faast et al. (2006), other studies in pigs have also reported higher blastocyst rates with porcine bone marrow MSCs (Kumar et al. 2007) or porcine skin-derived fetal stem cells (Zhu et al. 2004) than with fetal fibroblasts. All these reports provide evidence that the efficiency of SCNT with MSCs can be different not only between laboratories but also within the same laboratory and species. The differences might also be the result of the respective nuclear transfer protocol used in either study.

5.1.2 Effect of passage number of MSCs on in vitro development of NT embryos

Production of transgenic cloned rabbits requires long term culture of donor cells for stable integration of a transgene and selection of transfected cells prior to NT. In this study, MSCs were cultured until passage 23 or 24 (about 60 PDs) in a specific culture medium, and this proliferation capacity was higher than that of bovine and porcine MSCs (50 and 40 PDs, respectively; Colleoni et al. 2005). The blastocyst rate of 40% obtained with passage 14-17 R8 MSCs was significally lower compared to that with these cells at passage 3-7, suggesting that later passage MSCs can be appropriate nuclear donos but short time cultured cells may be superior in supporting the developemt of cloned embryos.

5.1.3 Effect of storage of MSCs on in vitro development of NT embryos

A decline in developmental potential of MSC A/B after one-year storage in liquid nitrogen (76% vs. 45%, $P<0.05$) was observed, suggesting that environmental conditions may affect the stability, both genetic and epigenetic, of MSCs. Consistent with our observations, others groups have also shown that neither refrigeration of donor cells for 1-2 weeks nor their freezing with a controlled cooling rate for several weeks or months compromised development of NT embryos (Liu et al. 2001a; Hayes et al. 2005). However, it must be taken into account that cell cryopreservation as well as trypsinization can cause chromatin rearrangements and loss of nuclear and cytoplasmic proteins (Chu 1962; Maizel et al. 1975). Moreover, dimethyl sulfoxide

(DMSO), a cryoprotectant usually used for cell freezing, is a known methylating agent (Iwatani et al. 2006) that might affect the developmental capacity of frozen cells.

5.1.4 Effect of donor cell line of MSCs on in vitro development of NT embryos

As demonstrated in pigs and cattle (Kuhholzer et al. 2001; Poehland et al. 2007) not only different cell lines, but also clones derived from one primary cell line can differ in their developmental potential when used for NT, suggesting that a particular cell line, irrespective of its origin, has unique intrinsic characteristics, inherent or acquired, which may affect its potential for NT. The results of the present study are in good agreement with numerous studies that argue for an unpredictable outcome when using MSCs as nuclear donors. In fact, our study, which to our knowledge is the first to assess the developmental potential of rabbit MSCs as nuclear donors, yielded all possible scenarios including a similar, lower or higher developmental potential of MSCs compared to fibroblasts suggesting that either cell line of MSCs has unique intrinsic characteristics affecting its ability for reprogramming following NT.

5.1.5 Fibroblast cells

Unlike MSCs, two fibroblast cell lines tested in the present study showed similar developmental potential suggesting that fibroblast cells are more stable than cells of stem cell origin. Embryonic stem (ES) cells, the best studied type of stem cells, show a remarkable epigenetic and genetic instability and even sister cells of freshly subcloned ES cells can exhibit substantial variation in gene expression (Humpherys et al. 2002). Pre-existing epigenetic errors in combination with faulty reprogramming during NT may cause gene expression abnormalities in ES cell derived clones, which are specific to the particular donor nucleus and the differences appear to reflect the epigenetic state of the respective donor nucleus. Whether this is the case with adult stem cells remains to be clarified. Presently, the epigenetic state of adult stem cells is not well known and molecular events regarding epigenetic reprogramming of the chromatin of adult stem cells are poorly understood. It remains also debatable whether adult stem cells are better donors than differentiated cells for cloning by NT (Hochedlinger and Jaenisch 2007). When testing the hypothesis as to whether SCNT clones can be generated directly from truly differentiated somatic cells with mouse

