Ricardo Cardoso de Almeida Ribas dos Santos

Factors Influencing the Development of Cloned Embryos

Instituto de Ciências Biomédicas de Abel Salazar

Universidade do Porto

Porto, 2005

Ricardo Cardoso de Almeida Ribas dos Santos

Factors Influencing the Development of Cloned Embryos

Instituto de Ciências Biomédicas de Abel Salazar

Universidade do Porto

Porto, 2005

Ricardo Cardoso de Almeida Ribas dos Santos

Factors Influencing the Development of Cloned Embryos

Dissertação de candidatura ao grau de Doutor em Ciências Veterinárias submetida ao Instítuto de Ciências Biomédicas de Abel Salazar, Universidade do Porto (ICBAS, UP)

Orientador-	Professor Doutor Ian Wilmut
Co-Orientadores-	Prof. Doutora Ana Colette Maurício
	Professor Doutor Mário Sousa

I declare that this thesis has been composed by myself and has not been submitted for any previous degrees. The work described herein is my own and all work and other authors are duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

All the work presented in this thesis was performed at Roslin Institute, Edinburgh, United Kingdom and is now being submitted to Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto (ICBAS, UP).

Publications

According to the Portuguese Law (nº2 do artigo 8º do Decreto_lei nº 388/70), results obtained from the following published (or submitted for publication) papers were introduced in this thesis:

Ribas, R.; Oback, B.; Taylor, J.; Mauricio, A.; Sousa, M.; Wilmut, I. (2005) "Cloned mouse produced using a zona-free method of nuclear transfer". Reproduction, Fertility and Development, 17 (1,2): 180-181. In Proceedings of the annual conference of the International Embryo Transfer Society, Copenhagen, Denmark, 8-12 January 2005

Ribas, R.; Oback, B.; Ritchie, W.; Chebotareva, T.; Ferrier, T.; Clarke, C.; Taylor, J.; Gallangher, E.J.; Mauricio, A.C.; Sousa, M.; Wilmut, I. (2005). "Development of a zona-free method of nuclear transfer in the mouse". Cloning and Stem Cells, 7 (2): 126-138.

Ribas, R.; Oback, B.; Ritchie, W.; Chebotareva, T.; Taylor, J.; Maurício, A.C.; Sousa, M.; Wilmut, I. (2006). "Modifications to improve the efficiency of zona-free mouse nuclear transfer". Cloning and Stem Cells (*in print*).

Ribas, R.; Taylor, E.; McCorquodale, C.; Maurício, A.C.; Sousa, M.; Wilmut, I. (2006). "Effect of zona pellucida removal on DNA methylation in early mouse embryos." Biology of Reproduction (*in print*).

Acknowledgements

Many thanks to Professor Ian Wilmut for accepting to be my supervisor and for all the help and support given to me during all my PhD project. I thank him for the supervision of all experimental work and the writing of this thesis, and for all the time he spent with me no matter how busy he was.

I am very grateful to both my co-supervisors, Professor Ana Colette Mauricio and Professor Mario Sousa, for the help regarding the organization of my PhD and all the support, encouragement and precious help during all the PhD.

I would like to thank Bjorn Oback and his group from New Zealand for having the patience to teach me their new technique of nuclear transfer in cattle and help me during the time I spent there.

I would like to thank Dr. Jane Taylor for all the help and supervision during the carry of all of the lab work, and for teaching lots of things about molecular biology.

A special thanks to Bill Ritchie, Alison Ainslie and Tatiana Chebotareva for all the technical help during the long cloning days. Without them most of the work wouldn't be possible. I am very grateful to all of you.

I would like to thank Michelle McGarry for teaching me lots of things about mouse nuclear transfer and for the help during the time I was starting my work with mouse.

Many thanks to Paul deSousa, Tricia Ferrier, Catriona Clarke, Judy Fletcher, Linda Harkness and John Gardner, for the cell culture, and the technical support concerning the donor cells that have been used for nuclear transfer. Without them the work wouldn't be possible either.

I am very grateful to Ms. Lynne Elvin for all the support, assistance and for saving my life so many times in my day-by-day problems and for making my days much nicer from the morning.

Thank you very much to Caroline McCorquodale for the extremely help concerning the statistical analysis of my results.

Many thanks to all the Small Animal Unit Staff, particularly to Dave Davies and Sally Frost. Without them none of this would be ever possible.

Thank very much to the GABBA Program for select me in the program. It was an amazing opportunity in my life.

A very special thank to all my friends, which share with me the good and bad moments of my life. You know who you are and how special all of you are to me. Without you my life would not be possible at all.

Finally, I own a sincere thanks to all my family; especially my mother and father that always have done everything for me to make me feel the happiest person ever. Lots of love for you both and thank you for everything.

Thank very much to FCT (Fundação para a Ciência e para a Tecnologia), for funding my project (SFRH/BD/5807/2001).

Abbreviations

AT	Acid Tyrode's
BS	Block solution
BSA	Bovine serum albumin
Ca_2Cl	Calcium chloride
cDNA	Complementary DNA
cm	centimeters
COC's	Cumulus oocyte complexes
CO_2	Carbone dioxide
Cyt B	Cytochalasin B
DAPI	4,6-diamino-2-phenylindole
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Desoxyribonuclease
Dnmt1o	DNA methyltransferases 1- oocyte form
Dnmt1s	DNA methyltransferase 1- somatic form
dNTPs	Deoxynucleotides
dsRNA	double strand RNA
DTT	Dithiothreitol
eCG	Equine choriogonadotrophin
EDTA	Ethylenediaminetetraacetic acid
ES cells	Embryonic stem cells
Eth	Ethanol
FCS	Fetal calf serum
Fgf4	Fibroblast growth factor 4
FITC	Fluorescein isothyocyanate
g	gram
GLMM	Generalised linear mixed model
GVBD	Germinal vesicle breakdown
G0	Cell cycle quiescent phase
G1	Cell cycle gap 1 phase
G2	Cell cycle gap 2 phase

h	hours
H1, H2A, H2B, H3, H4	Histone 1, 2A, 2B, 3, 4, respectively
HCG	Human choriogonadotrophin
hCZB	Hepes buffered CZB medium
HDAC	Histone deacetylase
ICM	Inner cell mass
Ion	Ionomycin
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IU	International units
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
KV	Kilovolts
LOS	Large offspring syndrome
M	Cell cycle mitosis phase/ Molar
MBD	Methyl-binding protein
$MgSO_4$	Magnesium sulfate
mg	milligrams
min	minutes
ml	millilitre
mm	millimetres
mM	millimolar
mOsm	Milliosmolarity
MPF	Meiosis/ Mitosis/ Maturation promoting factor
MR	Mechanical removal
mRNA	Messeger RNA
NaCl	Sodium chloride
NEBD	Nuclear envelope breakdown
ng	nanograms
nM	nanomolar
$NaHCO_3$	Sodium bicarbonate
NT	Nuclear transfer
O_2	Oxygen
Oct4	Octamer-binding transcription factor-4

PBS	Phosphate buffered saline
PCC	Premature chromosome condensation
PFA	Paraformaldehyde
PHA-P	Phytohaemaglutinin-P
Pr	Pronase
PrP	Prion protein gene
PVA	Polyvinyl alcohol
REML	Residual maximum likelihood
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	Rotations per minute
R-Point	Restriction point
RT-PCR	Reverse transcription- polymerase chain reaction
S	Cell cycle DNA synthesis phase
sec	seconds
SEM	Standard error of the mean
$SrCl_2$	Strontium chloride
TE	Trophectoderm
TF	Transcription factors
TRD	Transcription repression domain
UV	Ultraviolet
vs.	Compared to or versus
ZF	Zona-free
ZP	Zona pellucida
ZI	Zona-intact
WOW	Well of the well culture system
5-Metc	5-Methylcytosine
β-ME	β- mercaptoethanol
µg	Micrograms
µl	microlitres
µm	micrometer
µsec	microseconds

Contents

Abstract	xvi
Resumo	xviii
Résumé	xx
Chapter I- Literature Review	1
1. Nuclear Transfer (NT)	2
1.1. The History of NT	2
1.2. What has been achieved with NT?	3
1.3. NT Efficiency	4
1.4. Fetal Losses/ Offspring Problems	5
1.5. NT Technologies	7
1.5.1. NT technologies in the mouse	12
1.6. Approaches to Improve Efficiency	12
1.6.1. Type of donor cells and plasticity	13
1.6.2. Cell cycle and coordination	13
1.6.3. Recipient oocyte size	16
1.6.4. Nuclear recycling, serial NT and delayed activation	17
1.6.5. Pretreatment to change chromatin	18
2. Zona Pellucida (ZP)	20
2.1. What is ZP?	20
2.2. ZP Functions	21
2.3. Mechanisms of ZP removal	22
2.4. Perivitelline Space and Cortical Granules Envelope	23

3. Epigenetic Modifications and Reprogramming 25

3.1. Nucleus/ Chromatin Structure 25

3.2. Epigenetic Modifications 26

3.3. DNA Methylation 27

3.3.1. What is DNA methylation? 27

3.3.2. Roles of DNA methylation 28

3.3.3. DNA methyltransferases 29

3.3.4. DNA methylation and transcription repression 32

3.3.5. DNA methylation patterns in embryos 34

3.3.6. DNA methylation and reprogramming in NT embryos 37

3.4. Other Epigenetic Modifications 38

3.4.1. Histone modifications 38

3.4.1.1. Histone acetylation 39

3.4.1.2. Histone phosphorylation 39

3.4.1.3. Histone methylation 40

3.4.1.4. Histone ubiquitination 40

3.4.2. Higher order chromatin structure 40

3.4.3. RNA interference (RNAi) 41

4. Thesis Aims and Objectives 42

4.1. Specific Aims 42

Chapter II- Development of a zona-free method of nuclear transfer in the mouse 45

Abstract 46

Introduction 48

Material and Methods 49

Results 56

Discussion 61

References 65

Figures and Tables 70

Chapter III- Modifications to improve the efficiency of zona-free mouse nuclear transfer 84

Abstract 85

Introduction 86

Material and Methods 87

Results 88

Discussion 91

References 95

Figures and Tables 98

Chapter IV- Effect of zona pellucida removal on DNA methylationin early mouse embryos 102

Abstract 103

Introduction 104

Material and Methods 106

Results 111

Discussion 115

References 120

Figures and Tables 126

Chapter V- General discussion and future expectations 133

References 142

Appendices 173

Abstract

Nuclear transfer consists of transference of a nucleus from a donor cell to an oocyte previously enucleated. Different techniques of nuclear transfer are now available, in order to simplify and increase efficiency. However, in the mouse, previous methods depend upon expensive equipment and require a great deal of experience and manipulation skills. Several laboratories have attempted to use the Honolulu method, but with no success despite being successful with cell fusion in other species. Cloning mice from differentiated somatic cells is still far from being a routine procedure, even though it was reported as early as 1998. Very few laboratories world-wide have been able to reproducibly generate cloned viable offspring through direct transfer of adult somatic cells. In the present study, a zona-free nuclear transfer technique, which had been originally developed in cattle, was modified for the mouse. Steps involved in this approach include removing the zona pellucida and enucleating without a holding pipette; sticking donor cells to the cytoplast before electric pulses are applied to fuse them and culturing reconstructed embryos individually in single droplets, to prevent aggregation. Finally, some adjustments on the technique were performed in order to optimise this technique and improve results in the mouse. The birth of a healthy cloned mouse from zona-free NT provides 'proof of principle' of a technology that promises to increase throughput and ease of operation.

At the same time, studies were performed with control zona-free and zona-intact embryos from mated donors showing no significant difference in development to blastocyst, but did show reduced development to term. For this reason, the effect of removing the zona pellucida of mouse embryos fertilized *in vivo* and cultured *in vitro* was assessed in terms of blastocyst quality, DNA methylation, histone acetylation and onset of transcription of some regulatory genes. Results have shown a significant

reduction in the level of DNA methylation when the zona pellucida was removed at the time of the fertilization, but no differences at the levels of histone acetylation or at the time of gene expression of selected genes.

Keywords: Nuclear Transfer, Mice, Zona-Free, Zona Pellucida, DNA Methylation

Resumo

A técnica de transferência nuclear consiste na introdução de um núcleo de uma célula dadora para um oócito previamente enucleado. Diferentes técnicas de transferência nuclear estão agora disponíveis, de forma a simplificar o processo e aumentar a sua eficiência. No entanto, no ratinho, os métodos ainda estão dependentes de equipamento muito dispendioso, requerem ainda grande experiência e conhecimento das técnicas de manipulação. Vários laboratórios ainda não obtiveram sucesso ao usar o método de Honolulu, tendo no entanto obtido sucesso quando usam métodos de fusão de células em outras espécies. A clonagem de ratinhos através da utilização de células somáticas diferenciadas está ainda longe de ser uma técnica de rotina, apesar do primeiro animal já ter sido clonado em 1998. Muitos poucos laboratórios em todo mundo conseguiram repetidamente clonar animais saudáveis através da transferência directa de células somáticas adultas. Neste projecto, uma técnica de transferência nuclear em embriões "zona-free", previamente desenvolvida na espécie bovina, foi modificada e aplicada em ratinhos. Esta técnica incluí a remoção da zona pelúcida e a enucleação dos oócitos sem pipeta de sucção; a "colagem" da célula dadora ao citoplasto antes da aplicação dos pulsos eléctricos para a fusão e finalmente a cultura individual dos embriões reconstruídos em pequenas gotas para prevenir agregação. Por fim, foram testadas, algumas modificações e ajustes à técnica de forma a permitir a sua optimização e melhorar os resultados, no ratinho. O nascimento de um ratinho clonado e saudável usando esta técnica de transferencia nuclear em embriões "zona-free" é a prova real de uma tecnologia que promete um aumento da eficiência e sua simplificação.

Simultaneamente, outros estudos foram feitos em grupos de controlo de embriões com e sem zona pelúcida, provenientes de animais fertilizados. Estes embriões não

mostraram diferenças significativas no desenvolvimento *in vitro* até a fase de blastocisto, mas verificou-se uma redução do desenvolvimento até termo naqueles em que a zona pelúcida foi removida. Deste modo, o efeito da remoção da zona pelúcida em embriões de ratinhos fertilizados *in vivo* e em cultura *in vitro* foi analisada em termos de qualidade dos blastocistos, metilação do DNA, acetilação das histonas e iniciação da transcrição de genes reguladores. Os resultados obtidos mostraram um redução significativa nos níveis de metilação do DNA quando a zona pelúcida foi removida logo após fertilização, mas não foram encontradas diferenças nos níveis de acetilação das histonas ou no início da expressão dos genes reguladores seleccionados.

Palavras-Chave: Transferência Nuclear, Ratinhos, Zona-Free, Zona Pelúcida, Metilação do DNA

Résumé

Le transfert de noyau repose sur le transfert d'un noyau de cellule donneuse dans un ovocyte préalablement énucléé. Différentes techniques de transfert de noyau sont actuellement disponibles, notamment afin d'en simplifier la procédure et d'augmenter l'efficacité. Cependant, chez la souris, les techniques utilisées jusqu'à présent nécessitent un équipement coûteux ainsi qu'une grande expérience et habileté manuelle. Plusieurs laboratoires ont tenté d'utiliser la méthode d'Honolulu, mais sans succès et ce malgré une expertise en clonage par fusion cellulaire dans d'autres espèces. Le clonage de la souris à partir de cellules somatiques différenciées est donc loin d'être une procédure de routine, bien qu'elle existe depuis 1998. Très peu de laboratoires de par le monde ont été capables de générer de manière reproductible des clones viables par transfert direct de cellules somatiques adultes. Dans cette étude, la technique de transfert de noyau « zona-free », développée en premier chez le bovin, a été adaptée à la souris. Cette approche est basée sur le retrait de la zone pellucide et l'énucléation sans pipette de maintien; l'adhésion des cellules donneuses au cytoplasme avant de les faire fusionner par pulses électriques et la culture des embryons reconstitués en gouttes individuelles, afin d'empêcher l'agrégation. Quelques ajustements supplémentaires ont permis d'optimiser la technique et d'améliorer les résultats chez la souris. Finalement, la naissance d'une souris clonée saine à partir de cette technique de transfert de noyau « zona-free » apporte la preuve de la faisabilité de cette technologie prometteuse, plus efficace et aisée.

Par ailleurs, des expériences réalisées sur des témoins, embryons issus d'accouplements naturels, avec ou sans zone pellucide ont montré qu'il n'y avait aucune différence jusqu'au stade blastocyste, mais une réduction du taux de développement à terme. C'est pourquoi les effets dus aux retrait de la zone pellucide

ont été évalués, sur des embryons de souris fécondés *in vivo* et cultivés *in vitro*, en terme de qualité des blastocystes, méthylation de l'ADN, acétylation des histones et démarrage de la transcription de gènes régulateurs. Les résultats montrent une réduction significative du niveau de méthylation de l'ADN lorsque la zone pellucide est retirée après fécondation, mais aucune différence du niveau d'acétylation ni d'expression des gènes sélectionnés.

Mot Clé: Transfert de Noyau, Souris, Zona-Free, Zone Pellucide, Méthylation de l'ADN

Chapter I

Literature Review

1. Nuclear Transfer (NT)

1.1.The History of NT

Since 19th century scientists started to use procedures based in nuclear transfer. At the time, techniques were very rudimentary and were not based on micromanipulators. The first experiment was performed by accident by Jacques Loeb in sea urchin by osmotic blebbing of the cytoplasm (Loeb, 1884). Last century, a German scientist Hans Spemann used a more consistent model using a hair to make a transient and complete separation in salamander embryos with a noose. Later, he carried out an experiment to transfer one cell's nucleus into an egg without a nucleus, which is the basic method of cloning (Spemann, 1914; Spemann, 1938). In 1952, some other attempts of nuclear transfer were performed using hand pipettes in frog eggs. Initially the use of micromanipulators was not necessary because of the large size of the eggs and the lack of zona pellucida (Briggs & King, 1952; reviewed by Vajta et al., 2005). In 1962, John Gurdon announced that he had used a nucleus from fully differentiated adult intestinal cells to produce tadpoles (Gurdon, 1962). But it was with the beginning of mammalian cloning that the problems started to appear, as it was believed that zona pellucida was indispensable for early development, enabling the scientists to enucleate the oocytes without micromanipulators. The first example of mammal cloning using the process of nuclear transfer was reported by Willadsen in 1986, after introduce a technique of bisection of single-cell oocytes following selection of enucleated halves and reconstruction with single blastomeres by fusion (Willadsen, 1986). But we needed to wait 10 years more until the birth of "Dolly", the first animal to be cloned by a fully differentiated somatic cell (Wilmut et al., 1997). Since then, NT technologies have been modified and other species have been cloned with success from differentiated donor cells, such as cattle (Kato et al., 1998) and

mouse (Wakayama et al., 1998; Wakayama & Yanagimachi, 1999) in 1998; pig (Polejaeva et al., 2000a) in 2000; cat (Shin et al., 2002) in 2001; goat (Keefer et al., 2002) in 2002; mule (Woods et al., 2003), horse (Galli et al., 2003), rabbit (Challah-Jacques et al., 2003) and rat (Zhou et al., 2003) in 2003; and finally the dog in 2005 (Lee et al., 2005). Meanwhile, some other work has been done in buffalos however no development to term has occurred until this moment (Saikhun et al., 2004).

In June 2005, Hwang and co-workers reported that pluripotent human embryonic stem (ES) cells could be efficiently generated by NT from a wide variety of patients: male vs. females between 2 to 56 years of age. They transferred somatic-cell nuclei from donors who had conditions that are potentially amenable to stem-cell therapy (congenital hypogammaglobulinemia, spinal cord injury, juvenile diabetes) and transferred them into oocytes whose nuclear genomes had been removed, opening windows for the future of the cloning therapy in humans (Hwang et al., 2005). A detailed re-assessment of this research is being carried out in the light of suggestions that some of Professor Hwang claims are fraudulent. This is not yet complete, but recent analysis showed that no patient specific lines are still growing in the laboratory. Further studies are being carried out to discover exactly what had been achieved.

1.2. What has been achieved with NT?

Cloning by NT is a technique that consists of the transference of nuclei from one donor cell to a previously enucleated oocyte (cytoplast). Donor cells with four different origins have been used with success, producing live and healthy offspring:

a) Blastomeres: cells derived by zygote cleavage (amphibians: Briggs & King, 1952; sheep: Willadsen, 1986; cattle: Prather et al., 1987);

b) Embryonic cells, like embryonic stem cells (mouse: Wakayama et al., 1999; Rideout et al., 2000; Amano et al., 2001) or epithelial-like cells (sheep: Campbell et al., 1996; Wilmut et al., 1997; Wells et al., 1998a);

c) Fetal cells, like fetal fibroblasts (sheep: Wilmut et al., 1997; Schieke et al., 1997; McCreath et al., 2000; cattle: Cibelli et al., 1998; Zathartchenko et al., 1999);

d) Adult or differentiated cells, such as mammary gland cells, used to produce "Dolly" the sheep, the first animal cloned from an adult cell (Wilmut et al., 1997). However, other adult cells were used with success, such as cumulus cells (Kato et al., 1998; Wakayama et al., 1998); granulosa cells (Well et al., 1998a Wells et al., 1999); lymphocytes (Galli et al., 1999; Hochendlinger & Jaenisch, 2002); oviduct cells (Kato et al., 1998); ear skin cells (Kubota et al., 2000); muscle cells (Shiga et al., 1999); Sertoli cells (Ogura et al., 2000) and others.

1.3. NT Efficiency

Although healthy and fertile offspring have been produced, cloning is still an inefficient technique with only a small percentage (0-4%) of the reconstructed embryos (using adult or fetal somatic cells) developing to become live offspring. This rate is independent of species, donor cell, method of nuclear transfer and even on each laboratory (Oback & Wells, 2002; Wilmut I., et al., 2002; Wilmut I. & Peterson L.A., 2002 [on line]). However, pre-implantation development does not seem to be a problem, with laboratories reaching 20-70% of development to blastocysts in mice (Wakayama et al., 1998; Ono et al., 2001; Ono et al., 2001a; Gao et al., 2003; Sakai et al., 2005), 20-60% in cattle (Booth, et al., 2001; Oback et al., 2003) and 20-40% in sheep (Campbell et al., 1996; Wilmut et al., 1997). The majority of the losses occur during the post implantation period and after birth. It has been reported in some

experimental studies, that only 2-3% of the transferred embryos develop to term in the mouse (Wakayama et al., 1998; Ono et al., 2001a; Gao et al., 2003; Sakai et al., 2005), 5-6% in cattle (Sakai et al., 2005); 3-6% in sheep (Wilmut et al., 1997) when reconstructed by NT using adult somatic cells. These results are significantly lower than those obtained after natural mating (92% in sheep) (Walker et al., 1992) or even by *in vitro* fertilisation (IVF) (39% in cattle) (Wells et al., 1998). This inefficiency is the cumulative result of losses observed during all the stages of pregnancy and after birth due to a large number of deficiencies. This is a consequence of inappropriate expression of some genes that harmfully can affect development at different stages (Han et al., 2003).

Many factors can affect NT efficiency, involving the embryonic ploidy (cytogenetic effect) or the reprogramming process of the donor nucleus (epigenetic effect), such as developmental stage, cell cycle stage (Oback & Wells, 2002; Wells et al., 2003), cell type (Eggan et al., 2001; Oback & Wells, 2002), genetic background of the donor cell (Oback & Wells, 2002), as well as the cell cycle stage of the recipient oocyte (Bordignon & Smith, 1998). During the zygotic genome activation (transition from maternal to zygotic control of development), the epigenetic constrains imposed by the donor cell genome during the differentiation need to be deleted in order to convert it back to the embryonic stage. So, the more differentiated the donor nucleus, the less plasticity it has, leading to a lower reprogramming efficiency (Wells et al., 2003).

1.4. Fetal Losses/ Offspring Problems

Losses in pregnancy after NT can occur in early or late pregnancy (Young et al., 1998; Hill et al., 2000; Peterson et al., 2000; De Sousa et al., 2001). In cattle and

sheep, the majority of losses are observed in the first third of pregnancy, which have been mainly associated with placental vascularization deficiencies (Young et al., 1998; Hill et al., 2000; Peterson et al., 2000; De Sousa et al., 2001; Chavatte-Palmer et al., 2002).

Large Offspring Syndrome (LOS) has been described in late gestation of mice, sheep and cattle and causes important perinatal deaths in NT (Young et al., 1998; reviewed by Sakai et al., 2005) and *in vitro* produced animals (Behboodi et al., 1995). LOS is characterized by excess fetal size, abnormal placental development (such as hydroallantois, enlarged edematous placentomes in reduced number and deficiencies in chorioallantoic vascularization) and asynchronous growth of organs (Farin & Farin, 1995; Wilson et al.; 1995; Walker et al., 1996; Kruip et al., 1997; Heyman et al., 1999; Hill et al., 1999; De Sousa et al., 2001; Tanaka et al., 2001; Chavatte-Palmer et al., 2002). The culture conditions used during *in vitro* development, such as addition of serum (Thompson et al., 1990) and the use of co-culture systems (Farin & Farin, 1995), may contribute to the observed anomalies. Interestingly, LOS appears with higher incidence in clones produced from somatic cells than those produced from embryonic cells (Chavatte-Palmer et al., 2002; Heyman et al., 2002). In mice, placentomegaly is a striking and persistent characteristic, with the placenta being enlarged 2- to 3-fold, when compared with controls. This has also been found in cattle (reviewed by Sakai et al., 2005).

Some problems also occurred in the live offspring (postnatal development). The most common are respiratory distress syndrome (with the appearance of froth in the airways) and left heart insufficiency (Hill et al., 1999; Chavatte-Palmer et al., 2002), resulting in death in the most of the cases, during the first 6 months after birth (sheep: Wilmut et al.; 1997; Wells et al., 1998a; cattle: Cibelli et al., 1998; Wells et

al., 1998; mouse: Eggan et al., 2001). Abnormalities in kidney development and liver steatosis are also frequently (Sakai et al., 2005), and some other have been reported, such as:

- Abnormalities in the immune system, seen as thymic aplasia (Renard et al., 1999) and increase susceptibility for infections such as ruminitis and abomasitis in calves (Well et al., 1998a), coccidiosis or infection after trauma (Vignon et al., 1998; Lewis et al., 2000);

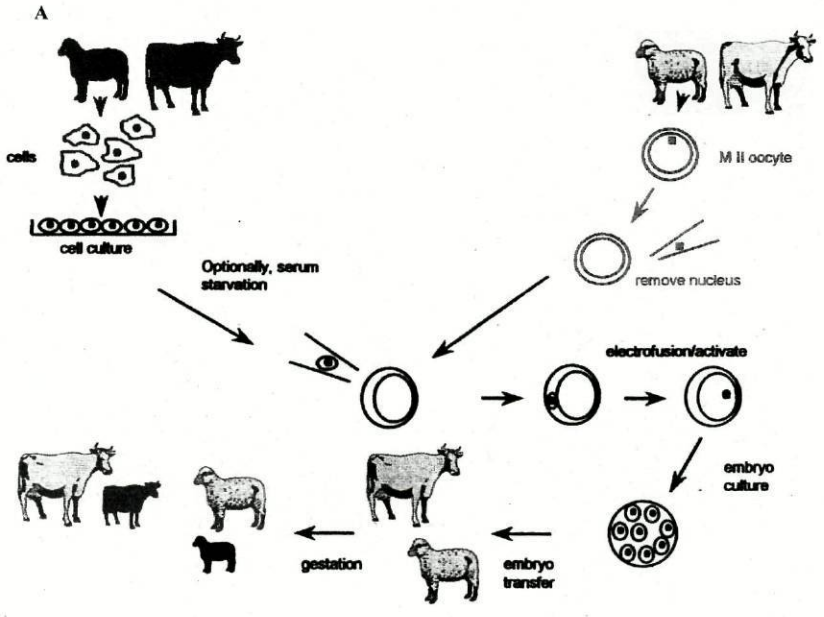
- Alterations in the behaviour have been also tested in mouse and cattle. Data suggests that cloned mice produced with adult somatic cells show a delay in appearance of few developmental milestones, but it does not adversely affects the postnatal behaviour (Tamashiro et al., 2000). Similarly, no large evident alterations in behaviour were found in cattle. Animals react normally to farm equipment and development of social dominance hierarchy, but showed greater curiosity, grooming activities and were more aggressive than control animals (Lanza et al., 2001; Savage et al., 2003). Further and more conclusive studies should be done in the future.

- Increase in the weight and obesity were also reported in mice produced by NT technologies (Tamashiro et al., 2000; Ogura et al., 2002; Tamashiro et al., 2002)

1.5. NT Technologies

Nuclear transfer consists of the transfer of a nucleus from a donor cell to an oocyte previously enucleated, followed by subsequent parthenogenic activation in order to mimic the sperm effect and initiate embryo development. There are currently several techniques of nuclear transfer, however mainly two different types have been used extensively and applied with success:

1) Fusion method: Placing the donor cell in the perivitelline space of the previously enucleated oocyte, before electric pulses are used to fuse the donor cell to the cytoplasm. This is a common technique applied to large animals such as cattle (Cibelli et al., 1998; Kato et al., 1998; Kubota et al., 2000), sheep (Wilmut et al., 1997), goat (Keefer et al., 2002) and pig (Polejaeva et al., 2000) (Fig. 1). Recently have been also used with success in dog (Lee et al., 2005) and cat (Shin et al., 2002).



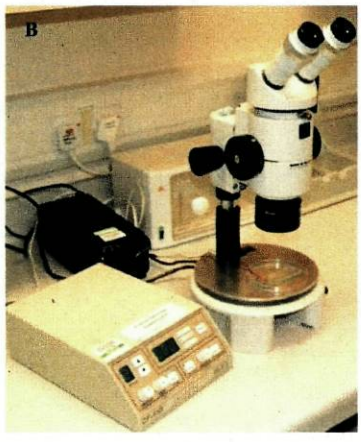


Fig.1. A) Diagrammatic representation of fusion method of nuclear transfer (modified by Colman, 1999/2000); B) Electrofusion machine and chamber

2) Non-fusion (or Honolulu) method: This method involves the intracytoplasmic injection of the donor cell nuclei into enucleated oocytes, usually by a piezo-actuated microinjection system. This is a common procedure in mouse, however it has been used in other animals such as pig and rat (mouse: Wakayama et al., 1998; pig: Onishi et al., 2000; rats: Zhou et al., 2003) (Fig. 2).

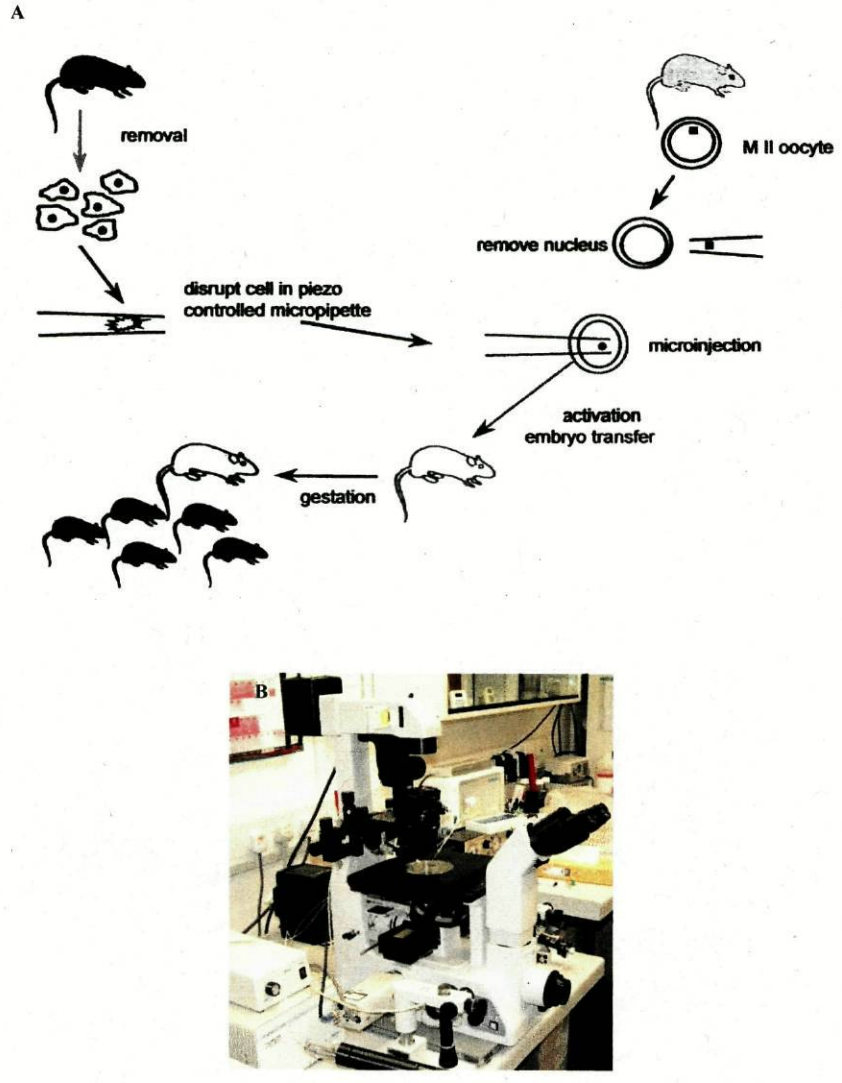


Fig.2. A) Diagrammatic representation of injection method of nuclear transfer (modified by Colman, 1999/2000); B) Inverted microscope used for enucleation and injection steps.

Both of these methods involve skill and experience working with micromanipulators, so some steps of each of these two procedures have been combined and tested with success:

(1) A new method of injection of whole donor cell inside the cytoplasm has also been tested with success in pigs. This technique involves direct injection of whole cell into a cytoplasm, bypassing both fusion and nucleus isolation, turning it into a simple, less labour-intensive technique, requiring no special micromanipulation equipment and as efficient in the generation of cloned piglets as previous methods. However, the mechanisms for dissolution of the plasma membrane are unknown and it seems that different donor cells may require different amounts of time for dissolution (Lee et al., 2003). This method has not yet been proved to work in any other animals.

(2) New protocols of nuclear transfer involving fusion have been also used. Bisection and New Zealand techniques are performed with zona-free (ZF) embryos:

a) Bisection technique: This procedure was first performed in cattle (Booth et al., 2001), and then in pigs (Booth et al., 2001a) by oocyte bisection with a microsurgical knife mounted in a micromanipulator. However, later in the same year, Vajta et al., reported "hand-made cloning" in which no micromanipulators are used (Vajta et al., 2001). This involves the manually bisection of zona-free oocytes, using an ultra sharp blade, and selection under UV light of the demi-oocytes without metaphase plate after DNA staining with Hoechst. Two half cytoplasts plus one donor cell are then adhered together using phytohemaglutinin (PHA-P) and electrofused in two steps. This technique has some advantages, such the low cost of the equipment,

the reduced requirement for preparative work, is easier to learn and faster than previous methods. Disadvantages include loss of half of each oocyte, exposure of the cytoplast to the potentially harmful UV light, and the production of embryos with chimeric mitochondrial DNA (Vajta et al., 2001). So far, it has not been possible to use this technique in mouse due to the small size of the oocytes.

b) New Zealand novel ZF technique of NT: Oocytes are enucleated under UV light with a help of a blunt aspiration pipette and a simple separation pipette (with closed firepolished tip), instead of a holding pipette, which helps to easily separate cytoplast and karyoplast. The reconstructed embryos are cultured in microdrops. This new method is faster, simpler and more efficient than the older ones. It is easier and quicker to learn than zona-intact (ZI) technologies, even for somebody with no micromanipulation experience and virtually no lyses of cytoplasts occurs during or after the process (Oback et al., 2003). So far this technique has only been tested in cattle.

c) In the past, other techniques of donor cell fusion to the cytoplasm used to be performed, using a virus-mediated cell fusion technique with Sendai virus (Giles & Ruddle, 1973; McGrath & Solter, 1983). Technique involves placing inactive Sendai virus in the perivitelline space together with the donor cell. However effective for NT in rodents (McGrath & Solter, 1984), this method was never efficient in other species. Studies in cattle have shown no efficacy of this procedure (Robl et al., 1987), but in horses, reports shown that combination of both procedures increased significantly the fusion rates (82%), when compared with use of Sendai virus (16%) or electrical stimulation (57%) individually (Li et al., 2002).

1.5.1. NT technologies in the mouse

The mouse has many advantages for research concerned with nuclear transfer because of shorter gestational and generation periods when compared with large animals and the detailed knowledge of genetic background. However, mouse nuclear transfer is far from an easy procedure. Previous methods depend upon expensive equipment and require a great deal of experience and micromanipulation skill. Several laboratories have attempted to use the Honolulu method (Wakayama et al., 1998) without success, despite being successful in other species (Zhou et al., 2000). The first cloned mouse produced from differentiated somatic cells was reported as early as 1998; however this is still far from a routine procedure. Only few laboratories have been able to reproducibly generate cloned viable offspring through direct transfer of adult somatic cells (Ogura et al., 2000; Yamazaki et al., 2001; Gao et al., 2003). However, only 0-4% of the reconstructed embryos from adult cells develops to become live offspring.

1.6. Approaches to Improve Efficiency

In the initial stage of development, embryos are under the control of maternally derived proteins and transcripts accumulated in the oocyte, until the activation of zygotic genome, after which time the embryo starts to be controlled by its own nucleus. This change, which may affect the subsequent gene expression pattern, is called zygotic genome activation and is preceded by altering the configuration of chromatin (Kikio & Wolffe, 2000; Kim, et al., 2002). It is generally accepted that inefficiency in development of NT derived embryos is related to alterations during reprogramming. In these embryos, reprogramming of gene

expression occurs by epigenetic mechanisms and does not involve modifications at the level of DNA sequence (reviewed by Campbell et al., 2005).

