

Infra-red laser applications in the
reproductive sciences: improving safety
for assisted reproductive technology and
developing novel research tools



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Abstract

Assisted reproductive technology (ART) has been rapidly expanding since the birth of Louise Brown, the first test tube baby, in 1978. Although an increasingly complex array of laboratory skills and procedures have been developed for infertility treatments, the success rate of ART remains low. In an attempt to make ART safer and more efficient, international medical practice is trending towards single embryo transfers and the use of innovative, sophisticated technologies to identify promising gametes and embryos with the highest potential to generate a pregnancy. Laser technology is increasingly being used to accomplish these aims. The application of lasers for ART has been successfully employed in clinical practice for some time now and is continually the subject of investigative research in order to generate new methods to improve operations. Moreover, lasers serve as a powerful tool at the forefront of investigative research in the reproductive sciences, assisting in broadening our understanding of reproductive and developmental biology. Nevertheless, there is a paucity of literature pertaining to the safe standardisation of such laser procedures with evidence at the molecular level. The primary aim of this thesis was to optimise applications of laser technology for clinical ART and research applications in the reproductive sciences. This thesis utilised the mouse embryo model to investigate potential deleterious effects of different laser treatment applications, both by the operator and hardware manufacturer. Safe and unsafe laser operator parameters were elucidated by assessing deleterious effects to the plasma membrane integrity, blastocyst survival rate, DNA fragmentation levels, and changes in gene expression of key developmental genes. The effect of altering the laser hardware to lower the power output was evaluated and it was determined that if a lower power laser is used to deliver a set amount of energy over a longer period of time, a smaller amount of damage is incurred. Work in this thesis also established a new method in which laser technology can be used as a research tool for the reproductive sciences, by creating a novel stimuli-responsive laser-activated nanoparticle delivery system with spatial control and increased efficiency in a mammalian cell model. The field of reproductive science continues to benefit greatly from laser application clinically to improve infertility treatments, and in research, to elucidate mechanisms underlying infertility, with a hope of increasing our understanding and eventually developing new treatment options.

Keywords: *Infra-red laser; Assisted reproductive technology; Infertility; Preimplantation embryo; Mouse embryo; Mesoporous silica nanoparticle*

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List of Abbreviations

ANOVA		Analysis of variance
APTES		3-aminopropyltriethoxysilane
ART		Assisted reproductive technology
ASRM		American Society of Reproductive Medicine
Bax		Bcl-2-associated X protein gene
Bcl2l2		Bcl-2-like protein 2 gene
BSA		Bovine serum albumin
cDNA		Complementary DNA
CFSE		Carboxyfluorescein succinimidyl ester di-acetate
CRISPR		Clustered regularly interspersed short palindromic repeats
CTAB		Cetyltrimethylammonium bromide
DAPI		4',6-diamidino-2-phenylindole
DMEM		Dulbecco's Modified Eagle Medium
DNA		Deoxyribonucleic acid
Dnmt3a		DNA methyltransferase 3A gene
EDTA		Ethylenediaminetetraacetic acid
ESC		Embryonic stem cell
FISH		Fluorescence <i>in situ</i> hybridisation
Gapdh		Glyceraldehyde-3-phosphate dehydrogenase
GFP		Green fluorescent protein
Gpx1		Glutathione peroxidase 1 gene
HCL		Hydrochloric acid
HEK293T		Human embryonic kidney 293T cells
HSE		Heat-shock element
HSF		Heat-shock factor
HSP		Heat-shock protein promoter
HSP70		Heat-shock protein 70

ICSI		Intra-cytoplasmic sperm injection
ICM		Inner cell mass
IR		Infra-red
IVF		<i>In vitro</i> fertilisation
KSOM		Potassium simplex optimization medium
LAH		Laser-assisted hatching
lncRNA		Long non-coding RNA
mJ		Millijoules
MSNP		Mesoporous silica nanoparticle
NGS		Next generation sequencing
NIR		Near infra-red
NICE		National Institute For Health and Clinical Excellence
OD		Optical density
OF		Oxford Fertility, United Kingdom
PEI		Polyethyleneimine
PGD		Preimplantation genetic diagnosis
PGS		Preimplantation genetic screening
PI		Propidium iodide
PTM		Post-transcriptional modification
qPCR		Quantitative real-time polymerase chain reaction
RNA		Ribonucleic acid
SCNT		Somatic cell nuclear transfer
siRNA		Small interfering RNA
Sod1		Superoxide dismutase 1 gene
TALEN		Transcription activator-like effector nuclease
TE		Trophectoderm
TEOS		Tetraethylorthosilicate
3-THPMP	..	3-(trihydroxysilyl)propyl methylphosphonate
TUNEL		Terminal deoxynucleotidyl transferase dUTP nick end labelling
WHO		World Health Organisation
UV		Ultraviolet
ZFN		Zinc finger nuclease
ZP		Zona pellucida

1

Introduction

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The increasing number of couples seeking infertility treatment, the medical imperative to improve success rates, and the short interval of time with which to manipulate gametes and embryos necessitate that embryologists are able to swiftly identify the healthiest and strongest gametes for procedural intervention. Assisted reproductive technology (ART) is the collective term for the technologies and procedures performed on sperm, oocytes, and embryos to help sub-fertile couples potentially achieve a pregnancy. The field of ART has undergone rapid expansion over the past decades, largely owing to revolutionary techniques such as *in vitro* fertilisation (IVF), intra-cytoplasmic sperm injection (ICSI), genetic screening, and cryopreservation. Laser manipulation of gametes and embryos represents a powerful advancement in ART, rendering procedures more efficient and thus reducing the exposure time of gametes and embryos to sub-optimal culture conditions outside of the incubator (Montag et al., 2009). The application of lasers for ART has been successfully employed in clinical practice for some time now and is continually the subject of investigative research in order to generate new methods to improve operations. Revolutionary methods such as mitochondrial replacement therapy, recently approved in the United Kingdom (UK)(Castro, 2016; Hyslop et al., 2016), and the editing of human embryo genomes, first reported for humans in China in 2015 (Liang et al., 2015), and recently approved in the UK (Callaway, 2016), represent powerful breakthroughs and highlight the potential that this exciting technology has to offer.

This Chapter begins by introducing infertility and the field of assisted reproduction. It highlights the key benefits and applications of laser technology in ART, and discusses its emerging uses in the clinic and for research into fundamental reproductive biology. It addresses benefits and concerns associated with the use of laser technology in a highly delicate system involving gametes and embryos, and discusses some of the controversies arising from different laser-mediated applications. Finally, this Chapter introduces nanotechnology in the reproductive sciences as a platform with which to pair laser technology to develop promising novel laser-mediated research tools for reproductive biology and medicine.

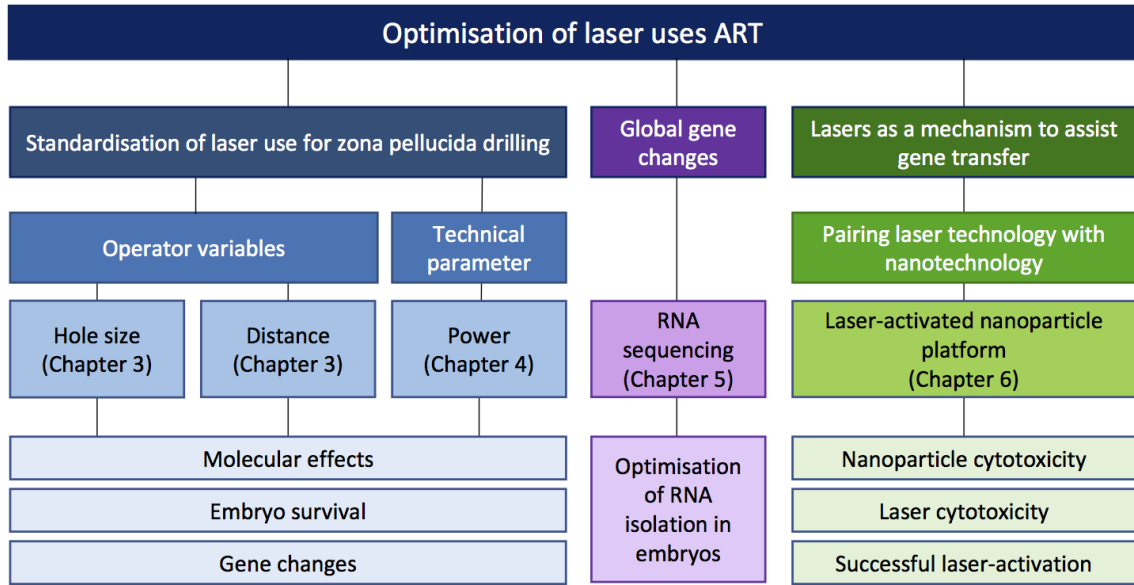


Figure 1.1: A schematic of the contents and structure of the data presented in this thesis.

This thesis examines the current status of cutting-edge laser-assisted reproductive technologies and explores the optimisation of laser technology for applications in the reproductive sciences. Figure 1.1 illustrates the general breakdown of the data presented in this thesis. The safety of laser application in terms of laser operator variables was investigated in Chapter 3 in an effort to refine safe and unsafe working parameters. The effects of altering the power setting of the laser technology hardware to improve the safety of application upon embryos was assessed in Chapter 4. An attempt to explore global changes to gene expression following laser treatment was undertaken in Chapter 5. Lastly, the pairing of laser technology with nanotechnology was carried out in Chapter 6, with the successful generation of a novel laser-activated research tool.

1.1 Infertility and assisted reproduction

An estimated one in seven couples experience difficulty conceiving, with many different reasons as to why conception may not be occurring naturally (NICE, 2013). A combination of factors within the male, female, or both, can contribute to infertility problems, such as a lack of oocytes or sperm, reduced oocyte quality,

the inability of sperm to meet and fertilise the oocyte, or the inability of fertilised embryos to implant or grow in the uterus. In around 15-30% of cases, however, a clear cause is never established, leading to the diagnosis of ‘unexplained’ or ‘idiopathic’ infertility (Gelbaya et al., 2014).

1.1.1 Types of infertility and investigations

For natural conception to occur, a woman must ovulate, have at least one patent fallopian tube, and exhibit a receptive uterine environment. Additionally, a sufficient quantity of healthy motile sperm must be deposited into the vagina. Infertility is defined as the failure to conceive naturally with 12 months of regular unprotected sexual intercourse (WHO, 2010). There are many pathological reasons as to why conception may not be occurring, leading to the exploration of assisted reproductive treatments to help attain a pregnancy (Table 1.1).

1.1.2 Preimplantation embryo development

The development of the human embryo begins with fertilisation of the oocyte by the sperm, fusion of the pronuclei, and a series of cleavage divisions (Figure 1.2). At the 4-8 cell stage, the embryonic genome is activated (Figure 1.3)(Dobson et al., 2004; Niakan et al., 2012). After embryonic genome activation the embryo undergoes compaction to form a morula, and then cells divide further to form a blastocyst, which is comprised of an inner cell mass, a fluid filled blastocoel cavity, and a trophectoderm outer layer. At approximately day 5-6 post-fertilisation, the blastocyst hatches out of the zona pellucida for implantation into the uterus (Niakan et al., 2012). The zona pellucida is a 13-15 μ m thick acellular matrix composed of glycoproteins, carbohydrates and proteins that surrounds the oocyte and embryo and holds structural and functional importance in sperm binding, acrosome reaction induction, blocking polyspermy, assisting in oviductal transport, and maintaining the integrity of the pre-compacted embryo (Gupta and Mullins, 2010).

The current understanding of human preimplantation embryo development has largely been derived from investigations involving surplus embryos from IVF

Table 1.1: Example of common causes of infertility. Adapted from Ehrmann (2005), Child (2013), and Punab et al. (2017).

Factor	Type	Cause(s)
Female factor	Hypogonadotropic hypogonadism	Lack of hypothalamic pituitary drive
	Polycystic ovarian syndrome	Peripheral insulin resistance
	Premature ovarian failure	Menopause before age 40
	Hyperprolactinaemia	Raised prolactin levels usually due to benign pituitary tumours
	Tubal damage	Infection; chlamydia; adhesions; cysts; fibroids
	Endometriosis	Presence and proliferation of endometrial tissues outside the uterine cavity
	Aneuploid oocytes	Increased maternal age
Male factor	Primary testicular disease	Genetic maldevelopment; torsion; trauma; cancer
	Obstructive male infertility	Inflammatory or iatrogenic causes; retrograde ejaculation
	Varicocoele	Varicose vein in scrotum
	Endocrine disorder	Absence of pituitary hormonal drive secondary to cranial tumour; drugs; trauma
	Environmental	Exposure to chemicals, ionising radiation, excessive heat
	Drugs affecting spermatogenesis	Steroids; opiates; chemotherapy; alcohol; tobacco; marijuana
	Autoimmune	Development of antibodies to own sperm after trauma or scrotal surgery
Idiopathic		Unknown cause



Figure 1.2: Normal human *in vitro* embryo development from day 1 to day 5. After fertilisation, embryos can be manipulated *in vitro* at days 3 and 5. Figure reproduced from Gardner et al. (2010).

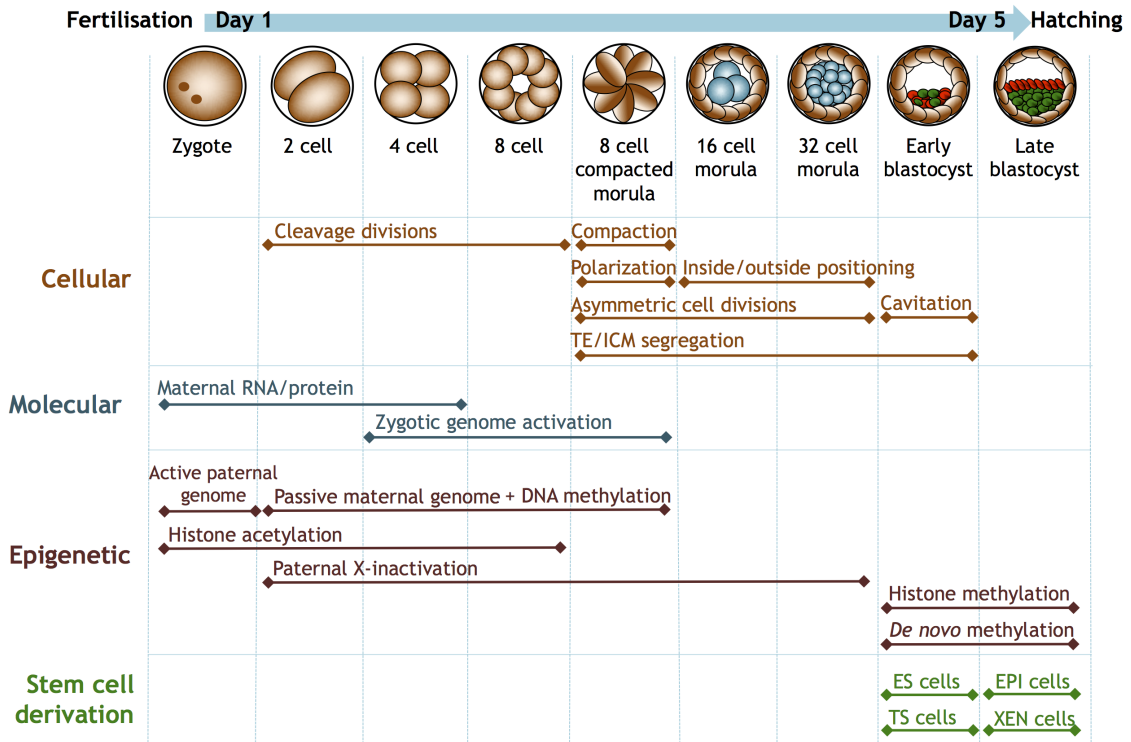


Figure 1.3: A schematic of the development of a mouse preimplantation embryo from fertilisation to hatching. Associated cellular, molecular, epigenetic, and stem cell derivation changes throughout blastocyst development, where TE = trophectoderm, ICM = inner cell mass, ES cells = embryonic stem cells, TS cells = trophoblast stem cells, EPI cells = embryonic epithelial cells, XEN cells = extraembryonic endoderm stem cells. Image created by author and adapted from Oron and Ivanova (2012).

procedures. Predominantly, such studies have focused upon morphological patterns and factors that affect *in vitro* culture and procedures, although more recently, important information has been elucidated using advanced imaging technology and gene expression profiling techniques (Niakan et al., 2012). There are many factors which affect the growth of preimplantation embryos, with many theories of indicators of early embryo health. For example, it has been suggested that timings and synchronicity of the first three mitotic divisions can predict successful development to the blastocyst stage (Wong et al., 2010), that embryos with less metabolic hyperactivity are more viable (Leese, 2002), and that levels of proteins of embryonic origin in the blastocoel cavity are quantifiable and correlate with aspects of embryo viability, such as chromosomal status (Poli et al., 2015). The continuation

of research into our understanding of the development of human preimplantation embryos is paramount to improving our understanding and treatment of infertility.