hematopoietic stem cells at different differentiation stages, Sung *et al*. (2006) found that cloning efficiency increases over the differentiation hierarchy, and the greatest cloning efficiency was achieved using terminally differentiated postmitotic granulocytes. Unlike using hematopoietic stem cells, a high efficiency of blastocyst development and ES cell derivation was demonstrated when neural stem cells were used for SCNT (Blelloch et al. 2006) thus arguing that some lineages somatic stem cells might be more efficiently reprogrammed that their differentiated counterparts. Comparison of the expression pattern of genes implicated in transcription and pluripotency, DNA methylation and histone deaacetylation, growth factor signaling, imprinting and apoptosis revealed that porcine embryos cloned from MSCs were more similar to *in vivo* derived embryos than embryos cloned from fetal fibroblasts (Kumar et al. 2007) suggesting that MSCs with a relatively undifferentiated genome are more efficiently reprogrammed and might be better donors for NT. In general, it can be assumed that different types of adult stem cells differ in the degree of genomic plasticity and therefore might not be equal in terms of reprogrammability following NT. The question whether the genomes of adult stem cells are similar to ES cells in that they are easier to reprogram than the genomes of terminally differentiated cells might not be easily answered and might in fact be dependent on the specific lineage.

5.2 Reprogramming of histone modifications in embryos cloned from MSCs and RAFs

It was believed that histone methylation is an extremely stable modification. However, recent discoveries of several classes of lysine demethylase, the LSD1/BHC110 class (which removes H3K4me1/2) (Lee et al. 2005b) and the jumonji class (which removes H3K4me2/3, H3K9me2/3, and H3K36me2/3) (Cloos et al. 2006; Tsukada et al. 2006; Christensen et al. 2007; Park et al. 2007) have put to an end debates about the reversibility of histone lysine methylation. Demethylation and re-methylation of histone lysine residues following NT appear to be critical for the regulation of gene expression and for the establishment of totipotency of cloned (Oback and Wells 2002). Since the somatic gene expression program has to be turned off before embryonic gene expression is established, repressive histone methylation that occurs after NT would facilitate somatic gene silencing. In the present study, which to our knowledge is the first report addressing the epigenetic status of embryos

cloned from MSCs, we investigated the changes of H3K27m3 and H3K4m2/3 in SCNT embryos compared to *in vivo* embryos and found that these two histone modifications are reprogrammed to a certain degree but the dynamics of their reprogramming significantly differ.

5.2.1 Reprogramming of H3K27m3 modifications in cloned embryos

Like in *in vivo* embryos, with minor differences in zygotes and blastocysts, H3K27m3 was undetectable at all stages of NT embryos except for one-cell embryos and blastocysts suggesting faithful reprogramming of this modification. In line with our findings in rabbit embryos, bovine embryos have shown a low level of the repressive marker H3K27m3 at the stage of major genome activation (MGA) (Ross et al. 2008). However, unlike in our study, they found H3K27m3 at all stages during preimplantation development, with the highest fluorescent intensity in immature oocytes, followed by a steady decrease after fertilization to a minimum at the 8-cell stage, at the time of MGA, with a subsequent increase until the blastocyst stage. In zygotes they could recapitulate an observation that had already been described for mouse embryos (Santos et al. 2005; van der Heijden et al. 2005), that only the maternal pronuclear chromatin is decorated with H3K27m3, while the male chromatin is essentially devoid of it. In contrast to their findings, we could not detect H3K27m3 in any of the pronuclei in rabbit *in vivo* zygotes, although the modification was clearly present in MII oocytes.

The reason for this discrepancy remains elusive, however species specific differences of early epigenetic reprogramming events might account for it. In fact such differences might represent a more general phenomenon, given that the dynamic of other epigenetic marks such as DNA methylation has been shown to vary considerably between species (Beaujean et al. 2004). In accordance to the presented data of *in vivo* rabbit embryos, we and others have observed a generally low level various repressive histone marks at the stage of MGA in bovine (Santos et al. 2003) and mouse (Liu et al. 2004; Huang et al. 2007; Ooga et al. 2008) embryos, suggesting that removal of such repressive epigenetic modifications may be an essential part of embryonic genome activation.

Moreover, our finding of a low/undetectable level of H3K27m3 until the blastocyst stage parallels a continuous decrease of 5-methyl cytosine, a DNA modification that

is usually associated with transcriptional silencing and which in several mammalian species including rabbits has been shown to be actively (paternal genome) or passively (maternal genome) removed during preimplantation development (Mayer et al. 2000; Oswald et al. 2000; Dean et al. 2001a).