1.6.1. Type of donor cells and plasticity

The type of donor cell is very important. The cells within an individual are very different, even though their genomes are similar. The phenotypic differences are dependent on changes in gene activity, referred to as epigenetics. Epigenetics describes the mechanisms that bring about the differences in gene expression and function, not affecting the DNA sequences (reviewed by Oback & Wells, 2002). In nuclear transfer embryos, during reprogramming, the epigenetic constraints imposed by the donor cell genome during differentiation need to be deleted in order to convert it back to the embryonic stage. In a traditional view, the more differentiated the donor nucleus, the less plasticity the cells have leading to a lower reprogramming efficiency, due to the accumulation of epigenetic changes during differentiation (Wells et al., 2003). Donor cells derived from blastomeres or embryonic cells undergo easier reprogramming than fetal cells, which in turn are easier to reprogram than adult/ differentiated cells.

1.6.2. Cell cycle and coordination

A critical aspect of the success of the NT is the cell cycle stage of the donor cell, as shown by Wilmut et al. in 1997 (Wilmut et al., 1997). Since then it is known that some stages of the cell cycle are more effective than others for NT efficiency. But is not only the stage of the donor cell that is important. Coordination between cell cycle of donor cell and recipient oocyte are also critical for maintenance of a normal chromosome constitution (ploidy) of the embryo. The cell cycle is constituted by four

phase: G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis). Following mitosis, cells enter in G1 phase, during which increase size and respond to extracellular growth factors that regulate the synthesis of G1 cyclins and the restriction point (R-point) of the cell cycle. As soon as the cells pass the restriction point, and the later point of the G1 phase, they start another DNA replication in S phase. So, the length of the cell cycle between different cells types or even between individual cells in the same population is determined by the early G1 period. In unfavorable conditions, such as withdrawal of serum growth factors, the cells do not go through the R-point and can eventually progress to G0 quiescent phase. As soon as the favorable conditions return, the cells leave G0 and re-enter G1 and continue the cell cycle (Oback & Wells, 2002). First cloned animals produced using cultured cells suggested the importance of using cells in G0 (Campbell et al., 1996a). Nowadays it is known that other combinations of donor cell and recipient cell cycle are possible to retain normal ploidy. Some reports have shown that it is possible to produce normal diploid embryos from nuclei arrest at any stage of the cell cycle (Kono, 1997) however; cells in G0 or G1 are the ones achieving better results in most of the studies. Wakayama et al., have reported same development to viable offspring when using mouse ES cells either in G1 and G2/M-phases (Wakayama et al., 1999b). Zhou et al. in 2001 have described an increase in the development to blastocyst if ES cells were in metaphase, comparing with G1 or G2 cells (Zhou et al., 2001). So, different treatments can be used to stop the donor cells in a particular stage of the cell cycle, such as the withdrawal of serum growth factors (serum deprivation), that leads the cells to exit the cell cycle and enter in the quiescent state, termed G0 (Oback and Wells, 2002; Wells et al., 2003). Alternatively, cells can be synchronized in G1 (non-quiescent stage) by using inhibitors of tubulin polymerization, such as nocodazole,

that blocks cells in M phase and when released, will synchronizes cells in G1 (Otaegui et al., 1994). Accurate comparison between the two phases remains to be described.

The oocyte cytoplasm plays an important role during donor nuclei reprogramming so its quality or competence should be the best to allow a normal reprogramming and early embryonic survival; establishment and maintenance of pregnancy and fetal development; and health of the offspring over its lifetime. The cell cycle is one of the most important factors to improve its reprogramming competence. Presently, metaphase II is the most commonly used source of cytoplasts, because it contains two crucial cytoplasmic factors: MPF (maturation/meiosis/mitosis-promoting factor) and cytostatic factor. Previous studies have shown that high levels of MPF activity are required for cell reprogramming. MPF is an important factor for oocyte maturation and is responsible for nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) (reviewed by Fulka et al., 2001). Oocyte maturation consists in the reinitiating and completion of the first meiotic division, and progression to metaphase II, preparing the nuclear and cytoplasmic compartments of the oocyte for fertilization and early embryo development. Oocyte quality is dependent on the type of maturation system employed. *In vivo* systems using ovulated oocytes are usually of superior quality than the *in vitro* maturation systems (Onishi et al., 2000; Polejaeva et al., 2000). When maturation is performed *in vitro*, media composition, particularly the addition of hormones and culture conditions are crucial factors affecting future oocyte quality (Naito et al., 1992).

Telophase II oocytes can also be used as cytoplast recipients. These are oocytes that undergo the second meiotic division after activation. The enucleation procedure is easier than with metaphase II oocytes, because mechanical aspiration of the extruded second polar body and surrounded cytoplasm can be done without exposure to the UV light (reviewed by Campbell et al., 2005). Additionally, a significantly smaller portion of cytoplasm is removed compared with enucleation of metaphase II oocytes (Bordignon & Smith, 1998). However, when pre-activated oocytes are used as cytoplasts donors, NEBD and PCC do not occur due to the low activity of MPF, but DNA synthesis occurs in relation to the cell cycle stage of the nucleus at the time of nuclear transfer (Campbell et al., 1996a). Activated oocytes receiving nuclei of preimplantation embryos also divide normally and develop to offspring. This indicates that the reprogramming of such donor nuclei might occur, without NEBD and PCC, in activated oocytes during the expansion of donor nuclei (reviewed by Tani et al., 2001). Later, studies carried out using cattle NT embryos reconstructed from cumulus cells and oocytes in telophase II showed failure to reprogram the nuclei and did not develop beyond the 8-cell stage, regardless the cell cycle stage of the donor cell (Tani et al., 2001).

1.6.3. Recipient oocyte size

During mammalian oogenesis, developing oocytes accumulate mRNA, proteins and protein precursors in the cytoplasm that are crucial after fertilization in order to support and regulate preimplantation embryonic development prior to embryonic genome activation. Oocyte quality depends on this effect and is responsible for early embryonic survival; establishment and maintenance of pregnancy and fetal development; and subsequent health of the offspring (reviewed

by Krisher, 2004). Studies have shown that the size of the cytoplast is important to embryo development. Zakhartchenko et al. (1997) have reported a decrease in the blastocyst development rate in nuclear transfer embryos when the cytoplasmic volume of the recipient oocyte was significantly reduced (Zakhartchenko et al., 1997). Other studies, demonstrated that increasing the volume of the cytoplasm resulted in no differences in rates of fusion, cleavage or development to blastocyst in embryos when comparisons were made using oocytes containing 75 and 150% of the original volume. However, mean cell number of resulting blastocysts was lower in the group with the smaller cytoplasmic volume (75%) (Peura et al., 1998a). These results indicate that the ratio between nucleus and cytoplasm is important, and that a reduction in cytoplasm size, as occurs during enucleation, may be detrimental to development.

1.6.4. Nuclear recycling, serial NT and delayed activation

A prerequisite for success of NT lies in the ability of the donor nucleus to be reprogrammed back to the early zygotic stage. It is known that in some species exposing the transferred nucleus to an early oocyte cytoplast for a prolonged period of time helps the reprogramming. This can be done by allowing cloned embryos to develop through cleavage divisions and then use the blastomeres as donor cells for a subsequent round of NT (nuclear recycling) or by placing a donor cell into a oocyte for a short period of time and then re-transferring it to into another cytoplasm (serial NT) (Peura & Trounson, 1998; Peura et al., 2001). Nuclear recycling technique presents an extra advantage by increase the absolute number of identical offspring. In mice a high embryonic and development to live offspring rates were achieved after serial transferring of nuclei derived from the fusion of metaphase stage 4-cell

karyoplasts into enucleated zygotes, demonstrating a beneficial effect of serial NT to genome reprogramming (Kwon & Kono, 1996). However, others studies in cattle have shown a deleterious effect of nuclear recycling, with a reduction of *in vivo* and *in vitro* developmental capacity of embryo-derived bovine clones after several rounds of nuclear recycling (Peura et al., 2001).

In 2003, experiments designed by Yin et al., have shown an improvement in development of reconstructed NT embryos to blastocyst and to term when these were submitted to a delayed-activation of 1 hour compared with simultaneously activated during NT reconstruction. These results suggest that activation during NT probably relates to the nuclear remodelling process, which can affect the ability of embryos to develop (Yin et al., 2003).

1.6.5. Pre-treatment to change chromatin

The ability of the donor cell to be reprogrammed in the oocyte during nuclear transfer involves the suppression of genes related to differentiation and reactivation of genes previously silent. When the reprogramming does not occur or it occurs in an inadequate way, this leads to an abnormal pattern of gene expression and silencing that does not allow the development of the cloned embryos. Previously reports have demonstrated that the nuclei of embryos produced by nuclear transfer have significant higher levels of DNA methylation compared with control embryos produced *in vivo* or *in vitro* (cattle: Dean et al., 2001; sheep: Beaujean et al., 2004a). DNA methylation is usually associated with transcription repression. Enright et al. in 2003, have used 5-aza-2'-deoxycytidine and trichostatin A in cattle in order to decrease the levels of DNA methylation and increase histone acetylation, respectively. 5-aza-2'-deoxycytidine is a DNA methyltransferases inhibitor preventing the transfer of methyl

groups to the DNA. Trichostatin A is a histone deacetylase inhibitor, which enhances the pool of acetylated histones, facilitating the binding of some transcriptional factors to nucleosome, thus increasing the transcriptional activity. The authors have treated adult fibroblasts cells with different concentration of Trichostatin A and 5-aza-2'-deoxycytidine and selected the ideal concentrations to either increase levels of histone acetylation or decrease chromatin methylation, respectively. These cells were then used as nuclear donors for NT, but no clear improvement in development was achieved (Enright et al., 2003). Previously studies in mouse have shown an induction of overexpression of imprinting genes when the same inhibitors were used (El Kharroubi et al., 2001).

2. Zona Pellucida (ZP)

2.1. What is ZP?

Oocytes of all vertebrates are surrounded by an extracellular matrix denominated chorion in fish (Cotelli et al., 1988), vitelline envelope in frogs, perivitelline membrane in birds (Bain & Hall, 1969) and zona pellucida in mammals (Rankin & Dean, 2000). The size of the matured zona pellucida varies between species. In mouse, its width is 7μm (Rankin et al., 2001), 13μm in humans (Austin, 1961), 14.5μm in sheep (Wright et al., 1977), and 27μm in cow (Wright et al., 1977). In mouse, the zona pellucida starts to be produced during folliculogenesis, process responsible for the follicle growth and development. This process starts just after birth and continues during the reproductive life of the females, in order to prepare the eggs for fertilization (Rankin et al., 1996). After fertilization, the zona pellucida surrounds the embryo until blastocyst stage, when the embryo must escape from the ZP in order to establish direct contact with endometrium, allowing implantation (Cole, 1967). The process in which the blastocyst escapes from the zona pellucida is known as hatching. Studies in humans have shown that the zona pellucida becomes thinner and ruptures due to blastocyst expansion (Cohen, 1991). Montag et al., in 2000 have shown similar results in *in vitro* mouse blastocysts with hatching being dependent on the number of embryonic cells, but *in vivo* hatching being mainly caused by uterine and trophectodermal factors (which are absent under *in vitro* conditions) (Montag et al., 2000).

In mouse, ZP is composed of three sulfated glycoproteins called ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980) encoded by single copy genes *Zp1* in chromosome 19 (Epifano et al., 1995); *Zp2* in chromosome 7 (Liang et al., 1990; Lunsford et al.,

1990) and *Zp3* located in chromosomes 5 (Kinloch et al., 1988; Chamberin and Dean, 1989; Liang et al., 1990; Lunsford et al., 1990).

Zp1-null mice form zona pellucida with only *Zp2* and *Zp3*, but the matrix is flawed and around 10% of growing follicles presented ectopic granulosa cells in the perivitelline space. In later folliculogenesis, the follicle showed an increase in the perivitelline space before ovulation. Females were fertile, but less fecund due to precocious hatching of early embryos because of the compromised structure of the zona matrix (Rankin et al., 1999).

Zp3 null mice presented the most severe phenotype, with zona-free oocytes, disorganised corona radiata, disruption of the cumulus-oocyte complexes and sterility (Rankin et al., 1996).

In Zp2-null mice a thin zona matrix developed in early follicles, but this was not sustained in pre-ovulatory follicles. There was a significant decrease in the number of antral follicles. No 2-cell embryos were recovered from the oviducts of females mated with normal males, although development to blastocyst occurred when zona-free oocytes were matured and fertilised *in vitro*, showing that the structural defects are more severe than *Zp*1-null mice but not as severe as *Zp*3-null mice (Rankin et al., 2001).

2.2. ZP Functions

A number of functions of the ZP have been described. Recently, studies have shown that the ZP's roles are much wider than just a membrane to maintain the

structural integrity of the oocyte and embryo. These include mechanical protection for the oocyte and embryo until blastocyst stage; development of tight junctions between blastomeres especially during compaction (Wassarman et al., 1999; Breed et al., 2002); protection of the early embryo from potential immunological rejection (Ozgur et al., 1998) and preventing embryo adherence. Zona-free mouse embryos transferred at the 1-cell, 2-cell and 4-cell stage did not implant and seemed to adhere to the oviduct wall and/or to each other in groups (Modlinski, 1970).

Fertilization is one of the main roles in which ZP is involved. It provides binding sites to sperm receptors promoting initiation of the acrosome reaction before fusion to the oocyte's oolemma and prevents polyspermy after spermatozoan entry through a mechanism of exocytosis of cortical granule contents (Breed et al., 2002).

2.3. Mechanisms of ZP Removal

There are three main methods to remove the zona pellucida: mechanical removal; chemical lysis with acidic Tyrode's solution; and enzymatic digestion using pronase. Pronase has usually been the preferred method, as mechanical removal is a very slow procedure and acidic Tyrode's can cause irreversible damage to the oocytes. The only advantage of the use of pronase is that no individual treatment is required and the procedure can easily be performed in groups of 100 to 200 oocytes, although the period for zona pellucida digestion can vary widely between 30 sec to 5 min. Initially a transitory misshaping of the oocytes occurs; however this effect reverts after pronase removal (reviewed by Vajta et al., 2005). Back in 1983, studies in mouse by Tsunoda and McLaren have shown that pronase appeared to have little or no adverse effect in the development of 8-cell embryos to blastocyst *in vitro*, however it showed an adverse effect on the development to term (Tsunoda & McLaren, 1983).

2.4. Perivitelline Space and Cortical Granules Envelop

The space between the zona pellucida and the oocyte membrane or oolemma is called the perivitelline space. This is an extracellular matrix produced by the oocyte and contains materials that change during development, according to the roles before, during and after fertilization. Fertilization, initiates a cortical reaction resulting in the release of cortical granules in the perivitelline space, leading to an increase of the space (Cherr and Ducibella, 1990; Hoodbhoy and Talbot, 1994). Some of these contents are released through the zona pellucida in order to set up the zona reaction as a block to polyspermy, but some of the cortical granules contents remain in the perivitelline space forming the cortical granule envelope (Dandekar & Talbot, 1992; Talbot & Dandekar, 2003). Little is known about the proteins present in this extracellular matrix. Two proteins have been identified in the perivitelline space and oolemma of fertilized mouse oocytes with molecular weights of 62 (p62) and 56kDa (p56) using an ABL2 antibody in Western blots (Hoodbhoy et al., 2001). These proteins are linked to the regulation of blastomere cleavage divisions, since treatment of fertilized oocytes and cleavage-stage embryos with a polyclonal antibody against p62/p56 inhibits cell division (Hoodbhoy et al., 2000). Proteins p62 and p56 were also found in the cortical granules of unfertilized mouse and hamster oocytes. It is believed that one protein is present in the perivitelline space and the other in the oolemma. When the zona pellucida was removed, ZF fertilized oocytes expressed lower levels of p62 and p56 proteins compared with ZI ones, showing that loss of these proteins occurs after zona removal (Hoodbhoy et al., 2001; reviewed by Talbot and Dandekar, 2003). In 1985, Polak-Charcon et al., reported that new synthesis of ABL2 binding antigens occurs after cleavage stage at about the 2-cell stage with greatest production at the 8-cell stage in mice. This suggests that the cortical granule envelope material

recognized by the ABL2 antibody is released and assembled at fertilization but that it is replenished and its amount is augmented during preimplantation development (Polak-Charcon et al., 1985; reviewed by Talbot & Dandekar, 2003). Other proteins have been found in the cortical granules, such as p32, p75, n-acetylglucosaminidase, ovoperoxidase, calreticulin, tissue plasminogen activator, heparin binding placental protein and other proteinases (reviewed by Liu et al., 2003), but none yet in the cortical granules envelope.

3. Epigenetic Modifications and Reprogramming

3.1. Nucleus/ Chromatin Structure

The nucleus contains the majority of the DNA present in the cells. The DNA is complexed with a wide variety of proteins that together form the chromatin. The nucleosome is the fundamental structure of the chromatin and is constituted of DNA bound to the histones (H1, H2A, H2B, H3 and H4), and other nuclear proteins which are important to organise the long DNA chains into a compact form allowing it to fit in the nucleus. However there are some other extra mechanisms in the nucleus involved in this compaction process. The nucleosome core is the association of two copies of each four histone (H2A, H2B, H3 and H4) and sealed on top by the histone H1 (Beaujean, 2002) (Fig. 3).

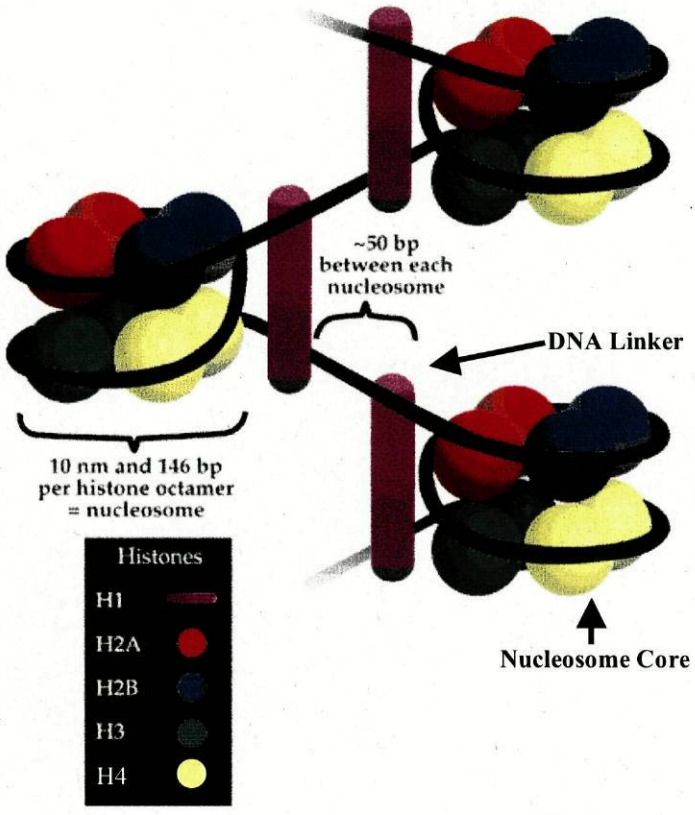


Fig. 3. Diagrammatic representation of chromatin structure and histones (modified from www.blackwellscience.com/11thhour/book5/about/ch5.html)

In the interphase nucleus (G1, S, G2, G0 phases), two types of chromatin regions exist: the euchromatin a descondensed region; and heterochromatin a condensed region which is darker in colour when observed under a microscope (Beaujean, 2002). Chromatin condensation is particularly evident during mitosis and cell death induced by apoptosis, whereas decondensation is observed during repair, replication and transcription. Histones are among the numerous DNA-binding proteins that control the level of condensation of the DNA. Modifications on the histone tails play an important role in the condensation/decondensation dynamics that occurs during the cell cycle (reviewed by Prigent & Dimitrov, 2003). This can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, leading to dynamic transitions between transcriptionally active or silent chromatin stages, subsequently affecting gene expression (Jenuwein & Allis, 2001).

3.2. Epigenetic Modifications

Errors leading to inappropriate expression can arise at any place at which regulation of gene expression occurs, such as organization of nucleus (nucleus structure), availability of regulatory molecules or chromatin structure (reviewed by Wilmut et al., 2002).

An increasing interest has been given recently to the effect of epigenetic modifications to the DNA or histones and subsequent changes in the chromatin structure and gene function. These chemical modifications are mitotically and/ or meiotically heritable, but do not change the nucleotide sequence of the DNA. DNA is folded into a compact and orderly structure that can affect the accessibility of proteins to the DNA including transcription factors and RNA polymerase. If the region is inaccessible to the transcription machinery, any gene within it will be silenced and so

transcriptionally silent. It is known that epigenetic modifications are highly related to this process by altering the chromatin structure. These include modifications to the DNA itself (like methylation- reviewed by Bird, 2002), histones modifications (as methylation, acetylation, phosphorylation and ubiquitination; reviewed by Beaujean, 2002), higher order chromatin structure- involving specific multiprotein complexes (reviewed by Lyko and Paro, 1999) and RNA interference (Matzke et al., 2001).

3.3. DNA Methylation

3.3.1. What is DNA methylation?

The most characterized modification to DNA is its methylation, characterized by a covalent addition of a methyl group mainly in the cytosine residues, when they are adjacent to a guanidine (position 5 at CpG dinucleotides) becoming 5-methylcytosine. About 70-80% of the CpG dinucleotides are methylated in a typical differentiated human cell and the most unmethylated CpG residues are located in GC-rich areas, called CpG islands (Bird, 1986). Around 60% of the human genes are predicted to have a significant number of CpG islands, which remain unmethylated throughout the development and adult tissues (Antequera & Bird, 1993). However, in 2000, Ramsahoye et al. found also methylation in the cytosine residues at CpA and CpT dinucleotides in mouse ES cells, but the significance of this is not yet understood (Ramsahoye et al., 2000).

DNA methylation is prevalent in both prokaryotic and eukaryotic organisms. The main role of the DNA methylation in prokaryotes is to work as a defence system, in order to protect the host genome from destruction by its own endonucleases (reviewed by Hubacek, 1992). In eukariotes, DNA methylation is present in all the

four groups: plants, fungi, protist and animals, although, levels of 5-methylcytosine differs between groups. Vertebrates have high levels throughout the genome; however, methylation patterns in invertebrates are more restricted to non-coding regions (Tweedie et al., 1997). Even amongst vertebrate species, some differences in the DNA methylation distribution occur, which has been proposed to reflect an evolutionary system for implementation of alternative functions (Colot & Rossignol, 1999).

3.3.2. Roles of DNA methylation

In 1980 Razin and Riggs showed the relation between DNA methylation and gene silencing (Razin & Riggs, 1980). This occurs when expression is not required or may be detrimental to genomic stability (reviewed by Beaujean et al., 2004). In somatic cells DNA methylation is known to have other functions, including X-chromosome inactivation (Panning & Jaenisch, 1996), genomic imprinting (Li et al., 1993), inactivation of retroviral sequences, heritability of transcription depression (Jaenisch & Bird, 2003; Young & Beaujean, 2004), tumour formation (Robertson and Jones, 2000) and ageing (Issa, 2004). There is also evidence that is required for the tissue-specific regulation, cellular differentiation and development of stage-specific genes (Jackson-Grusby et al., 2001; Stancheva et al., 2002). Some recent reports indicate that the loss of DNA methylation might be a fundamental mechanism to limit longitudinal bone growth, thereby determining the overall adult size of the organism (Nilsoon et al., 2005). It has also been shown that defects in methylation can lead to diverse disorders from mental retardation to immune deficiencies (Liu et al., 2003a). There is strong evidence that these defects create a favorable environment for malignant transformation (Belinsky et al., 1998; Baylin et al., 2001)

3.3.3. DNA methyltransferases

DNA methylation is catalysed by a family of enzymes known as DNA methyltransferases (Dnmts). These enzymes are responsible for tranferring a methyl group to a carbon 5 of the cytosine residue, using S-adenosylmethionine as a substrate and creating a covalent modification to the DNA. The most abundant and first enzyme to be cloned in the mouse was Dnmt1 (Bestor et al., 1988), which is believe to be responsible for most DNA methylation. Dnmt1 is also known as the "maintenance enzyme", due to its 5- to 30-fold preference for methylating hemi-methylated DNA substrates, over unmethylated substrates, allowing the maintenance of methylation patterns in the newly replicated DNA (Bestor, 2000; Ratnam et al., 2002). Deletion of *Dnmt1* in mouse leads to global demethylation and embryonic death (Li et al., 1992). There are three isoforms of Dnmt1, which are expressed, in sequential pattern during development. Dnmt1o is a variant protein of Dmnt1 with a 175,000 relative molecular weight, which is present in the cytoplasm of the matured metaphase II oocyte and the preimplantation embryo, but actively enters the nucleus of the blastomeres only at 8-cell stage. This transient nuclear localization suggests that Dnmt1o maintains methyltransferase activity specifically on alleles of imprinted genes during the fourth embryonic S phase (Howell et al., 2001). Dmnt1o protein is synthesised from an oocyte-specific transcript, which is translated into a truncated version of Dnmt1s that is missing its N-terminal 118 amino-acids (Fig.4). The somatic isoform of Dnmt1 (or Dnmt1s), with 190,000 relative molecular weight replaces the Dmnt1o after implantation (Trasler et al., 1996; Ratnam et al., 2002). Finally Dnmt1p is exclusive present in male cells, particularly in pachytene spermatocytes (Ko et al., 2005).

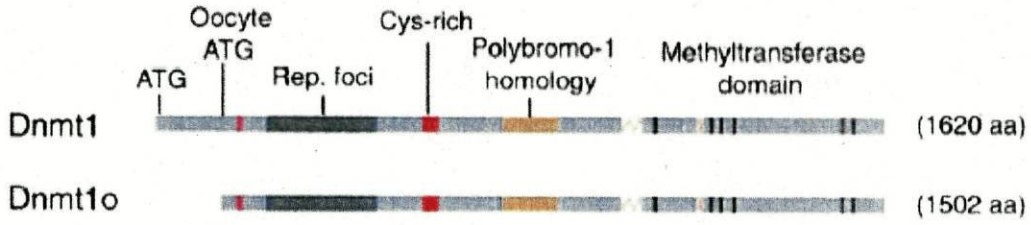


Fig. 4. Comparison of Dnmt1s and Dnmt1o. Dmnt1o protein is synthesised from an oocyte-specific transcript, which is translated into a truncated version of Dnmt1s that is missing its N-terminal 118 amino-acids (from Ratnam et al., 2002).

The establishment of new patterns of methylation is called *de novo* methylation and is performed by different enzymes other than Dnmt1. Four potential candidates were identified: Dmnt2, Dnmt3a, Dnmt3b, Dnmt3L, but only the Dmnt3a and Dnmt3b have been demonstrated to be required for genome-wide *de novo* methylation *in vitro* (Okano et al., 1998; Okano et al., 1999), although the mechanisms by which they target specific DNA sequences are still unknown. More recently, it has been shown by biochemical analysis of Dnmt3a that this protein will methylate hemi-methylated DNA, but that it prefers unmethylated DNA substrates more than 3-fold, to hemimethylated DNA (Yokochi & Robertson, 2002). Other results indicate that Dnmt3a together with Dnmt3L is required for methylation of most imprinted *loci* in germ cells (Kaneda et al., 2004). Embryos lacking both copies of Dnmt3b were stunted, delayed in development, and did not survive past mid-gestation, whereas Dnmt3a-null survive about 4 weeks after birth. Heterozygous mutants appear to be normal and fertile (Li et al., 1992; Okano et al., 1999; Liu et al., 2003a).

Dnmt2 protein contains the conserved methyltransferase motifs identified in eukaryotic and prokaryotic methyltransferases (Yoder & Bestor, 1998), but it was demonstrated that is not required for global *de novo* or maintenance methylation of DNA in ES cells (Okano et al., 1998b; Hermann et al., 2003).

Dnmt3L was the most recent enzyme to be identified. The lack of methyltransferase motifs suggests that it does not have enzymatic activity, but nonetheless is required as a regulatory element for the establishment of proper methylation in oocytes and embryos (fig. 5) (Aapola et al., 2000). Targeted disruption of this protein in mice leads to a loss of methylation imprints, indicating that is necessary for *de novo* methylation of maternal methylation imprinting genes, possibly by interacting with Dnmt3a and/or Dnmt3b (Bourc'his et al., 2001; Hata et al., 2002; Suetake et al., 2004).

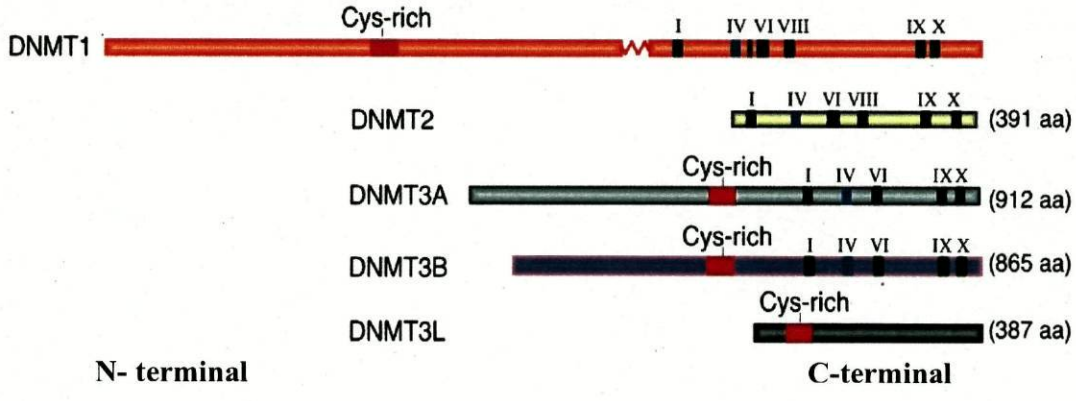


Fig.5. Schematic representation of comparison between DNA methyltransferases identified in mammals demonstrating the presence of canonical motifs at the C-terminal, except in Dnmt3L which does not have methylating activities (Bestor, 2000).

There are some other proteins that are related to maintenance or establishment of DNA methylation, although without an obvious methylating activity, such as DDM1 (when mutated in *Arabidopsis thaliana* causes a severe reduction in DNA methylation; Vongs et al., 1993); ATRX (Gibbons et al., 2000) or LSH (targeted disruption in mice causes loss of methylation and lethality; Dennis et al., 2001). All these proteins belong to the SNF2-like helicase family that shares a highly conserved zinc finger domain with the Dnmt3 family (Aapola et al., 2000).

3.3.4. DNA methylation and transcription repression

The roles of DNA methylation vary according with the eukaryotic organisms, although they all involve a transcription repression. Approximately 70-80% of the CpG dinucleotides are methylated in the mammalian genome, and most unmethylated CpG residues are located in GC-rich areas, called CpG islands (Bird, 1986). CpG islands tend to be localised in the promoter regions of many RNA polymerases transcribed genes (Antequera & Bird, 1993).

DNA methylation can affect gene expression by direct or indirect mechanisms (Fig. 6). One of the mechanisms by which DNA methylation can cause transcriptional repression is by directly interfering with the binding of sequence-specific transcription factors to DNA. Some transcriptional factors like Sp1 can interact directly only with non-methylated DNA sequences, whereas methylation of cytosine abolishes interaction (Clark et al., 1997; Hendrich & Bird, 1998), leading to less effective transcription of certain genes. Several other models have been proposed to explain how DNA methylation can repress transcription by indirect mechanisms. The model most currently accepted is the one that explains DNA methylation mediated repression through the recruitment of the so-called MBD (methyl-DNA-binding

domain) proteins (Hendrich & Bird, 1998). These proteins specifically recognize modified sequences and attract large multiprotein complexes that can change chromatin conformation from "opened" to "closed". The first and best-described methyl-binding protein was MeCP2 (Meehan et al., 1989), followed by MeCP1 (Lewis et al., 1992). MeCP2 contains both a MBD which preferentially binds methylated DNA and a transcription repression domain (TRD), which interacts with a co-repressor Sin3a, which binds histone deacetylase (HDAC), leading to a formation of a silent chromatin state (Hendrich & Bird, 1998; Nan et al., 1998) (Fig. 6). MeCP2 is capable of binding a single symmetrically methylated CpG pair (Nan et al., 1996), however MeCP1 requires more than 10 methyl CpG to bind DNA (Meehan et al., 1989), making it more likely to be involved in DNA methylation-mediated transcriptional repression of several genes (Hendrich & Bird, 1998).

More recently, other methyl-binding proteins such as MBD1, MBD2, MBD3 and MBD4 have been identified, all of them containing the highly conserved methyl-CpG-binding domain (Hendrich & Bird, 1998). Together with MeCP2, MBD1, MBD2, and MBD3 have been characterized as transcriptional repressors and MBD4 has been characterized as a thymine DNA glycosylase involved in DNA repair of deamination products at methyl-CpG sites (Watanabe et al., 2003; Ichimura et al., 2005). All these proteins demonstrate affinity for methylated DNA and bind specifically to the methyl-CpG pairs, except MBD3, which localizes in methylated DNA regions by associating with MBD2. MBD1, MBD2 and MBD3 are also involved in histone deacetylase-associated transcription repression, leading to a formation of a silent chromatin state (Ng et al., 2000).

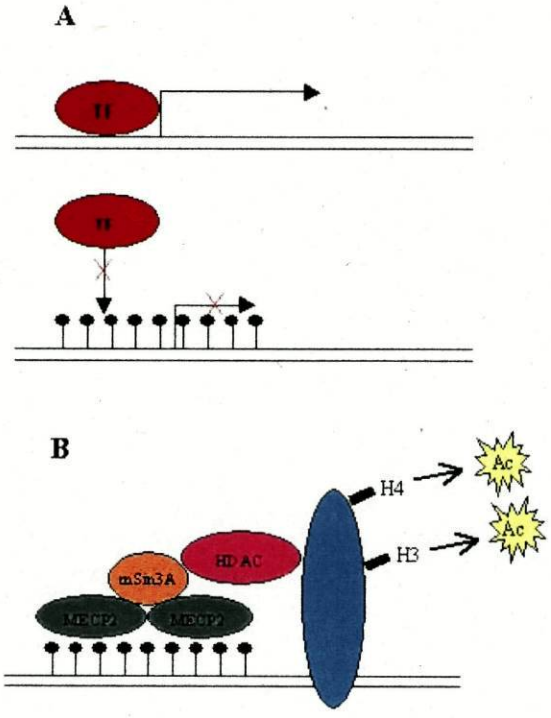


Fig. 6. Theories for transcription repression by DNA methylation (A) Direct mechanism: DNA methylation of CpG dinucleotides prevents the transcription factors (TF) or proteins associated with transcription activation to bind the promoting region; (B) Indirect mechanism: Methylated DNA binds methyl-binding proteins which will bind transcription repression complexes such as mSin3A/HDAC complexes, leading to a deacetylation of the histone tails (H3 and H4), and consequent formation of a compact and inaccessible chromatin structure (modified from Fairburn, 2002).

3.3.5. DNA methylation patterns in embryos

Although stable in somatic cells, DNA methylation has a dynamic pattern in embryos (Reik et al., 2001). As DNA methylation is related to transcription repression, it follows that demethylation of the DNA is linked to gene activation. Before fertilization, sperm and metaphase II oocytes genome are transcriptionally inactive. During mouse embryo development, demethylation occurs in a different way for maternal and paternal genome. In the paternal pronucleus, an active demethylation

wave and DNA decondensation (through removal of protamines and replacement by acetylated histones) involving an enzymatic process occurs within hours after fertilization (reviewed by Reik et al., 2001) (Fig. 7). Much experimental work has been done in order to find the enzymes responsible for this process, although the search is still in progress. In 1996, an RNA-associated enzyme activity was linked to demethylation in rat myoblast cells (Weiss et al., 1996) and in 1999 a MBD2B (truncated variant of the methyl-binding protein) demonstrated an ability to cleave methyl groups, from cytosine residues (Bhattacharya et al., 1999). However, in both cases, further experiments demonstrated no function in demethylation (Santos et al., 2002). Demethylation can also occurs by initial deamination of 5-methylcytosine followed by mismatch repair. The identification of a MBD4 human protein with G/T mismatch DNA glycosylase activity was a potential candidate (Oudejans et al., 2001). After years of experiment, the search for the mammalian so-called "demethylase" is still in progress. However, the active demethylation in the male pronuclei does not occur in the same way in all species. In 2001, Dean et al., showed a loss of cytosine methylation in male pronuclei in mouse, rat, pig and cow (Dean et al., 2001), however Beaujean et al. did not observe this loss in sheep, rabbit and only a partial asymmetry in the cattle (Beaujean et al., 2004b). Interestingly, in conditions permissive to polyspermy, up to five male pronuclei underwent demethylation in the same oocyte, suggesting that the demethylation activity is not limiting and that the activity is intimately associated with the sperm (Santos et al., 2002).

After the completion of the first cell cycle, DNA methylation continues to decline but now by a passive mechanism, due to the absence of the maintenance methylase, Dnmt1, from the nucleus (Fig. 7). At 8-cell stage, an alternative transcript of Dnmt1 (Dnmt1o) appears again in the mouse nucleus and it is believed to be

crucial for maintaining the methylation levels of imprinted genes (Howell et al., 2001). Because before and after this stage Dnmt1o is present in the cytoplasm, is possible that other proteins maintain the imprinted methylation (Reik et al., 2001).

In 2002, Santos et al., showed that in mouse *de novo* methylation occured in the blastocyst in the inner cell mass but not in the trophectoderm (Santos et al., 2002). Enzymes responsible for *de novo* methylation are Dnmt3a and Dnmt3b (Fig. 7) (Okano et al., 1999). The basic reprogramming events of paternal demethylation, passive demethylation and *de novo* methylation are conserved in mammals, although the timing may vary between species. In mouse, *de novo* methylation occurs in the inner cell mass at the blastocyst stage, as previously referred, although in cow, this occurs at the 8 to 16-cell stage (Dean et al., 2001), as well as in sheep (Beaujean et al., 2004a). Differences in the timing of key events such as zygotic genome activation (the switch from maternal to zygotic control of development: 2-cell in mouse; 8-16 cell in cow and sheep), compaction, implantation and gastrulation are likely to be reflected in the gene expression and epigenetic processes (Telford et al., 1990).

