University of Pretoria
Faculty of Health Sciences
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Optimization of techniques required for somatic cell nuclear transfer using a mouse model

by
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DEDICATION

To my parents, thank you for giving me everything I have ever needed in this life, and more! Thank you for your endless love and support, and for guiding me towards the life I am meant to live and the person I am meant to be. I am forever grateful for you both.

DECLARATION BY CANDIDATE

'I hereby declare that the dissertation submitted for the degree MSc Reproductive Biology, at the Faculty of Health Sciences, University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.'

CHANTEL GOUVEIA

Name in block letters

Signature

31 August 2018

Date

ETHICAL CLEARANCE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

31/05/2018

Chantel Gouveia
Health Sciences Dean's Office
University of Pretoria

Dear Chantel Gouveia

RE.: 358/2015 ~ Letter dated 17 Apr 2018

Protocol Number	**358/2015**
New Title dd May 2018	Optimisation of techniques required for somatic cell nuclear transfer using a mou se model.
Principal Investigator	Chantel Gouveia **Tel:** 765465119 **Email:** chantelgouveia5@gmail.com **Dept:** Health Sciences Dean's Office

We hereby acknowledge receipt of the following document:

- Extension until end of December 2018

which has been approved at 30 May 2018 meeting.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed; PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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SUMMARY

Introduction: Somatic cell nuclear transfer (SCNT) is the removal (enucleation) of chromosomes from a metaphase II oocyte, followed by the transfer and fusion of a donor somatic cell to the enucleated oocyte (cytoplast). The reconstructed oocyte is then activated to induce embryonic development. One of the main purposes of creating SCNT blastocysts is the derivation of embryonic stem cells (ESCs) for therapeutic cloning. The consensus for the low SCNT blastulation rate (12-15% in mice and 10% in humans) is epigenetic reprogramming failure of the donor somatic cell nucleus by the oocyte. The optimization and technical efficiency of SCNT was investigated in this study.

Methods: Female B6D2F1 mice were used as oocyte and cumulus cell (somatic cell) donors. In study 1, non-invasive spindle imaging by Hoffman modulation contrast microscopy was used to identify the spindle within 484 oocytes. Once located, the spindles were enucleated. Successful enucleation was confirmed through artificial activation (317 cytoplasts) using calcium ionophores and kinase or protein synthesis inhibitors that caused fragmentation of the cytoplasts; and Hoechst DNA staining (167 cytoplasts) to microscopically confirm the absence of chromosomes. Appropriate controls for the techniques were included.

In study 2, enucleation of 564 oocytes was followed by the transfer a single cumulus cell exposed to a membrane fusogen into close contact with the oolemma of the cytoplast. Post fusion, the reconstructed oocytes were artificially activated and subsequently cultured to the blastocyst stage, with the addition of a histone deacetylase inhibitor to aid epigenetic reprogramming. The reconstructed oocytes (80-90%) were expected to survive nuclear transfer; with 70-80% surviving activation, 60-70% pseudo-pronucleus formation; 50-60% cleavage to the 2-cell stage after 24 hours of nuclear transfer; and 30-50% development to the morula/blastocyst stage 72-96 hours post nuclear transfer.

Results: The first enucleation confirmation technique was the analysis of cytoplast fragmentation between 16-18 hours after activation, which indicated that **85%** of cytoplasts were effectively enucleated. A cohort of non-enucleated control oocytes confirmed the efficiency of the activation protocol by showing a pseudo-pronucleus

formation rate of **95.4%.** The second confirmation technique revealed an enucleation efficiency of **97.5%**, which was confirmed by the absence of chromosomes in stained cytoplasts. The staining protocol was verified using a group of non-enucleated control oocytes, resulting in **100%** of the oocytes presenting with stained and visible chromosomes.

In study 2, enucleation was performed with a survival rate of **99.1%**. The cytoplasts then underwent cumulus cell nuclear transfer with **100%** survival. Subsequently, a fusion rate of **72.3%** and an activation rate of **81.7%** was achieved in this study. Blastocyst formation by SCNT was significantly lower than that of the control group (**5.4%** vs. **55.1%**), and more poor-quality blastocysts were produced by SCNT (**63.6%**). Therefore, according to statistical analyses, the chance of forming a blastocyst by SCNT in this project was 0.041-fold that of the control group.

Discussion: Results revealed that the rates of enucleation and nuclear transfer survival, fusion efficiency, activation survival, pseudo-pronucleus formation, cell division, compaction and morula development of the SCNT embryos were as good as those reported. However, the formation of SCNT blastocysts was below the published average, which may be a consequence of epigenetic reprogramming failure. Experimental adaptations such as medium supplementation or nuclear reprogramming strategies can be applied to improve epigenetic reprogramming by SCNT, which could be evidenced by a greater number of embryos progressing to good-quality blastocysts.

In humans, use of SCNT as a tool to generate specific ESCs from the somatic cells of an individual could ultimately lead to the analysis of disease mechanisms, as well as improve the efficiency of cell-based therapies with a negligible risk of immune rejection in the treatment of degenerative diseases. SCNT is a cutting-edge technique that can offer innovative clinical applications in the field of assisted reproduction such as preventing the transmission of mitochondrial DNA diseases from mother to child, as well as the treatment of ooplasm pathologies.

Keywords: SCNT, oocyte, B6D2F1, activation, enucleation, somatic cell, spindle, fusion, TSA, cloning, epigenetic

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RESEARCH OUTPUTS

Article

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Poster presentation

Gouveia C, Huyser C, Pepper MS. Experimental setup of Somatic Cell Nuclear Transfer: Procedural optimization. National SASREG (Southern Africa Society of Reproductive Medicine and Gynaecological Endoscopy) Congress: New Debates in Endoscopy and Infertility. Investec Conference Centre, Johannesburg (October 2015).

Award: Best oral presentation in Hons/MSc category

Posters

Gouveia C, Huyser C, Pepper MS. Experimental setup of Somatic Cell Nuclear Transfer: Procedural optimization. National SASREG (Southern Africa Society of Reproductive Medicine and Gynaecological Endoscopy) Congress: New Debates in Endoscopy and Infertility. Investec Conference Centre, Johannesburg (October 2015).

Award: Best electronic poster in Hons/MSc category

Gouveia C, Huyser C, Pepper MS. Experimental setup of Somatic Cell Nuclear Transfer: Procedural optimization. Faculty of Health Sciences Faculty Day. University of Pretoria, Pretoria (August 2016)

Award: Third place for Junior Researcher in Basic Science

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ASRM:	American Society for Reproductive Medicine
[Ca^{2+}]:	Intracellular calcium concentration
CB:	Cytochalasin B
CB-GTH:	Cytochalasin B in Global® Total® w/HEPES
CD:	Cytochalasin D
CI:	Confidence interval
COC:	Cumulus-oocyte complexes
CO_2:	Carbon dioxide
CZB:	Chatot Ziomek Bavister
DC:	Direct current
6-DMAP:	6-Dimethylaminopurine
DNA:	Deoxyribonucleic acid
ESCs:	Embryonic stem cells
F:	Fusion
GT:	Global® Total®
GTH:	Global® Total® w/HEPES
hCG:	Human chorionic gonadotropin
HDACi:	Histone deacetylase inhibitors
HEPA:	High efficiency particulate air
H3K9me3:	Histone 3 lysine 9 trimethylation
HMC:	Hoffman modulation contrast
HN:	Hemagglutinin-neuraminidase
HVJ-E:	Hemagglutinating virus of Japan envelope
HVJ:	Hemagglutinating virus of Japan
ICM:	Inner cell mass
ICSI:	Intracytoplasmic sperm injection
iPSC:	Induced pluripotent stem cell
IVF*:*	*In vitro* fertilization
Kdm4d:	Lysine demethylase 4D
KSOM:	Kalium simplex optimized medium
Lat-A:	Latrunculin A

MAPK:	Mitogen-activated protein kinase
MPF:	Maturation promoting factor
mtDNA:	Mitochondrial DNA
MII:	Metaphase II
NEBD:	Nuclear envelope breakdown
N_2	Nitrogen
O_2:	Oxygen
PB:	Polar body
PCC:	Premature chromosome condensation
PVP	Polyvinylpyrrolidone
PVS:	Perivitelline space
RBL:	Reproductive Biology Laboratory
RNA:	Ribonucleic acid
SASREG:	Southern African Society for Reproductive Medicine and Gynaecological Endoscopy
SBAH:	Steve Biko Academic Hospital
SCNT:	Somatic cell nuclear transfer
si:	Short interfering
$SrCl_2$:	Strontium chloride
TE:	Trophectoderm
TSA:	Trichostatin A
UK*:*	United Kingdom
UPBRC:	University of Pretoria's Biomedical Research Centre
UP:	University of Pretoria
USA:	United States of America
UV:	Ultraviolet

LIST OF FIGURES

All graphical figures were designed by C Gouveia using Microsoft Word 2016 (Microsoft Office 365, 2016 Microsoft Corporation, Unites States of America).

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 1

Chapter 3

Chapter 4

Chapter 1: Literature review

1.1 Introduction

Somatic cell nuclear transfer (SCNT) has been an area of interest in the field of stem cell research for the past 15 years. The first step of SCNT is called enucleation and can be described as the removal of the haploid (1n) chromosomes which comprises of the meiotic spindle complex from a metaphase II (MII) stage oocyte.[1,2] Enucleation is then followed by the transfer and fusion of a diploid (2n) somatic cell (obtained from a suitable donor) into an enucleated oocyte, known as a cytoplast.[2,3] The manipulated oocyte is then artificially activated by means of either electric pulses or chemical stimulation, which induces subsequent development of the embryo (Figure 1.1).[2] Somatic cell nuclear transfer is used primarily for generating cells and tissues that are immunocompatible with the somatic cell donor, a concept known as therapeutic cloning. Originally suggested in 1999, producing patient-specific or genetically identical embryonic stem cells (ESCs) for research and therapeutic intentions emphasised the potential of SCNT as a unique technique.[4]

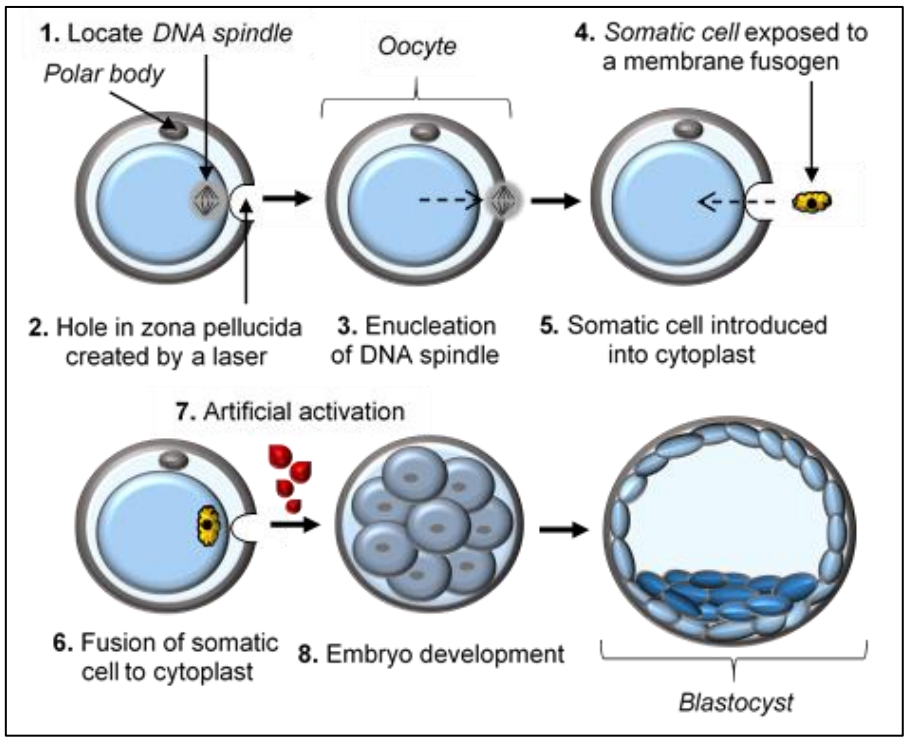

Figure 1.1: Graphic representation of the steps (1-8) involved in the **SCNT technique** performed in this research project. Dashed arrows represent removal of the spindle in step 3, and transfer of the somatic cell into the enucleated oocyte in step 5.

1.2 Background

1.2.1 A brief history of nuclear transfer

In 1938, the initial concept of nuclear transfer was suggested by the "father of cloning" Hans Spemann, who proposed that the nuclear genome of embryonic or somatic cells could be reprocessed and evaluated for the possibility of maintaining full-term development.[5] The first successful investigation of this concept was performed in amphibians in 1952, by the transfer of embryonic blastomere nuclei into enucleated eggs.[6] The first successful demonstration of nuclear transplantation to produce cloned frogs was achieved in 1962 and resulted in viable offspring.[7] This represents the first case to report the reprogramming of a somatic cell to a totipotent state via an enucleated egg. Due to technical and biological limitations related to oocyte manipulation, not until the early 1980s was nuclear transplantation reported in mammals. Eventually, a breakthrough was reached in 1996 when Dolly the sheep was the first progeny to be created by SCNT using an adult somatic cell.[8] These milestones in nuclear transfer are representative of *reproductive cloning*, which can be described as the transfer of a cloned embryo created by SCNT into the uterus of a surrogate mother, to ultimately achieve full-term development of the cloned offspring (Figure 1.2). The concept of *therapeutic cloning* was initially demonstrated in the mouse,[9] with the aim of creating and harvesting stem cells that could potentially be used to treat diseases (Figure 1.2). Subsequently, using similar SCNT techniques, many species including cattle, mouse, pig, rabbit, *Rhesus macaque*, and several more have been cloned successfully, producing viable offspring or ESCs for the purpose of reproductive or therapeutic cloning, respectively.[10] Despite previous achievements, the overall efficiency of creating viable offspring in animals by SCNT varies,[11] ranging between 0.8% and 33% according to Table 1.1. The realistic expected rate of blastocyst development in human oocytes after SCNT is 10%.[12]

Table 1.1: The success rates of blastocyst and full-term development in different species after SCNT

Species	Blastocyst development	Full-term development
Cow[13-17]	31-42%	6.5-13%
Mouse[18-23]	57-75%	0.9-9.8%
Pig[24-30]	21-81%	0.8-11%
Rabbit[31,32]	45-79%	5%
Rhesus macaque[33,34]	12-72.7%	9-33%
Human[12,35,36]	10-23%	*Not applicable*

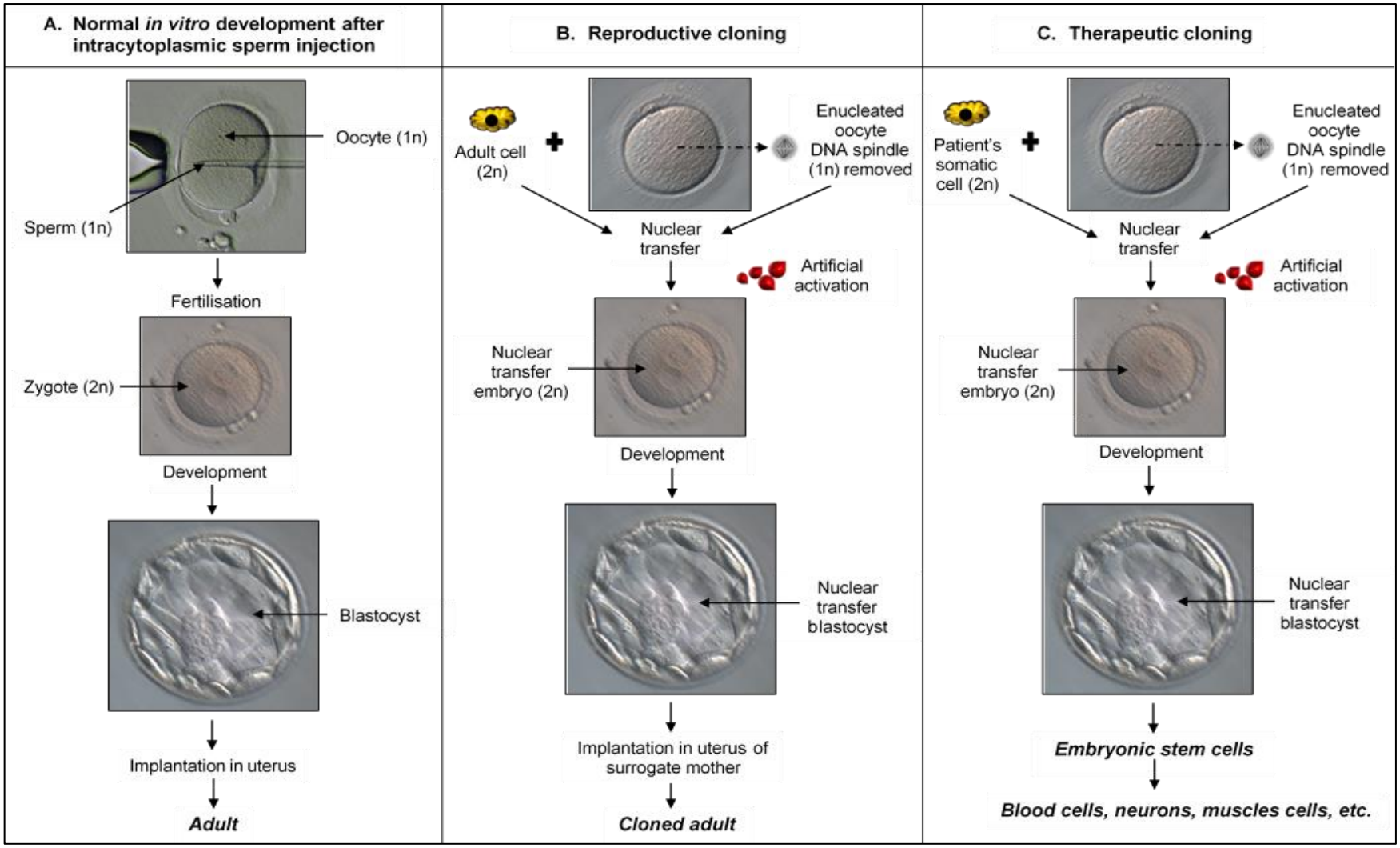

Figure 1.2: The different outcomes in A) **normal *in vitro* development** after intracytoplasmic sperm injection (ICSI), B) **reproductive cloning**, and C) **therapeutic cloning** (images captured at Reproductive Biology Laboratory [RBL], University of Pretoria [UP]).

1.2.2 SCNT protocol deficiencies and optimizations

To date, over 20 animal species have undergone cloning by nuclear transplantation; however, SCNT remains an inefficient process. According to Chia and colleagues,[37] developmental arrest of human SCNT embryos is correlated with genetic instability, in the forms of deoxyribonucleic acid (DNA) damage and mitotic errors related to chromosome segregation. In mice, abnormal epigenetic modifications including aberrant DNA methylation and histone modifications have been revealed in cloned embryos.[38-40] Furthermore, in addition to abnormal placentas[41,42] several abnormalities have been found in full-term mice offspring that have led to early death due to, respiratory failure or other deformities,[43,44] obesity,[45] liver necrosis, tumours and pneumonia.[46] Several factors that have contributed to this low efficiency include: *invasive micromanipulation, oocyte incompetence and variation in developmental efficiency,* as well as *in vitro culture inconsistencies*. However, the general accepted cause is abnormal gene expression due to **failure of epigenetic reprogramming** of the donor somatic cell nucleus by the oocyte.[11] The transferred somatic cell nucleus is expected to undergo a sequence of epigenetic changes caused by factors within the cytoplasm of the cytoplast. Ideally, this implies the thorough removal of the "somatic donor cell memory" followed by new zygotic chromatin being established.[10]

The additional invasive steps involved in SCNT may further induce damage and contribute to the low success rate. The procedure of SCNT involves the removal of the meiotic spindle complex of the oocyte. As opposed to interphase nuclei, meiotic spindles are not visible in the MII stage human oocyte using conventional light microscopy.[10] Originally, enucleation protocols **identified the spindles** by staining the chromosomes with fluorochromes followed by exposure to ultraviolet (UV) light. However, exposure of oocytes to UV light is detrimental and compromises the development of SCNT embryos.[10] Following the undesirable consequences of the initial SCNT protocol, additional improvements were introduced into the standard protocol and this led to enhanced procedural efficiency in various species. One of the first modifications was the introduction of a **non-invasive spindle imaging system** in the form of polarized light birefringence, which has dramatically improved blastocyst development.[47] In monkeys, this single modification to the SCNT procedure significantly improved the blastocyst formation rate from 1% to 16%.[48] Furthermore,

oocyte lysis during enucleation was a procedural issue; however this has been addressed by **thinning and creating a hole the zona pellucida** with a laser system.[49]

Transferring and fusion of the donor somatic cell nucleus into the cytoplast is another possible detrimental step. Fusion was regularly performed by whole cell electroporation, which has been reported to prematurely trigger cytoplast activation and extrusion of the second polar body (PB), followed by the continuation of meiosis in MII spindles.[50] An adjustment was also made to bypass the adverse effect of premature activation caused by electrofusion through testing a **membrane fusion** approach using an inactivated Sendai virus (also known as hemagglutinating virus of Japan [HVJ]) extract between the donor somatic cell and the cytoplast.[50] The viral envelope is made up of fusogenic proteins that promote successful fusion[49] and prevents not only premature cytoplast activation, but also the degradation of the maturation-promoting factor that is required for successful reprogramming.[10] A study in which monkey oocytes were exposed to caffeine, a protein phosphatase inhibitor, also reported effective protection of the cytoplast from premature activation, as well as improved development of SCNT embryos.[51] Another study investigated the effects of caffeine on human oocytes during spindle enucleation and fusion, and reported an enhanced blastocyst development rate and good blastocyst quality, characterized by noticeable and prominent inner cell masses (ICM), comparable to embryos produced by *in vitro* fertilization (IVF).[36]

Another downfall of the SCNT protocol yet to be corrected is the **cell-cycle stage incompatibility** between the donor somatic cell nucleus and the cytoplast that may cause irregular DNA replication and consequently aneuploidies.[52] The oocyte cytoplasm is paused at the metaphase stage, while the somatic cell nucleus is transferred at the G0/G1 (interphase) stage of the cell cycle.[53] Ideally, the somatic cell nucleus should be synchronised and transferred at the mitotic phase of the cell cycle. This can be achieved by exposing donor somatic cells to compounds (e.g. nocodazole) that hamper microtubule polymerisation.[10] However, even minimal exposure to these drugs can be toxic or cause irreparable damage and in most cases is detrimental to normal development.[54] Additionally, oocyte activation is an essential step for the continuation and completion of meiosis, which is naturally triggered upon the entry of sperm during fertilization. The zygote relies on activation, which triggers reprogramming and metabolic activity in the oocyte cytoplasm, which in turn is

required to maintain subsequent development.[55] In SCNT, artificial induction of the activation stimulus is needed since natural fertilization is sidestepped. As early as the 1980s, artificial chemical induction treatments were developed to mimic the biochemical processes activated by natural sperm stimulation.[55] Use of this treatment however does not always ensure complete activation. To optimize the activation protocol, cytoplasts are exposed to kinase or protein synthesis inhibitors in addition to induction of a calcium influx.[10,56] Interestingly, in human SCNT oocytes fused by the HVJ extract and activated via standard treatment, subsequent development to the blastocyst stage failed.[36] Therefore, as an additional activation stimulant and not as a cell fusion promoter, electrofusion is used to support proper activation and reprogramming of the cytoplast after human SCNT.[36]

Although the SCNT approach is continuously being improved, further studies should be performed to optimize protocol efficiency, understand the molecular mechanisms involved in reprogramming, and ultimately link the technique to clinical applications, including assisted reproductive options.

1.3 Technical aspects of SCNT techniques

All SCNT experiments use standard micromanipulation procedures, and further include microscopy, enucleation, cell transfer, fusion, and activation. Like many other technical procedures, good-quality micromanipulation systems and microtools contribute approximately 75% to successful SCNT experiments.[57]

1.3.1 Micromanipulation medium

Oocyte species is the primary determinant for culture medium choice for micromanipulation techniques. Even so, micromanipulation medium are generally HEPES-buffered alternatives of embryo culture medium (that are normally bicarbonate-buffered), which maintains the correct pH balance outside the incubator. Commercially available standard medium, such as M2 for mouse embryos[58] are preferred by many investigators. However, since the application and modifications of Kalium simplex optimized medium (KSOM)[59,60] for micromanipulation, this medium and an additional corresponding HEPES-buffered flushing medium have become commercially available. Additionally, species-specific culture medium for the optimal development of species-specific embryos have been developed in recent years, for example calcium-free Chatot Ziomek Bavister (CZB) medium for mouse embryos.[61] At

the same time, manipulation medium has been customized, to ensure that the environmental surroundings of the oocyte are maintained as consistently as possible inside and outside the incubator. An example of a customized medium is HEPES-buffered CZB which is specific for mice.[9] During nuclear transfer, the ooplasm is severely compromised. Modifications made to manipulation medium could become essential for improved survival.[57]

1.3.2 Preparation of the micromanipulation dish

Page and Malcuit[57] described that the micromanipulation setup contains microdrops of medium overlaid with mineral oil to reduce the risk of contamination and prevent evaporation. Using microdrops as opposed to an uncovered dish filled with medium conveniently permits the categorization and identification of different experimental oocytes with ease. The multi-microdrop arrangement allows for different types of medium, which may include a fluorescent dye or a microfilament inhibitor such as cytochalasin B (CB), to be used within the same micromanipulation dish. Therefore, time efficiency is improved by reducing the need to change dishes between specific techniques. The choice of a glass or plastic dish depends on the optical system that is used. In the case of differential interference contrast microscopy, an ordinary glass slide with elevated silicone edges that holds the mineral oil may be utilized. However, if a glass slide is used, it is important to have the slide siliconized to maintain the size/shape and prevent flattening of the microdrops. For Hoffman modulation contrast (HMC) microscopy, a 100x15 mm tissue culture dish lid is most frequently used (Figure 1.3). The edge of the lid is low which allows for movement and placement of microtools. The medium, which is generally HEPES-buffered, may be supplemented with 10% polyvinylpyrrolidone (PVP) to clean, lubricate and prevent blockage of the enucleation/cell transfer pipettes with cell debris.[57]

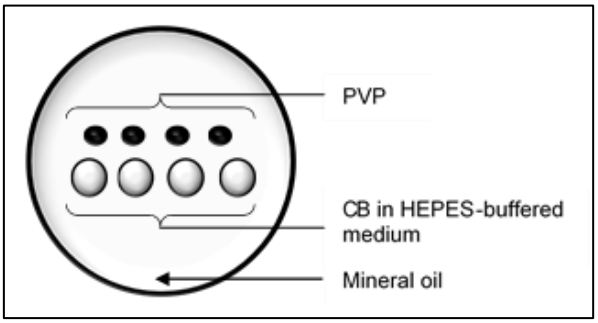

Figure 1.3: Schematic diagram of the micromanipulation dish and **medium preparation** required for nuclear transfer.

1.3.3 Enucleation of oocytes for SCNT

In SCNT, before the DNA from one cell can be transferred to another, the recipient cell should first have its own genetic material removed.[57] This is usually performed in oocytes arrested at the MII stage of meiosis.[57] During this stage, a metaphase plate meiotic spindle complex is formed by the condensed chromosomes, and is located at the border of the oocyte adjacent to the extruded PB.[62] In most mammalian species, including humans, the metaphase stage meiotic spindle complex of the oocyte is not visible under a light microscope due to dark cytoplasmic lipids.[57,63] Therefore, location and removal of the meiotic spindle complex are facilitated by using the intercalating dye bisbenzimide (Hoechst 33342) to label the oocyte DNA which is then made visible using UV light.[64-66] Although this is common practice and a useful tool for identifying the desired position for enucleation, exposure of an oocyte stained with a DNA fluorescent dye to epi-illumination is not recommended. This is because of the possible damage to the oocyte, which decreases developmental efficiency.[56,67-70] Despite the negative impact, in several cases the meiotic spindle may not always be located nearby the PB,[70,71] which necessitates the utilization of UV illumination to visualize the meiotic spindle complex. An alternative would be to make use of a narrow-band UV excitation filter to completely reduce exposure of the oocyte to high-energy UV.[57] Additionally, a method using an H3S10ph antibody-phycoerythrin conjugate coupled with low-energy halogen light results in lower phototoxic effects when compared to UV illumination.[72]

More commonly, studies have shown that non-invasive imaging is achieved by using polarized light microscopy, which maintains oocyte viability and has no negative effects on the developmental competence of the oocytes.[47,51] The principle of polarized light is based on the ability of submicroscopic molecular order to be imaged and measured by this microscope.[70,73] Molecular bonds or submicroscopic particles that are partially aligned cause birefringence, which changes the state of the polarized light that is passing through.[74] Since meiotic spindles are highly birefringent,[75] a microscopy technique based on detecting polarized light generated by birefringent cell structures, has proven reliable and advantageous for non-invasively visualizing the meiotic spindle.[47,73,76] Contrary to most mammalian species including humans, in mice (e.g. the BDF1 strain), the meiotic spindle complex containing the chromosomes in the metaphase stage oocyte is easily visible as a translucent region under a HMC

microscope.[3,9,70,77,78] Based on the above findings, SCNT experiments in mice do not require an identifying tool, which avoids the damage incurred by UV light or the need to purchase expensive equipment for polarized light birefringence.[76]

As reviewed by Page and Malcuit,[57] the preferred alignment for optimal enucleation occurs when the inner surface of the zona pellucida and the tip of the enucleation pipette are all in the same plane of focus (Figure 1.4). The optimal position is attained by using the holding pipette to lightly secure the oocyte, and the enucleation pipette to rotate the oocyte until the correct focal plane and spindle visualization are achieved.[12,56,76,79] Once the correct placement is reached, the suction on the oocyte is tightened and the enucleation pipette is introduced through an opening in the zona pellucida created by a laser, located close to the oolemma near to where the meiotic spindle should be.[79,80] Gentle aspiration of the meiotic spindle into the enucleation pipette is performed using a microinjector.[57] Subsequently, the enucleation pipette is gradually retracted, with the isolated meiotic spindle complex encapsulated inside a very small portion of ooplasm, known as a karyoplast.[76,78] During enucleation, the oocyte membrane is not compromised because of the addition of 5-10 µg/ml CB to the micromanipulation medium.[12,34,36,56,57,76,78,81] Cytochalasin B destabilizes the actin cytoskeleton rendering the oocyte more flexible therefore reducing the risk of lysis and damage during the process.[57] After enucleation, epi-illumination may be used to confirm the absence of the meiotic spindle complex and PB from the cytoplast.[2] Small batches of about 10-20 oocytes should be enucleated at a time to reduce exposure time to the sub-optimal micromanipulation environment.[78] Ideally, cytoplasts should be incubated in culture medium for at least 30 minutes prior to nuclear transfer, to allow for the recovery of the oolemma and cytoskeleton.[57,78]

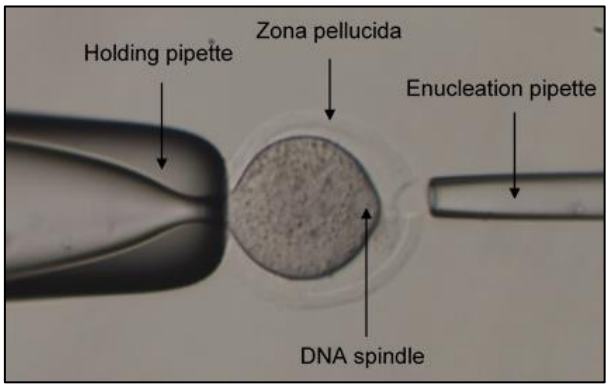

Figure 1.4: Photo image portraying the **preferred alignment and focal plane** for optimal enucleation (image captured at RBL, UP).