5.2.2 Reprogramming of H3K4m2/3 modifications in cloned embryos

H3K4m2/3 was found in all stages of *in vivo* as well as NT embryos, but the fluorescence intensity ratios in embryos cloned from either MSCs or RAFs were not similar to that of *in vivo* embryos suggesting an aberrant reprogramming of this chromatin modification. Our results corroborate previous observations that epigenetic reprogramming occurs abnormally in a large proportion of cloned embryos (Dean et al. 2001b; Kang et al. 2003; Santos et al. 2003; Hiendleder et al. 2004; Wee et al. 2006). Unlike with other specific histone modifications, which have been proposed as epigenetic markers to assess the process of nuclear reprogramming and predict SCNT efficiency (Santos et al. 2003; Yang et al. 2007) in the present study reprogramming of H3K4m2/3 modifications did not show clear correlation with the developmental potential of embryos cloned from any of the various utilized cell types. Moreover, embryos cloned from either cell type, irrespective of its origin, exhibited no close similarities in the reprogramming of H3K4m2/3 modification. NT embryos derived from MSC A/B differed from all others in the way that the fluorescence ratios were generally lower and remained in the range of the donor cells. It can be assumed that reprogramming of H3K4m2/3 is more aberrant with MSC A/B cells than with any other cell type used in this study. However, embryos cloned from MSC A/B cells developed to blastocysts with similar or higher rates than embryos derived from other cell types indicating little or no effect of H3K4m2/3 reprogramming on the subsequent development of reconstructed embryos. The rather stochastic than regular reprogramming of this chromatin modification in NT embryos might be also due to differential remodelling (histone methylation and demethylation) activities present in the recipient cytoplasm. This is supported by the fact that fluorescence levels of H3K4m2/3 fluctuated stochastically between embryos at different stages.

Patterns of epigenetic reprogramming of specific histone modifications appear to depend on the type of modification and species. In one study in mouse, histone H3K9m2/3 of somatic cells was gradually demethylated in the SCNT embryos

following activation (Wang et al. 2007). However, reprogramming patterns considerably differed for other epigenetic marks, as histones H3K9, H3K14, and H4K16 were quickly deacetylated following NT and then reacetylated following activation. In contrast, acetylation of histones H4K8 and H4K12 persisted in the genome of cloned embryos with only mild deacetylation during NT and activation. Histone H3K9 di- and trimethylation is correlated to gene silencing while histone H3K4 di- and trimethylation is involved in transcriptional competence and transcriptional activity, respectively (Santos-Rosa et al. 2002; Papp and Muller 2006).

When analyzing the dynamics of H3K4m2/3 in rabbit NT embryos reconstructed from cells of different type and origin, we could observe a general reprogramming of the donor chromatin towards a situation similar to that found in *in vivo* embryos. Similar to the situation in *in vivo* embryos, H3K27m3 was readily erased from the one-cell stage on and remained below detectable levels. Accordingly, H3K4m2/3 levels were initially elevated, in the one- or 2-cell stage and then declined rapidly to reach a minimum at the 4-cell stage, which is however anticipated compared to the *in vivo* situation where the minimum was reached at the 8-cell stage. The subsequent increase in *in vivo* embryos was also recapitulated in embryos reconstructed from all donor cells, although the extent varied considerably and in case of RAF 95 and RAF 93 this increase was followed by a final decline in morulae and blastocysts. This general recapitulation of H3K4m2/3 levels in NT embryos trying to mimic the situation *in vivo* argues for an important functional significance of correct H3K4m2/3 levels. The deviations observed in NT embryos might in the end be part of an aberrant reprogramming that leads to a high degree of developmental defects generally found in embryos reconstructed by nuclear transfer.

Similar to our results showing a continuously high level of the euchromatic mark H3K4m2/3 in rabbit embryos, Huang *et al.* (2007) have shown a constantly high level of histone H4 acetylation in the mouse preimplantation development, a modification that is associated with genetically active chromatin. The direct impact of H3K4 methylation on transcriptional activity during embryonic development was recently shown by Shao *et al.* (2008), who could demonstrate that hypermethylation of H3K4 induced by a specific histone de-methylation inhibitor, can lead to a precocious activation of genes in early mouse embryos (Shao et al. 2008). On the other hand we were surprised to find a minimum of the euchromatic marker H3K4m2/3 at the 8 cell

stage, a stage where MGA is expected to take place, i.e. the chromatin should adapt a most open chromatin conformation and transcription-prone constitution. Interestingly, we had already observed such a drop in the level of a marker for trancriptionally competent euchromatin, namely histone H3K9/K14 acetylation, in both rabbit (Yang et al. 2007) and bovine (Santos et al. 2003) embryos at the time point of MGA.