1.2 Assisted reproductive technology lasers

1.2.1 History of laser development for ART

The first solid state laser to be used on gametes was the neodymium:yttrium-aluminium garnet laser (Nd:YAG), with a wavelength of 1064nm, which was utilised by Tadir et al in 1989 to trap spermatozoa (Tadir et al., 1989; Tadir et al., 1990). In 1991, the same group coupled a Nd:YAG laser with a potassium titanyl phosphate crystal to double the working wavelength to 532nm to drill the zona pellucida, and observed well defined craters in the zona pellucida when applied tangential to the oocyte sphere (Tadir et al., 1991). Over the next five years, multiple groups explored the ability of different lasers to drill preimplantation embryos (Table 1.2).

When the use of lasers for ART was first proposed, there were understandable concerns about safety, due to their application at such a delicate stage of human development. Most concerns centred upon questions of DNA damage, failed embryo development and possible congenital disorders (Montag et al., 2009). While the initial lasers showed effective zona pellucida drilling in animal models (Palanker et al., 1991; Blanchet et al., 1992; el Danasouri et al., 1993), due to the laser working within the DNA-damaging UV range, clinical application was limited due to potential cytotoxic and mutagenic effects on DNA (Kochevar, 1989). The Er:YAG laser worked in the near infra-red range, however, it required direct contact of the laser fibre with the gametes or embryos, causing concern of damage and contamination. The following years saw the emergence of non-contact lasers working in the infra-red or near infra-red range (800-1500nm), further away from the DNA absorption peak of 260nm, eventually leading to clinical application and live births. Figure 1.4 illustrates a timeline of when the first laser-assisted clinical procedures were accomplished relative to milestones in the field of ART.

The first laser to have a major impact upon the advancement of ART was the indium-gallium-arsenic-phosphorous (InGaAsP) semiconductor 1.48 μ m wavelength

Table 1.2: History of laser development for ART.

Year	Laser	Wavelength	Application	Reference
1989	Nd:YAG	1064nm	Trap spermatozoa	Tadir et al. (1989)
1991	Nd:YAG	532nm	Drill zona pellucida	Tadir et al. (1991)
1991	ArF	193nm	Drill mouse zona pellucida, grow to blastocyst	Palanker et al. (1991)
1992	KrF	248nm	Drill zona pellucida of 2-cell mouse embryos, grow to blastocyst	Blanchet et al. (1992)
1992	Er:YAG	3000nm	Drill human oocyte zona pellucida prior to IVF	Feichtinger et al. (1992)
1993	XeCl	308nm	Drill mouse oocytes prior to improve fertilisation with vasectomised mice	el Dana-souri et al. (1993)
1994	InGaAsP	1480nm	Non-contact drilling of mouse oocyte zona pellucida	Rink et al. (1994)

(infra-red) diode laser, which is still used today (Rink et al., 1994; Rink et al., 1996). Rink et al. (1994) used the InGaAsP laser to drill mouse oocytes without the need for direct contact or micromanipulation to stabilise the oocytes. A beam emitted by the $1.48\mu\text{m}$ laser was coupled with a visible aiming pilot red-light laser beam (670nm), and the collinear beams were directed through an inverted microscope objective (45X), along its optical axis, being focused in $2\text{-}3\mu\text{m}$ diameter spots on the zona pellucida of oocytes ($n=150$). Laser pulses (10-20ms long, at a power of 60-70mW) were used to drill openings in the zona pellucida of $5\text{-}7\mu\text{m}$ in diameter. The authors found that 70% of drilled oocytes successfully developed into blastocysts, compared to control oocytes which underwent no intervention. Overall, this laser avoided the mutagenicity and contamination associated with UV contact lasers, making it a valuable tool for ART. Its unique absorption characteristics for water and culture media allows for non-contact application in standard culture dishes, enabling routine application without special equipment or additional steps (Montag et al., 2009).

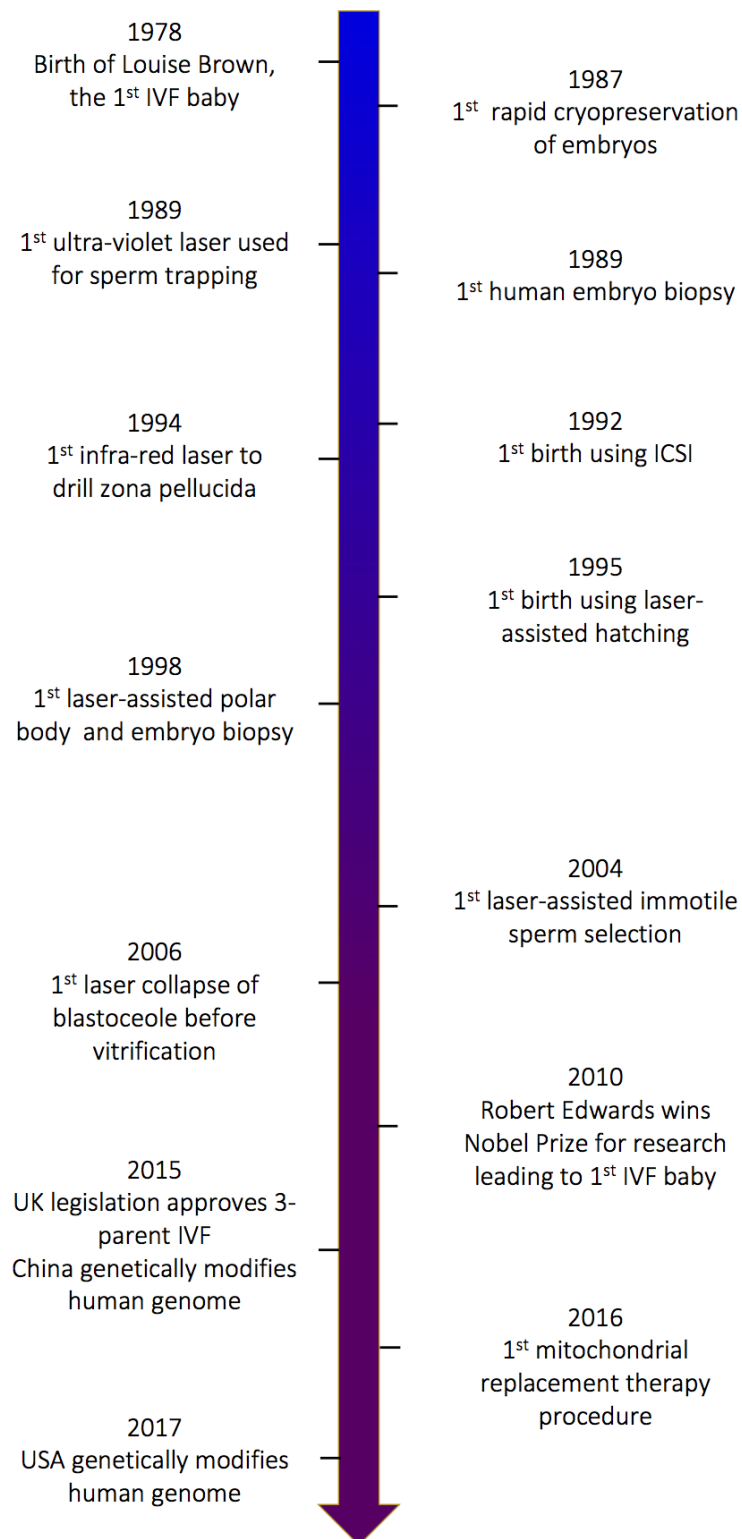


Figure 1.4: Milestones in assisted reproductive technology relating to clinical laser-assisted procedures on human embryo. Image created by author.

1.2.2 Current ART lasers

During ART manipulations, the embryo's culture environment must remain constant to optimise potential for a viable pregnancy. Therefore, to provide consistent, reliable, and reproducible results, the lasers must integrate well and efficiently into the equipment required for routine laboratory procedures. The inverted microscope is a versatile instrument used in ART which allows precise modifications to the stage to facilitate the incorporation and control of precision micro-tools, which are fundamental requirements for the micromanipulation of sperm, eggs, and embryos. Construction of the laser is such that the beam travels through a specialised microscope objective of 40X or 20X magnification. These objectives are placed into inverted microscopes with brightfield, phase contrast, and Hoffman modulation optics, which are used routinely in ART (Figure 2.1). This set up facilitates the application of laser pulses during normal manipulations. In addition to being fitted with lasers and micromanipulation tools, the micromanipulator systems can also be equipped with heated or cooled stages to allow cells and embryos to be maintained at constant physiological temperature throughout manipulation (Kashir et al., 2012). The current ART lasers available on the market are described in Table 1.3.

Three characteristics of lasers that can exert impact upon embryos or tissues are wavelength, power, and pulse length. The risk of thermal damage arising from the use of laser technology was cause for significant debate, as this effect could potentially heat and damage the surrounding cells. The ultra violet (UV) wavelength historically used in biomedical lasers posed significant concern, especially given their reputation to be potentially mutagenic to DNA (Richa et al., 2015), and as a result, laser technology in ART deliberately adapted to operate in the infra-red wavelength. The amount of power from a laser, while constant, can impact upon both the diameter of the hole created and the amount of heat emitted in the process; high power translates to a larger diameter and increased local heating (Taylor et al., 2010). Similarly, pulse length, which can vary from 20ms down to the femtosecond level, affects hole diameter, with a longer pulse correlating to a larger hole and more energy delivered (Ebner et al., 2005).



Figure 1.5: Research Instruments Ltd. Saturn 5 Active infra-red laser fitted on an Integra 3 micromanipulation station on an inverted microscope. (Image courtesy of Research Instruments Ltd.).

Table 1.3: Assisted reproductive technology lasers currently on the market.

Name	Company	Wavelength	Power	Objective	Approved work
Saturn 5	Research Instruments Ltd.	1480nm	400mW	40X	Clinical
Saturn 5-Active	Research Instruments Ltd.	1480nm	400mW	20X, 40X	Clinical
LYKOS	Hamilton Thorne	1460nm	300mW	40X	Clinical
ZILOS-tk	Hamilton Thorne	1460nm	300mW	40X	Clinical
XYRCOS	Hamilton Thorne	1460nm	300mW	20X, 40X	Research
XYClone	Hamilton Thorne	1460nm	300mW	40X	Research

1.3 Clinical laser ART applications

Laser technology is currently used in clinical ART practice to assist with embryo biopsies, assisted hatching, embryo cryopreservation, and sperm investigations (Figure 1.6).

1.3.1 Embryo biopsy

Assisted reproductive technologies are increasingly being used to screen embryos for preimplantation genetic screening (PGS) to investigate chromosomal disorders, or for preimplantation genetic diagnosis (PGD) to investigate hereditary genetic conditions. In order to perform these tests, embryonic cells must be acquired from the developing oocyte or live embryo which have been created through ART. There are three main types of cells that can be used for such biopsies: a polar body from unfertilised oocytes, a blastomere from a cleavage stage embryo, or trophectoderm cells from a blastocyst stage embryo (Montag et al., 2009).

Historically, for embryo biopsies, a hole was created in the zona pellucida by degradation with acid Tyrode's solution to allow a glass extraction pipette to reach the cells. The embryo was immobilised while acid in a microcapillary tube was gently blown over the embryo until a localised region of the zona pellucida began to dissolve, and a cell was suctioned out and used for genetic screening. The use of acid presents many technical challenges, exposes the embryo to abnormal pH levels, and requires a change of culture media afterwards to remove excess acid (Geber et al., 2011). On top of introducing potentially dangerous acid to the embryo's environment, the additional washing step required enhances the risk of contamination, which is detrimental for the accuracy of sensitive single cell biopsy genetic tests. Another method used to breach the zona pellucida for biopsy is to mechanically carve a hole using sharp glass pipettes (Tarín and Handyside, 1993). However, both acid and mechanical techniques are highly variable, lead to inconsistent hole sizes, and could easily result in damage or loss of cells.

In current clinical practice, laser embryo biopsy is the preferred method of choice, since it simplifies the process and reduces damage to the oocyte or embryo

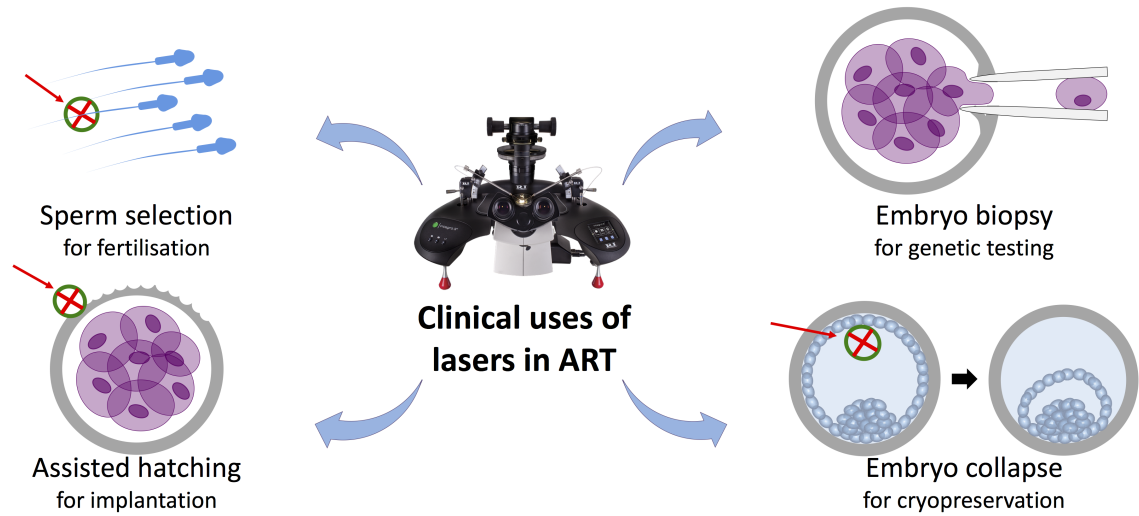


Figure 1.6: Applications of infra-red laser technology for clinical assisted reproductive technology. Red arrows indicating the crosshair positioning of the laser pulse. Image created by author.

while providing equal or increased success rates. Laser pulses are directed at the zona pellucida, creating a uniform hole through which a blunt glass capillary can be inserted to extract target cells. The ability to use a blunt capillary, rather than a sharp glass needle, to traverse the zona pellucida avoids damage such as mechanical strain and deformation of the embryo (Rienzi et al., 2001). Additionally, the laser equipment enables multiple embryos to be kept in the same dish as they do not need to be washed from the acid afterwards. Studies comparing the use of acid Tyrode's with laser zona drilling for embryo biopsy have shown that laser drilling is an easier and faster procedure, resulting in a greater number of intact cells post-procedure, and often increased pregnancy success rates (Joris et al., 2003; Chatzimeletiou et al., 2005; Geber et al., 2011). Prior to laser-assisted biopsy, the biopsy technique was limited by operator skill and non-standardised tunnel size, and embryos were subjected to a drastic pH change due to the introduction of acid into the environment. Now, more consistent holes can be created in embryos and the biopsy procedure takes far less time, thus minimising the time the embryo spends outside of the incubator.

1.3.1.1 Polar body biopsy

Polar bodies are small haploid cells extruded from the oocyte during oogenesis which, while considered genetic discards, contain valuable information which can help determine the health of a given embryo. Polar body biopsy is often used for PGD/PGS as genetic variants associated with human disease can be deduced by sequencing the polar body genome (Figure 1.7) (Hou et al., 2013). Polar bodies can be obtained and tested prior to fertilisation, however they only provide information about the maternal genome. In some countries such as Switzerland, Austria, Germany and Italy, polar body biopsy of unfertilised oocytes is the only legally acceptable method for genetic investigations (Kuliev et al., 2006; Coco, 2014; Guimaraes et al., 2016).

Laser-assisted polar body biopsy was first explored in 1997 by Montag and colleagues. Of 921 human oocytes which were laser-treated to open the zona pellucida to retrieve a polar body, 443 oocytes were identified as euploid using fluorescence *in situ* hybridisation (FISH), 293 were fertilised, 214 were transferred to the uterus, and 27 pregnancies resulted. The take home baby rate was 18.3% per transfer cycle, which was comparable to the mechanical drilling method (Montag et al., 1998). Hammoud et al., investigated the effects of using lasers for polar body biopsies and concluded that there were no deleterious effects of laser drilling for polar body biopsy in *in vitro* matured oocytes in term of oocyte lysis, oocyte activation, or embryo chromosome breakage (Hammoud et al., 2010).