1.3.4 Cell transfer into cytoplasts for SCNT

The aspects involved in cell transfer were discussed by Page and Malcuit[57] and will be summarised below. Different cell types can be used as nuclear donors, and the method of collection is based on the cell type. For example, adherent cells require trypsinization.[82] Centrifugation is used to remove culture medium and concentrate the cells.[82] The cell pellet is then suspended in medium, and placed into a clean microdrop along with previously enucleated oocytes. To maintain fine control movement, the interface created by oil and medium in the cell transfer pipette should be kept visible at the edge of the field of view when loading of the cells begins. When more cells are aspirated, fine control movement within the pipette is reduced as the oil/medium interface shifts from the tip into a larger diameter of the pipette.[57]

The same or a newly made opening created in the zona pellucida is accessed by the cell transfer pipette for the donor cell transfer into the perivitelline space (PVS).[2,12,34,36,76,79,80,83] Before entry through the opening, a single donor cell is placed at the tip of the pipette to limit the volume of culture medium that may also be blown into the PVS.[56,57,76,78] Access through the same opening reduces the risk of the ooplasm bulging out of the zona, in addition to preventing the blastocyst from hatching through two holes at a later stage of embryo development, which may be detrimental.[57] Once the pipette has passed through the hole in the zona pellucida, a microinjector is used to expel the cell out of the pipette and into close contact with the oolemma. About 20-40 oocytes may undergo cell transfer at a time, and fusion is initiated when contact occurs between the cell membranes.[57]

1.3.5 Cell fusion of cytoplasts and nuclear donor cells

As summarised by Page and Malcuit,[57] fusion may occur through adding polyethylene glycol, inactivated HVJ-envelope (HVJ-E), or electrical stimulation, the latter of which is the most commonly used. Electrofusion is accomplished by placing the cell-oocyte couplets in a mostly non-ionic, but marginally hypotonic medium in the middle of two electrodes and applying a high-voltage direct current (DC) pulse that breaks down the membranes.[84] Since different laboratories use different conditions, the strength and duration of the pulse must be investigated. An electrical pulse causes the breakdown of membrane phospholipids, creating holes in the membrane. Once healing occurs, the membranes between the two cells become fused. Thus, a very high voltage applied for a long period will cause lysis.[84,85] On the other hand, a low voltage for a

short duration will result in poor rates of fusion. Generally, the fusion parameters range between 1.25-1.5 kV/cm for 10-50 µs, although several researchers choose to apply a few shorter pulses instead of a single long pulse.[57]

In the early 1950s, HVJ was the first virus to be isolated in Japan. This is a mouse parainfluenza virus of the genus *Paramyxoviridae*, which is 150–600 nm in diameter and within its viral envelope contains negative-strand ribonucleic acid (RNA) (15,383 bases). On the outside of the viral envelope are two glycoproteins, named fusion (F) and hemagglutinin-neuraminidase (HN). Adhesion with the cell surface is achieved by the binding of HN to acetylated sialic acid receptors and fusion with the cell membrane is induced through the F protein (Figure 1.5).[86]

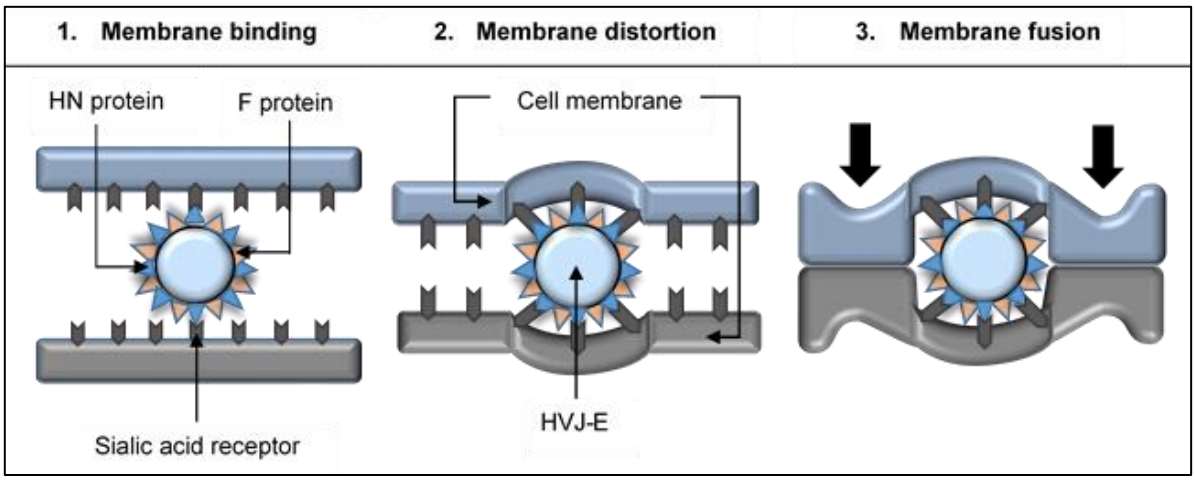

Figure 1.5: The **principles of HVJ-E-mediated** membrane fusion.[87]

As a result of the abovementioned properties, the inactivated HVJ-E was established as a drug-delivery vector or a cell fusion agent.[88] When HVJ is completely inactivated by UV-irradiation, the HVJ-E is prepared as a purified product (Figure 1.6). Only a vesicle that maintains the cell membrane fusion activity of the envelope protein is retained. Within the HVJ-E, the genomic RNA has been completely inactivated resulting in loss of viral genome replication and viral protein synthesis.[50,88] Therefore, all infective and proliferative potentials have been eradicated and the inactivated HVJ-E will not infect humans or experimental animals. No special procedures or facilities are required when working with HVJ-E, which means that purified HVJ-E is safe to use in general laboratories.[88]

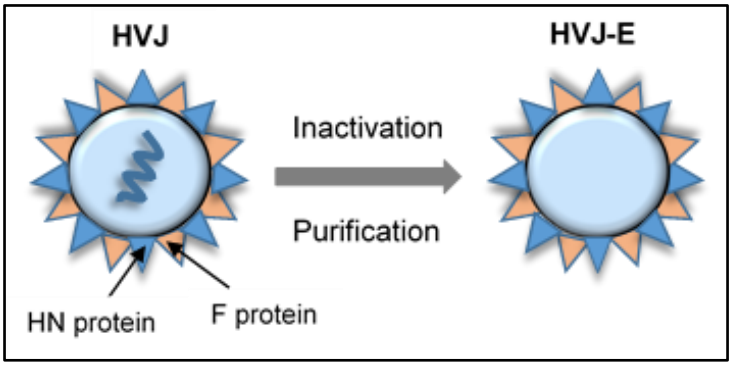

Figure 1.6: **Purification of HVJ-E** through complete inactivation of HVJ by UV-irradiation.[87]

Even though the method of choice for cell fusion is polyethylene glycol or electrical stimulation, severe toxicity may be induced by polyethylene glycol and special electrical equipment is required for electrical stimulation. A less toxic and more convenient alternative is the inactivated HVJ-E requiring no special instruments and can be used successfully as a cell fusion agent in SCNT (Figure 1.7).[12,36,49,50,76,81,83,89,90]

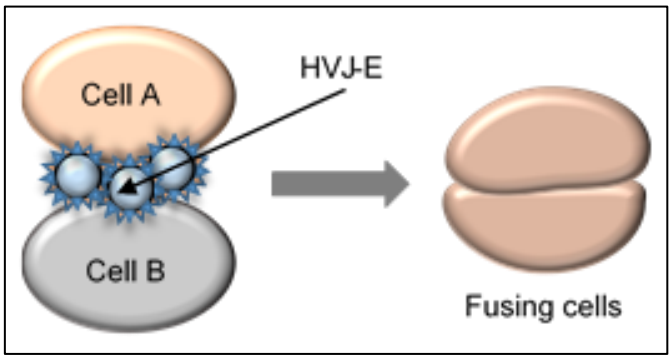

Figure 1.7: **Fusion** of two cells using inactivated HVJ-E as a cell fusion agent.[87]

1.3.6 Artificial activation of fused oocytes for SCNT

Oocyte activation due to sperm entry during fertilization is an essential step for the continuation and completion of meiosis.[91] Mammalian oocytes are arrested during the second phase of meiosis at the MII stage. Fertilization also induces other modifications that collectively denote oocyte activation, which include the release of cortical granules from the oocyte, second PB extrusion, and the formation of pronuclei.[92-94] The zygote relies heavily on activation, importantly triggering reprogramming and metabolic activity within the ooplasm, which is required to maintain subsequent development.[55] Oocyte activation in many mammalian species requires a rise in the intracellular calcium concentration ($[Ca^{2+}]$).[95] During fertilization, the signal of intracellular $[Ca^{2+}]$ is

made up of recurring surges that are generally known as [Ca^{2+}] oscillations.[96] The sperm component that is almost certainly responsible for these oscillations is a sperm-derived factor called phospholipase C zeta.[97-101] The recommencement of meiosis and the initiation of development are made possible through an association between the required [Ca^{2+}] oscillations and the capacity of the signal to activate the anaphase promoting complex[102,103] and subsequently, inactivate maturation promoting factor (MPF).[104,105]

In SCNT, artificial induction of the activation stimulus is required since natural fertilization is sidestepped. Several methods exist for artificial activation of mammalian oocytes, all inducing resumption of the arrested oocyte from MII and initiation into the embryonic cell development cycle. As early as the 1980s, artificial chemical induction treatments were developed to mimic the biochemical processes activated by natural sperm stimulation.[55] These include reproducing the sperm-induced [Ca^{2+}] oscillation responses or replicating the effects thereof, such as M-phase kinase inactivation promoting exit from MII arrest.[91] A standard oocyte activation protocol causes an individual and continuous increase in intracellular [Ca^{2+}], which adequately promotes the early phases of oocyte activation including the release from MII arrest. Oocytes are therefore treated with ethanol or [Ca^{2+}] ionophores such as A23187 and ionomycin.[106] In mammals however, the progress from MII to interphase is achieved over several hours. Therefore, during this changeover, the ongoing intracellular [Ca^{2+}] oscillations induced by the sperm are in control of breaking down cyclin B, which regulates MPF. Since the synthesis of cyclin B is consistent during this phase, its persistent degradation is necessary to inactivate MPF and to ensure MII stage exit.[107,108] However, a single increase in intracellular [Ca^{2+}] is not always sufficient for the complete degradation of cyclin B. An early stop to the [Ca^{2+}] surges followed by an initial decline, results in the recovery of MPF activity leading to irregular progression of the cell cycle, as well as in certain cases, meiotic re-arrest.[108] Therefore, in addition to the application of ethanol and [Ca^{2+}] ionophores, compounds that maintain low levels of MPF activity and ensure the switch to interphase are incorporated into the activation medium. These include supplementing the medium with broad-spectrum **kinase or protein synthesis inhibitors**, such as cycloheximide, 6-dimethylaminopurine (6-DMAP) and puromycin, for several hours following treatment with ionomycin for example, which induces a calcium influx.[10,56]

On the other hand, repetitive [Ca^{2+}] surges that persist for a few hours after artificial activation, mimicking signals more similar to the mechanism of sperm activation, can be obtained with the addition of strontium chloride ($SrCl_2$), thimerosal, or acetylcholine.[109] Furthermore, intracellular [Ca^{2+}] oscillations may be induced by injecting IP_3R agonists into oocytes, although this method is less appropriate when high numbers of oocytes are to be injected.[91] Finally, together with extracellular [Ca^{2+}], electrical DC pulses may be applied to induce several surges in [Ca^{2+}], resulting in high rates of embryo development to the blastocyst stage.[110,111] Nonetheless, refined equipment as well as optimization of the technique are necessary for the constant usage of electrical pulses, which is the reason for its limited adoption. Importantly, a widespread method of oocyte activation cannot be prescribed for all species. Species-specific treatments must be carefully chosen according to rates of successful oocyte activation, subsequent embryo development, zygote survival and efficiency of the application.[91]

General activation methods for mouse oocytes in SCNT

Amongst mammalian species, the SCNT technique in the mouse is one of the most advanced.[91] Numerous activation protocols which include treatment with ethanol, $SrCl_2$, and electrical pulses have been used to successfully obtain cloned pups.[112] Of the abovementioned treatments, the most common method for the creation of SCNT embryos is the application of $SrCl_2$. The mechanisms and channels by which extracellular $SrCl_2$ is transported into the cell are poorly understood;[112] however, it is believed that $SrCl_2$ treatment causes oscillations and promotes the release of [Ca^{2+}] from the endoplasmic reticulum into the cytosol.[113] According to Wakai and colleagues,[91] exposure to $SrCl_2$ induces oscillation patterns that have much longer rises than observed after normal fertilization, and are therefore noticeably different from the patterns caused by sperm. Nevertheless, all the events involved in oocyte activation are initiated and completed by the application of $SrCl_2$. Supplementation with protein synthesis or kinase inhibitors is unnecessary, thus the risks of affecting broader targets and subsequent non-specific effects are avoided. Oocyte activation by means of $SrCl_2$ satisfies the criteria of parthenogenesis, which aims to achieve high levels of oocyte activation along with continued support for high levels of pre- and post-implantation development of the SCNT embryos.[91]

1.3.7 Improving mouse cloning by technical developments

Since the first article in 1997 of the cloned sheep named Dolly,[8] numerous reports of successful cloning by SCNT in many mammalian species have been described. Despite these successes, the efficiency of cloning, particularly in mice, is still quite low. The blastulation rate of enucleated mouse oocytes that have undergone SCNT is 30-50%;[18] however the rate of full-term development of mice cloned by SCNT is only 5-6%.[63] Additionally, several studies have reported many abnormalities in cloned mice, including abnormal gene expression in embryos,[114-116] placental abnormalities,[41,42] obesity,[45,117] and premature death.[46] The laboratory mouse is the most popular species to investigate new avenues of SCNT, and is favourably comparable to expensive, highly outbred, and large farm animals. This is attributed to the short gestation period of 19.5 days, the short generation period of approximately 2 months, the affordability and small stature, as well as the reasonably good understanding of developmental biology of the mouse.[63] The unanswered question is, what causes such a low success rate in mouse cloning? Due to the relatively long SCNT procedure and the stressful implications that micromanipulation may have on oocytes and donor cell nuclei, several groups have attempted to improve mouse cloning efficiency[63,118,119] by concentrating on the technical aspects involved in SCNT. Despite altered methods and technical improvements, there has been no effect on the overall success rate of SCNT and ultimately on mouse cloning. However, there are several technical rules that should be followed when performing SCNT in mice; these will be reviewed in the paragraphs that follow.

1.3.7.1 Oocyte donor mouse strain

According to Kishigami and colleagues[78] and Ogura,[120] BDF1 is the best and preferred choice of mouse strain. First generation hybrid mice such as B6D2F1 (C57BL/6 x DBA/2) or B6CBF1 (C57BL/6 x CBA/J), between the ages of 8 and 12 weeks, are the most appropriate oocyte donors. Oocytes are produced efficiently by these strains and micromanipulation of these oocytes is relatively easy. Embryos derived by SCNT from these oocytes have shown better *in vitro* and *in vivo* development compared to oocytes from inbred and outbred mouse strains.[82,120]

1.3.7.2 Oocyte activation timing

Wakayama and colleagues[121] tested the microinjection of cumulus cell nuclei into oocytes that were activated prior to enucleation. This method resulted in high

chromosomal fragmentation and poor ongoing development. On the contrary, when activation was performed directly after or within a few hours of cumulus cell injection into cytoplasts, several cloned embryos reached full-term development.[9,121] Therefore, the timing of oocyte activation in SCNT is crucial.

1.3.7.3 Choice of activation protocol

Artificial activation is required to stimulate the developmental progression of SCNT manipulated oocytes. A study performed by Kishikawa and colleagues[122] compared four different oocyte-activating agents, which included treatments with ethanol and strontium, electrical stimulation, and the microinjection of sperm. The results between the four methods were not statistically significant regarding overall efficiency rates, suggesting that the choice of activation protocol used for mouse SCNT is not a critical determinant.[122]

1.3.7.4 Timing of oocyte spindle removal

Another possible factor is the effect of oocyte enucleation itself. It is unknown whether the enucleation technique impairs ongoing development or if certain unknown reprogramming factors are removed during enucleation. For this reason, Wakayama and colleagues[123] first studied the effects of injecting donor cell nuclei into non-enucleated oocytes, followed by the enucleation of the oocyte meiotic spindle complex. The success rates of this method were comparable to the original protocol. These results therefore suggested that the low success rate of SCNT (full-term development of 5-6%[63]) could not be caused by either oocyte chromosome enucleation or the possible removal of factors essential for epigenetic reprogramming.[123]

1.3.7.5 Consequence of cytokinesis inhibitors

In the original mouse cloning protocol by Wakayama and colleagues,[9] CB and cytochalasin D (CD) (which both disrupt F-actin filaments) were most commonly used during activation of SCNT manipulated mouse oocytes, in order to prevent inclusion of donor somatic cell nuclei into the extruded PB.[9] Nocodazole, which affects microtubule assembly, has also been used.[124] Activation of SCNT manipulated oocytes treated with a combination of nocodazole and CB or CD showed comparable success rates in the production of cloned mice, and therefore it was assumed that cytokinesis inhibitors did not negatively affect development.[121] On the other hand,

when Latrunculin A (Lat-A) (which affects actin filaments and binds G-actin) was used in place of CB, the success rate in terms of full-term development of cloned embryos was significantly improved.[125] During the standard activation protocol, SCNT embryos must be washed after exposure to CB for 6 hours due to the toxicity of CB, and are then cultured further for an additional several hours with just Trichostatin A (TSA) (which is a histone deacetylase inhibitor [HDACi]) to ensure efficient epigenetic reprogramming. Since Lat-A is less toxic that CB, a new protocol proposed by Terashita and colleagues[125] permits SCNT embryos to be cultured continuously with TSA for the entire 10 hour period, eliminating the one step that required washing of the oocytes, ultimately decreasing the time during SCNT experimentation (Figure 1.8). A study investigating the effect of supplementing culture medium with 100 µM vitamin C for at least 16 hours after activation, in combination with using Lat-A during micromanipulation and activation, reported significantly increased rates of mouse blastocyst formation. The mean number of ICM cells at 96 hours post activation and the total blastocyst cell number were also increased.[126] The abovementioned results suggest that by optimizing each technical step involved in the SCNT procedure in mice, an overall improvement in cloning efficiency can be achieved.

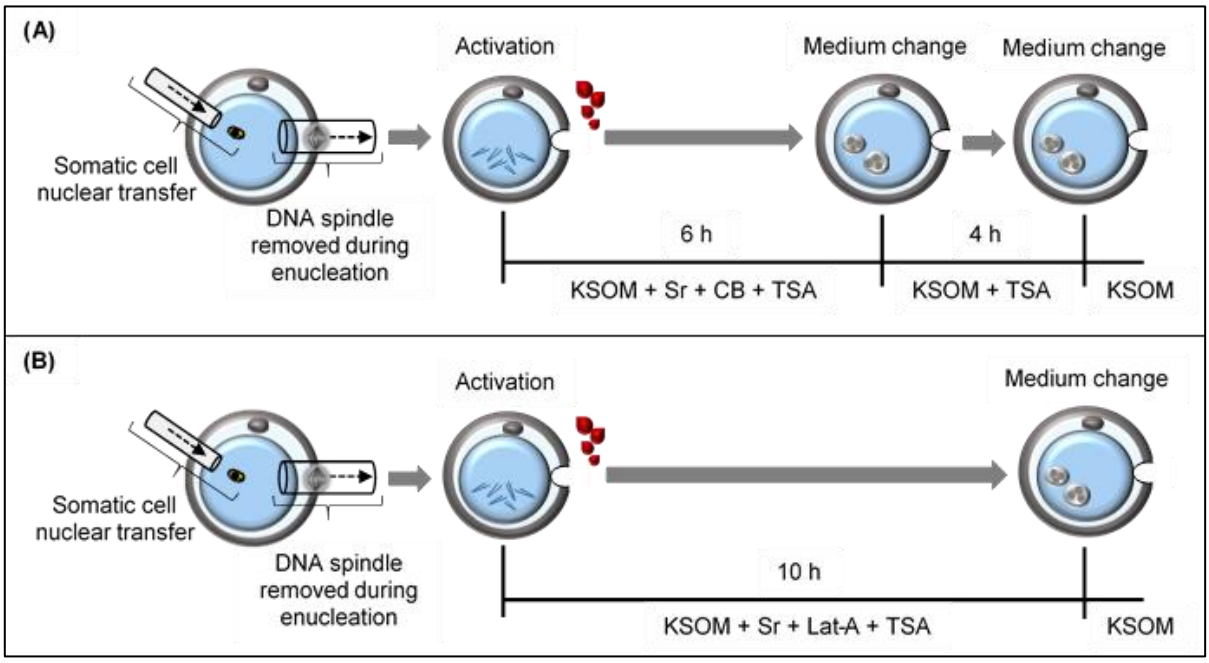

Figure 1.8: Schematic representation of (A) the standard SCNT activation protocol and (B) the new **Lat-A treatment protocol**.[63,125] Dashed arrows represent removal of the DNA spindle during enucleation, and transfer of the donor cell into the cytoplast during SCNT.

1.4 Donor cell type and cell cycle synchrony

In 1997, the first cloned mouse was born by the transfer of an adult cumulus cell nucleus using a novel single step SCNT technique known as piezoelectric nuclear transfer[a].[9] The application of piezo-actuated micromanipulation was discovered and reported by Yanagimachi and colleagues.[127] Lysis of many oocytes, due to the difficulty of injecting delicate mouse oocytes using standard micropipettes, was ultimately overcome by using piezo-manipulation. Technique effectiveness in preventing lysis during mouse oocyte injection was reported, and this new technique allowed for larger micropipettes to be used in nuclear transfer.[127]

To date, the somatic cell nuclear donors that are routinely used for the production of cloned mice include cumulus cells,[9] tail-tip fibroblast cells,[41] fetal fibroblast cells,[128] immature sertoli cells,[129] and ESCs.[44] The success rate of producing cloned mice is generally higher when ESC nuclei are used as opposed to somatic cell nuclei.[44,130] This suggests that the efficiency of cloning may increase if donor cells in an undifferentiated state are used. Several reports of full-term development following SCNT with undifferentiated donor cell types, such as neuronal,[131] hematopoietic,[132] mesenchymal[133] and keratinocyte stem cells,[134] have shown equivalent or lower efficiency rates compared to differentiated somatic cell nuclei data. One group reported that differentiated donor cells were more efficient than adult stem cells for cloning by SCNT.[135] In a recent study that cloned Macaque monkeys by SCNT using adult cumulus cells and fetal fibroblasts, SCNT efficiency using adult cumulus donor cells was lower than when fetal fibroblast were used as donor cells.[34] This may be a result of the less efficient reprogramming of adult nuclei as opposed to fetal nuclei, or it could be attributed to a difference in the type of somatic donor cell used.[34]

According to a study that compared the cloning efficiency of cattle using ovarian cumulus, mammary epithelial, and skin fibroblast donor cells, the type of donor cell can significantly affect embryo development;[136] in terms of *in vitro* and full-term development, cumulus donor cells were the most effective. The results suggest that cumulus cell DNA may be reprogrammed more effectively after SCNT. An earlier study

[a] *Piezoelectric nuclear transfer* offers direct nuclear injection with minimal transfer of cytoplasm from the donor cell, also bypassing the need for fusion. This is achieved by exposing the piezoelectric material to an electric field, which induces very powerful and accurate directional movement of the pipette tip to cut the zona during zona drilling and to penetrate the oolemma during nuclear transfer.[57]

in mice also reported an increase in live birth rate from cumulus donor cells when compared to sertoli and neuronal donor cells.[9] Numerous types of somatic cell donors have been investigated; the agreement from several laboratories is that cumulus donor cells achieve the highest cloning efficiency with the least abnormalities in cloned animals.[9,136-138] Additional studies are required to research the reprogramming of adult somatic cells for future use in SCNT. See Table 1.2 for success rates using various donor cell types.

Table 1.2: The success rate of full-term development of mice cloned from different donor cells

Donor cell type	Donor age	Success rate
Cumulus (B6D2F1)	Adult	2.5-4.5%[9,139]
Cumulus (129B6F1)	Adult	3.2%[140]
Cumulus (BDF1x129/Sv)	Adult	**15.6%**[125]
Tail-tip fibroblast	Adult	1.1-4.8%[41,141]
Fetal fibroblast	Fetus	3.0-3.7%[128,139]
Sertoli (B6D2F1)	Newborn	4.5%[129]
Sertoli (B6129F1)	Newborn	10.8%[140]
Neuronal stem cell	Newborn	0.5%[131]
Neuronal stem cell	Fetus	1.6%[133]
Hematopoietic stem cell	Adult	0.7%[132]
Keratinocyte stem cell	Adult	5.4%[134]
ESC (G1 phase)	Embryonic	12.3%[44]
ESC (G2/M phase)	Embryonic	6.4%[44]

Synchronization of the cell cycle between the recipient oocyte and the donor cell nucleus in SCNT is important to ensure successful epigenetic reprogramming and ultimately full-term development.[8] After the birth of Dolly, it was proposed that the donor somatic cell should be in the quiescent (G0) phase of the cell cycle during SCNT.[8] However, according to Wakayama and Yanagimachi,[41] the cell cycle phase of the donor does not affect cloning efficiency in mice. Several other studies have proposed that successful cloning can be achieved with donor cells in the G0, G1, G2, and M phases of the cell cycle.[44,142,143] In SCNT, the introduction of G0, G1, and M

phase donor cells into MII stage oocytes is performed routinely, preventing both DNA damage and unplanned DNA replication of the donor cell. Compatibility between the donor cell cycle stage and elevated MPF activity in MII oocytes is recommended. In MII stage oocytes, elevated MPF activity promotes the donor cell nucleus to undergo nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC).[53,144] Nuclear reprogramming in SCNT is believed to be promoted by PCC. Although the exact mechanism has not been identified, it has been proposed that NEBD and PCC may facilitate the release of somatic factors that are bound to chromatin. As a result, donor cell chromatin is more accessible to oocyte factors playing a role in reprogramming and DNA synthesis.[145] The remodelling and/or reprogramming of a somatic cell nucleus in SCNT are believed to be accelerated by high levels of MPF and mitogen-activated protein kinase (MAPK) activity; however, a recent study found that both MPF and MAPK activity are not necessary for the initial step in nuclear reprogramming and/or remodelling of the chromatin.[146]

1.5 Epigenetic reprogramming in SCNT

Epigenetics can be defined as the study of phenotypic deviations that occur in cells controlled by gene expression and the modifications made to the chromatin structure, which may switch genes on or off, without altering the genotype of the cell.[11,147,148]

Numerous reprogramming events occur during cell differentiation, and SCNT is one of the most effective techniques for studying this phenomenon. Cloning with somatic cell nuclei has shown that epigenetic modifications within a differentiated genome can be altered to the totipotent state.[7] However, despite the fact that nearly 20 years have passed since the first mammal was successfully cloned from an adult somatic cell,[8] the complete reprogramming of a differentiated somatic cell nucleus remains inefficient and the mechanisms by which this phenomenon is achieved are not yet fully understood.

To recognize the essential mechanisms involved in embryo development following SCNT, gene expression during normal development and regulation must be discussed. Chromatin architecture is very complex and plays a vital role in regulating gene expression. Changes in chromatin structure, and ultimately patterns of gene expression, are modulated by DNA methylation, histone subunits and the composition of nuclear lamins, and are followed by histone post-translational modifications

including acetylation, phosphorylation, and methylation. Nuclear composition alters drastically during embryogenesis as well as during the specialization of nuclei in specific tissues. To ensure successful SCNT, the pattern of epigenetic modifications in the differentiated nucleus of the donor cell should undergo remodelling to replicate the pattern present in the nucleus of a fertilized embryo. In addition, the cytoplasm of the arrested MII oocyte should assist the remodelling process. Nuclear remodelling is defined as a change in chromatin structure and is known to alter the pattern of genes that are to be transcribed, which is known as nuclear reprogramming. The difference between nuclear remodelling and nuclear reprogramming is emphasised to avoid misperception, as remodelling refers to the structural rearrangement of the DNA, while reprogramming is a consequence of those physical changes.[147,149]

1.5.1 Nuclear remodelling and reprogramming in embryogenesis

During SCNT, the nucleus undergoes structural modifications, which are better understood by describing the general structure of the somatic cell nucleus, as well as that of the pronuclei in a zygote. The pronuclei are surrounded by a unique environment where very little to no transcription occurs within the zygotic cytoplasm, where factors are contained directing the first few cell divisions after fertilization.[147] Embryonic genome activation eventually kicks in once the embryo begins producing sufficient amounts of RNA, at which point significant transcription can begin.[147] This occurs at species-specific cell stages, for example at the 2-cell stage in mice[150] and during the 4 to 8-cell stage in humans.[151] At this stage, true control over embryo development is maintained by the developing embryo's own nuclei. As the embryo passes through each developmental stage, protein associations with the nuclei change.[152] For example, when the ICM and the trophectoderm (TE) (which are the first two distinguished cell types) are formed, a specific set of genes is transcribed along with specific proteins associated with the nuclei for each different cell type.[153] Subsequently, tissue formation and specialization occur, each with their own unique nuclear structure and set of genes that are transcribed.[147]

1.5.1.1 DNA methylation

DNA methylation and histone post-translational modifications occur after SCNT. This includes methylation, acetylation, phosphorylation and ubiquitination. In mice, DNA methylation and histone modifications have been well described during normal embryo development. However, since several species-specific differences exist, one should

be mindful that patterns of development in one species do not necessarily replicate those in another species. Nonetheless, the arrangement of local chromatin is altered by DNA methylation, which is generally associated with the inhibition of transcription.[13] Patterns of DNA methylation differ between early mammalian embryos and maternal and paternal genomes.[154] At fertilization, high levels of DNA methylation are present in the DNA of sperm and oocytes. During preimplantation development in the mouse, total DNA demethylation occurs as the paternal genome undergoes active demethylation after fertilization, which causes the decondensation of sperm DNA and establishment of the paternal pronucleus.[155] In addition, and contrary to the paternal genome, the maternal genome undergoes passive demethylation during the first few cell divisions.[155,156] At the approximate time of ICM and TE specialization, new patterns of DNA methylation are regulated by DNA methyltransferases, followed by lineage-specific methylation as cell specialization is determined.[147]

1.5.1.2 Histone modifications

DNA compaction is achieved through interaction with histone proteins. Acetylation and methylation are generally the most extensively investigated histone modifications. Histone acetylation reduces the association between the histone and tightly packed heterochromatin, which usually results in the newly accessible euchromatin which can undergo active transcription. Transcriptional activation or repression may be a result of histone methylation, dependent on the histone residue altered.[157] In conjunction with DNA methylation, histone modifications are also changed during normal embryogenesis. Directly after fertilization in mice, the histone modification patterns of the paternal and maternal genomes are irregular. The paternal pronuclei histone H4 is hyperacetylated in comparison to that of the maternal pronuclei.[158] In contrast, the maternal pronuclei contain high levels of other histone residues which are not present in the paternal pronuclei. At the blastocyst stage, deviation in the histone modification profiles of the ICM and TE are noted.[27] Histone modification reprogramming is more complicated than that of DNA methylation; however similar to DNA methylation, stage-specific and cell type-specific changes occur.[147,148]

1.5.1.3 Associations of epigenetic events

The array of epigenetic modifications and the control of transcription during normal embryo development are complex, with optimal epigenetic regulation being reliant on the interaction between DNA methylation and histone modifications.[159] An inverse

relationship exists between histone acetylation at certain sites of DNA and the resultant methylation of surrounding chromatin. Transcription is affected by several mutually supporting interactions between DNA methylation and histone modifications (Figure 1.9).[160] An example of the multiple levels of epigenetic regulation in embryogenesis is the remodelling of the paternal chromatin that occurs after fertilization until the first cell division. Sperm DNA is highly compacted due to its interaction with protamine. After fertilization, protamine is removed and replaced with acetylated histones to help maintain the newly folded DNA in an open, loosely packed conformation.[161] To prepare the DNA for transcription, reprogramming of the genome is performed through the combination of histone modifications, progressive DNA demethylation, loss of oocyte-specific histones, and the acquisition of non-histone proteins.[147,149,161]

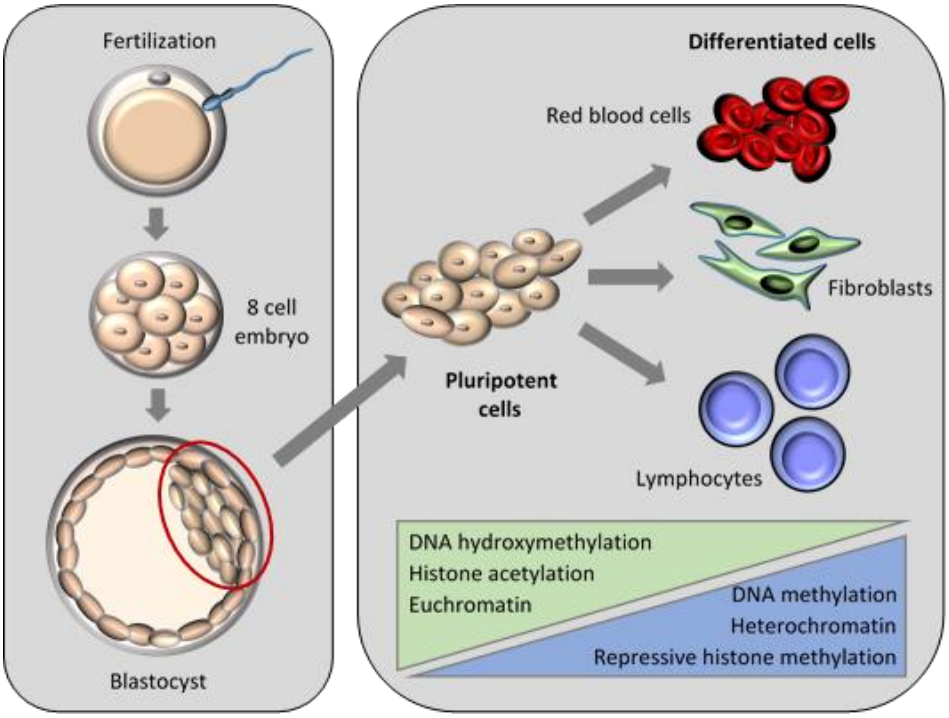

Figure 1.9: **Epigenetic interactions** during the progression from pluripotent to differentiated cells *(adapted from reference* [148]*)*.