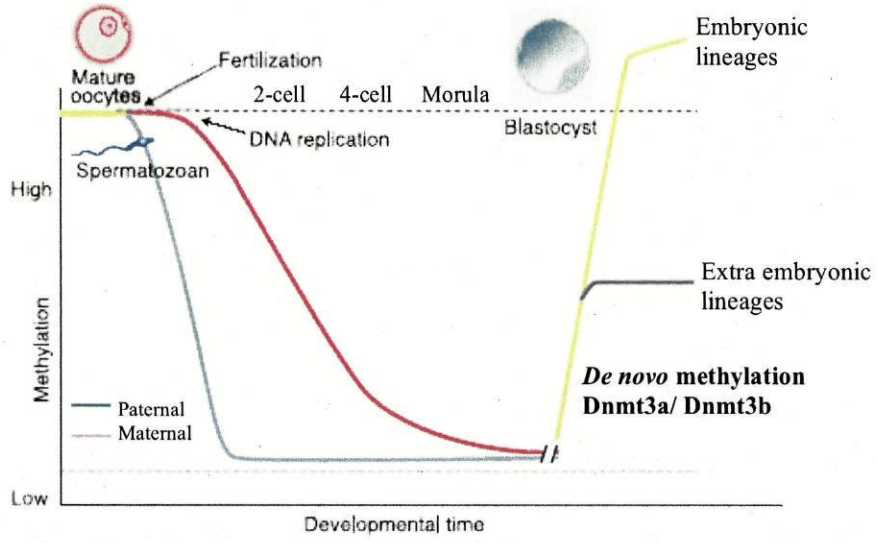


Fig. 7. DNA methylation pattern in mouse preimplantation embryos: the paternal genome is active demethylated, whereas maternal is demethylated by a passive mechanism that depends on DNA replication. Both are *de novo* methylated around the time of implantation to different extents in embryonic and extraembryonic lineages (modified from Reik et al., 2001).

3.3.6. DNA methylation and reprogramming in NT embryos

It is generally accepted that in NT embryos, reprogramming of gene expression occurs by epigenetic mechanisms and does not involve modifications at level of DNA sequence (reviewed by Campbell et al., 2005). During nuclear transfer techniques, chromosomes in the transferred nucleus have an extensively methylated DNA. The successful production of cloned animals from adult cells has shown that a highly methylated and differentiated somatic cell is capable of being reprogrammed and sustaining embryonic development to term, allowing the animal to become an adult (Wilmut et al., 1997). However, experiments comparing levels of DNA methylation in NT and control embryos have shown a higher methylation level in cloned embryos, either by immunocytochemistry (cattle: Dean et al., 2001; sheep: Beaujean et al.,

2004a), or bisulfite analysis (Kang et al., 2001) resembling the methylation levels of the donor cell genome. When nuclei from fibroblasts were transferred to enucleated oocytes, some methylation was lost, however no further demethylation occurred and *de novo* methylation started earlier (Dean & Ferguson-Smith, 2001; Reik et al., 2001). Demethylation in cloned embryos seems to be inefficient, perhaps because the somatic nuclei only contain the somatic form of Dnmt1 (Dnmt1s), which, unlike the oocyte form (Dnmt1o), is capable of maintaining methylation levels.

Additionally epigenetic reprogramming is also inefficient or partial in most cloned embryos, leading to developmental problems especially if *de novo* methylation events are linked to nuclear organization and cellular differentiation. This may be responsible for death at pre- and post-implantation stages and even for the specific abnormalities (particularly placental) in those that develop to term. Finally, some other epigenetic modifications such as histone acetylation or histone methylation may also be difficult to reprogram in nuclear transfer embryos (reviewed by Reik et al., 2001).

3.4. Other Epigenetic Modifications

3.4.1. Histones modifications

Histones are not only an inert DNA-packaging material as previously believed. They are a dynamic component of the chromatin. Distinct amino-terminal covalent modifications in histone tails can also occur, generating synergistic or antagonistic interaction affinities for chromatin-associated proteins, leading to dynamic transitions between transcriptionally active or silent chromatin stages, subsequent affecting gene expression (Jenuwein & Allis, 2001). These modifications include acetylation, phosphorylation, methylation and ubiquitination.

3.4.1.1. Histone acetylation

Acetylation of the histones is the best-known regulatory modification consisting in a transfer of acetyl groups on lysine residues of core histones (Eberharter & Becker, 2002). This is a regulatory modification that occurs predominantly at the level of the lysines in the amino-terminal tail of histone 3 and 4. It plays an important role in gene expression by altering the accessibility of DNA to proteins such as transcription factors. Some studies in mouse particularly with histone H4 have shown that the increase of acetylation can facilitate the binding of some transcriptional factors to nucleosome, increasing the transcriptional activity and so, leading to an over expression of some genes (Worrad, 1995). Usually, genes that are undergoing transcription are hyperacetylated, and those that are repressed are deacetylated (Hebbes et al., 1988; Ng & Bird, 1999).

3.4.1.2. Histone phosphorylation

Histone phosphorylation was firstly observed in the 1960's (Gutierrez & Hnilica, 1967) and the kinase responsible is the AMP-dependent kinase (Langan, 1968). Phosporylation, particularly involving serine residue in position 10 (ser-10) on histone H3 has been extensively studied. *In vitro*, phosphorylation is catalysed by a cAMP-dependent kinase (Taylor, 1982). This modification is related to both transcription and cell division, which require opposite levels of chromatin condensation. It seems that during interphase phosphorylation of H3 leads to chromatin decondensation, whereas during mitosis it is associated with chromatin condensation (Cheung et al., 2000; Nowak & Corces, 2000; reviewed by Prigent & Dimitrov, 2003).

3.4.1.3. Histone methylation

There are two types of histone methylation, targeting either arginine or lysine residues within the tails of H3 or H4. In the case of the histone H3, the lysine residue K9 (9^{th} amino acid from the amino terminal end) is acetylated in active chromatin, but methylated in regions of the genome that are silenced (Bird, 2001). When methylated by Suv(3)9h1, a binding site for HP1 (protein associated with condensed/silenced heterochromatin) is created, resulting in the formation of a repressive protein complex (Cowell et al., 2002). There are also some studies suggesting that histone modifications can lead to the methylation of nearby DNA, by affecting directly or indirectly DNA methyltrasferases (Ng & Bird, 1999; Bird, 2001).

3.4.1.4. Histone ubiquitination

Some studies have shown that histone alteration by ubiquitination in *Saccharomyces cerevisae*, especially on lysine 123 of histone H2B tail, is critical to mitotic and meiotic growth (Beaujean, 2002; Shekhar et al., 2002). Moreover, other works have shown that ubiquitination of histone H2B controls methylation of lysine 79 and lysine 4 in histone 3, both mediating gene silencing (Dover et al., 2002).

3.4.2. Higher order chromatin structure

There is evidence to suggest that some specific multiprotein complexes constitute one device to establish and maintain epigenetic marks. Defined chromosomal elements conferring epigenetic inheritance of transcriptional expression states, have also been identified. During the period where the difference in expression of identical genes is established, these sequences appear to be used as negative and positive regulators. Once the epigenetic mark is "set", the elements maintain either the

silenced or the activated expression state over many cell generations (Lyko & Paro, 1999).

3.4.3. RNA interference (RNAi)

Since the late 1980's, it has been described, both in plants and fungi that interactions between homologous DNA and/or RNA sequences can silence genes and induce DNA methylation (reviewed by Fagard & Vaucheret, 2000). In some species, small RNAs derive from cleavage of double strands RNA (dsRNA). RNA interference (RNAi) is usually a post-transcriptional gene-silencing phenomenon in which dsRNA triggers degradation of homologous mRNA in the cytoplasm. Small RNAs can cause post-transcriptional degradation of messenger RNAs and in plants transcriptional gene silencing by methylation of homologous DNA sequences (Matzke et al., 2001).

4. Thesis Aims and Objectives

The purpose of this study was to investigate the feasibility of using a simplified method of nuclear transfer using zona-free embryos in the mouse. In cattle, zona-free NT is simpler, faster, easier to learn and more reproducible than zona-intact NT. As this was the first time that a technique using zona-free embryos for nuclear transfer had been performed in the mouse, parallel studies were carried out in order to understand the effect of removing the zona pellucida on the development of mouse embryos, DNA methylation, histone acetylation and gene expression of some regulatory genes during preimplantation development

4.1. Specific Aims

a) *To assess and learn the new zona-free technique of nuclear transfers in cattle at Agresearch, New Zealand and establish at Roslin.*

The novel zona-free technology of nuclear transfer was developed in cattle in the Agresearch laboratory in New Zealand (Oback et al., 2003). The first aim of this project was to learn the technical skills using bovine oocytes in this laboratory. In Roslin, the method was first developed in the cattle to allow some familiarity with the technique and achieve some development to blastocyst. Unfortunately, our laboratory does not have a licence for embryo transfer of bovine embryos to surrogate mothers, which is the reason why the development to term was not assessed.

b) *To assess the zona-free method of nuclear transfer in mouse.*

Techniques of NT in mouse still depend upon expensive equipment and require a great deal of experience and micromanipulation. Several laboratories have attempted to use the Honolulu method (Wakayama et al., 1998) without success,

despite their success using other techniques cloning other species (Zhou et al., 2000). Our objective was to simplify the technique of NT in the mouse, making it less expensive and easier to perform. This was the first time a zona-free technology had been attempted in the mouse.

c) *To adjust and optimise zona-free technique for the mouse in order to improve success rates.*

After development of the technique, some adjustments and modifications to the method were made in order to improve overall efficiency and development rates.

d) *To investigate the effect of the zona pellucida removal on in vivo fertilized mouse embryos on development to blastocyst and subsequent development to term.*

This is the first time that a zona-free nuclear transfer has been applied in the mouse. Some previous studies have reported a reduction of development to term of *in vivo* fertilized zona-free embryos when compared with zona-intact, but no differences in development to blastocyst. Our objective was to determine if this effect also occurs in our laboratory. Blastocyst quality was also assessed by cell number.

e) *To analyse the effect of zona pellucida removal on DNA methylation, histone acetylation and gene expression of some regulatory genes during preimplantation development.*

Reprogramming is an important step during development of normal embryos and even more important in NT embryos. Analysis of DNA methylation, histone acetylation and gene expression, by techniques of immunofluorescence and RT-PCR were performed to understand the effect of the zona removal in the reprogramming.

CHAPTER II

Development of a zona-free method of nuclear transfer in the mouse

R. Ribas[1,6], B. Oback[2], W. Ritchie[1], T. Chebotareva[3], P. Ferrier[1], C. Clarke[1], J. Taylor[1], E. J. Gallagher[4], A. C. Maurício[5,6], M. Sousa[6], I. Wilmut[1]

[1] Department of Gene Function and Development, Roslin Institute, Roslin, Midlothian EH25 9PS, Scotland, United Kingdom

[2] Reproductive Technologies, AgResearch Ltd., Ruakura Research Centre, East Street, Private Bag 3123, Hamilton, New Zealand

[3] Molecular Physiology Laboratory, The Wilkie Building, University of Edinburgh, Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland, United Kingdom

[4] MRC Technology, Crewe Road South, Edinburgh EH4 2PS, Scotland, United Kingdom

[5] Centro de Estudos de Ciência Animal (CECA)- Instítuto de Ciências e Tecnologicas Agrárias e Agro- Alimentares (ICETA), Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal

[6] Instítuto Ciências Biomédicas Abel Salazar (ICBAS), Largo Prof. Abel Salazar, 2, 4099-003 Porto, Portugal

Published in Cloning and Stem Cells 2005, 7(2): 2005

ABSTRACT

In the present study, a zona-free nuclear transfer technique, which had been originally developed in cattle, was modified for the mouse. Steps involved in this approach include removing the zona pellucida and enucleating without a holding pipette; sticking donor cells to the cytoplast before electric pulses are applied to fuse them and culturing reconstructed embryos individually in single droplets, to prevent aggregation. Control zona-free and zona-intact embryos from mated donors showed no significant difference in development to blastocyst, but did show reduced development to term. Removal of the zona pellucida affected the response to activation by strontium in the absence of calcium as a significant proportion of zona-free control oocytes and embryos reconstructed by nuclear transfer lysed during this treatment. A comparison between cumulus and ES cells as donor cells revealed significant differences in fusion efficiency (58.1± 4.0%, n= 573 vs. 42.9± 2.2%, n= 2064, respectively, $P< 0.001$), cleavage (77.2± 3.4%, n= 334 vs. 40.8± 2.7%, n= 903, respectively, $P<0.001$) but not for development to morula/blastocyst (8.7± 2.1%, n= 334 vs. 13.9± 1.8%, n= 903, respectively, $P<0.1$). The stage at which embryo development arrested was also affected by donor cell type. A majority of embryos reconstructed from cumulus cells arrested at 2-cell stage, usually with two nuclei, whereas those reconstructed from ES cells arrested at 1-cell stage, usually with two pseudo-pronuclei. After transfer of ES cell-derived NT embryos, a viable cloned mouse was produced (3.0% of transferred embryos developed to term). These observations establish that a zona-free cloning approach is possible in the mouse, although further research is required to increase the efficiency.

Keywords: Nuclear Transfer, Mice, Zona-Free, Zona-Intact, Calcium, Parthenogenic Activation

INTRODUCTION

Two methods for mammalian nuclear transfer (NT) have been used extensively. In the fusion method, the donor cell is placed in the perivitelline space of the previously enucleated oocyte (cytoplast), before electric pulses are used to fuse the donor cell to the cytoplast (cattle: Cibelli et al., 1998; Kato et al., 1998; Kubota et al., 2000; mouse: Ono et al., 2001; sheep: Wilmut et al., 1997; pig: Polejaeva, 2000). By contrast, in the non-fusion or Honolulu method the nucleus of the donor cell is isolated and injected into the cytoplast, usually by piezo actuated microinjection system (mouse: Wakayama et al., 1998; pig: Onishi et al., 2000).

Cattle have been cloned using a new zona-free technique where oocytes are enucleated under the UV light with a help of a blunt aspiration pipette and a simple separation pipette (with closed fire-polished tip), instead of a holding pipette. As this procedure is quick it can be observed under UV illumination to ensure that enucleation is achieved. Lectin is used to promote adherence of donor cell to cytoplast before electric pulses are used to fuse the two cells. This method is simpler, faster, easier to learn and equally efficient as zona-intact NT (Oback et al., 2003). We describe the first application of this procedure to NT in the mouse.

Previous NT methods in mouse depend upon expensive equipment and require a great deal of experience and manipulation skills. Several laboratories have attempted to use the Honolulu method (Wakayama et al., 1998), but with no success despite being successful by cell fusion in other species (Zhou, et al., 2000). Cloning mice from differentiated somatic cells is still far from being a routine procedure, even though it was reported as early as 1998. Very few laboratories world-wide have been able to reproducibly generate cloned viable offspring through direct transfer of adult somatic

cells (Wakayama & Yanagimachi, 1999; Ogura et al., 2000; Yamazaki et al., 2001). In our study, several factors were examined during the adaptation of the zona-free procedure for the mouse including the effect of removing the zona pellucida on development, method of activation and choice of nuclear donor cell type. In our procedure, UV illumination was not necessary during enucleation because the mouse cytoplasm is very clear. The birth of a healthy cloned mouse from zona-free NT provides 'proof of principle' of a technology that promises to increase throughput and ease of operation.

MATERIAL AND METHODS

All chemicals were supplied by Sigma-Aldrich (Dorset, UK) and all embryo manipulation was performed at 37°C unless indicated otherwise.

Collection of matured oocytes and zona removal

Cumulus oocytes complexes (COC's) were recovered at metaphase II stage (13-13.5 hours after injection of human chorionic gonadotrophin- [hCG] Chorulon- Intervet) from oviducts of 8-10 weeks old B6D2F1 females (C57BL/6J×DBA/2) that had been superovulated by intraperitonial injections of 5 IU eCG (Folligon- Intervet) and 5 UI of hCG given 48 hours apart. The cumulus cells were removed with hyaluronidase (300 Units/ml) in hepes-buffered CZB media- [hCZB] (Appendix I) (Gao et al., 2003a). Oocytes that had even cytoplasm and a first polar body were selected.

A comparison was made between removal of the zona pellucida by exposure to acid Tyrode's or exposure to pronase. Acid Tyrode's digestion of zona pellucida was carried out by incubation in acid Tyrode's solution (pH 2.5 ± 0.3) for 3-5 minutes. Pronase digestion was achieved by 3-5 minutes incubation in 5mg/ml of pronase diluted in hCZB containing 0.1 mg/ml of PVA (Polyvinyl Alcohol- ICN; M_r: 10,000-30,000). Oocytes were then washed thoroughly in hCZB to remove all traces of pronase. To confirm that zona removal did not induce activation, oocytes were examined 6 h after zona removal for metaphase II and the following day for signs of cleavage.

Staining and enucleation of zona-free murine oocytes

Zona-free oocytes were enucleated according to the procedure developed for cattle (Oback et al., 2003) with some modifications. All the micromanipulation tools were made with borosilicate capillaries (Harvard Apparatus- GC100T15- 1.0 mm outer diameter × 0.78 mm inner diameter × 150 mm length), using a horizontal moving coil microelectrode puller (Campden Instruments Limited- Model 753) and a microforge (Research Instruments Limited- MF1). Metaphase II chromosome spindle complex and polar body (if not removed with the zona pellucida) were removed at 40× magnification. Contrary to the bovine oocyte, the murine metaphase chromosomes are easily visible in oocytes without UV light illumination and with experience enucleation can be done using only differential interference contrast (Nikon- Eclipse TE300), avoiding potential harmful effects of UV light on the cytoplasm. Aspiration was performed using an enucleation pipette (16-20 μm outer diameter, perpendicular break without spike or jagged edges). Instead of a holding pipette we used a

separation pipette closed at the end, to both prevent the oocytes from rolling away and to separate karyoplast from cytoplast by pushing the cytoplast away from the enucleation pipette (Fig. 1c, d, e). Since mouse oocytes are more fragile than bovine, cytochalasin B at 5 μg/ml (diluted in hCZB) was crucial during enucleation steps, to prevent oocyte lysis. Accuracy of chromosomal removal was verified by illuminating the metaphase spindle inside the enucleation pipette under UV illumination.

Donor cells

Two types of donor cells were used: 1) cumulus cells obtained from B6D2F1 (C57BL/6J×DBA/2) oocyte donors immediately after being stripped from the oocytes, and kept in hCZB for 1 hour until being attached to cytoplasts and 2) embryonic stem (ES) cells (ED_2). ED_2 cells were derived from a 3.5-day blastocyst produced by natural mating of 129/Sv female with C57BL/6J male mice. ED_2 cells at passage 10 were cultured for 3 days in GMEM media (Gibco) supplemented with LIF (leukaemia inhibitor factor) and 15% of fetal calf serum (FCS) until they reached 90% confluency and then serum starved for 16h in 5% FCS at 37°C and 5% CO_2. While this treatment does not modify the cell cycle of the ES cells, it does enhance their response to NT (Gao et al., 2003). Cells from this line had previously yielded offspring using the piezo procedure in our laboratory (unpublished observations).

Lectin-mediated attachment of donor cells to zona-free oocytes

Donor cells were attached to the cytoplasts with phytohaemaglutinin (PHA-P) (10 μg/ml) in hCZB. Donor cells were aspirated with a mouth pipette and added to a drop of PHA-P. Cytoplasts were then added individually to the same drop and a single cell was pushed against oocytes until it adhered. Usually spherical and bright cells were selected as nuclear donors (Fig. 1f). Groups of 5-10 couplets were transferred to an hCZB drop to be fused.

Electrofusion

Donor cells were electrically fused to the cytoplasts in a hypo-osmolar fusion buffer (200-210 mOsm) with 0.2 M mannitol, supplemented with 0.1 mM of Mg_4SO_2, 0.5 mM of hepes and 0.05% of fatty acid-free bovine serum albumin with a pH of 7.3. Each couplet was covered with fusion buffer in a glass fusion chamber (BLS, Hungary) with wires 250 μm apart, connected to a CF-150 fusion machine (BLS, Hungary). Fusion was performed at 30°C. Orientation of the couplets was such that the plane of contact between karyoplast and cytoplast was perpendicular to the direction of the electric current. Each couplet was manually aligned and a direct current (2.00 kV/cm) was applied during 2 × 16 μsec pulses. Couplets were then removed from fusion chamber, washed in hCZB without calcium and incubated for 1-2 hours in CZB (Appendix I) (Gao et al., 2003a) at 37°C, 5% CO_2 in air. Fusion was assessed 30- 60 minutes later.

Artificial activation

Reconstructed embryos were activated individually 1-2 hours after fusion (17-18h post-hCG), by culture for 5-6 hours in either CZB or CZB calcium-free medium containing 10 mM strontium chloride and 5 μg/ml cytochalasin B.

In vitro culture (IVC) and assessment of development

Following activation, embryos were washed in CZB to remove all the traces of strontium and then cultured *in vitro* in 5 μl droplets under paraffin oil (Fluka) at 37°C and 5% CO_2 for 4 days (day 0 = fusion), according to the protocol previously established in our laboratory (Gao et al., 2003). Cleavage was recorded 24 hours after first exposure to strontium to induce activation. Development was assessed daily.

Embryos that reached morula and blastocyst stages were transferred into the uteri of day 2.5 pseudopregnant surrogate mothers (C57BL/6J×CBA/2J) using a siliconised injection pipette (Nagy et al., 2003a). Recipient mothers were sacrificed at 19.5 days postcoitum and pups removed. Airway passages were cleaned to remove any fluid and the pups were held in a warm box at 37°C before being fostered by a lactating mother (Balb/c).

Parthenogenically activated controls

COC's were recovered at metaphase II and the cumulus cells removed as described previously. The zona pellucida was removed from half the oocytes, using methods described above. Both intact and zona-free oocytes were cultured in CZB until

activation 17-18 hours after hCG injection, which is the same age as reconstructed oocytes at the time of activation. Activation was accomplished by incubation for 5-6 hours in CZB media containing 10 mM strontium chloride and 5 μg/ml of cytochalasin B. All embryos, with and without the zona, were cultured individually for 4 days (day 0 = activation) as described above. The effect of presence and absence of calcium in the CZB during activation was assessed.

In vivo fertilised controls and assessment of development

Eight to ten week old B6D2F1 (C57BL/6J×DBA/2) females were superovulated by intraperitoneal injections of 5 IU eCG (Folligon- Intervet) followed by 5 IU hCG (Chorulon- Intervet) 48 hours later and mated with B6D2F1 males. Embryos were recovered approximately 16 hours after hCG injection. Cumulus cells were removed with hyaluronidase (300 Units/ml) in hCZB and zona pellucida digested from half of the embryos, using methods described above. All embryos were cultured individually for 4 days (day 0 = recovery) as described above. Development was assessed daily and zona-free and zona-intact morulae and blastocyst were transferred into the uteri of surrogate mothers as described above. Number of pups born was assessed.

Genomic DNA analysis of cloned mouse by micro-satellite analysis

The genotype of mice was confirmed by polymerase chain reaction (PCR) amplification of the microsatellite MapPairs (Invitrogen) mouse markers: D4Mit204, D9Mit207 and D12Mit182 as previously reported (Wakayama et al., 1999). Genomic DNA was extracted from tail tips of the cloned mouse, recipient mother and oocyte

donor and ES cell pellets using a DNeasy tissue kit (Qiagen). PCR was carried out using Thermostart® PCR mastermix (Abgene, UK) in a total reaction volume of 25 µl containing PCR master mix (1.5 mM of magnesium chloride, 0.8 mM dNTP, 1 unit thermostar taq polymerase), 250 mM each of forward and reverse primers and 250 ng of template DNA. After a 15 min initial incubation at 94°C, reactions were subjected to 30 cycles of 45 sec at 94°C, 45 sec at 57°C, 60 sec at 72°C and a final extension time of 7 min at 72°C. The PCR products were separated on a 4% agarose gel and visualized after staining with ethidium bromide.

DNA staining of NT embryos

Embryos that failed to develop to the morula stage were fixed and stored in 4% paraformaldehyde in PBS. Groups of embryos were washed in 0.05% Tween-20 in PBS and permeabilized for 30 min in 0.2% Triton-X 100 in PBS. After washing twice in 0.05% Tween-20, embryos were mounted on a slide with 4,6-diamidino-2-phenylindole staining (DAPI) (Vectashield- Vector Labotarories). Fluorescence observation was performed on a Nikon upright microscope (Microphot), using a filter wheel equipped with standard filters for FITC and DAPI emissions. Images were captured through a Nikon Fluar 40x objective (NA 0.75) by a cooled CCD Digital Pixel camera with a KAF 1600 sensor coupled to the IPLab Digital Pixel software.

Statistical analysis

Percentages were analyzed using a generalized linear mixed model and the method of Breslow and Clayton (Breslow & Clayton, 1993). This method took account of the binomial distribution of the numbers of embryos reaching the various stages of development and any extra variation between repeat runs of the experiment and between treatment groups of embryos within runs. The effect of the treatments was estimated in the logistic scale by this method and statistical significance was determined approximately using Student's t tests on treatment means in this scale. Deviations to the means were represented by standard errors (SEM).

RESULTS

Technique development

In the present experiments oocytes exposed to acid Tyrode's were found to be more fragile during subsequent manipulation, particularly during enucleation (data not shown). In addition, more of the first polar bodies remained attached when acid Tyrode's was used, compared with pronase, which makes enucleation more difficult and slower as the polar body needs to be removed as well as the metaphase spindle. There was no evidence that treatment with pronase to remove the zona induces activation of the oocytes. When examined 6 h after zona removal 95 of 97 (97.9%, 3 replicates) oocytes were at metaphase II and only a small proportion (3/129= 2.3%, 4 replicates) of non-activated oocytes cleaved following zona removal and overnight incubation. The comparison of parthenogenic activation when the zona pellucida was removed with pronase or acid Tyrode's showed that there was no significant effect

upon either activation rate (98.2± 1.1%, n= 74 for pronase and 96.2± 2.3%, n= 89 for acid Tyrode's, 2 replicates, P>0.2). The effect on the proportion of embryos that developed into blastocysts approached significance (54.2± 11.4%, n= 74 for pronase and 20.0± 7.4%, n=89 for acid Tyrode's, 2 replicates, P=0.052). Pronase was selected for future work. Representative oocytes treated by this method are shown in Fig. 1b.

Average enucleation time was approximately 20-30 seconds per oocyte, being similar to the procedure in bovine oocytes, and reduction of the oocyte volume was minimal. Accuracy of chromosomal removal by UV light illumination of the material in the enucleation pipette was practically 100%.

Two different types of nuclear donor cell were used: embryonic stem (ES) cells and fresh cumulus cells. Both cell types adhered firmly to the cytoplast and usually remained attached during subsequent pipetting through different media. During fusion procedures, significant differences appeared between different types of donor cells. Cumulus cells showed 58.1± 4.0% fusion, n= 573, 14 replicates, against 42.9± 2.2%, n= 2064 for ES cells, 42 replicates (P< 0.001) (Table 1). With experience using ES cells, this proportion was 52.2 ± 3.6% (n= 458, 9 replicates) towards the end of the study.

Couplets were manually aligned, as aligning with alternating current was not satisfactory. If couplets touched the wires they usually lysed. After preliminary studies the protocol that was adopted was as follows. Two pulses of direct current of 2.0 KV/cm for 16 μseconds were applied in a hypo-osmotic fusion buffer with 0.2 M mannitol and no calcium, at 30°C. There was no significant effect on fusion of varying pulse duration from 12 to 16 μseconds when using ES cells (fusion

percentage 44.4± 6.5%, n= 189; 49.0± 5.1%, n= 144; 56.3± 6.3%, n= 155, 10 replicates, for 12, 14 and 16 μseconds, respectively).

Comparison between parthenogenic activation in media with and without calcium in zona- free and zona-intact embryos

The effect of calcium in the activation medium was assessed because initial observations suggested that zona-free oocytes and embryos died in calcium free medium.

Removal of the zona pellucida changed the response to activation treatments. A large proportion of zona-free oocytes lysed when activated with strontium diluted in calcium free CZB. This was also true for control parthenogenetically activated oocytes (52.9± 6.5%, and 3.9± 1.6%, lysis of oocytes activated in calcium free media and media containing calcium, respectively, $P<0.001$) and NT reconstructed embryos (34.1± 14.9%, and 0.3± 0.3%, lysis when zona-free NT embryos were activated in calcium-free media and media containing, respectively, $P<0.01$). By contrast, when zona-intact embryos were activated both in media without (1.9± 1.4%) and with calcium (0.0± 0.0%), virtually no cell death was seen, $P>0.2$ (Table 2)

Considering the response of only those oocytes that were intact after the treatment, the presence of calcium in the activation medium influenced cleavage and development to the blastocyst stage (Table 3). The presence of calcium reduced cleavage of both zona-intact control embryos (97.1± 1.9% and 64.7± 8.1%, for embryos without and with calcium, respectively; $P<0.01$) and zona-free control embryos (96.1± 2.1%, and 82.9± 4.5%, of zona-free control embryos activated without and with calcium

respectively, P<0.05). However the effect was not statistically significant in NT embryos (33.9± 6.9%, and 41.3± 3.9%, of embryos activated without and with calcium respectively, P>0.2) (Fig. 2a, b). Similarly, a significantly lower proportion of zona-intact control embryos reached the blastocyst stage after activation in the presence of calcium (82.0± 4.9%, and 32.5± 6.4%, of embryos activated without and with calcium respectively, P<0.001). By contrast, development to blastocyst of control zona-free or NT embryos was not influenced by the presence of calcium (56.5± 7.0%, and 49.1± 6.1%, of zona-free control embryos activated without and with calcium respectively, P>0.2; and 12.7± 5.4%, and 13.2± 3.3%, of NT embryos activated without and with calcium respectively, P>0.2) (Fig. 2a, b).

After taking into account both cell death and differences in development after activation, a decrease in the proportion of blastocysts was seen in the group of zona-free oocytes activated without calcium (25.8± 4.6%, and 47.5± 5.8%, for parthenote controls activated without and with calcium respectively, P<0.001). These results suggest that although the presence of calcium in the activation media prevents death of oocytes it is detrimental to activation. However, this effect was not seen after NT (5.8± 2.4%, and 9.3± 2.1%, for NT activated without and with calcium, respectively, P>0.2) (Table 3).

Comparison of development of zona-free cloned embryos cultured with different oxygen concentration

There was no statistical effect of varying oxygen concentration from 5% to 20% on the cleavage (66.9± 7.9%, n= 114, 9 replicates, and 58.2± 8.7%, n= 104, 9 replicates in 20% and 5% O_2 respectively, P>0.2) or development to blastocyst (11.1± 4.3%, n=

114, 9 replicates, and 5.82± 2.8%, n= 104, 9 replicates, in 20% and 5% O_2 respectively, P>0.2) in NT embryos (Fig. 3). As previous results have shown a benefit in development to term of using the higher concentration (Gao et al., 2003) we used 20% of oxygen in all subsequent experiments.

Comparison between two different types of donor cells

During the development of this technique, the frequency of fusion, cleavage of reconstructed embryos and development to morula/blastocyst stage was assessed for two types of donor cells (cumulus and ES cells). Fusion (58.1± 4.0 %, vs. 42.9± 2.2%, P< 0.001) and cleavage (77.2± 3.4%, vs. 40.8± 2.7%, P<0.001) were higher when cumulus cells were used as donor cells. However, the percentage of embryos developing to morula/ blastocyst stage was greater when ES cells were used (13.9 ± 1.8% vs. 8.7 ± 2.1%, P<0.1) (Table 1). Interestingly, the stage at which embryos tended to block differed with choice of nuclear donor cell. Table 4 shows that reconstructed embryos derived from cumulus cells tend to stop at 2- 3 cell stage (64.9± 3.5% vs. 26.8± 2.2% in ES cells, P<0.001) usually with two nuclei (72.4± 3.3%, n= 150), whereas the majority of those derived from ES cells arrested at 1-cell stage (70.7 ± 2.4%, vs. 23.3± 3.2% in cumulus cells, P<0.001) usually with two pseudo-pronuclei (56.0± 8.6%, n= 188).

In vivo fertilised controls and assessment of development

There was no significant difference in development to blastocyst of *in vivo* fertilised embryos cultured with and without zona pellucida (66.3± 7.5%, n= 535, and 57.7± 8.2%, n= 498, 6 replicates, P>0.2) (Fig. 4). By contrast, there was a significant reduction in development to term after removal of the zona (28.4± 4.7%, n= 624 for zona-intact, 28 replicates and 11.8± 2.7%, n= 540 for zona-free embryos, 27 replicates, P<0.001).

Assessment of ability to develop to term

An assessment was made of the ability to develop to term of embryos produced by the selected procedure: ES cells were used as nuclear donors, the zona was removed with pronase, electrical pulses for fusion 16μseconds, activation in the presence of calcium, culture in 20% oxygen. A total of 272 embryos were produced in 19 replicates, 120 (44.1%) cleaved and of these 36 (30%) reached compact morula/blastocyst stage. After transfer of 33 embryos to recipients, one pup was born (3.0%). The pup was apparently larger and heavier than other pups of the same age (2.16 g vs. 1.24 g), but no controls for breed and litter size were available for an accurate assessment (Fig. 5a). The pup is healthy, fertile and now 7 months old. Genotype was confirmed by microsatellite analysis (Fig. 6).

DISCUSSION

These observations establish that a zona-free NT approach is possible in the mouse, although further research is required to establish a reliable procedure. In particular, in

these studies mouse oocytes were fragile after the treatments used to remove the zona pellucida and they responded differently to activation treatments. In comparison to the previous zona-intact methods of enucleation, this is a simpler approach being easier to learn specially for those without any previous experience in micromanipulation or making microtools. In addition, each of the enucleation tools can reproducibly be made in less than 2 minutes. The necessary equipment is cheaper because a piezo micromanipulation system, frequently used in mice, is not required.

Electrofusion has been used previously to fuse adult somatic cells to zona-intact mouse oocytes (Wakayama & Yanagimachi, 1999). The efficiency was similar to that obtained in these studies (68.8% vs. 58.1%, for tail tip and cumulus cells, respectively). Several factors have been known to influence the efficiency of fusion. Previous experiments in cattle have shown a 1.4 fold improvement in the fusion rate when using a hypo-osmotic buffer of 207 mOsm instead of a 318 mOsm for oocyte-oocyte pairs (Oback et al., 2003). Similar results were reported in mouse with a two-fold increase in hybrid production efficiency when osmolarity was reduced (Stenger et al., 1988). The field, strength, duration and number of pulses have been shown to influence fusion in several species (Mouse: Jiang et al., 2005; Elsheikh et al., 1995; Bovine: Van Stekelenburg-Hamers et al., 1993). Further research may be necessary to optimize procedures using a mouse zona-free protocol.

One practical problem in the present experiments was the reduction in survival of zona-free oocytes. Development to term was reduced simply by removal of the zona with pronase, as has been reported previously (Tsunoda & McLaren, 1983). However, in these studies there was a marked change in the response to activation with strontium. Typically, if mouse oocytes are activated by exposure to strontium the

response is reduced if calcium is included in the medium (Nagy et al., 2003), as was confirmed in the present studies. By contrast, in these experiments there was significant death of zona-free control oocytes and of embryos reconstructed by NT if they were activated in the absence of calcium. The increased cell death always occurred in the first hour after activation. This response is not understood, but it dramatically reduced the number of embryos produced by NT.

The choice of nuclear donor cell influenced the outcome at several stages in the process. The size of the cells and their pretreatment can influence fusion simply because larger cells fuse more efficiently than smaller cells, perhaps explaining in part the better fusion of cumulus cells. In these studies better development to morula/ blastocyst stage was obtained with the ES cells than cumulus cells. ES cells are unusual in that cell cycle stage is not influenced by confluence and serum starvation (Gao et al., 2003b). As a result, more than 50% are expected to be in S-phase and not expected to be suitable for transfer into oocytes at metaphase II. One third of the cleaved embryos reconstructed from ES cells reached the morula/ blastocyst stage, against one tenth in cumulus cells. This contrasts with previous comparisons with adult somatic cells, when a smaller proportion of embryos produced by transfer of ES cell nuclei developed to the blastocyst stage, but a greater proportion of these developed to term (Rideout et al., 2001). A comparison with published results suggests that, procedure used in the present studies were not suitable for cumulus cells.

Cattle, and pigs have already been successfully cloned using either this technique or another zona-free technology, named handmade cloning (Booth et al., 2001; Booth et al., 2001a; Vajta et al., 2001). The main objectives of these new NT technologies are

to improve throughput and to eliminate the requirement for expensive equipment. In addition, the procedure is easier to learn even for somebody without micromanipulation experience.

In the mouse, there is an extra advantage of being able to see the metaphase plate by differential interference contrast microscopy, removing the need to use UV light, which is not possible in cattle oocytes. However, there are some disadvantages to the use of zona-free methods as the embryos are more fragile. It is assumed that if the embryo is to be transferred to a recipient then it must be cultured to morula or blastocyst stage when compaction has taken place (Bronson & McLaren, 1970). In all of the present studies embryos were cultured individually in microdroplets according to the original cattle protocol (Oback et al., 2003). Recently it has been shown that development is enhanced if embryos are cultured in groups in larger droplets (Fleur Oback, personal communication). Aggregation is avoided by separating the embryos within the drop and culturing within a vibration-free incubator.