The value of polar body biopsy remains controversial, however, as polar bodies do not always accurately reflect the status of the oocyte (Harper et al., 2010). Scott and colleagues demonstrated that half of the oocytes associated with single chromatid errors detected in the first polar body resulted in the development of healthy embryos which would have otherwise been discarded (Scott et al., 2012). Levin et al. further demonstrated conflicting results, reporting that laser-treated embryos had a higher rate of cleavage arrest, higher fragmentation, and lower rates of good cleavage; however, despite these findings, the laser-treated groups, when

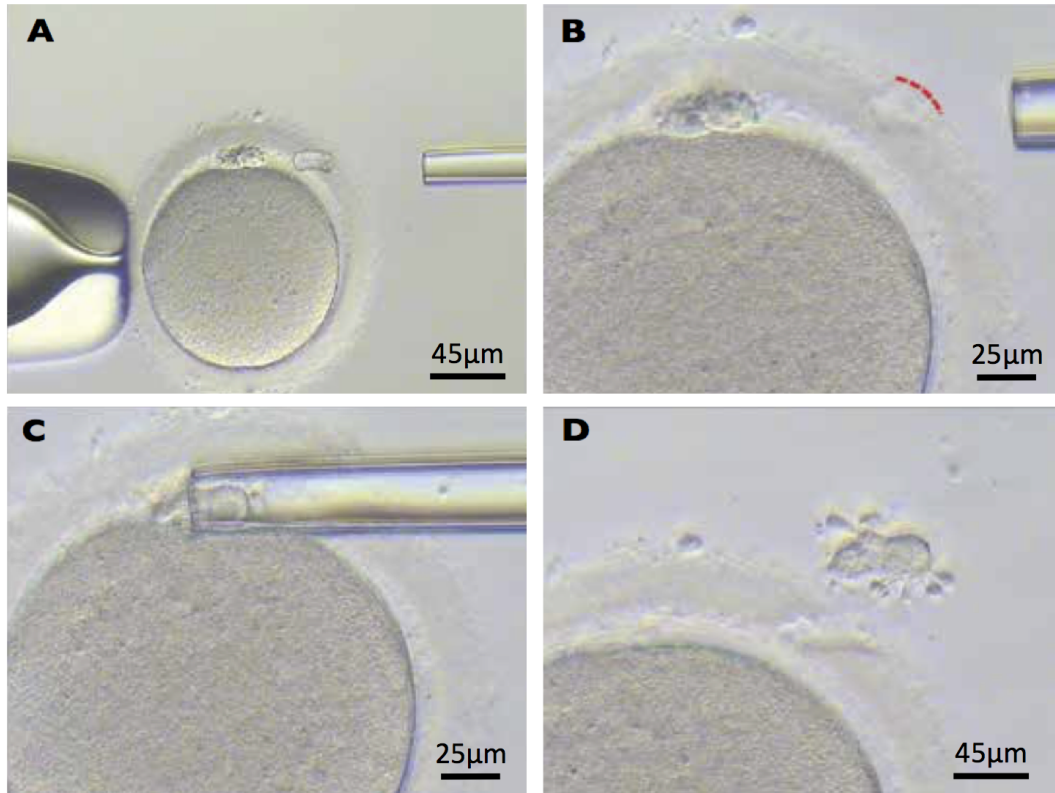


Figure 1.7: Images showing a polar body biopsy for genetic screening. (A) Laser pulse path alignment. (B) Red line indicating where the zona pellucida has been treated with laser pulses. (C) Insertion of a blunt capillary tube to reach the polar body cell. (D) Extracted polar body outside of the zona pellucida ready for collection and genetic analysis. Image courtesy of Tracey Griffiths and reproduced from Davidson et al. (2015a).

compared to controls, showed significantly higher chemical pregnancy rates, clinical pregnancy rates, and live birth rates (Levin et al., 2012).

1.3.1.2 Blastomere biopsy

Blastomere biopsy for PGD/PGS is a well accepted method of choice on the general international level due to the diagnostic possibilities to detect both maternal and paternal contributions to the embryo. Performed on cleavage stage embryos following zona pellucida opening, a blastomere is removed for analysis. Older, less common, methods are based on extrusion of the blastomeres without touching the cell itself. One method is by injecting culture medium into the embryo through another site which pushes the blastomeres near the biopsy hole to protrude past the zona (Pierce et al., 1997). Another method involves squeezing a blastomere out of the zona

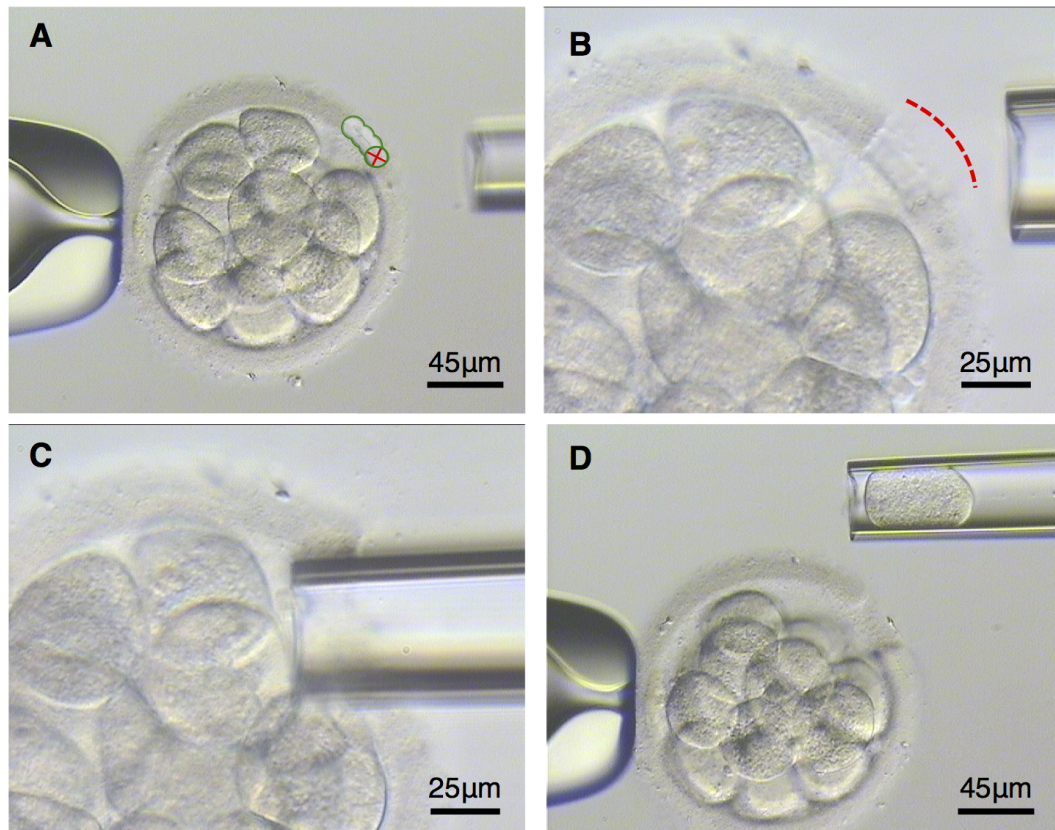


Figure 1.8: Images showing laser-assisted blastomere biopsy from a day 3 embryo for genetic screening. (A) Laser pulse path alignment along the zona pellucida. The red cross marks the laser pulse location, and the green outline specifies the path over which a series of laser pulses will be administered. (B) Red line indicating where the zona pellucida has been treated with laser pulses. (C) Insertion of a blunt glass capillary tool to reach a blastomere near the periphery of the embryo. (D) Glass capillary tool containing the extracted blastomere ready for genetic analysis. Image courtesy of Tracey Griffiths and reproduced from Davidson et al. (2015a).

using a pressure point distant to the hole (Tarín and Handyside, 1993). Currently, removal of blastomeres is mainly performed by gentle aspiration (Figure 1.8). If the first cell is clearly seen to be non-viable, a second blastomere may be aspirated for testing, however there is conflicting data on whether the removal of two blastomeres reduces the chance of pregnancy success (Brodie et al., 2012).

Boada et al (1998) published the first case report of laser-assisted blastomere biopsy for PGD in two haemophiliac patients, which resulted in a single successful pregnancy. Two to four laser pulses were applied to 13 embryos, three normal female embryos were replaced in the uterus on day 3, and one pregnancy resulted (Boada

et al., 1998). Since then, numerous studies have reported the use of laser-assisted cleavage stage embryo biopsies with successful PGD/PGS analysis (Jones et al., 2006). Joris and colleagues report that using a laser to open the zona pellucida for blastomere biopsy is an easier procedure which resulted in significantly more intact blastomeres compared to acid Tyrode's opening, and no difference between pregnancy and implantation rates (Joris et al., 2003). Jones et al. reported that laser biopsy does not impair embryonic development to the blastocyst stage in terms of expansion and cell numbers in the inner cell mass and trophectoderm (Jones et al., 2006). Investigations by Kahraman et al. further showed that the use of laser-assisted blastomere biopsy on day 3 is a useful tool for aneuploidy testing in couples with poor prognosis due to factors such as advanced maternal age, recurrent implantation failure, repeated spontaneous abortion, and abnormal gamete cell morphology (Kahraman et al., 2004). While cleavage-stage biopsy has the ability to detect chromosomal aberrations or single-gene disorders of both maternal and paternal origin, concerns include cellular mosaicism in embryos, whether the analysed cell is representative of the rest of the embryo, and the limited amount of tissue available for diagnosis (Adler et al., 2014).

1.3.1.3 Trophectoderm biopsy

The blastocyst stage embryo is divided into two sections: trophectoderm cells which form the placenta, and the inner cell mass, which forms the embryo. The advantage of blastocyst biopsy is that multiple trophectoderm cells can be collected, thus allowing for more reliable results, and that no cells that form the embryo are compromised. Historically, the zona pellucida was breached and trophectoderm cell samples were obtained by mechanical excision. Alternatively, with laser application, a small opening is made in the zona pellucida on day 3, and as the blastocyst grows and expands, part of the embryos herniates out of the hole, and on day 5, these cells can be excised using laser pulses (Figure 1.9). The laser procedure is much faster, requires less mechanical skill, and imposes less mechanical stress on the blastocyst (Montag et al., 2009). The use of lasers has become the accepted

convention for embryo biopsy and has been used to investigate differences between blastocyst trophectoderm biopsy and cleavage stage blastomere biopsy. Kokkali et al., compared laser blastomere biopsy versus blastocyst biopsy for beta-thalassaemia PGD and found that blastocyst biopsies resulted in significantly higher diagnosis rates and implantation rates compared to blastomere biopsies (Kokkali et al., 2007). In support of this finding, many other studies have shown laser trophectoderm biopsy to be more reliable and successful than blastomere biopsy for PGD (Adler et al., 2014). Internationally, clinics are moving towards elective blastocyst stage biopsy to allow for more accurate tests and thus improved success rates. Laser application remains the method of choice for embryo biopsies to ensure the least invasive and most efficient procedure for the embryo, and is reliably used for polar body, blastomere, or trophectoderm biopsy. Table 1.4 illustrates some of the main advantages and disadvantages of the three biopsy methods.

1.3.2 Assisted hatching

To implant into the uterus, the embryo needs to escape from the zona pellucida at the blastocyst stage, a process referred to as 'hatching'. Hatching *in vivo* is dependent on embryonic and uterine factors, however the physiological course of hatching, and the mechanisms preventing normal hatching and implantation in some patients, is still poorly understood. While exact causes of failed implantation are unknown, increased maternal age, reduced oocyte quality, and poor embryo morphology are all known to be contributing factors (Das and Holzer, 2012; Simon and Laufer, 2012). Moreover, it has been hypothesised that exposure of oocytes and embryos to the artificial conditions of *in vitro* culture may have negative effects upon the embryo's ability to undergo the normal hatching process. Consequently, assisted hatching is often offered to patients with recurrent ART failure in an attempt to increase implantation potential, to patients of advanced maternal age to help overcome age-related zona pellucida hardening from endocrine disruptions, and to frozen-thawed embryos where excessive *in vitro* culture and cryopreservation procedures lead to glycoprotein alterations and zona hardening (Martins et al., 2011).

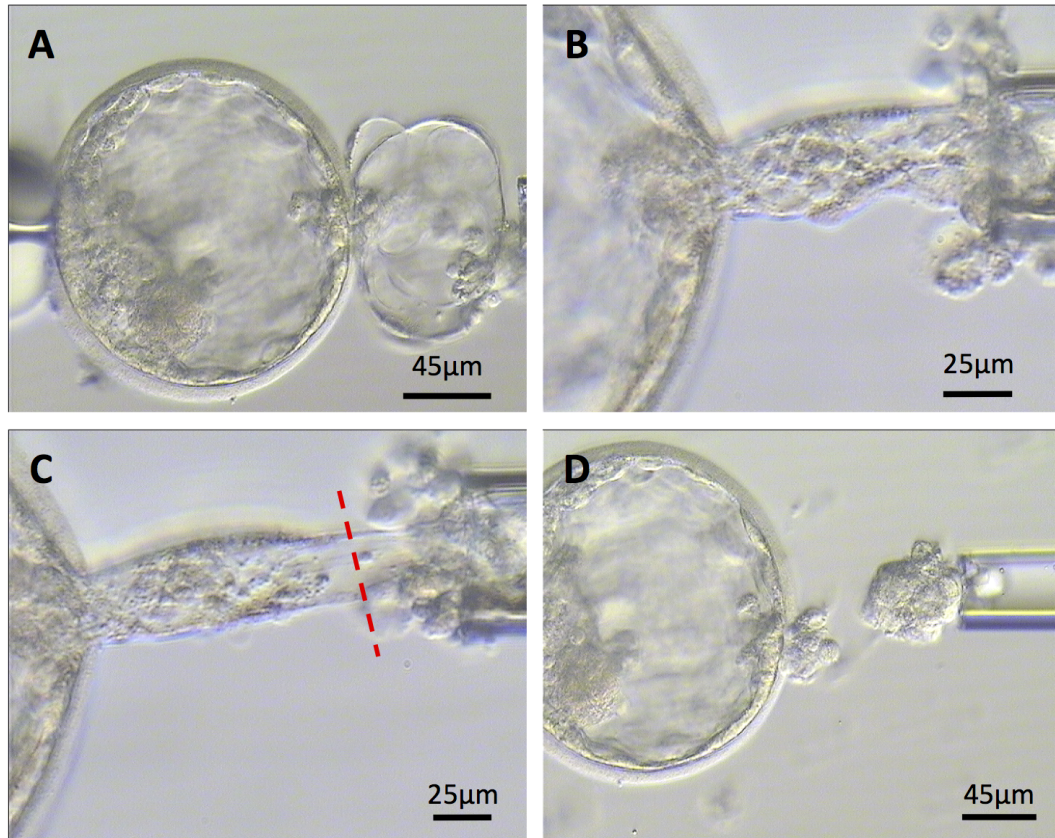


Figure 1.9: Images showing laser-assisted trophectoderm biopsy from a blastocyst embryo for genetic screening. (A) A hole was created in the zona pellucida on day 3, and as the blastocyst expands, part of the trophectoderm begins to herniate out of the zona pellucida (B) An aspiration pipette is used to hold the herniated cells in place and create tension between the cells. (C) Laser pulses are applied at the cell junctions between trophectoderm cells to separate trophectoderm cells from the embryos (laser firing location indicated by the red line). (D) Glass capillary tool containing the extracted trophectoderm cells ready for genetic analysis. Image courtesy of Tracey Griffiths, Oxford Fertility.

The different methods of *in vitro* assisted hatching are mechanical hatching, chemical drilling with Tyrode's acid, and laser-assisted hatching, to create a hole to act as an initial hatching site in the zona pellucida through which the blastocyst can escape (Figure 1.10). It has been postulated that the artificial gap formed via assisted hatching may serve as a channel for the exchange of metabolites and growth factors between the embryo and endometrium prior to hatching, and that subsequent hatching is accelerated and allows earlier blastocyst-endometrium contact, thus facilitating implantation (Hammadeh et al., 2011; Wan et al., 2014). A meta-analysis

Table 1.4: Advantages and disadvantages of polar body, blastomere, and trophectoderm biopsy. Adapted from Rink et al. (1994) and Thornhill et al. (2005).