1.5.2 Nuclear remodelling and reprogramming in SCNT embryos

Once a somatic cell nucleus is introduced into an oocyte, several sequential events must occur to ensure successful reprogramming. The structure of the chromatin within the nucleus is remodelled, consequently erasing the differentiated epigenetic markers of the somatic cell. This is completed by reprogramming the developmental gene

expression pattern to one that mimics that of a normally fertilized embryo. Following appropriate activation, the reconstructed SCNT embryo undergoes the equivalent developmental sequence and subsequent embryonic gene expression as observed in a normal zygote.[147] The transcriptional silencing of the somatic nucleus requires structural remodelling. This includes nuclear membrane breakdown, chromatin condensation, spindle assembly, the release of somatic cell-specific proteins from the nucleus into the ooplasm, the acquisition of certain ooplasmic-specific proteins from the ooplasm by the transferred nucleus, and the establishment of a structure comparable to a pronucleus after activation.[162]

Well-known examples of protein exchange after SCNT in mice include the histone variants H1FOO and MacroH2A. Histone subunits are linked together by histone H1 which make up the nucleosome. An oocyte-specific alternative of histone H1 exists; H1FOO that quickly replaces histone H1 when a somatic cell nucleus is introduced into the ooplasm.[163] MacroH2A is present in somatic cells and absent from the nuclei of fertilized embryos until the first few cell divisions. After SCNT, MacroH2A is eliminated from the chromatin and broken down. At the morula stage of embryo development, MacroH2A is then re-established and accumulates into the chromatin, as in normally fertilized embryos.[164]

For effective reprogramming, it is assumed that the somatic cell pattern of epigenetic modifications must be reversed prior to embryonic genome activation[165], at the 2-cell stage in mice[150] and the 4-cell stage in humans.[151] Incomplete epigenetic remodelling and aberrant patterns of DNA methylation or histone acetylation in SCNT embryos have been identified in numerous studies, and all contribute to the inefficiency of SCNT.[18,38,166] Following SCNT, the somatic cell genome does not react to the ooplasmic activity of dynamic demethylation, therefore SCNT embryos have increased DNA methylation levels as opposed to normal embryos.[167] Rapid deacetylation of histones is another result of SCNT, as well as abnormal patterns of histone methylation in SCNT embryos.[27,168]

1.5.3 Improving SCNT with chromatin remodelling agents

Reprogramming is reliant on chromatin remodelling, emphasising the need to improve the modification process. Although ooplasmic factors present in the oocyte can facilitate remodelling to some extent, they cannot modify all nuclei. One of the key

events for successful remodelling is the unrestricted exchange of proteins between the ooplasm and the transferred nucleus that takes place at the DNA level.[147] Chromatin is tightly packed resulting in physical limitations to protein exchange; any approaches that would unravel this structure may be beneficial to the modification process.[147] Treatments that encourage the somatic genome to imitate normal DNA methylation and chromatin remodelling have been investigated to aid epigenetic reprogramming and ultimately cloning efficiency. These treatments entail preparing donor cells or treating SCNT embryos with a reagent that decreases DNA methylation, such as 5-aza-20-deoxycytidine, or HDACi that increases histone acetylation. Improved SCNT embryo development and cloning efficiency have been described in several studies following only a few of these methods; however, no improvement has been reported following 5-aza-20-deoxycytidine treatment of donor cells before SCNT.[21,169]

Reprogramming of somatic nuclei should occur before embryonic genome activation, therefore the unwinding of chromatin by increasing histone acetylation may effectively assist this process.[147,170] Global histone acetylation is achieved with HDACi that functions by inhibiting histone deacetylases, causing chromatin structure alteration which allows proteins such as RNA polymerases to easily infiltrate the DNA and begin transcription.[19] Histone acetylation in HDACi-treated SCNT embryos is increased, and is beneficial after the reconstructed embryos have undergone activation.[171] Trichostatin A is a commonly used HDACi in SCNT that enhances DNA demethylation.[172] In 2006, two independent groups established the appropriate conditions for TSA treatment of SCNT mouse embryos including concentration, time point, and period of exposure.[18,173] One group reported a fivefold increase in mouse clone survival by enhancing oocyte activation with TSA.[18,119] Blastocyst development rates in SCNT monkey embryos treated with TSA have improved from 4% to 18%.[174] Despite the enhanced blastocyst development rate, blastocyst quality and the possible establishment of stable ESCs remained unknown.[174] A recent study also confirmed the improved blastocyst development and pregnancy rates of monkey SCNT embryos, after treatment with TSA at the one-cell stage.[34] In a different study, an assumption was made that high levels of TSA may have a negative impact on blastocyst quality, even though blastocyst formation was promoted with TSA treatment.[36] According to these studies, the most effective TSA treatment protocol in mice includes at least 10

hours of continuous TSA exposure of the reconstructed oocytes from the time of oocyte activation, but before the first cell division.[18,173] The suggested TSA concentration is 5-50 nM, since TSA shows effectiveness from 5 nM but becomes toxic at 500 nM.[18] Since the narrow window for successful reprogramming occurs before embryonic genome activation, the timing of TSA treatment is vital to its effectiveness on the developmental potential of the reconstructed embryos.[170]

Although the rate of blastocyst formation is improved, another study reported that none of the SCNT embryos treated with TSA gave rise to animals that persisted through to adulthood.[32] In addition, TSA is teratogenic[175] since use of high concentrations may be detrimental to the quality of blastocysts and will significantly reduce normal development of the embryo.[19] Scriptaid, another potent HDACi, has a lower toxicity level than TSA and through the ability to increase transcription and protein expression, has resulted in significant improvement in the creation of cloned mice[19] and pigs.[26,27] Many studies have attempted to use other HDACi to improve SCNT; however, TSA currently remains the best approach for mouse cloning, despite controversial outcomes in farm animals.[176,177] The mechanism through which HDACi improves the efficiency of cloning is most likely related to the capability to encourage the synthesis of nascent mRNA, following the increase in histone acetylation.[19] Even though incomplete, histone acetylation remodelling in reconstituted embryos is enhanced with HDACi treatment after SCNT.[27]

1.6 SCNT applications

In humans, the idea of SCNT as a technique to generate specific ESCs from the somatic cells of an individual could ultimately lead to the understanding of disease mechanisms, as well as improve the efficiency of cell-based therapies for the treatment of degenerative diseases with a negligible risk of immune rejection.[4,178-180] The potential to create new gametes for animals, and in future for human patients, was made more realistic by Hayashi and colleagues.[181,182] This group accomplished the production of viable sperm and oocytes from ESCs and induced pluripotent stem cell (iPSC) derived germ cells. The gametes successfully completed development and produced several generations of offspring. Research comparing the possibility of SCNT and iPSC derivations to produce gametes is of great interest and will continue in the future.

Other potential clinical applications include assisted reproductive procedures that prevent the transmission of mitochondrial DNA (mtDNA) diseases from a mother to her offspring, as well as the treatment of infertility due to defects in the cytoplasm of oocytes.[76] Mitochondrial transfer, also known as three-parent IVF, is performed using the same techniques applied in SCNT, by transferring the patient's meiotic spindle complex into enucleated donor oocytes which have healthy mitochondria (Figure 1.10).[183,184]

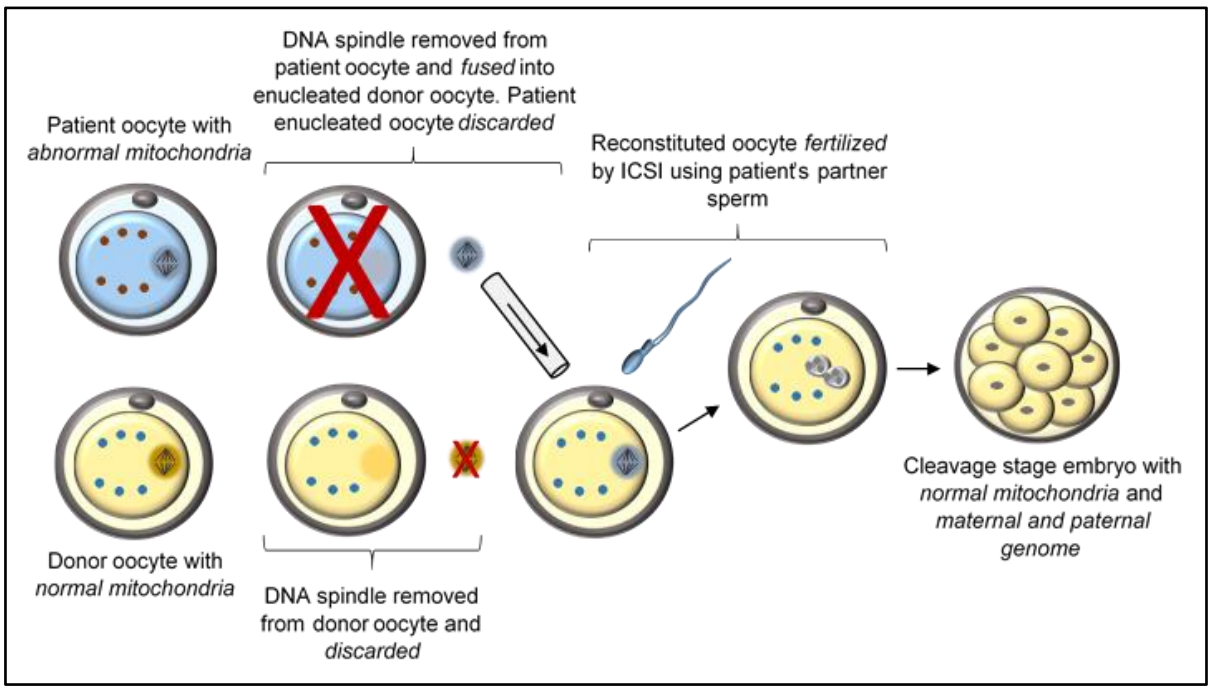

Figure 1.10: Spindle transfer technique in **three-parent IVF**.[183]

1.7 Ethics and legalities of SCNT in humans

Many countries and organisations have prohibited human reproductive cloning.[185] Human cloning in all forms has been banned by the United Nations, emphasising the incompatibility of human cloning with human dignity and the protection of human life.[186] Creating a human being that shares the same nuclear genome as another living or dead being is also prohibited by the European Council.[187] A recommendation by the International Society for Stem Cell Research is that the gestation or transfer of a human embryo created by SCNT or by other nuclear reprogramming techniques into a uterus should be banned.[188] Reproductive cloning is also prohibited in several countries that take part in large stem cell research programs, including the United States of America (USA), China, Germany, and South Korea.[185] The use of reproductive SCNT as an option for the treatment of infertility has sparked widespread

debate. The American Society for Reproductive Medicine (ASRM) has presented valid arguments against reproductive SCNT and concluded that it is unethical to use it as an assisted reproductive technology due to safety concerns and the undefined effect on children, relatives and humanity.[189]

As reviewed by Cervera and Mitalipov,[10] several ethical and legal implications are associated with SCNT. One of the main drawbacks with SCNT experimentation in humans is evidently the availability of oocytes. In addition to this limitation, there are financial and ethical implications related to obtaining human oocytes for research purposes.

The technique of SCNT is presently performed in several laboratories worldwide to create human stem cells. In the United Kingdom (UK), human SCNT research is legal and in 2001 was included in the Human Fertilization and Embryology Act 1990.[190] However, before performing SCNT, it is necessary to obtain permission from the Human Fertilization and Embryology Authority.[191] In the USA, SCNT research is also legal but is not allowed to be funded by the national government because of the Dickey-Wicker Amendment bill passed in 1995.[10] In the USA, the Department of Health and Human Services, and the National Institutes of Health forbid the use of funds for research studies involving the creation of human embryos and the destruction thereof.[10] Nonetheless, SCNT research aimed at producing human ESCs may be legally performed when funded by private or non-governmental organisations.[192] With regards to the techniques involved in mitochondrial transfer, the Nuffield Council on Bioethics of the UK[193] provided overall ethical approval for the use of such techniques to prevent the transmission of mtDNA disorders in humans. After much debate beginning in 2012, in February 2015 the UK parliament voted in support of mitochondrial replacement legislation for human clinical application.[194,195] Despite the approval and apparent clinical utility, Yamada and colleagues[196] highlighted a concern that mitochondrial transfer may lead to reversion of the mtDNA genotype, even with the smallest amount of mtDNA carried over during the procedure. To steadily prevent mtDNA disease transmission, this concern would need to be monitored in all patients and ultimately sidestepped in the clinical setting.

Strict regulation of human SCNT research should however be maintained by local institutional research ethics boards, as well as by ethical guidelines that have been

established by the USA National Academy of Science, the International Society for Stem Cell Research, and the ASRM.[192] Local laws regulating compensation for oocyte donors may also govern the procurement of human oocytes for research purposes. In California for example, patients donating oocytes for research purposes are covered for certain expenses, but are not reimbursed for "time, effort and inconvenience".[192] In Oregon on the other hand, research oocyte donors are fully compensated in a manner that is equal to reproductive oocyte donors.[197]

In the South African context, reproductive cloning is banned. Therapeutic cloning and research involving human oocytes and embryos including SCNT, are all acceptable with the requirement that ministerial authorisation needs to be obtained.[198] However, the National Health Act does not address matters regarding oocyte donation specifically for research purposes. Regarding the payment of oocyte donors in South Africa, details are provided in the 2008 guidelines of the Southern African Society for Reproductive Medicine and Gynaecological Endoscopy (SASREG).[199] According to these guidelines, "monetary compensation of the donor should reflect the time, inconvenience, financial costs to the donor - e.g. travel, loss of income and childcare costs, physical and emotional demands and risks associated with oocyte donation and should be at a level that minimizes the possibility of undue inducement of donors and the suggestion that payment is for the oocytes themselves. The monetary compensation should not be predicated on the clinical outcome (no. of oocytes or pregnancy outcome) but rather on fair compensation for the procedure of donating eggs. Donors should only receive financial compensation via fertility clinics and not receive any compensation directly from the recipients or other third parties". An amendment was made to the SASREG Guidelines for Gamete Donation on the 25 November 2014, which now states that "egg donors should not be compensated more than R 7 000.00 per procedure from 1 January 2015".[200]

Alternative sources of human oocytes have been investigated for SCNT research because of the financial and ethical burdens related to reimbursement of oocyte donors. Immature oocytes, which are generally discarded in assisted reproductive procedures, are voluntarily donated by patients for research purposes.[10] However, *in vitro* maturation, fertilization, and subsequent development of these immature oocytes to the blastocyst stage are highly compromised following SCNT, and are therefore not appropriate for optimization of the SCNT procedure.[201] An ideal approach would be to

collect donors willing to donate oocytes solely for research purposes without reimbursement.[10] However, women are simply not prepared to undergo ovarian stimulation and invasive oocyte retrieval without being reimbursed for their efforts.[202]

It should be noted that even the use of high quality human oocytes in SCNT does not guarantee successful embryo development to the blastocyst stage.[83] In a study conducted by Noggle and colleagues,[83] successful blastocyst development and subsequent isolation of ESCs was only observed in those embryos that had somatic cells transferred to non-enucleated oocytes. This observation may imply that unknown factors essential for proper reprogramming of the somatic cell nucleus may be removed during enucleation and are retained by the presence of the oocyte's own meiotic spindle complex. Another study reporting early failure in monkey SCNT embryo development also assumed the cause was related to the removal of reprogramming factors during enucleation.[1] Subsequent research has however shown that the oocyte meiotic spindle complex is not a prerequisite for the successful reprogramming of the somatic cell nuclear genome.[48]

Nevertheless, many studies encourage that each step in SCNT be thoroughly optimized and adapted specifically for human oocytes. However, a major drawback related to rigorous testing on human oocytes is the requirement for many good-quality human oocytes, which remains limited.[10]

1.8 Conclusion

Considering theoretical and epigenetic perspectives, mouse SCNT has provided important information necessary for the overall technical improvements of SCNT techniques.[203] The cytoplast plays an important role in SCNT by preventing premature activation. Additionally, careful fusion of the somatic cell and efficient activation are required to effectively reprogram the somatic cell nucleus to the pluripotent state.[180] The quality of oocytes has always been important and appreciated, where positive SCNT outcomes have correlated with excellent donor oocytes in both non-human primates[48] and humans.[36,81] In the near future, oocyte quality may be considered a less detrimental factor as continual protocol modifications are made.[180] The poor success rates, in terms of SCNT embryo development, may be attributed to the incomplete or inefficient reprogramming of the somatic cell nucleus required to support

ongoing development.[148,161,180,204] Continued research and protocol optimization should be encouraged for improvement of reprogramming efficiency by SCNT.

Despite the many technical, ethical, and legal implications associated with SCNT, this field of research holds great potential. In addition to the extensive practical applications of SCNT, the technique may offer unique and intriguing experimental approaches to genomic research, and more specifically to epigenetics.[149] Research in this field may provide insight into the reprogramming ability of the somatic cell genome to a totipotent state, identical to that of a fertilized oocyte.[176] The importance of SCNT research in the mouse is emphasised and encouraged by the potential to understand underlying principles of nuclear reprogramming. The reversibility of these epigenetic processes facilitates many new prospects in basic research, and almost certainly in time to come, cell transplantation and regenerative medicine.[56,180,203]

1.9 Aim

The aim of this study was to optimize and determine the efficiency of the techniques involved in SCNT using a B6D2F1 mouse model.

1.10 Objectives

The objectives of the following studies were to:

1.10.1 Study 1

- Enucleate the DNA spindle complex from the mouse oocyte without lysis of the oocyte.
- Artificially induce activation in the manipulated oocytes by using chemical reagents that mimic natural sperm-induced fertilization.

1.10.2 Study 2

- Enucleate mouse oocytes followed by the transfer of a somatic cell into the PVS of the cytoplast.
- Induce fusion of the cells using a specific cell-fusion mediator known as the inactivated envelope of the HVJ.
- Artificially induce activation in manipulated oocytes by using chemical reagents that mimic natural sperm-induced fertilization.
- Achieve *in vitro* development of at least 20 blastocysts.

Chapter 2: Materials and Methods

2.1 The path to SCNT travelled

This project was undertaken based on the knowledge gained from the Department of Pediatrics and the Naomi Berrie Diabetes Center, Columbia University, New York, USA. Observation and training in laboratory techniques involving nuclear transfer and the development of SCNT protocols was necessary to initiate this research. Dr Dietrich M. Egli, Assistant Professor of Developmental Cell Biology and head of the Egli Lab at Columbia University, supervised the training. Courses were attended at Columbia University on infection control, biological safety/blood borne pathogen training, lab safety, chemical hygiene and hazardous waste management. Related to the SCNT procedure, oocyte retrieval from mice (cervical dislocation, dissection etc.), medium preparation, setup of the micromanipulator for the procedure, spindle enucleation and nuclear transfer techniques in mouse oocytes were observed. Dr Egli also demonstrated human oocyte to oocyte spindle enucleation and transfer at The New York Stem Cell Foundation, where he is a Senior Research Fellow.

2.2 Importation of DBA/2 and C57BL/6 mice to breed B6D2F1 hybrids

Ten DBA/2 mice (5 male and 5 female) and 10 C57BL/6 mice (5 male and 5 female) were acquired from Jackson Laboratory, Bar Harbor, Maine, USA. Following approval from the UP Faculty of Health Sciences Research Ethics Committee (358/2015) in August 2015 and the Animal Ethics Committee (H010-15 extension period: June 2017 - June 2018) initially obtained in September 2015, an application for permission under section 20 of the animal diseases act (Act 35 of 1984) to perform research/study was submitted to the Department of Agriculture, Forestry and Fisheries (DAFF), and approved in November 2015. An import permit was then received in May 2016. The mice were ordered in June 2016 and delivered on the 21st of July 2016. A period of 2 months was needed to establish the mouse colony before the B6D2F1 hybrids could be bred at the University of Pretoria's Biomedical Research Centre (UPBRC) situated at the Onderstepoort campus. The first hybrid mating pairs (C57BL/6 x DBA/2) were set up at the beginning of October 2016 and the first hybrid pups (B6D2F1) were born on the 19th of October 2016. The appropriate age of mice for superovulation is 8-12-week-old, therefore the first experiments of this research project were initiated in January 2017.

2.3 Quality control

Medium and oocyte preparations for all experimentation were performed within the sterile environment of an IVF workstation with a heated surface (Mobile IVF workstation L13, K-Systems Kivex Biotec Ltd, Birkerod, Denmark) to ensure an aseptic technique. Equipment, disposables and medium used were certified and validated for IVF use (CE-marked, FDA cleared and 1-cell stage Mouse Embryo Assay-tested). All equipment used was calibrated and tested during the annual maintenance services of the assisted reproduction laboratory (*SOP A2.1.1 General maintenance procedures, SOP A2.2.1 Equipment maintenance – Embryology and SOP A2.3.1 Equipment maintenance - Spermatology*). Cell culture, micromanipulation, activation and staining dishes were prepared with appropriate culture medium overlaid with mineral oil (FertiPro, Beernem, Belgium) prior to oocyte/embryo culture or SCNT experimentation, and pre-equilibrated in a humidified conventional embryo culture K-Minc incubator (Cook Medical, Bloomington, Indiana, USA) using pre-mixed tri-gas at 7.35% carbon dioxide (CO_2), 5% oxygen (O_2) and nitrogen (N_2) balance at 37°C. Ongoing embryo culture was further maintained in a K-Minc incubator at 7.35% CO_2, 5% O_2 and 37°C. Air quality was maintained by working in a certified IVF workstation equipped with a high efficiency particulate air (HEPA) filter. Tri-gas was also filtered through in-line HEPA filters before flowing into the incubator. Additionally, air quality was preserved by the physical barrier of the mineral oil overlay in the culture dishes, protecting the embryos from any volatile organic compounds. At the same time, the oil overlay prevented medium evaporation and was also a safeguard against unexpected temperature and gas composition fluctuations. All quality control measures of experimental equipment formed part of the daily quality control program at the laboratory (*SOP C1.3.2 Quality Control*). The pH values of culture medium samples were measured at 7.2-7.4 using a calibrated blood gas analyser (Radiometer ABL 800 Flex, Radiometer Inc., Brea, California, USA). All experimentation was performed under sterile conditions on a 37°C heated microscope stage (Tokai Hit, Shizuoka-ken, Japan). Following nuclear transfer, SCNT embryos were co-cultured for up to 5 days in 20 µl microdrops (n = 20-25 embryos per microdrop). Control embryos were cultured in a similar manner (n = 15-20 embryos per microdrop). A single-step embryo culture medium was used, and no medium changes were performed on day 3 of culture.

2.4 Collection of oocytes

Female B6D2F1 (C57BL/6 x DBA/2) mice between 8 and 15 weeks old were restrained by the veterinary technologist at the UPBRC, and superovulation was induced by an intraperitoneal injection of 5 IU Pregnant Mare Serum Gonadotropin (Sigma-Aldrich® Co, St Louis, Missouri, USA), followed approximately 48 hours later by an intraperitoneal injection of 2.5 IU human chorionic gonadotropin (hCG) (Sigma-Aldrich® Co, St Louis, Missouri, USA).[205] The animals were humanely sacrificed by cervical dislocation 10-13 hours post hCG injection.[206] The oviducts and uterus were collected,[206] and the carcasses incinerated. Cumulus-oocyte complexes (COC) (Figure 2.1) were recovered from the oviduct ampullae and placed into 1 ml of pre-heated M2 medium (Sigma-Aldrich® Co, St Louis, Missouri, USA).[207] The COC were then transported in a portable warming oven (G95E Portable Incubator, K-Systems Kivex Biotec Ltd, Birkerod, Denmark) at 37ºC to RBL at Steve Biko Academic Hospital (SBAH).

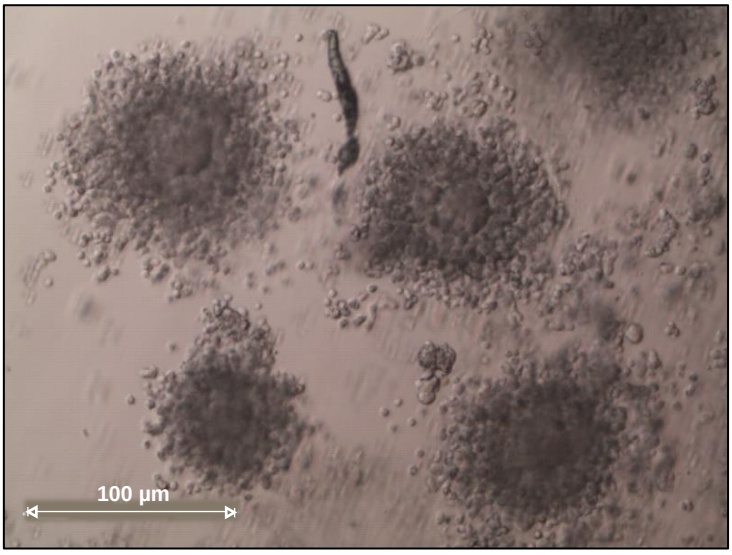

Figure 2.1: **Cumulus-oocyte complexes** (image captured at RBL, UP).

A 35x10 mm Nunc IVF tissue culture dish (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was prepared a day before experimentation with eleven 20 µl microdrops of Global® Total® (GT) medium (LifeGlobal® Group, Guilford, Connecticut, USA) and covered with 3 ml of mineral oil. On the morning of experimentation, a 60x15 mm Nunc IVF tissue culture dish (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was prepared with four 50 µl microdrops of Global® Total® w/HEPES (GTH) medium (LifeGlobal® Group, Guilford, Connecticut, USA) and one 80 µl microdrop of 80 IU/ml hyaluronidase (LifeGlobal® Group, Guilford,

Connecticut, USA) diluted in 120 µl of GTH medium (2:3 dilution ratio) for enzymatic denudation of oocytes. Subsequently, the denudation dish was covered with 6 ml of mineral oil and placed into a 37ºC heated warming oven (Labcon, Petaluma, California, USA). Once the dish had reached 37ºC after at least 30 minutes of placement in the warming oven, oocytes were enzymatically denuded of their cumulus cells by gentle pipetting of the COC in the diluted hyaluronidase microdrop on the heated IVF workstation at 37^0C for no longer than 1 minute.[207] The oocytes were subsequently washed several times in the three GTH microdrops, and then rinsed further in the three GT microdrops and incubated in a single 20 µl GT microdrop in the tissue culture dish that had been prepared and pre-equilibrated the day before experimentation. The culture dish containing the denuded oocytes was stored in the K-Minc incubator until enucleation was performed after an oocyte recovery period of at least 30 minutes. See Figure 2.2 and 2.3 for a detailed timeline of experimentation for study 1 and 2.

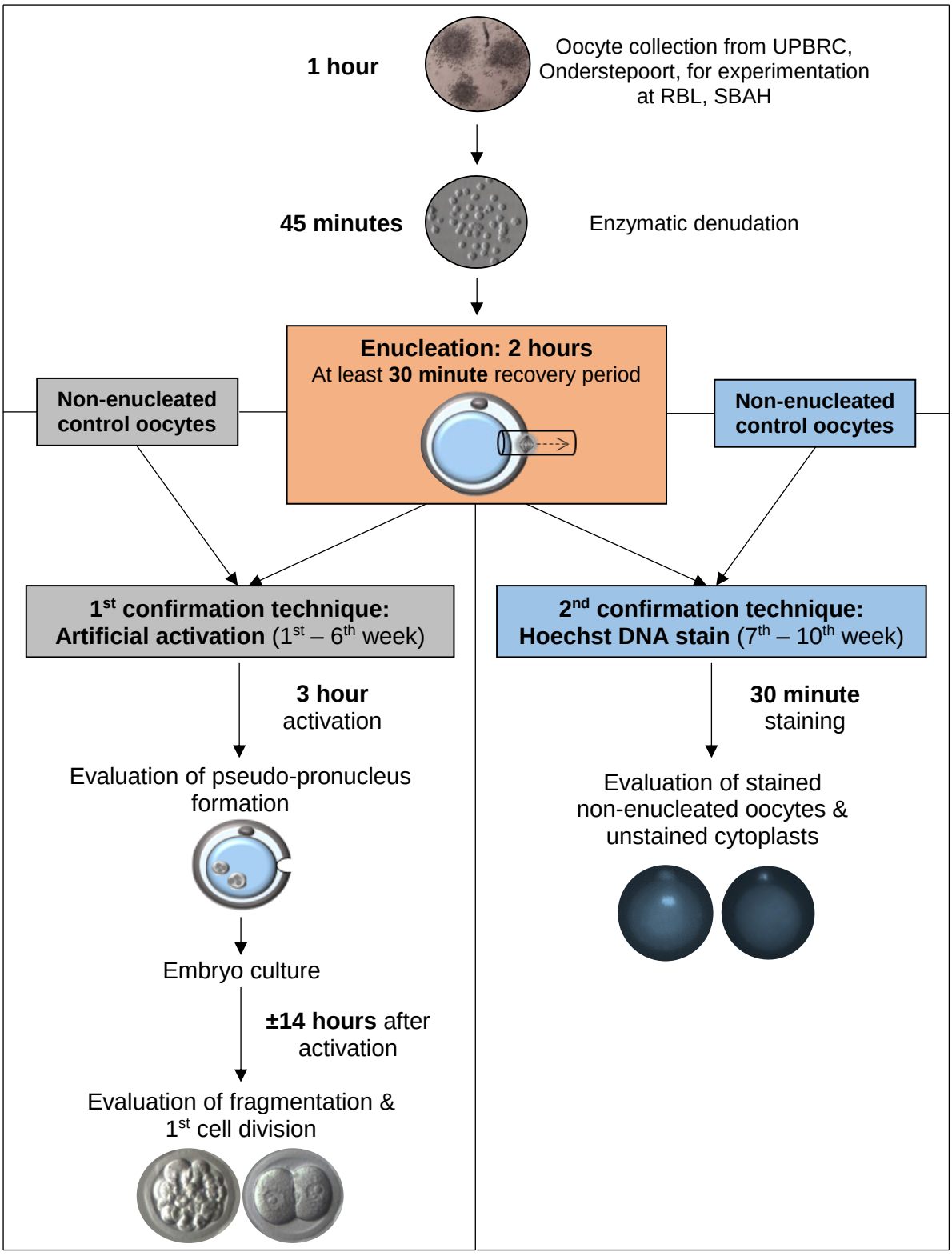

Figure 2.2: Flow diagram depicting the **timeline of experimentation for study 1**. Timing varied slightly between experiments depending on the number of oocytes used (images captured at RBL, UP).

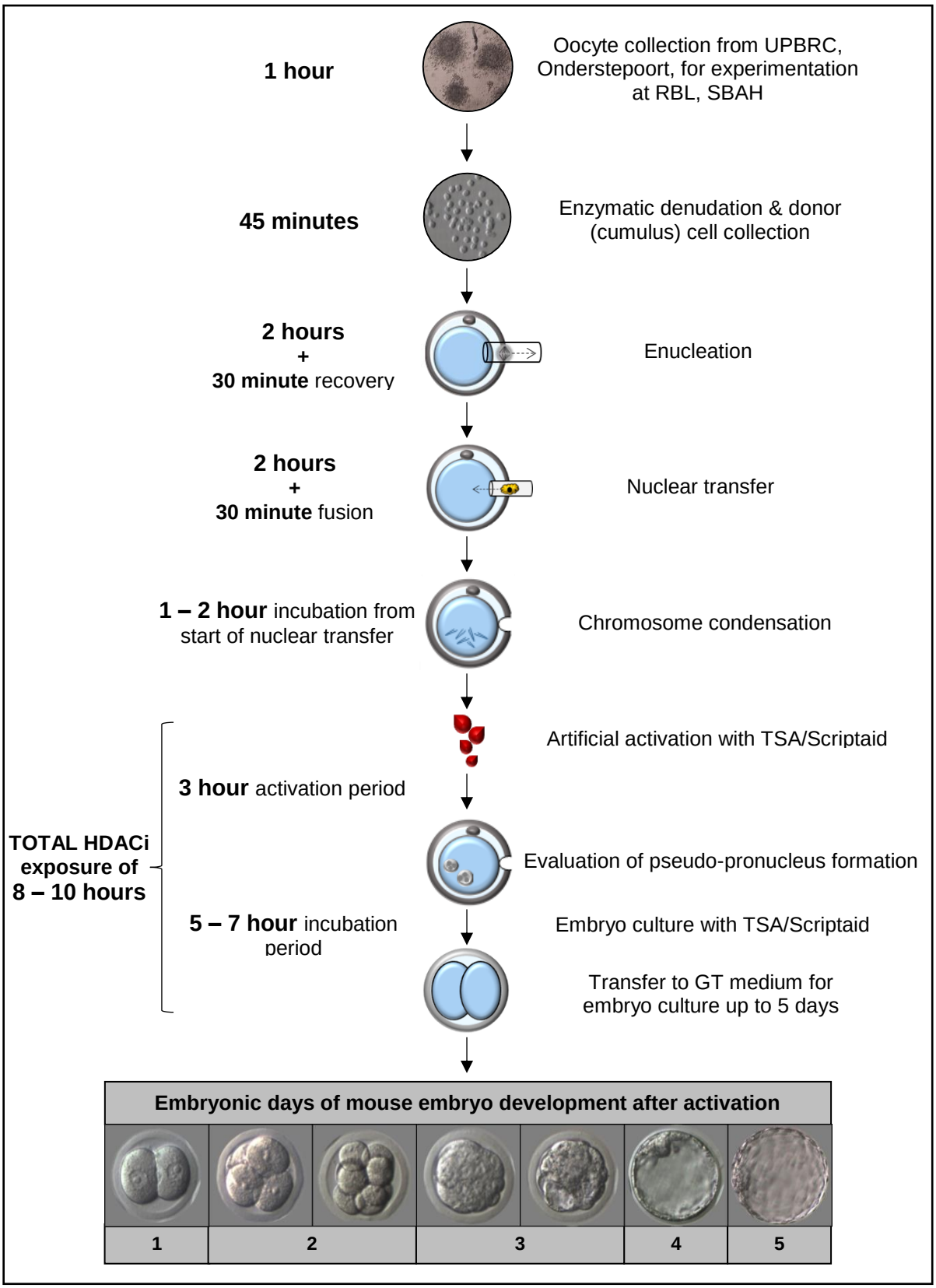

Figure 2.3: Flow diagram depicting the **timeline of experimentation for study 2**. Timing varied slightly between experiments depending on the number of oocytes used (images captured at RBL, UP).