The birth of a healthy cloned mouse pup from zona-free NT provides 'proof of principle' of a technology that promises to increase throughput and ease of operation.

ACKNOWLEDGEMENTS

Many thanks to all the members of the cloning group at AgResearch Ltd., Ruakura Research Centre in New Zealand; All Ian Wilmut's group, Caroline McCorquodale for statistical analysis; and all the staff from the small animal unit at Roslin Institute.

This worked was supported by Fundação para a Ciência e para a Tecnologia, Portugal.

REFERENCES

1. Breslow, N. E.; Clayton, D. G. (1993). "Approximate inference in generalized linear mixed models." **Journal American Statistical Association**, 88: 9-25.

2. Booth, P.J.; Tan, S.J.; Reipurth, R.; Holm, P.; Callesen, H. (2001). "Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique." **Cloning Stem Cells**, 3(3): 139-150.

3. Booth, P.J.; Tan, S.J.; Holm, P.; Callesen, H. (2001a). "Application of the zona-free manipulation technique to porcine somatic nuclear transfer." **Cloning Stem Cells**, 3(4): 191-197.

4. Bronson, R. A. & McLaren, A. (1970). "Transfer to the mouse oviduct of eggs with and without the zona pellucida." **J. Reprod. Fert.**, 22: 129-137.

5. Cibelli, J. B.; Stice, S. L.; Golueke, P. J.; Kane, J. J.; Jerry, J.; Blackwell, C.; Ponce de Leon, A.; Robl, J. M. (1998). "Cloned transgenic calves produced from non-quiescent foetal fibroblasts". **Science**, 280: 1256-1258.

6. Elsheikh, A.S.; Takahashi, Y.; Tanaka, H.; Hishinuma, M.; Kanagawa, H. (1995). "Electrofusion of zona-free mouse embryonic cells in electrolytes and their development *in vitro*." **Jpn J Vet Res.,** 43(3-4): 125- 134.

7. Gao, S.; McGarry, M.; Priddle, H.; Ferrier, T.; Gasparrini, B.; Fletcher, J.; Harkness, L.; De Sousa, P.; McWhir, J.; Wilmut, I. (2003). "Effects of donor oocytes and culture conditions on development of cloned mice embryos." **Mol Reprod Dev.**, 66(2): 126- 133.

8. Gao, S.; McGarry, M.; Latham, K.E.; Wilmut, I. (2003a). "Cloning of mice by nuclear transfer." **Cloning Stem Cells**, 5(4): 287- 294.

9. Gao, S.; McGarry, M.; Ferrier, T.; Pallante, B.; Priddle, H.; Gasparrini, B.; Fletcher, J.; Harkness, L.; De Sousa, P.; McWhir, J.; Wilmut, I. (2003b). "Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line." **Biol Reprod.**, 68(2): 595- 603.

10. Jiang, P.Z.; Shen, X.M.; Qiao, G.L.; Huang, H.; Yao, K.T. (2005). "Effects of different electrofusion parameters on activation and early development of mouse embryos reconstructed by cumulus cell nuclear transfer. **Di Yi Jun Yi Da Xue Xue Bao.**, 25(2): 156- 159.

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

12. Kubota, C.; Yamakuchi, H.; Todoroki, J.; Mizoshita, K.; Tabara, N.; Barber, M.; Yang, X. (2000). "Six cloned calves produced from adult fibroblasts cells after long-term culture" **Proc. Nat. Acad. Sci. USA**, 97: 990-995.

13. Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. (2003). "Parthenogenesis, pronucler transfer and mouse cloning". **In Manipulating the mouse embryo- a laboratory manual**, 3rd Edition, Cold Spring Harbor Laboratory Press: 562.

14. Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. (2003a). "Recovery and *in vitro* culture of preimplantation- stage embryos". **In Manipulating the**

mouse embryo- a laboratory manual, 3rd Edition, Cold Spring Harbor Laboratory Press: 191.

15. Oback, B.; Wiersema, A.T.; Gaynor, P.; Laible, G.; Tucker, F.C.; Oliver, J.E.; Miller, A. L.; Troskie, H. E.; Wilson, K. L.; Forsyth, J. T.; Berg, M. C.; Cockrem, K.; McMillan, V.; Tervit, H. R.; Wells, D. N. (2003). "Cloned cattle derived from a novel zona-free embryo reconstruction system". **Cloning and Stem Cells,** 5 (1): 3-12.

16. Ogura, A.; Inoue, K.; Ogonuki, N.; Noguchi, A.; Takano, K.; Nagano, R.; Suzuki, O.; Lee, J.; Ishino, F.; Matsuda, J. (2000). "Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells." **Biol Reprod.**, 62(6): 1579- 1584.

17. Onishi, A.; Iwamoto, M.; Akita, T.; Mikawa, S.; Takeda, K.; Awata, T.; Hanada, H.; Perry, A. C. (2000). " Pig cloning by microinjection of fetal fibroblasts nuclei". **Science**, 289: 1188-1190.

18. Ono, Y.; Shimozawa, N.; Ito, M.; Kono, T. (2001). "Cloned mice from fetal fibroblasts cells arrested at metaphase by serial nuclear transfer". **Biol. Reprod.**, 64: 44-50.

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

20. Rideout, W.M. 3rd; Eggan, K.; Jaenisch, R. (2001). "Nuclear cloning and epigenetic reprogramming of the genome." **Science,** 293(5532): 1093- 1098.

21. Stenger, D.A.; Kubiniec, R.T.; Purucker, W.J.; Liang, H.; Hui, S.W. (1988). "Optimization of electrofusion parameters for efficient production of murine hybridomas." **Hybridoma**, 7(5): 505-518.

22. Tsunoda, Y. & McLaren, A. (1983). "Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres." **J. Reprod. Fert.**, 69: 315- 322.

23. Vajta, G.; Lewis, I.M.; Hyttel, P.; Thouas, G.A.; Trounson, A.O. (2001). "Somatic cell cloning without micromanipulators." **Cloning**, 3(2): 89-95.

24. Van Stekelenburg-Hamers, A.E.; Van Inzen, W.G.; Van Achterberg, T.A.; Kruip, T.A.; de Laat, S.W.; Weima, S.M. (1993). "Nuclear transfer and electrofusion in bovine *in vitro*-matured/*in vitro*-fertilised embryos: effect of media and electrical fusion parameters." **Mol Reprod Dev.**, 36(3): 307- 312.

25. Wakayama, T.; Rodriguez, I.; Perry, A. C.; Yanagimachi, R.; Mombaerts, P. (1999). "Mice cloned from embryonic stem cells." **Proc Natl Acad Sci U S A.**, 96(26): 14984- 14989.

26. Wakayama, T. & Yanagimachi, R. (1999). "Cloning of male mice from adult tail-tip cells." **Nat Genet**: 22(2): 127- 128.

27. Wakayama, T.; Perry, A. C. F.; Zuccotti, M.; Johnson, K. R.; Yanagimachi, R. (1998). "Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei". **Nature**, 394: 369-374.

28. Wilmut, I.; Schnieke, A. E.; McWhir, J.; Kind, A. J.; Campbell, K. H. S. (1997). "Viable offspring derived from foetal and adult mammalian cells. **Nature**, 385: 810-813.

29. Yamazaki, Y.; Makino, H.; Hamaguchi-Hamada, K.; Hamada, S.; Sugino, H.; Kawase, E.; Miyata, T.; Ogawa, M.; Yanagimachi, R.; Yagi, T. (2001). "Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer." **Proc Natl Acad Sci U S A.,** 98(24): 14022- 14026.

30. Zhou, Q. I.; Boulanger, L.; Renard, J. P. (2000). "A simplified method for recontruction of fully competent mouse zygotes from adult somatic donor nuclei. " **Cloning**, 2 (1): 35- 44.

FIGURES AND TABLES

Figure 1.

Zona-Free Technique of Nuclear Transfer. **(a)** Mouse oocytes after removal of cumulus cells by hyaluronidase (zona-intact embryos). **(b)** Oocytes after digestion of zona pellucida with pronase (zona-free oocytes). **(c)** Oocytes being manipulated using both pipettes until the metaphase plate was close to enucleation pipette. **(d)** Metaphase plate being aspirated with a small amount of cytoplasm. (e) Separation pipette pushing cytoplast away from the enucleation pipette in order to separate karyoplast from cytoplast. (f) Cumulus cells attached to cytoplasm with phytohaemaglutinin. Bars≈ 85μm

Note: Pictures of enucleation c) and d) were taken with UV light to help locate the metaphase spindle, however UV light was not used during enucleation procedure. UV light was only used to check accuracy of chromosomal removal inside the enucleation pipette

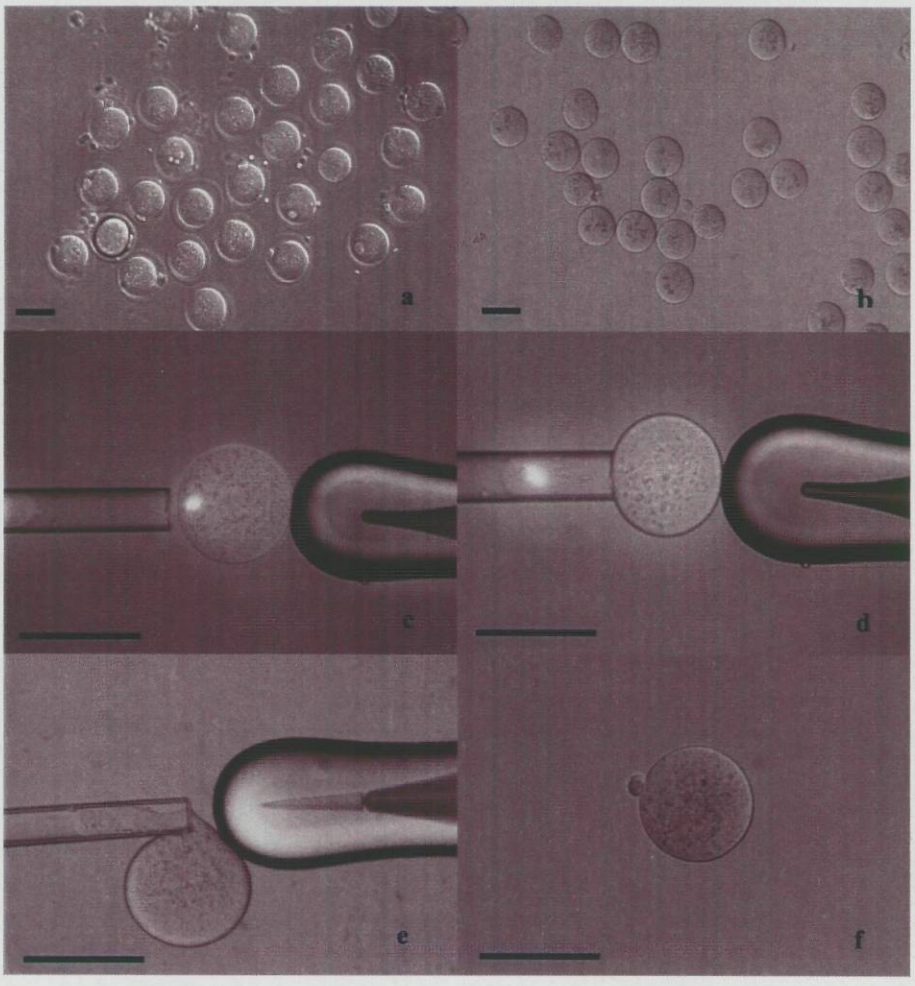

a
b
c
d
e
f

Table 1.

Comparison between two different donor cell types used for zona-free NT.

	No. of Replicates	**No. of cytoplasts submitted to fusion**	**% of Fusion**	**No. of embryos fused**	**% of Cleavage of fused embryos**	**% of Development to Morula/ Blastocyst of fused embryos**
Cumulus Cells	14	573	58.1± 4.0	334	77.2±3.4	8.7±2.1
ED2 cells	42	2064	42.9±2.2	903	40.8±2.7	13.9±1.8
P value			P<0.001		P<0.001	P<0.1

Table 2.

Percentage of lysed oocytes and reconstructed embryos during parthenogenic activation of zona-free (ZF) and zona-intact (ZI) control oocytes and nuclear transfer (NT) embryos reconstructed with ES cells. [a,a'] Significant difference (P<0.001); [b,b'] Significant difference (P>0.2); [c,c'] Significant difference (P<0.01)

	Type of Activation	**No. Replicates**	**No. Oocyte/ NT reconstructs activated**	**No. Lysed oocytes/ NT reconstructs during activation (%)**
ZF oocytes	+ calcium	9	205	9 (3.9± 1.6%)[a]
	- calcium	9	217	117 (52.9± 6.5%)[a']
ZI oocytes	+ calcium	6	114	0 (0.0± 0.0%)[b]
	- calcium	6	105	2 (1.9± 1.4%)[b']
ZF-NT embryos	+ calcium	23	397	1 (0.3± 0.3%)[c]
	- calcium	11	129	49 (34.1± 14.9%)[c']

Table 3.

Percentage of cleavage and development to blastocyst in zona-free (ZF), zona-intact (ZI) controls and nuclear transfer (NT) embryos reconstructed with ES cells after activation in a presence or absence of calcium during parthenogenic activation. [a,a'] Significant difference ($P<0.05$); [b,b'] Significant difference ($P<0.01$); [c,c'] Significant difference ($P>0.2$); [d,d'] Significant difference ($P<0.001$); [e,e'] Significant difference ($P<0.001$); [f,f'] Significant difference ($P>0.2$); [g,g'] Significant difference ($P>0.2$); [h,h'] Significant difference ($P<0.001$); [i,i'] Significant difference ($P>0.2$).

TABLE 3. PERCENTAGE OF CLEAVAGE AND DEVELOPMENT TO BLASTOCYST IN ZONA-FREE (ZF), ZONA-INTACT (ZI) CONTROLS, AND NUCLEAR TRANSFER (NT) EMBRYOS RECONSTRUCTED WITH ES CELLS AFTER ACTIVATION IN A PRESENCE OR ABSENCE OF CALCIUM DURING PARTHENOGENC ACTIVATION

	Type of activation	*No. replicates*	*No. oocyte/ NT reconstructs*	*No. oocytes/ NT reconstructs surviving activation (%)*	*No. oocytes/ NT reconstructs in culture*	*No. cleaved per total oocytes (%)*	*Percentage cleaved per successfully activated embryos*	*No. blastocysts per total oocytes (%)*	*Percentage blastocysts per successfully activated embryos*
ZF embryos	+ Calcium	9	205	196 (96.1 ± 1.6%)	196	164 (80.3 ± 4.6%)	82.9 ± 4.5%[a]	97 (47.5 ± 5.8%)[d]	49.1 ± 6.1%[g]
	− Calcium	9	217	100 (47.1 ± 6.5%)	100	96 (45.2 ± 6.6%)	96.1 ± 2.1%[a']	55 (25.8 ± 4.6%)[d']	56.5 ± 7.0%[g']
ZI embryos	+ Calcium	6	114	114 (100 ± 0.0%)	114	74 (64.3 ± 7.6%)	64.7 ± 8.1%[b]	38 (32.9 ± 6.3%)[e]	32.5 ± 6.4%[h]
	− Calcium	6	105	103 (98.1 ± 1.4%)	103	100 (95.3 ± 2.4%)	97.1 ± 1.9%[b']	85 (80.8 ± 5.0%)[e']	82.0 ± 4.9%[h']
NT embryos	+ Calcium	23	397	396 (99.7 ± 0.3%)	282	118 (29.2 ± 3.2%)	41.3 ± 3.9%[c]	33 (9.3 ± 2.1%)[f]	13.2 ± 3.3%[i]
	− Calcium	11	129	80 (65.9 ± 14.9%)	66	20 (16.5 ± 3.9%)	33.9 ± 6.9%[c']	7 (5.8 ± 2.4%)[f']	12.7 ± 5.4[i']

[a,a']Significant difference ($p < 0.05$); [b,b']significant difference ($p < 0.01$); [c,c']significant difference ($p > 0.2$); [d,d']Significant difference ($p < 0.001$); [e,e']significant difference ($p < 0.001$); [f,f']significant difference ($p > 0.2$); [g,g']Significant difference ($p > 0.2$); [h,h']significant difference ($p < 0.001$); [i,i']significant difference ($p > 0.2$).

Figure 2a.

Embryo development of zona-free (ZF) and zona-intact (ZI) oocytes after parthenogenic activation with strontium in CZB medium with (+Ca) or without calcium (-Ca). Development is expressed as a proportion of those oocytes that survived activation treatment.

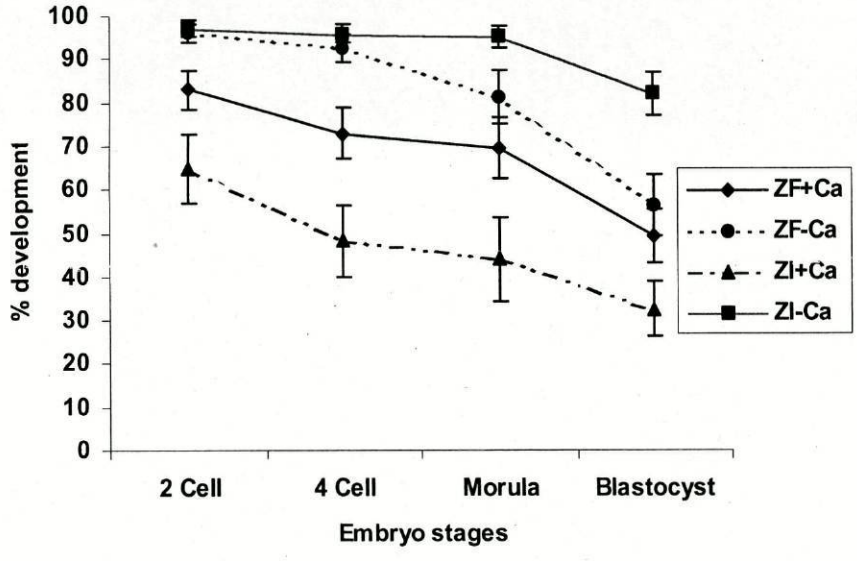


Figure 2b.

Embryo development of zona-free (ZF) NT embryos reconstructed with ES cells after activation with strontium in CZB medium with (+Ca) and without calcium (-Ca). Development is expressed as a proportion of those reconstructed embryos that survived activation treatment.

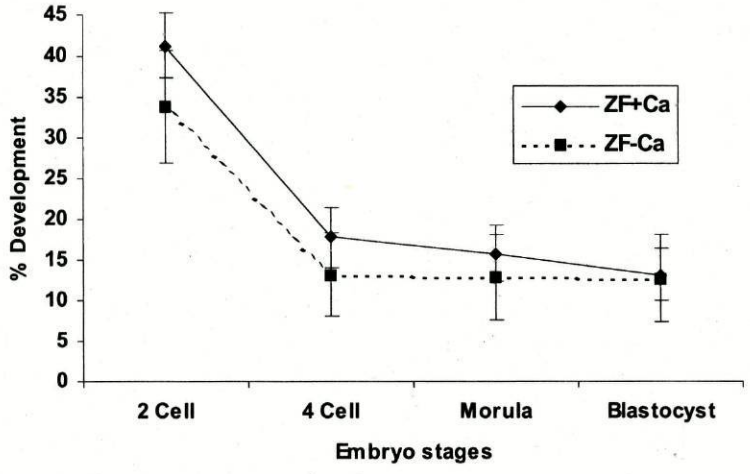


Figure 3.

Embryo development of zona-free NT embryos reconstructed with ES cells cultured in 5% and 20% oxygen concentration.

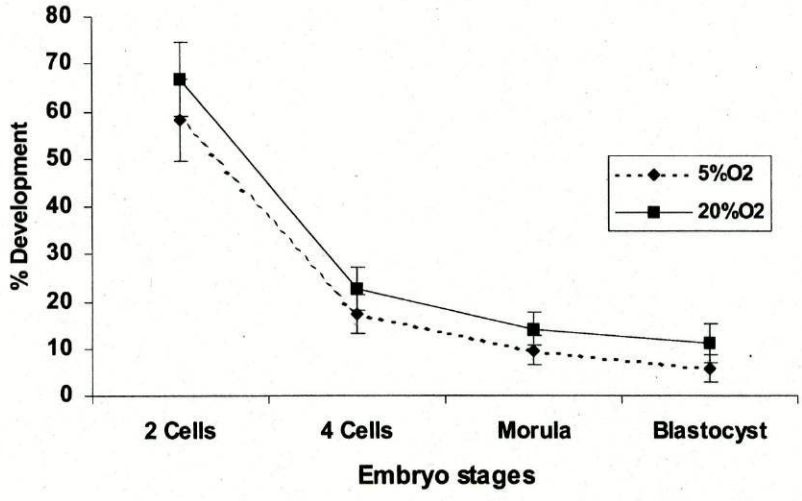


Table 4.

Effect of donor cell type on the stage at which embryos stopped development.

	No. Replicates	**No. Embryos**	**% Block at 1 cell stage**	**% Block at 2-3 cell stage**	**% Block at 4-8 cell stage**
Cumulus Cells	14	242	23.3 ± 3.2	64.9 ± 3.5	11.7 ± 2.6
ED2 cells	42	519	70.7 ± 2.4	26.8 ± 2.2	2.5 ± 0.7
P value			P<0.001	P<0.001	P<0.001

Figure 4.

Development of 2-cell Zona-Free (ZF) and Zona-Intact (ZI) *in vivo* fertilised embryos.

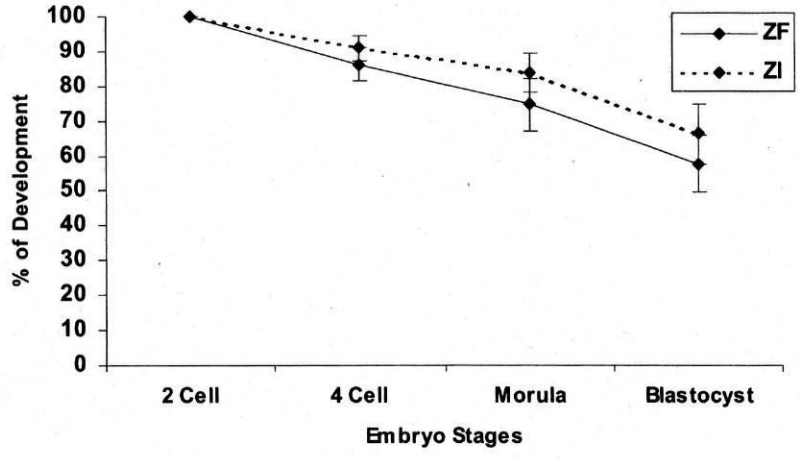


Figure 5.

Cloned mouse at birth (a) and 6 weeks (b). At birth the cloned mouse (left) is larger than another mouse born on the same day, although, they belong to different strains.

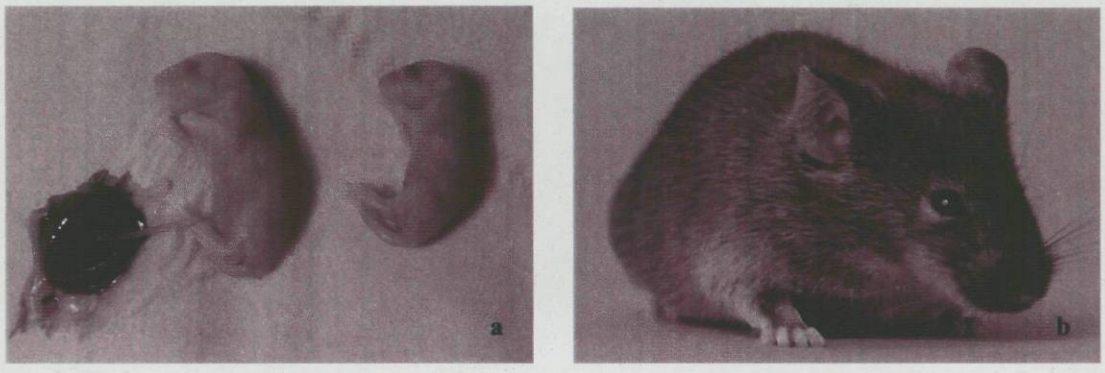
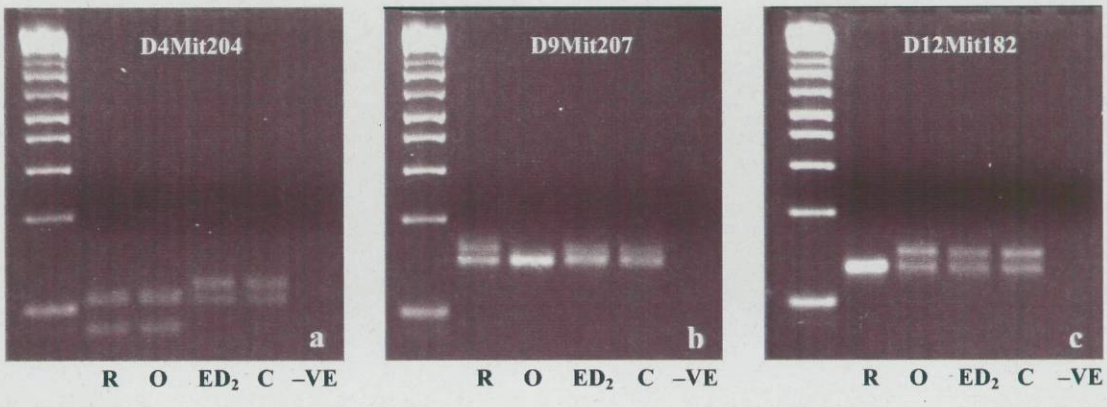


Figure 6.

Results from genomic DNA test of cloned mouse derived from ED_2 ES cells. R- Surrogate mother B6CBAF1 (C57BL6 × CBA); O- oocyte donor B6D2F1; ED_2- Donor Cells; C- Cloned mouse; -VE- negative control (water). (a) Result for polymorphic DNA marker on chromosome 4, (b) chromosome 9 and (c) chromosome 12.



CHAPTER III

Modifications to improve the efficiency of zona-free mouse nuclear transfer

R. Ribas[1,4], B. Oback[2], W. Ritchie[1], T. Chebotareva[1,5], J. Taylor[1,5], A. C. Maurício[3,4], M. Sousa[4], I. Wilmut[1,5]

[1]Department of Gene Function and Development, Roslin Institute, Roslin, Midlothian EH25 9PS, Scotland, UK

[2] Reproductive Technologies, AgResearch Ltd., Ruakura Research Centre, East Street, Private Bag 3123, Hamilton, New Zealand

[3] Centro de Estudos de Ciência Animal (CECA)- Instítuto de Ciências e Tecnológicas Agrárias e Agro- Alimentares (ICETA), Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal

[4] Instítuto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto (UP), Largo Prof. Abel Salazar, 2, 4099-003 Porto, Portugal

[5] The Queen's Medical Research Institute, Reproductive and Development Sciences, University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK

Cloning and Stem Cells (*in print*)

ABSTRACT

In the present study, some modifications were made to the zona-free nuclear transfer technique in the mouse in order to achieve greater efficiency. Firstly, a one-hour interval was allowed between cumulus removal and zona pellucida digestion. Secondly, acid Tyrode's was selected for zona pellucida removal because, contrary to pronase, it allows embryo survival during parthenogenic activation in the absence of calcium. Even when the exposure time to pronase was reduced to as little as one minute, or washed with fetal calf serum to inhibit the enzyme, the percentage of lysis during activation in the absence of calcium was still very high. Thirdly, electrofusion was performed at room temperature (21°C) instead of 30°C as in our previous experiments. Finally, embryos were cultured in groups of 12-15, instead of individually, using a 'well of the wells' system during activation and culture. When compared, parthenogenic activated control embryos showed an increase in the development to blastocyst when cultured in pairs instead of individually. By the end of the experiments and using embryonic stem (ES cells), there was a significant increase in fusion rate (1.5-fold increase) and in development to morula/ blastocyst from cleaved reconstructed embryos (1.5-fold increase) when compared with the results before the modifications. A 2.4-fold increase in overall efficiency was achieved from the oocyte to morula/blastocyst stages.

Keywords: Nuclear Transfer, Mice, Acid Tyrode's, Parthenogenic Activation, Calcium, Zona-Free

INTRODUCTION

Efficient methods of nuclear transfer (NT) following removal of the zona pellucida (ZP) have been established for the cow (Booth et al., 2001; Vajta et al., 2001; Oback et al., 2003) and the pig (Booth et al., 2001a). However, during an attempt to develop a zona-free (ZF) method for NT in the mouse, a deleterious effect of activation in the absence of calcium was apparent (Ribas et al., 2005). In those studies, pronase was used to remove the zona pellucida.

In the present studies, the effect of two changes to the protocol was examined. 1) Acid Tyrode's (AT) was used to remove the zona before parthenogenetic activation or nuclear transfer. 2) Embryos were activated and cultured in groups of 12-15 instead of individually, using a well of the wells system (WOW) (Vajta et al., 2000). Culture of zygotes individually or in small groups results in a lower rate of development than culture in large groups (Lane and Gardner, 1992). In our initial studies, ZF embryos were cultured individually to prevent aggregation between zygotes at compaction stage. However, the WOW system has been developed in cattle, allowing the culture of several embryos (one per each small well) within the same medium droplet (Vajta et al., 2000). This makes it possible to have the benefits of co-culture, with no risk of aggregation.

There were two other changes in the procedure. A delay of one hour between cumulus removal and zona pellucida digestion was introduced, as it appears to contribute to a better development of cloned embryos (unpublished information). Secondly, fusion was induced at room temperature rather than 37°C. When performed at temperatures

higher than 30°C resealing of the individual membranes is accelerated such that intermingling of the attached membranes cannot occur (Neil & Zimmermann, 1993).

MATERIAL AND METHODS

All animal procedures were under strict accordance with UK Home Office guidelines and within a project license issued under the Animal (Scientific Procedures) Act of 1986.

Modifications to the procedure

The methods used in these experiments were as described previously (Ribas et al., 2005) except for the specific changes. Zona pellucida removal was performed one hour after treatment with hyaluronidase to remove cumulus cells, rather than immediately afterwards. A comparison was made between removal of the zona pellucida by exposure to acid Tyrode's or to pronase. Digestion of zona pellucida was carried out by incubation in acid Tyrode's solution (pH 2.5 ± 0.3) until total removal (1-2min). Pronase digestion (Sigma, P8811) was achieved with 5mg/ml of pronase diluted in hCZB containing 0.1 mg/ml of PVA (Polyvinyl Alcohol; ICN; M_r: 10,000-30,000; hCZB-PVA) until total removal (5-7 min). Oocytes were then washed thoroughly in hCZB to remove all traces of pronase/ acid Tyrode's.

Electrofusion was performed as described previously except that it was at room temperature (21°C) rather than 30°C.

Following activation, embryos were washed in CZB to remove all traces of strontium and then cultured *in vitro* using the WOW system in groups of 12-15 in 30 µl droplets under paraffin oil (Fluka, UK) at 37°C and 5% CO_2 for 4 days (day 0 = fusion).

Statistical analysis

Percentages were analyzed using a generalized linear mixed model and the method of Breslow and Clayton (Breslow & Clayton, 1993). This method took account of the binomial distribution of the numbers of embryos reaching the various stages of development and any extra variation between repeat runs of the experiment and between treatment groups of embryos within runs. The effect of the treatments was estimated in the logistic scale by this method and statistical significance was determined approximately using Student's t tests on treatment means in this scale. Deviations to the means were represented by standard errors of the mean (SEM). The statistical significance was accepted at $P<0.05$.

RESULTS

Effect zona removal by acid Tyrode's or pronase on lysis during parthenogenic activation of zona-free oocytes

A comparison was made between removal of the zona pellucida by exposure to acid Tyrode's or to pronase. Acid Tyrode's digestion of the zona pellucida was carried out by incubation in acid Tyrode's solution (pH 2.5 ± 0.3) for 1-2min until total removal (AT). Pronase digestion was achieved by either 1min (P1), 3min (P3), 3min followed by 10% fetal calf serum (FCS) in hCZB to inhibit the enzyme (Pfcs) or until total

removal (6-7 min; Pt) with 5mg/ml of pronase diluted in hCZB-PVA. Oocytes were then washed thoroughly in hCZB to remove all traces of pronase/acid Tyrode's. Untreated zona-intact (ZI) oocytes served as controls.

Both ZI and ZF oocytes were cultured in CZB until activation 17-18 hours after hCG injection, which is the same age as reconstructed oocytes at the time of activation. All embryos were cultured in groups of 12-15 in the WOW system for 4 days (day 0 = activation). Percentage of lysis and development to blastocyst was assessed for the groups in which the zona was removed with pronase or acid Tyrode's.

There were no significant differences in the proportion of embryos that lysed when ZI embryos were compared with ZF embryos after ZP removal with acid Tyrode's: AT (4.7±2.7%, n=83 vs. 6.1±3.2%, n= 83, respectively, P>0.2, Fig. 1).

By contrast, there was a significant incidence of lysis when the zona was removed with pronase (P<0.001 for the followed combinations: P1 vs. ZI, P3 vs. ZI, Pt vs. ZI, Pfcs vs. ZI, P1 vs. AT, P3 vs. AT, Pt vs. AT, Pfcs vs. AT). A small but not statistical increase in lysis occurred with the increase of exposure time to pronase (65.3±8.6, n=88; 78.2±7.0, n=81; 81.4±6.4, n= 79 for oocytes submitted to pronase for 1min, 3 min and until total digestion respectively, P>0.1). Finally, the treatment with fetal calf serum (FCS) after pronase did not protect the oocytes from lysis (78.2±7.0, n=81 vs. 74.7±8.3, n=70 for P3 and Pfcs respectively, P>0.2, Fig. 1). Development to morula/blastocyst was determined for surviving parthenogenic embryos. No correlation was seen for development rate and type of zona pellucida removal procedure, when compared with ZI controls.

Effect of culture singularly or in pairs on in vitro development of parthenogenotes

The zona pellucida was removed by exposure to 5mg/ml pronase before oocytes were washed thoroughly in hCZB to remove all traces of pronase. The oocytes were cultured in CZB until activation 17-18 hours after hCG injection, which is the same age as reconstructed oocytes at the time of activation. Activation was accomplished by individually incubation for 5-6 hours in 5µl droplets CZB media containing 10 mM strontium chloride and 5 µg/ml of cytochalasin B. Embryos were then cultured either individually in 5µl or in pairs in 10µl droplets of CZB media for 4 days (day 0= activation). When cultured in pairs, embryos were placed opposite to each other to prevent aggregation during compaction stages.

There was no effect upon development to 4-cell and morula stage being cultured individually or in pairs, but cleaved parthenotes showed a significant higher development to blastocyst when cultured in pairs (35.6±6.7%, n= 134, for individually cultured vs. 53.2±7.2%, n= 135, when embryos were cultured in pairs, $P<0.05$; Fig. 2).

Modifications and adjustments to the zona-free nuclear transfer technique in the mouse

The effect of several changes to the nuclear transfer protocol was determined by comparison with the results of previous experiments. First acid Tyrode's was selected for zona removal procedure in order to allow parthenogenic activation of nuclear transfer reconstructed embryos in the absence of calcium. Secondly, a one-hour delay between removal of cumulus cells and ZP was performed, due to previous work

showing a beneficial effect of this delay in development (unpublished information). Third, fusion pulses were applied at room temperature. Finally, embryo activation and culture was performed in groups of 12-15 using the WOW system. The results are summarised in Table 1.

There was significantly more lysis when NT embryos were submitted to parthenogenic activation after ZP removal with pronase (P3) than acid Tyrode's (41.3±1.5%, n= 40 of lysis for pronase vs. 1.5± 1.6%, n= 45 of lysis for acid Tyrode's, $P<0.02$).

There was an increase of approximately 1.5-fold in fusion percentage after the technique modifications using ES cells as donors (42.9±2.0%, n=2064; 62.7±4.2%, n=360, before and after the alterations, respectively, $P<0.001$).

No statistical difference was seen in cleavage either before or after the modifications (40.8±2.8%, n= 903; 32.5±4.9%, n= 224, respectively, $P>0.2$), however an increase of over 1.5-fold was reached in development to morula/blastocyst from cleaved embryos (33.7±3.6%, n= 903; 50.9±7.8%, n= 224, respectively, $P>0.05$).

Overall there was an increase in efficiency of the process, as 9.2% of oocytes became morulae or blastocysts in comparison with 3.9% with the initial procedures.

DISCUSSION

In this study, modifications to the mouse zona-free NT approach (Ribas et al., 2005) led to significant improvements in development to the blastocyst stage. As several factors were changed at the same time it is not possible to know which change led to

the improvement. However, the major concern in the earlier zona-free protocol was the unusual response to activation in strontium chloride, when it was found that a majority of the oocytes lysed if activation was in the absence of calcium (Ribas et al., 2005). This presented a difficulty because development is reduced if calcium is included in the media, when mouse oocytes are activated by exposure to strontium (Nagy et al., 2003).

The present results show that it is not the absence of the zona that makes the embryos more susceptible to lysis, but the process used to remove the zona pellucida. When the zona pellucida was removed with acid Tyrode's, the rate of lysis during parthenogenic activation in the absence of calcium, approached the values for zona-intact control (approximately 5%). Results were similar for either control oocytes or NT reconstructed embryos.

However when the zona pellucida was removed using pronase, a majority of the oocytes lysed. The duration of exposure to pronase does not seem to have any effect in the lysis rates. Even when exposed for only one minute (time not sufficient to complete zona removal) the percentage of lysis during activation in the absence of calcium reaches approximately 65% for control embryos. When FCS was added after pronase treatment to inhibit the enzyme, no significant reduction occurred in the percentage of lysis. Interestingly, the cell death occurred within the first hour of activation. The mechanisms that cause this lysis are not understood. When the same groups of embryos were placed in culture, the rate of development of 2-cells to morula/blastocyst stage was not affected by the zona removal method.