Biopsy	Day post fertilisation	Sample type	Advantages	Disadvantages
Polar body	Day 0-1	Polar body	-Maximum time available between fertilisation and transfer -Does not compromise any components which form the embryo -Minimal invasion	-Genetic analysis limited to maternal lineage -Increase labour intensity and expense due to increased sample size
Cleavage stage	Day 3	Blastomere	-Both maternal and paternal lineages examined -Majority of embryos reach cleavage stage	-Maximum of 2 cells can be retrieved -Removal of 2 cells can be detrimental -Minimal room for error
Blastocyst stage	Day 5-7	Trophectoderm	-Multiple cells (3-10) can be retrieved for more robust analysis -Reduced cost and time -ICM kept intact	-Short time frame between biopsy and fresh embryo transfer -Not all embryos reach blastocyst stage

of randomised controlled trials investigating the benefits of assisted hatching was performed in 2003, however the results were not universally accepted due to the heterogeneity of the techniques being used (Sallam et al., 2003). The introduction of laser technology created an option for standardised and reproducible hatching for the first time, which was paramount for analysing results from multi-centre trials and comparing results from different studies.

The use of lasers is the most consistent and reproducible method for embryo manipulation and is therefore the most dependable method to conduct comparison studies. However, the current literature on laser-assisted hatching (LAH) reveals conflicting results causing the benefits of assisted hatching to remain under debate. Numerous studies have indicated that LAH provides better clinical pregnancy, implantation, and live birth rates compared to no hatching, especially in patients

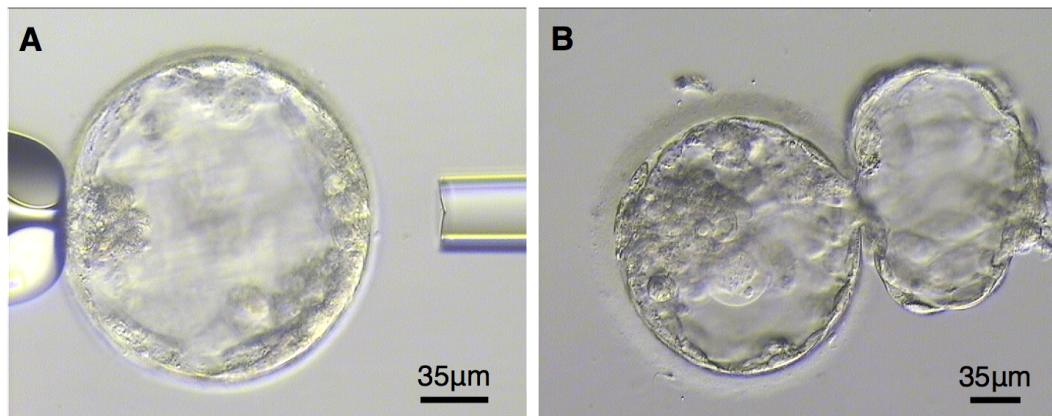


Figure 1.10: Blastocyst embryo hatching from the zona pellucida. (A) An unhatched blastocyst stage embryo. (B) Blastocyst in the midst of hatching out through a hole in the zona pellucida shell. Image courtesy of Tracey Griffiths, Oxford Fertility, United Kingdom (reproduced with permission).

with increased age, low grade embryos, recurrent miscarriages, or frozen embryos (Wan et al., 2014; Zhou et al., 2014). A randomised control trial investigating LAH compared to no assisted hatching initially found no significant differences between clinical pregnancy rates and implantation rates; however, when the results were categorised based on patient's age, there was a significant improvement in clinical pregnancy rates following LAH (70.0% *versus* 25.0%) in patients 35 years or older (Elhussieny et al., 2013). In contrast, other studies report that while LAH is not detrimental, it is also not significantly beneficial. Kutlu et al., found that in women aged 35 and under, and in women aged 35 and over, LAH on day 3 embryos did not significantly increase clinical pregnancy rates (Kutlu et al., 2010). Similarly, a randomised controlled trial investigating LAH showed no improvement with respect to implantation rate or live birth rate (Debrock et al., 2011). The conflicting nature of the results in the available literature further defines the present debate of whether LAH is a beneficial, neutral, or detrimental technique. There are conflicting studies relating to whether LAH shows better outcomes than acid Tyrode's hatching. While multiple studies report no significant differences in embryo development and clinical pregnancy rate between LAH and the acid Tyrode's method, the ease of use, efficiency, and reproducibility of the laser technique still renders it the more favourable option (Wong et al., 2003; Lanzendorf et al., 2007). Other studies have

also shown that the laser technique shows significantly improved ART outcomes compared to the acid technique. Hsieh et al., for example, reported that laser-assisted hatching in women aged 38 years or older showed significantly higher implantation rate, clinical pregnancy (Hsieh et al., 2002).

There are three different LAH methods of varying invasiveness, with the optimal method still being debated (Figure 1.11). First, the laser can be used to create a hole completely penetrating through the whole zona pellucida layer. Secondly, a small hole through most of the zona pellucida can be created to partially hatch the embryo. Thirdly, the laser can be used to thin a large inner or outer area of the zona pellucida around the embryo to facilitate natural hatching. The precise effects of different types of laser assisted hatching techniques still remain controversial. Comparative studies and meta-analyses have, in the past, suggested that there are minimal differences in outcome from different types of laser-assisted zona thinning, partial hatching and full hatching that are available at present (Lanzendorf et al., 2007; Martins et al., 2011). However, one study directly comparing all three laser methods indicated that zona thinning should be performed rather than total dissection (Mantoudis et al., 2001). More recent comparative studies support this finding, reporting that zona pellucida thinning is the least invasive method and increases pregnancy rates compared to other laser methods in patients with a history of repeated implantation failure (Wan et al., 2014).

One hypothesis as to why zona thinning of large areas may show increased success rates is that a more extensive area of ablation increases the chance that the resultant hatching site corresponds to the naturally predisposed hatching site (Sathananthan et al., 2003). There have been few follow up reports following the health of children born following laser-assisted hatching. Kanyo and Konc followed the health of babies up to 1 year post-delivery and reported no increase in the rate of major or minor congenital malformation, or the rate of chromosomal aberrations in 134 children born following LAH (Kanyó and Konc, 2003). Supporting these results, a subsequent study discussed unpublished data relating to 62 children born following LAH with no evident increase in congenital malformation or chromosomal

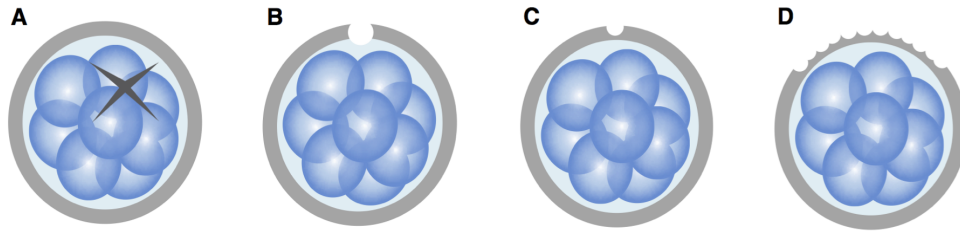


Figure 1.11: Cleavage stage embryo assisted-hatching methods. (A) Mechanical cross section for assisted hatching or embryo biopsy. (B) Full laser-assisted hatching penetrating through the whole zona pellucida layer. (C) Partial laser-assisted zona pellucida hatching. (D). Quarter laser-assisted zona thinning. Image created by author.

abnormality (Primi et al., 2004). Further large-scale studies are now required to fully investigate the health of children born following laser treatment.

1.3.3 Cryopreservation

Cryopreservation allows the storage of surplus gametes and embryos. Preimplantation embryos are comprised mostly of water, and as adjacent volumes of liquid water within cells solidify to ice, the expansion causes pressure and shearing forces on intracellular organelles causing damage to the embryos (Jain and Paulson, 2006). There still exists the debate between fresh *versus* frozen embryo transfer for assisted reproduction. A recent Cochrane review concluded with moderate-quality evidence that neither fresh nor frozen embryo transfer is superior to the other in terms of cumulative live birth rates, and concluded with low-quality evidence that fresh transfers increases the risk of ovarian hyperstimulation syndrome (OHSS) in women at risk of developing OHSS (Wong et al., 2017). There are, however, some patients who require cryopreservation; for example, patients who are undergoing gametotoxic procedures, such as chemotherapy and radiation, who wish to have the option of achieving pregnancy in the future (Mahajan, 2015).

The two cryopreservation methods routinely employed in ART are slow-freezing and vitrification. Slow-freezing embryos allows the embryos to freeze at a sufficiently slow rate such that adequate cellular dehydration occurs while minimising intracellular ice formation (Granne et al., 2008; Rienzi et al., 2017). Vitrification uses

high concentrations of cryoprotectants and rapid cooling at a rate of $-20,000^{\circ}\text{C}$ per minute to help prevent ice crystal formation in cells by allowing the solidification of the cells and extracellular matrix into a glass-like state (Craig and Turner, 2013). Vitrification has been reported to be more efficient than slow freezing of embryos in terms of post-warming survival rate in cleavage stage embryos and lower apoptosis rates in blastocyst embryos (Fasano et al., 2014; Song et al., 2016). A recent systematic review and meta-analysis of randomised control trials investigating oocyte, embryo, and blastocyst cryopreservation comparing slow-freezing *versus* vitrification concluded that vitrification and warming is superior to slow-freezing and thawing with regard to clinical outcomes and cryosurvival rates for oocytes, cleavage stage embryos, and blastocysts, suggesting that ART laboratories which continue to use slow-freezing should transition to using vitrification methods (Rienzi et al., 2017).

1.3.3.1 Blastocoel collapse for vitrification

At the blastocyst stage, the embryo has a fluid filled cavity called the blastocoel. Artificial shrinkage of the blastocoel cavity to reduce fluid volume prior to vitrification has been shown to increase embryo survival (Vanderzwalmen et al., 2002; Chen et al., 2005; Granne et al., 2008; Van Landuyt et al., 2015). Shrinking of the blastocoel can be performed in four different ways. First, via mechanical puncture with a sharp glass needle, where the blastocyst collapses over a period of 30 seconds to 2 minutes. Secondly, by manually aspirating the blastocyst in and out of tight pipettes, causing the blastocoel to collapse (Hiraoka et al., 2004). Thirdly, by osmotic shock, which uses high sucrose concentrations to induce the transport of water out of cells and dehydrate the embryo. Fourthly, the fastest method, by a short laser pulse, shrinking the cavity immediately without any other form of manipulation or mechanical strain upon the embryo (Figure 1.12)(Iwayama et al., 2011).

Using laser technology to artificially shrink the blastocoel was first introduced by Mukaida et al (2006) when the authors compared laser collapse with mechanical puncturing using a micro-needle. A single 200ms laser pulse was targeted at a cellular junction in the trophectoderm creating a hole, which induced collapse of

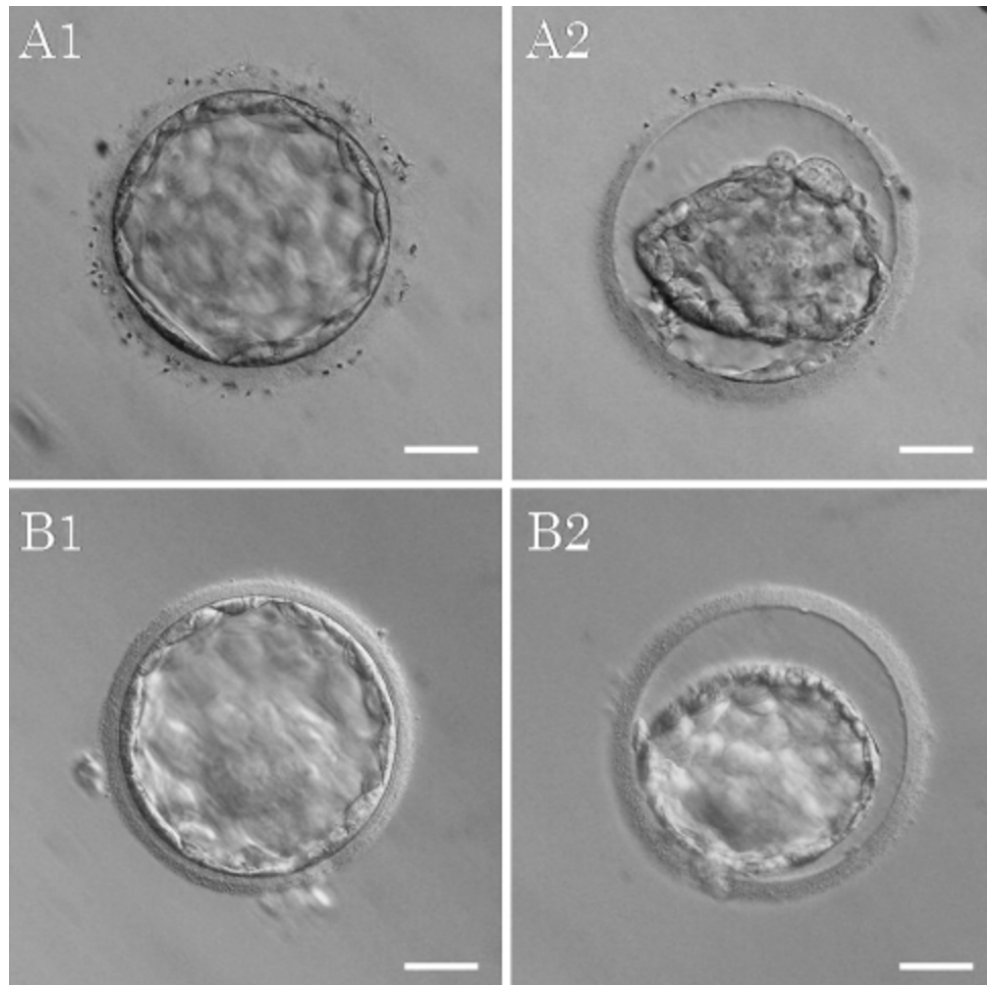


Figure 1.12: Artificial shrinkage of blastocoelic cavity in human expanded blastocysts. (A) Treatment by a laser pulse or (B) treatment with hyperosmotic sucrose solutions (1) before and (2) after the treatment. Scale bar = $50\mu\text{m}$. Figure reproduced from Iwayama et al. (2011).

the blastocoel cavity. No statistical difference in terms of clinical pregnancy was seen between the two methods and the laser procedure eliminated the need for holding pipettes and micromanipulation tools, rendering the procedure more efficient (Mukaida et al., 2006). Further studies have shown that human blastocysts vitrified after mechanical and laser collapse show fewer damaged cells and significantly higher pregnancy rates compared to non-collapsed blastocysts (Hur et al., 2011; Cao et al., 2014; Min et al., 2014). Desai et al., found that laser-collapsed blastocysts showed significantly higher rates of expansion upon thawing, and also lower levels of DNA damage (Desai et al., 2008). Furthermore, laser treatment of expanded

blastocysts prior to vitrification has been shown to exhibit lower rates of apoptosis compared to non-laser treated vitrified embryos and fresh non-vitrified embryos (Song et al., 2016). Cao et al., reported similar results between mechanical collapse and laser collapse, with both resulting in significantly better blastocyst hatching, implantation, clinical pregnancy, gestational duration, and birth weight compared to controls. However, the authors also reported that the laser collapse method resulted in a significantly higher blastocyst hatching rate, longer average gestational duration, and lower premature birth rate, suggesting that laser collapse is a superior method (Cao et al., 2014).

1.3.3.2 Necrotic blastomere removal

Cryopreservation procedures at the day 3 cleavage stage can impart damage to embryos via several mechanisms including solution effects, osmotic effects, or physical damage by growing ice crystals (Hendriks et al., 2014). Frozen-thawed embryos are considered to have survived if at least half of the blastomeres remain intact. It has been shown that partially-damaged embryos after thawing have lower implantation rates than fully-intact embryos (Guerif et al., 2002; El-Toukhy et al., 2003). The poor implantation rates associated with partially-damaged embryos may be due to the release of toxic metabolites from necrotic blastomeres, and the action of such metabolites upon the remaining healthy blastomeres. It has been suggested that the up-regulation of preimplantation embryo metabolism is associated with a reduction in viability (Leese, 2002). It has been further hypothesised that blastomeres neighbouring necrotic cells may have to engage in detoxification mechanisms, which could deplete energy and resources needed for further development and differentiation (Rienzi et al., 2002).

After thawing, necrotic blastomeres can be removed from the embryo by aspiration via a small hole created by a laser through the zona pellucida. Removal of necrotic blastomeres from partially-damaged embryos has been shown to significantly increase clinical pregnancy rates and implantation rates by more than double compared to no removal (Rienzi et al., 2002; Liu et al., 2005; Liu et al., 2007). Rienzi

et al. showed that the developmental potential of partially-damaged frozen/thawed embryos can be equivalent to normally healthy embryos if necrotic blastomeres are removed with the aid of laser technology, even if multiple blastomeres need to be removed (Rienzi et al., 2005). While not yet widely utilised in clinics for cryopreservation, laser treatment represents a powerful means with which to improve embryo survival and is suspected to increase accordingly as clinics are moving towards immediate vitrification and frozen embryo transfers following PDG/PGS.