2.5 Preparation of donor somatic cells

Cumulus cells were collected in the abovementioned step from the digested COC. The cells were resuspended and then washed in GTH medium three times to remove residual hyaluronidase. A concentrated suspension of cells was placed into an unused microdrop of GTH in the denudation dish and kept at 4ºC for approximately 2 hours before nuclear transfer (Figure 2.3).[82] More than 80% of cumulus cells are arrested at the G phase (G0/G1) of the cell cycle, and thus are suitable donor cells that can be used without any selection and do not require further *in vitro* culture to ensure cell cycle synchronization.[78,208]

2.6 Micromanipulation system setup and medium preparation for study 1

Micromanipulation was performed on an Axiovert 200 inverted microscope equipped with HMC optics (Zeiss, Jena, Germany) and micromanipulators (Research Instruments Ltd, Falmouth, UK). The holding pipette (outer diameter 120 µm, inner diameter 15 µm; standard 30º bend) (The Pipette Company, Cooper Surgical, Målov, Denmark) and the enucleation (biopsy) pipette (outer diameter 20 µm, inner diameter 16 µm; bevel 90º; standard 30º bend) (The Pipette Company, Cooper Surgical, Målov, Denmark) were placed into the pipette holders and set up according to the manufacturer's guidelines (Research Instruments Ltd). The OCTAX Laser SHOT™ system (MTG, Bruckberg, Germany) was used to create an opening in the zona pellucida of the oocyte. A 51x9 mm Nunc ICSI dish (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was prepared with ten 20 µl microdrops of 0.5 µl of 10 mg/ml CB (Sigma-Aldrich® Co, St Louis, Missouri, USA) diluted in 1 ml of GTH medium (CB-GTH) (Figure 2.4). The micromanipulation dish was overlaid with 3 ml of pre-heated (37ºC) mineral oil and placed on a 37ºC heated stage of an Axiovert 200 inverted microscope.

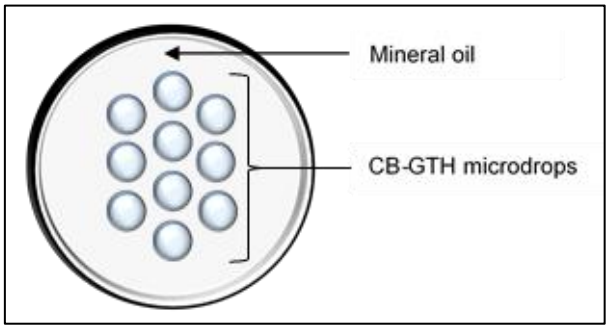

Figure 2.4: Schematic diagram of the **medium preparation** required for oocyte enucleations performed in study 1.

2.7 Medium preparation for study 2

A 51x9 mm Nunc ICSI dish was prepared with one 20 µl microdrop of GTH, three 20 µl microdrops CB-GTH (as prepared in Section 2.4), as well as a single 20 µl microdrop of inactivated HVJ-E solution (Ishihara Sangyo Kaisha Ltd, Osaka, Japan) (Figure 2.5). The dish was overlaid with 3 ml of pre-heated (37ºC) mineral oil and placed on a 37ºC heated stage.

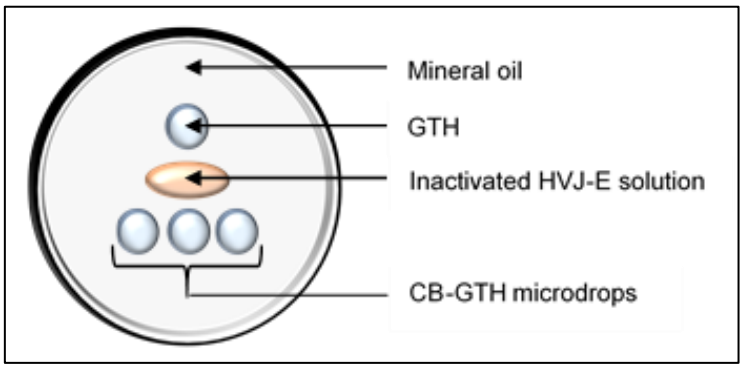

Figure 2.5: Schematic diagram of the **medium preparation** required for oocyte enucleations, somatic cell transfers, and fusion performed in study 2.

2.8 Study 1: Enucleation

The enucleation protocol followed was adapted from procedures described in literature[12,76,79,80] and by Dr Dietrich M. Egli from Columbia University (personal communication). Before manipulation, the enucleation pipette was lubricated by moving it into a CB-GTH microdrop in the dish illustrated in Figure 2.4. The medium was then aspirated, expelled, and aspirated again to ensure equilibrium within the enucleation pipette. The same procedure was repeated between groups of oocyte enucleations, to prevent blockage of the needle.

Maintaining oocytes out of an incubator for periods longer than 20-30 minutes is not recommended,[56] therefore a maximum of five oocytes were enucleated at a time. The denuded oocytes were placed into a CB-GTH microdrop. The holding and enucleation pipettes were moved into the same microdrop and aligned with the equatorial plane of the first oocyte to be enucleated. The oocyte was then rotated using the pipettes until the metaphase spindle was visualized. In mice, the spindle can be visualized using HMC microscopy, and identified as a smooth, translucent clump with no surface granularity.[9,56] After identification, the spindle was placed at the 3 o'clock position, and the oocyte was kept secure by applying suction to the oocyte using the holding pipette (Figure 2.6 A). One or two laser pulses of low intensity (shot duration: 4.5 ms ~ø25

µm) were applied to create an opening in the zona pellucida next to the spindle (Figure 2.6 B). Once the enucleation pipette made contact with the spindle and showed resistance, the spindle was aspirated, and the pipette retracted (Figure 2.6 C). The oocyte has a fluidic cytoplasm; however, the spindle moved as a unit when manipulated with the pipette.[56] The cytoplast was then moved aside, and the same steps were performed for the remaining oocytes within the specific batch. Following enucleation, the oocytes were rinsed and incubated in a single pre-equilibrated 20 µl GT microdrop in the K-Minc incubator for a recovery period of at least 30 minutes before artificial activation and Hoechst 33342 DNA staining (Figure 2.2).

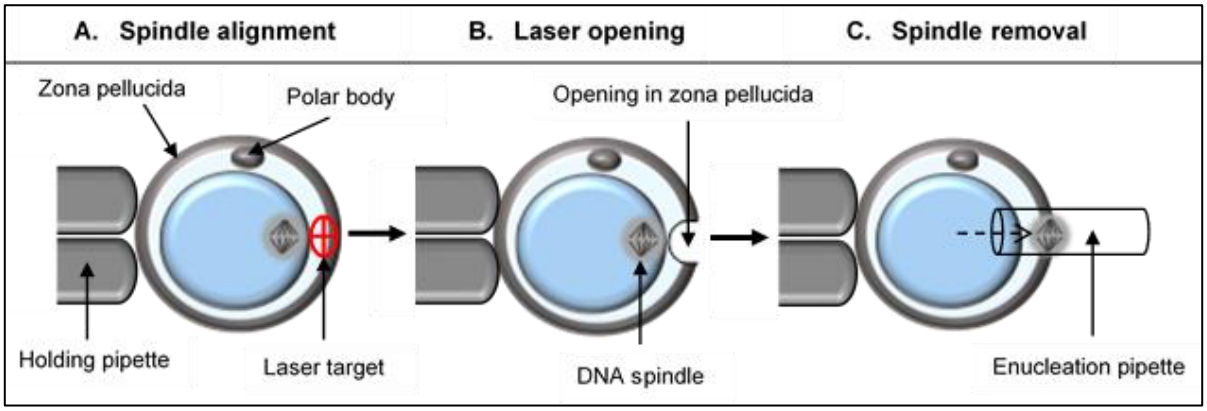

Figure 2.6: Schematic representation of the steps involved in the **enucleation technique**. Dashed arrow represents removal of the DNA spindle.

2.8.1 Quality control

For study 1, the karyoplast was removed and discarded and the cytoplast was the product of interest. The key to successful enucleation is the complete removal of the meiotic spindle complex with very little surrounding cytoplasm.[76,78,79] Two confirmation techniques, namely artificial activation and Hoechst 33342 DNA stain, were used to determine the efficiency of enucleation in study 1. Figure 2.7 illustrates the experimental design for study 1. A total of at least 450 oocytes were required in this study to account for statistical significance (see section 2.10). Experimentation was performed in batches over several weeks to achieve the numbers required.

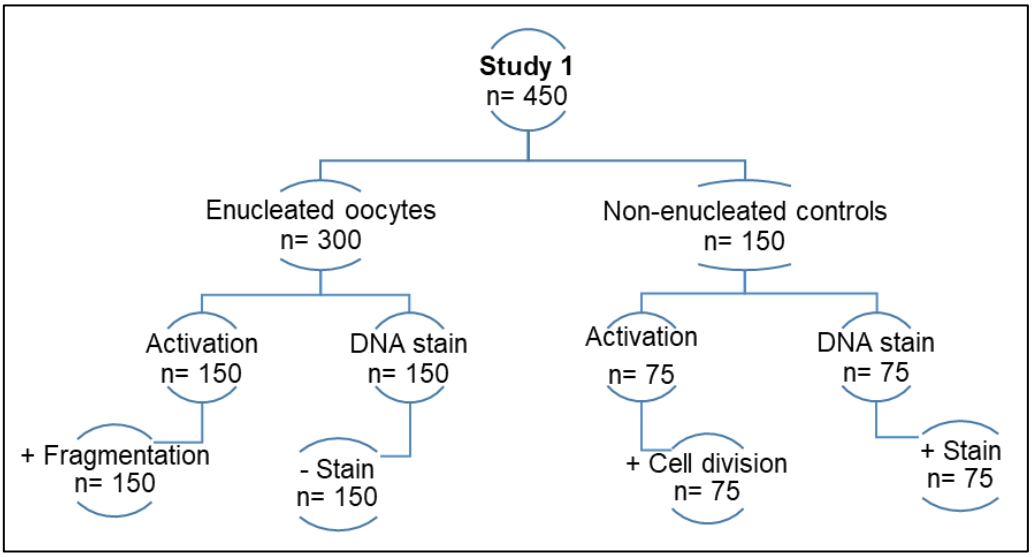

Figure 2.7: Flow diagram of **experimental design for study 1**.

2.8.1.1 Artificial activation

Approximately 14 hours after activation the cytoplasts will be fragmented, thereby confirming successful enucleation[56]. As a control for enucleation, cytoplasts were artificially activated. A Nunc 4-well dish (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was prepared with 750 µl of GT medium in each well. As indicated in Figure 2.8, 2 µl of 1 mM ionomycin (Sigma-Aldrich® Co, St Louis, Missouri, USA) was added to the GT medium in the 1st well, followed by the addition of 0.75 µl of 10 mg/ml puromycin (Sigma-Aldrich® Co, St Louis, Missouri, USA), 0.75 µl of 10 mg/ml CB, and 3.75 µl of 0.4 M 6-DMAP (Sigma-Aldrich® Co, St Louis, Missouri, USA) to the GT medium in the 4th well. The cytoplasts were incubated in well 1 for 5 minutes, which induced a calcium influx.[106] The cytoplasts were then washed in well 2 and 3, followed by incubation in well 4 in the K-Minc incubator for 3 hours to induce activation.

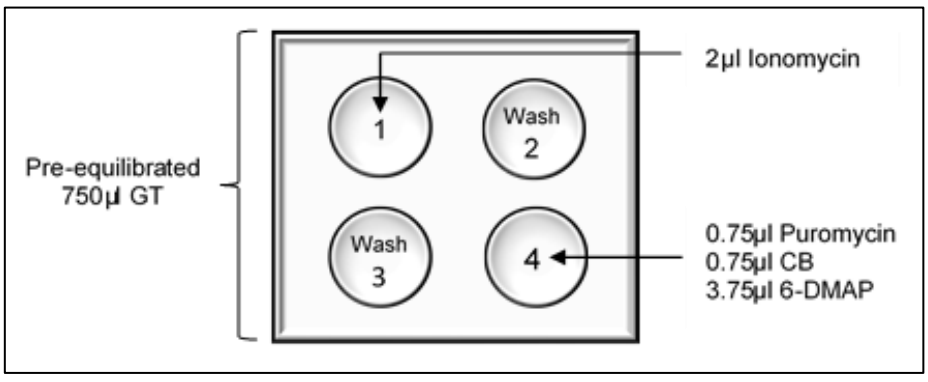

Figure 2.8: Schematic diagram of the medium and reagent preparations required for oocyte **activation**.

After the 3 hour activation period, the cytoplasts were washed through three microdrops of pre-equilibrated GT medium and then placed in a single 20 µl microdrop for evaluation of pseudo-pronucleus formation, using an Axiovert 200 inverted microscope. To verify successful activation, a cohort of non-enucleated oocytes served as controls and were activated as described above, demonstrating pseudo-pronucleus formation and further cell division as a result of parthenogenesis[b].[56,82] Fragmentation of the cytoplasts and parthenogenesis of the non-enucleated control oocytes were analysed approximately 14 hours after activation (Figure 2.9 and Figure 2.2). All oocyte images were captured using an Axiovert 200 inverted microscope.

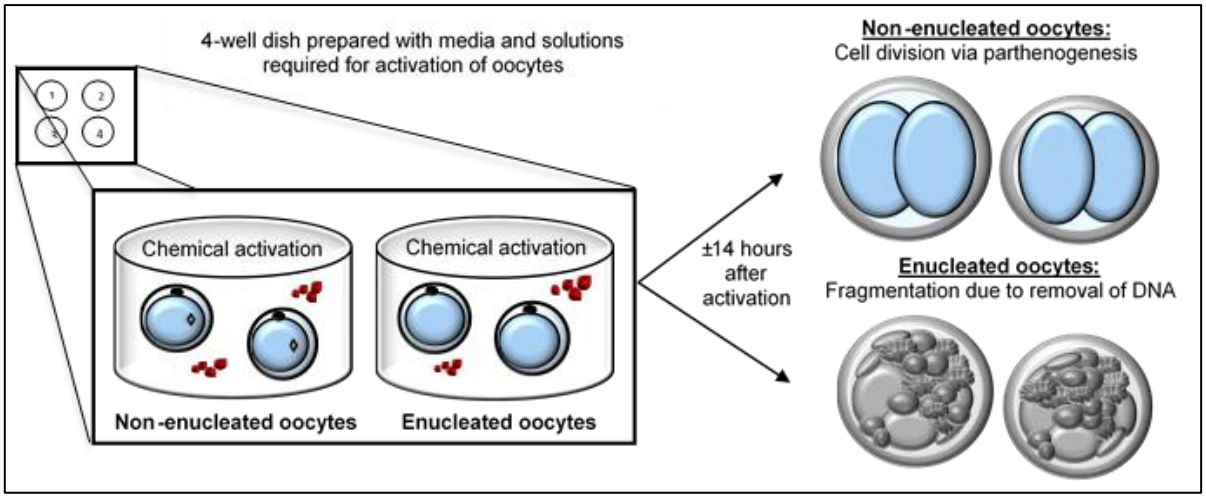

Figure 2.9: Diagram illustrating the **quality control for enucleation**: fragmentation of enucleated oocytes and parthenogenesis of non-enucleated control oocytes following chemical activation.

2.8.1.2 Hoechst 33342 DNA stain

Hoechst 33342 (Sigma-Aldrich® Co, St Louis, Missouri, USA) is cell membrane permeable and stains chromosomes blue.[80] Therefore, to confirm successful enucleation the stain was used as a control to visualize the absence of the meiotic spindle complex within the remaining cytoplasts. Two wells of a Nunc 4-well dish were prepared with 500 µl of GT medium. After enucleation, 2.5 µl of 1 mg/ml Hoechst 33342 was added to the two wells containing the pre-equilibrated GT medium.[2,64,70,209] The cytoplasts were placed into one well, and a group of non-enucleated control oocytes (to verify the staining technique) were placed into the other well. The cytoplasts and non-enucleated control oocytes were incubated with the stain for 30

[b] *Parthenogenesis*: Development of an oocyte without fertilization

minutes in the K-Minc incubator. [2,64,70,209] After the 30 minute staining incubation, the cytoplasts and non-enucleated control oocytes were washed through three microdrops of pre-equilibrated GT medium and then placed in two separate 20 µl microdrops for evaluation of unstained cytoplasts and stained non-enucleated oocytes (Figure 2.2). All evaluations were performed using an Axiovert A1 fluorescent microscope (Zeiss, Jena, Germany).

2.9 Study 2: Enucleation, nuclear transfer and fusion, followed by activation and embryo culture to the blastocyst stage

The nuclear transfer protocol was adapted from procedures described in the literature[12,36,49,76,81,83,89,90] and by Dr Dietrich M. Egli from Columbia University (personal communication), and was performed by fusion of somatic cells to cytoplasts using the inactivated HVJ-E. Enucleation was performed as described in Section 2.8 of study 1 (Figure 2.6). To avoid potential fusion of the PB during HVJ-E exposure, the PB (if not degenerated) was removed.[49,83] Subsequent to enucleation, the cytoplasts (already incubated for approximately 1-2 hours in the K-Minc incubator) were subjected to nuclear transfer of the donor somatic cells (see timeline in Figure 2.3).

A cohort of five cytoplasts were transferred into a clean microdrop of CB GTH in the micromanipulation dish, along with a concentrated suspension of cumulus cells which were transferred to the GTH microdrop of the same dish (Figure 2.5). A single cumulus cell was selected with the transfer pipette and moved to the HVJ-E solution for 20 seconds of exposure. The holding and transfer pipettes were then moved to the microdrop containing the cytoplasts and aligned with the equatorial plane of the first cytoplast to undergo transfer. The cytoplast was rotated using the pipettes until the opening, previously created in the zona pellucida during enucleation, was placed at the 3 o'clock position. Suction was then applied with the holding pipette to firmly secure the cytoplast. The transfer pipette was inserted through the opening under the zona pellucida, and the cumulus cell was expelled into the PVS and manoeuvred into close contact with the cell membrane of the cytoplast (Figure 2.10). The manipulated cytoplast was then secluded at the bottom of the microdrop, and the same steps were repeated for the remaining cytoplasts within the cohort. Finally, the manipulated cytoplasts were washed and incubated in a single pre-equilibrated 20 µl GT droplet. Fusion was confirmed by the disappearance of the somatic cell into the cytoplast using an Axiovert 200 inverted microscope within 30 minutes of nuclear transfer. Between

1-2 hours post nuclear transfer (see timeline in Figure 2.3), the reconstituted oocytes were artificially activated as described in Section 2.8.1.1 of study 1. Specific to study 2, the HDACi TSA (Sigma-Aldrich® Co, St Louis, Missouri, USA) or Scriptaid (Sigma-Aldrich® Co, St Louis, Missouri, USA) were added into well 4 of the activation dishes (Figure 2.8).

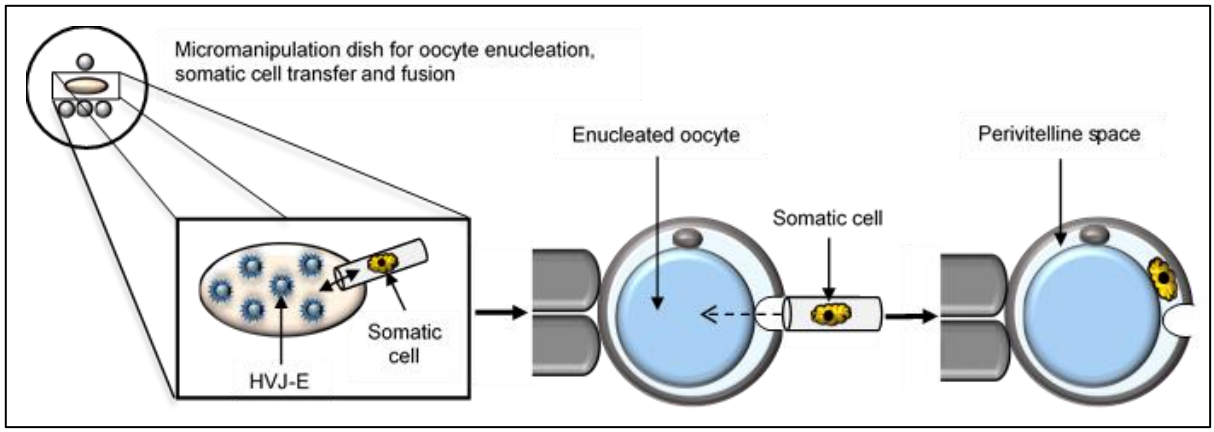

Figure 2.10: Schematic representation of the steps involved in the **transfer and fusion** of the somatic cell into the enucleated oocyte (refer to Figure 2.5 on page 39 for medium preparation details in the micromanipulation dish). Dashed arrow represents transfer of the somatic cell into the enucleated oocyte.

2.9.1 Quality control

According to literature, the most effective TSA treatment protocol in mice is a TSA concentration of between 5-50 nM, and this requires continuous exposure of the reconstructed oocytes to TSA for no less than 8 hours from the time of oocyte activation, but before the first cell division.[18,173] In order to define the optimal TSA protocol specific to this study, three different concentrations of TSA (5, 25 and 50 nM) were tested at two different exposure times (8 and 10 hours), and were compared to determine the most effective exposure combination (Figure 2.11). In addition, Scriptaid (250 nM)[19] was tested at a total exposure of 8 hours and the results were compared to TSA.

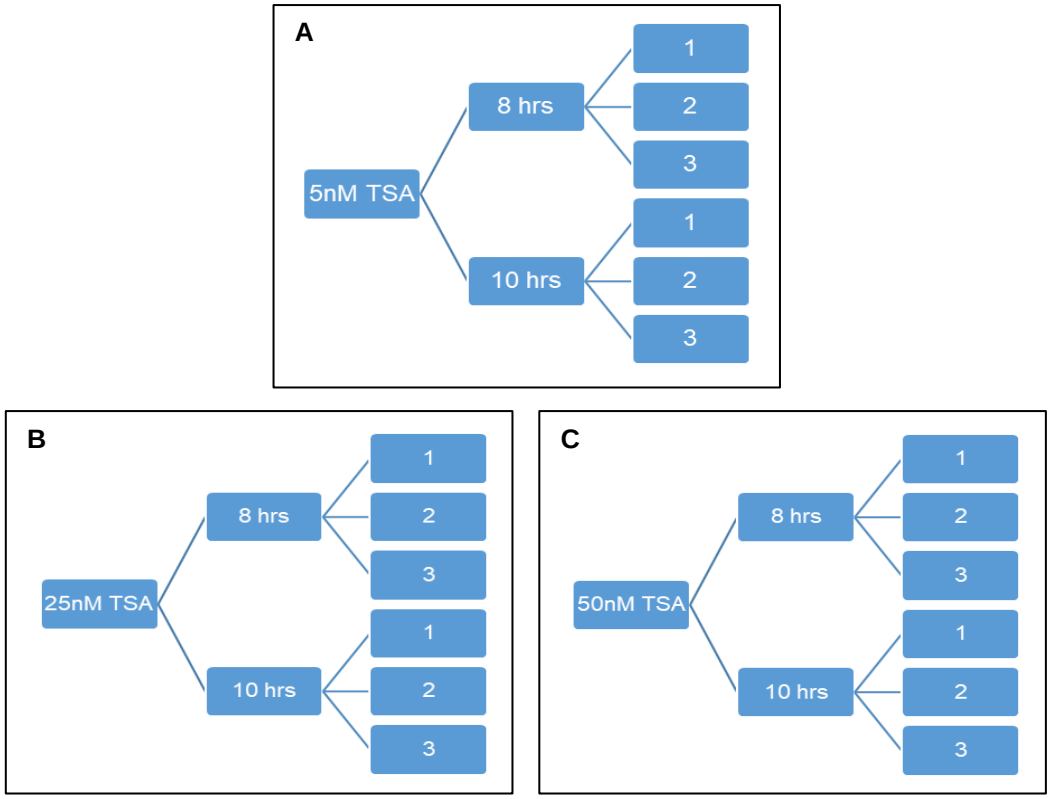

Figure 2.11: Flow diagram depicting the experimental setup to compare 3 concentrations of **TSA**, i.e. (A) 5 nM (B) 25 nM and (C) 50 nM at 2 exposure times (experiment repeated 3 times).

After the 3 hour activation period with a specific concentration of TSA or 250 nM Scriptaid added to the 4th well of the activation dish, the manipulated SCNT oocytes were washed through 3 microdrops of pre-equilibrated GT medium and then placed in a single 20 µl microdrop supplemented with the specific TSA concentration being investigated or 250 nM Scriptaid. The SCNT oocytes were then co-cultured in the abovementioned supplemented medium for either an additional 5 hours (for the 8 hour total TSA and Scriptaid exposure period), or for an additional 7 hours (for the 10 hour total TSA exposure period) (see timeline in Figure 2.3). After the continuous exposure of the reconstructed oocytes to TSA or Scriptaid for a total of either 8 or 10 hours from the start of activation, the SCNT oocytes were washed through 3 microdrops of pre-equilibrated GT medium and then placed in a single 20 µl GT microdrop. The SCNT embryos were co-cultured in a single microdrop in the K-Minc incubator until the blastocyst stage, which occurred between approximately 72-96 hours post activation (see Figure 2.3 on page 37 for timeline).

An Axiovert 200 inverted microscope was used to confirm successful fusion of the cumulus cells and cytoplasts after 30 minutes of nuclear transfer. Non-fused oocytes were excluded from further data analysis. The formation of pseudo-pronuclei indicates successful nuclear transfer and activation.[56] To verify activation of the SCNT oocytes, the number of oocytes with pseudo-pronuclei were counted and images were taken using an Axiovert 200 inverted microscope directly after the 3 hour activation period (see Figure 2.3 on page 37 for sequential steps). Ongoing cell division and subsequent embryo development to the blastocyst stage were monitored and photographed daily using an Axiovert 200 inverted microscope and an EmbryoScope™ time-lapse system[210-212] (Vitrolife, Göteborg, Sweden), for 72-96 hours post activation. The EmbryoScope™ is an incubator with a built-in microscope equipped with HMC optics that acquires images automatically in seven focal planes every 15 minutes. The tissue culture dish used in the EmbryoScope™ is called an EmbryoSlide® (Vitrolife, Göteborg, Sweden), which contains 12 individual wells. The wells were filled with 25 µl of GT medium and were covered with 1.2 ml of mineral oil (LifeGuard® Oil, LifeGlobal® Group, Guilford, Connecticut, USA) to prevent evaporation (*SOP A1.20.3.1 Preparation of an Embryoscope slide and operation of the Embryoscope*). The EmbryoSlides® were prepared in advance and were left in the incubator to pre-equilibrate at 7% CO_2, 5% O_2, 88% N_2 and 37^0C. For this study two embryos were cultured per well in the EmbryoSlide®.

As controls for activation, HDACi exposure and cell culture conditions, non-enucleated oocytes were activated and cultured as described above in Section 2.9.1 of study 2 and exhibited further development due to parthenogenesis. According to Kishigami and colleagues,[78] when a highly skilled operator performs mouse SCNT experiments, 80-90% of the oocytes should survive nuclear transfer; 70-80% should survive activation; pseudo-pronucleus formation should be visible in 60-70%; cleavage to the 2-cell stage after 24 hours of nuclear transfer should occur in 50-60%; and finally 72 hours post nuclear transfer 30-50% should develop to the morula/blastocyst stage (Figure 2.12). A total of at least 300 oocytes were required in this study to account for statistical significance (Figure 2.12). Experimentation was performed in batches over several weeks to achieve the numbers required.

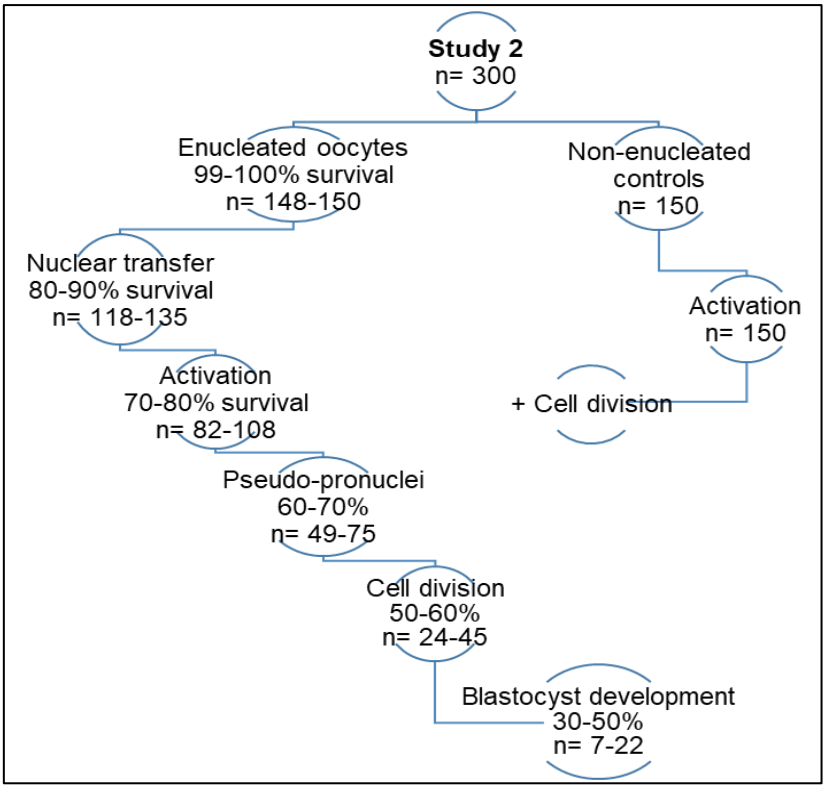

Figure 2.12: Flow diagram of **experimental design for study 2**. Success rates are based on piezoelectric nuclear transfer of BDF1 mice performed by a highly skilled operator, using cumulus cell nuclei under optimal conditions.[78]

2.10 Statistical analyses

Morphological (qualitative) data was obtained by HMC and fluorescence microscopy, as well as time-lapse microscopy using the EmbryoScope™ system.

Due to the nature of the experiments in study 1, categorical data was obtained as proportions or percentages of successful enucleation (positive fragmentation and negative DNA stain) vs unsuccessful enucleation (positive cell division and positive DNA stain) (Figure 2.7) to provide descriptive statistics. In study 2, categorical data was also obtained as proportions or percentages for the successful formation of blastocysts (Figure 2.12). To compare the efficiency of the enucleation procedure and blastocyst development, a Fisher's exact test was used, depending on the sample size. The significance level was set at $\alpha = 0.05$, a p value <0.05 and a confidence interval (CI) of 95% was considered. Statistical significance was indicated by an asterisk (*). Statistical analyses were performed by Prof P Becker from the Faculty of Health Sciences, UP using Microsoft Excel, Statistix and STATA 14 statistical software.