The increase of 1.5-fold in the fusion rate in the present study may not only be due to the different temperature during the fusion procedure, but also may reflect other

modifications in the technique, such as the one hour interval between hyaluronidase and ZP removal or the ZP removal procedure. Previous reports suggest that temperatures higher than 30°C accelerate the resealing process of the individual membranes not allowing the merge of the attached membranes to occur (Neil & Zimmermann, 1993).

Finally the culture procedure seems to have an important role during the development to morula/blastocyst. A particular problem for culture of zona-free embryos is the risk of aggregation of embryos cultured in groups. However, previous data showed a beneficial effect of culture in groups during *in vitro* development of preimplantation embryos (Wiley et al., 1986; Canseco et al., 1992). Here, we show significantly better development to blastocyst when parthenotes were cultured in pairs rather than singly. This is believed to be attributed to the influence of embryotrophic factors such as growth factors, cytokines, antioxidants or chelators (reviewed by Donnay et al., 1996), that act in a paracrine-autocrine fashion, creating a mutual stimulation (Gandolfi, 1994). The WOW system was established to permit culture of zona-free bovine embryos in groups where it has shown beneficial results (Vajta et al., 2000). Here we showed a benefit during culture of cloned mouse embryos.

In conclusion, an increase of 1.5-fold in fusion rate and over 1.5-fold development of cleaved embryos to morula/blastocyst were achieved. The potential of embryos produced in this way to develop to term remains to be confirmed.

ACKNOWLEDGEMENTS

The authors would like to thank all the members of the cloning group at AgResearch Ltd., Ruakura Research Centre in New Zealand; all members of Ian Wilmut's group especially to Tricia Ferrier and Catriona Clarke for help with cell culture; Caroline McCorquodale for statistical analysis; and the staff from the small animal unit at Roslin Institute.

This worked was supported by Fundação para a Ciência e a Tecnologia (FCT-MCES), Portugal.

REFERENCES

1. Booth, P. J.; Tan, S.J.; Reipurth, P.; Holm, P.; Callesen, H. (2001). "Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique". **Cloning and Stem Cells**, 3 (3): 139-150.

2. Booth, P. J.; Tan, S.J.; Holm, P.; Callesen, H. (2001a). "Application of zona-free manipulation technique to porcine somatic nuclear transfer". **Cloning and Stem Cells**, 3 (4): 191-197.

3. Breslow, N. E. & Clayton, D. G. (1993). "Approximate inference in generalized linear mixed models." **Journal American Statistical Association**, 88: 9-25.

4. Canseco, R.S.; Sparks, A.E.; Pearson, R.E.; Gwazdauskas, F.C. (1992). "Embryo density and medium volume effects on early murine embryo development." **J Assist Reprod Genet.**, 9(5): 454-457.

5. Donnay, I; Van Langendonckt, A.; Auquier, P.; Grisart, B.; Vansteenbrugge, A.; Massip, A.; Dessy, F. (1996). "Effects of co-culture and embryo number on the in vitro development of bovine embryos". **Theriogenology**, 47 (8): 1549-1561.

6. Gandolfi, F. (1994). "Autocrine, paracrine, and environmental factors influencing embryonic development from zygote to blastocyst". **Theriogenology**, 41: 95-100.

7. Lane, M.; Gardner, DK. (1992). " Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro." **Hum Reprod.**, 7(4): 558-562.

8. Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. (2003). "Parthenogenesis, pronucler transfer and mouse cloning". **In Manipulating the mouse embryo- a laboratory manual**, 3rd Edition, Cold Spring Harbor Laboratory Press: 562.

9. Neil, G. A.; Zimmermann, U. (1993). "Electrofusion." **Methods Enzymol**, 220, 174-96.

10. Oback, B.; Wiersema, A.T.; Gaynor, P.; Laible, G.; Tucker, F.C.; Oliver, J.E.; Miller, A. L.; Troskie, H. E.; Wilson, K. L.; Forsyth, J. T.; Berg, M. C.; Cockrem, K.; McMillan, V.; Tervit, H. R.; Wells, D. N. (2003). "Cloned cattle derived from a novel zona-free embryo reconstruction system". **Cloning and Stem Cells,** 5 (1): 3-12.

11. Ribas, R.; Oback, B.; Ritchie, W.; Chebotareva, T.; Ferrier, P.; Clarke, C.; Taylor, J.; Gallagher, E.J.; Mauricio, A.C.; Sousa, M.; Wilmut, I. (2005). "Development of a Zona-Free Method of Nuclear Transfer in the Mouse." **Cloning Stem Cells,** 7 (2): 126-138.

12. Vajta, G.; Peura, T.T.; Holm, P.; Paldi, A.; Greve, T.; Trounson, A.O.; Callesen, H. (2000). "New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system." **Mol Reprod Dev.,** 55(3): 256-264.

13. Vajta, G.; Lewis, I. M.; Thouas, G. A; Trouson, A.O. (2001). " Somatic cell cloning without micromanipulators". **Cloning and Stem Cells**, 3 (2): 89-95.

14. Wiley, L.M.; Yamami, S.; Van Muyden, D. (1986). "Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development in vitro." **Fertil Steril.,** 45(1):111-119.

FIGURES AND TABLES

Figure 1.

Percentage of lysis in activation and development to morula/blastocyst stage of zona-intact (ZI) and zona-free parthenogenic embryos after zona removal with: incubation in acid Tyrode's (AT) until total zona removal (1- 2min) or pronase digestion for either 1min (P1), 3min (P3), until total removal (Pt) or 3min followed by fetal calf serum (Pfcs). Development was expressed as proportion of the embryos placed into *in vitro* culture.

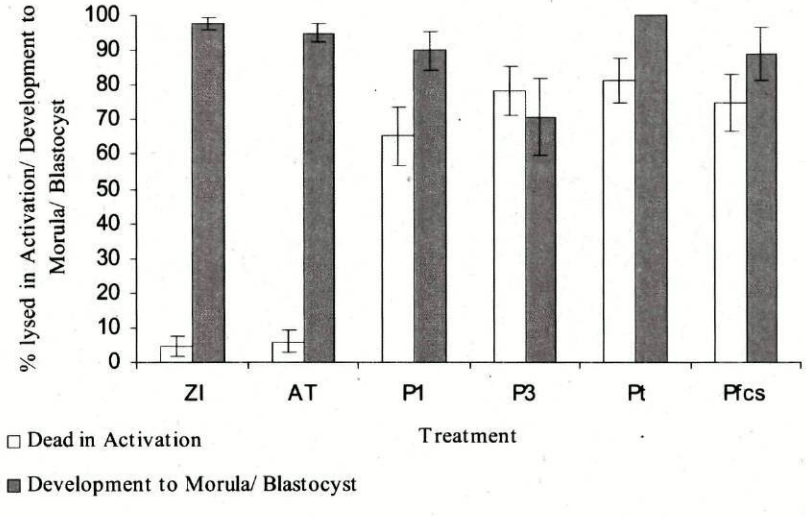


Figure 2.

Percentage of development to blastocyst of parthenogenic-activated embryos cultured *in vitro* individually in 5µl droplets (Single) or in groups of two in 10µl droplets (Pairs).

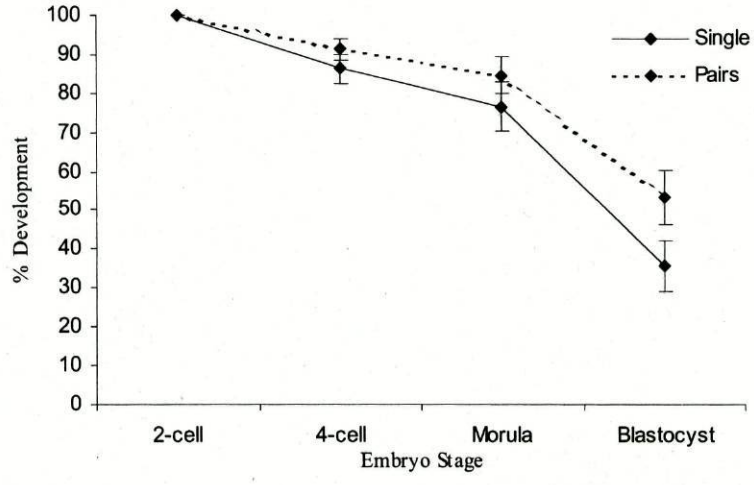


Table 1.

Comparison between zona-free NT output before and after several modifications to the technique using ES cells as donors.

	No. of Replicates	**No. of cytoplasts submitted to fusion**	**% Fusion**	**% Cleavage of fused embryos**	**% Development to Morula/ Blastocyst of fused embryos**	**% Development to Morula/ Blastocyst of cleaved embryos**
Before Modifications	42	2064	42.9±2.0 (n= 903)	40.8±2.8 (n= 243)	13.9±1.8 (n= 81)	33.7±3.6 (n= 81)
After Modifications	9	360	62.7±4.2 (n= 224)	32.5±4.9 (n= 64)	16.5±3.7 (n= 33)	50.9±7.8 (n= 33)
P value			P<0.001	P>0.2	P>0.2	P<0.05

CHAPTER IV

Effect of zona pellucida removal on DNA methylation in early mouse embryos

Ricardo C. Ribas[2,4], Jane E. Taylor[2,5], Caroline McCorquodale[2], Ana C. Maurício[3,4], Mário Sousa[4], Ian Wilmut[2,5]

[2]Roslin Institute, Roslin, Midlothian EH25 9PS, United Kingdom

[3]Centro de Estudos de Ciência Animal (CECA)- Instítuto de Ciências e Tecnologicas Agrárias e Agro- Alimentares (ICETA), Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal

[4]Instituto Ciências Biomédicas Abel Salazar (ICBAS), Largo Prof. Abel Salazar, 2, 4099-003 Porto, Portugal.

[5]The Queen's Medical Research Institute, Centre for Reproductive Biology, Reproductive and Developmental Sciences, University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK

Biology of Reproduction (*in print*)

ABSTRACT

Removal of the zona pellucida is known to affect mouse development to term. Zygotes were recovered immediately after fertilization and their zona pellucida removed by exposure to pronase before culture and comparison with zona-intact embryos. The effect of removing the zona pellucida was assessed in terms of embryo development to blastocyst, DNA methylation, histone acetylation and expression of three developmentally regulated genes. No significant differences were seen in percentage of embryos that developed to the blastocyst stage. However, zona-free embryos showed a significant reduction in the DNA methylation level at 2-cell and 4-cell stages, but no differences at pronuclear, morula and blastocyst stage, as observed by immunofluorescence. Mechanical or enzymatic removal of the zona pellucida showed similar DNA methylation staining patterns at the 2-cell stage. The time when the zona pellucida was removed appears to influence the levels of DNA methylation. When zona removal was delayed for eight hours, there was no difference in DNA methylation levels between zona-free and zona-intact 2-cell embryos, indicating that the critical time is early on between one and eight hours post-fertilization. In contrast, when immunofluorescence analysis of histone acetylation was performed, no significant differences were seen between zona-free and zona-intact embryos at any of the developmental stage. Similarly, no differences were found regarding the onset of transcription of *Dnmt1s*, *Nanog* and *Fgf4* genes.

Keywords: Zona Pellucida Removal, DNA Methylation, Mice, Embryo Development

INTRODUCTION

Oocytes of all vertebrates are surrounded by an extracellular matrix. This is known as chorion in fish [1], perivitelline membrane in birds [2] and zona pellucida (ZP) in mammals [3]. In mouse, the ZP starts to be produce during folliculogenesis. This process starts just after birth and continues during the reproductive life of the female [4], in order to prepare the eggs for the fertilization. After fertilization, the ZP surrounds the embryo during early development, until the blastocyst stage. At this point, embryos must hatch from the ZP in order to establish direct contact with endometrium, allowing implantation to take place [5]. Other suggested functions of the ZP include mechanical protection for the oocyte and embryo until blastocyst stage; facilitating the development of tight junctions between blastomeres during compaction [6, 7]; protection of the early embryo from potential immunological rejection [8] and preventing embryo adherence. Zona-free (ZF) mouse embryos transferred to recipient mice at the 1-cell, 2-cell and 4-cell stage do not implant and seem to adhere to the oviduct wall and/or to each other in groups [9]. Fertilization is one of the main roles in which the ZP is involved: it provides binding sites to sperm receptors promoting initiation of the acrosome reaction before fusion to the oocyte's oolemma and prevents polyspermy after spermatozoa entry [6].

During early embryo growth, development and differentiation, gene expression is highly dependent on epigenetic modifications of the genome (reviewed by [10]). These modifications can occur either at the amino terminal tail of the core histones as methylation, acetylation, phosphorylation, ubiquitination; but also in the DNA itself as methylation (reviewed by [11]). The most described modification in the DNA is its methylation, which is characterized by a covalent addition of a methyl group mainly in the cytosine residues at position 5 in CpG dinucleotides becoming 5-

methylcytosine. A relationship between DNA methylation and gene silencing has been shown [12], for example when expression is not required or may be detrimental to genomic stability (reviewed by [13]). The enzymes responsible for this process are known as DNA methyltransferases (*Dnmts*). The most abundant is *Dnmt1*. Deletion of *Dnmt1* in mouse leads to global demethylation and embryonic death [14]. In somatic cells, DNA methylation is known to have other functions in X-chromosome inactivation, genomic imprinting, inactivation of retroviral sequences (reviewed by [10]; [15]), tumour formation (reviewed by [16]) and ageing process (reviewed by [17]).

Although stable in somatic cells, DNA methylation changes during early development [18]. During mouse embryo development an active demethylation wave and DNA decondensation, seen as an increase in pronuclei size, occurs during in the male pronucleus within hours after fertilization, but in the female pronucleus demethylation occurs passively in subsequent cleavage divisions. Santos et al. in 2002, showed that in mouse *de novo* methylation occurs in the blastocyst in the inner cell mass but not in the trophectoderm [19]. Enzymes responsible for *de novo* DNA methylation are *Dnmt3a* and *Dnmt3b* [20]. However, this pattern varies within different species [21].

Histone acetylation is a regulatory histone modification that occurs predominantly at the level of the lysines in the amino-terminal tail of histone 3 and 4. It plays an important role in gene expression by altering the accessibility of DNA to proteins such as transcription factors. In the majority of cases, genes that are undergoing transcription are hyperacetylated, and those that are repressed are deacetylated [22, 23].

In view of previous results showing that removal of the zona pellucida affects mouse development to term [24], and because normal regulation of DNA methylation is

essential for normal development, the effect of the zona pellucida removal on DNA methylation of preimplantation mouse embryos was performed by 5-methylcytosine antibody. Histone 4 lysine 5 (H4K5) acetylation antibody was also used to study the effect of zona removal on histone acetylation.

MATERIAL AND METHODS

All chemicals were supplied by Sigma-Aldrich (Dorset, UK) and all embryo manipulation was performed at 37°C temperature unless indicated otherwise.

Production and collection of murine in vitro fertilized embryos

All animal procedures were under strict accordance with UK Home Office regulations and within a project license issued under the Animal (Scientific Procedures) Act of 1986. Eight to ten week old B6D2F1 (C57BL/6J×DBA/2) mouse females were superovulated by intraperitoneal injections of 5 IU eCG (Folligon- Intervet) followed by 5 IU hCG (Chorulon- Intervet) 48 hours later and mated with B6D2F1 mouse males. Zygotes were recovered approximately 16 hours after hCG injection. At this point, a majority of the oocytes should have been fertilized for 1 hour [25]. Immediately after recovery, cumulus cells were removed by incubation with hyaluronidase (300 Units/ml) in 4-(2-hydroxyethil- 1-piperazinethane sulfonic acid) (hepes)-buffered CZB media- [hCZB] [26] and zona pellucida digested from half of the embryos, using 5mg/ml pronase in hCZB. In later studies, the zona pellucida was removed using pronase 8 hours after recovery from the oviduct. In some cases the zona pellucida was removed mechanically using a Nikon Eclipse TE300 microscope with a holding pipette and a sharp tool, which is used to remove the ZP. All embryos were cultured individually *in vitro* in 5 μl droplets of CZB [26] under paraffin oil

(Fluka) at 37°C and 5 % CO_2 in air for 4 days (day 0= recovery). Embryo development was assessed daily.

The zona pellucida was removed from control zona-intact (ZI) embryos before fixation using the methods described above to prevent differences in immunostaining techniques resulting from the presence of a zona pellucida.

Number of cells per blastocyst

Some ZF and ZI embryos that reached the blastocyst stage were stained using Hoechst (5µl/ml). The mean number of cells per blastocyst was determined after three consecutive counts for each blastocyst.

5-Methylcytosine and H4 lysine 5 acetylation immunodetection in embryos

Embryos were washed in phosphate buffer solution (PBS) and fixed overnight at 4°C in 4% paraformaldehyde. In order to compare several development stages in the same immunostaining experiment, some embryos were kept in 4% paraformaldehyde at 4°C for 1 to 6 days (control experiments showed no differences in staining within embryo from the same stage when processed within 7 days intervals [13]).

Polyvinyl alcohol (PVA) at concentration of 0.1mg/ml was added to the media to prevent ZF embryos sticking to the dishes during the staining procedure. After fixation, embryos were washed extensively in 0.05% Tween-20 (VWR) and permeabilized in 0.2% Triton X-100 (VWR) for 30 min at room temperature. After extensive washes in 0.05% Tween-20, embryos were subjected to 2M hydrochloric acid for 1 hour at 37°C to denature the DNA, and then washed in 0.05% Tween-20. After blocking for 1 hour in 2% bovine serum albumin (BSA) at room temperature, methylated DNA was visualized with a mouse monoclonal antibody against 5-

methylcytosine (Eurogentec, Belgium). Incubation with this antibody was performed at 37°C for 1 hour (1:400 dilution in Block Solution (BS): 1% BSA, 0.05% Tween-20 in PBS). Embryos were washed four times in BS for 15 min each and incubated in donkey anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch) at a concentration of 1:200 for 1 hour at room temperature. After extensive washes in BS, embryos were post-fixed in 4% PFA for 30 min and mounted on multiwell slides (VWR) with Vectashields containing 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories Ltd.).

The staining protocol for Acetylation of H4 lysine 5 was as described above for 5-methylcytosine, without the denaturation step. Acetylation at histone 4 at lysine 5 was visualized with an anti-H4Ac5 antibody (R40; kind gift from Bryan Turner). Incubation in primary antibody (1:200 dilution in BS) as well as in secondary antibody fluorescein coupling (Jackson ImmunoResearch) was performed at room temperature for 1 hour. Comparisons were made between ZF and ZI embryos for each stage of development. Control embryos were prepared by omitting the primary antibody, or both the primary and secondary antibodies.

Whole-mount fluorescence microscopy and quantitative analysis of nuclei fluorescence

Fluorescence observation was performed on a Nikon upright microscope (Microphot), using a filter wheel equipped with standard filters for FITC, and DAPI emissions. Images were captured through a Nikon Fluar 40x objective (NA 0.75) by a cooled CCD Digital Pixel camera with a KAF 1600 sensor coupled to the IPLab Digital Pixel software. The same software was used for quantification of total embryonic fluorescence intensities.

Nuclear fluorescence intensities of nuclei in cleavage stage embryos were measured by manually outlining all nuclei. For blastocysts at least 15 nuclei were randomly selected from inner cell mass and trophectoderm cell populations based on cellular morphology (small and compact cells in inner cell mass compared to elongated cells in trophectoderm). A total fluorescence intensity emitted by each individual nucleus was measured using IPLab Digital Pixel software and averaged per each embryo.

Assessment of gene expression

Total RNA was extracted from groups of six preimplantation embryos using the Qiagen QIAshredder kit and RNeasy kit, following manufacturer's instructions. Reverse transcription was performed immediately after RNA extraction using a random hexamer ($pd(N)_6$) primer and the First-Strand cDNA Synthesis kit (Amersham Biosciences). Polymerase Chain Reaction (PCR) was carried out using Thermo-Start® PCR master mix (ABgene, UK) in a total reaction volume of 25 µl containing PCR master mix (1.5 mM magnesium; 0.8 mM dNTP, 1U Thermo-Start® *Taq* polymerase), 250 nM each of forward and reverse primers, and 2 µl template cDNA. After a 5 min initial incubation at 95°C, reactions were subjected to 40 cycles of denaturation (94°C for 30 sec), annealing (58°C: *Dnmt1s*, *Ezh2*, *Nanog*; and 60°C: *Fgf4* for 30 sec) and extension (72°C for 45 sec), with a final extension time of 10 min at 72°C (Appendix V). All PCR products were separated on a 2 % (w/v) agarose gel, visualized after staining with ethidium bromide, cloned into the pCR4-TOPO vector (TA cloning kit; Invitrogen, The Netherlands) and sequenced by MWG Biotech, UK. Primers used were 5'-AAGCACAATGCAACACCAAA-3' and 5'-AGACGGTGCCAGCAGTAAGT-3' for *Ezh2*; 5'-GACTACCTGCTGGGCCTCAAAAG-3' and 5'-

TTGGTCCGCCCGTTCTTACTGAG-3' for *Fgf4*; 5'-CTTACAAGGGTCTGCTAC-3' and 5'-CTGAAACCTGTCCTTGAG-3' for *Nanog*; and Dnmt1s [27]. Each experiment was repeated at least four times for each gene and Fig. 5 shows a representative PCR.

Statistical analysis

Percentages- Percentages of embryos reaching the 2 cell, 4 cell, morula and blastocyst stages were analysed using a generalised linear mixed model (GLMM) and the method of Breslow and Clayton [28]. This method took account of a binomial distribution of the numbers of embryos reaching the various stages of development and any extra variation between repeat runs of the experiment and between treatment groups of embryos within runs. Treatment means and standard errors of differences were estimated in the logistic scale by this method and statistical significance of differences between these treatment means was determined approximately using Student's t tests with degrees of freedom equal to those associated with random interaction of runs and treatments.

Blastocyst Cell Number- REML (residual maximum likelihood) was used to estimate the treatment effects of zona type on blastocyst cell number taking into account random variation between experimental runs, between treatment groups within runs and between blastocysts within groups. Cell numbers were left untransformed as there was little evidence of the variance changing with the mean. Statistical significance was determined approximately as described earlier.

Quantification- REML (residual maximum likelihood) [29] was also used to estimate the effects of zona pellucida removal, development stage and blastocyst cell type taking into account random variation between experimental runs, between treatment

groups within runs, between embryos within groups and between cells within embryos. Quantification was transformed to the logarithmic scale before analysis, as there was evidence of variation increasing with the mean. In addition, the variance between cells within embryos appeared to change with development stage and this was also accounted for in the REML analysis.

Statistical significance of differences between means in the logarithmic scale was established approximately using Student's t tests as above. REML is an extension of the analysis of variance for observations with unequal replication and a nested structure as described above. Exact significance tests are not generally available with REML or GLMM.

Units for area and total nuclear fluorescence are arbitrary units given by the software. Deviations to the means or back-transformed means where the logistic or logarithmic scale was used were represented by standard errors (SEM).

RESULTS

In vivo fertilized control development until blastocyst and blastocyst quality

There was no significant difference in development to blastocyst, for *in vivo* fertilized embryos cultured with and without zona pellucida (70.3± 8.0% vs. 61.0± 9.1% respectively; P>0.2 Fig. 1). Although, previous results from our group have shown a significant reduction in development to term after removal of the ZP [24].

Blastocyst quality was assessed by number of cells per blastocyst. Morphologically, ZF blastocyst look smaller, less expanded and more irregular than ZI (Fig. 2), however the number of cells per blastocysts was not significantly different (37.5 ± 2.21, n= 118, 37.8 ± 2.36, n= 73; P>0.2, for blastocysts cultured with and without zona pellucida, respectively).

Effect of zona removal on DNA methylation in 2-cell, 4-cell, morula and blastocyst stage embryos

After ZP removal and culture of *in vivo* fertilized embryos, 5-methylcytosine fluorescence was quantified in 2-cell, 4-cell, morula and inner cell mass and trophectoderm in the blastocyst. Interestingly, ZF embryos showed a 30 % reduction in the levels of DNA methylation compared with ZI embryos at 2-cell (2.04 ± 0.36 vs. 2.90 ± 0.51 respectively; $P<0.05$) and 43 % reduction at 4-cell stage (1.32 ± 0.23 vs. 2.33 ± 0.41 respectively; $P<0.01$) (Fig. 3a, b). At morula stage however, the reduction in DNA methylation of ZF embryos had lessened compared with ZI embryos, and was not statistically different (1.46 ± 0.26 vs. 1.81 ± 0.32 respectively; $P> 0.1$). At the blastocyst stage the levels of fluorescence were very similar for ZF and ZI embryos in both inner cell mass (1.35 ± 0.24 vs. 1.43 ± 0.25 respectively; $P>0.2$) and trophectoderm (0.90 ± 0.16 vs. 0.92 ± 0.16 respectively; $P>0.2$)(Fig. 3a,b).

Effect of mechanical zona removal on DNA methylation in 2-cell stage embryos

To exclude the possibility that enzymatic digestion with pronase was in some way responsible for the changes observed in DNA methylation levels, the ZP was removed by an alternative method. Mechanical removal of the ZP resulted in a similar reduction in DNA methylation levels in 2-cell embryos as seen after removal with pronase digestion compared with ZI embryos (Fig. 4). However, there was no difference between the two methods of zona pellucida removal (2.10± 0.44 vs. 2.06± 0.44; $P>0.2$ for pronase and mechanical removal respectively- Appendix VI).

Effect of delaying zona pellucida removal on DNA methylation

To determine if the time of ZP removal was critical for normal development zygotes were collected from mothers and cultured for eight hours before zona pellucida removal with pronase incubation. Eight hours culture time was chosen to ensure that the zygotes were still in the first cell cycle, before DNA replication. Two-cell ZF embryos showed a non-significant reduction in 5-methylcytosine fluorescence intensity compared with ZI (2.23± 0.28 vs. 2.68± 0.34; P>0.2 respectively), indicating that the critical period is between one hour and eight hours post-fertilization (Appendix VI).

Effect of zona removal on DNA methylation in male and female pronuclei

Seven hours after zona removal male and female pronuclei were analyzed for DNA fluorescence intensity and size. Determination of pronulear area is necessary because the male pronucleus increases in size in the first hours after fertilization, due to DNA decondensation. The aim was to investigate the effect of the ZP removal on DNA methylation at an earlier stage of development. The male pronuclei of ZF embryos were smaller and more methylated than those of ZI embryos, although not significantly different. Similarly, female pronuclei size and DNA methylation levels were similar for ZF and ZI groups (Table 1; Appendix VI).

Effect of zona removal on H4K5 acetylation in 2-cell, 4-cell, morula and blastocyst stage embryos

Histone acetylation plays an important role in gene expression by altering the accessibility of DNA to proteins such as transcription factors. In the majority of cases, genes that are undergoing transcription are hyperacetylated, and those that are

repressed are deacetylated. Comparisons were made between ZF and ZI embryos at each stage of development (2-cell, 4-cell, morula and blastocyst inner cell mass and trophectoderm) to assess the acetylation of lysine 5 on histone 4. Similar levels of fluorescence were observed for ZF and ZI embryos for each stage of development: 2-cell (5.27± 0.53, n= 21 for ZF and 4.69± 0.47, n= 21 for ZI; P>0.2); 4-cell (2.05± 0.21, n= 20 for ZF and 2.40±0.24, n= 21 for ZI; P>0.1); morula (1.7± 0.17, n= 20 for ZF and 2.02± 0.2, n= 22 for ZI; P>0.1); and blastocyst, including inner cell mass (1.38± 0.15, n= 15 for ZF and 1.23± 0.13, n= 16 for ZI; P>0.2) and trophectoderm (0.93± 0.10, n= 15 for ZF and 0.93± 0.10, n= 16 for ZI; P>0.2- Appendix VI).

Effect of zona removal on expression of Dnmt1s, Nanog and Fgf4

To address the possibility that reduced levels of DNA methylation in ZF embryos at the time of genome activation may result in altered gene expression, onset of transcription was observed for three genes essential for early mouse development. *Ezh2* was used as a positive control as it is ubiquitously expressed during preimplantation development from oocytes to blastocyst. Onset of transcription of the somatic form of *Dnmt1 (Dnmt1s)*, *Nanog* and *Fgf4* occurs after embryonic gene activation between 4-cell and morula stage and no maternal mRNA is carried from the oocyte [27, 30, 31]. Fig.5 shows RT-PCR results for the three different genes. Transcription for *Nanog* started to occur for the majority of the embryos at morula stage, although the *Nanog* transcript was observed in some embryos at the 8-cell stage. Similar results were seen in ZF and ZI embryos. *Dnmt1s* and *Fgf4* mRNA expression began at the 4-cell stage and continued through to the blastocyst stage. Despite changes in methylation of DNA, the onset of genome activation for these three genes was similar for embryos with or without zona pellucida (Fig. 5).

Although, small but meaningful differences in gene expression between ZF and ZI embryos may have been missed by used a non-quantitative RT-PCR assay employing 40 amplification cycles.

DISCUSSION

In the present study, removal of the zona pellucida immediately after recovery of fertilized eggs caused a reduction in DNA methylation at the 2 and 4-cell stages. As the same effect was seen when the zona was removed mechanically or by exposure to pronase it does not reflect effects of an enzyme on the cells. There was no reduction if the zona was removed 8 hours after zygote recovery, suggesting that the effect can only occur during a very brief period. DNA methylation levels were restored to normal in the morula and blastocyst stages. It is important that confirmation of these effects is sought by molecular analyses. Despite this transient reduction in DNA methylation, ZP removal had no effect upon development to the blastocyst stage or cell number in blastocysts, although in an earlier study was observed a reduced development to term [24].

The time when the ZP was removed appears to influence the levels of DNA methylation. When zona removal was delayed for 8 hours, there was no difference in DNA methylation levels between ZF and ZI 2-cell embryos, indicating that the critical time is early on between 1 and 8 hours post-fertilization. This suggests that the period immediately after the fertilization is the most sensitive. At this time, the male DNA is very close to the oolemma, which may make it more vulnerable to changes during zona pellucida removal and possibly affecting DNA methylation as well. ZF and ZI embryos were observed at the pronucleus stage, approximately 7 hours after zona removal. Zona removal had no effect on male or female pronuclear area or

pronuclear DNA methylation level. Hence, visible changes arise between 7 and 24 hours (2-cell stage) post-fertilization.

During early stages of development in the mouse, demethylation of the DNA begins at fertilization and continues until the blastocyst stage. This occurs firstly in an active way in the paternal genome [32-34] and secondly in a passive way during the first cleavage divisions from 2-cell to morula stage [32, 35]. Our ZI DNA methylation staining pattern confirms these results, showing passive demethylation until the blastocyst stage and *de novo* methylation in the inner cell mass of the blastocyst as previously shown [19]. By contrast, a reduction in the ZF fluorescence emission was observed when compared with ZI controls. Substantial differences in DNA methylation occurred at 2-cell and 4-cell stage, contrasting with no differences at morula or blastocyst stage (either in inner cell mass or trophectoderm). However, small differences may not have been detected because these may be masked by differences in access of antibody to the interphase nuclei. There is also a possibility at the later stages of development that nuclei may overlie one another causing mis-leading measurments. As removal of the zona did not affect development to the blastocyst stage or the number of cells in the blastocyst ZF embryos are able to recover the normal level of DNA methylation. It is interesting to note that DNA methylation in zona-free embryos remained relatively constant for 4-cell, morula and blastocysts inner cell mass stages.

Interestingly, no differences were seen when acetylation at lysine 5 in histone 4 was analyzed in ZF or ZI embryos at any of the stages of the development, but there is a possibility that small differences may not have been detected. The lysine 5 is the final lysine to be acetylated in histone H4, after lysine 16, 8 and 12, reflecting the hyperacetylated state of H4, and is strongly correlated with the active states of the

genes (reviewed by [36]; [37]). This could explain our data showing that no differences were found regarding onset of transcription for *Dnmt1s*, *Nanog* and *Fgf4*, although, small differences in expression would not have been detected by the assay method used in this study. The present observations provide no indication of the sites at which DNA methylation was lost. This information could be obtained by molecular analyses of DNA methylation within regulatory regions of critical genes and in repeat sequences. Disturbance of DNA methylation at both regulatory regions and repeat sequences has been shown after embryo culture and manipulation [38, 39].

There are earlier observations showing epigenetic effects of zona removal or culture environment upon embryo development and in some cases this has been shown to be associated with changes in DNA methylation. Removal of the zona pellucida from mouse zygotes influences development to term. This effect was associated with differences in cell contact at the 4-cell stage and the authors suggested that cell association influences formation of the inner cell mass [40]. The health of offspring of several species is prejudiced by culture in inappropriate conditions, such as use of serum in the media [38, 39]. Gross abnormalities in gene expression have been observed in embryos, foetuses and offspring produced by nuclear transfer, in same cases has been associated with abnormal DNA methylation [41]. In some species the inefficiency of nuclear transfer is associated with epigenetic effects on expression of imprinted genes [42]. The mechanisms that bring about these epigenetic effects are not known. Several possible causes of the transient reduction in DNA methylation level in the present studies can be considered.

The mechanisms that regulate DNA methylation in the preimplantation embryos are not yet fully established in any species, although one important gene is *Dmnt1o*, which is synthesized in the oocyte, but it resides in the cytoplasm and is functional in

preimplantation embryos to maintain genomic imprinting [43]. It is also possible that the pronase, as a protease enzyme, could in some way affect some of the cytoplasmic enzymes of the zygote/ embryo, which may have a downstream effect on DNA methylation, altering patterns of the pre-implantation embryos. However, the fact that mechanical removal of the zona pellucida had a similar effect suggests that it is not the pronase that is responsible for the altered levels of DNA methylation, but the absence of the zona itself.

Fertilization causes a release of cortical granules to the perivitelline space by exocytosis. Some of the proteins expelled by the cortical granules help the zona pellucida to prevent polyspermy, but others remain in the perivitelline space forming the cortical granule envelope [44, 45]. The premature loss of the proteins from the cortical granule envelope by the time of the zona removal may be responsible for the alterations of DNA methylation levels. A variety of proteins have been shown to be present in the cortical granules, including p62/p56, p32, p75, n-acetylglucosaminidase, ovoperoxidase, calreticulin, tissue plasminogen activator, heparin binding placental protein and other proteinases, (reviewed by [46]). Little is known about the proteins present in the extracellular matrix. However, Hoodbhoy et al., in 2001 have identified two proteins in the extracellular matrix with molecular weights of 62 kDa (p62) and 56 kDa (p56) using Western blots. ZF fertilized oocytes expressed lower levels of p62 and p56 proteins compared with ZI ones, showing that loss of these proteins occurs after zona removal [47]. It was also shown in mice that new synthesis of some of these proteins such as p62 or p56 occurs at 2-cell stage, with greatest production at 8-cell stage, suggesting that they are replenished after the first cleavage[47, 48].

Premature loss of proteins from the cortical granule envelope may account for the difference in DNA methylation seen in this study. This hypothesis could be tested by assessing the effect upon DNA methylation of using RNA interference to reduce the level of these candidate proteins in ZI embryos.

ACKNOWLEDGEMENTS

The authors would like to thank to all the members of the Ian Wilmut group at Roslin Institute, specially to William Ritchie for the help with mechanical removal of zona pellucida; all the staff from the small animal unit at Roslin Institute and to Bryan Turner for the H4K5 acetylation antibody gift.

REFERENCES

1. Cotelli F, Andronico F, Brivio MF, Lora Lamia C. Structure and composition of the fish egg chorion (Carassius auratus). J Ultrastruct Mol Struct Res 1988; 99: 70-78.
2. Bain JM, Hall JM. Observations on the development and structure of the vitelline membrane of the hen's egg: an electron microscope study. Aust J Biol Sci. 1969; 22: 653-665.
3. Rankin T, Dean J. The zona pellucida: using molecular genetics to study the mammalian egg coat. Rev Reprod 2000; 5: 114-121.
4. Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J. Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. Development 1996; 122: 2903-2910.
5. Cole RJ. Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst. J Embryol Exp Morphol 1967; 17: 481-490.
6. Breed WG, Hope RM, Wiebkin OW, Spargo SC, Chapman JA. Structural organization and evolution of the marsupial zona pellucida. Reproduction 2002; 123: 13-21.
7. Wassarman P, Chen J, Cohen N, Litscher E, Liu C, Qi H, Williams Z. Structure and function of the mammalian egg zona pellucida. J Exp Zool 1999; 285: 251-258.
8. Ozgur K, Patankar MS, Oehninger S, Clark GF. Direct evidence for the involvement of carbohydrate sequences in human sperm-zona pellucida binding. Mol Hum Reprod 1998; 4: 318-324.

9. Modlinski JA. The role of the zona pellucida in the development of mouse eggs in vivo. J Embryol Exp Morphol 1970; 23: 539-547.

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

11. Beaujean N. Fundamental features of chromatin structure. Cloning Stem Cells 2002; 4: 355-361.

12. Razin A, Riggs AD. DNA methylation and gene function. Science 1980; 210: 604-610.

13. Beaujean N, Taylor JE, McGarry M, Gardner JO, Wilmut I, Loi P, Ptak G, Galli C, Lazzari G, Bird A, Young LE, Meehan RR. The effect of interspecific oocytes on demethylation of sperm DNA. Proc Natl Acad Sci U S A 2004; 101: 7636-7640.

14. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 1992; 69: 915-926.

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

16. Robertson KD, Jones PA. DNA methylation: past, present and future directions. Carcinogenesis 2000; 21: 461-467.

17. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004; 4: 988-993.

18. Monk M, Boubelik M, Lehnert S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 1987; 99: 371-382.

19. Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 2002; 241: 172-182.

20. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999; 99: 247-257.

21. Beaujean N, Taylor J, Gardner J, Wilmut I, Meehan R, Young L. Effect of Limited DNA Methylation Reprogramming in the Normal Sheep Embryo on Somatic Cell Nuclear Transfer. Biol Reprod 2004.