1.3.4 Applications involving sperm

There are many different aspects of sperm which can contribute to male factor infertility, such as low sperm count, abnormal morphology, and poor motility. In such cases, if the sperm cannot fertilise normally, a procedure known as intracytoplasmic sperm injection (ICSI) can be performed, which involves selecting a single sperm and manually injecting it into an oocyte to induce fertilisation (Lee et al., 2017). The ICSI technique has revolutionised the treatment of male factor infertility and is now used in over half of the treatment cycles taking place in Europe, Australia, New Zealand, and the United States of America (Nyboe et al., 2008; Merchant et al., 2011).

1.3.4.1 Intracytoplasmic sperm injection (ICSI)

Prior to injection for ICSI, the sperm tail must be immobilised so that its beating does not cause damage inside the oocyte. Traditionally, sperm tail immobilisation is performed using a fine-tipped glass micro-tool, which is manually brought down and across the tail, thus causing the tail to break. Immobilisation of sperm can be simplified by using low level laser pulses on the sperm tail to incapacitate its beating without affecting viability (Vizziello et al., 2005).

Conventional ICSI is associated with mechanical damage to the oocyte caused by deformation of the zona pellucida and exposure to negative pressure during injection (Figure 1.13). In particular, fragile oocytes show high zona pellucida

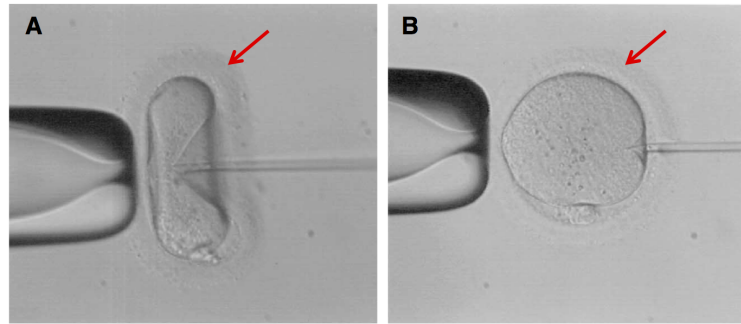


Figure 1.13: Conventional ICSI and laser-assisted ICSI. (A) Conventional ICSI into an oocyte with a hardened zona pellucida showing oocyte deformation. (B) Laser-assisted ICSI showing no oocyte deformation. Red arrows indicate the zona pellucida. Figure reproduced from Rienzi et al. (2001).

resistance to ICSI injection needle penetration, showing unusual extensive deformation and causing abnormal patterns of oocyte membrane breakage, subsequently compromising survival (Danfour and Elmahaishi, 2010). In laser-assisted ICSI, a precise controlled hole can be drilled into the zona pellucida leaving the innermost layer of the zona intact, allowing easy access for the needle though the mechanical barrier with minimal deformation.

Multiple studies have supported the notion that creating a micro-hole through the zona pellucida of the oocyte by a laser beam prior to ICSI results in less traumatic penetration of the injection needle, thereby resulting in lower oocyte and embryo degeneration, as well as higher fertilisation, embryonic development, and pregnancy rates compared to conventional ICSI for patients with fragile oocytes (Rienzi et al., 2001; Lu et al., 2015). Nonetheless, laser-assisted ICSI is not yet widely adopted in clinics, as there is some debate regarding the overall benefit of the procedure. Some embryologists believe that it is the resistance and stretching ability of the membrane under the zona pellucida and around the cells, and not the zona pellucida itself, that relates to oocyte degeneration and embryo developmental competence following conventional ICSI, and that such damage can be reduced by simply using good injection needles and technique (Danfour and Elmahaishi, 2010).

1.3.4.2 Laser selection of immotile sperm

The selection of a viable sperm is required for successful fertilisation with ICSI. Usually, motility is the parameter used to select sperm, however there are cases where none or only a few sperm are motile. This is most often seen in cases where sperm has had to be surgically retrieved from the testes, but can also be seen in ejaculated samples (Nordhoff, 2015). It has been reported that immotile sperm can still successfully fertilise oocytes, albeit creating lower quality embryos and achieving lower pregnancy rates (Montjean et al., 2014). Laser-assisted sperm selection identifies viable sperm using a single pulse to the tip of the sperm tail, causing the tail of live immotile sperm to curl but without effect upon non-viable sperm.

Interestingly, the latest World Health Organization (WHO) manual (2010) for the examination and processing of human semen lacks recommendations for the selection of immotile viable sperm. There are four main tests that have been developed to identify viable immotile sperm: the hypo-osmotic swelling test, chemical induction of tail movements, sperm tail flexibility test, and laser-assisted immotile sperm selection. The main disadvantages of the first three techniques are that they are time consuming, require additional chemicals, and cannot all be used on cryopreserved sperm. In contrast, laser-assisted sperm selection can be used on all samples and also on patients with immotile cilia syndrome (Nordhoff, 2015). Laser-assisted sperm selection has shown the same level of detection as the hypo-osmotic swelling test, while increasing the rates of fertilisation and live births (Aktan et al., 2004). The main advantages of lasers over the other methods is that the test can be conveniently performed immediately before the ICSI injection, does not require the use of additional incubation steps or chemicals which may cause side effects, and does not cause damage to sperm DNA.

1.3.5 Mitochondrial replacement therapy

An exciting application of lasers in reproductive medicine is to facilitate spindle and pronuclear transfer processes for mitochondrial replacement therapy, recently approved in the UK to treat women at risk of passing on mitochondrial diseases to

their children. Of the approximate 25,000 genes in the human genome, mitochondrial DNA (mtDNA) encodes just 37. Mitochondrial disease, which can be caused by mutations in both nuclear DNA and mtDNA, is estimated to affect 1 in 5000 births (Haas et al., 2007). Certain features of mitochondrial biology such as the maternal inheritance, heteroplasmy, and haplotypes, render traditional PGD screening methods ineffectual for such cases.

Mitochondria are semi-autonomous organelles which regulate energy production via oxidative phosphorylation and possess their own genomes and transcriptional machinery. Clinical phenotypes of mitochondrial disease are related to heteroplasmy and thus the proportion of mitochondrial DNA in the affected cells. Due to their central role in cellular metabolism, the consequences of mitochondrial disorders upon tissues and organs with the highest energy demands, such as the brain, heart, muscle, liver, kidney, and central nervous system, are catastrophic (Poulton et al., 2009). Since mitochondria are only inherited from the mother, the mitochondrial replacement therapy technique involves the use of nuclear material from a man and woman (the genetic parents) and cellular material from a third woman (Figure 1.14). The spindle transfer process is carried out by transferring the nucleus, containing DNA, from the afflicted maternal oocyte, into an enucleated donor oocyte containing healthy mitochondria. Sperm can then fertilise the donor oocyte containing the transferred maternal DNA to create an embryo with the desired genetic parents, but with the mitochondria of the donor (Bredenoord et al., 2008). A second method, referred to as pronuclear transfer, occurs after fertilisation and involves transferring both pronuclei from a zygote containing mutant mtDNA into a donated enucleated zygote. Lasers are deployed in both methods to assist in removing and transferring the nuclei from both cells in a faster and more efficient manner, causing less mechanical stress on the embryo (Li et al., 2009). A recent study highlighting the importance of nuclear transfer for mitochondrial disease reported the requirement of laser-assisted thinning of the zona pellucida in order to prevent lysis of the oocyte during the enucleation process (Greggains et al., 2014). Moreover, the first preclinical study reported investigating pronuclear transplantation in normally

fertilised zygotes to prevent mitochondrial disease utilised infra-red laser technology to create an opening in the zona pellucida. The laser technology was utilised for creating an opening in the zona pellucida of both zygotes, for the insertion of the enucleation/fusion pipette for aspiration of the pronuclei and insertion through to expel the pronuclei into the perivitelline space to allow fusion with the cytoplasm (Hyslop et al., 2016). These specialised techniques benefit immensely from the assistance of lasers to perform fragile embryo manipulations, and represent only a fraction of the future clinical and research potential of this exciting technology.

It should be noted that while mitochondrial replacement therapy represents a plausible therapeutic for women with mitochondrial disease, the technique raises risks and ethical concerns worldwide. Both methods carry a risk of still transmitting a variable fraction of mutant DNA during the transfer. It also raises a challenge to the definition of biological parentage due to the three separate origins of genetic material. Moreover, there are concerns regarding social impacts, legal issues, and profit-oriented behaviour, which still need to be addressed (Liu and Chu, 2014).

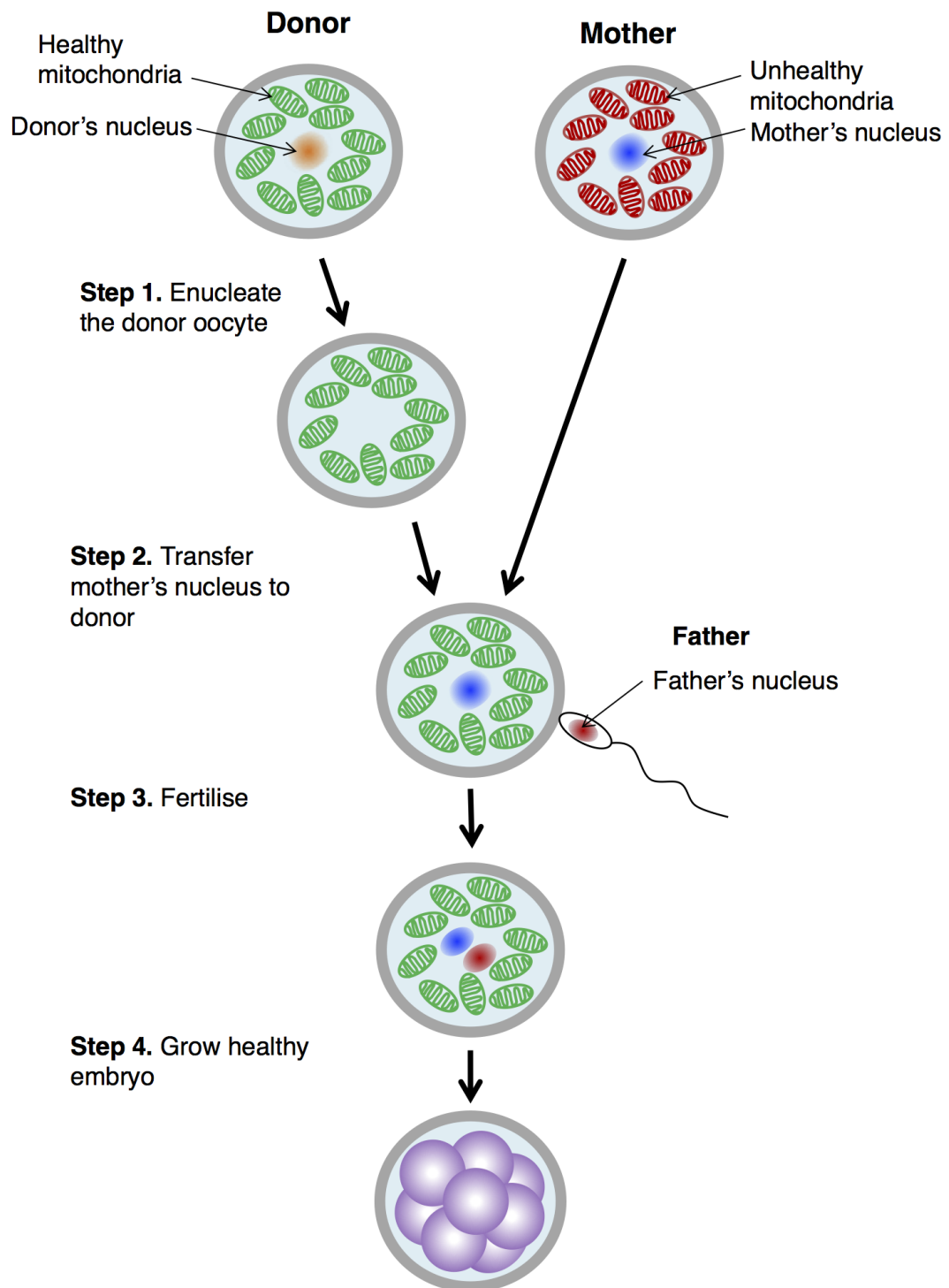


Figure 1.14: Schematic of the mitochondrial replacement therapy procedure using spindle transfer. The nucleus of the mother who has a mitochondrial disease is transferred into an enucleated donor oocyte which contains healthy mitochondria. The oocyte is fertilised and the resulting embryo contains genetic nuclear DNA from the desired parents, and healthy mitochondrial DNA from the enucleated donor oocyte. Image created by author.

1.4 Safety concerns

Infra-red laser technology is increasingly being deployed in the ART sector for both clinical and research purposes. There is, therefore, an important need to understand more thoroughly the safety of such technology upon embryos and research models. There is currently a paucity of critical literature pertaining to the specific molecular effects of laser treatment upon embryos, particularly in relation to developing optimised and standardised methods for clinical and research use (Wong and Mills, 2017). Investigations later in this thesis will explore the safety of laser technology upon ART models, to help elucidate potentially damaging effects upon vulnerable embryos and improve reproductive research and infertility treatments.

1.5 Infra-red lasers as a research tool in the reproductive sciences

Despite the rapidly advancing nature of ART, there are still many unknown factors that are believed to contribute to male and female infertility. The success rates of pregnancy and healthy delivery for IVF and ICSI rarely exceed 30% and 23% respectively (de Mouzon et al., 2010). While the evolution of laser application is translating to increased ART efficiency and clinical success rates, another exciting prospect of this technology lies in the advancements that can be made as part of investigative research in the reproductive sciences. ART lasers represent powerful tools with which to help investigate and elucidate potential mechanisms underlying unknown or unexplained causes of infertility. Fine physiological processes in cells and organisms such as protein pathways, genetic modifications and mutations, or pathological disruptions to normal developmental biology pathways, can be investigated down to the molecular level using this technology.

1.5.1 Embryonic stem cells

Inner cell mass (ICM) cells can be isolated and extracted from blastocyst embryos using laser technology for the purpose of preparing and acquiring embryonic stem

cell (ESC) lines for research purposes (Filliers et al., 2011; Niakan et al., 2012; Xu et al., 2014). While ESC lines have been created by seeding and flattening whole blastocysts, it is often desirable to isolate and seed only inner cell mass cells. Inner cell mass cells are seeded onto cell culture dishes where they lose their three-dimensional shape and are no longer surrounded by trophectoderm cells, thus rendering them accepted cell tissue for research purposes. Historically, ICM separation was performed using immunosurgery, whereby trophoblast cells were killed via complement-dependent antibody cytotoxicity (Solter and Knowles, 1975). Currently, ICM separation is usually performed and is simplified with laser micro-dissection (Chen et al., 2009). Due to their qualities of pluripotency, plasticity, and capacity for unlimited self-renewal, ESCs are highly useful for investigations in regenerative medicine for many disease types, and with genetic manipulation, as models of genetic disorders (Niakan et al., 2012). Moreover, investigations using ESCs can help to further understand the organised interactions and mechanisms involved in cell fate, embryo development, vital tissue interactions and developmental biology to eventually translate to human medical research to help develop new treatments for infertility and reproductive pathologies. The creation of ESCs is of high ethical concern, however, as it requires the destruction of a blastocyst.

1.5.2 Somatic cell nuclear transfer

Laser technology opens up new paths for improvements in the derivation of human embryonic stem cells, which are currently mostly derived from frozen, poor-quality embryos. Somatic cell nuclear transfer (SCNT) is a method used to create viable embryos from an oocyte cell and a somatic cell for therapeutic and research purposes. The use of SCNT helps overcome many ethical issues concerning the use of embryonic stem cells, which otherwise requires the destruction of new viable embryos. The process consists of removing the nucleus from an oocyte and replacing it with the nucleus from a regular somatic cell which already has a full set of DNA from two parents (Campbell et al., 2007). Where legislative considerations permit, these clones can be used to create transgenic organisms and to establish stem cell lines

for research applications such as regenerative medicine and genetic reprogramming (Simerly et al., 2004).

1.5.3 Editing the human genome

Established research techniques involving injections into oocytes and embryos, such as pronuclear, embryonic stem cell, and viral vector injections have been shown to benefit from laser assistance to traverse the zona pellucida with minimal strain and deformation (Tachibana et al., 2013). It is hypothesised that lasers might also benefit the cytoplasmic injection of site-specific nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The most recent addition to site-specific nucleases for genetic modification, which are suspected to further benefit from laser manipulation, are the clustered regularly interspersed short palindromic repeats (CRISPRs), which provide an easy, cheap and efficient alternative to ZFNs and TALENs. CRISPR/Cas9 is a bacteria-derived system that uses RNA molecules to recognise specific DNA sequences to match nucleotides to desired locations in the human genome (Ledford, 2015).