Chapter 3: Results

3.1 Study 1: Overview

Enucleation is the first step in SCNT and is defined as the removal of the meiotic spindle complex containing the chromosomes from an oocyte arrested at the MII stage of meiosis.[1,2,57] In contrast to most mammalian species including humans, the meiotic spindle complex in the mouse oocyte is easily visible as a translucent region under a HMC microscope.[3,9,70,77,78] In this study, occasionally the translucent meiotic spindle complex was also noticeable as a bulge (see Figure 3.1). Mice oocytes (B6D2F1) were used in this project and no other identifying tools were therefore required, eliminating possible damage to the oocytes caused by UV light and the need for expensive polarized light birefringence equipment.[76]

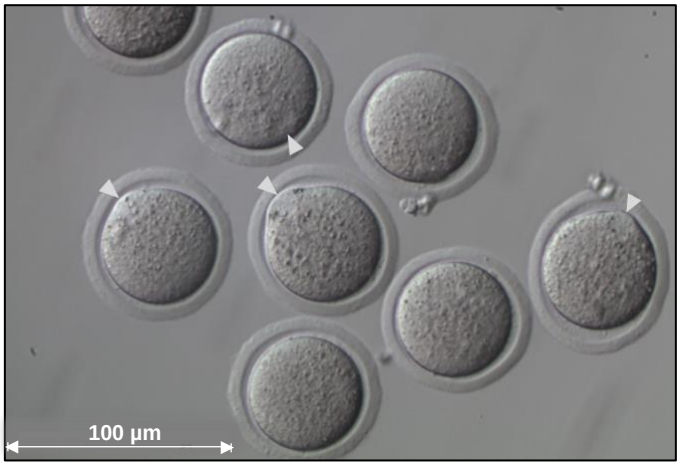

Figure 3.1: Photo image of **B6D2F1 mouse oocytes**. Meiotic spindle complexes (visible as a translucent region with an occasional bulge) are indicated by white arrowheads (image captured at RBL, UP).

The hybrid mouse strain BDF1, is a popular and preferred choice for SCNT experiments.[78,120] First generation hybrid mice such as B6D2F1 (C57BL/6 x DBA/2), between the ages of 8 and 12 weeks, are the most suitable oocyte donors. In addition to these strains producing oocytes efficiently, the oocytes are relatively easy to micromanipulate.[82] This specific mouse strain as well as other similar strains are not available nationally. Therefore, breeding pairs were imported from the USA to establish a mouse colony, and to eventually breed the B6D2F1 hybrids required for this research project. See section 2.2 in Chapter 2 for details on importation and breeding of the mice.

In study 1, oocyte retrievals were performed on 53 BDF1 mice (average age of 12.9 weeks). Approximately 4 mice (median) were superovulated per day of oocyte retrievals. The average number of oocytes retrieved per mouse in this study was 23.7 (min = 52; max =181). Approximately 11% (139/1254) of the total number of oocytes retrieved were parthenogenetic or abnormal. Abnormal oocytes were classified as those with abnormally large PBs, a high degree of fragmentation, vacuoles or any other obvious cytoplasmic anomalies. See Table 3.1 for an overview of the mice used and the allocation of oocytes in each repeat performed in study 1. The oocytes were allocated randomly between the control and experimental groups.

Table 3.1: A detailed summary of the superovulated mice and oocytes retrieved in each repeat performed in the two quality control sections of study 1

Repeat	Age of BDF1 mice (weeks)	No. of mice used	Number (No.) of oocytes			
			Retrieved	Parthenogenetic/ abnormal	Enucleated (experiment)	Non-enucleated (control)
First confirmation technique: artificial activation						
1	15	6	113	28	35	35
2	15	6	171	18	50	30
3	15	6	52	8	25	20
4	8	4	77	5	50	22
5	9	4	79	9	50	16
6	11	4	181	16	59	25
7	9 & 10	4	103	10	48	32
Subtotal		**34**	**776**	**94**	**317**	**180**
Second confirmation technique: Hoechst DNA staining						
1	13	4	94	14	30	20
2	13	3	96	8	22	13
3	12	4	97	3	40	25
4	12	4	105	13	35	25
5	13	4	86	7	40	23
Subtotal		**19**	**478**	**45**	**167**	**106**
TOTAL		**53**	**1254**	**139**	**484**	**286**
% Oocytes retrieved per group				**11.1%**	**38.6%**	**22.8%**
% Oocytes used in Study 1					**61.4%**	
% Unused oocytes in Study 1: **27.5%**						
Average oocytes retrieved per mouse in Study 1: **23.7**						

3.1.1 Enucleation procedure

Enucleation is an invasive step that, if not performed optimally, may induce damage to the oocytes and contribute to the low success rate of SCNT. Preliminary experimentation to verify spindle identification and the enucleation technique was practiced on 88 oocytes from 16 BDF1 mice (8-14-week-old). Initially, spindle visualization was challenging. However, through continuous rotation of the oocyte, the eye was trained to identify the translucent meiotic spindle complex. At first, it took 40 minutes to enucleate 7 oocytes, which is approximately 5.7 minutes per oocyte. No immediate oocyte lysis was noted during the first practice round, which confirmed that the correct concentration of CB had been utilized. However, during the second practice round several oocytes lysed during rotation with the pipettes, confirming their fragility. From the practical experience gained during preliminary experimentation, the handling procedure was adjusted. The fragile oocytes were rotated more gently and only 5 oocytes were enucleated at a time to limit exposure outside of the incubator to 20-30 minutes.

A total of 484 B6D2F1 oocytes were enucleated in study 1 (Table 3.1). It was noticed in the study that depending on the rotation of the oocyte and location of the spindle inside the oocyte, the translucent meiotic spindle complex was occasionally noticeable as a bulge (refer back to Figure 3.1). Identification was made easier by a clear bulge in the oocyte; however, if no bulge was present the meiotic spindle complex was identified by its translucency and rotated relatively easily to the 3 o'clock position (Figure 3.2 A). In most cases, one or two laser pulses were applied to create an opening in the zona pellucida next to the spindle, with minimal to no oocyte lysis (data to follow in the chapter) (Figure 3.2 B). The enucleation pipette was moved into contact with the spindle and after resistance of the pipette against the spindle was "felt", the spindle was aspirated, and the pipette retracted (Figure 3.2 C, D and E). Complete removal of the spindle as a single unit (known as a karyoplast) from the oocyte, was achieved (Figure 3.2 F).

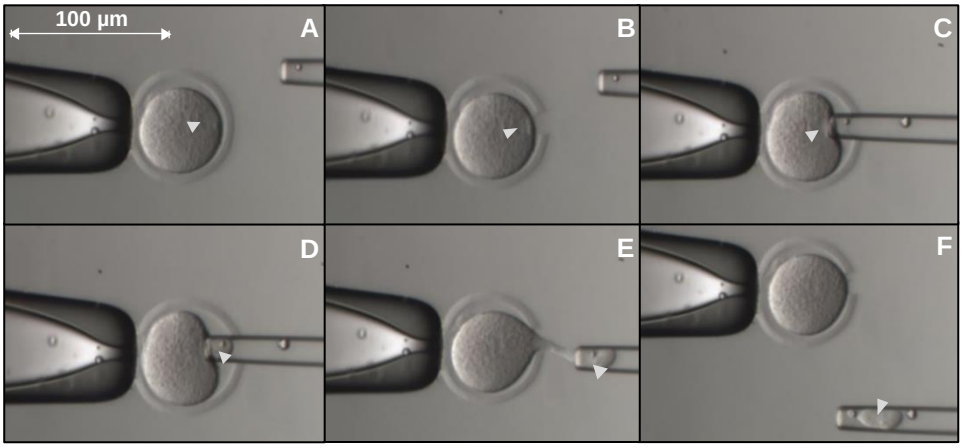

Figure 3.2: The steps involved in the **enucleation procedure** (A-F). Meiotic spindle complexes are indicated by white arrowheads (images captured at RBL, UP).

3.1.2 Quality control

The cytoplasts were used for further experimentation in study 1. Two confirmation techniques, namely artificial activation and Hoechst 33342 DNA staining were used to determine the efficiency of enucleation. Four hundred and eighty-four oocytes were enucleated in study 1, of which 317 were enucleated and artificially activated during the first 6 weeks of experimentation, and 167 were enucleated and stained with Hoechst 33342 during the last 4 weeks of experimentation.

3.1.2.1 Artificial activation

Artificial activation was the first technique used to confirm enucleation. This was achieved by inducing fragmentation of cytoplasts due to the removal of DNA (Figure 3.3).

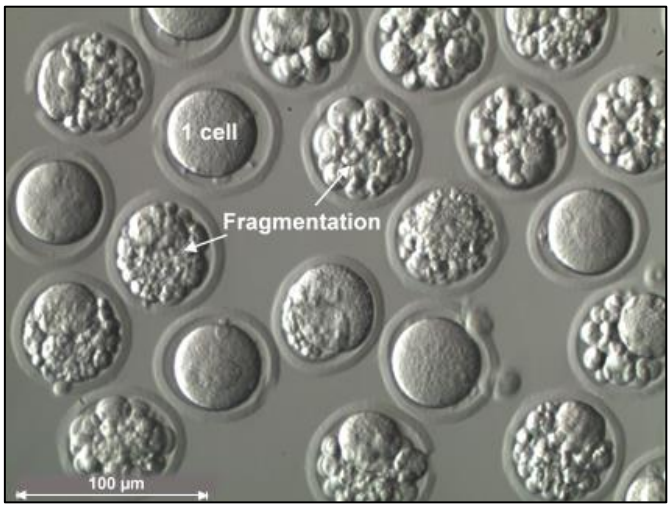

Figure 3.3: Image of **fragmented cytoplasts** ±16-18 hours post activation (image captured at RBL, UP).

Cytoplasts and non-enucleated control oocytes were transferred from the activation dish to a tissue culture dish after the 3 hour activation period. Immediately thereafter, HMC microscopy analysis was performed to identify the absence (Figure 3.4 A.i) or presence (Figure 3.4 B.i) of pseudo-pronuclei in the cytoplasts and non-enucleated control oocytes, respectively. Fragmentation of cytoplasts (Figure 3.4 A.ii) and parthenogenesis of non-enucleated control oocytes showing the first cell division (Figure 3.4 B.ii) were analysed between 16-18 hours after activation. In Figure 3.4 A.i and A.ii, the encircled oocyte with the presence of pseudo-pronuclei and the encircled 2 cell embryo show examples of unsuccessful enucleation within the cohort of oocytes that were enucleated. One cell cytoplasts noted in Figure A.ii did not react to the activation protocol, and therefore were not included in further data analysis.

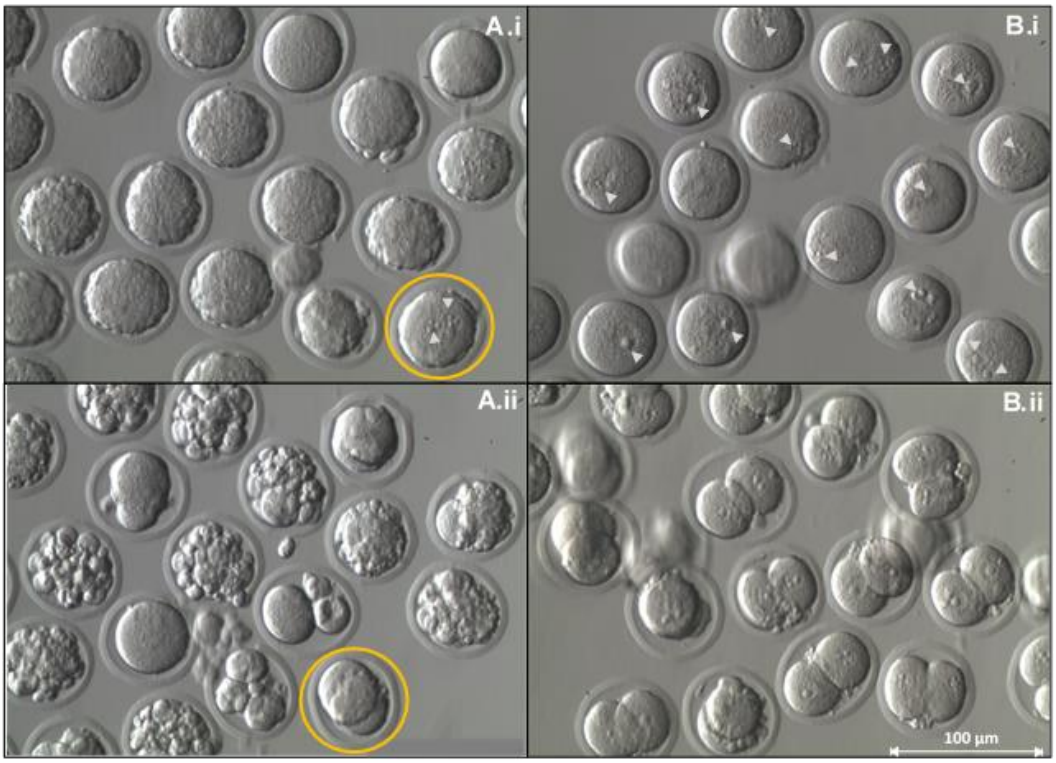

Figure 3.4: Photo images illustrating the **outcomes of activation.** Cytoplasts at 3 (A.i) and 16-18 (A.ii) hours; and non-enucleated control oocytes at 3 (B.i) and 16-18 (B.ii) hours post activation. Pseudo-pronuclei are indicated by white arrowheads (images captured at RBL, UP).

To achieve the number of at least 150 enucleated and activated oocytes required for statistical significance, 7 repeats were performed in this section of study 1. Table 3.2 is an overview of the outcomes of cytoplasts at 3 and 16-18 hours after activation for each experimental repeat.

Table 3.2: A summary of the outcomes of enucleated oocytes at 3 and 16-18 hours post activation of 7 repeats

Repeat	Total oocytes enucleated & activated	Cytoplasts "lost" due to handling	Cytoplasts remaining	3 hours				16-18 hours							
				Degen	Lysis	PN visible	No PN	Frag	Degen	1C No PN	1C PN	2C	3C	5C	Lysis
1	35	0	35		1	1	33	27		4	2	1			1
2	48	0	48	48					48						
3	27	0	27	27					27						
4	50	1	49				49	35		7	5	1		1	
5	50	1	49			1	48	34		12		1	2		
6	59	0	59				59	16		37		4	2		
7	48	0	48			1	47	18		27		2	1		
TOTAL	**317**	**2**	**315**	**75**	**1**	**3**	**236**	**130**	**75**	**87**	**7**	**9**	**5**	**1**	**1**

Degen = degenerated, PN = pronuclei, Frag = fragmentation, C = cell

Orange highlighted rows indicate the repeats wherein all cytoplasts degenerated after activation

Orange highlighted columns indicate the number of degenerated cytoplasts in each repeat

Three hundred and seventeen oocytes were enucleated and activated. Of the 317 activated cytoplasts, 75 within experimental group 2 and 3 degenerated either during or directly after the activation period (indicated by the orange highlighted sections in Table 3.2). The exact cause of degeneration is unknown. Due to degeneration (Figure 3.5), these repeats were excluded from the final data analyses.

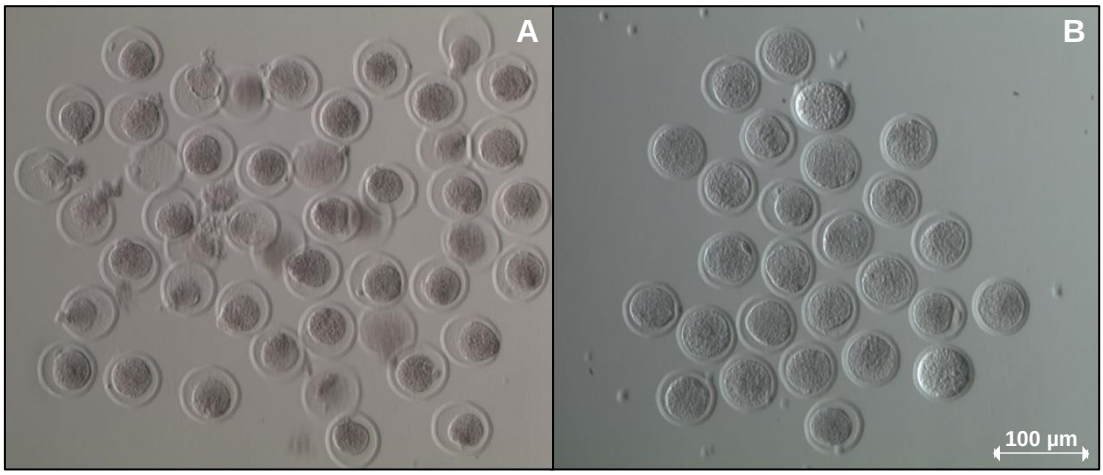

Figure 3.5: Photo images illustrating **degeneration of cytoplasts** 3 hours post activation in repeat 2 (A) and repeat 3 (B) (images captured at RBL, UP).

Table 3.3 is an overview of the outcomes of cytoplasts at 3 and 16-18 hours after activation of the 5 included experimental repeats, excluding repeat 2 and 3 due to the degeneration of cytoplasts in these cohorts. At 3 hours post activation, **0.4%** (1/240) of the cytoplasts had lysed, **1.3%** (3/240) showed visible pseudo-pronuclei and **98.3%** (236/240) presented with absent pseudo-pronuclei. The latter result was promising, because absent pseudo-pronuclei would generally imply successful removal of chromosomes from the oocyte. However, the highlighted vertical column in Table 3.3 indicates that **36.3%** (87/240) of the enucleated oocytes remained at the 1 cell stage with no visible pseudo-pronuclei at 16-18 hours post activation. This suggests that these cytoplasts did not react to the activation protocol. Since efficient enucleation is determined solely by the fragmentation of cytoplasts, the 1 cell stage oocytes with no visible pseudo-pronuclei at 16-18 hours post activation could not be grouped as fragmented cytoplasts and were therefore also excluded from the final data analyses. At 16-18 hours post activation, 1 cell stage oocytes with visible pseudo-pronuclei or oocytes showing cell division indicated that chromosomes were present in the oocytes at the time of activation, confirming unsuccessful enucleation.

Table 3.3: A summary of the outcomes of enucleated oocytes at 3 and 16-18 hours post activation of 5 repeats

Repeat	Cytoplasts remaining	3 hours			16-18 hours						
		No PN	PN visible	Lysis	Frag	1C No PN	1C PN	2C	3C	5C	Lysis
1	35	33	1	1	27	4	2	1			1
4	49	49			35	7	5	1		1	
5	49	48	1		34	12		1	2		
6	59	59			16	37		4	2		
7	48	47	1		18	27		2	1		
TOTAL	**240**	**236**	**3**	**1**	**130**	**87**	**7**	**9**	**5**	**1**	**1**
	%	**98.3**	**1.3**	**0.4**	**54.2**	**36.3**	**2.9**	**3.8**	**2.1**	**0.4**	**0.4**

PN = pronuclei, Frag = fragmentation, C = cell

Orange highlighted column indicates the number of cytoplasts in each repeat that did not react to activation

Due to the degeneration of cytoplasts in repeats 2 and 3 and oocytes that were non-reactive to activation in all included repeats, a larger number of oocytes were used in this section of study 1 to reach the number of at least 150 enucleated and activated oocytes required for statistical significance. Once the 1 cell stage oocytes with no visible pseudo-pronuclei were excluded, fragmentation of cytoplasts was analysed between 16-18 hours after activation and this indicated that **85%** (130/153) of cytoplasts with a 95% CI [79.3;90.6] were effectively enucleated (Table 3.4). A cohort of non-enucleated control oocytes confirmed effectiveness of the activation protocol by showing a pseudo-pronucleus formation rate of **95.4%** (124/130) with a 95% CI [90.5;98.5] (Table 3.5).

Table 3.4: Summary of the final data of included cytoplasts showing fragmentation at 16-18 hours post activation of the 5 repeats

Repeat	Cytoplasts remaining	1C No PN cytoplasts excluded	Final cytoplasts included	16-18 hours
				Fragmented
1	35	4	31	27
4	49	7	42	35
5	49	12	37	34
6	59	37	22	16
7	48	27	21	18
TOTAL	**240**	**87**	**153**	**130 (85%)**

Table 3.5: Summary of the outcomes of non-enucleated control oocytes at 3 and 16-18 hours post activation

Repeat	Non-enucleated control oocytes activated	3 hours			16-18 hours								
		PN visible	No PN	Degen	Frag	Degen	1C NO PN	1C PN	2C	3C	4C	5C	16C
1	35	29	3	3	2	3	3	20	6	1			
4	22	22						5	13	3	1		
5	16	16						4	11	1			
6	25	25					2	4	16		1	1	1
7	32	32							24	2	4		2
TOTAL	**130**	**124**	**3**	**3**	**2**	**3**	**5**	**33**	**70**	**7**	**6**	**1**	**3**
	%	**95.4 95% CI [90.5;98.5]**	**2.3**	**2.3**	**1.5**	**2.3**	**3.8**	**25.4**	**53.8**	**5.4**	**4.6**	**0.8**	**2.3**

Degen = degenerated, PN = pronuclei, Frag = fragmentation, C = cell

3.1.2.2 Hoechst 33342 DNA stain

Hoechst 33342 stain was the second technique used to confirm the presence or absence of the meiotic spindle complex after enucleation. Fluorescence microscopy was used to investigate positively and negatively stained non-enucleated control oocytes and cytoplasts, respectively. In positively stained non-enucleated control or unsuccessfully enucleated oocytes, fluorescent signals from the stained chromosomes were clearly and easily observed (Figure 3.6 A). In contrast, negatively stained cytoplasts showed no fluorescent signals due to the absence of chromosomes, which confirmed efficient enucleation (Figure 3.6 B). Karyoplasts, encapsulating the isolated meiotic spindle complex after enucleation, were distinguished by the presence of fluorescent signals emitted from the stained chromosomes within a very small portion of ooplasm (Figure 3.6 B).

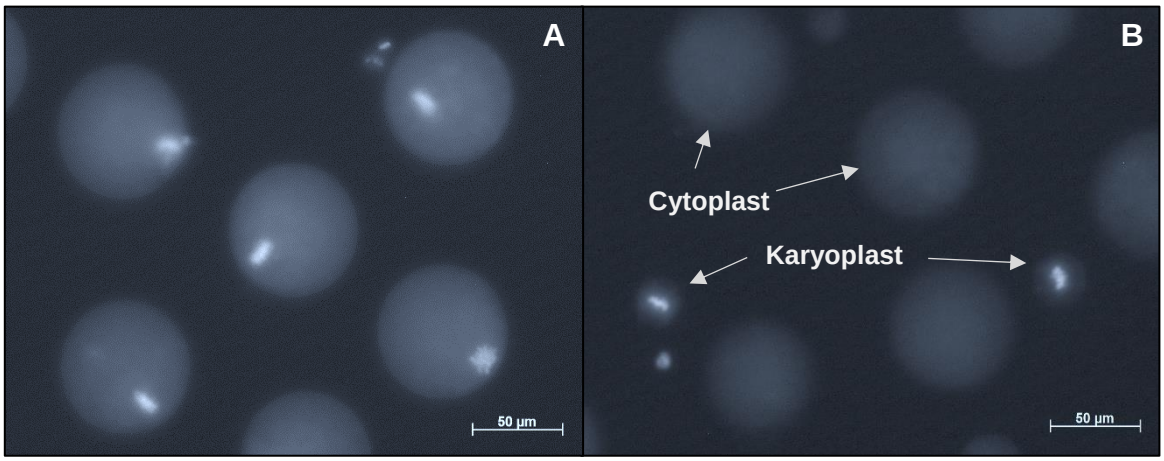

Figure 3.6: Fluorescent microscopy images of positively stained non-enucleated control oocytes (A) and negatively stained cytoplasts and positively stained karyoplasts (B) (images captured at RBL, UP).

To achieve the number of at least 150 enucleated and stained oocytes required for statistical significance, 5 repeats were performed in this section of study 1. Table 3.6 is a summary of the outcomes of cytoplasts and non-enucleated control oocytes after Hoechst 33342 DNA staining for 5 repeats. A total of 167 oocytes were enucleated and stained. An enucleation efficiency of **97.5%** (156/160) with a 95% CI [95.1;99.9] was confirmed by negatively stained cytoplasts (Table 3.6). The staining protocol was verified using a group of non-enucleated control oocytes in parallel, with a result of **100%** (101/101) positively stained oocytes (Table 3.6).

Table 3.6: Overview of the outcomes of cytoplasts and non-enucleated control oocytes after Hoechst 33342 DNA staining

Repeat	Enucleated oocytes stained	Cytoplasts "lost" due to handling	Cytoplasts included	Pos	Neg	% Neg	Non-enucleated control oocytes stained	Oocytes "lost" due to handling	Oocytes included	Neg	Pos	% Pos
1	30	1	29	0	29	100	20	1	19	0	19	100
2	22	0	22	1	21	96,0	13	0	13	0	13	100
3	40	1	39	1	38	97,4	25	4	21	0	21	100
4	35	3	32	2	30	94,0	25	0	25	0	25	100
5	40	2	38	0	38	100	23	0	23	0	23	100
TOTAL	**167**	**7**	**160**	**4**	**156**	**97,5 95% CI [95.1;99.9]**	**106**	**5**	**101**	**0**	**101**	**100**

Pos = positive stain, Neg = negative stain

3.2 Study 2: Overview

In study 2, oocyte retrievals were performed on 43 BDF1 mice (average age of 10.2 weeks). Mice aged between 8-12 weeks were used in study 2. Approximately 3 mice (median) were superovulated per day of oocyte retrievals. The average number of oocytes retrieved per mouse in this study was 39.5 (min = 58; max = 215). Of the total number of oocytes retrieved, 11% (186/1698) were parthenogenetic or abnormal. See Table 3.7 for an overview of the mice used and the allocation of oocytes in each repeat performed in study 2. The oocytes were allocated randomly between the control and experimental groups.

Table 3.7: A detailed summary of the superovulated mice and oocytes retrieved in each repeat performed in study 2

Repeat	Age of BDF1 mice (weeks)	No. of mice used	Number (No.) of oocytes			
			Retrieved	Parthenogenetic/ abnormal + (degenerated)	SCNT (experiment)	Non-enucleated (control)
1	12	2	58	5	30	20
2	12	2	59	8	23	20
3	8	2	100	10	40	20
4	8	2	60	6	17	10
5	8	2	80	15	20	20
6	8	4	140	10	60	50
7	9 & 10	4	205	24 + (12)	64	60
8	10	4	192	16 + (12)	64	60
9	8	4	215	10	52	45
10	8	3	60	4	40	15
11	9	3	141	14	50	24
12	8	3	65	13	30	15
13	8 & 9	4	138	17	39	12
14	8	4	185	10	35	12
TOTAL		**43**	**1698**	**186**	**564**	**383**
% Oocytes retrieved per group				**11%**	**33.2%**	**22.6%**
% Oocytes used in Study 2					**55.8%**	
% Unused oocytes in Study 2: **33.2%**						
Average oocytes retrieved per mouse in Study 2: **39.5**						

3.2.1 Enucleation, nuclear transfer and fusion, followed by activation and embryo culture to the blastocyst stage

A standard enucleation protocol is followed by the transfer and fusion of a somatic cell (obtained from a suitable donor) into a cytoplast.[2,3] The manipulated oocyte is then artificially activated by means of chemical stimulation, which induces subsequent development of the embryo to the blastocyst stage.[2] The latter was the ultimate objective of study 2.

Enucleation in study 2 was performed as in study 1 (section 3.1.1 and Figure 3.2); however, with the additional step to remove the PB if viable and not degenerated (Figure 3.7). This was done to avoid potential fusion of the PB during HVJ-E exposure in this study.

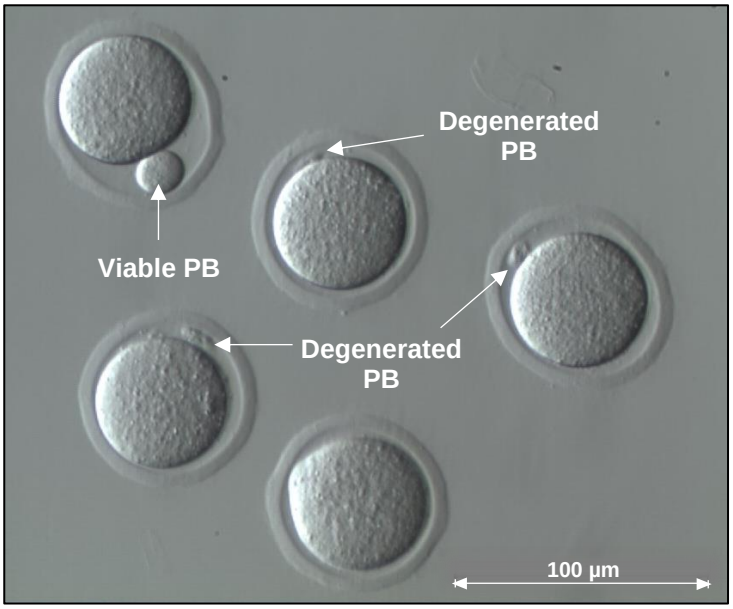

Figure 3.7: Photo images showing a **viable** and several **degenerated polar bodies** in B6D2F1 mouse oocytes (image captured at RBL, UP).

Nine hundred and forty-seven B6D2F1 oocytes were used in study 2 (Table 3.7 and Figure 3.8). A breakdown of the number of oocytes utilized and remaining in each step of the SCNT procedure in study 2 is depicted in Figure 3.8. Of the 383 non-enucleated control oocytes that were exposed to the activation protocol, 375 successfully activated as seen by the formation of pseudo-pronuclei. The first cell division due to parthenogenesis was noted in 325 of the successfully activated oocytes, of which 211 developed to the blastocyst stage. Five hundred and sixty-four oocytes were enucleated in study 2. Enucleation was performed with a survival rate of **99.1%**

(559/564) and a lysis rate of **0.9%** (5/564). After enucleation, 559 cytoplasts underwent cumulus cell nuclear transfer with 100% (559/559) survival. Subsequently, 404 reconstructed oocytes showed fusion and were artificially activated. Successful activation was achieved in 330 fused oocytes, of which 225 showed the first cell division of embryo development. From those that cleaved, only 22 SCNT embryos reached the blastocyst stage. The abovementioned results will be discussed in more detail in the sections to follow.

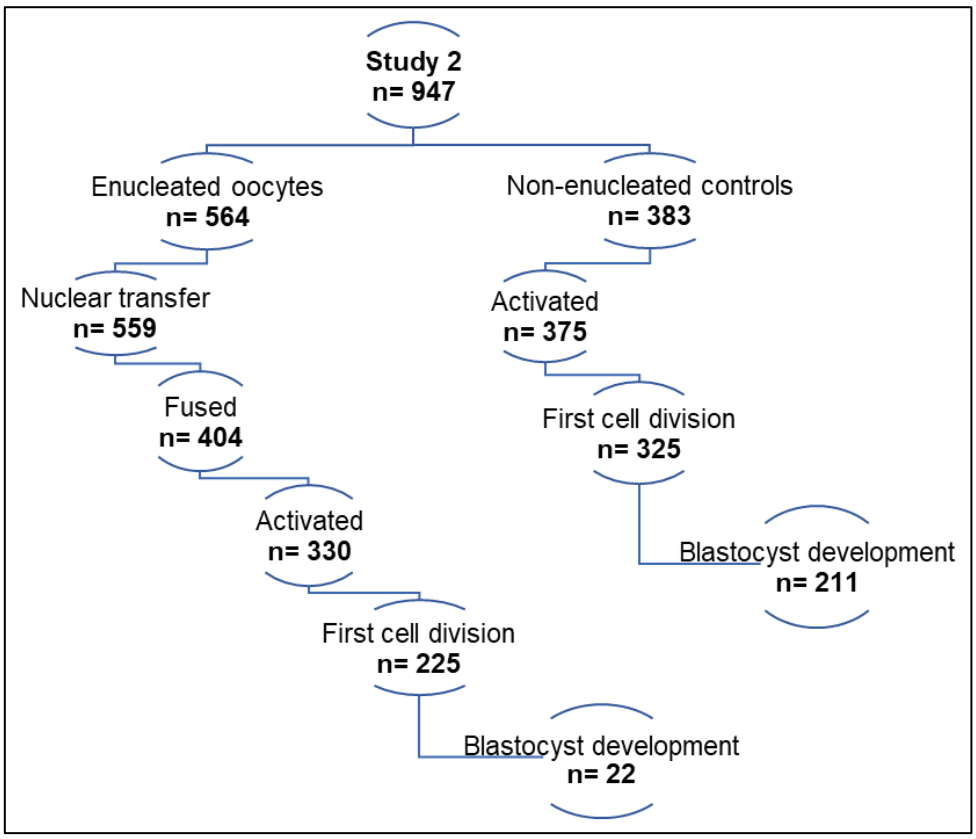

Figure 3.8: Flow diagram depicting the distribution of oocytes used as non-enucleated controls and in each step of the SCNT procedure in study 2.