22. Hebbes TR, Thorne AW, Crane-Robinson C. A direct link between core histone acetylation and transcriptionally active chromatin. Embo J 1988; 7: 1395-1402.

23. Ng HH, Bird A. DNA methylation and chromatin modification. Curr Opin Genet Dev 1999; 9: 158-163.

24. Ribas R, Oback B, Ritchie W, Chebotareva T, Ferrier P, Clarke C, Taylor J, Gallagher EJ, Mauricio AC, Sousa M, Wilmut I. Development of a Zona-Free Method of Nuclear Transfer in the Mouse. Cloning Stem Cells 2005; 7: 126-138.

25. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Summary of the mouse development. In: Nagy A, Gertsenstein M, Vintersten K, Behringer R (eds.), Manipulating the mouse embryo- a laboratory manual: Cold Spring Harbor Laboratory Press; 2003: 49.

26. Gao S, McGarry M, Latham KE, Wilmut I. Cloning of mice by nuclear transfer. Cloning Stem Cells 2003; 5: 287-294.

27. Ratnam S, Mertineit C, Ding F, Howell CY, Clarke HJ, Bestor TH, Chaillet JR, Trasler JM. Dynamics of Dnmt1 methyltransferase expression and

intracellular localization during oogenesis and preimplantation development. Dev Biol 2002; 245: 304-314.

28. Breslow NE, Clayton DG. Approximate inference in generalized linear mixed models. Journal American Statistical Association 1993; 88: 9-25.
29. Patterson HD, Thompson R. Recovery of inter-block information when block sizes are unequal. Biometrika 1971; 58: 545- 554.
30. Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003; 113: 643-655.
31. Rappolee DA, Basilico C, Patel Y, Werb Z. Expression and function of FGF-4 in peri-implantation development in mouse embryos. Development 1994; 120: 2259-2269.
32. Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci U S A 2001; 98: 13734-13738.
33. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J. Active demethylation of the paternal genome in the mouse zygote. Curr Biol 2000; 10: 475-478.
34. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T. Demethylation of the zygotic paternal genome. Nature 2000; 403: 501-502.
35. Rougier N, Bourc'his D, Gomes DM, Niveleau A, Plachot M, Paldi A, Viegas-Pequignot E. Chromosome methylation patterns during mammalian preimplantation development. Genes Dev 1998; 12: 2108-2113.

36. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997; 389: 349-352.

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

38. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998; 3: 155-163.

39. Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B, Gutierrez-Adan A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A 2004; 101: 5880-5885.

40. Suzuki H, Togashi M, Adachi J, Toyoda Y. Developmental ability of zona-free mouse embryos is influenced by cell association at the 4-cell stage. Biol Reprod 1995; 53: 78-83.

41. Wrenzycki C, Niemann H. Epigenetic reprogramming in early embryonic development: effects of in-vitro production and somatic nuclear transfer. Reprod Biomed Online 2003; 7: 649-656.

42. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001; 27: 153-154.

43. Howell CY, Bestor TH, Ding F, Latham KE, Mertineit C, Trasler JM, Chaillet JR. Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. Cell 2001; 104: 829-838.

44. Talbot P, DiCarlantonio G. Ultrastructure of opossum oocyte investing coats and their sensitivity to trypsin and hyaluronidase. Dev Biol 1984; 103: 159-167.

45. Dandekar P, Talbot P. Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization. Mol Reprod Dev 1992; 31: 135-143.

46. Liu M, Sims D, Calarco P, Talbot P. Biochemical heterogeneity, migration, and pre-fertilization release of mouse oocyte cortical granules. Reprod Biol Endocrinol 2003; 1: 77.

47. Hoodbhoy T, Dandekar P, Calarco P, Talbot P. p62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development. Mol Reprod Dev 2001; 59: 78-89.

48. Talbot P, Dandekar P. Perivitelline space: does it play a role in blocking polyspermy in mammals? Microsc Res Tech 2003; 61: 349-357.

FIGURES AND TABLES

Figure 1.

Development of Zona-Free (ZF; n=410) and Zona-Intact (ZI; n= 449) *in vivo* fertilized embryos. Bar graphs represent mean ± SEM.

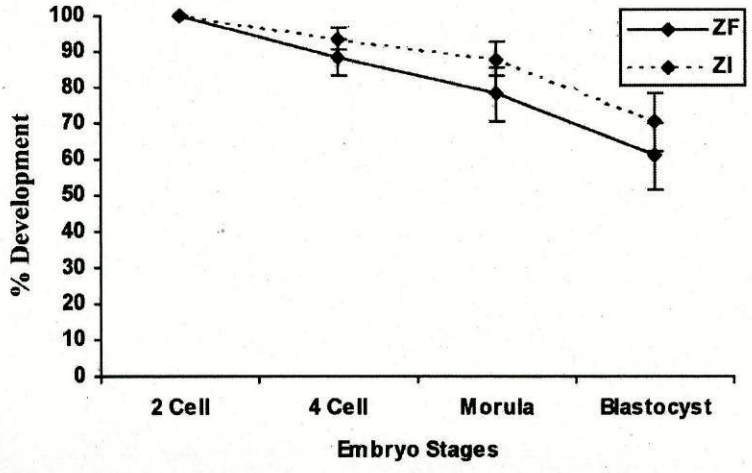


Figure 2.

Blastocyst morphology of zona-free and zona-intact embryos. Blastocysts were fertilized *in vivo* and then cultured without (a) and with (b) zona pellucida. Zona-free blastocysts look smaller, less expanded and more irregular than zona-intact ones. Scale bar= 90μm

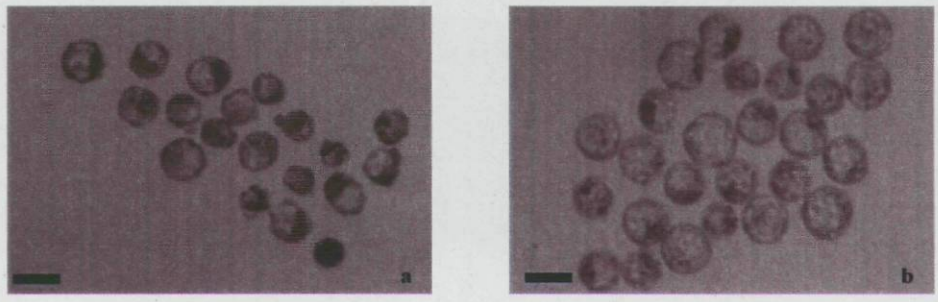


Figure 3.

Methylation analysis in preimplantation zona-free and zona-intact mouse embryos. A) 5-Methylcytosine Immunofluorescence of *in vivo* fertilized 2-cell (a, b), 4-cell (c, d), morula (e, f) and blastocyst (g, h) embryos, cultured with (ZI) and without (ZF) zona pellucida. Scale bar= 20μm. B) Quantification of total nuclear DNA methylation intensities in *in vivo* fertilized 2-cell, 4-cell, morula and blastocyst embryos (ICM- inner cell mass and TE- trophectoderm), cultured with (ZI) and without (ZF) zona pellucida. Each column represents the back-transformed mean value of these intensities per developmental stage except for blastocysts, where we distinguished between ICM and TE. Samples sizes (n) are indicated above the corresponding column. Bar graphs represent mean ± SEM. Significant differences are shown by * P< 0.05 and ** P< 0.01

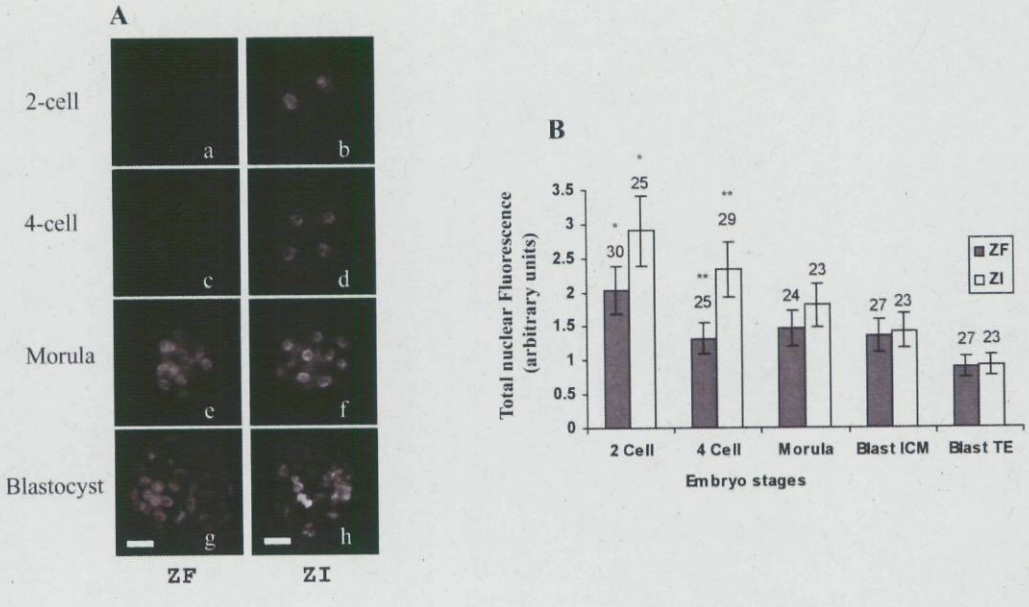


Figure 4.

5-Methylcytosine total nuclear fluorescence of *in vivo* fertilized 2-cell embryos, cultured with and without zona pellucida from the zygotes stages. Zona pellucida was removed either with pronase (Pr; n= 87) and mechanically (MR; n=38). ZI represents control zona-intact embryos (n=62). Bar graphs represent mean ± SEM. [a,b], $P<0.02$; [a,c], $P>0.2$; [b,c], $P<0.01$

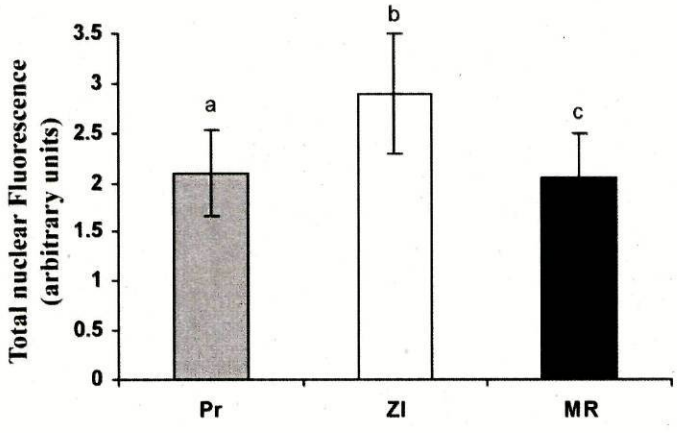


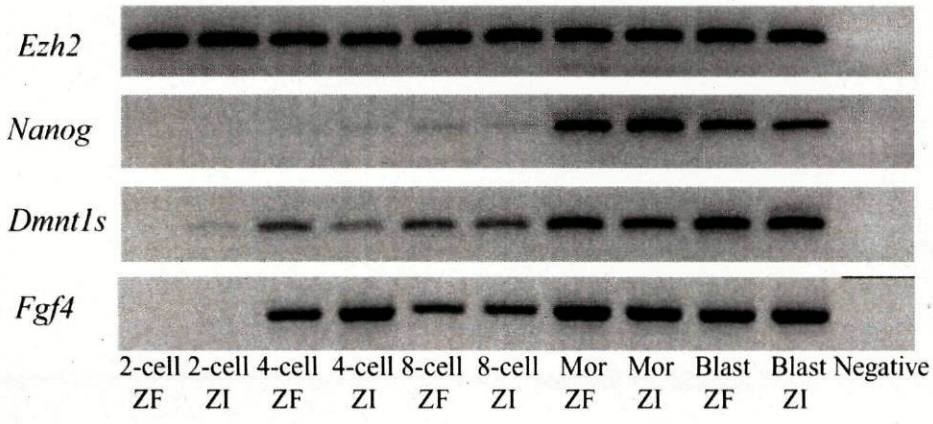
Table 1

5-Methylcytosine total nuclear fluorescence and size of *in vivo* fertilized pronuclear stage embryos approximately 7 hours after fertilization, cultured with (ZI) and without (ZF) zona pellucida.

		Total Nuclear Fluorescence (arbitrary units)		**Nuclear Area (arbitrary units)**	
	No. Embryos	**Male Pronucleus**	**Female Pronucleus**	**Male Pronucleus**	**Female Pronucleus**
ZF	28	2.82± 0.51	3.92± 0.71	3.29± 0.29	2.89± 0.25
ZI	50	2.56± 0.46	3.33± 0.59	3.90± 0.32	3.03± 0.25
		P>0.2	P>0.2	P>0.2	P>0.2

Figure 5.

Gene expression of *Nanog*, *Dnmt1s* and *Fgf4* during mouse preimplantation development of *in vivo* fertilized embryos, cultured with and without zona pellucida. Gene expression was assayed by RT-PCR of pool of 6 embryos from: 2-cell ZF, 2-cell ZI, 4-cell ZF, 4-cell ZI, 8-cell ZF, 8-cell ZI, Morula ZF, Morula ZI, Blastocysts ZF and Blastocyst ZI. *Ezh2* served as a ubiquitously expressed positive control.



Chapter V

General discussion and future expectations

Since the birth of Dolly, nuclear transfer (NT) has been applied with success in some other species and adapted for the production of transgenic offspring after genetic manipulation of the donor cells, combining techniques of cell culture with molecular biology (reviewed by Campbell et al., 2005). These genetic modifications include gene addition (Schnieke et al., 1997), knock out and gene knock-in (McCreath et al., 2000) and have been developed to produce transgenic animals with a wide range of applications, such us:

- Pharmaceutical industry: production of human proteins such as human factor IX in sheep milk for managing hemophilic patients (Schnieke et al., 1997); alpha-1-antitrypsin for treatment of emphysema (reviewed by Polejaeva and Campbell, 2000) or antithrombin III to supplement to individuals deficient in this anticoagulant (Baguisi et al., 1999);
- Modification of animal products: such as increasing the level of beta and kappa casein content in cattle milk (Brophy et al., 2003);
- Xenotransplantation: removal of potential antigens 1-3 galactosyltransferases in knock out pigs (Lai et al., 2002);
- Research of animal pathologies: such as deletion of prion protein (*PrP*) gene in sheep, which is the directly associated with spongiform encephalopathies in humans and animals (Denning et al., 2001).

Others believe in the potentialities of cloning for propagation of endangered species or family pets such as dogs and cats (reviewed by Sakai et al., 2005).

But it is the use of stem cells derived from cloned human embryos that has created more expectations because of their potential use for research and therapy. Dolly (Wilmut et al., 1997) and other cloned animals have shown that it is possible to reverse the development by creating embryos and fetus from adult cells. Recently,

human ES cells have been isolated from the blastocyst of human embryos produced by nuclear transfer from cumulus cells (Hwang et al., 2004). In 2005, the same group reported the production of 11 other human ES cell lines derived from blastocysts produced by NT using skin cells of patients with conditions such as spinal cord injury, juvenile diabetes, or congenital hypogammaglobulinemia (Hwang et al., 2005). This provides us with the possibility to produce stem cells for therapeutic use or stem cell lines and embryos from genetically manipulated or deficient cells for research purposes (reviewed by Campbell et al., 2005). Differentiation of stem cells derived from nuclear transfer blastocysts to certain tissues can open up opportunities to treat important diseases, not previously possible, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, motor neuron disease, muscular dystrophy, paralyses, spinal cord injuries, hepatitis, diabetes, hepatic cirrhosis, cancer, AIDS and others.

With these objectives in mind, methods of nuclear transfer need to be adjusted and improved in order to increase its efficiency. Technologies of NT are constantly changing to improve throughput, simplify methods and decrease costs by eliminating the requirement for expensive equipment. In this project we have contributed to some of these objectives. Particularly in the mouse, NT technologies have not changed since the first cloned mice reports in 1998 using the Honululu method (Wakayama et al., 1998). This method depends upon expensive equipment and requires a great deal of experience and manipulation skills. Additionally, very few laboratories world-wide have been able to reproducibly generate cloned viable offspring through direct transfer of adult somatic cells (Wakayama & Yanagimachi, 1999; Ogura et al., 2000; Yamazaki et al., 2001). Contrasting with the mouse, NT techniques have been simplified in other species, such as cattle (Booth et al., 2001; Vajta et al., 2001; Oback

et al., 2003) and pig (Booth et al., 2001a). The most important step in the simplification process occurred when the scientists started to remove the zona pellucida before the enucleation step. In 2001, Vajta et al. reported the first completely hand-made method in cattle in which the oocytes were bisected by hand, also known as "hand-made cloning" (Vajta et al., 2001). Our project reports the first use of a ZF technology of NT in the mouse. Hand-made cloning is not possible in mice due to the small size of the oocytes, so we adapted another ZF technology previously reported in cattle by a New Zealand group (Oback et al., 2003). This method is simpler, faster, easier to learn (as would take in mean approximately only 2-3 weeks to learn, instead of 6 months) and equally as efficient as zona-intact NT in cattle. Since the piezzo micromanipulation system is no longer necessary, it makes this technique cheaper than the previous technologies.

Some previous experiments were performed to optimise the technique in the mouse. Firstly, some work was performed in bovine oocytes using the skills learned in AgResearch, New Zealand in order to test the technique in our laboratory. A regular development to blastocyst in each experiment was obtained, (reaching around 16% of the cleavage embryos) giving us some confidence to start developing the technique in the mouse (Appendix II). Secondly, some basic experiments were performed in mouse using zona-free control parthenogenic activated oocytes/ embryos to optimize the NT conditions. These conditions included establishing the most suitable culture medium (CZB vs. M16 vs. KSOM), the type of mineral oil, and procedures for parthenogenic activation (ethanol, ionomycin or strontium) (Appendix II). Additionally, we found a statistical significant correlation between the number of

oocytes recovered per female (aged between 8-10 weeks) and their weight. Based on these results, no animals were used as oocyte donors if less than19-20g of weight.

During development of the NT zona-free technique, a healthy male pup was produced, however larger and 1.7-fold heavier than other pups of the same age. The placenta was also enlarged. Placentomegaly is a striking and consistent characteristic of cloned mice. Large offspring syndrome (LOS) is also a very frequent characteristic of cloned animals from several different species. The mouse was fertile and had 28 offspring from 3 females. Offspring showed normal weight at birth and during development, confirming that LOS condition in cloned animals is not hereditable, because is a result of epigenetic and not of genetic modifications (Appendix III). At eight months of age, the mouse was euthanased due to a signs of left eye conjunctivitis, left ear haemorrhage and abnormal gait. Necropsy analysis showed normal morphology of all major organs, with the exception of a small enlargement in the thymus. Histopathology revealed a classic mouse polyarteritis *nodosa* (Appendix IV). This is a relatively common age-dependent condition of several inbred strains including B6. It is thought to be an autoimmune, probably immune complex disease and as such, nothing to link it to cloning *per se*. The birth of a healthy cloned mouse from zona-free NT provides 'proof of principle' of a technology that promises to increase throughput and ease of operation.

The effects of zona removal in the mouse are still not well understood. In our hands, zona-free oocytes and embryos appear to be more fragile than zona-intact, particularly during parthenogenic activation in the absence of calcium. Later we showed that the lysis during activation was related to the process used to remove the zona pellucida. Better understanding of zona-free embryos and some technical

modifications resulted in an increase of approximately 1.5-fold in the fusion rate and an increase of over 1.5-fold in the percentage of cleaved embryos developing to morula/blastocyst stage when compared with the results achieved before the modifications. An increase in overall efficiency of 2.4-fold was achieved from the oocytes enucleated to development to morula/blastocyst stages.

Tsunoda & McLaren showed in 1983 that development to term was reduced simply by removing the zona with pronase (Tsunoda & McLaren, 1983). We also showed a similar effect, confirming those results. Our next objective was to try to understand if zona pellucida removal would cause any effect on gene expression of preimplantation embryos. Interestingly, the tests showed that removal of the zona pellucida immediately after recovery of fertilized eggs causes a reduction in DNA methylation at the 2 and 4-cell stages, however, normal levels are restored at the morula and blastocyst stages. Despite this transient reduction in DNA methylation, ZP removal had no effect upon development to the blastocyst stage or cell number in blastocysts. No effect was seen on male or female pronuclear area or pronuclear DNA methylation level; hence, visible changes arise between 7 and 24 hours post-fertilization. The same effect was seen when the zona was removed mechanically or by exposure to pronase, demonstrating that the changes in DNA methylation level are not the result of the activity of an enzyme, on the zygotes. When zona removal was delayed for 8 h after zygote recovery, there was no reduction in DNA methylation, suggesting that the critical time is early on between 1 and 8 h post-fertilization. Interestingly, no apparent effect was seen on acetylation of lysine 5 in histone 4 or onset of transcription for *Dnmt1s*, *Nanog* and *Fgf4* at any of the stages of development when compared with ZI embryos. In summary, zona pellucida removal seems to create changes in the

epigenetics at the level of DNA but this effect seemed to be compensated and no large effect is seen during development to blastocysts and gene expression. However, is still not known yet if this will interfere with development to term. We have proposed that a premature loss of proteins from the cortical granule envelope may account for the difference in DNA methylation seen in this study. This hypothesis could be tested by assessing the effect upon DNA methylation of using RNA interference to reduce the level of these candidate proteins in ZI embryos.

Previous work reported that the nuclei of embryos produced by NT have significantly higher levels of DNA methylation than *in vivo* or *in vitro* produced control embryos (cattle: Dean et al., 2001; sheep: Beaujean et al., 2004a). Some strategies have been studied pre-treating donor cells for nuclear transfer with drugs such as trichostatin A or 5-aza-2'deoxycytidine (Enright et al., 2003) in order to reduce levels of DNA methylation. Our studies have shown that zona pellucida removal creates a similar effect as the ones induced by the drugs. It would be interesting study to look at the differences in development of nuclear transfer embryos reconstructed with donor blastomeres derived by both zona-free and zona-intact *in vivo* fertilized embryos. This could be a good model to test the possible effect of pre-treatment of donor cells with drugs for uses in nuclear transfer.

These project objectives were achieved by creating an open door for the future of the cloning. Mouse is usually a good animal model for reproductive studies, because is cheap choice, with short pregnancy periods and easy to work with and. Lot of work still need to be done in order understand and increase cloning efficiency, however, the development of simplified techniques permit broader and cheaper

opportunities for NT work in laboratories world-wide. Although zona removal leads to a reduction in development to term, and levels of DNA methylation, no effect in gene expression of some important genes for early development was found. This does not mean that nuclear transfer reconstructed embryos would be also affected, because some of the early development mechanisms would be altered, such as absence of the sperm and oocyte pronuclei. For the other side, has been previously shown an abnormal higher DNA methylation levels in the nuclei of NT embryos. The reduction effect on DNA methylation after zona pellucida can in some way compensate this hypermethylation, allowing better development to term.

References

1. Aapola, U.; Kawasaki, K.; Scott, H.S.; Ollila, J.; Vihinen, M.; Heino, M.; Shintani, A.; Minoshima, S.; Krohn, K.; Antonarakis, S.E.; Shimizu, N.; Kudoh, J.; Peterson, P. (2000). "Isolation and initial characterization of a novel zinc finger gene, DNMT3L, on 21q22.3, related to the cytosine-5-methyltransferase 3 gene family." **Genomics**, 65:293-298
2. Amano T.; Kato, Y.; Tsunoda Y. (2001). "Full-term development of enucleated mouse oocytes fused with embryonic stem cells from different cell lines". **Reproduction**, 12: 729-733.
3. Antequera, F.; Bird, A. (1993). "Number of CpG islands and genes in human and mouse." **Proc Natl Acad Sci U S A** 90:11995-11999.
4. Austin, C.R. (1961). "Fertilization of mammalian eggs in vitro.". **Int Rev Cytol.,** 12: 337- 359.
5. Baguisi, A.; Behboodi, E.; Melican, D.T.; Pollock, J.S.; Destrempes, M.M.; Cammuso, C.; Williams, J.L.; Nims, S.D.; Porter, C.A.; Midura, P.; Palacios, M.J.; Ayres, S.L.; Denniston, R.S.; Hayes, M.L.; Ziomek, C.A.; Meade, H.M.; Godke, R.A.; Gavin, W.G.; Overstrom, E.W.; Echelard, Y. (1999). "Production of goats by somatic cell nuclear transfer." **Nat Biotechnol.**, 17(5):456-61.
6. Bain, J.M.; Hall, J.M. (1969). "Observations on the development and structure of the vitelline membrane of the hen's egg: an electron microscope study." **Aust J Biol Sci.**; 22(3): 653- 665.
7. Baylin, S.B.; Esteller, M.; Rountree, M.R.; Bachman, K.E.; Schuebel, K.; Herman, J.G. (2001). "Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer." **Hum Mol Genet.**, 10(7):687-92.

8. Beaujean, N. (2002). " Fundamental features of chromatin structure". **Cloning and Stem Cells**, 4: 355-361.

9. Beaujean, N.; Taylor, J.E.; McGarry, M.; Gardner, J.O.; Wilmut, I.; Loi, P.; Ptak, G.; Galli, C.; Lazzari, G.; Bird, A.; Young, L.E.; Meehan, R.R. (2004). "The effect of interspecific oocytes on demethylation of sperm DNA." **Proc Natl Acad Sci U S A.,** 101(20): 7636- 7640.

10. Beaujean, N.; Taylor, J.; Gardner, J.; Wilmut, I.; Meehan, R.; Young, L. (2004a). "Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer." **Biol Reprod.**, 71(1):185- 193.

11. Beaujean, N.; Hartshorne, G.; Cavilla, J.; Taylor, J.; Gardner, J.; Wilmut, I.; Meehan, R.; Young, L. (2004b). "Non-conservation of mammalian preimplantation methylation dynamics." **Curr Biol.**, 14(7):R266-7.

12. Belinsky, S.A.; Nikula, K.J.; Palmisano, W.A.; Michels, R.; Saccomanno, G.; Gabrielson, E.; Baylin, S.B.; Herman, J.G. (1998). "Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis." **Proc Natl Acad Sci U S A.,** 95(20):11891-6.

13. Behboodi, E.; Anderson, G. B.; BonDurant, R. H.; Cargill, S. L.; Kreuscher, B. R.; Medrano, J. F.; Murray, J. D. (1995). "Birth of large calves that developed from in vitro-derived embryos". **Theriogenology**, 44: 227-232.

14. Bestor, T.H. (1988). "Cloning of a mammalian DNA methyltransferase." **Gene,** 74:9-12

15. Bestor, T.H. (2000). "The DNA methyltransferases of mammals." **Hum Mol Genet**, 9:2395-2402.

16. Bhattacharya, S.K.; Ramchandani, S.; Cervoni, N.; Szyf, M. (1999). "A mammalian protein with specific demethylase activity for mCpG DNA." **Nature**, 397:579-583

17. Bird, A.P. (1986). "CpG-rich islands and the function of DNA methylation." **Nature**, 321:209-213.

18. Bird, A. (2001). "Methylation talk between histones and DNA". **Science**, 294: 2113-2115.

19. Bird, A. (2002). "DNA methylation patterns and epigenetic memory." **Genes Dev**, 16: 6- 21.

20. Bleil, J.D.; Wassarman, P.M. (1980). "Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida." **Dev Biol.,** 76(1): 185- 202.

21. Booth, P. J.; Tan, S.J.; Reipurth, P.; Holm, P.; Callesen, H. (2001). "Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique". **Cloning and Stem Cells**, 3 (3): 139-150.

22. Booth, P. J.; Tan, S.J.; Holm, P.; Callesen, H. (2001a). "Application of zona-free manipulation technique to porcine somatic nuclear transfer". **Cloning and Stem Cells**, 3 (4): 191-197.

23. Bordignon, V.; Smith, L. C. (1998). " Telophase enucleation: an improvement method to prepare recipient cytoplasts for use in bovine nuclear transfer". **Molecular Reproduction and Development**, 49: 29-36.

24. Bourc'his, D.; Xu, G.L.; Lin, C.S.; Bollman, B.; Bestor, T.H. (2001). "Dnmt3L and the Establishment of Maternal Genomic Imprints." **Science**, 294:2536-2539.

25. Breed, W.G.; Hope, R.M.; Wiebkin, O.W.; Spargo, S.C.; Chapman, J.A. (2002). "Structural organization and evolution of the marsupial zona pellucida." **Reproduction.,** 123(1): 13- 21.

26. Briggs, R.; King, T.J. (1952). "Transplantation of living nuclei from blastula cells into enucleated frogs' eggs." **Proc. Natl. Acad.Sci. USA** 38: 455-463

27. Brophy, B.; Smolenski, G.; Wheeler, T.; Wells, D.; L'Huillier, P.; Laible, G. (2003). "Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein." **Nat Biotechnol.**, 21(2):157-62.

28. Campbell, K. H. S.; McWhir, J.; Ritchie, W. A.; Wilmut, I. (1996). "Sheep cloned by nuclear transfer from cultured cell line". **Nature**, 380: 64-66.

29. Campbell, K.H.; Loi, P.; Otaegui, P.J.; Wilmut, I. (1996a). " Cell cycle co-ordination in embryo cloning by nuclear transfer." **Rev Reprod.,** 1(1):40-6.

30. Campbell, K.; Alberio, R.; Choi, I.; Fisher, P.; Kelly, R.; Lee, J.H.; Maalouf, W. (2005) "Cloning: eight years after Dolly." **Reprod Domest Anim.,** 40(4): 256-68.

31. Challah-Jacques, M.; Chesne, P.; Renard, J. P. (2003). "Production of cloned rabbits by somatic nuclear transfer." **Cloning Stem Cells**; 5(4): 295-9.

32. Chamberlin, M.E.; Dean, J. (1989). "Genomic organization of a sex specific gene: the primary sperm receptor of the mouse zona pellucida." **Dev Biol.,** 131(1): 207- 214.

33. Chavatte-Palmer, P.; Heyman, Y.; Richard, C.; Monget, P.; LeBourhis, D.; Kann, G.; Chilliard, Y.; Vignon, X.; Renard, J. P. (2002). "Clinical, hormonal, and hematologic characteristics of bovine calves derived from Nuclei from Somatic Cells". **Biology of Reproduction**, 66: 1596-1603.

34. Cherr, G.N.; Ducibella, T. (1990). "Activation of the mammalian egg: cortical granule distribution, exocytosis, and the block to polyspermy." In: Bavister B.D., Cummins, E.R.S.; Roldan, E.R., editors. **Fertilization in mammals**. Norwell. M.A.: Serono Symposium: 309-334.

35. Cheung, P., Tanner, K. G.; Cheung, W. L. et al. (2000). "Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation". **Mol. Cell**, 5: 905- 915.

36. Cibelli, J. B.; Stice, S. L.; Golueke, P. J.; Kane, J. J.; Jerry, J.; Blackwell, C.; Ponce de Leon, A.; Robl, J. M. (1998). "Cloned transgenic calves produced from non-quiescent foetal fibroblasts". **Science**, 280: 1256-1258.

37. Clark, S.J.; Harrison, J.; Molloy, P.L. (1997). "Sp1 binding is inhibited by (m)Cp(m)CpG methylation." **Gene**, 195(1):67-71.

38. Cohen, J. (1991). "Assisted hatching of human embryos." **J In Vitro Fert Embryo Transf.,** 8(4): 179- 190.

39. Cole, R.J. (1967). "Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst." **J Embryol Exp Morphol.,**, 17(3): 481- 490.

40. Colman, A. (1999/2000). "Somatic cell nuclear transfer in mammals: progress and applications." **Cloning**, 1(4): 185-200.

41. Colot, V.; Rossignol, J-L. (1999). "Eukaryotic DNA methylation as an evolutionary device." **Bioessays**, 21:402-411.

42. Cotelli, F.; Andronico, F.; Brivio, M.F.; Lora, Lamia, C. (1988). "Structure and composition of the fish egg chorion (*Carassius auratus*)." **J Ultrastruct Mol Struct Res**, 99: 70-78.

43. Cowell, I.G.; Aucott, R.; Mahadevaiah, S. K.; Burgouyne, P. S.; Huskisson, N.; Borgiorni, G.; Prantera, G.; Fanti, L.; Pimpinelli, S.; Wu, R.; Gilbert, D. M.; Shi, W.; Fundele, R.; Morrison, H; Jeppesen, P; Singh, P.S. (2002). "Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals". **Chromosoma**, 111 (1): 22-36.

44. Dandekar, P.; Talbot, P. (1992). "Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization." **Mol Reprod Dev**., 31(2):135-143.

45. Dean, W.; Ferguson-Smith, A. (2001). "Genomic imprinting: mother maintains methylation marks." **Curr Biol.,** 11(13):R527-30.

46. Dean, W.; Santos, F.; Stojkovic, M.; Zakhartchenko, V.; Walter, J.; Wolf, E.; Reik, W. (2001). "Conservation of methylation reprogramming in mammalian development; aberrant reprogramming in cloned embryos." **Proc Natl Acad Sci U S A.** , 98(24): 13734-8.

47. Denning, C.; Burl, S.; Ainslie, A.; Bracken, J.; Dinnyes, A.; Fletcher, J.; King, T.; Ritchie, M.; Ritchie, W.A.; Rollo, M.; de Sousa, P.; Travers, A.; Wilmut, I.; Clark, A.J. (2001). "Deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep." **Nat Biotechnol**, 19(6):559-62.

48. Dennis, K.; Fan, T.; Geiman, T.; Yan, Q.; Muegge, K. (2001). "Lsh, a member of the SNF2 family, is required for genome-wide methylation." **Genes Dev.,** 15(22):2940-4.

49. De Sousa, P. A.; Walker, S.; King, T. J.; Young, L. E.; Harkness, L.; Ritchie, W. A.; Travers, A.; Ferrier, P.; Wilmut, I. (2001). "Evaluation of gestational deficiencies in cloned sheep fetuses and placenta". **Biol Reprod**, 65: 23-30.

50. Dover, J.; Schneider, J.; Tawiah-Boateng, M.A.; Wood, A.; Dean, K.; Johnston, M.; Shilatifard, A. (2002). "Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6." **J Biol Chem.,** 277(32):28368-71.

51. Eberharter, A.; Becker, P. B. (2002). "Histone acetylation: a switch between repressive and permissive chromatin". **EMBO Rep.**, 3: 224-229.

52. Eggan, K.; Akutsu, H.; Loring, J.; Jackson-Grusby, L.; Klemm, M.; Rideout, W. M.; Yanagimachi, R.; Jaenisch, R. (2001). "Hybrid vigor, foetal overgrowth and viability of mice derived by nuclear cloning and tetraploid embryo complementation". **Proc. Natl. Acad. Sci. USA**, 98: 6209-6214.

53. El Kharroubi, A.; Piras, G.; Stewart, C.L. (2001). "DNA demethylation reactivates a subset of imprinted genes in uniparental mouse embryonic fibroblasts." **J Biol Chem.**, 276(12): 8674-80.

54. Enright, B.P.; Kubota, C.; Yang, X.; Tian, X.C. (2003). "Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine." **Biol Reprod.**, 69(3): 896-901.

55. Epifano, O.; Liang, L.F.; Dean, J. (1995). "Mouse Zp1 encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish." **J Biol Chem.,** 270(45): 27254- 27258.

56. Fagard, M.; Vaucheret, H. (2000). "(Trans)gene silencing IN plants: How Many Mechanisms?" **Annu. Rev. Plant Physiol. Plant Mol. Biol**,. 51: 167-194.

57. Fairburn, H.R. (2002). "Dynamics of DNA methylation in normal and cloned sheep development". Edinburgh, UK, **University of Edinburgh**. Thesis.

58. Farin, P. W.; Farin, C. E. (1995). "Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development". **Biol Reprod**, 52: 676-682.

59. Fulka, J. Jr.; Loi, P.; Ledda, S.; Moor, R.M.; Fulka, J. (2001). "Nucleus transfer in mammals: how the oocyte cytoplasm modifies the transferred nucleus." **Theriogenology,** 55(6): 1373-80.

60. Galli, C.; Duchi, R.; Moor, R. M.; Lazzari, G. (1999). "Mammalian leukocytes conatin all the genetic information necessary for the development of a new individual". **Cloning**, 1: 161-170.

61. Galli, C.; Lagutina, I.; Crotti, G.; Colleoni, S.; Turini, P.; Ponderato, N.; Duchi, R.; Lazzari, G. (2003). "A cloned horse born to its dam twin". **Nature**, 424: 635.

62. Gao, S.; McGarry, M.; Ferrier, T.; Pallante, B.; Gasparrini, B.; Fletcher, J.; Harkness, L.; De Sousa, P.; McWhir, J.; Wilmut, I. (2003). "Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line". **Biology of Reproduction**, 68: 595-603.

63. Gibbons, R.J.; McDowell, T.L.; Raman, S.; O'Rourke, D.M.; Garrick, D.; Ayyub, H.; Higgs, D.R. (2000). "Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation." **Nat Genet,** 24:368-371

64. Giles, R.E.; Ruddle, F.H. (1973). "Production of Sendai virus for cell fusion." **In Vitro,** 9(2): 103-7.

65. Gurdon, J. B. (1962). " The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles." **J Embryol Exp Morphol.,** 10:622-40.

66. Gutierrez, R.M.; Hnilica, L.S. (1967). "Tissue specificity of histone phosphorylation." **Science.**, 157(794):1324-5.

67. Han, Y.M.; Kang, Y. K.; Koo, D. B.; Lee, K. K. (2003). "Nuclear reprogramming of cloned embryos produced in vitro". **Theriogenology,** 59: 33-44.

68. Hata, K.; Okano, M.; Lei, H.; Li, E. (2002). "Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice." **Development**, 129(8):1983-93.