Liang et al., (2015), using the CRISPR/Cas9 system, described for the first time the modification of the human genome to alter a gene that causes a fatal blood disorder. These scientists used embryos that were accidentally fertilised by two sperm, and were thus non-viable regardless of the CRISPR modification, and would have otherwise been discarded. While the CRISPR/Cas9 technique already represents a very powerful tool for research in animal embryos or human adult cells, the Liang et al. study was the first to report the intentional editing of a human embryo (Liang et al., 2015). The research team identified off-target mutations, which underlie the significant safety concerns surrounding germline editing, as these unintended mutations are unpredictable and could be harmful. The main conclusion drawn was that the technique is feasible, but presently too immature for clinical use in human embryos, as only half of the embryos were correctly edited, and very few contained the appropriate machinery to repair the DNA. It is suspected that the lower success of the procedure in human embryos was

due to the fact that the embryos used were created from two sperm and one egg, and inherently not representative of the normal functioning of a healthy embryo. It has further been pointed out that Liang and colleagues did not use the most up to date CRISPR/Cas9 methods and that many of the reported problems could have been mitigated (Cyranoski and Reardon, 2015).

Very recently, a new study showed for the first time that CRISPR/Cas9 could be applied to human embryos to correct a pathogenic gene mutation (Ma et al., 2017). The authors suggest the successful correction of a heterozygous mutation in human preimplantation embryos using CRISPR/Cas9 and activating an endogenous, germline-specific DNA repair response. Unlike the first report by Liang and colleagues in 2015, these authors were able to avoid mosaicism in cleaving embryos and achieve a high yield of homozygous embryos carrying the wild-type gene without evidence of off-target mutations. The authors still caution that much remains to be considered before clinical applications of this technique (Ma et al., 2017). Ma et al. infer that a double stranded break generated by Cas9 in human gametes and zygotes can be efficiently repaired by homology-directed repair of the mutant paternal allele using the wild-type maternal allele. The suggestion of correction of the pathogenic allele reported arose from the inability to detect the mutant paternal allele following the procedure; however, this has raised some controversy. A publication by Egli et al. (2017) emphasise the importance of needing direct evidence for the correction to be provided, rather than an inference from a lack of detectable mutations, due to the fact that mechanisms other than recombination between homologues are possible to have occurred, especially since the cell biology of fertilised eggs are thought to preclude the direct interaction between the maternal and paternal genomes which would have been required for homology-directed repair. For example, one point raised by Egli et al. (2017) is that the physical separation of the maternal and paternal genomes into pronuclei up until metaphase of the first mitosis would prevent the direct interactions of the genomes required for inter-homologue repair (Egli et al., 2017). The authors suggest exploring methods to rule out other types of events, including a more detailed genotyping investigation to investigate long deletions.

Since the mutation was approximately 200bp from the primer-binding site, the genotyping performed by Ma et al. (2017) could have resulted in the deletion of the paternal primer-binding site, leading to the amplification of only the maternal allele, resulting in a misleading appearance of a corrected paternal allele(Egli et al., 2017).

The ability to modify the genome in humans could answer many basic science questions, relevant to both clinical and research applications. However, researchers in the field themselves appear divided on whether or not to support this controversial form of research. Some do not oppose the research reported by Liang et al., as the embryos used were ones that could not have led to a live birth, stating it is no worse than what happens in IVF clinics on a day-to-day basis - that non-viable embryos are discarded (Ledford, 2015). Others have expressed concerns that the fast paced nature of the field allows for little time to address ethical and safety concerns in an appropriate manner for the potential clinical application of such procedures. Some further argue that any form of gene modification research in human embryos could lead to a slippery slope towards unethical, unsafe, or non-medical uses of the technique, and even go as far to call for a moratorium (Lanphier et al., 2015). While there are still many unanswered questions and ethical issues surrounding the development of this technology for potential future clinical use against monogenic diseases, the technique remains promising from a basic research standpoint. Gene editing could be used to reveal specific functions of different genes, or to engineer disease-related mutations in an embryo which can be used to produce embryonic stem cells to act as models for testing drugs and interventions for diseases.

The application of CRISPR/Cas9 technology is also continuing to be explored in other animal models for research purposes. To reduce the rate of embryo lysis, studies are employing laser-assisted cytoplasmic microinjection for the CRISPR/Cas9 procedure. The zygote is immobilised and a laser is used to create a hole in the zona pellucida. A blunt-end glass micropipette is introduced and used to break the plasma membrane via aspiration of the ooplasm. Then, the aspirate ooplasm and gene-editor contents are injected back into the cytoplasm (Gil et al., 2017). Laser assistance is reported to help cytoplasmic microinjection for CRISPR/Cas9

in porcine embryos (Gil et al., 2017), livestock embryos (Bogliotti et al., 2016), and bovine embryos (Bogliotti et al., 2014).

1.6 Potential novel laser-mediated research tools

As the rates of idiopathic infertility remain worrying at levels of 15-30%, there is a growing need to develop novel research methods to investigate and selectively manipulate the molecular pathways associated with fertilisation, gametogenesis, and early embryogenesis (Ji et al., 2012). One promising avenue for laser technology as a research tool lies in its pairing with nanotechnology. Nanoparticles represent an efficient delivery platform for molecular research in the reproductive sciences, which can target localised physiological pathways directly in a non-invasive manner to mark, augment, or suppress endogenous functional activity (Petros and DeSimone, 2010; Kunzmann et al., 2011). Part of this thesis intends to explore the combined application of infra-red laser technology with nanotechnology to create a novel research tool for the reproductive sciences.

1.6.1 Nanotechnology for research in the reproductive sciences

Nanotechnology has revolutionised medical treatments over the last decade, however, developments in this area remain challenging due to the complexity of the human system. Nanotechnology has been applied to certain areas of biomedical research, notably oncology; however, only scant attention has been paid to the potential for using nanomedicine in the reproductive sciences. It is entirely conceivable that nanotechnology could be used to study early embryogenesis and development, as well as endogenous deficiencies associated with infertility (Davidson et al., 2015b; Davidson et al., 2016). Furthermore, the growing need for fundamental research in the fields of reproductive biology and ART for the management of reproductive pathologies cannot be achieved without developing novel research methods to investigate and selectively manipulate the sensitive physiological mechanisms underlying gametogenesis, fertilisation, and early embryo development.

There is an acute need, therefore, to develop new and more efficient methods for exploring and manipulating molecular pathways associated with fertilisation, gametogenesis, and early embryogenesis.

Fertility medicine is increasingly focusing on developing non-invasive methods of detection or treatment of disease, while minimising the need for surgical intervention. Nanotechnology is already aiding in the biodetection, diagnostic imaging and nanomediated delivery of chemotherapeutics for reproductive cancers to improve general medical treatment efficiency. Additionally, a growing body of research is investigating the benefits of nanotechnology for the diagnosis and treatment of non-cancerous conditions, such as using nanoparticles as novel contrast agents to extend magnetic resonance imaging (MRI) detection of endometriosis (Lee et al., 2012; Zhao et al., 2012), as delivery vessels for anti-tumour cytokines for uterine fibroids (Ali et al., 2013), and as tags to label preimplantation embryos during fertility treatment (Fynnewever et al., 2007).

The small size of nanoparticles, similar to that of biomolecules, allows for integration into physiological cellular processes, such as innate internalisation via endocytosis or selective targeting of endogenous functional pathways (Petros and DeSimone, 2010; Kunzmann et al., 2011). Nanoparticles are intrinsically stable thus providing capability for systemic administration, long distance action, and targeted delivery of large cargo volumes. Notably, they can be tailored to facilitate both cargo and target specificity, by manipulating numerous physical and chemical properties such as size, surface charge and polarity, and functional group or ligand attachments (Tarn et al., 2013). Nanocarriers thus present a promising means to manipulate and investigate specific molecular pathways or deliver personalised medical treatments in the future (Figure 1.15).

In particular, mesoporous silica nanoparticles (MSNPs) are universally recognised as a powerful biomedical nanomaterial distinguished for their low cytotoxicity across a variety of cell types (Tarn et al., 2013). MSNPs are synthetically modified colloidal silica with highly ordered meso-scale sized pores (2-50nm)(Figure 1.15)(Rosenholm et al., 2012), and exhibit many favourable characteristics for use as a targeted

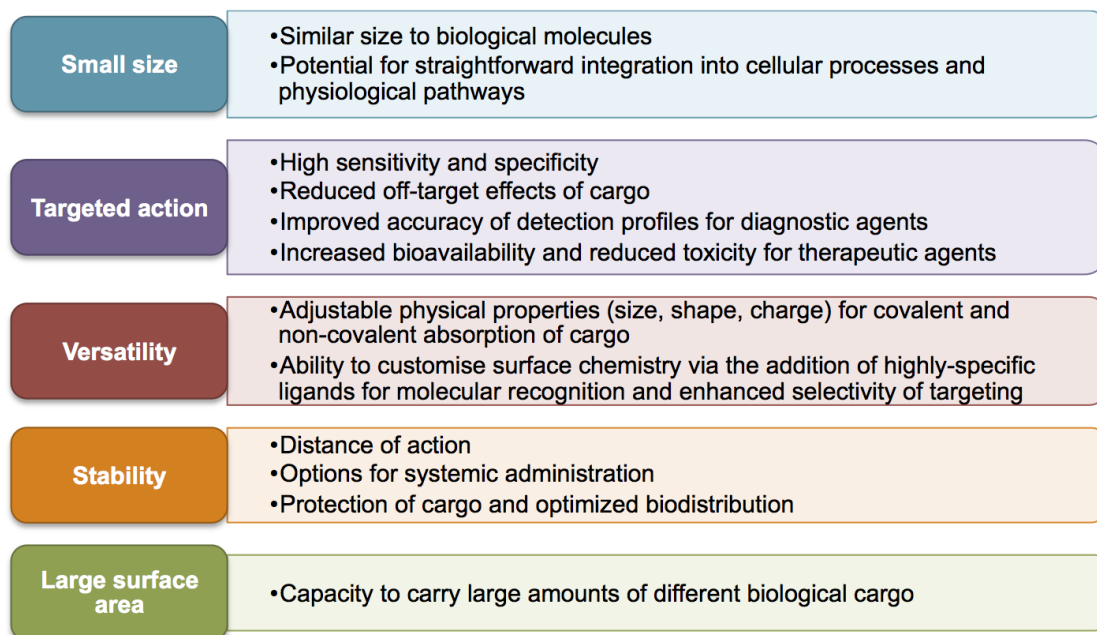


Figure 1.15: Key features of nanomaterials favouring their use in biomedicine. Image created by author and adapted from Barkalina et al. (2014).

delivery vector for reproductive biology. MSNPs are robust with a high surface-area to volume ratio, possess an increased likelihood to persist in target cell populations following internalisation, exhibit high loading capacity, and therefore require reduced doses of nanovector. Moreover, MSNPs, and their degradation products, are chemically inert and not prone to induce free radical formation. They are also versatile and easily customisable, allowing for specific loading and targeted delivery (Rosenholm et al., 2012).

Mesoporous silica has been extensively studied in somatic cell types, and data is now emerging concerning its biocompatibility and potential with gametes and embryos. The use of non-toxic MSNPs for delivery into gametes is highly promising, compared to other types of nanoparticle, for example gold or silver, which are widely accepted for use in somatic cells but exhibit toxicity when applied to gametes (Zakhidov et al., 2010; Zakhidov et al., 2012). Barkalina et al., reported that the exposure of mammalian sperm to MSNPs under conditions similar to those used during routine sperm processing for ART resulted in the binding of MSNPs to sperm without any deleterious effects upon motility, viability, acrosome

morphology, or levels of DNA fragmentation (Barkalina et al., 2014a). MSNPs have also been investigated as a delivery system in zebrafish embryos, a common and powerful model organism for reproductive biology investigations, with eminently positive reports of safety and efficacy (Nadrah et al., 2013). The development of an efficient active targeting and internalisation strategy for the transport of biological compounds into gametes and embryos could represent a milestone in enhancing the efficacy of current research methodologies associated with gene transfer. Such a delivery system with temporal and spatial control would be highly useful for more specific investigations into embryo development. For example, refined temporal control would allow for improved precision in investigations into time-dependent events, such as embryonic genome activation and blastulation. Moreover, improved spatial control of cargo delivery would allow for localisation investigations, also in a temporal manner. Mesoporous silica, with its inherent high loading capacity, low toxicity, and tuneable affinity, appears to represent a very promising candidate for these advancements in reproductive biology.

1.6.1.1 Mesoporous silica nanoparticle applications with sperm

Targeted transgenesis is a crucial component of research in the reproductive sciences. Unfortunately, the laboratory methods developed thus far remain inefficient and costly in terms of equipment, time, and labour. Safe and efficient gene transfer could be used to precisely manipulate gene expression in gametes and embryos in order to investigate the role of specific proteins and molecular pathways during gametogenesis, fertilisation and early embryogenesis. In theory, sperm could be used to deliver various forms of biological cargo into oocytes, and could be enhanced by coupling cargo with nanocarriers. Sperm-mediated nanoparticle delivery could transport cargo such as proteins, peptides, antibodies, fluorescent markers, or any agents designed to enhance, suppress or detect endogenous biological activity (Barkalina et al., 2014c; Davidson et al., 2015b). The development of such a powerful system could provide invaluable insight into the molecular pathways associated with fertilisation and early embryo development. Several studies have emerged indicating the use of

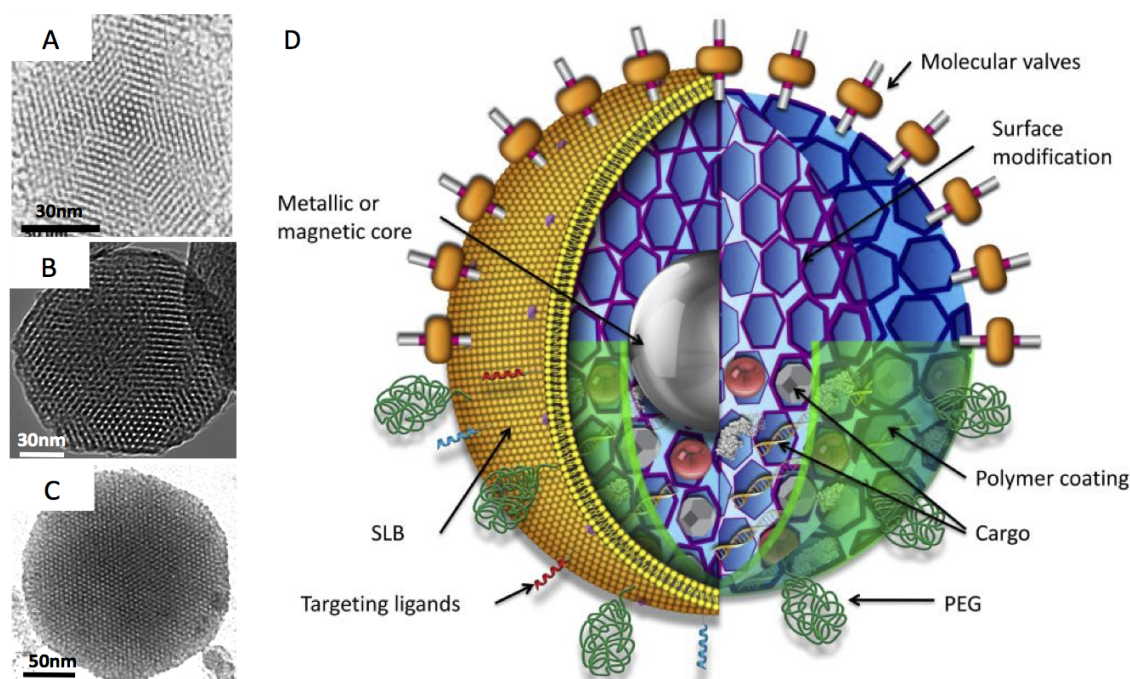


Figure 1.16: (A-C) Transmission electron micrographs of mesoporous silica nanoparticles (MSNP) of varying diameters. A. 70nm. B. 120nm. C. 200nm. (D) Multi-functional mesoporous silica nanoparticle displaying different core and shell designs, surface modifications, and multiple types of cargo. SLB = supported lipid bilayer; PEG = polyethylene glycol. Both particle and pore diameters of MSNP can be customised to suit various uses. Adapted from Gao et al. (2012) and Tarn et al. (2013).

nanoparticles to enhance sperm-mediated cargo delivery. Makhluf et al demonstrated the successful delivery of an antibody raised against protein kinase C into bovine sperm using polyvinylalcohol-coated magnetic iron oxide nanoparticles, without the antibody losing any functional activity (Makhluf et al., 2008). Barkalina et al. demonstrated that mesoporous silica nanoparticles (MSNPs), functionalised with polyethileneimine (PEI) and aminopropyltriethoxysilane (APTES), and optionally loaded with common types of nucleic acid or protein cargo, form strong associations with boar sperm following incubation *in vitro* (Barkalina et al., 2014a). Barkalina and colleagues further demonstrated that these associations do not exert any negative effects upon the main parameters of sperm function such as motility, viability, acrosomal status, and DNA fragmentation index (Barkalina et al., 2014a).