The nuclear transfer procedure was performed by the fusion of cumulus cells to cytoplasts using inactivated HVJ-E. Cumulus cells from B6D2F1 female mouse COCs were the somatic donor cells of choice (illustrated in Figure 3.9 A). A single cumulus cell was randomly selected with the transfer pipette (Figure 3.9 B), and fully immersed in the HVJ-E solution for an exposure period of 20 seconds (Figure 3.9 C). Using the holding and transfer pipettes, the selected cytoplast was rotated until the hole in the zona pellucida (created for enucleation) was located and manoeuvred to the 3 o'clock position (Figure 3.9 D). While securing the cytoplast using the holding pipette, the

transfer pipette was inserted deep into the cytoplast (to ensure good contact) without penetrating the oolemma, followed by expulsion of the cumulus cell (Figure 3.9 E and F). Immediately after nuclear transfer it was noticed that the cumulus cells would adsorb to the outer PVS region of the cytoplast, which was expected. As was previously stated, donor somatic cells when entirely exposed to HVJ-E, may become sticky and as a result, difficult to handle.[12] Stickiness of cumulus cells after complete exposure to HVJ-E, and difficulty in manipulation thereof, were only experienced during a few attempts in this study.

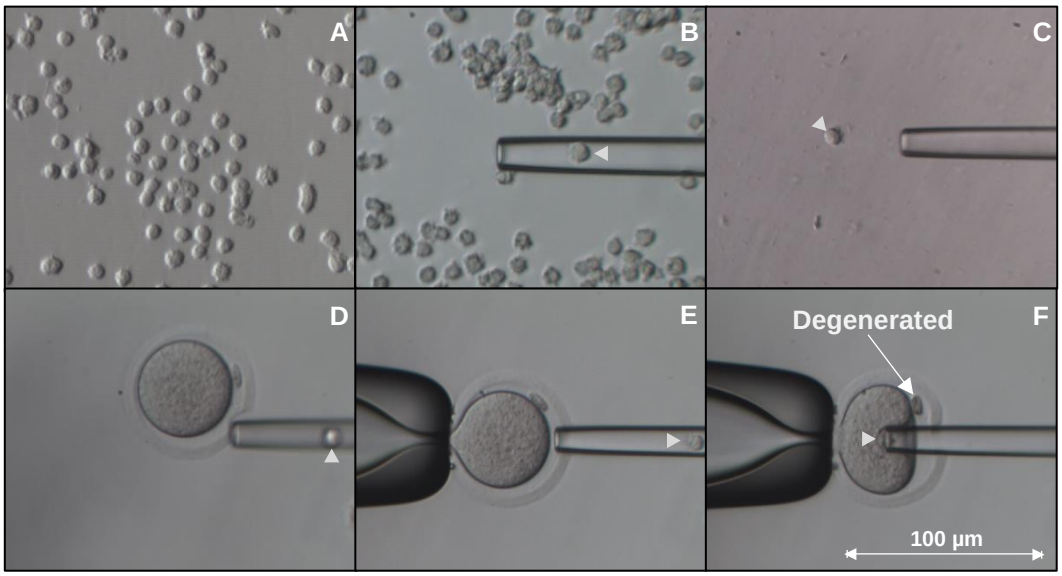

Figure 3.9: Step-wise photos indicating the **nuclear transfer procedure** (A-F). Selected and transferred cumulus cell is indicated by white arrowheads (images captured at RBL, UP).

3.2.1.1 Fusion

Hoffman modulation contrast microscopy was used to confirm fusion of the cumulus cells into the cytoplasts 30 minutes after nuclear transfer. Directly following nuclear transfer, the cumulus cells had adsorbed to the outer PVS region of the cytoplasts (Figure 3.10: 0 min). Figure 3.10 is a time-lapse example of a cumulus cell fading into a cytoplast within a short period of 15 minutes. In this study, fusion of donor cells and cytoplasts by HVJ-E was completed within 15 to 30 minutes after nuclear transfer.

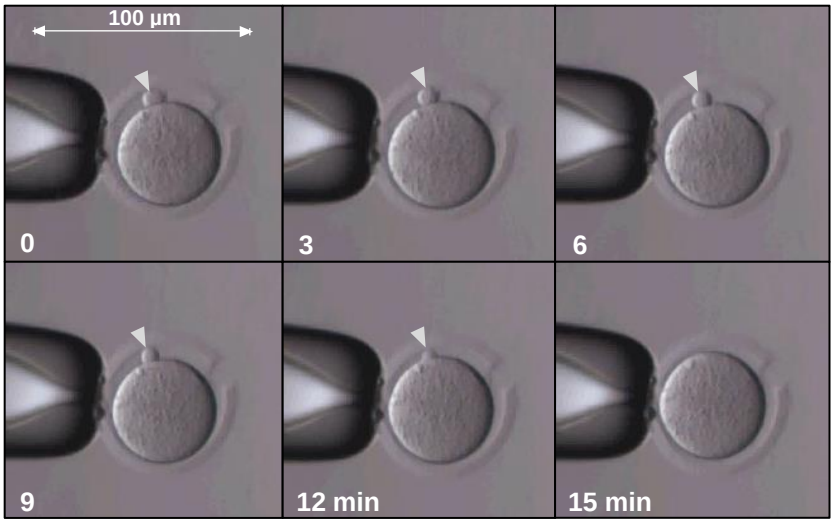

Figure 3.10: Time-lapse images showing **fusion** of a cumulus cell into a cytoplast within 15 minutes. The transferred cumulus cell is indicated by white arrowheads (images captured at RBL, UP).

Successful fusion was confirmed by the complete disappearance of cumulus cells into cytoplasts, which were sometimes visible as bulges (Figure 3.11 A). Unsuccessful fusion was indicated by the adsorption of cumulus cells to the outer PVS region of cytoplasts (Figure 3.11 B). A fusion rate of **72.3%** (404/559) with a 95% CI [68.8;75.8] was achieved in this study. Fused oocytes were used for further SCNT experimentation including activation, embryo culture and final data analysis. Non-fused oocytes were discarded and excluded from further experimentation and final data analysis.

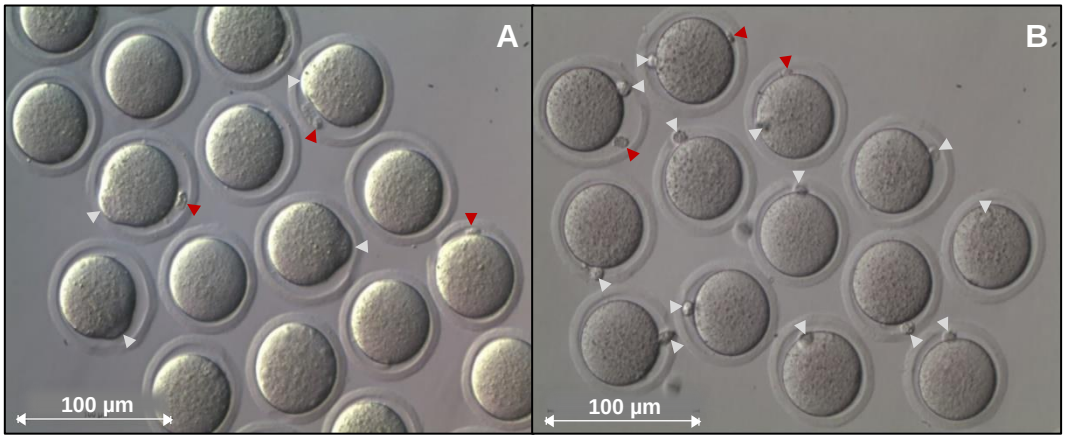

Figure 3.11: Images demonstrating **fused** (A) and **non-fused cumulus cells and cytoplasts** (B). Bulges in figure A and non-fused cumulus cells in figure B are indicated by white arrowheads. Degenerate PBs are indicated by red arrowheads (images captured at RBL, UP).

3.2.1.2 Activation

Fused oocytes were artificially activated as described in section 2.8.1.1 of study 1, with the addition of TSA in well 4 of the activation dishes (Figure 2.8, page 41). An activation survival rate of **99.8%** (403/404) with a **0.2%** (1/404) degeneration rate was achieved. As a verification of the activation protocol with TSA, a cohort of non-enucleated control oocytes was activated and showed a pseudo-pronucleus formation rate of **97.9%** (375/383) with a 95% CI [96.5;99.3]. Efficient activation of the fused SCNT oocytes was analysed by the number of oocytes with visible pseudo-pronuclei immediately after the 3 hour activation period. An activation rate of **81.7%** (330/404) with a 95% CI [77.9;85.5] was achieved in the SCNT group. The difference between the pseudo-pronucleus formation rate of the SCNT group versus that of the control group was statistically significant (Figure 3.12).

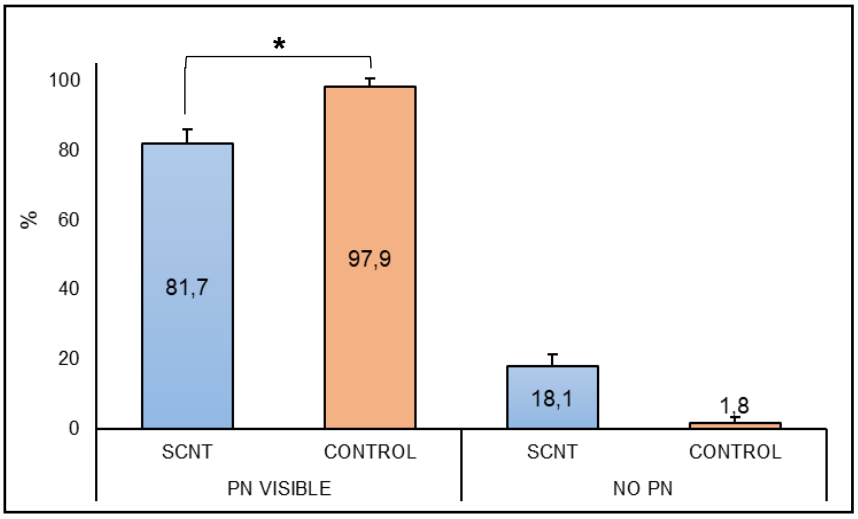

Figure 3.12: Bar graph representing the percentages of visible and absent **pseudo-pronuclei** in the SCNT group versus the non-enucleated control group. *P*-value < 0.001.

3.2.1.3 SCNT embryo development to the blastocyst stage

TSA treatment protocol and blastocyst formation

Twenty-six experimental repeats totalling 404 fused SCNT oocytes underwent activation and further culture with the addition of TSA treatment. Based on recommendations in the literature, three different concentrations of TSA (5 nM, 25 nM and 50 nM) were tested at two different exposure times (8 and 10 hours) and compared to determine the most effective exposure combination (Table 3.8). The most efficient TSA protocol for this study was determined by the number of blastocysts

formed by at least 96 hours post activation from the total number of fused SCNT oocytes. A trend towards increased blastocyst formation was noticed when SCNT embryos were treated with 50 nM TSA for 8 hours. According to statistical analyses, the difference in blastocyst formation between the 8 and 10 hour exposure group was statistically significant (**6.8%** vs. **2.9%**, $p < 0.001$). However, there were no statistically significant differences in blastocyst formation between 5 nM, 25 nM and 50 nM TSA (**4.3%** vs. **1.5%** vs. **7.5%**).

To reach at least 20 blastocyst stage embryos required for the final objective of study 2, more experiments were performed with the preferred treatment protocol, namely 50 nM TSA at 8 hours exposure from the start of activation. Blastocyst formation by SCNT was significantly lower than that of the control group (**5.4%** [22/404] vs. **55.1%** [211/383]). Therefore, the chance of forming a blastocyst by SCNT was **0.041-fold** that of the control group, according to statistical analyses. See Table 3.8 for a summary of the total blastocyst formation outcomes of the 26 experiments performed with the three concentrations of TSA at two exposure periods.

Table 3.8: Overview of total blastocyst formation investigating three concentrations of TSA at two exposure times when comparing the SCNT and control groups

Concentration of TSA [ ]	#	Hour exposure	SCNT blastocyst formation	Control blastocyst formation
5 nM	4	8	5/64	39/57
	5	10	1/75	33/80
25 nM	3	8	0/35	23/40
	3	10	1/30	20/40
50 nM	8	8	13/167	72/116
	3	10	2/33	24/50
Total blastocyst formation			**22/404**	**211/383**
% blastocyst formation			**5.4**	**55.1**
***P*-value**			*< 0.001*	

 # = number of experiments performed in each hour of exposure group

Analysis of blastocyst formation using a different histone deacetylase inhibitor (HDACi), Scriptaid (250 nM) at 8 hours of total exposure was tested in triplicate and compared in parallel to TSA (50 nM). The blastocyst formation findings with Scriptaid did not differ significantly from those with TSA (**2.5%** vs. **7.7%**; Table 3.9). To investigate the possible negative impact of these HDACis on embryo development, a control group of non-enucleated oocytes was also activated using TSA and Scriptaid, and cultured further with these HDACis as described for the SCNT embryos. Efficient blastocyst development with no significant difference was achieved in both the groups exposed to TSA and Scriptaid (**56.4%** vs. **61.5%**; Table 3.9) and no toxic effects of the HDACis were noted.

Table 3.9: Blastocyst formation rates of 250 nM Scriptaid versus 50 nM TSA at 8 hours exposure

HDACi	Blastocyst formation	% Blastocyst formation/HDACi	Control blastocyst formation	% Control blastocyst formation/HDACi
250 nM Scriptaid	1/40	**2.5**	24/39	61.5
50 nM TSA	5/65	**7.7**	22/39	56.4
***P*-value**	*0.404*		*0.837*	

SCNT embryo development

Non-enucleated oocytes were activated and cultured as controls for activation and cell culture conditions with TSA treatment, and exhibited further development due to parthenogenesis. Ongoing cell division and subsequent embryo development of the SCNT and control groups were monitored and photographed daily, from immediately after activation for up to 5 days of embryo culture post SCNT.

Significant differences were noted between the SCNT and control groups regarding the expected stage of embryo development on specific embryonic days[213] post SCNT. These results are summarised in Table 3.10 and depicted in Figure 3.13. On embryonic day 1, mouse embryos were expected to have undergone their first cell division to the 2 cell stage. More control embryos were at the 2 cell stage than SCNT embryos (67.6% vs 48.8%, p = 0.018). The control group also had more embryos at the 3 cell (6.5% vs 3.2%, p = 0.035) and 4 cell (10.4% vs 1.7%, p = 0.001) stages compared to SCNT embryos on day 1. Despite the majority of the SCNT embryos

cleaving to the 2 cell stage on day 1, more SCNT embryos had arrested at the 1 cell stage with visible pseudo-pronuclei compared to the control group (20.3% vs 1%, $p < 0.001$).

Day 2 of embryonic development was expected to reveal embryos at the 4 and 8 cell stages. No statistical difference was noted between the control and SCNT groups at the 4 cell stage (30.5% vs 26.5%, $p = 0.687$). However, there was a noticeable difference between the control and SCNT groups at the 8 cell stage (14.6% vs 0.7%, $p < 0.001$). Additionally, more control embryos were at the 5 cell (9.1% vs 3.2%, $p = 0.020$), 6 cell (6.3% vs 1.5%, $p = 0.006$) and 13-16 cell (3.9% vs 0%, $p = 0.036$) stages compared to SCNT embryos on day 2.

Compacting cells, morulae, starting (early) blastocysts and blastocysts were expected on day 3 of embryonic development. No difference in compacting cells was noted between the control and SCNT groups (13.8% vs 18.8%, $p = 0.207$). On the contrary, a higher number of control embryos compared to SCNT embryos were morulae (26.4% vs 13.6%, $p = 0.033$), starting blastocysts (10.2% vs 0.7%, $p = 0.001$) and blastocysts (2.3% vs 0%, $p = 0.042$) on day 3 of embryonic development. Compared to control embryos, SCNT embryos were still arrested at the 1 cell (16.8% vs 4.2%, $p < 0.001$), 2 cell (14.1% vs 6.0%, $p = 0.004$) and 3 cell (7.2% vs 3.7%, $p = 0.045$) stages on this day.

Expanded blastocysts were expected on day 4 of embryonic development. A significant difference was observed between the control and SCNT group at this stage of development (32.6% vs 0%, $p < 0.001$). More control embryos were also at the blastocyst stage compared to SCNT embryos (9.1% vs 1.7%, $p = 0.010$). A higher number of SCNT embryos were arrested at the 1 cell (12.1% vs 1.3%, $p < 0.001$) and 2 cell (10.6% vs 2.6%, $p = 0.001$) stages. However, most of the SCNT embryos had degenerated on day 4 compared to the control group (32.45 vs 12%, $p = 0.002$).

On day 5 of embryonic development, hatching or fully hatched blastocysts were expected. There was no significant difference in the hatching blastocysts between the control and SCNT group (6.8% vs 2.7%, $p = 0.144$). However, there was an apparent difference between the control and SCNT blastocysts that were fully hatched on day 5 (26.4% vs 2.5%, $p < 0.001$). In addition, a larger number of control embryos were blastocysts (5.2% vs 1%, $p = 0.012$) and expanded blastocysts (16.7% vs 0.2%, $p <$

0.001) compared to SCNT embryos. Yet again, more SCNT embryos compared to control embryos were arrested at the 1 cell (5.7% vs 0%, $p = 0.003$), 2 cell (6.7% vs 1%, $p < 0.001$) and 4 cell (2% vs 0.3%, $p = 0.023$) stages. On day 5, more SCNT starting blastocysts were present compared to the control group (7.2% vs 2.1%, $p = 0.003$). Nevertheless, degeneration of the majority of the SCNT embryos was once again observed when compared to the control group on day 5 (61.4% vs 34.2%, $p < 0.001$).

Table 3.10: The rate of expected stages of embryo development on specific embryonic days post SCNT comparing the SCNT group and the control group

Embryonic days post SCNT	Expected stage of embryo development[213]	SCNT	Control	*P*-value
Day 1	2C	**48.8%** 197/404)	**67.6%** 259/383)	*0.018**
Day 2	4C	**26.5%** 107/404)	**30.5%** 117/383)	*0.687*
	8C	**0.7%** (3/404)	**14.6%** (56/383)	*<0.001**
Day 3	Compacting cells	**18.8%** (76/404)	**13.8%** (53/383)	*0.207*
	Morula	**13.6%** (55/404)	**26.4%** 101/383)	*0.033**
	Starting blastocyst	**0.7%** (3/404)	**10.2%** (39/383)	*0.001**
	Blastocyst	**0%** (0/404)	**2.3%** (9/383)	*0.042**
Day 4	Expanded blastocyst	**0%** (0/404)	**32.6%** 125/383)	*<0.001**
Day 5	Hatching blastocyst	**2.7%** (11/404)	**6.8%** (26/383)	*0.144*
	Hatched blastocyst	**2.5%** (10/404)	**26.4%** 101/383)	*<0.001**

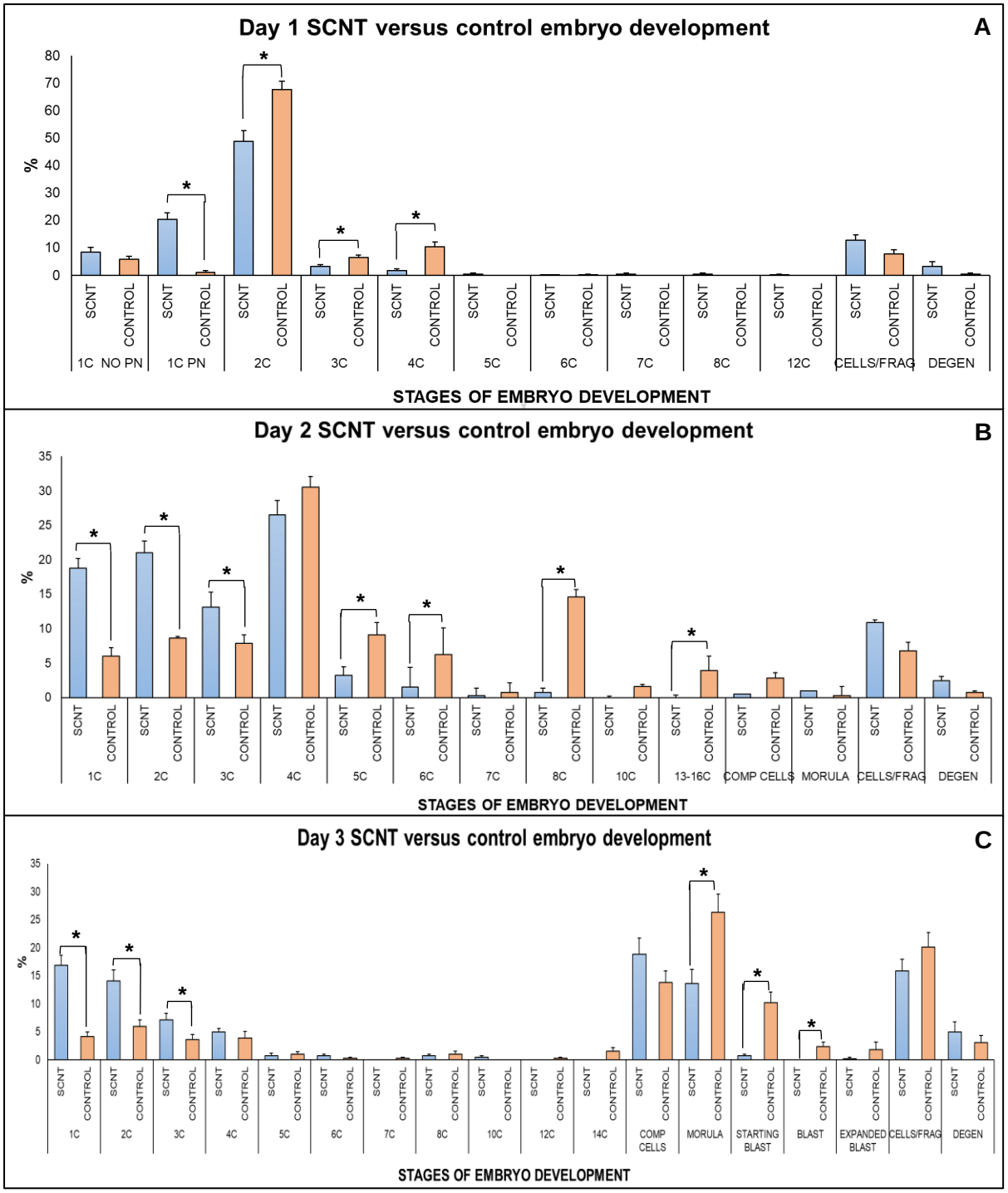

Figure 3.13: Bar graphs depicting the **embryo development of SCNT and control groups** on specific embryonic days after SCNT. *P*-value < 0.05.

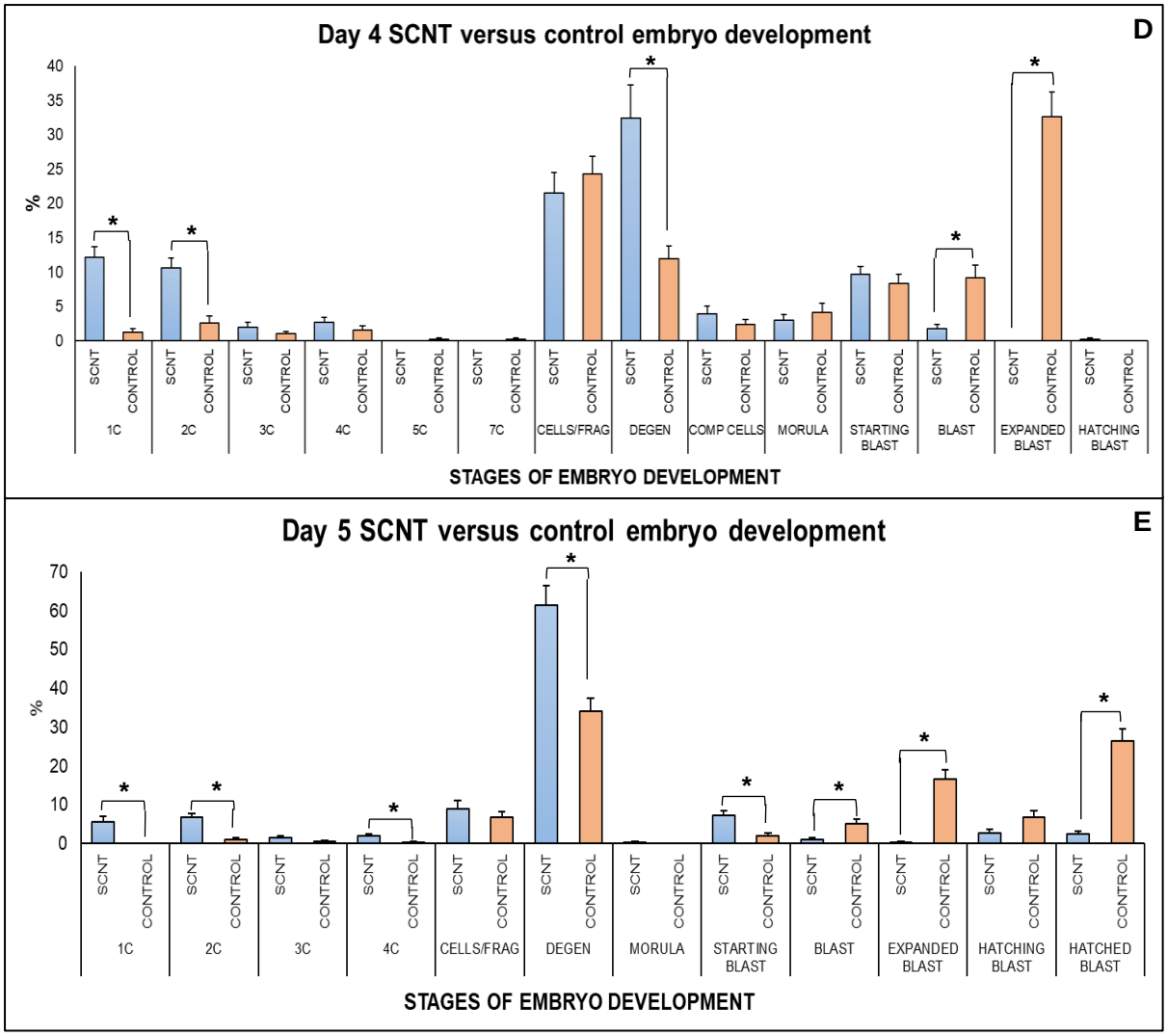

Figure 3.13 (continued): Bar graphs depicting the **embryo development of SCNT and control groups** on specific embryonic days after SCNT. *P*-value < 0.05.

Expanded or hatching SCNT blastocysts had only developed on day 5 (approximately 120 hours post nuclear transfer), whereas the control group achieved fully expanded blastocysts on day 4 (approximately 96 hours post nuclear transfer). Figures 3.14 and 3.15 are examples of timelines of SCNT and control embryo development**.** These figures show the difference in blastocyst expansion of SCNT and control blastocysts noted on days 4 and 5, which can be seen between 98-114.6 hours post nuclear transfer in Figure 3.14, and between 85-107.4 hours post the 8 hour TSA exposure in Figure 3.15. Figure 3.14 also highlights the formation of SCNT starting blastocysts and the high degree of degeneration on days 4 and 5 of embryonic development. Figure 3.15 illustrates the development of SCNT expanding and hatching blastocysts on embryonic days 4 (85 hours) and 5 (107.4 hours).

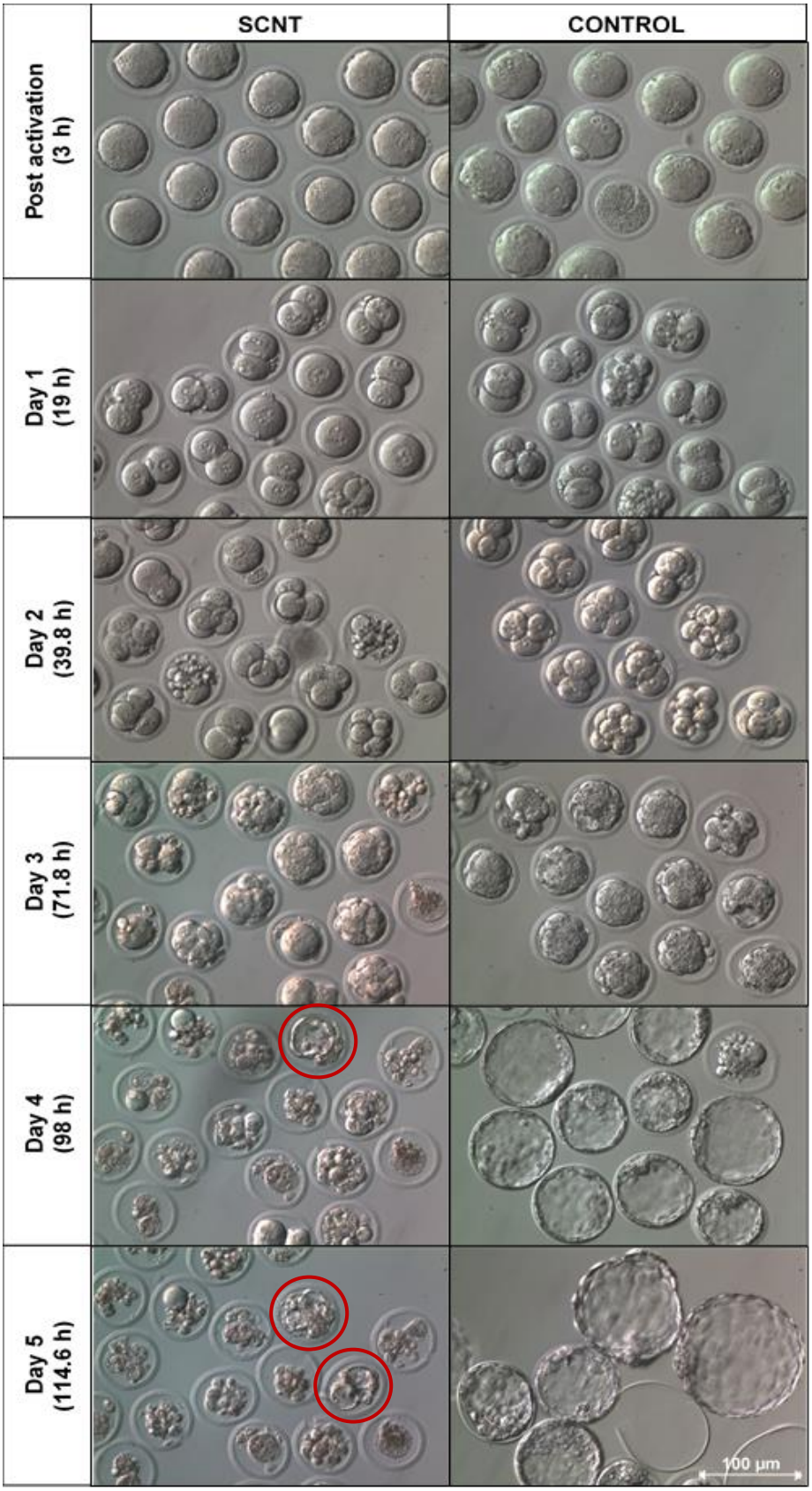

Figure 3.14: An example of a **timeline of SCNT versus control embryos development** in hours (h) post nuclear transfer. Starting blastocysts in the SCNT group are encircled (images captured at RBL, UP).

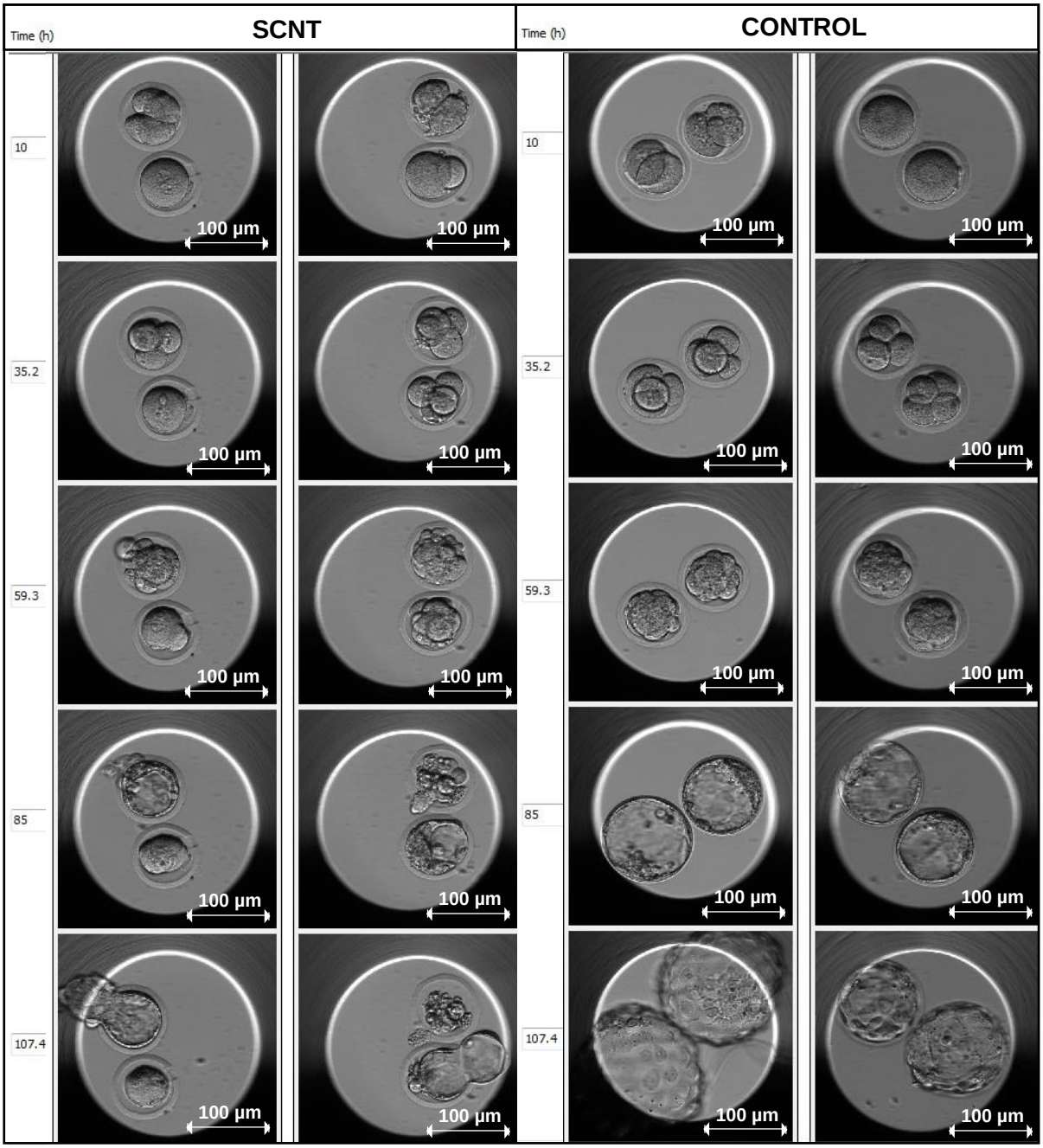

Figure 3.15: EmbryoScope™ time-lapse system images comparing the **timelines of SCNT versus control embryos development** in hours (h) post TSA exposure period (images captured at RBL, UP).