69. Hebbes, T.R.; Thorne, A.W.; Crane-Robinson, C. (1988). "A direct link between core histone acetylation and transcriptionally active chromatin." **Embo J**, 7: 1395-1402.

70. Hendrich, B.; Bird, A. (1998). "Identification and characterization of a family of mammalian methyl-CpG binding proteins." **Mol Cell Biol.** 18(11):6538-47.

71. Hermann, A.; Schmitt, S.; Jeltsch, A. (2003). "The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. **J Biol Chem.,** 22;278(34):31717-21.

72. Heyman, Y.; Chavatte-Palmer, P.; Lebourhis, D.; Deniau, F.; Laigre, P.; Vignon, X.; Renard, J. P. (1999). "Evolution of pregnancies after transfer of cloned bovine blastocysts derived from fetal or adult somatic cells". **Proceedings of the 15th AETE meeting**, Lyon, France. Abstract 166.

73. Heyman, Y.; Chavatte-Palmer, P.; LeBourhis, D.; Camous, S.; Vignon, X.; Renard, J. P. (2002). "Frequency and occurrence of late-gestation losses from cattle cloned embryos". **Biol Reprod**, 66: 6-13.

74. Hill, J. R.; Roussel, A.; Cibelli, J. B.; Edwards, J. F.; Hooper, N. L.; Miller, M. W.; Thompson, J. A.; Looney, C. R.; Westhusin, M. E.; Robl, J. M.; Stice, S.

L. (1999). "Clinical and pathological features of cloned transgenic calves and fetuses (13 case studies)". **Theriogenology**, 51: 1451-1465.

75. Hill, J.R.; Long, C. R.; Looney, C. R.; Winger, Q. A.; Spencer, T. E.; Bazer, F. W.; Burghardt, R. C.; Westhusin, M. E. (2000). "Placental abnormalities in first trimester somatic cell cloned fetuses". **Theriogenology**, 53: 218.
76. Hochedlinger, K.; Jaenish, R. (2002). "Monoclonal mice generated by nuclear transfer from mature B and T- cells". **Nature**, 718: 1-4.
77. Hoodbhoy, T.; Talbot, P. (1994). "Mammalian cortical granules: contents, fate, and function." **Mol Reprod Dev.**, 39(4):439-448.
78. Hoodbhoy, T.; Carroll, E.J. Jr.; Talbot, P. (2000). " Relationship between p62 and p56, two proteins of the mammalian cortical granule envelope, and hyalin, the major component of the echinoderm hyaline layer, in hamsters." **Biol Reprod.**, 62(4):979-87.
79. Hoodbhoy, T.; Dandekar, P.; Calarco, P.; Talbot, P. (2001). "p62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development." **Mol Reprod Dev.**, 59(1):78-89.
80. Howell, C.Y.; Bestor, T.H.; Ding, F.; Latham, K.E.; Mertineit, C.; Trasler, J.M.; Chaillet, J.R. (2001). "Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene." **Cell.,** 104(6):829-38.
81. Hubacek, J. (1992). "Biological function of DNA methylation." **Folia Microbiol (Praha)**, 37:323-329.
82. Hwang, W.S.; Ryu, Y.J.; Park, J.H.; Park, E.S.; Lee, E.G.; Koo, J.M.; Jeon, H.Y.; Lee, B.C.; Kang, S.K.; Kim, S.J.; Ahn, C.; Hwang, J.H.; Park, K.Y.;

Cibelli, J.B.; Moon, S.Y. (2004). "Evidence of a pluripotent human embryonic stem cell line derived from a cloned\blastocyst." **Science**, 303(5664):1669-74.

83. Hwang, W.S.; Roh, S.I.; Lee, B.C.; Kang, S.K.; Kwon, D.K.; Kim, S.; Kim, S.J.; Park, S.W.; Kwon, H.S.; Lee, C.K.; Lee, J.B.; Kim, J.M.; Ahn, C.; Paek, S.H.; Chang, S.S.; Koo, J.J.; Yoon, H.S.; Hwang, J.H.; Hwang, Y.Y.; Park, Y.S.; Oh, S.K.; Kim, H.S.; Park, J.H.; Moon, S.Y.; Schatten, G. (2005). "Patient-specific embryonic stem cells derived from human SCNT blastocysts." **Science**, 308(5729):1777-83.
84. Ichimura, T.; Watanabe, S.; Sakamoto, Y.; Aoto, T.; Fujita, N.; Nakao, M. (2005). "Transcriptional repression and heterochromatin formation by MBD1 and MCAF/AM family proteins." **J Biol Chem.,** 280(14):13928-35.
85. Issa, J.P. (2004). "CpG island methylator phenotype in cancer." **Nat Rev Cancer**, 4(12): 988- 993.
86. Jackson-Grusby, L.; Beard, C.; Possemato, R.; Tudor, M.; Fambrough, D.; Csankovszki, G.; Dausman, J.; Lee, P.; Wilson, C.; Lander, E.; Jaenisch, R. (2001). "Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation." **Nat Genet** 27:31-39.
87. Jenuwein, T.; Allis, D. (2001). "Translating the histone code". **Science**, 293: 1074- 1080.
88. Jaenisch, R.; Bird, A. (2003). "Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals." **Nat Genet.,** 33 Suppl: 245- 254.
89. Kaneda, M.; Okano, M.; Hata, K.; Sado, T.; Tsujomoto, N.; Li, E.; Sasaki, H. (2004). "Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting." **Nature**, 429(6994):900-3.

90. Kang, Y.K.; Koo, D.B.; Park, J.S.; Choi, Y.H.; Chung, A.S.; Lee, K.K.; Han, Y.M. (2001). "Aberrant methylation of donor genome in cloned bovine embryos." **Nat Genet.,** 28(2):173-7.

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

92. Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS, Zhou FJ, Kafidi N, Wang B, Baldassarre H, Karatzas CN. (2002). "Production of cloned goats after nuclear transfer using adult somatic cells". **Biol Reprod.**, 66(1): 199-203.

93. Kikio, N.; Wolffe, A. P. (2000). "Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons". **J. Cell Sci**, 113: 11-20.

94. Kim, J-M.; Ogusa, A.; Nagata, M; Aoki, F. (2002). "Analysis of the mechanism for chromatin remodeling in embryos reconstructed by somatic nuclear transfer". **Biology of Reproduction**, 67: 760-766.

95. Kinloch, R.A.; Roller, R.J.; Fimiani, C.M.; Wassarman, D.A.; Wassarman, P.M. (1988). "Primary structure of the mouse sperm receptor polypeptide determined by genomic cloning." **Proc Natl Acad Sci U S A.,** 85(17): 6409-6413.

96. Ko, Y.G.; Nishino, K.; Hattori, N.; Arai, Y.; Tanaka, S.; Shiota, K. (2005). "Stage-by-stage change in DNA methylation status of Dnmt1 locus during mouse early development." **J Biol Chem.**, 280(10):9627-9634.

97. Kono, T (1997). "Nuclear transfer and reprogramming". **Rev. Reprod.**, 2: 74-80.

98. Krisher, R.L. (2004). "The effect of oocyte quality on development." **J Anim Sci.**, 82 E-Suppl: E14-23.

99. Kruip, T. A. M.; den Daas, J. H. G. (1997). "In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring". **Theriogenology**, 278: 2130-2133.

100. Kubota, C. Yamakuchi, H.; Todoroki, J.; Mizoshita, K.; Tabara, N.; Barber, M.; Yang, X. (2000). "Six cloned calves produced from adult fibroblasts cells after long-term culture" **Proc. Nat. Acad. Sci. USA**, 97: 990-995.

101. Kwon, O. Y.; Kono, T. (1996). "Production of identical sextuplets mice by transferring metaphase nuclei from four-cell embryos". **Proc. Natl. Acad. Sci. USA**, 93: 13010-13013.

102. Lai, L.; Kolber-Simonds, D.; Park, K.W.; Cheong, H.T.; Greenstein, J.L.; Im, G.S.; Samuel, M.; Bonk, A.; Rieke, A.; Day, B.N.; Murphy, C.N.; Carter, D.B.; Hawley, R.J.; Prather, R.S. (2002). " Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning." **Science**, 295(5557):1089-92.

103. Langan, T.A. (1968). "Histone phosphorylation: stimulation by adenosine 3',5'-monophosphate."**Science.**, 162(853):579-80.

104. Lanza, R.P.; Cibelli, J.B.; Faber, D.; Sweeney, R.W.; Henderson, B.; Nevala, W.; West, M.D.; Wettstein, P.J. (2001). "Cloned cattle can be healthy and normal". **Science**, 294: 1893-1894.

105. Lee, B.C.; Kim, M.K.; Jang, G.; Oh, H.J.; Yuda, F.; Kim, H.J.; Shamim, M.H.; Kim, J.J.; Kang, S.K.; Schatten, G.; Hwang, W.S. (2005). "Dogs cloned from adult somatic cells". **Nature**, 436: 641.

106. Lee, J-W.; Wu, S-C.; Tian, X. C.; Barber, M.; Hoagland, T.; Riesen, J.; Lee, K-H; Tu, C-F.; Cheng, W. T. K.; Yang, X. (2003). "Production of cloned pigs by whole cell intracytoplasmic microinjection". **Biology of Reproduction**, 69: 995- 1001.

107. Lewis, I. M.; Peura, T. T.; Owens, J. L.; Ryan, M. F.; Diamente, M. G.; Pushett, D. A.; Lane, M. W.; Jenkin, G.; Coleman, P. J.; Trounson, A. O. (2000). "Outcomes from novel simplified nuclear transfer techniques in cattle". **Theriogenology**, 53: 233.

108. Lewis, J.D.; Meehan, R.R.; Henzel, W.J.; Maurer-Fogy, I.; Jeppesen, P.; Klein, F.; Bird, A. (1992). "Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA." **Cell**, 69(6):905-14.

109. Li, E.; Bestor, T.H.; Jaenisch, R. (1992). "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." **Cell**, 69(6): 915-926.

110. Li, E.; Beard, C.; Jaenisch, R. (1993). "Role for DNA methylation in genomic imprinting." **Nature**, 366:362-365.

111. Li, X.; Morris, L.H.; Allen, W.R. (2002). " In vitro development of horse oocytes reconstructed with the nuclei of fetal and adult cells." **Biol Reprod.**, 66(5): 1288-1292.

112. Liang, L.F.; Chamow, S.M.; Dean, J. (1990). "Oocyte-specific expression of mouse Zp-2: developmental regulation of the zona pellucida genes." **Mol Cell Biol.**, 10(4): 1507- 1515.

113. Liu, K.; Wang, Y.F.; Cantemir, C.; Muller, M.T. (2003a). "Endogenous assays of DNA methyltransferases: Evidence for differential

activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo." **Mol Cell Biol.**, 23(8):2709-19.

114. Liu, M.; Sims, D.; Calarco, P.; Talbot, P. (2003). "Biochemical heterogeneity, migration, and pre-fertilization release of mouse oocyte cortical granules." **Reprod Biol Endocrinol.**, 1(1):77.

115. Loeb, J. (1984). "Über eine einfache Methode, zwein oder mehr zusammengesachsene Embryonen aus einem Ei hervorzubringen." **Pflugers Arch.** 55, 525-530.

116. Lunsford, R.D.; Jenkins, N.A.; Kozak, C.A.; Liang, L.F.; Silan, C.M.; Copeland, N.G.; Dean, J. (1990). "Genomic mapping of murine Zp-2 and Zp-3, two oocyte-specific loci encoding zona pellucida proteins." **Genomics**, 6(1): 184- 187.

117. Lyko, F.; Paro, R. (1999). "Chromosomal elements conferring epigenetic inheritance." **Bioessays**, 21:824-832

118. Matzke, M.; Matzke, A.J.; Kooter, J.M. (2001). "RNA: guiding gene silencing." **Science**, 293:1080-1083

119. McCreath, K. J.; Howcroft, J.; Campbell, K. H. S.; Colman, A.; Schnieke, A. E.; Kind, A. J. (2000). "Production of gene-targeted sheep by nuclear transfer from cultured somatic cells". **Nature**, 405: 1068-1069.

120. McGrath, J.; Solter, D. (1983). "Nuclear transplantation in the mouse embryo by microsurgery and cell fusion." **Science**, 220(4603):1300-2.

121. McGrath, J.; Solter, D. (1984). "Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro." **Science.**, 226(4680): 1317-1319.

122. Meehan, R.R.; Lewis, J.D.; McKay, S.; Kleiner, E.L.; Bird, A.P. (1989). "Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs." **Cell.**, 58(3):499-507.

123. Modlinski, J.A. (1970). "The role of the zona pellucida in the development of mouse eggs in vivo." **J Embryol Exp Morphol.**, 23(3): 539-547.

124. Montag, M.; Koll, B.; Holmes, P.; van der Ven, H. (2000). "Significance of the number of embryonic cells and the state of the zona pellucida for hatching of mouse blastocysts in vitro versus in vivo." **Biol Reprod.,** 62(6): 1738- 1744.

125. Naito, K.; Daen, F.P.; Toyoda, Y (1992). "Comparison of histone H1 kinase activity during meiotic maturation between two types of porcine oocytes matured in different media in vitro." **Biol Reprod.,** 47(1): 43-7.

126. Nan, X.; Tate, P.; Li, E.; Bird, A. (1996). "DNA methylation specifies chromosomal localization of MeCP2." **Mol Cell Biol.**, 16(1):414-21.

127. Nan, X.; Ng, H.H.; Johnson, C.A.; Laherty, C.D.; Turner, B.M.; Eisenman, R.N.; Bird, A. (1998). "Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex." **Nature**, 393(6683):386-9.

128. Ng, H.; Bird, A. (1999). "DNA methylation and chromatin modification". **Current Opinion in Genetics & Development**, 9: 158- 163.

129. Ng, H.H.; Jeppesen, P.; Bird, A. (2000). "Active repression of methylated genes by the chromosomal protein MBD1." **Mol Cell Biol**, 20:1394-1406.

130. Nilsoon, O.; Mitchum, R.D. Jr.; Schrier, L.; Ferns, S.P.; Barnes, K.M.; Troedle, J.F.; Baron, J. (2005). "Growth plate senescence is associated with loss of DNA methylation."**J Endocrinol.**, 186(1): 241-9.

131. Nowak, S. J.; Corces, V. G. (2000). " Phosphorylation of histone H3 correlates with transcriptionally active loci". **Genes Dev.**, 14: 3003- 3013.

132. Oback B.; Wells, D (2002). "Donor cells for nuclear cloning: many are called, but few are chosen". **Cloning and Stem Cells**, 4 (2): 147-168.

133. Oback, B.; Wiersema, A.T.; Gaynor, P.; Laible, G., Tucker, F.C.; Oliver, J.E.; Miller, A. L.; Troskie, H. E.; Wilson, K. L.; Forsyth, J. T.; Berg, M. C.; Cockrem, K.; McMillan, V.; Tervit, H. R.; Wells, D. N. (2003). "Cloned cattle derived from a novel zona-free embryo reconstruction system". **Cloning and Stem Cells,** 5 (1): 3-12.

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

135. Ogura, A.; Inoue, K.; Ogonuki, N.; Lee, J.; Kohda, T.; Ishino, F. (2002). "Phenotic effects of somatic cell cloning in mice". **Cloning Stem Cells**, 4: 397-405.

136. Okano, M.; Xie, S.; Li, E. (1998). "Cloning and characterization of a novel mammalian DNA (cytosine-5) methyltransferases." **Nature Genetics**, 19, 219-220.

137. Okano, M.; Xie, S.; Li, E. (1998b). "Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells." **Nucleic Acids Res,** 26:2536-2540

138. Okano, M.; Bell, D.W.; Haber, D.A.; Li, E. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." **Cell**, 99(3): 247- 257.

139. Onishi, A.; Iwamoto, M.; Akita, T.; Mikawa, S.; Takeda, K.; Awata, T.; Hanada, H.; Perry, A. C. (2000). " Pig cloning by microinjection of fetal fibroblasts nuclei". **Science**, 289: 1188-1190.

140. Ono, Y.; Shimozawa, N.; Ito, M.; Kono, T. (2001). "Cloned mice from fetal fibroblasts cells arrested at metaphase by serial nuclear transfer". **Biol. Reprod.**, 64: 44-50.

141. Ono, Y.; Shimozawa, N.; Muguruma, K.; Kimoto, S.; Hioki, K.; Tachibana, M.; Shinkai, Y.; Ito, M.; Kono, T. (2001a). "Production of cloned mice from embryonic stem cells arrested at metaphase". **Reproduction**, 122: 731-736.

142. Oudejans, C.B.; Westerman, B.; Wouters, D.; Gooyer, S.; Leegwater, P.A.; van Wijk, I.J.; Sleutels, F. (2001). "Allelic IGF2R repression does not correlate with expression of antisense RNA in human extraembryonic tissues." **Genomics,** 73(3):331-7.

143. Otaegui, P. J.; O'Neill, G. T.; Campbell, K. H.; Wilmut, I. (1994) "Transfer of nuclei from 8-cell stage mouse embryos following use of nocodazole to control the cell cycle". **Mol Reprod Dev.**, 39(2): 147-52.

144. Ozgur, K.; Patankar, M.S.; Oehninger, S.; Clark, G.F. (1998). "Direct evidence for the involvement of carbohydrate sequences in human sperm-zona pellucida binding." **Mol Hum Reprod.,** 4(4): 318- 324.

145. Panning, B.; Jaenisch, R. (1996). "DNA hypomethylation can activate Xist expression and silence X-linked genes." **Genes Dev**, 10:1991-2002

146. Peterson, A. J.; McMillan, W. H.; Thompson, J. G. (2000). "Various allantoic pathologies are associated with malformation of allantoic development of the IVP bovine embryo". **Proc. 14th Int. Cong. An. Rep.**, Stockholm, Sweden. Abstract 159.

147. Peura, T. T.; Trounson, A. O. (1998). "Recycling bovine embryos for nuclear transfer". **Reprod. Fertil Dev.**, 10 (7-8): 627-632.

148. Peura, T. T.; Lewis, I. M.; Trounson, A. O. (1998a). "The effect of recipient oocyte volume on nuclear transfer in cattle". **Molecular Reproduction and Development**, 50: 185- 191.

149. Peura, T. T.; Lane, M. W.; Lewis, I. M; Trounson, A. O. (2001). "Development of bovine embryo-derived clones after increasing rounds of nuclear recycling". **Molecular Reproduction and Development**, 58: 384-389.

150. Polak-Charcon, S; Calarco-Gillam, P; Johnson L.V. (1985). "Intracellular localization and surface expression of a stage-specific embryonic glycoprotein. " **Gamete Res**, 12: 329-343

151. Polejaeva, I.A.; Campbell, K.H. (2000). "New advances in somatic cell nuclear transfer: application in transgenesis." **Theriogenology**, 53(1):117-26.

152. Polejaeva, I. A.; Chen, S-H.; Vaught, T. D.; Page, R. L.; Mullins, J.; Ball, S.; Dai, Y.; Boone, J.; Walker, S.; Ayares, D. L.; Colman, A.; Campbell' K. H. S. (2000a). "Cloned pigs produced by nuclear transfer from adult somatic cells". **Nature**, 407: 86-90.

153. Prather, R.S.; Barnes, F.L.; Sims, M.M.; Robl, J.M.; Eyestone, W.H.; First, N.L. (1987). "Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte." **Biol Reprod.**, 37(4): 859- 866.

154. Prigent, C,; Dimitrov, S. (2003). "Phosphorylation of serine 10 in histone H3, what for?" **J Cell Sci.,** 116(Pt 18):3677-85.

155. Ramsahoye, B.H.; Biniszkiewicz, D.; Lyko, F.; Clark, V.; Bird, A.P.; Jaenisch, R. (2000). "Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a." **Proc Natl Acad Sci U S A** 97:5237-5242.

156. Rankin, T.; Familari, M.; Lee, E.; Ginsberg, A.; Dwyer, N.; Blanchette-Mackie, J.; Drago, J.; Westphal, H.; Dean, J. (1996). "Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile." **Development,** 122(9): 2903- 2910.

157. Rankin, T.; Talbot, P.; Lee, E.; Dean, J. (1999). "Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss." **Development.,** 126(17):3847-55.

158. Rankin, T.; Dean, J. (2000). "The zona pellucida: using molecular genetics to study the mammalian egg coat." **Rev Reprod.,** 5(2): 114-121.

159. Rankin, T.L.; O'Brien, M.; Lee, E.; Wigglesworth, K.; Eppig, J.; Dean, J. (2001). "Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development." **Development.**: 1119- 1126.

160. Ratnam, S.; Mertineit, C.; Ding, F.; Howell, C.Y.; Clarke, H.J.; Bestor, T.H.; Chaillet, J.R.; Trasler, J.M. (2002). "Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development." **Dev Biol.** 245(2): 304-14.

161. Razin, A.; Riggs, A.D. (1980). "DNA methylation and gene function." **Science,** 210(4470): 604- 610.

162. Reik, W.; Dean, W.; Walter, J. (2001). "Epigenetic reprogramming in mammalian development." **Science**, 293(5532):1089-93.

163. Renard, J. P.; Chastant, S.; Chesne, P.; Richard, C.; Marchal, J.; Cordonnier, N.; Chavatte, P.; Vignon, X. (1999). " Lymphoid hypoplasia and somatic cloning". **The Lancet**, 353: 1489-1491.

164. Rideout, W. M.; Wakayama, T.; Wutz, A.; Eggan, K.; Jackson-Gruskby, L.; Dausman, J. Yanagimachi, R.; Jaenish, R. (2000). "Generation of mice from wild type and targeted ES cells by nuclear cloning". **Natute Genetics**, 24: 109-110.

165. Robertson, K.D.; Jones, P.A. (2000). "DNA methylation: past, present and future directions." **Carcinogenesis**, 21(3): 461- 467.

166. Robl, J.M.; Prather, R.; Barnes, F.; Eyestone, W.; Northey, D.; Gilligan, B.; First, N.L. (1987). "Nuclear transplantation in bovine embryos." **J Anim Sci.**, 64(2):642-647.

167. Saikhun, J.; Kitiyanant, N.; Songtaveesin, C.; Pavasuthipaisit, K.; Kitiyanant, Y. (2004). "Development of swamp buffalo (Bubalus bubalis) embryos after parthenogenetic activation and nuclear transfer using serum fed or starved fetal fibroblasts". **Reprod Nutr Dev.**, 44(1): 65-78.

168. Sakai, R.R.; Tamashino, K.L.K.; Yamazaki, Y.; Yanagimachi, R. (2005). "Cloning and assisted reproductive techniques: influence on early development and adult phenotype". **Birth Defects Research**, 75: 151-162.

169. Santos, F.; Hendrich, B.; Reik, W.; Dean, W. (2002). "Dynamic reprogramming of DNA methylation in the early mouse embryo." **Dev Biol.**, 241(1): 172- 182.

170. Savage, A.F.; Maull, J.; Tian, X.C.; Taneja, M.;Katz, L.; Darre, M.; Yang, X. (2003). "Behavioral observations of adolescent Holstein heifers cloned from adult somatic cells". **Theriogenology**, 60: 1097-1110.

171. Schnieke, A. E.; Kind, A. J.; Ritchie, W. A.; Mycock, K, Scott, A. R.; Rithchie, M; Wilmut, I.; Colman, A.; Campbell, K. H. S (1997). "Human factor IX transgenic sheep produced by transfer of nuclei from transfected foetal fibroblasts". **Science**, 278: 2130-2133.

172. Shekhar, M. P.; Lyakhovich, A.; Visscher, D. W.; et al. (2002). "Rad6 overexpression induces multinucleation, centrosome amplification, abnormal mitosis, aneuploidy and transformation". **Cancer Res.**, 62: 2115- 2124.

173. Shiga, K.; Fujita, T; Hirose, K; Sasae, Y.; Nagai, T. (1999). "Production of calves by transfer of nuclei from cultured somatic cells from Japanese black bulls". **Theriogenology**, 52: 527-535.

174. Shin, T.; Kraemer, D.; Pryor, J.; Liu, L.; Rugila, J.; Howe, L.; Buck, S.; Murphy, K.; Lyons, L.; Westhusin, M. (2002). "A cat cloned by nuclear transplantation". **Nature**, 415(6874): 859.

175. Spemann, H. (1914). "Über verzögerte Kernversorung von Keimteilen. **Verh. Dtsch. Zool. Ges. Leipzig (Freiburg)**, 24, 216-221.

176. Spemann, H. (1938). "Embryonic Development és Induction." **Yale University Press: New Haven, CT, USA.**

177. Stancheva, I.; El-Maarri, O.; Walter, J.; Niveleau, A.; Meehan, R.R. (2002). "DNA methylation at promoter regions regulates the timing of gene activation in Xenopus laevis embryos." **Dev Biol**, 243:155-165.

178. Suetake, I.; Shinozaki, F.; Takeshima, H.; Tajima, S. (2004). "DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction." **J Biol Chem.**, 279(26):27816-23.

179. Talbot, P.; Dandekar, P. (2003). "Perivitelline space: does it play a role in blocking polyspermy in mammals?" **Microsc Res Tech.**, 61(4):349-57.

180. Tamashiro, K. L. K.; Wakayama, T.; Blanchard, R. J.; Blanchard, D. C. (2000). "Postnatal growth and behavioral development of mice cloned from adult cumulus cells". **Biology of Reproduction**, 63: 328-334.

181. Tamashiro, K. L. K.; Wakayama, T.; Akutsu, H.; Yamazaki, Y.; Lachey, J. L.; Wortman, M. D.; Seeley, R. J.; D'Alessio, D.; Woods, S. C.; Yanagimachi, R.; Sakai, R. R. (2002). "Cloned mice have an obese phenotype not transmitted to their offspring". **Nature Medicine**, 8 (3): 262- 267.

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

183. Tani, T.; Kato, Y.; Tsunoda, Y. (2001). "Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming." **Biol Reprod.**, 64(1):324-30.

184. Taylor, S.S. (1982). "The in vitro phosphorylation of chromatin by the catalytic subunit of cAMP-dependent protein kinase." **J Biol Chem.**, 257(11): 6056-63.

185. Telford, N.A.; Watson, A.J.; Schultz, G.A. (1990). "Transition from maternal to embryonic control in early mammalian development: a comparison of several species. " **Mol Reprod Dev,** 26:90-100

186. Thompson, J. G.; Gardner, D. K.; Pugh, P. A.; McMillan, W.H.; Tervit, H. R. (1990). "Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos". **Biol Reprod.**, 53(6): 1385-91.

187. Trasler, J.M.; Trasler, D.G.; Bestor, T.H.; Li, E.; Ghibu, F. (1996). "DNA methyltransferase in normal and Dnmtn/Dnmtn mouse embryos." **Dev Dyn.,** 206(3):239-47.

188. Tsunoda, Y.; McLaren, A. (1983). "Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres. **J Reprod Fertil.**, 69(1): 315-22.

189. Tweedie, S.; Charlton, J.; Clark, V.; Bird, A. (1997). "Methylation of genomes and genes at the invertebrate-vertebrate boundary." **Mol Cell Biol** 17:1469-1475

190. Vajta, G.; Lewis, I. M.; Thouas, G. A; Trouson, A.O. (2001). " Somatic cell cloning without micromanipulators". **Cloning and Stem Cells**, 3 (2): 89-95.

191. Vajta, G.; Kragh, P.M.; Mtango, N. R.; Callesen, H. (2005). "Hand-made cloning approach: potentials and limitations." **Reprod Fertil Dev.**, 17(2): 97-112.

192. Vignon, X.; Chesné, P.; Lebourhis, D.; Fléchon, J. E.; Heyman, Y.; Renard, J. P. (1998). "Developmental potential of bovine embryos

reconstructed with enucleated matured oocytes fused with cultured somatic cells". **CR Acad Sci Paris**, 321: 735-745.

193. Vongs, A.; Kakutani, T.; Martienssen, R.A.; Richards, E.J. (1993). Arabidopsis thaliana DNA methylation mutants. **Science,** 260:1926-1928

194. Walker, S. K.; Heard, T. M.; Seamark, R. F. (1992). "In vitro culture of sheep embryos without co-culture: siccess and perspectives". **Theriogenology**, 37: 111-126.

195. Walker, S. K.; Hartwich, K. M.; Seamark, R. F. (1996). "The production of unusually large offspring following embryo manipulation: concepts and challenges". **Theriogenology**, 45: 111-120.

196. Wassarman, P.; Chen, J.; Cohen, N.; Litscher, E.; Liu, C.; Qi, H.; Williams, Z. (1999). "Structure and function of the mammalian egg zona pellucida." **J Exp Zool.**, 285(3): 251- 258.

197. Watanabe, S.; Ichimura, T.; Fujita, N.; Tsuruzoe, S.; Ohki, I.; Shirakawa, M.; Kawasuji, M.; and Nakao, M. (2003). "Methylated DNA-binding domain 1 and methylpurine-DNA glycosylase link transcriptional repression and DNA repair in chromatin." **Proc. Natl. Acad. Sci. U. S. A.,** 100, 12859–12864

198. Wakayama, T.; Perry, A. C. F.; Zuccotti, M.; Johnson, K. R.; Yanagimachi, R. (1998). "Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei". **Nature**, 394: 369-374.

199. Wakayama, T.; Yanagimachi, R. (1999). "Cloning of male mice from adult tip cells". **Nature Genetics**, 22: 127-128.

200. Wakayama, T.; Rodriguez, I.; Perry, A.C.; Yanagimachi, R.; Mombaerts, P. (1999). "Mice cloned from embryonic stem cells." **Proc Natl Acad Sci U S A.**, 96(26): 14984- 14989.

201. Weiss, A.; Keshet, I.; Razin, A.; Cedar, H. (1996). "DNA demethylation in vitro: involvement of RNA." **Cell**, 86:709-718.

202. Wells, D. N.; Misica, P. M.; Tervit, H. R.; Vivanco W. H. (1998). "Adult somatic nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed". **Rep. Fertil. Dev.**, 10: 369-378.

203. Wells, D. N.; Misica, P. M.; Day, A. M.; Peterson, A. J.; Tervit, H. R. (1998a). "Cloning sheep from cultured embryonic cells". **Rep. Fert. Deve.**, 10: 615-626.

204. Wells, D.; Misica, P. M; Tervit, H. R. (1999). "Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells". **Biology of Reproduction**, 60, 996- 1005.

205. Wells, D. N.; Laible, G.; Tucker, F. C.; Miller, A. L.; Oliver, J. E.; Xiang, T.; Forsyth, J. T.; Berg, M. C.; Cockrem, K.; L'Huillier, P. J.; Tervit, H. R.; Oback, B. (2003). "Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency". **Theriogenology**, 59: 45-59.

206. Willadsen, S.M. (1986). "Nuclear transplantation in sheep embryos." **Nature**, 320(6057): 63- 65.

207. Wilmut, I.; Schnieke, A. E.; McWhir, J.; Kind, A. J.; Campbell, K. H. S. (1997). "Viable offspring derived from foetal and adult mammalian cells. **Nature**, 385: 810-813.

208. Wilmut, I.; Beaujeau, N; de Sousa, P. A.; Dinnyes, A.; King, T. J.; Paterson, L. A.; Wells, D. N.; Young, L. E. (2002). "Somatic cell nuclear transfer". **Nature**, 149: 583-586.

209. Wilmut, I.; Peterson, L.A. (2002). "Somatic cell nuclear transfer (cloning) efficiency", online: http://www.roslin.ac.uk/public/ webtableGR.df

210. Wilson, J. M.; Williams, J. D.; Bondioli, K. R.; Looney, C. R.; Westhusin, M. E.; McCalla, D. F. (1995). "Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning) embryo transfer and natural mating". **Ani. Reprod Sci**, 38: 73-83.

211. Woods, G. L.; White, K. L.; Vanderwall, D. K.; Li, G-P.; Aston, K. I.; Bunch, T. D.; Meerdo, L. M; Pate, B. J. (2003). "A mule cloned from fetal cells by nuclear transfer". **Science** , 301(5636): 1063.

212. Worrad, D.M.; Turner, B. M.; Schultz, R. M. (1995). "Temporally restricted spatial localization of acetylated isoforms of histone H4 and RNA polymerase II in the 2-cell mouse embryo". **Development**, 121: 2949- 2959.

213. Wright, R.W. Jr.; Cupps, P.T.; Gaskins, C.T.; Hillers, J.K. (1977). "Comparative solubility properties of the zona pellucidae of unfertilized murine, ovine and bovine ova." **J Anim Sci.,** 44(5): 850- 853.

214. Yamazaki, Y.; Makino, H.; Hamaguchi-Hamada, K.; Hamada, S.; Sugino, H.; Kawase, E.; Miyata, T.; Ogawa, M.; Yanagimachi, R.; Yagi, T. (2001). "Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer." **Proc Natl Acad Sci U S A.,** 98(24): 14022- 14026.

215. Yin, X.J.; Cho, S.K.; Park, M.R.; Im, Y.J.; Park, J.J.; Jong-Sik-Bhak; Kwon, D.N.; Jun, S.H.; Kim, N.H.; Kim, J.H. (2003). "Nuclear remodelling and the developmental potential of nuclear transferred porcine oocytes under delayed-activated conditions." **Zygote**, 11(2):167-74.

216. Yoder, J.A.; Bestor, T.H. (1998). "A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast." **Hum Mol Genet**, 7:279-284

217. Yokochi, T., Robertson, K.D. (2002). "Preferential methylation of unmethylated DNA by mammalian de novo DNA methyltransferase Dnmt3a." **J Biol Chem**, 277:11735-11745

218. Young, L. E.; Sinclair, K. D.; Wilmut I (1998). "Large offspring syndrome in cattle and sheep". **Rev Reprod**, 3: 155-163.

219. Young, L.E.; Beaujean, N. (2004). "DNA methylation in the preimplantation embryo: the differing stories of the mouse and sheep." **Anim Reprod Sci.**, 82-83: 61- 78.

220. Zakhartchenko, V.; Stojkovic, M.; Brem, G.; Wolf, E. (1997). "Karyoplast-cytoplast volume ratio in bovine nuclear transfer embryos: effect on developmental potential". **Mol Reprod Dev.**, 48(3): 332-8.

221. Zakhantchenko, V.; Durcova-Hills, G.; Stojkovic, M; Schernthaner, W.; Prelle, K.; Steinborn, R.; Muller, M.; Brem, G.; Wolf, E. (1999). "Effect of serum starving and re-cloning on the efficiency of nuclear transfer using bovine foetal fibroblasts". **J. Rep. Fert.**, 115: 325-331.

222. Zhou, Q. I.; Boulanger, L.; Renard, J. P. (2000). "A simplified method for recontruction of fully competent mouse zygotes from adult somatic donor nuclei. " **Cloning**, 2 (1): 35- 44.

223. Zhou, Q.; Jouneau, A.; Brochard, V.; Adenot, P.; Renard, J.P. (2001). "Developmental potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei." **Biol Reprod.**, 65(2): 412-419.

224. Zhou, Q.; Renard, J. P.; Le Friec, G.; Brochard, V.; Beaujean, N.; Cherifi, Y.; Fraichard, A.; Cozzi, J. (2003). "Generation of fertile cloned rats by regulating oocyte activation". **Science**, 302(5648): 1179.

Appendices

Appendix I- Mouse media and stock solutions

1. Stock Media

Component	Volume/ Weight for 500ml (mg or ml)	MW or Units/ml	Final Concentration (mM, Units)	Supplier
Ultra- Pure Water	495ml	-	-	Speciality Media (TMS-006-A)
NaCl	2380mg	58.4	81.5mM	Sigma (S0390)
KCl	180mg	74.6	4.8mM	Sigma (P5405)
$MgSO_4.7H_2O$	145mg	246.5	1.2mM	Sigma (M1880)
EDTA	20mg	372.2	0.1mM	Sigma (E6635)
Lactate Acid	2.65ml	112.1	47.0mM	Sigma (L7900)
D-Glucose	500mg	180.2	5.5mM	Sigma (G6152)
KH_2PO_4	80mg	136.1	1.2mM	Sigma (P5655)
Strep/ Pen	5ml	5.000 Units/ml	50Units/ml	Gibco (15070-022)

Osmolarity should be approximately between 235-245mOsm and medium should be filtered. It can be stored at 4°C up to 1 month.

2. Stock Calcium Chloride ($CaCl_2$- 100× concentration)

Component	Volume/ Weight for 10ml (mg or ml)	MW or Units/ml	Final Concentration (mM)	Supplier
Ultra- Pure Water	10ml	-	-	Speciality Media (TMS-006-A)
$CaCl_2.2H_2O$	249mg	147.0	169.3mM	Sigma (C7902)

Medium should be filtered and it can be stored at 4°C up to 2 weeks.

3. Stock Strontium Chloride ($SrCl_2$- 100mM)

Component	Volume/ Weight for 10ml (mg or ml)	MW or Units/ml	Final Concentration (mM)	Supplier
Ultra- Pure Water	10ml	-	-	Speciality Media (TMS-006-A)
$SrCl_2.2H_2O$	266mg	266.6	100.0mM	Sigma (25,552-1)

Medium should be filtered and it can be stored at 4°C up to 2 weeks.

4. Hepes buffered CZB Medium (hCZB)

Component	Volume/ Weight for 100ml (mg or ml)	MW, Units/ml	Final Concentration (mM, Units)	Supplier
Stock Medium	99ml	-	-	-
Hepes	476mg	238.3	20.0mM	Sigma (H4034)
$NaHCO_3$	42mg	84.0	5.0mM	Sigma (S5761)
$CaCl_2.2H_2O$ (100× stock)	1ml	169.3	1.7mM	-
Pyruvic Acid	3mg	110.0	0.3mM	Sigma (P4562)
Glutamine-L	15mg	146.2	1.0mM	Sigma (G8540)
PVA	10mg	15,000	residual	ICN (102787)

Osmolarity should be approximately between 275-280mOsm, pH= 7.4 and medium should be filtered. It can be stored at 4°C up to 2 weeks.