The reasons for this apparent contradiction are still unclear, but a possible explanation for this reduction in global euchromatic modifications could be that extensive histone modifications could hinder this first burst of embryonic transcription of the embryonic genome. In fact, the recruitment and binding of essential transcriptional constituents such as the basal transcription machinery or additional auxiliary transcription factors might be counteracted by too extensive histone modifications. Another possible explanation is that the presence of the transcription machinery and/or huge amounts of nascent RNA might mask antigens on the histone tails that would become less accessible for the antibodies used to visualize them. In fact in one of our previous studies on histone modifications dynamics during preimplantation development we have witnessed a low level of both, repressive and active marks at the time point of MGA (Santos et al. 2003). A somewhat similar, contracting finding recently presented by Ooga *et al.* (2008) who have shown that in the mouse embryo H3K79m2, a histone modification associated with active genes is rapidly lost from the maternal oocyte chromatin after fertilization and remains low at the 2 cell stage, i.e. during MGA.

In conclusion, our study demonstrates that while H3K27m3 was faithfully reprogrammed following NT with all cell lines examined, patterns of H3K4m2/3 differed between either group of cloned embryos irrespective of the origin or type of donor cells and did not resemble that of *in vivo* fertilized embryos, suggesting that reprogramming of this modification involves a more complex mechanism. Reprogramming of H3K4m2/3 did not correlate with developmental potential of embryos cloned from either type of donor cells. The criteria of cloning efficiency used in our study, i.e. development of cloned embryos to blastocysts and reprogramming of H3K27m3 and H3K4m2/3 modifications, could not provide convincing evidence for a correlation between the differentiation status of a donor nucleus and its epigenetic reprogramming.

6 SUMMARY

The main aim of this thesis was to find out which donor cells would be most suitable for production of cloned rabbits with a targeted modification of their genome. Such donor cells must follow a number of criteria including high developmental potential for NT, high proliferation capacity and genetic stability during their manipulation and culture. To follow these criteria we have chosen mesenchymal stem cells (MSCs) isolated from a bone marrow, the cell type which is considered to be less differentiated than any somatic cells of non-stem cell origin. We have established several MSC lines and assessed their developmental potential and epigenetic reprogramming of the specific histone modifications compared to genetically matched rabbit adult fibroblasts (RAFs) isolated from the same rabbits. As a "golden standard" for epigenetic reprogramming of histone modifications we used in vivo fertilized embryos. Histone modifications were used as epigenetic markers which appear to be reasonable for evaluating the efficiency of nuclear reprogramming.

As epigenetic markers for transcriptionally competent, as well as for silent chromatin, we have chosen antibodies that recognize specifically di- and tri-methylated lysine 4 on histone H3 (H3K4m2/3) and tri-methylated lysine 27 on histone H3 (H3K27m3), respectively. Using three MSC lines, MSC 93, 95 and MSC A/B, and two RAF lines, RAF93 and 95, which are genetically matched to MSC 93 and 95, we analysed reprogramming of these epigenetically and functionally different chromatin modifications following nuclear transfer into enucleated oocytes. We also assessed whether histone methylation patterns of cloned embryos correlate with their developmental potential. Additionally we transferred some embryos cloned from MSCs into recipients to test their potential to develop to offspring.

MSC 93 and 95 tended to differ in the ability to support the development of cloned embryos to blastocyst (29% and 44%, respectively) whereas similar (46% and 50%) blastocyst rates were obtained with RAF cell lines. Embryos cloned from fresh or long-term stored MSC A/B developed to blastocysts with significantly higher or similar rates (76% and 45%) compared to other cell lines. Transfer of 404 embryos cloned from cell lines MSC R3 and R8 into 10 recipients resulted in 5 pregnancies and the birth of one rabbit which lived only for a few hours. Neither transfer of cloned embryos at the one-cell stage nor co-transfer with parthenogenic embryos improved pregnancy or offspring rates. Transfer of 357 embryos cloned from cell line MSC A/B

into 10 recipients resulted in four pregnancies and one cloned rabbit which lived for three days. When other three recipients were slauthered on day 13 after embryo transfer, nine implantations were found.