SCNT blastocyst quality

More poor-quality SCNT blastocysts without distinct ICMs and the presence of excluded and degenerated cells were produced than good or average quality blastocysts (**63.6%** vs **18.2%** vs **18.2%**, $p < 0.001$) (Figure 3.16). All blastocysts were evaluated using the grading system described by Gardner and Schoolcraft.[214] Blastocyst grading was defined as follows: AA was considered "good" quality; BB was "average" quality; BC, CB and CC were "poor" quality. No AB, AC, BA or CA graded blastocysts were observed. The first and second letters in the grading system represent the grades of the ICM and the TE, respectively. Twenty-two SCNT expanding or hatching blastocysts were obtained and graded. Of the 22 blastocysts that developed through the course of this study, 13 (**59.1%**) were cultured in a K-Minc incubator (Figure 3.17) and the remaining 9 (**40.9%**) were cultured in the EmbryoScope™ time-lapse system (Figure 3.18).

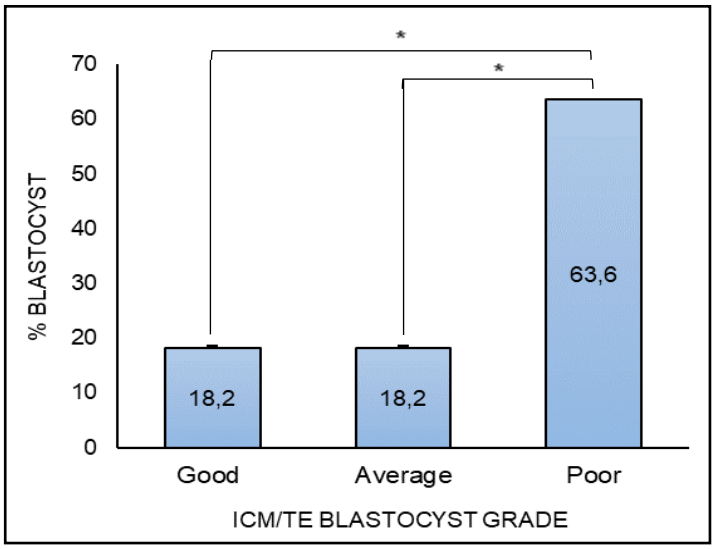

Figure 3.16: Bar graph representing the ICM/TE quality of SCNT blastocysts. *P*-value < 0.001.

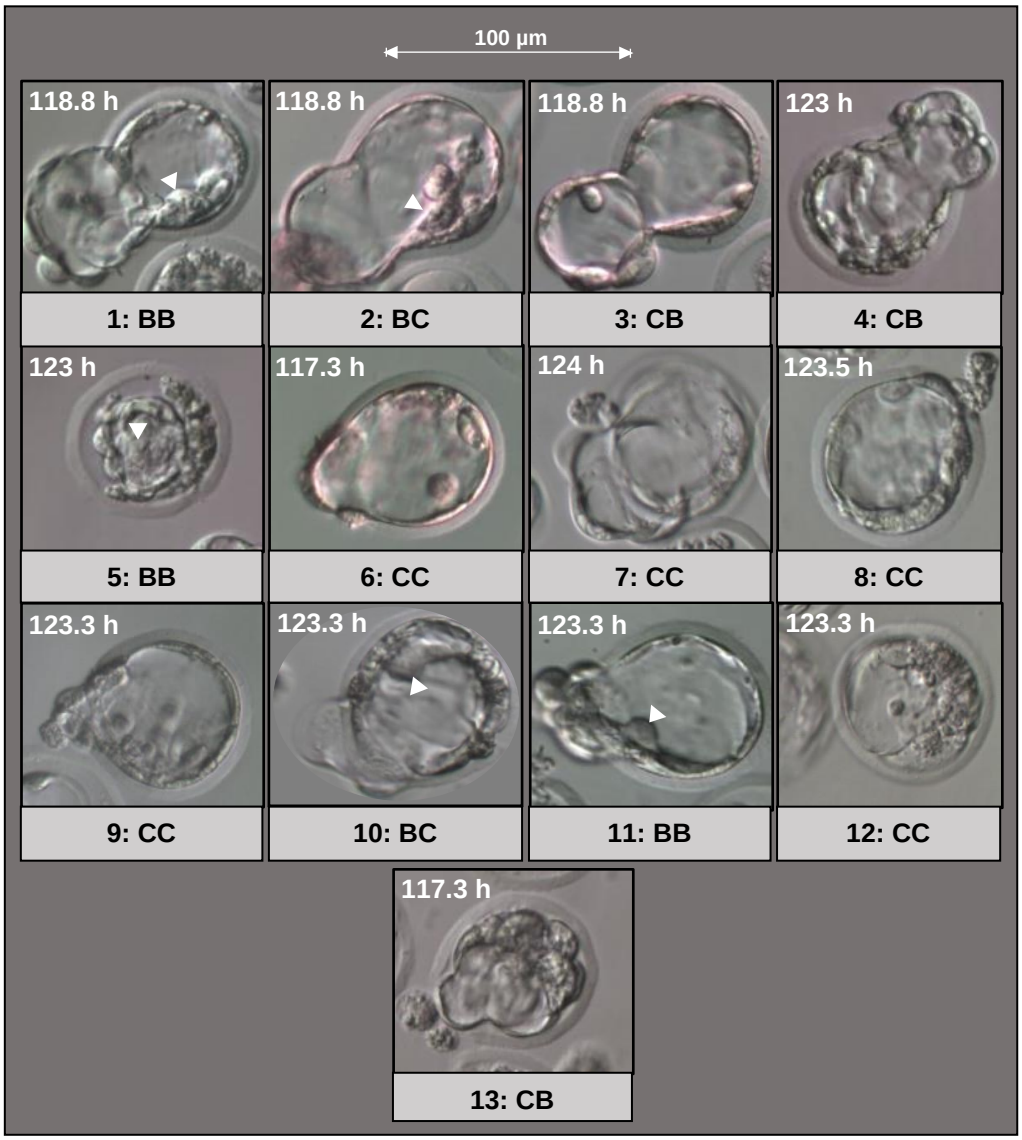

Figure 3.17: Images of 13 **expanded or hatching SCNT blastocysts** (numbered and graded) cultured in the **K-Minc incubator**. Photos were taken on Day 5 at different hours (h) post nuclear transfer. Visible ICMs are indicated by white arrowheads (images captured at RBL, UP).

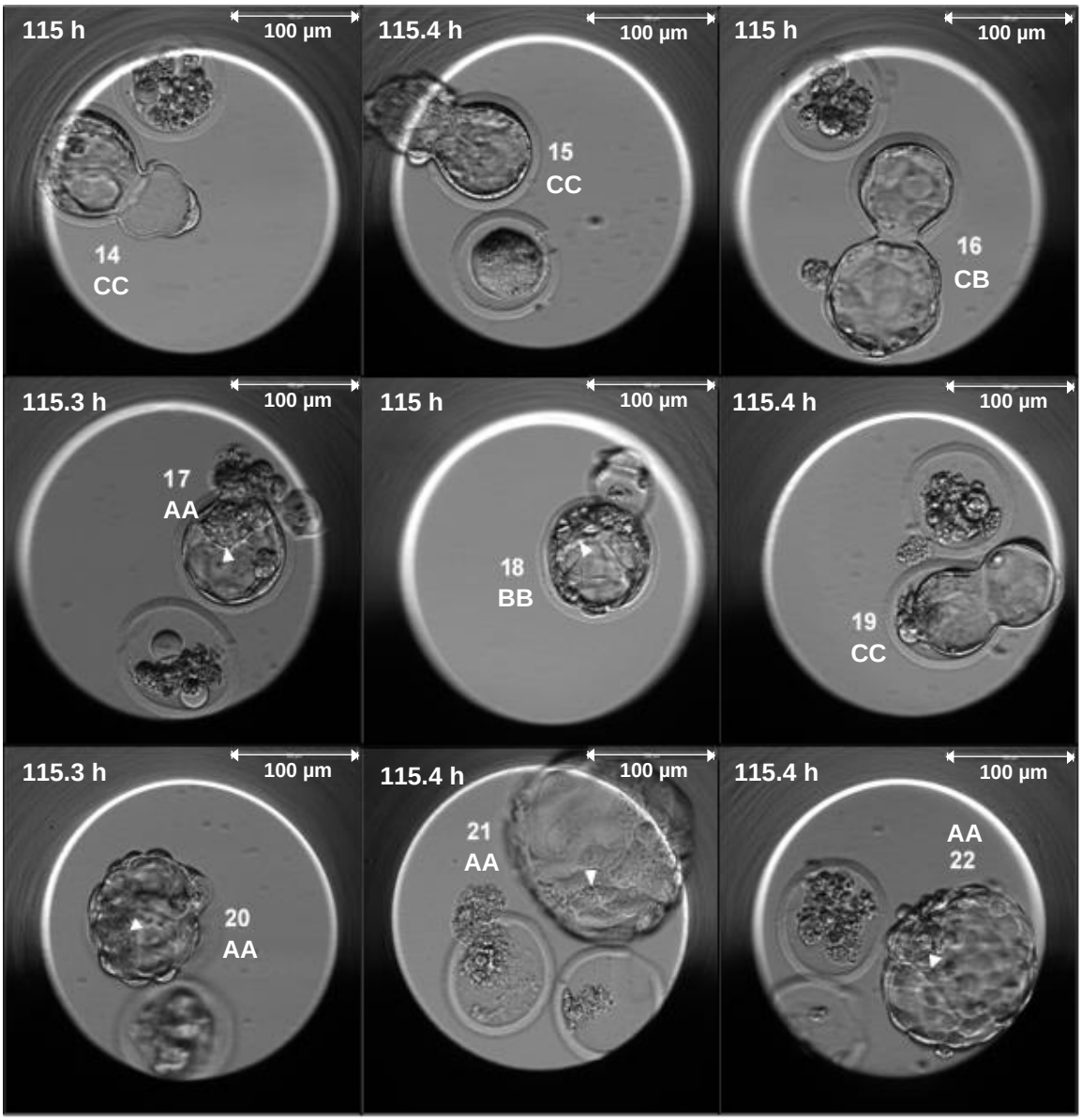

Figure 3.18: **EmbryoScope™ time-lapse system** images of 9 **SCNT hatching or hatched blastocysts** (numbering continued from Figure 3.17 and graded) taken on Day 5 at different hours (h) post nuclear transfer. Visible ICMs are indicated by white arrowheads (images captured at RBL, UP).

A significant increase in the number of poor-quality blastocysts was obtained from those cultured in the K-Minc incubator as opposed to the EmbryoScope™ (**45.5%** vs **18.2%**, p = 0.002). In contrast, a higher percentage of good-quality blastocysts was obtained from those cultured in the EmbryoScope™ when compared to the K-Minc incubator (**18.2%** vs **0%**, p = 0.025). Figure 3.19 summarises the gradings of the blastocysts cultured in the K-Minc incubator and the EmbryoScope™ time-lapse system.

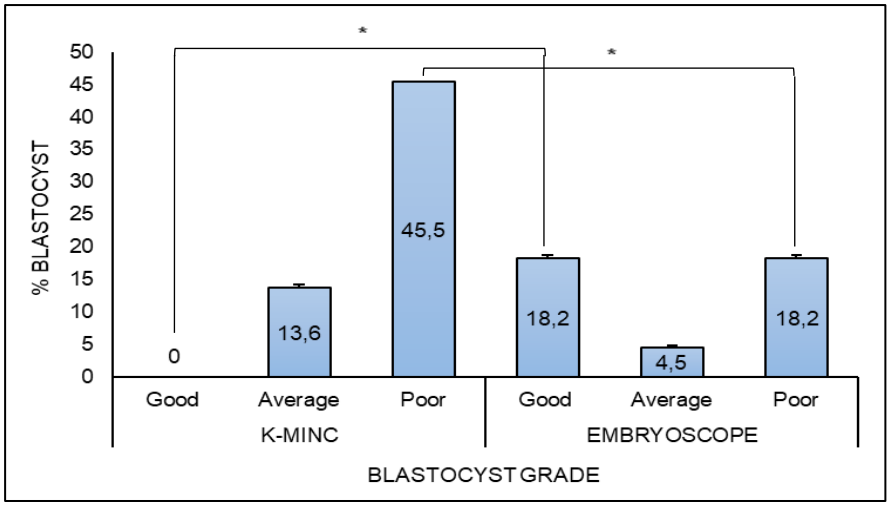

Figure 3.19: Bar graph of **SCNT blastocysts quality** cultured in the K-Minc incubator and the EmbryoScope™ time-lapse system. *P*-value < 0.001.

Overview of SCNT success rates

Despite the significantly lower rates of development in the SCNT group compared to the control group, most of the SCNT outcomes were either higher than or within the success rates recommended by Kishigami (Table 3.11).[78] Blastocyst development was the only outcome after SCNT that was not within the proposed success rate. This finding may be attributed to the significantly higher degeneration rates observed in SCNT embryos on embryonic days 4 and 5 (Figure 3.13 D/E and Figure 3.14 Day 4/Day 5).

Table 3.11: Comparison of the outcomes after laser-assisted SCNT between the SCNT and control groups, and the success rates described by Kishigami *et al.*[78] using piezoelectric nuclear transfer

SCNT outcomes	Kishigami *et al.* success rates	Study 2	
		SCNT	Control
Enucleation survival	99-100%	**99.1%**	-
Nuclear transfer survival	80-90%	**100%**	-
Fusion efficiency	70-80%	**72.3%**	-
Activation survival	70-80%	**99.8%**	99.7%
Pseudo-pronucleus formation	60-70%	**81.7%**	97.9%
Cleavage (1st cell division)	50-60%	**55.7%**	84.9%
Compaction/morula	**30-50%**	**32.4%**	40.2%
Blastocyst development		**5.4%**	55.1%

Figure 3.20 illustrates that over a 5 month period of experimentation more blastocysts were produced during the last 50% of experimental repeats that were performed. It was noted that more successful repeats were achieved towards the end of the study, which was most likely due to the experience gained in the technique.

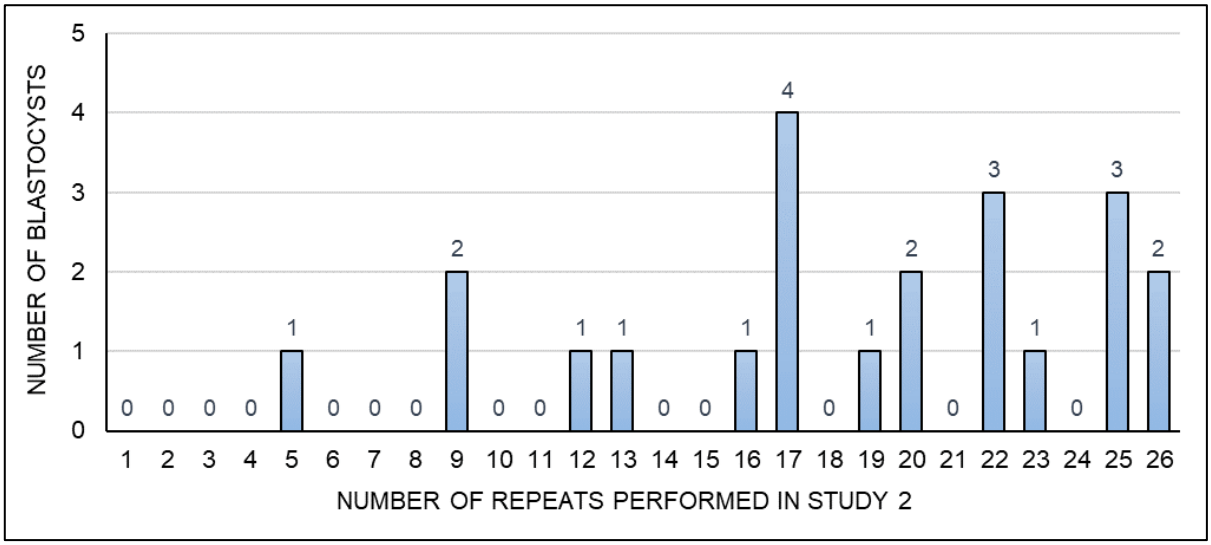

Figure 3.20: Bar graph demonstrating the **number of SCNT blastocysts produced in each SCNT experimental repeat** performed in study 2.

Chapter 4: Discussion and conclusion

4.1 Discussion

The importance of SCNT research in the mouse is highlighted by its distinct advantages over other experimental animal models and the potential thereof to understand underlying principles of nuclear reprogramming. The reversibility of these epigenetic processes facilitates many new prospects in basic research.[56,180,203] Furthermore, the integration of SCNT and ESC techniques is known as therapeutic cloning[4] and provides an opportunity to treat untreatable degenerative diseases, such as Parkinson's disease and many others. The amalgamation of these techniques refers to the injection of a patient's somatic cell into an enucleated oocyte followed by the isolation of ESCs (which can differentiate into any cell type) from the cloned blastocysts. To finalise this process, the differentiated cells are then grafted into the affected patient who donated the healthy somatic cell.[203] In 2012, the Nobel Prize for Physiology and Medicine was jointly awarded to Sir John Gurdon, the inventor of SCNT, and Shinya Yamanaka, the pioneer of induced pluripotent stem cells.[203]

The derivation of human ESCs has been achieved at high rates from SCNT-produced blastocysts, which is critical to the potential use of SCNT as a method for therapeutic cloning.[36,81,215] However, despite technical advancements in the SCNT protocol including polarized light imaging for the removal of the meiotic spindle complex from the oocyte, incubation of the donor somatic cell with a membrane fusogen (e.g. HVJ-E), laser-assisted nuclear transfer to facilitate donor cell insertion into the PVS and improvements to human oocyte activation,[34,203] poor success rates have been observed in terms of SCNT embryo development, which may be attributed to the incomplete or inefficient reprogramming of the somatic cell nucleus.[148,161,180,204]

In mouse SCNT, various factors can affect the success rate of blastocyst development, including the condition of the mice and oocyte quality.[76,78] Multiple factors, depicted in Figure 4.1, including one or more of the invasive steps performed during SCNT, may have negative effects on cytoplast quality, causing inefficient reprogramming and ultimately influencing the success of SCNT.[36]

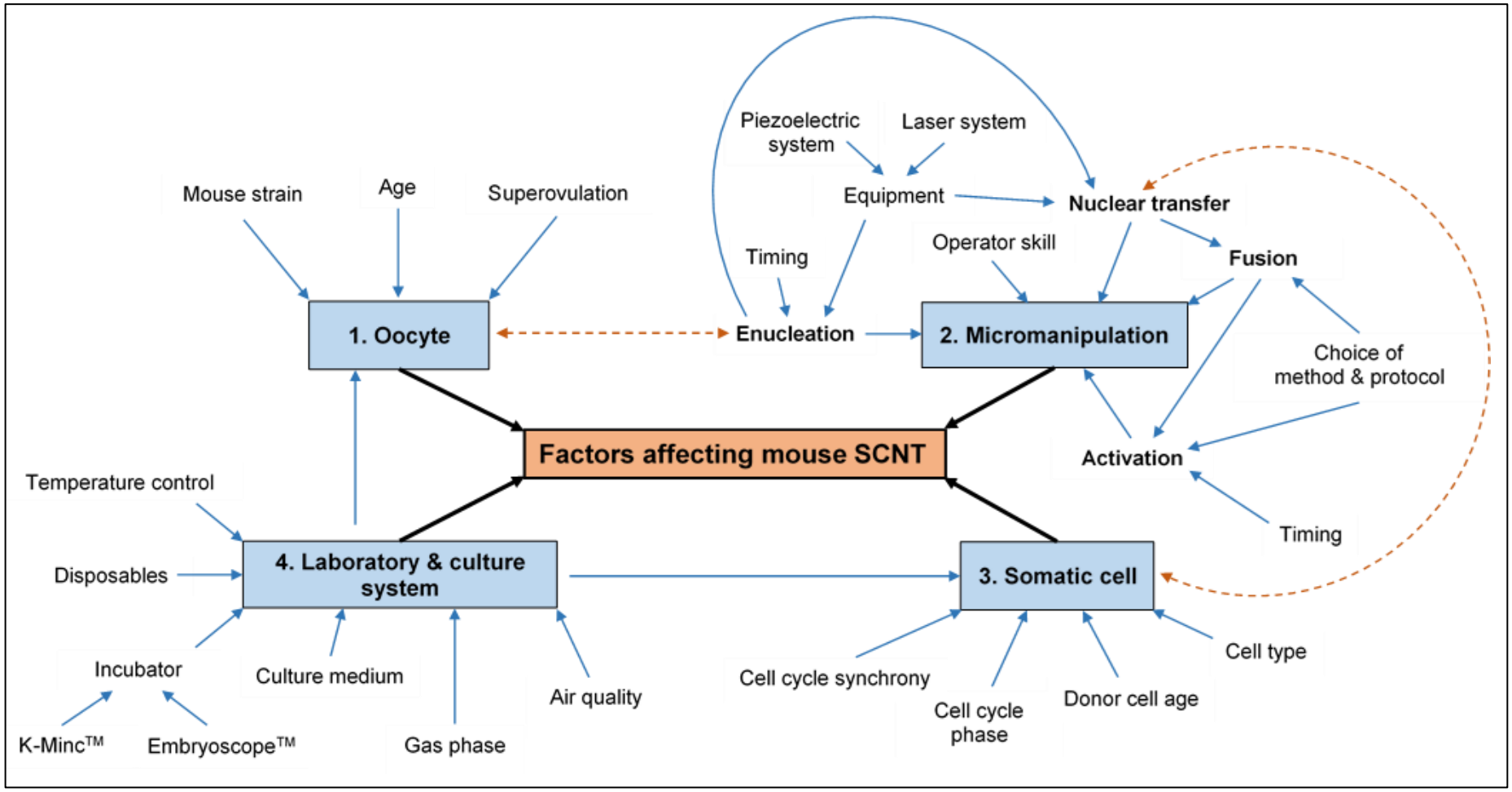

Figure 4.1: Summary of the numerous **factors** that could impact on **affecting the efficiency of SCNT** in the mouse.

4.1.1 Strain characteristics of the oocytes

The mouse strain from which oocytes were collected was selected based on developmental competence, susceptibility to *in vitro* handling and micromanipulation, and visibility of the meiotic spindle complex of the oocytes. Donor oocytes with these advantages (requirements) are oocytes from the **B6D2F1 (C57BL/6×DBA/2)** mouse strain.[78,82,120] Several other strains also fulfil these requirements and can be selected based on the purpose of the research. Ogura[120] provided a short guideline on the applicable strain characteristics as described below:

1. High developmental capability: B6D2F1, B6C3HF1, B6CBAF1, and other F1 hybrids
2. Susceptibility to ICSI procedure: DBA/2 and B6D2F1
3. Visibility of meiotic spindle complex: B6D2F1, DBA/2, ICR, and 129

Superovulation and handling of the mice were performed by a trained veterinary technologist at the UPBRC without difficulty. Superovulation of female B6D2F1 mice between 8 and 15 weeks was induced by an intraperitoneal injection of 5 IU Pregnant Mare Serum Gonadotropin, followed approximately 48 hours later by an intraperitoneal injection of 2.5 IU hCG.[205] The animals were humanely sacrificed by cervical dislocation 10-13 hours post hCG injection to retrieve the COCs.[206]

In **study 1**, a total of 1254 oocytes were harvested from 53 BDF1 mice (±12.9 weeks of age). The average number of oocytes retrieved per mouse in this study was 23.7. Approximately **11%** (139/1254) of the total number of oocytes retrieved were parthenogenetic or abnormal and were therefore not suitable for experimentation. Abnormal oocytes were classified as those with abnormally large PBs, a high degree of fragmentation, vacuoles or any other obvious cytoplasmic or morphological anomalies. A total of **38.6%** (484/1254) and **22.8%** (286/1254) of the overall number of oocytes retrieved were used for enucleation and as non-enucleated controls, respectively. In summary therefore, **61.4%** (770/1254) of the total number of oocytes retrieved were used for experimentation in this study. This was primarily due to time constraints as no more than 50 oocytes could be enucleated per day (processing time ±2 hours) due to the level of difficulty of the technique. In addition, oocyte collection from the UPBRC (±1 hour), preparation of the medium, oocytes and micromanipulation system (±1 hour), performing the confirmation techniques by including artificial

activation (3 hours) and Hoechst 33342 DNA staining (30 minutes), and evaluation of the cytoplasts and non-enucleated controls thereafter (±1 hour), were also taken into consideration with regards to timing of the experiments (see timeline for study 1 in Figure 2.2 on page 36).

In **study 2**, a total of 1698 oocytes were produced from 43 BDF1 mice (±10.2 weeks of age). The average number of oocytes retrieved per mouse in this study was 39.5. Of the total number of oocytes retrieved, **11%** (186/1698) were parthenogenetic or abnormal. Furthermore, **33.2%** (564/1698) and **22.6%** (383/1698) of the overall number of oocytes retrieved were used for SCNT and as non-enucleated controls, respectively. In summary, **55.8%** (947/1698) of the total number of oocytes retrieved were used for experimentation in this study. Fewer oocytes were used for experimentation in study 2 than study 1. This was due to the procedural timeline described above for study 1, as well as the additional nuclear transfer step (±2 hours) and the 5-7 hour incubation period with TSA after activation, which needed to be considered with regards to timing of the experiments (see timeline for study 2 in Figure 2.3 on page 37).

4.1.2 Micromanipulation

Direct nuclear injection using single step piezoelectric nuclear transfer has proven to be a valid and first choice technique to clone mice for reproductive and therapeutic purposes.[9,56,78,82] This method bypasses the need for a laser system and a membrane fusogen or electrofusion, and also combines enucleation and donor somatic cell injection in a one-step manipulation method.[82] Due to the unavailability of a piezoelectric system at the University of Pretoria and the high expense thereof, laser-assisted nuclear transfer in combination with a membrane fusogen and artificial activation were used to investigate and optimize the SCNT procedure as the main purpose of this project.

4.1.2.1 Study 1: Enucleation

Enucleation is an invasive step that may further induce damage to the oocytes and contribute to the low success rate of SCNT if not performed optimally. Importantly, clear spindle visualization allows for effective isolation of the spindle into a karyoplast, surrounded by very little cytoplasm. Preliminary experimentation revealed the difficulty of spindle visualization. The initial prolonged period to enucleate a single oocyte (±5.7

minutes per oocyte) was attributed to inexperience in spindle identification, including the need to rotate and position the oocyte for complete removal of the meiotic spindle complex with very little surrounding cytoplasm. Technical similarities in terms of micromanipulation were evident between the enucleation procedure and the biopsy technique performed in human blastocysts. With 3 years of background experience as an embryologist including micromanipulation skills, the time to enucleate a single oocyte was reduced to a third of the initial procedure over a period of 3 months during the progression of the first part of this study.

No oocyte lysis was observed during the first practice round, which confirmed the concentration of CB to be optimal. During enucleation, the oocyte membrane is not compromised because of the addition of CB to the micromanipulation medium.[12,34,36,56,57,76,78,81] Cytochalasin B destabilizes the actin cytoskeleton rendering the oocyte more flexible and therefore reducing the risk of lysis and damage during the process.[57] On the contrary, several oocytes were susceptible to damage during handling and lysed when rotated with pipettes in the second practice round. Based on the practical experience gained during preliminary experimentation, the oocytes were rotated more gently and fewer oocytes (n = 5) were enucleated at a time to limit exposure outside of the incubator to 20-30 minutes or preferably less.

In the first study, 484 B6D2F1 MII stage oocytes were enucleated. Laser-assisted enucleation was performed by firstly rotating and identifying the meiotic spindle complex in the oocytes. It was noted that depending on the rotation of the oocyte and location of the spindle, the translucent meiotic spindle complex was occasionally visible as a bulge. Identification was made simple by the appearance of a clear bulge; however, if no bulge was present the meiotic spindle complex was then identified by its translucency. The karyoplast was removed and discarded, and the cytoplast was used for further investigation.

4.1.2.1.1 Artificial activation

Two confirmation techniques were applied to determine efficient enucleation in study 1, namely artificial activation and Hoescht 33342 DNA stain, as recommended by Dr Egli (Senior Research Fellow at The New York Stem Cell Foundation, Assistant Professor of Developmental Cell Biology and head of the Egli Lab at Columbia University). Artificial activation, the initial technique, caused cytoplasts to undergo

fragmentation within a few hours (±14 hours) following effective enucleation.[56] An enucleation rate of **85%** (130/153) with a lysis rate of **0.4%** (1/240) was obtained. According to literature, when a highly skilled operator (who has mastered the technical skills required) performs SCNT micromanipulations, spindle visualization and successful laser-assisted enucleation can be achieved at a rate of **90%** or higher with *Rhesus macaque* MII stage oocytes.[76] Furthermore, using a piezoelectric system, an enucleation rate of **99-100%** with mouse MII stage oocytes can be reached.[78] In this study, the reported below average rate of enucleation may be attributed to the initial difficulty in spindle visualization and removal. Spindles may have been partially enucleated or completely missed due to ineffective spindle identification. Initially, recognition of the spindle proved challenging because of its transparent nature. Therefore, continuous rotation of the oocyte was performed to visualize the spindle and ensure that the spindle was positioned in the correct focal plane for enucleation.

Degeneration of all cytoplasts within 2 repeats of the artificial activation experimental group was noted. The cause of degeneration was initially based on an assumption that the 15-week-old mouse oocytes used in the unsuccessful repeats 2 (n = 48) and 3 (n = 27) were “old” oocytes, which are more sensitive to the activation medium after the enucleation procedure. This assumption was invalid as 15-week-old mouse oocytes were used successfully in repeat 1 with no degeneration. Additionally, literature does not define 15-week-old mouse oocytes as “old”. One study categorized young, mature and aged mice as 7-8, 20-24, and 40-48 weeks old respectively.[216] In another study, mice at 5-9-weeks were defined as juvenile and 50-62-week old mice were described as climacteric.[217] The recommended age of mice for SCNT oocyte collection is 8-12 weeks;[78] oocytes from mice of this age were therefore used for the remaining experiments performed in this project. The most probable cause for the degeneration may be attributed to the sensitivity of the specific oocyte cohort to ionomycin used in the activation protocol (Dr Gloryn Chia, Columbia University, personal communication). A study by Uranga and colleagues showed that when oocytes were treated with either 1 mM or 5 mM ionomycin for 5 or 10 minutes, oocyte sensitivity to ionomycin was indicated by the 50-60% embryo mortality at 24 hours after activation.[218] Exposure to 2.7 µM ionomycin for 5 minutes was used in this project; the precise cause of the degeneration remains unresolved.

4.1.2.1.2 Hoechst 33342 DNA staining

Experimental cytoplasts and non-enucleated control oocytes were stained with Hoechst 33342 to further confirm successful enucleation. Fluorescence microscopy was used to distinguish between positively stained control oocytes and negatively stained cytoplasts, which confirmed the presence and absence (successful enucleation) of DNA, respectively. The data showed a successful enucleation rate of **97.5%** (156/160) with **no lysis**. This result was comparable to the **99-100%**[78] success rate discussed previously and was a higher enucleation success rate without lysis, compared to the first set of enucleation experiments performed. The improved result was most likely due to the experience gained and confidence acquired in identifying and removing the spindle during the first study.