5. CZB Medium

Component	Volume/ Weight for 100ml (mg or ml)	MW, Units/ml	Final Concentration (mM, %, Units)	Supplier
Stock Medium	99ml	-	-	-
$NaHCO_3$	211mg	84.0	25.1mM	Sigma (S5761)
Pyruvic Acid	3mg	110.0	0.3mM	Sigma (P4562)
$CaCl_2.2H_2O$ (100× stock)	1ml	169.3	1.7mM	-
Glutamine-L	15mg	146.2	1.0mM	Sigma (G8540)
BSA Fraction V	500mg	-	0.5%	Sigma (A3311)

Osmolarity should be approximately 290mOsm, and medium should be filtered. It can be stored at 4°C up to 2 weeks.

6. CZB calcium-free Medium

Component	Volume/ Weight for 100 ml (mg or ml)	MW, Units/ml	Final Concentration (mM, %, Units)	Supplier
Stock Medium	100ml	-	-	-
$NaHCO_3$	211mg	84.0	25.1mM	Sigma (S5761)
Pyruvic Acid	3mg	110.0	0.3mM	Sigma (P4562)
Glutamine-L	15mg	146.2	1.0mM	Sigma (G8540)
BSA Fraction V	500mg	-	0.5%	Sigma (A3311)

Osmolarity should be approximately 285mOsm, and medium should be filtered. It can be stored at 4°C up to 2 weeks.

Appendix II- Previous Experiments

A) Cattle Experiments

A.1. Zona-free technique of nuclear transfer in cattle

The first objective of this project was to assess and learn the new zona-free technique of nuclear transfer in cattle at AgResearch in New Zealand (Oback et al., 2003) and establish it at Roslin. At Roslin, the method was first developed in cattle to allow some familiarity with the technique for the future establishment in the mouse. Only development to blastocyst was assessed, as we do not hold currently a licence for bovine embryo transfer. All material and methods were performed as described by Oback et al. (2003). As these results were from small preliminary experiments, they were not included in the Chapter II. Figure II1 shows the development to blastocyst of NT reconstructed and parthenogenic activated embryos.

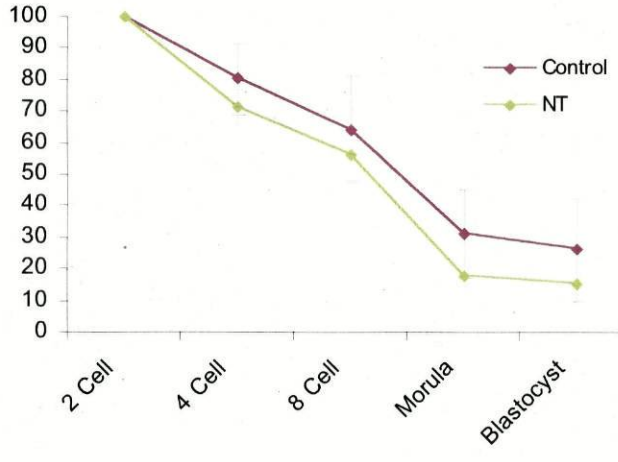


Fig. II1. Development to blastocyst of reconstructed NT and parthenogenic activated control bovine embryos in culture. NT embryos were reconstructed using the novel zona-free New Zealand method of NT, carried out at Roslin Institute (Oback et al., 2003). n_{NT}= 159, $n_{Control}$= 87, 7 replicates.

By the end of the study, 15.5± 3.2% of cleaved NT embryos reached blastocyst stage, against 26.0± 16.0% of parthenogenic activated oocytes. This demonstrates that the development of NT embryos is quite close to the controls, giving us confidence about this innovative technique of NT.

B) Mouse Experiments

Several previous and basic experiments were performed in mouse using zona-free control parthenogenic activated oocytes/ embryos in order to establish the better conditions for the use of zona-free mouse embryos for nuclear transfer.

B.1. Experiment to show how mouse weight correlates with oocyte number

As, the weight of the animals varies; we designed an experiment using mice with the same age but with different weights and compared the number of oocytes recovered. All oocytes donors were 8-10 weeks old B6D2F1 (C57BL/6J×DBA/2) females. Oocytes were recovered 13-13.5 h after injection of human chorionic gonadotrophin- [hCG] Chorulon- Intervet. Superovulation protocol includes intraperitonial injections of 5 IU eCG (Folligon- Intervet) followed by 5 UI of hCG given 48 hours later.

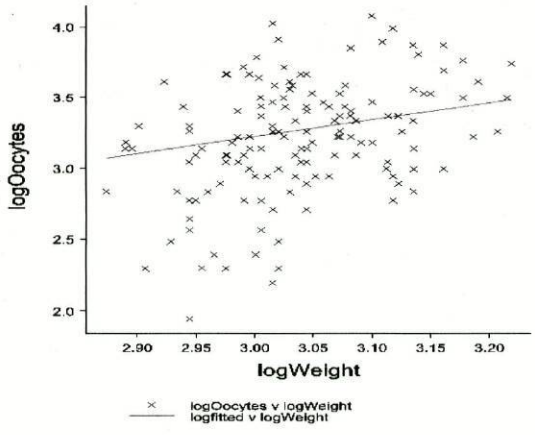


Fig. II2. Correlation between weight of 8-10 week old female mice and the number of oocytes recovered. Values are represented in logarithmic scales due to the nature of the statistical test used (GLMM- generalised linear mixed model). Statistical significance for the regression coefficient is $P<0.05$, n= 144.

Statistical significant correlation ($P<0.05$) between the number of oocytes recovered per females (with 8-10 weeks) and their weight was found, as seen in the Fig. II2. On the basis of these results, only animals over 19-20g of weight were used for our experiments.

B.2. Experiments to optimisation of culture and activation conditions of zona-free mouse embryos

Some other previous experiments were designed to optimise the conditions of culture and activation using zona-free embryos in mouse. For all the follow experiments, oocytes were recovered at 13- 13.5 hours after hCG injection. Cumulus cells removal and zona pellucida digestion with pronase were performed as previously reported (chapter II). Oocytes were cultured in CZB media until activation 17-18h

after hCG injection. All embryos that survived activation were washed and cultured for 4 days in CZB media 5µl droplets under paraffin oil (Fluka) at 37°C and 5% CO_2.

B.2.1. Establishment of activation protocol for zona-free mouse oocytes

Four protocols of activation were selected to test which would produce highest cleavage and development to blastocyst, with reduced death percentage during activation in zona-free mouse oocytes: a) 8% ethanol for 8min followed by 5µg/ml of Cyt B in calcium free CZB media for 5 hours (Eth); b) 5µM ionomycin for 5 min followed by 5µg/ml Cyt B in calcium free CZB media for 5 hours (Ion); c) 10mM strontium chloride plus 5µg/ml Cyt B in CZB media for 5 hours (St+Ca); d) 10mM strontium chloride plus 5µg/ml Cyt B in calcium free CZB media for 5 hours (St-Ca). Fig. II3a shows the percentage of dead embryos during activation and cleavage and Fig. II3b the percentage of embryos that develop to blastocyst, with the 4 different activation protocols tested.

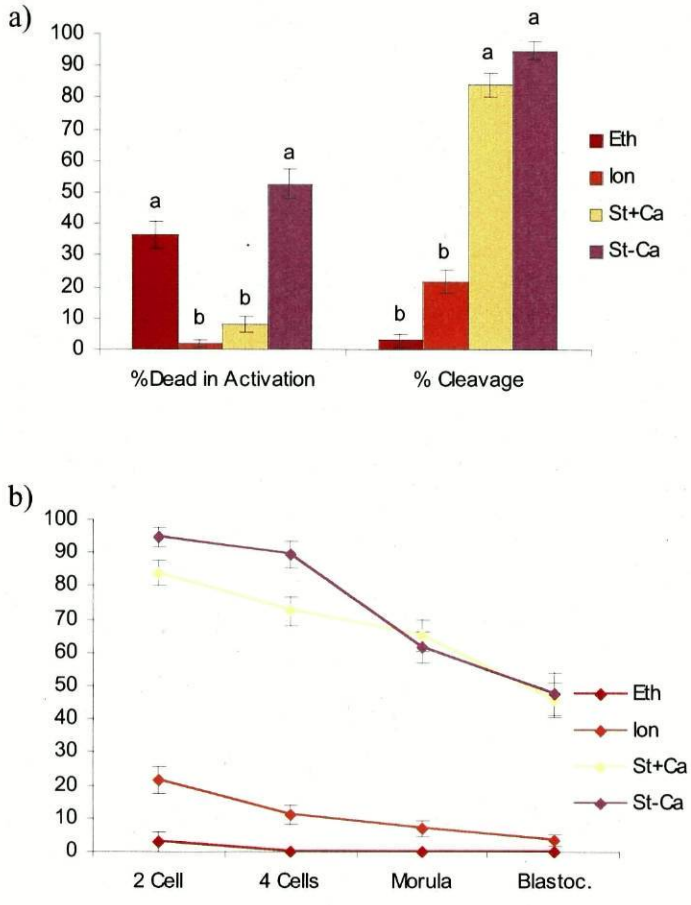


Fig. II3- a) Percentages of dead zona-free embryos, during activation and cleavage using four different protocols, ^{a,b}, $P<0.001$, ^{a,a}, $P>0.1$; b) Development to blastocyst in zona-free embryos activated using four different protocols. Ethanol (Eth), Ionomycin (Ion), Strontium diluted in CZB media (St+Ca), Strontium diluted in calcium free CZB media (St+Ca). $n_{Eth}= 118$, $n_{Ion}= 119$, $n_{St+Ca}= 115$, $n_{St-Ca}=120$, 3 replicates.

By the end of this experimental study, the method selected was the use of strontium chloride diluted in media containing calcium, because it resulted in fewer dead in activation yet achieved similar cleavage and development to blastocyst than when diluted in calcium-free medium. The cleavage percentages of oocytes activated with ethanol and ionomycin activation protocols were extremely low, reason why neither of these methods was selected for use in NT experiments (Fig. II3).

B.2.2. Establishment of culture media protocol for *in vitro* culture of zona-free mouse oocytes

Similar experiments were performed comparing three different media for embryo culture in mouse: CZB media, produced in our laboratory, M16 commercial media (Sigma) and KSOM commercial media (Specialty Media). Previous experiments have shown that M16 medium is inferior to the CZB medium during the culture of mouse embryos (Chatot et al., 1989). KSOM is a modified version of a simplex optimized medium (SOM) with a high potassium concentration. For that purpose we have used oocytes that were parthenogenic activated using the protocols selected above. Embryos were cultured for 4 days in CZB, M16 and KSOM media 5µl droplets under paraffin oil (Fluka) at 37°C and 5% CO_2.

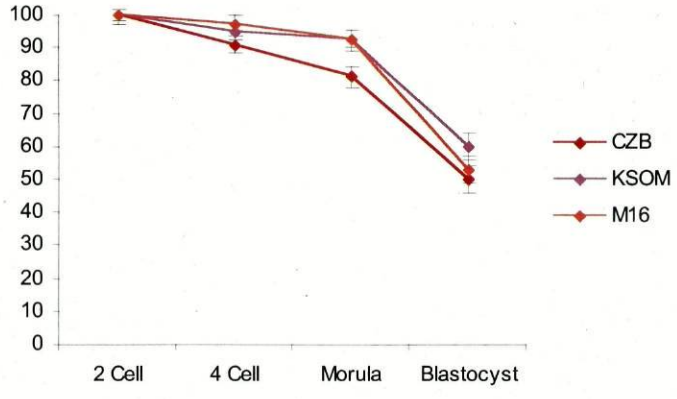


Fig. II4. Development to blastocyst parthenogenic activated embryos cultured *in vitro* using three culture media systems: CZB, KSOM and M16. n_{CZB}= 155, n_{KSOM}= 153, n_{M16}= 155, 5 replicates.

Analysis of the results has shown no statistical differences in the development to blastocyst for any of the culture used. P> 0.1 for comparison between: CZB vs. KSOM; CZB vs. M16 and KSOM vs. M16. Previous unpublished results in our

laboratory have show better results for NT embryo development produced by zona-intact techniques when cultured in CZB medium, reason why we select this one for future experiments (Fig. II4).

B.2.3. Establishment of covering oil protocol for *in vitro* culture of zona-free mouse oocytes

It has been shown that the type of oil used in culture to cover the media is extremely important. For this reason, experiments were drawn with the objective to select the best commercial oil to be used for culture systems of zona-free mouse embryos. For that purpose we have used oocytes that were parthenogenic activated using the protocols selected above. Embryos were cultured for 4 days in CZB media in 5µl droplets under: Squibb (Sq) mineral oil (Roberts Pharmaceutical Corp.); Liquid paraffin (PL) (Unichem Ltd.); Sigma embryo tested oil (Sig); Cook Oil (Cook); paraffin oil (Fluka) at 37°C and 5% CO_2.

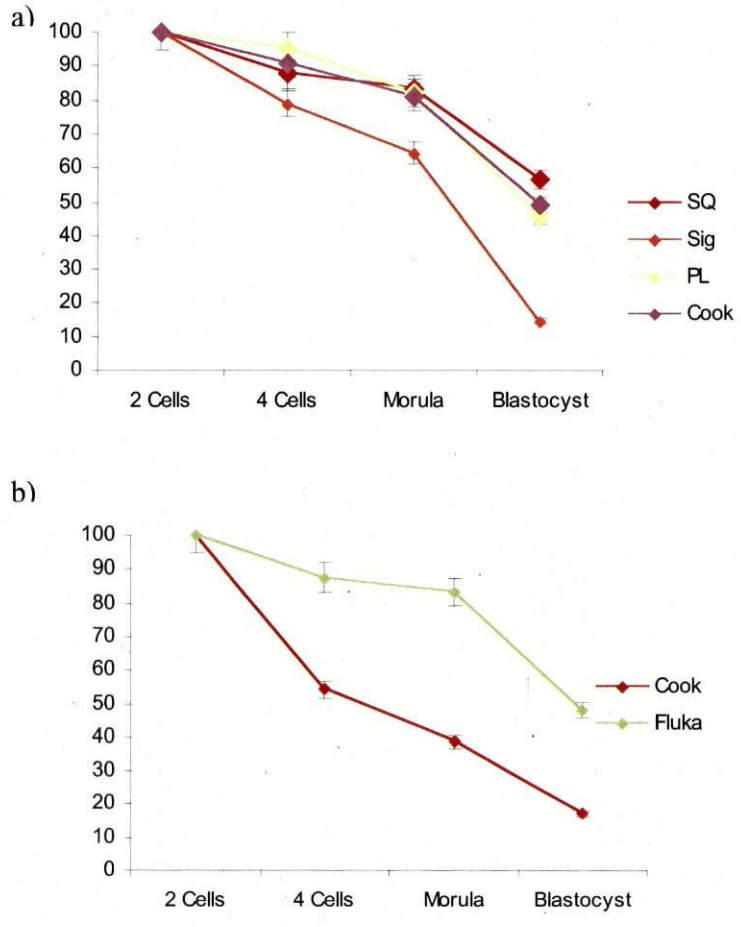


Fig. II5. Development to blastocysts of parthenogenic activated embryos kept in culture using CZB media, comparing the effect of different oils to cover the dish and droplets. a) Comparison of Squibb (Sq); Liquid paraffin (PL); Sigma (Sig) and Cook (Cook) mineral oils, n_{Sq}= 88, n_{Sig}=91, n_{PL}= 89, n_{Cook}= 88, 4 replicates; b) comparison between Cook (Cook) and Fluka (Fluka) mineral oils, n_{Cook}=54, n_{Fluka}= 37, 2 replicates.

The first experiment showed no differences in development to blastocyst of three of the mineral oils selected, but we rejected the Sigma oil. Squibb and Cook mineral oils are expensive, and liquid paraffin has the inconvenience of being very watery, leading to problems with dish manipulation. So a second experiment (b) was performed to test

a cheaper and thicker mineral oil commercially available (Fluka). This experiment was performed to compare it against Cook oil, as it achieved the best results in previous experiment (Fig. II5a). Fluka oil achieved even better embryo development than Cook oil, so it was selected for use in future experiments (Fig. II5b).

Appendix III- Weight after birth of nuclear transfer, NT offspring and control ZF *in vivo* fertilized mouse

The cloned mouse produced using this novel zona-free technique of NT was born apparently larger and 1.7-fold heavier than other pups of the same age. The placenta was also enlarged. This is a usual characteristic of cloned embryos and it has been associated with epigenetic disturbances. However, the offspring of the cloned mouse showed normal weight at birth and during development, showing that the large offspring syndrome (LOS) is not a heritable condition, but that is a result of epigenetic and not genetic modifications (Fig. III1).

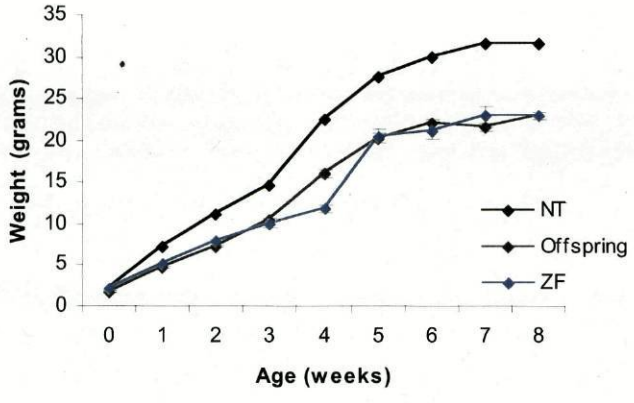


Fig.III1. Weight from birth to 8-weeks of age of mouse produced by nuclear transfer (NT), nuclear transfer mouse offspring (Offspring) and mice derived from control zona-free *in vivo* fertilized embryos (ZF). N_{NT}= 1, $n_{Offspring}$= 29, n_{ZF}= 13.

Appendix IV- Cloned Mouse Pathology Report

VETERINARY PATHOLOGY UNIT

Royal (Dick) School of Veterinary Studies

Easter Bush Veterinary Centre

Easter Bush

Midlothian EH25 9RG

Telephone 0131 650 6265

Fax 0131 445 5770

DIAGNOSTIC PATHOLOGY REPORT

Case Number	Penny 170205	*Owner:*	Ricardo Ribas
Pathology Number	BB197/05		
Sender	Lesley Penny		
Species	Mouse		
Breed	C57Bl/6Jx129/Sv		
Submission type	Necropsy		

Report 1

Date of report 2nd March 2005

Histopathology of sections from submitted fixed mouse.
Major changes are noted in relation to the vasculature. There is a prominent large vessel arteritis with active suppurative inflammation affecting the vascular walls and adjacent perivascular tissue. These changes are particularly severe in the heart where the reaction extends into the myocardium.
Elsewhere in tongue musculature similar generic change affecting arterioles is evident with striking fibrinoid necrosis and perivascular inflammation.

Spleen: white pulp prominent and active; otherwise unremarkable
Pancreas: unremarkable
CNS: periarteritis; meningitis.
Urogenital tract – unremarkable
Lungs: congested; otherwise NAD
Gastrointestinal tract: unremarkable with exception of those areas where the associated lymphoid tissue and vasculature exhibits vasculitis as evident in association with the heart.

Diagnosis
Arteritis and periarteritis with fibrinoid necrosis; suppurative; severe
Mycocarditis; suppurative, locally extensive; severe

Comment
The morphological features identified are consistent with classic mouse polyarteritis nodosa. This is a relatively common age-dependent condition of several inbred strains including B6. It is thought to be an autoimmune, probably immune complex disease and as such, there is nothing to link it to cloning per se.

Pathologist Dr S Rhind

Page 1 of 1

Fig. IV1. Cloned mouse diagnostic pathology report.

Appendix V- Techniques of RNA Extraction, RT-PCR and Agarose Gel Electrophoresis

1. RNA Extraction

1.1. RNA Extraction from Single Embryos with Absolutely RNA® Nanoprep Kit

a. Reagents

1. **RNase-Free DNase I:** Reconstitute the lyophilised RNase-Free DNase I by adding 290µl of DNase Reconstitution Buffer to the vial. Mix the contents thoroughly to ensure that all the powder goes into solution. Do not introduce air bubbles into solution. Store the reconstituted RNase-Free DNase I at –20°C
2. **High-Salt Wash Buffer:** Prepare 1× High-Salt Wash Buffer by adding 16ml of 100% ethanol to the bottle of 1.67× High-Salt Wash Buffer. Store at room temperature
3. **Low-Salt Wash Buffer:** Prepare 1× Low-Salt Wash Buffer by adding 68ml of 100% ethanol to the bottle of 5× Low-Salt Wash Buffer. Store at room temperature
4. **β- mercaptoethanol:** Once opened, store at 4°C

b. Protocol

1. Add 0.7µl of β- mercaptoethanol (β-ME) to 100µl of Lysis Buffer to 100µl of Lysis Buffer for each sample up to 1×10^4 cells. A fresh mixture of lysis buffer and β-ME should be done just before each use.
2. Add 100µl of the Lysis Buffer-β-ME mixture to each embryo sample and vortex or pipette the sample repeatedly until homogenized. Ensure that the

viscosity of the lysate is low. High viscosity causes a decrease in RNA yield and an increase in DNA contamination. Viscosity can be reduced by additional vortexing, pipetting and/or increasing the volume of Lysis Buffer (up to 200µl).

3. Add an equal volume (usually 100µl) of 70% ethanol (stored at room temperature) to the cell lysate and mix thoroughly by vortexing for 5 seconds.
4. Transfer this mixture to an RNA-binding nano-spin cup that has been seated within a 2ml collection tube and snap the cap onto the top of the spin cup.
5. Spin the sample in the microcentrifuge at 13000× g for 60 seconds
6. Remove and retain the spin cup and discard filtrate. Re-seat the spin cup in the same 2ml collection tube. Up to this point, the RNA has been protected from RNases by the presence of *guanidine thiocyanate*.
7. Optional DNase treatment: This procedure is recommended if DNA-free RNA is required. If DNA removal is not necessary, omit the DNase treatment and proceed directly to step 8.
    a. Add 300µl of 1×Low-Salt Wash Buffer to the spin cup. After capping, spin the sample in the microcentrifuge at 13000× g for 60 sec.
    b. Remove and retain the spin cup and discard the filtrate. Re-seat the spin cup in the collection tube, cap the spin cup, and spin the sample in a microcentrifuge at 13000× g for 2 min to dry the fiber matrix
    c. Prepare the DNase solution by a gentle mixing 2.5µl of reconstituted RNase-Free DNase I with 12.5µl of DNase Digestion Buffer for each sample. Gentle mixing is necessary because the DNase I is very sensitive to denaturation

d. Add the 15µl of DNase solution directly onto the fiber matrix inside the spin cup and cap the spin cup.

e. Incubate the sample at 37°C for 15 min.

8. Add 300µl of 1×High-Salt Wash Buffer to the spin cup, cap the spin cup, and spin the sample in a microcentrifuge at 13000× g for 60 sec

9. Remove and retain the spin cup, discard the filtrate, and re-seat the spin cup in the collection tube. Add 300µl of 1×Low-Salt Wash Buffer. Cap the spin cup and spin the sample in a microcentrifuge at 13000× g for 60 sec

10. Perform a second low salt wash (Remove and retain the spin cup, discard the filtrate, and re-seat the spin cup in the collection tube. Add 300µl of 1×Low-Salt Wash Buffer. Cap the spin cup and spin the sample in a microcentrifuge at 13000× g for 60 sec)

11. Remove and retain the spin cup, discard the filtrate and re-seat the spin cup in the collection tube. Cap the spin cup and spin the sample in a microcentrifuge at 13000× g for 3 min to dry the fiber matrix.

12. Transfer the spin cup to a fresh 2ml collection tube.

13. Add 10µl of Elution Buffer directly onto the fiber matrix inside the spin cup. Cap the spin cup and incubate the sample at room temperature for 2 min. The Elution Buffer must be added directly onto the fiber matrix to ensure that the buffer permeates the entire fiber matrix. RNA yield may be increased by using Elution Buffer warmed at 60°C.

14. Spin the sample in a microcentrifuge at 13000× g for 5 min. This elution step may be repeated to increase the yield of total RNA. The purified RNA is in the eluate in the collection tube. Transfer the eluate to a capped microcentrifuge

tube to store the RNA. The RNA can be stored at –20°C for up to one month or at –80°C for long term storage.

1. 2. RNA Extraction from multiple embryos, tissues and cells with Qiashredder® and Rneasy® Kits

a. Reagents

Some information related to the protocol and buffer can be found in the Rneasy Micro® Handbook.

b.1. Protocol for Embryos

1. Add 10µl of β-mercaptoethanol (Sigma) to every 1 ml of Buffer RLT before use.
2. Add 350µl Buffer RLT to the tube containing embryo. Vortex for a few seconds to mix.
3. Pipette cell lysate onto QIAshredder column and spin for 2 min at maximum speed to homogenize.
4. Add 350µl of 70% ethanol (made in RNase-free water) to homogenised lysate and mix well by pipetting.
5. Apply the 700µl sample to an Rneasy spin column in a 2 ml collection tube and centrifuge for 1min at 10.000rpm. Discard the flow-through.
6. Transfer the column to a fresh 2 ml collection tube. Pipette 700µl Buffer RW1 onto column and centrifuge for 1 min at 10.000rpm.
7. Transfer the column to a new collection tube. Pipette 500µl Buffer RPE onto column and centrifuge for 1 min at 10.000rpm. Discard the flow-through.

8. Pipette another 500µl Buffer RPE onto Rneasy column and centrifuge for 2min at 10.000rpm to dry the column. Discard the flow through.
9. Transfer the column to a fresh 1.5ml tube (not provided in the kit- so use a 1.5ml tube with the lid cut off). Centrifuge for 1 min at max speed to remove all the traces of ethanol.
10. Transfer column into a new 1.5ml collection tube from the kit and pipette 50µl RNase free water/ Diethylpyrocarbonate (DEPC) water onto a column. Centrifuge for 1 min at 10.000rpm to elute.

b.2. Protocol for Tissues and Cells

1. Add 10µl β-mercaptoethanol for each 1ml Buffer RLT required.
2. As quick as possible, add 75mg tissue to 1.75ml Buffer RTL in green rybolyser tube. Ensure lids are tight. Keep tubes on ice.
3. Homogenize samples in Hybaid Rybolyser (power setting 6.5 for 30sec). Place tubes on ice.

All steps from now are carried out at room temperature.

4. Remove 700µl of homogenate and place in clean 1.5ml tube.
5. Centrifuge at 13.000rpm for 3 min. Transfer supernatant to fresh 1.5ml tube.
6. Add 700µl of 70% ethanol (made in DEPC water). Mix well by pipetting (do not vortex)
7. Apply 700µl to Rneasy column including any precipitate, which may have formed. Centrifuge for 30sec at 10.000rpm. Discard the flow through. Repeat with the remaining 700µl to homogenate.
8. Add 700µl of Buffer RW1 to the Rneasy column. Centrifuge for 30sec at 10.000rpm to wash the column. Discard the flow through and collection tube

9. Transfer the Rneasy column into a new 2ml collection tube. Pipet 500µl Buffer RPE onto a Rneasy column. Centrifuge for 30sec at 10.000rpm to wash the column. Discard the flow through.
10. Add another 500µl Buffer RPE onto a Rneasy column. Centrifuge for 2min at 10.000rpm to wash the column. Discard the flow through.
11. Transfer the Rneasy column into a new 2ml collection tube. Centrifuge for 1min at 13.000rpm to remove all traces of ethanol.
12. To elute RNA, transfer the Rneasy column to a new 1.5ml tube and pipette 50µl RNase-free water directly onto the membrane. Centrifuge for 1 min at 10.000rpm to elute. If yield is expected to be >30µg, repeat elution in second 1.5ml tube.
13. RNA Quantification: Prepare 1:100 dilution of each sample and measure OD at 260nm and 280nm. µg/µl= (40×dilution×OD)/1000, so 4×OD (for 1:100 dilutions).
14. Purity of RNA: The ration between the readings at 26 and 280nm (A_{260}/A_{280}) provides an estimate of the purity of the RNA with respect to protein contaminates. Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1

c. RNA Precipitation using a Pellet Paint

1. Bring pellet aliquot to room temperature. Invert several times to form a uniform suspension.
2. Add 2µl pellet paint to each tube of embryo RNA.
3. Add 0.1 volumes (5µl) of 3M sodium acetate, mix briefly.
4. Add 2 volumes (100µl) of 100% ice cold ethanol, vortex briefly.
5. Incubate samples at room temperature for 2 min.

6. Spin sample at 14.000rpm for 30min at 4°C, carefully remove supernatant.
7. Rinse pellet with 500µl of ice cold 70% ethanol (made in DEPC water), vortex briefly and spin at 14.000rpm, for 25min at 4°C.
8. Remove supernatant, spin again briefly and remove as much ethanol as possible with P10 tip. Dry the pellet on ice in fume hood for approximately 10-15min.
9. Re-suspend pellet in 8µl RNAse-free water. Keep tubes on ice.
10. Proceed to cDNA synthesis

2. First Strand cDNA Synthesis (Amersham® Kit)

All steps should be performed on ice.

1. Heat RNA to 65°C for 10min, then chill on ice.
2. Make mastermix of bulk first-strand reaction mix, primer ($pd(N)_6$) and DTT (ditriotreitol) solution: for each tube add 5µl of bulk first-strand reaction mix, 1µl of primer and 1µl of DTT
3. Add 7µl mastermix to denatured RNA and mix gently.
4. Incubate for 1h at 37°C, cool to 4°C.
5. The reaction product is now ready for PCR amplification.
6. If more than one embryo per tube was submitted to RNA extraction, dilution of cDNA was performed to achieve the same concentration as in individually embryos.

3. Polymerase Chain reaction (PCR) amplification

3.1. Protocol

1. In sterile PCR flow hood, prepare a master mix containing: 22.5µl PCR Buffer (1.1×ReddyMix PCR Master Mix containing 1.5mM MgCl2, dNTP's and Taq DNA polymerase); 0.125µl of each primer; distilled water to give the final volume of 23µl. Mix well.
2. Add 23µl of mix to each tube.
3. On lab bench add 2µl of cDNA (to prevent contamination of PCR hood with cDNA).
4. Spin tubes briefly.
5. Switch on PCR machine and select required program. Allow heated lid to pre warm.
6. Place samples in Hybaid PCR block and press enter.

3.2. PCR Program

The exact parameters depend on the PCR primer pairs and need to be optimized accordingly. Generally the cycling conditions were the following. The annealing temperature varied depending on the primers.

- 1 cycle of 95°C 5 min
- 30-40 cycles of 94°C for 30 sec; annealing temperature for 30sec; 72°C for 45sec.
- 1 cycle 72°C for 10min
- Cool to 4°C. Hold

4. Agarose Gel Electrophoresis

4.1. Reagents

1. **Running Buffer (1× Tris Borate EDTA Buffer- TBE):** Prepare a 1:10 dilution of 10× TBE buffer with distilled water. Add Ethidium bromide (Sigma) to give the final concentration of 0.2μg/ml.
2. **Agarose Gel (1.5-2%):** Weigh required amount of agarose (BDH) in a flask, add 100ml of running buffer and mix. Heat until boiling in microwave. Cool to approximately 50°C and pour into a prepared gel tray. Allow gel to set.

4.2. Protocol

1. Place gel into a electrophoresis tank and cover with the remaining 900ml of running buffer.
2. Load 20μl of sample into each well of the gel (the 1.1×ReddyMix PCR Master Mix already contains the dye and precipitant to facilitate gel loading).
3. Load 5μl of low molecular weight PCR size marker (1Kb Plus DNA Ladder-Invitrogen) to one well.
4. Apply voltage of 10V per cm to the gel for 1-1.5 h.

Appendix VI- Supplementary immunocytochemistry images for Chapter IV

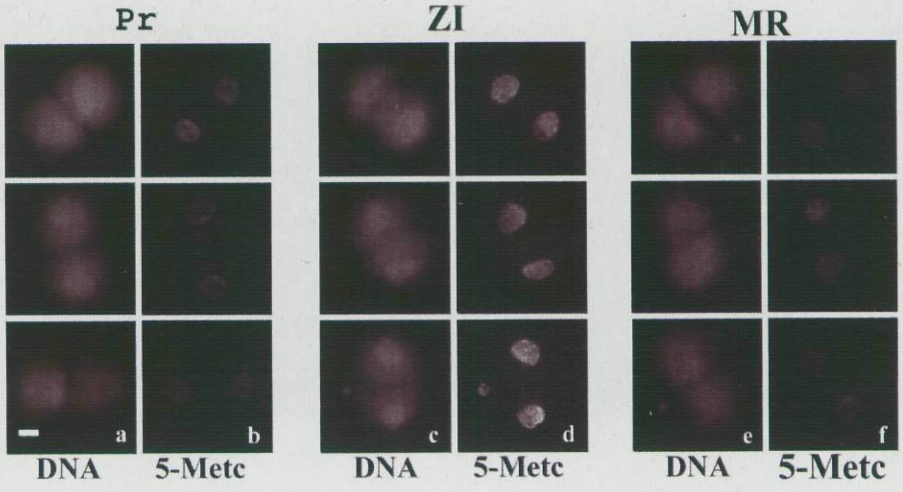


Fig. VI1. Methylation analysis of 2-cell zona-intact (ZI) and zona-free mouse embryos after zona removal with either pronase (Pr) or mechanically (MR). Columns a, c and e represents DAPI (DNA) staining and b, d, f represent 5-methylcytosine expression (5-Metc). Due to the fact that the DNA has been denaturated, DAPI fluorescence is not very bright. Figure of 5-methylcytosine quantification is shown in Chapter IV. Scale bar= 20μm.

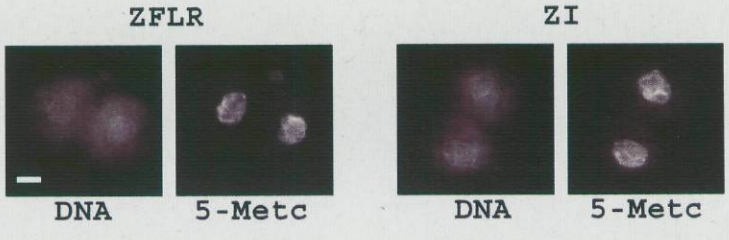


Fig. VI2. 5-methylcytosine fluorescence in *in vivo* fertilized 2-cell mouse embryos cultured with and without zona pellucida from the zygotes stages. Zona pellucida was removed 8 hours after fertilization and embryos cultured until 2-cell stage. Due to the fact that the DNA has been denaturated, DAPI fluorescence is not very bright. Quantification analysis is shown in Chapter IV. Scale bar= 20μm.

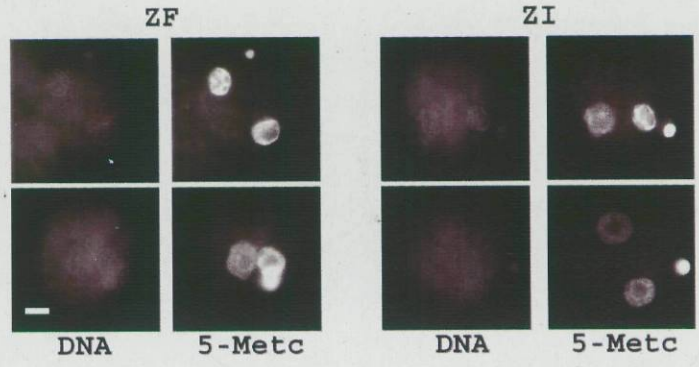


Fig. VI3. 5-methylcytosine fluorescence of *in vivo* fertilized pronuclear stage mouse embryos approximately 7 hours after fertilization, cultured with and without zona pellucida. Due to the fact that the DNA has been denaturated, DAPI fluorescence is not very bright. Figure of 5-methylcytosine quantification chart is shown in the Chapter IV. Scale bar= 20μm.

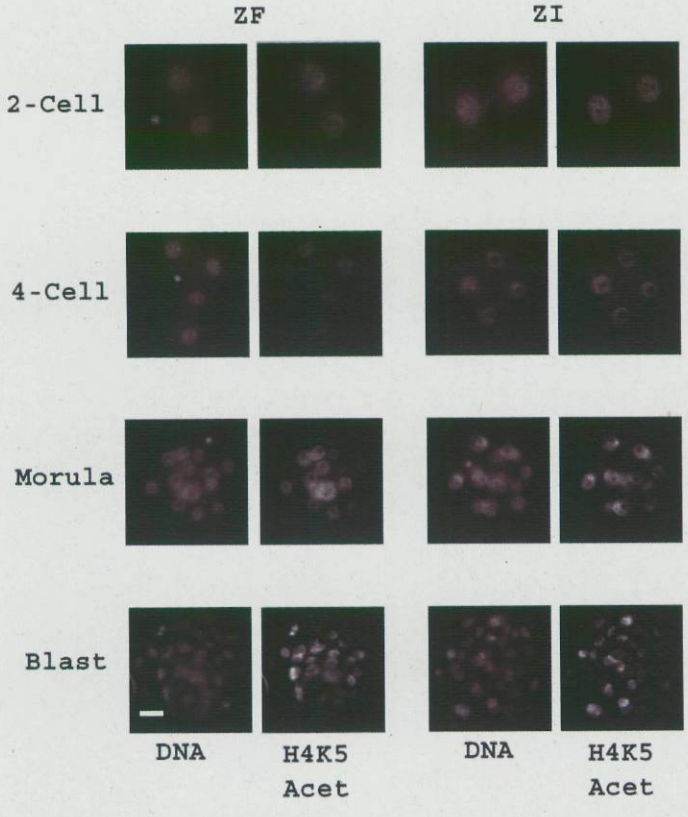


Fig. VI4. Histone 4 lysine 5 acetylation and DAPI immunofluorescence in *in vivo* fertilized 2-cell, 4-cell, morula and blastocyst zona-free and zona-intact mouse embryos. Scale bar= 20μm.