H3K27m3 was not detectable from the zygote to morula stages in *in vivo* embryos. Only in blastocysts a weak staining of a single domain in some embryos in a few cells was visible presumably resembling the inactive X chromosome. In all stages of nuclear transfer embryos except for one cell stage and blastocysts H3K27m3 was undetectable. It seems that H3K27m3 is faithfully reprogrammed in transferred nuclei of all donor cell types, with minor differences in zygotes and blastocysts. Strong signals for H3K4m2/3 were detected at the one to two-cell stages of *in vivo* embryos with a slight decrease at the 4-cell stage, followed by a more drastic decrease at the 8-cell stage, where the signal minimum was reached. In 16-cell embryos signals slightly increased and then reached in morulae and blastocysts the levels observed in one-two cell embryos. In all types of nuclear transfer embryos fluorescence intensity ratios differed from that of *in vivo* embryos. The minimum was not reached at the 8-cell stage but at the 4-cell stage. Reprogramming of H3K4m2/3 modification occurred quite differently with either type of cells irrespective of the cell origin or type and no close similarities in the patterns of this reprogramming was observed between *in vivo* and nuclear transfer embryos. Embryos cloned from MSC A/B differed from all others in the way that H3K4m2/3 was generally lower and remained in the range of the donor cells. This suggests that reprogramming of H3K4m2/3 modification is more aberrant with MSC A/B cells than with any other cell type used in this study. However, with MSC A/B cells, a significantly higher or similar proportion of cloned embryos developed to blastocysts indicating that reprogramming of H3K4m2/3 modification does not correlate with developmental potential of donor cells.

In conclusion, our study provides evidence that histone modifications for heterochromatin are faithfully reprogrammed during NT of rabbit somatic cells, while patterns of epigenetic reprogramming of euchromatic histone modifications differ between individual cell lines irrespective of their origin or type and are not correlated with their developmental potential. Although MSCs were not superior to RAFs in respect to the criteria tested in our study they may be suitable nuclear donors for generating transgenic cloned rabbits due to their high developmental plasticity.

7 ZUSAMMENFASSUNG

Reprogrammierung von mesenchymalen Stammzellen und reifen Fibroblasten durch Kerntransfer im Kaninchen

Ziel dieser Arbeit war die Identifizierung von Kernspenderzellen, die für die Erzeugung klonierter Kaninchen mit einer gezielten genetischen Modifikation am geeignetsten sind. Kriterien für solche Kernspenderzellen umfassen ein hohes Entwicklungspotential nach dem Kerntransfer, eine hohe Proliferationskapazität sowie genetische Stabilität während der Manipulation und Kultur. Auf der Basis dieser Kriterien wurden mesenchymale Stammzellen (MSCs) aus dem Knochenmark isoliert, die als weniger differnziert gelten als alle somatischen Zellen ohne Stammzelleigenschaften. Im Rahmen dieser Arbeit wurden mehrere MSC Linien etabliert und hinsichtlich ihrer Entwicklungskapazität und epigenetischen Reprogrammierung hinsichtlich spezifischer Histonmodifikationen nach dem Kerntransfer untersucht. Als Vergleich dienten Fibroblasten (RAFs), die von den gleichen Spendertieren isoliert wurden. Als Goldstandard für die epigenetische Reprogrammierung der Histonmodifikationen während der frühen Embryonalentwicklung wurden Embryonen aus in vivo fertilisierten Eizellen untersucht. Histonmodifikationen wurden untersucht, da sie als epigenetische Marker für die Evaluierung der Effizienz der Kern-Reprogrammierung angesehen werden.
Zur Detektion epigenetischer Marker für transkriptionsaktives bzw. –inaktives Chromatin wurden Antikörper gewählt, die spezifische di- und tri-methylierte Varianten des Lysin 4 im Histonprotein H3 (H3K4m2/3) bzw. tri-methyliertes Lysin 27 im Histonprotein H3 (H3K27m3) erkennen. Für drei MSC Linien, MSC 93, 95 und MSC A/B, sowie zwei RAF Linien, RAF93 und 95, die isogenetisch zu den MSC Linien 93 und 95 sind, wurde die Reprogrammierung dieser epigenetisch und funktionell unterschiedlichen Chromatinmodifikationen nach dem Kerntransfer in enukleierte Eizellen analysiert. Zudem wurde untersucht, ob die Histon-methylierungsmuster mit der Entwicklungskapazität der Kerntransferembryonen korrelieren. Schließlich wurden Kerntransferembryonen aus MSCs auch auf Embfängertiere übertragen, um zu testen, ob sie sich zu lebenden Nachkommen entwickeln können.