4.1.2.2 Study 2: Somatic cell nuclear transfer

Laser-assisted enucleation was performed on 564 MII stage oocytes with a survival rate of **99.1%** (559/564) and a lysis rate of **0.9%** (5/564). The PB was removed (if not degenerated) to avoid potential resorption of the PB during HVJ-E exposure,[49,83] and to prevent confusion with the donor somatic cell during fusion.[12] Nuclear transfer was achieved by the fusion of B6D2F1 cumulus cells into 559 cytoplasts using the inactivated HVJ-E membrane fusogen.[12,36,49,50,76,81,83,89,90] Cumulus cells from B6D2F1 female mouse COCs were selected as the somatic cell donor. Numerous types of somatic cell donors have been investigated in the literature, however several laboratories concur that cumulus donor cells achieve the highest cloning efficiency with the least abnormalities in cloned animals.[9,136-138] Cumulus cells were also chosen based on their accessibility. Additionally, more than 80% of cumulus cells are arrested at the G0/G1 phase of the cell cycle, and thus are suitable donor cells that can be used without any selection criteria and do not require further *in vitro* culture to verify cell cycle synchronization.[78,208] Synchronization of the cell cycle between the recipient MII stage cytoplast and the donor cell nucleus in SCNT is important to ensure successful epigenetic reprogramming, and ultimately full-term development.[8] After the birth of Dolly the sheep, the donor cell was suggested to be in the quiescent (G0) phase of the cell cycle during SCNT.[8] Several other studies have proposed that successful cloning can be achieved with donor cells in the G0, G1, G2, and M phases of the cell cycle.[44,142,143] In SCNT, the introduction of G0, G1, and M phase donor cells

into MII stage cytoplasts is routinely performed, preventing both DNA damage and unplanned DNA replication of the donor cell.

4.1.2.2.1 Fusion by HVJ-E exposure

Fusion was verified by the disappearance of cumulus cells into cytoplasts after 30 minutes, with a success rate of **72.3%** (404/559). According to Egli and Chia,[12] the HVJ-E fusion efficiency of mouse oocytes with somatic cells is poor, and so direct injection for nuclear transfer using a piezoelectric system is recommended. However, according to the HVJ-E cell fusion protocol, the fusion effectiveness specifically for cumulus cells as the donor cell type is expected to be between **70-80%**.[219] Therefore, the successful result obtained in this study supports the reported efficiency of the HVJ-E fusion protocol for mouse oocytes. When exposed entirely to HVJ-E, donor somatic cells become quite sticky and are difficult to manipulate according to Dr Egli (personal communication).[12] All donor cumulus cells were fully exposed to HVJ-E for 20 seconds in this project. After exposure to HVJ-E, stickiness of donor cells and difficult handling were only experienced during a few attempts in this study. These cells would stick to the inside of the nuclear transfer pipette making it difficult to release the cells into contact with the cytoplasts. Membrane fusogen agents such as HVJ-E function by distorting the plasma membrane integrity, subsequently increasing the absorbency of ions within the culture medium.[12,86,87] As a result, cells that are exposed to HVJ-E are prone to lysis due to the increased fragility of the cell membrane, with this negative effect persisting longer than the 20 second exposure period to the fusogen.[12] Therefore, literature by Egli and Chia[12] recommended the donor somatic cell remain in the pipette opening when exposed to HVJ-E resulting in minimal contact, which is reported to be sufficient to achieve fusion and at the same time protect the plasma membrane integrity of the oocyte.[12] In this study however, cumulus cells were fully exposed to HVJ-E for 20 seconds, and no oocyte lysis was observed following this method of fusion. Our findings therefore differed from those observed by Egli and Chia regarding fusion of mouse oocytes using HVJ-E.[12]

4.1.2.2.2 Activation of fused oocytes

Artificial activation is necessary to stimulate the development of SCNT manipulated oocytes. Only fused SCNT oocytes were used for subsequent activation in study 2. When activation is performed within a few hours of cumulus cell injection into cytoplasts, full-term development of cloned embryos is a reality.[9,121] Oocyte lysis may

occur if activation is performed less than 30 minutes after nuclear transfer; therefore recovery of manipulated oocytes before activation, for at least 30 minutes, is recommended.[78] Thus, timing of oocyte activation in SCNT is crucial.[63] The recovery period was complied with in study 2 and activation was induced approximately 1-2 hours after nuclear transfer as previously described for study 1. The 1-2 hour period before activation is a result of performing enucleation and nuclear transfer on a large cohort of oocytes in a single day (average n = 40 per day). Cytoplasts underwent nuclear transfer in groups of 8-10. Once nuclear transfer was performed in each group, the reconstituted oocytes were incubated until nuclear transfer was completed on all cytoplasts for that day. Thus, the first set of cytoplasts of the larger cohort were subjected to a longer incubation after nuclear transfer and were activated within the maximum 2 hour period. On the other hand, the cytoplasts that underwent nuclear transfer last were incubated for a shorter time and thus were activated closer to 1 hour post nuclear transfer. According to Kishigami and colleagues, an activation survival rate of **70-80%** was expected after SCNT.[78] However in this study, a **99.8%** (403/404) activation survival rate was achieved. Pseudo-pronucleus formation, which confirms successful nuclear transfer and activation of fused oocytes,[56] should be visible in at least **60-70%** of activated oocytes as expected by Kishigami and colleagues.[78] This outcome was analysed using HMC microscopy directly after the 3 hour activation period, and qualitative data obtained demonstrated a successful activation rate of **81.7%** (330/404)**.** As a control for the activation protocol, a group of non-enucleated oocytes were similarly activated, with a pseudo-pronucleus formation rate of **97.9%** (375/383). Despite the statistical significance between the SCNT group and the control group, the rate of survival and pseudo-pronucleus formation after activation is higher than the accepted success rate reported by Kishigami and colleagues.[78] The **99.8%** (403/404) activation survival and **81.7%** (330/404) pseudo-pronucleus formation rates of the SCNT embryos are indicative of an efficient activation protocol in this study. This protocol success further reinforces the suggestion made by Kishikawa and colleagues that the choice of activation method used for mouse SCNT is not the most critical determinant in the procedure.[122] The latter was proposed based on the comparison of four different oocyte-activating agents including ethanol, strontium, electrical stimulation and the microinjection of sperm; no statistically significant difference was observed in the overall efficiency rates between the groups.[122]

4.1.2.2.3 Histone deacetylase inhibitor exposure to improve blastocyst formation

In study 2, the histone deacetylase inhibitor (HDACi) TSA was added to the activation protocol and culture medium to promote histone acetylation, and ultimately improve SCNT embryo development.[9,18,21,119,121,147,170,171,173] The mechanism through which the HDACi improves the efficiency of cloning is probably related to the capacity to encourage the synthesis of nascent mRNA, following the increase in histone acetylation.[19] Even though incomplete, histone acetylation remodelling in reconstituted embryos is enhanced with HDACi treatment after SCNT.[27] Trichostatin A is a commonly used HDACi in SCNT[172] and currently remains the best approach for mouse cloning.[176,177] The most effective treatment protocol in mice is a TSA concentration between 5-50 nM, with constant exposure of the manipulated oocytes to TSA for at least 8-10 hours from the start of activation, but before the first cell division.[18,173] Should TSA exposure of manipulated SCNT embryos exceed 12 hours from the start of activation (specifically very close to the first cleavage stage), the embryos will arrest (Dr Dietrich M. Egli, Columbia University, personal communication). A narrow window for successful reprogramming in mouse oocytes occurs before embryonic genome activation, which is at the 2-cell stage. Timing of TSA treatment is therefore vital for its effectiveness on the developmental potential of the reconstructed embryos.[170] Based on the abovementioned guidelines of TSA treatment, protocols may differ in concentration and extent of TSA exposure.

To establish the most effective TSA protocol specific to this study, three different concentrations of TSA (5, 25 and 50 nM) were tested at two different total exposure times from the start of activation (8 and 10 hours). Effectiveness of TSA treatment was based on the number of blastocysts formed in each group by 96 hours post activation. No statistically significant differences were noted in the blastocyst formation rates between 5 nM, 25 nM and 50 nM TSA (**4.3%** vs. **1.5%** vs. **7.5%**). This result may indicate why a specific concentration of TSA has not yet been established. Therefore, it may be suggested to test different concentrations of TSA to elucidate the concentration for the best results of SCNT embryo development and blastocyst formation specific to a given laboratory. On the other hand, a significant increase in blastocyst formation was achieved during the total exposure period of 8 hours compared to 10 hours (**6.8%** vs. **2.9%**, $p < 0.001$). This outcome indicates that a

shorter period of TSA exposure is preferable, especially since TSA is known to be toxic at long exposure periods (>12 hours), with subsequent negative effects on blastocyst quality and normal embryo development.[19,175] As an alternative HDACi which has a lower toxicity level, use of Scriptaid has been shown to result in a significant improvement in the creation of cloned mice[19] and pigs.[26,27] Scriptaid (250 nM) was therefore compared to TSA (50 nM) at 8 hours of total exposure from the start of activation. Scriptaid did not differ significantly from TSA regarding blastocyst formation rates (**2.5%** vs. **7.7%**, $p = 0.404$). Despite several studies using other HDACi to improve SCNT, TSA currently remains the best approach for mouse cloning.[203] Based on the above findings, 50 nM TSA at 8 hours of total exposure from the start of activation was selected as the optimal TSA treatment protocol specifically for this project.

4.1.3 Troubleshooting

Kishigami and colleagues[78] discussed the success rates of SCNT when all conditions had been optimized. All techniques described in this project required specific micromanipulation skills, accompanied by meticulous practice before attempting the challenging procedure of SCNT. Troubleshooting was an inherent element of this study, whereby results were benchmarked to peer-reviewed outcomes. Table 4.1 provides an overview of troubleshooting advice that was considered during experimentation in this project.

Table 4.1: Troubleshooting for SCNT protocols provided by the groups of Kishigami[78] and Tachibana[76]

Problem	Possible reasons	Solutions
Inability to identify the meiotic spindle during enucleation	a) Inaccurate microscope setting b) Incorrect mouse strain c) Low temperature	a) Request microscope company to correct the instrument b) Oocyte transparency and meiotic spindle visualisation relies on mouse strain c) Spindle microtubule assembly may be disordered and become cloudy at room temperature. Oocytes should be incubated at 37ºC for at least 30 minutes

Difficulty in creating a hole in the zona with the laser	a) Incorrect laser settings b) Low laser power level	a) Ensure all cables of the laser system are connected, or contact laser company to correct the instrument b) Gradually increase laser output
Difficulty in isolating the spindle	a) Incorrect focal plane of the enucleation pipette b) Enucleation pipette is too small	a) Align the spindle to an equatorial plane close to the 3 o'clock position and adjust the pipette tip to the same focal plane. Correct alignment can be tested by gently poking the spindle through the zona with the pipette. The spindle should move away as a unit b) If the pipette is too small, the spindle may not fit. However, if the pipette is too large, excess volume of ooplasm may be removed during enucleation
Lysis of oocytes during enucleation	a) Incorrect concentration of CB	a) Recheck concentration of CB and be sure to use fresh stock
Lysis of oocytes after nuclear transfer	a) Large pipette b) High temperature	a) Use a smaller diameter pipette b) Oocyte lysis is increased by warm temperatures, therefore decrease the room temperature
Low fusion rates	a) Inadequate contact between the donor cell membrane and the oolemma b) Inactive HVJ-E	a) Ensure sufficient membrane contact between the two cells b) Ensure that HVJ-E is thawed directly before use
Oocyte degeneration during activation	a) Bad stock of activation medium b) TSA concentration too high c) Activation step performed too quickly	a) Make a fresh dish of activation medium b) High levels of TSA are toxic, recheck TSA concentration c) The reconstructed oocytes should recover for at least 30 min before activation

Extrusion of a PB	a) Premature activation caused by a bad stock of CB	a) Fresh stock of CB is required
No pronucleus formation	a) Inadequate fusion b) Failed oocyte activation	a) See solutions for “Low fusion rates” b) Test activation medium using non-enucleated oocytes
No embryo development	a) Donor somatic cell type b) Low TSA concentration c) Inadequate culture conditions	a) Ensure G0/G1 or G2/M phase of cell cycle b) Optimal TSA concentration is reliant on donor cell type c) Check the quality of all reagents, medium and culture conditions using parthenogenetic embryos

4.1.4 SCNT embryo development and blastocyst formation

The final objective of study 2 was to achieve *in vitro* development of at least 20 blastocyst stage embryos after SCNT. The majority of the SCNT experiments were performed with the selected TSA treatment protocol of 50 nM TSA for a total exposure of 8 hours from the start of activation. Based on the stage of mouse embryo development that is expected on specific embryonic days,[213] significantly lower rates of development were noted in the SCNT group compared to the control group in this study. Despite this result, the rates of enucleation and nuclear transfer survival, fusion efficiency, activation survival, pseudo-pronucleus formation, first cell division, compaction and morula development of the SCNT group were either higher than or within the success rates described by Kishigami and colleagues.[78] Blastocyst development was the only outcome after SCNT that was not within the success rate suggested by literature. A blastocyst formation rate of **30-50%** is expected after SCNT in the mouse,[78] however this project demonstrated an overall rate of **5.4%** (22/404). This result was significantly lower than the control group that produced a blastocyst formation rate of **55.1%** (211/383). The outcome is a consequence of the significantly higher degeneration rates in SCNT embryos compared to control embryos on embryonic days 4 and 5. Embryonic development failure to the blastocyst stage may be a result of the inability of the somatic cell nucleus to completely reprogram and activate important embryonic genes, i.e. epigenetic reprogramming failure.[54,83] Based on these findings, the efficiency of producing a blastocyst by means of SCNT in this

study was **0.041-fold** that of the parthenogenetic control group. In addition to the low blastocyst development rate, poor-quality SCNT blastocysts without distinct ICM, few TE cells and the presence of excluded or degenerated cells were also noted. Kishigami and colleagues[78] did not specify the blastocyst quality or grade to be expected after mouse SCNT. However, in a study deriving human ESCs by SCNT, poor-quality blastocysts with the same undesirable characteristics were produced because of suboptimal protocols.[36] This study aimed to improve the protocol of mice SCNT based on current standards. All the steps involved in the SCNT protocol were positive, except for the final stage of blastocyst formation. The unforeseen outcome was probably due to epigenetic reprogramming failure, requiring further research and investigation.

4.1.5 Culture systems

4.1.5.1 Embryo density

Overall, a significantly higher number of poor-quality SCNT blastocysts, identified as those without distinct ICMs and the presence of excluded and degenerated cells, were obtained compared to good or average quality blastocysts. Of the total SCNT blastocysts formed in this study, **59.1%** (13/22) were cultured in the K-Minc incubator (n = 20-25 SCNT oocytes per 20 µl microdrop) and **40.9%** (9/22) were cultured in the EmbryoScope™ time-lapse system (n = 2 SCNT oocytes per 25 µl well). A substantially larger percentage of poor-quality blastocysts were obtained from those cultured in the K-Minc in comparison to the EmbryoScope™ (**45.5%** vs **18.2%**, p = 0.002). Correspondingly, a higher number of good-quality blastocysts were cultured in the EmbryoScope™ when compared to the K-Minc incubator (**18.2%** vs **0%**, p = 0.025). The same batch of Global® Total® medium was used for continuous culture in both incubation systems; however, the embryo density per microdrop (number of embryos/volume of culture medium) differed between the two culture systems. Densities of approximately 0.8-1 µl/embryo and 12.5 µl/embryo were utilized in the K-Minc and EmbryoScope™, respectively.

Several studies have reported the benefit of mouse embryo group culture with differing optimal embryo densities.[220-224] Wiley and colleagues cultured 20 embryos per 10-12 µl microdrop with a density of 0.5-0.6 µl/embryo.[220] Paria and Dey cultured 5-10 embryos per 25-50 µl microdrop with a density of 2.5-10 µl/embryo.[221] Canseco and colleagues cultured 5 embryos per 10 µl microdrop with a density of 2 µl/embryo.[222]

Lane and Gardner cultured 2-16 embryos per 5-320 µl microdrop with a density of 0.3-40 µl/embryo.[223] Kato and Tsunoda cultured 20 embryos per 10 µl microdrop with a density of 0.5 µl/embryo.[224] According to the abovementioned studies, the main advantage of group culture is that adjacent embryos may secrete embryotrophic or positive factors that give rise to faster rates of embryo development,[220,225] higher rates of blastocyst formation[221,223,225] and better-quality embryos.[226] On the other hand, a concerning disadvantage of group culture is the emission of embryotoxic factors from poor-quality or degenerating embryos producing a possible negative effect by decreasing the competence of healthy companion embryos.[227] Salahuddin and colleagues[225] investigated the effects of embryo density and co-culture of unfertilized (degenerating) oocytes on embryonic development of *in-vitro* fertilized mouse embryos. In their study, zygotes were cultured for 168 hours at 38.7°C in 5%CO_2 either singularly or in cohorts of 5, 10 and 20 in 20 µl microdrops of human tubal fluid medium under mineral oil, with densities of 20 µl, 4 µl, 2 µl and 1 µl/embryo, respectively. In terms of increased embryo density (group culture with viable IVF zygotes), enhancement of mouse embryo development was reported. However, the results showed a significant decrease in the number of embryos that reached the blastocyst stage and the number of cells per blastocyst when co-cultured specifically with unfertilized (degenerating) oocytes. These results suggest that the development of mouse embryos is impaired by group culture with degenerating oocytes and embryos.[225]

Similarly, most of the poor-quality blastocysts that were cultured in the K-Minc incubator in this research project were subjected to group culture with degenerated oocytes and embryos. The higher rate of poor-quality blastocysts produced in the K-Minc incubator compared to the EmbryoScope™ system (**45.5%** vs **18.2%**) may therefore be the result of the high embryo density (n = 20-25 SCNT oocytes/20 µl) through embryotoxic and detrimental factors secreted by degenerating oocytes and embryos. The good-quality blastocysts obtained were also co-cultured in the EmbryoScope™; however, with only a single degenerating companion embryo, embryotoxic factors were likely at a minimum. Based on the above literature, the higher rate of good-quality blastocysts achieved in the EmbryoScope™ may be explained by a lowered embryo density (n = 2 SCNT oocytes/25 µl) with less embryotoxic factors released from a single degenerating oocyte or embryo.

Additionally, the embryos cultured in the EmbryoScope™ were never removed from the incubation system and remained in a consistent culture environment during evaluation of the embryos. On the contrary, embryos cultured in the K-Minc had to be taken out of the incubator for static evaluations and were therefore exposed to the outside environment with fluctuating temperatures and gas compositions. Therefore, the incubation systems may have also played a role in determining the quality of the SCNT blastocysts obtained in this study.

4.1.5.2 Culture medium

Another variable to consider when trying to optimize group culture is the culture medium utilized.[227] In recent years, species-specific culture medium have been developed to enhance the development of embryos from different species, for example KSOM for mouse embryos.[59,60] However, Global® Total® and Global® Total® with HEPES medium from the LifeGlobal® Group were selected for micromanipulation and culture in this project. This medium was selected based on its certification and validation for IVF use through quality control with a 1-cell stage Mouse Embryo Assay test. A minimum of 80% mouse blastocyst development is required for passing the tests for non-toxicity and functionality of all LifeGlobal® medium. A study performing a side-by-side comparison of five different commercial embryo culture systems found that KSOM and Global® Total® medium performed similarly and are the most alike based on their constituents.[228] The amino acid L-glutamine is a component of KSOM, whereas glycyl-L-glutamine is a constituent of Global® Total® medium. A study replacing L-glutamine with glycyl-L-glutamine in a KSOM-type medium found that the development of more mouse embryo ICM cells was favoured than TE cells.[229] The latter implies that the replacement of glutamine with glycyl-L-glutamine (which is present in Global® Total® medium) may be beneficial due to the greater stability of glycyl-L-glutamine *in vitro*.

4.1.6 Current protocol optimizations

One of the main purposes of creating SCNT blastocysts is isolation of the ICM to derive ESCs. However, poor-quality SCNT blastocysts will prevent ESC isolation.[83] Even though the rate of blastocyst development is encouraging, protocol optimizations are continuously being developed to focus primarily on improving SCNT embryo quality. For example, monkey and human SCNT studies supplementing spindle enucleation and fusion medium with caffeine have reported improved SCNT embryo development,

blastocyst quality (characterized by visible and prominent ICM formation) and subsequent ESC line derivation.[36,51] Regarding the use of cytokinesis inhibitors during enucleation and activation, when Lat-A was used instead of CB in a study by Terashita and colleagues, the success rate in terms of full-term development of cloned mouse embryos was significantly improved (9% vs 4.5%).[125] In a study by Mallol and colleagues, the effect of supplementing culture medium with vitamin C for at least 16 hours after activation, in combination with Lat-A during micromanipulation and activation, significantly increased rates of mouse blastocyst formation.[126] The mean number of ICM cells at 96 hours post activation and the total blastocyst cell number were also increased.[126]

Nuclear reprogramming strategies

Several factors that may impact on the outcome of SCNT include: *invasive micromanipulation, oocyte incompetence and variation in developmental efficiency,* as well as *in vitro culture inconsistencies* (Figure 4.1). As illustrated in Figure 4.2, all these factors can contribute to abnormal gene expression caused by **epigenetic reprogramming failure** of the donor somatic cell nucleus by the oocyte.[11] Continued research and protocol optimization should therefore be encouraged to improve the efficiency of nuclear reprogramming by SCNT.[203]

Histone deacetylase inhibitors including TSA and Scriptaid have successfully been used to improve the efficiency of SCNT blastocyst development in several species, including human, monkey, mouse, bovine and pig.[15,18,26,34,36,174] Nonetheless, the resulting blastocyst quality and the possible establishment of stable ESCs remains unknown.[174] In addition, TSA toxicity[175] may be detrimental to the quality of the blastocyst, and may significantly reduce normal development of the embryo.[19,36] Based on gene expression analysis, Inoue and colleagues discovered the downregulation of X-linked genes (required for embryonic development) in SCNT mouse embryos; this is caused by the ectopic expression of the *Xist* gene that is responsible for X chromosome inactivation.[230] The birth rate and cloning efficiency in mice were dramatically improved by complete deletion of *Xist* from the donor genome or repression of the gene by injecting short interfering (si) RNA into SCNT embryos.[230,231] Furthermore, next-generation sequencing of donor cells and SCNT embryos identified histone 3 lysine 9 trimethylation (H3K9me3) of the somatic cell genome as the main obstacle preventing nuclear reprogramming.[232] A significant

improvement in both mouse and human SCNT cloning competence was achieved by expressing H3K9me3 demethylase lysine demethylase 4D (Kdm4d) in SCNT embryos to reduce the H3K9me3 level.[215,232-234] The abovementioned studies suggest that by implementing nuclear reprogramming strategies into the SCNT procedure, an overall improvement in cloning can be achieved (see Figure 4.2).

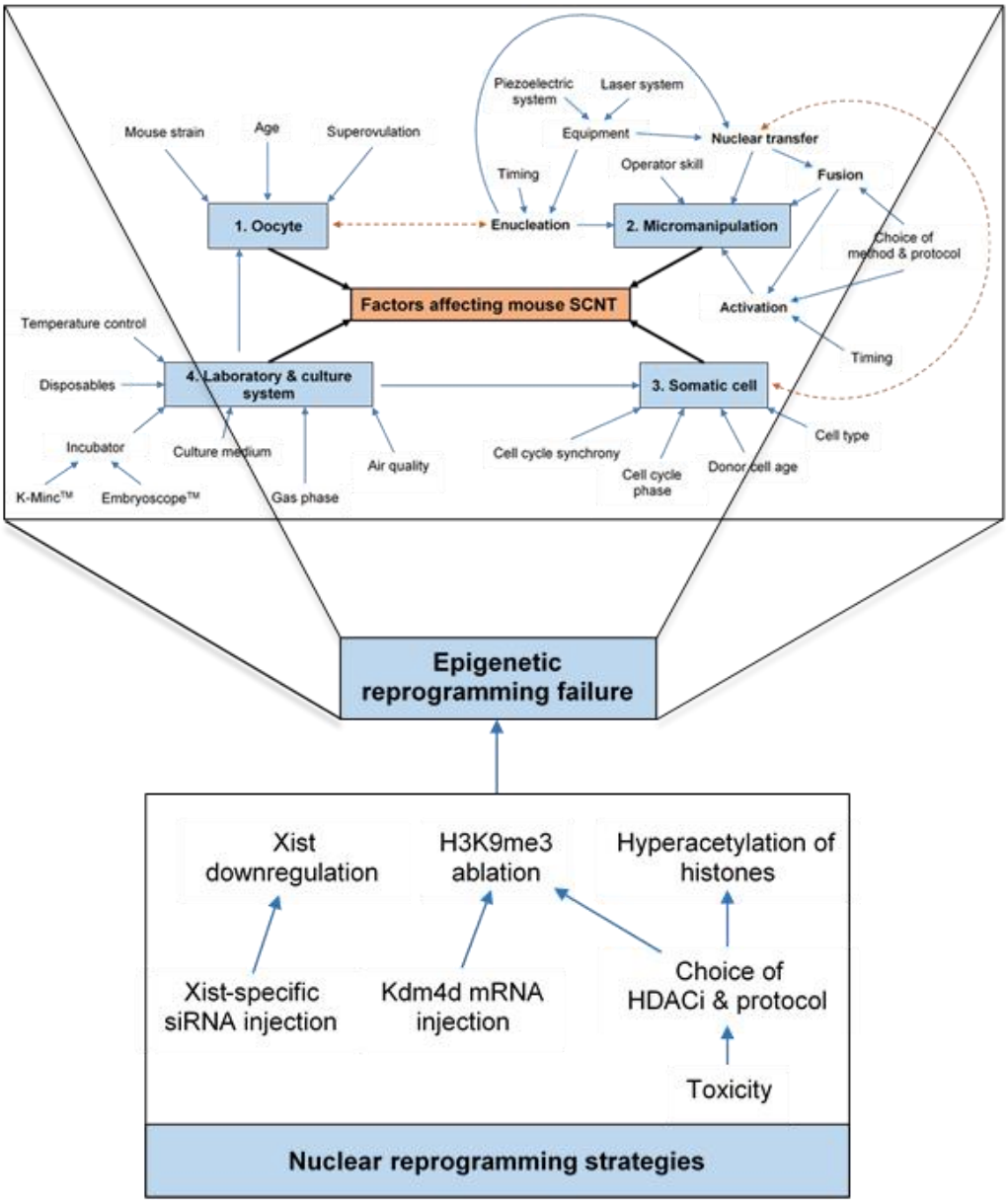

Figure 4.2: Diagram illustrating the **impact of many factors on epigenetic reprogramming failure** and the most recent and applicable **nuclear reprogramming approaches** developed to improve epigenetic reprogramming.

4.2 Recommendations

"Establishing this protocol requires a significant institutional commitment and a dedicated research program with researchers skilled at manipulating oocytes and embryos", as stated by Dr Egli.[12] Even though the technique is constantly developing, based on the current research and literature, the following recommendations for an optimized SCNT procedure using a mouse model can be made:

4.2.1 Equipment and oocyte handling

- The piezoelectric micromanipulation system may be used for single step nuclear transfer to improve time management and avoid the purchase of a laser system and a membrane fusogen kit.[9,56,78,82]
- Considering Figure 1.4 on page 9 and Figure 3.9 E on page 62, a reduced suction force by the holding pipette (no bulging) should decrease physical stress to the oocyte and may increase blastocyst rates.
- The optimal donor oocytes to use for high developmental competence, susceptibility to *in vitro* handling and micromanipulation, and visibility of meiotic spindle complex are 8-12-week-old B6D2F1.[78,82,120]
- Undesirable fluctuations to the culture environment of oocytes through exposure outside of the incubator for longer than 20-30 minutes can be prevented by adjusting the number of oocytes undergoing experimentation to the level of experience of the operator.[56]
- If the meiotic spindle complex cannot be visualized at first, it can be identified using Hoechst and UV illumination, which would be for practice purposes only.[56]

4.2.2 Medium supplementation

- Regarding the use of cytokinesis inhibitors other than CB during enucleation and activation, Lat-A should be investigated further as an option to improve the success rate of full-term development of cloned mice.[125]
- The use of $SrCl_2$ in the activation protocol, which sidesteps the need to purchase additional reagents such as protein synthesis or kinase inhibitors, should be tested.[91]
- Supplementing the culture medium with an antioxidant such as vitamin C (that protects cells against reactive oxygen species) for at least 16 hours after

activation, in combination with Lat-A during micromanipulation and activation should be considered which may increase the rates of blastocyst formation.[126]

- As an alternative histone deacetylase inhibitor, Scriptaid may be added to the activation protocol and culture medium as an alternative to TSA to promote histone acetylation and ultimately improve SCNT embryo development, due to its lower toxicity.[19,26,27]

4.2.3 Quality control and training

- It is important to create laboratory based standard operating procedures and training manuals for the established and optimized methods of SCNT.
- Maintaining a stable environment for embryo development; including temperature, pH and medium optimization, should never be underestimated and is of utmost importance.
- Non-enucleated oocytes should be activated and cultured in parallel with reconstructed oocytes as parthenogenetic controls for the activation protocol and culture conditions.[56]
- To ensure optimal use of this advanced technique, adequate training of embryologists in ICSI and biopsy procedures is essential and a prerequisite.[76,78]
- Many hours of experimentation and practice are required to perform the difficult SCNT protocol.[76,78]

4.3 Conclusion

The aim of this study was to determine the efficiency of the techniques involved in SCNT using a B6D2F1 mouse model. Results revealed that the rates of enucleation and nuclear transfer survival, fusion efficiency, activation survival, pseudo-pronucleus formation, cell division, compaction and morula development of the SCNT embryos were as good as those reported elsewhere. The formation of blastocysts derived by means of SCNT was below the published average, which may be a consequence of epigenetic reprogramming failure of the donor somatic cell nucleus.[148,161,180,204]

Despite the reduced rate of blastocyst development achieved after SCNT in this project, Figure 3.20 (page 77) illustrates that over a 5 month period of experimentation, most blastocysts were produced during the last 50% of experiments performed. This demonstrates the observation from the groups of Dr Egli, Kishigami and Tachibana, that a certain practical skill in manipulating oocytes and embryos is required in order

to perform SCNT successfully.[12,76,78] Whether using a piezoelectric or laser-assisted micromanipulation system, a beginner with no embryology skills would most likely need several months of practice to become familiar with the techniques involved in SCNT, and attain the essential technical skills needed to obtain good, reliable and useful results. Without these skills, producing SCNT blastocysts and establishing ESCs may not be possible. A recommendation is made to gain experience in embryology-related experiments such as ICSI or gamete cryopreservation prior to attempting SCNT.[78] This will allow familiarity and understanding of the micromanipulation system used for enucleation and nuclear transfer. A considerable amount of time, resources and perseverance must be invested into the SCNT procedure, irrespective of the micromanipulation system used.[12,78,82] In the South African context, the resources and skilled embryologists are available, however the lack of expertise in the field of SCNT required for training is a major setback.

An important purpose of creating SCNT blastocysts is to derive ESCs through isolating the ICM. The derivation of human ESCs has been achieved from laser-assisted SCNT-produced blastocysts.[36,81,215] The required blastocyst quality expected after SCNT for efficient ESC derivation has not been specified in literature. However, poor-quality SCNT blastocysts will prevent ESC isolation.[36,83] In this research project, poor-quality SCNT blastocysts without distinct ICM, few TE cells and the presence of excluded or degenerated cells were produced. Similarly, in a study deriving human ESCs by laser-assisted SCNT, poor-quality blastocysts with the same undesirable characteristics had failed to derive stable ESCs.[36] Therefore, the poor-quality blastocysts produced by laser-assisted SCNT in this research study would most likely not be able to support the establishment of stable ESCs. Nevertheless, SCNT protocol optimizations focussed on improving blastocyst quality would in turn lead to improved ESC derivation.[36]

In humans, SCNT as a tool to generate specific ESCs from the somatic cells of an individual is possible and could ultimately lead to the understanding of disease mechanisms, as well as improve the efficiency of cell-based therapies for the treatment of degenerative diseases with a negligible risk of immune rejection.[4,178,179] Other clinical applications include assisted reproductive procedures that prevent the transmission of mitochondrial DNA diseases from a mother to her offspring, as well as the treatment of infertility due to defects in the cytoplasm of oocytes.[76] The latter is

achieved using the same techniques applied in SCNT, instead in this case the patient's chromosomes would be transferred to donor oocytes with healthy mitochondria. The SCNT approach is continuously improving; however continued research and protocol optimization in terms of improving the reprogramming efficiency by SCNT is necessary. In terms of future studies, it may also be of value to follow the fusion rate and outcome of the oocytes. Understanding the molecular mechanisms involved in reprogramming and ultimately linking the technique to clinical applications, including assisted reproductive options, is the eventual goal.

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