Since MSNPs have shown promise as a nanocarrier in the reproductive sciences, part of this thesis intended to investigate the pairing of this nanotechnology with

infra-red laser technology to develop a novel research tool with more refined spatial and temporal control for applications in the reproductive sciences.

1.7 The mouse as a preimplantation embryo model

This thesis set out to use the mouse embryo as a model to gain an understanding and foundation of the effects of laser treatment on embryos. Due to the scarcity, ethical and logistical challenges of undertaking research with human embryos, the mouse embryo model has played a significant role in the development of IVF and ART (Summers and Biggers, 2003; Woods et al., 2004; Edwards, 2005; Taft, 2008). The mouse embryo is an ideal model for ART due to its small size, prolificacy, ease of handling, high fecundity, ease of breeding, and short generation interval time. Moreover they present highly useful genetic power in that 99% of mouse genes possess human homologs, gene order is conserved between mice and humans, and mutations in mouse genes often show similar consequences in human homologs (Taft, 2008). Both mouse embryos and human embryos develop from a one-cell zygote into a blastocyst, undergo hatching, and implantation. Nonetheless, limitations of animals models should still be kept in mind when performing research with the mouse embryo model; it should be noted that the timings of these events vary between mice and humans, and human embryos undergo an additional round of cell division before implantation compared to mice (Niakan et al., 2012). Zygotic gene activation is initiated at the 4-8 cell stage in humans, however this process is initiated earlier in mouse embryos, at the 1-2 cell stage, meaning there is more transcriptional silence in the human preimplantation embryo before embryonic gene activation (Wang et al., 2004; Vassena et al., 2011). Additionally there can be differences in gene expression patterns, frequency of chromosome mis-segregation, and epigenetic modification patterns, and implantation patterns (Niakan et al., 2012; Davidson and Coward, 2016). Nevertheless, the mouse remains one of the most powerful models for mammalian preimplantation embryo development, which can provide a strong foundation to enhance our understanding of biological processes.

1.8 Aims of this thesis

The primary aim of this thesis was to optimise applications of laser technology for ART and research purposes. To achieve this aim the following study objectives were pursued:

- Refine safe and unsafe operator variables when applying laser pulses to mouse embryos, evidenced by potential damage incurred at the molecular level, with an aim to promote standardisation of laser applications for zona pellucida drilling.
- Evaluate the effects of altering the laser hardware to lower the power output for application upon mouse embryos, to determine whether clinical laser manufacturing can be further improved.
- Explore global changes in gene expression in mouse embryos following laser treatment mimicking clinical biopsy conditions, to potentially uncover any novel genes or pathways to be further explored.
- Develop a method in which laser technology can be used as a novel research tool for the reproductive sciences, by creating an efficient stimuli-responsive laser-activated nanoparticle delivery system in a mammalian cell model.

2

General Materials and Methods

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2.1 Infra-red laser and embryo micromanipulator system

The work carried out in this thesis investigated different applications and safety profiles of infra-red laser technology for assisted reproductive technology and research in the reproductive sciences. The laser apparatus used in these investigations was a 1.48 μ m diode non-contact infrared laser (Saturn 5 Active Laser System; Research Instruments Ltd., UK) which was attached to a standard embryo micromanipulator (Integra Micromanipulation System; Research Instruments Ltd., UK) fitted onto an inverted microscope (Nikon, UK)(Figure 1.5). The embryo micromanipulator featured a heated stage to maintain a constant temperature for the samples while they were outside of the incubator. RiViewer software (Research Instruments Ltd, UK) was used for all laser applications, including regularly calibrating the laser alignment, measuring distances in the field of view, and determining accurate laser pulse position. Different laser treatments and parameters were achieved by altering the pulse length parameter, resulting in different hole sizes produced in the sample tissue. For the experiments described in Chapters 3, 5, and 6, the commercial clinically-approved standard Saturn 5 Active Laser system, which operates at a constant power of 400mW, was utilised. In Chapter 4, which explored differing laser powers, the laser system was purposefully altered by engineers at Research Instruments Ltd to be able to also operate at a constant power of 100mW.

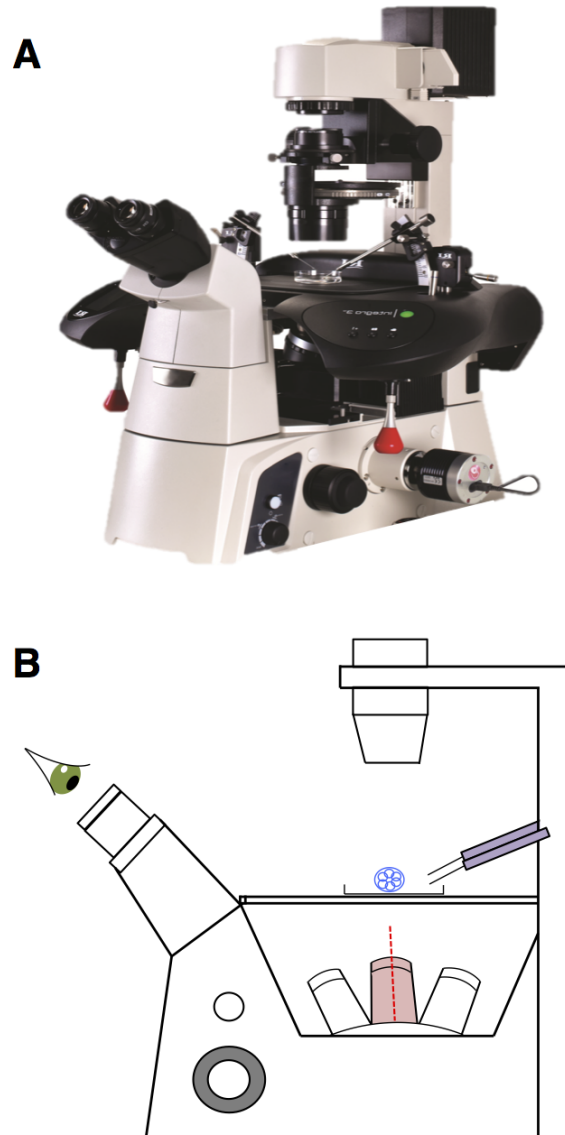


Figure 2.1: (A) Microscope and manipulator set up for *in vitro* fertilisation and laser treatment. (Image courtesy of Research Instruments Ltd.). (B) Schematic diagram of microscope and manipulator set-up. The eggs, sperm or embryos (blue) are placed in a petri dish on top of an inverted microscope. Glass micro-tools (purple) are then used to manipulate and orient the specimen in the dish. A specialised objective (red) allows for an infra-red laser pulse to be delivered to the specimen from below. Image created by author.

2.2 Mouse embryo manipulations

2.2.1 Mouse embryo acquisition

All animal procedures were carried out by trained technicians under an appropriate licence in accordance with the Animals (Scientific Procedures) Act 1986 at the Biomedical Services Unit at the John Radcliffe Hospital (Oxford, UK). Three to 4-week-old female C57BL/6 mice were super-ovulated by an intraperitoneal injection of 0.1mL pregnant mare serum gonadotrophin (PMSG, Sigma-Aldrich, UK), followed by 0.1ml human chorionic gonadotrophin (hCG, Sigma-Aldrich, UK) 48h later. Super-ovulated female mice were mated with C57BL/6 males following hCG. Mice were subsequently culled via cervical dislocation at approximately 20h post-hCG (to obtain 2-cell embryos), or at 72h post-hCG (to obtain cleavage stage embryos), and reproductive tracts were harvested and stored in M2 medium (Sigma-Aldrich, UK) for collection and transfer to the Institute of Reproductive Sciences (Oxford, UK), where all subsequent experiments were carried out. C57BL/6 mice were selected as this is the most widely used mouse strain for *in vitro* fertilisation and embryo research (Takahashi and Liu, 2010).

2.2.2 Mouse embryo manipulation and culture

At the Institute of Reproductive Sciences, oviducts were placed under pre-warmed M2 media under a stereomicroscope and embryos were harvested using forceps. M2 media, a modified Krebs-Ringer bicarbonate solution containing HEPES buffer, was used as recommended for collecting and handling embryos for prolonged periods outside a CO₂ incubator (Piliszek et al., 2011). Embryos were assessed during the harvest and only those which were at the desired 2-cell stage or cleavage stage with evenly-sized blastomeres were retained for further investigation, and transferred to fresh M2 media droplets for laser manipulations. Following experimental treatments, in groups of nine, embryos were transferred into 15 μ l drops of KSOM + 1/2 amino acids (EmbryoMax, Merck Millipore, UK) under mineral oil. Embryos were cultured in groups as evidence supports the notion that group culture may

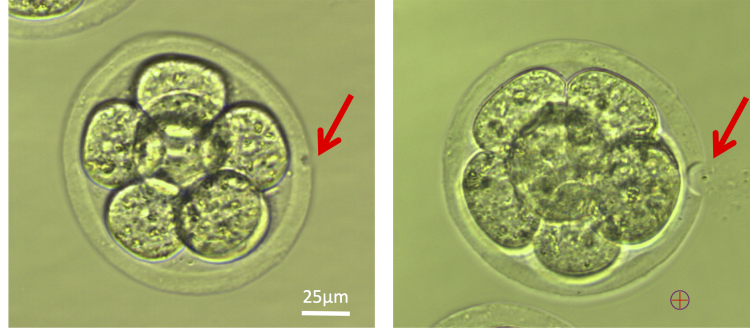


Figure 2.2: Brightfield images of different hole sizes created by a single infra-red laser pulse. The red arrow indicates the hole created.

produce better quality embryos due to the secretion of embryotrophic factors (Reed et al., 2011). Embryos were either used immediately for experiments, or cultured to the blastocyst stage in a Planer Benchtop Incubator BT37 (Origio, UK) with 5%CO₂, 5%O₂, and 90%N₂ gas.

2.2.3 Laser treatment of mouse embryos

Each embryo was individually laser-treated for the investigations described herein. Using the Integra Micromanipulation system, each embryo was held in position using a holding pipette (32μm diameter, 15μm inner diameter, 35° bend angle; Research Instruments Ltd., UK) and a pilot laser was used to position the laser beam at the chosen position on the zona pellucida. The laser was then fired for the specified pulse duration to create the desired ablation hole size (Figure 2.2). The embryo was then released from the holding pipette and either subjected to experimental investigation immediately, or cultured for 72h to the blastocyst stage. Embryos which were not used for investigations immediately following laser treatment were cultured for 72h to the blastocyst stage and either 1) used immediately for investigations, 2) frozen at -80° in 3μl RNAlater (Invitrogen, UK) for future qPCR investigations, or 3) treated for RNA extraction before freezing at -80°C for future RNA sequencing investigations.

2.2.4 Cell viability staining

To assess the viability of embryo blastomeres, two fluorescent markers of cell viability were applied immediately following laser treatment, as previously described by Oktay et al. (1997) and Chatzimeletiou et al. (2001). These markers included: (i) carboxyfluorescein-diacetate succinimidylester (CFSE; Molecular Probes Inc., Cambridge Bioscience, Cambridge, UK) which binds covalently in the cytoplasm of viable cells indicating healthy hydrolase metabolic activity, and (ii) propidium iodide (PI)(Life Technologies Invitrogen, UK), which is excluded from viable cells but enters cells with damaged plasma membranes, binding to double-stranded DNA in the nucleus. Laser-treated embryos were first incubated in CFSE at a concentration of $20\mu\text{g/ml}$ in PBS for 30min at 37°C and then transferred into a well containing $100\mu\text{g/ml}$ PI in PBS for 8min at 37°C , in the dark. Each embryo was then washed three times in PBS, transferred to a microscope slide in $10\mu\text{l}$ PBS, and a coverslip was carefully positioned on top of rubber solution (Elmer's, USA) which was placed in a circle around the drop, as described by Chatzimeletiou et al. (2001). The slides were then examined by fluorescence microscopy as described in Section 2.5.

2.2.5 Embryo grading

Using the inverted microscope, embryos were assessed at 40X magnification and graded according to the Gardner grading system as outlined in Figure 2.3 (Gardner and Schoolcraft, 1999). Embryos which reached a grade of 3-5AA or 3-5AB were considered 'good quality', and blastocyst survival rate was determined as the percentage of overall cultured embryos which reached 'good quality' grading. Only 'good quality' embryos were used for subsequent investigations involving blastocysts.

2.2.6 Determination of apoptotic index

To assess the apoptotic index of the blastocysts cultured following laser treatment, grade 3-5AA and 3-5AB embryos were evaluated using the TUNEL assay (*In-situ* Cell Death Detection System; Roche Diagnostic) as described by Castillo et al.

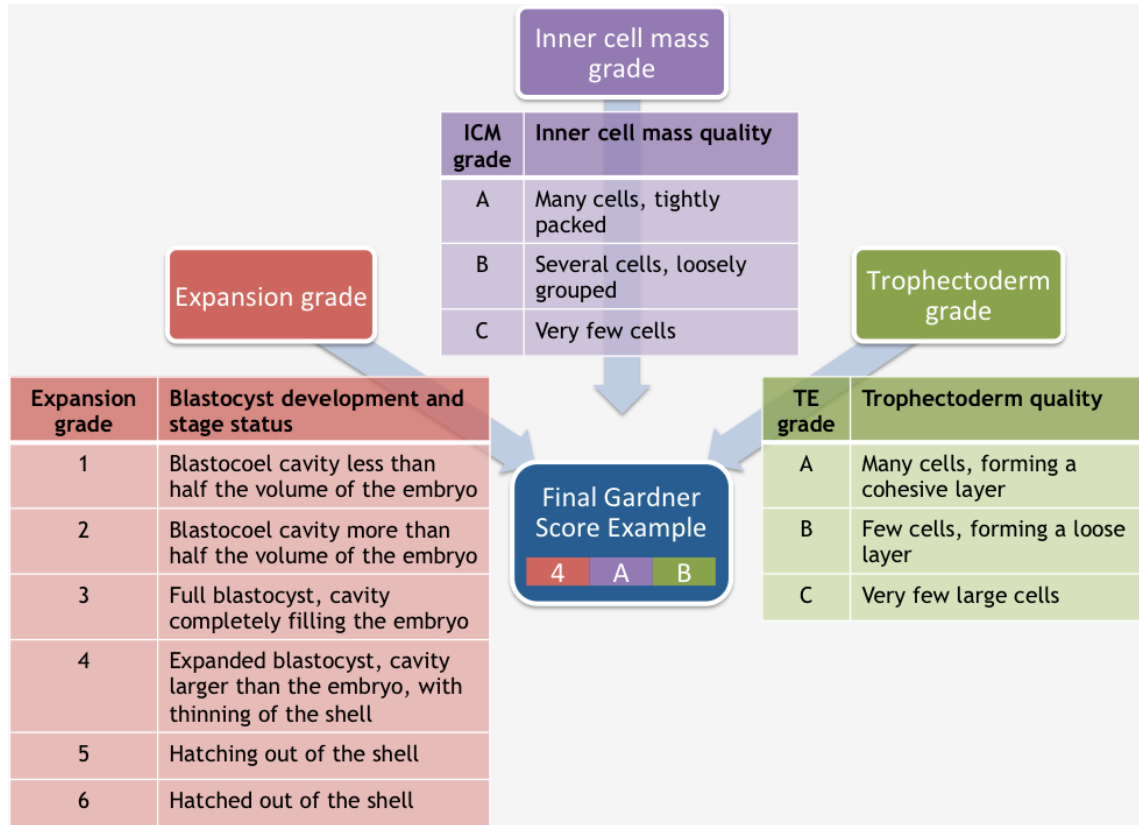


Figure 2.3: Gardner grading of blastocysts. Image created by author and modified from Gardner and Schoolcraft (1999).