MSC 93 und 95 zeigten nach dem Kerntransfer eine Tendenz zu unterschiedlichen Blastozystenraten (29% bzw. 44%), während für die beiden RAF Zellkulturen ähnliche Blastozystenraten erhalten wurden (46% bzw. 50%). Kerntransferembryonen aus frischen bzw. kryokonservierten MSC A/B entwickelten sich zu einem signifikant höheren bzw. ähnlichen Prozentsatz (76% bzw. 45%) zu Blastozysten wie die Klonembryonen aus den anderen Zelllinien. Der Tansfer von 404 Kerntransfer-embryonen aus den MSC Linien R3 und R8 in 10 Empfängertiere resultierte in 5 Trächtigkeiten und einem geborenen Nachkommen, der nur für wenige Stunden lebte. Weder der Transfer von Embryonen im Einzellstadium noch der Kotransfer mit parthenogenetischen Embryonen verbesserten die Trächtigkeitsrate bzw. die Ausbeute an Nachkommen. Der Transfer von 357 Kerntransferembryonen aus der Linie MSC A/B in 10 Empfängertiere resultierte in 4 Trächtigkeiten sowie in der Geburt eines geklonten Nachkommen, der drei Tage lebte. Die übrigen 3 Empfängertiere wurden am Tag 13 nach dem Embryotransfer geschlachtet. In den Uteri fanden sich insgesamt 9 Implantationsstellen.

H3K27m3 war in *in vivo* Embryonen vom Zygoten- zum Morulastadium nicht nachweisbar. Lediglich in einigen Blastozysten zeigten einige Blastomeren eine schwache Färbung einer einzelnen Domäne, bei der es sich vermutlich um das inaktivierte X-Chromosom handelte. In allen Stadien der Kerntranferembryonen, mit Ausnahme des Einzellstadiums und der Blastozyste, war ebenfalls kein H3K27m3 nachweisbar. Offenbar wurde H3K27m3 in den transferierten Kernen aller Spenderzelltypen bis auf kleine Unterschiede im Einzell- und Blastozystenstadium korrekt reprogrammiert. Für H3K4m2/3 wurden starke Signale in *in vivo*-Embryonen im Ein- bis Zweizellstadium detektiert, die zum Vierzellstadium leicht und dann stärker abnahmen, um im Achtzellstadium einen Tiefpunkt zu erreichen. Im Sechzehnzellstadium stieg die Markierung dann wieder an und erreicht schließlich im Morula und Blastozystenstadium ähnliche Intensitäten, wie sie im Ein- und Zweizellstadium zu beobachten waren. In Kerntransferembryonen aller Spenderzelltypen unterschied sich das Fluoreszenzmuster von dem der *in vivo* Embryonen. So wurde das Minimum der Markierung nicht im Acht-, sondern bereits im Vierzellstadium erreicht. Die Reprogrammierung der H3K4m2/3 Modifikation unterschied sich unabhängig von der verwendeten Spenderzelllinie deutlich zwischen den Kerntransferembryonen und den *in vivo* erzeugten Embryonen. MSC A/B-

Kerntransferembryonen unterschieden sich von allen anderen dadurch, dass die H3K4m2/3 Markierung generell schwächer war und im Bereich der Markierung der Kernspenderzellen blieb. Dies weist darauf hin, dass die Reprogrammierung der H3K4m2/3 Modifikation in den MSC A/B Zellen weniger ausgeprägt als bei den anderen Spenderzellen verlief. Trotzdem entwickelte sich aus den MSC A/B Zellen nach dem Kerntransfer ein ähnlicher, teilweise sogar höherer Anteil der Embryonen zu Blastozysten als bei den anderen Spenderzellen. Dies weist darauf hin, dass die Reprogrammierung der H3K4m2/3 Modifikation nicht unbedingt mit dem Entwicklungspotential der Kernspenderzellen korreliert.

Zusammenfassend zeigt diese Studie, dass Histonmodifikationen, die für Heterochromatin charakteristisch sind, nach dem Kerntransfer somatischer Zellen beim Kaninchen korrekt reprogrammiert werden, während sich die Muster von euchromatischen Histonmodifikationen zwischen den Spenderzelllinien stark unterscheiden können und nicht mit der Entwicklungskapazität korrelieren. Obwohl die MSCs den RAFs nach den in dieser Studie untersuchten Parametern den RAFs als Kernspenderzellen nicht überlegen waren, könnten sie für die Erzeugung transgener geklonter Kaninchen aufgrund ihrer höheren Entwicklungsplastizität besser geeignet sein.

8 PUBLICATIONS

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