(2014). Blastocysts were washed in PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich, UK) at 37°C for 40min. After three washes in PBS, blastocysts were permeabilised with 0.5% Triton X-100 in PBS for 10 min. Embryos were washed three times in PBS and incubated in TUNEL reaction cocktail at 37°C for 1h in the dark. Embryos treated with 50U/mL DNase 1 in PBS at 37°C for 20 min in the dark were used as a positive control. Samples were washed three times in PBS and mounted on slides with 10µl Vectashield Mounting medium containing 4',6'-diamidino-2-phenylindole (Vectashield H-1200; DAPI concentration:1.5µg/ml; Vector Laboratories, UK) and covered with a coverslip. Coverslips were maintained in position by placing Elmer's rubber solution around the edges of the coverslip. Slides were examined by fluorescence microscopy as described in Section 2.5, and the apoptotic index was calculated as:

$$\text{apoptotic index} = \frac{\text{TUNEL stained nuclei}}{\text{DAPI stained nuclei}} \times 100\%$$

2.2.7 cDNA production by reverse transcription

All cDNA and RNA work was carried out in a dedicated hood (VWR PCR workstation, UK) under decontaminated conditions with all equipment being UV light-treated and wiped with DNAaway (ThermoScientific, UK). To analyse gene expression from blastocysts, cDNA was generated by reverse transcription from 27 blastocysts per laser treatment group (pools of 9 embryos) using the Cells to cDNA II Kit (Ambion, Life Technologies, UK), as described in (Poli et al., 2015). The protocol was employed as described by the manufacturer, apart from the cell lysis step, where a reduced volume of lysis buffer (50 μ l) was used to increase final RNA concentration, as recommended by (Poli et al., 2015).

Briefly, cells from a pool of 9 blastocysts were lysed in 50 μ l lysis buffer for 10 min at 75°C then incubated with 0.04U/ μ l DNase for 15 min at 37°C to degrade genomic DNA in the sample. DNase was subsequently inactivated by heating the samples to 75°C for 5 min. Reverse transcription was performed by mixing 10 μ l cell lysate with 4 μ l dNTP mix, and 2 μ l random sequence oligonucleotide primers provided with the commercial kit, before heating for 3 min at 70°C. Next, 2 μ l of 10 $\times$ reverse transcription buffer, 1 μ l M-MLV reverse transcriptase, and 1 μ l RNase inhibitor were added to the samples. The samples were incubated at 42°C for 60 min to allow reverse transcription to occur, then heated to 95°C for 10 min to inactivate the reverse transcriptase. No template controls and no enzyme controls were performed in triplicate. Amounts of cDNA were quantified using the Qubit Assay (Invitrogen, UK) according to the manufacturer's instruction. The reaction tubes containing cDNA were stored at -20°C until all samples were collected.

2.2.8 Quantitative real-time polymerase chain reaction

Quantitative assessment of changes in gene expression following laser treatment in mouse embryos was performed using real-time polymerase chain reaction (qPCR) (Table 2.1). Primers for β -actin, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), homeobox transcription factor Nanog (*Nanog*), BCL-2 associated X protein (*Bax*), BCL2-like 1 (*Bcl2l2*), DNA methyltransferase 3A (*Dnmt3a*), superoxide dismutase

1 (*Sod1*), and glutathione peroxidase 1 (*Gpx1*) genes were custom designed using PrimerBLAST, Oligocalc, and Primer3 softwares (Table 2.2). All primers were designed to span exon-exon boundaries and were verified using GenBank BLAST Primer database for sequence specificity, primer dimer, and loop formation. T_m for both inner and outer primers were empirically validated by gradient PCR.

Singleplex qPCR for amplification of each target gene was set up in triplicate in 96-well plates (MicroAmp Fast Optical, Life Technologies, UK) by mixing 5 μ l Power SYBR Green Master Mix (Life Technologies, UK) with 0.5 μ l forward and reverse primers (100nM each), 50-100ng template cDNA, and nuclease-free water to a final volume of 10 μ l. No template controls and no enzyme controls were included for the qPCR reaction and from the reverse-transcription step. Amplification was carried out using a StepOnePlus Real-Time PCR system (Life Technologies, UK) as outlined in Table 2.3. To quantify the amount of cDNA in each sample group, the comparative cycle threshold (Ct) method was applied using the equation $2^{-\Delta\Delta Ct}$. The $\Delta\Delta Ct$ was calculated using the following equation: $\Delta\Delta Ct = (\text{mean Ct (LaserTreatment)} - \text{mean Ct (HouseKeepingTreatment)}) - (\text{mean Ct (LaserControl)} - \text{mean Ct (HouseKeepingControl)})$. Melting curve analysis was performed to ensure specificity of the generated amplicons. The housekeeping values were calculated by averaging the two means of the GAPDH and β -actin values in triplicate.

Table 2.1: Basic information regarding the genes selected for quantitative RT-PCR.

Gene	Role	Reference
Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>)	Housekeeping gene	(Jeong et al., 2014)
Beta actin (<i>Actb</i>)	Housekeeping gene	(Jeong et al., 2014)
Nanog homeobox (<i>Nanog</i>)	ICM stem cell gene, widely recognised as important for preimplantation development	(Castillo-Martin et al., 2015b)
BCL2-associated X protein (<i>Bax</i>)	Pro-apoptotic gene participating in the extrinsic apoptosis pathway	(Castillo-Martin et al., 2015b)
BCL2-like 2 (<i>Bcl2l2</i>)	Anti-apoptotic gene participating in the extrinsic apoptosis pathway	(Castillo-Martin et al., 2015a; Castillo-Martin et al., 2015b)
DNA methyltransferase 3 (<i>Dnmt3</i>)	Gene involved in increased levels of <i>de novo</i> methylation.	(Li et al., 1992)
Superoxide dismutase 1 (<i>Sod1</i>)	Gene indicative of reactive oxygen species (ROS) detoxification; over-expression of <i>Sod1</i> in mice has been shown to protect embryos against oxidative damage and from malformation in a diabetic environment	(Takahashi, 2012; Wang et al., 2013a; Castillo-Martin et al., 2014; Castillo-Martin et al., 2015b)
Glutathione peroxidase (<i>Gpx1</i>)	Gene indicative of ROS detoxification; higher expression of <i>Gpx1</i> is associated with higher blastocyst survival following vitrification thawing	(Castillo-Martin et al., 2014)

Table 2.2: Primers used for quantitative RT-PCR.

Gene		Primer sequences (5'-3')	Amplicon size (bp)	RefSeq Entry	Uniprot Entry
<i>β-actin</i>	Reverse	TCGTAGATGGGCACAGTGT	155	NM007393	P60710
	Forward	CGTGAAAAGATGACCCAGATCAT			
<i>Gapdh</i>	Reverse	ATCGAAGGTGGAAGAGTGGG	140	NM008084	P16858
	Forward	AGAAACCTGCCAAGTATGATGAC			
<i>Nanog</i>	Reverse	TGCTGAGCCCTTCTGAATCA	167	NM028016.3	Q80Z64
	Forward	AGCAGAAGTACCTCAGCCTC			
<i>Bax</i>	Reverse	GCAATCATCCTCTGCAGCTC	165	NM007527	Q07813
	Forward	GCTACAGGGTTTCATCCAGG			
<i>Bcl2l2</i>	Reverse	CTGTGAACTCCGCCCAGC	151	NM007537	P70345
	Forward	TTCTTTGTCTTTGGGGCTGC			
<i>Dnmt3a</i>	Reverse	GCTGATGTAGTAGGGGTCCC	157	NM007872	O88508
	Forward	AGGAGAGGGCAAAGAACAGA			
<i>Sod1</i>	Reverse	TCTTGTTTCTCATGGACCACC	140	NM011434	P08228
	Forward	GAGAGGCATGTTGGAGACCT			
<i>Gpx1</i>	Reverse	TGTTGGTGTCTGGTTCCTGT	160	NM024198	Q3TNK3
	Forward	GGCTCGGTTTCCCTGGTG			

Table 2.3: Cycling conditions for the qPCR amplification of mouse embryo cDNA.

	Cycles	Time	Temperature
Initial denaturation	1	2 min	94°C
Denaturation	40	30 s	94°C
Annealing		30 s	54°C
Elongation		30 s	72°C
Full elongation	1	5 min	72°C

2.3 Global gene analysis via RNA sequencing

2.3.1 RNA extraction and isolation

Total RNA was isolated from batches of 12 pooled embryos using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, UK) according to the manufacturer's instructions. Samples were frozen at -80°C until all samples were collected. From each sample, 1 μ l was analysed to determine RNA concentration and purity using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). The remaining sample volumes were stored at -80°C until they could be sent off to the Wellcome Trust Centre for Human Genetics for RNA sequencing.

2.3.2 RNA sequencing

For the RNA sequencing project we liaised with the High-throughput Genomics centre at Wellcome Trust Centre for Human Genetics. Dr Sarah Lamble operated as the Project Manager for our project and performed the quality control assessment on our isolated RNA samples.

2.4 Mesoporous silica nanoparticle (MSNP) delivery system

The Coward group have a long history of using mesoporous silica nanoparticles as research tools in the reproductive sciences. The application of infra-red lasers as a novel research tool for the reproductive sciences was investigated by developing a novel system combining the use of infra-red lasers with mesoporous silica nanoparticles (MSNPs). A system was developed where MSNPs were used to deliver a specialised heat-shock promoter-driven plasmid cargo into mammalian cells, and the plasmid gene expression would be induced by infra-red laser treatment.

2.4.1 HEK293T mammalian cell model

For these nanoparticle investigations, human embryonic kidney cells (HEK293T) were used as a well characterised mammalian cell model. HEK293T cells were obtained from the American Type Culture Collection (ATCC CRL-3216) and maintained at 37°C with 5%CO₂ in a humidified chamber in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum (FBS), 1%v/v 5000U Penicillin, 5mg/ml streptomycin, 2mM L-glutamine and sodium pyruvate. For all experiments, cells were seeded at 30% confluency on detachable 8-well tissue culture chambers on a polyolefin slide (Sarstedt, Germany).

2.4.2 Production of MSNPs

Spherical MSNPs with hexagonal pore symmetry (approximately 130nm) (Figure 2.4) were synthesised by a surfactant-templated base-catalysed sol-gel reaction in-house by Dr. Natalia Barkalina (Nuffield Department of Obstetrics and Gynaecology,

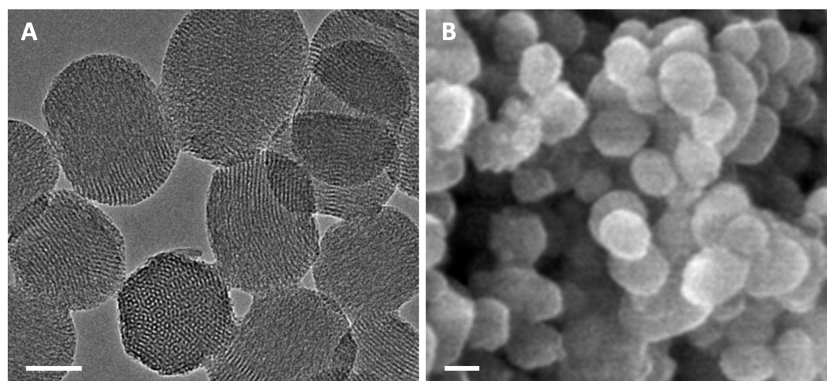


Figure 2.4: Mesoporous silica nanoparticles (MSNPs). A) Transmission electron microscopy image of unmodified MSNPs. Scalebar = $0.05\mu\text{m}$. B) Scanning electron microscopy image of unmodified MSNPs. Scalebar = $0.1\mu\text{m}$. Image reproduced from Barkalina et al. (2014).

University of Oxford), as previously described by Hom et al (2010). All synthesised MSNPs used in these experiments were functionalised with polyethileneimine (PEI, MW 1.3kD, Sigma-Aldrich, UK) to impart a positive charge to the surface. For confocal microscopy, MSNPs were functionalised with both PEI and fluorescein isothiocyanate (FITC), in order to allow fluorescent tracking. Please refer to Appendix A Supplemental Methods for a detailed description of MSNP production and functionalisation with PEI and FITC.

2.4.3 Internalisation of MSNPs in HEK293T cells

In order to investigate the internalisation of MSNPs into HEK cells, fluorescent FITC and PEI-coated MSNPs (FITC-MSNPs) were incubated with HEK293T cells for 24h and visualised using confocal microscopy. The variance in signal intensity of cells incubated with different FITC-MSNPs concentrations ($4.0\text{-}12.0\mu\text{gMSNPs}/10^6\text{cells}$) was assessed using fluorescence microscopy and ImageJ 1.49v software (National Institute of Health, USA).

2.4.4 Cytotoxicity of MSNPs in HEK293T cells

The cytotoxicity of MSNP internalisation upon HEK293T cells was determined by incubating cells at 37°C with increasing concentrations ($4.0\text{-}12.0\mu\text{gMSNPs}/10^6\text{cells}$), for 24-72h. The MSNP concentration range was derived from previous cytotoxicity

Table 2.4: PCR protocol for the amplification of target HSP70-1 gene fragment

	Cycles	Time	Temperature
Initial denaturation	1	2 min	94°C
Denaturation	10	10 s	94°C
Annealing		70 s	65°C
Elongation		1 min	72°C
Denaturation	20	15 s	94°C
Annealing		30 s	65°C
Elongation		1 min	72°C
Full elongation	1	7 min	72°C

studies in our group and publications using similar concentrations in human mammalian cells which have shown insignificant toxicity (Adiga et al., 2007a). Cells were stained with PI, then fixed and mounted with DAPI for fluorescence imaging, as described earlier, to assess cytotoxicity. Data was derived from three independent replications of 500 cells at each concentration of MSNPs for each time point.

2.4.5 Creation of a heat-shock inducible plasmid as MSNP cargo

A thermo-controlled nanoparticle cargo plasmid (pDRIVE-HSP70-GFP) was created by fusing a heat-shock promoter (HSP70) to the green fluorescent protein (GFP) reporter gene. The 471bp human HSP70-1 promoter was amplified from the pDRIVE01-hHSP70 plasmid (InVivoGen, UK) via polymerase chain reaction (PCR) using the High Fidelity PCR Master Kit (Roche, Germany) and primers designed in-house (forward: 5'- TAT ACC GGT ATG GTG AGC AAG GG CGA G -3' ; reverse: 5'-TAT GGT ACC CTT GTA CAG CTC GTC CAT -3'). Amplification was carried out in a G-storm Thermal Cycler (Model GS4, GSI, UK) using the protocol described in Table 2.4. PCR products were analysed for size by electrophoresis on a 1.3% TAE agarose gel pre-stained with ethidium bromide (final concentration: 0.5µg/ml). The gel was run in TAE at 100V for 1 hour, and imaged by a UVP GelDoc-It TS Gel Imaging System.

The amplified HSP70-1 promoter was cloned into a linear pcDNA3.1/CT-GFP-TOPO plasmid using a GFP Fusion TOPO TA Expression Kit (Life Technologies, UK) according to the manufacturer's protocol (Figure 2.5), transformed into competent cells, and grown on ampicillin-LB-agar plates overnight. Three colonies were selected from the plate to allow propagation and purification of plasmid DNA. Extraction and purification of plasmid DNA was performed using the QIAprep Spin Miniprep Kit (Qiagen, UK) and DNA yield was increased using the Qiagen Plasmid Maxi Kit (Qiagen, UK). Restriction analysis confirmed appropriate insertion of the target DNA into the plasmid. Each resulting plasmid was digested with either *Kpn1* only, *NotI* only, or both restriction enzymes. Cloned plasmids were also sequenced by Source BioScience (Oxford, UK), and the resultant sequences compared to the HSP70 sequence (pDRIVE01-hHSP70 sequence; InVivogen, UK) and GFP sequence (pcDNA3.1/CT-GFP-TOPO sequence; Life Technologies, UK) using ClustalW Multiple Sequence Alignment (BioEdit Sequence Alignment Editor Software version 7.2.5).

2.4.5.1 Verification of pDRIVE-HSP70-GFP plasmid expression

The cloned pDRIVE-HSP70-GFP plasmid cargo expression was verified by transfecting 1 μ g pDRIVE-HSP70-GFP plasmid, or 1 μ l linear pcDNA3.1/CT-GFP-TOPO plasmid (without the HSP promoter), into HEK293T cells for 24h using jetPEI DNA transfection agent (Polyplus Transfection, France) according to the manufacturer's instructions. Transfected cells were incubated at 37°C and monitored from 8-56h. In order to assess cytotoxicity via damage to the plasma membrane, cells were then stained with 10 μ M propidium iodide (PI; Life Technologies Invitrogen, UK), fixed with 4% paraformaldehyde (Sigma-Aldrich, UK), and mounted using DAPI (4'-6-diamidino-2-phenylindole)-containing medium (Vectashield H-1200; DAPI concentration: 1.5 μ g/ml; Vector Laboratories, UK). pDRIVE-HSP70-GFP expression and cytotoxicity were analysed using fluorescence imaging as described previously in Section 2.5.

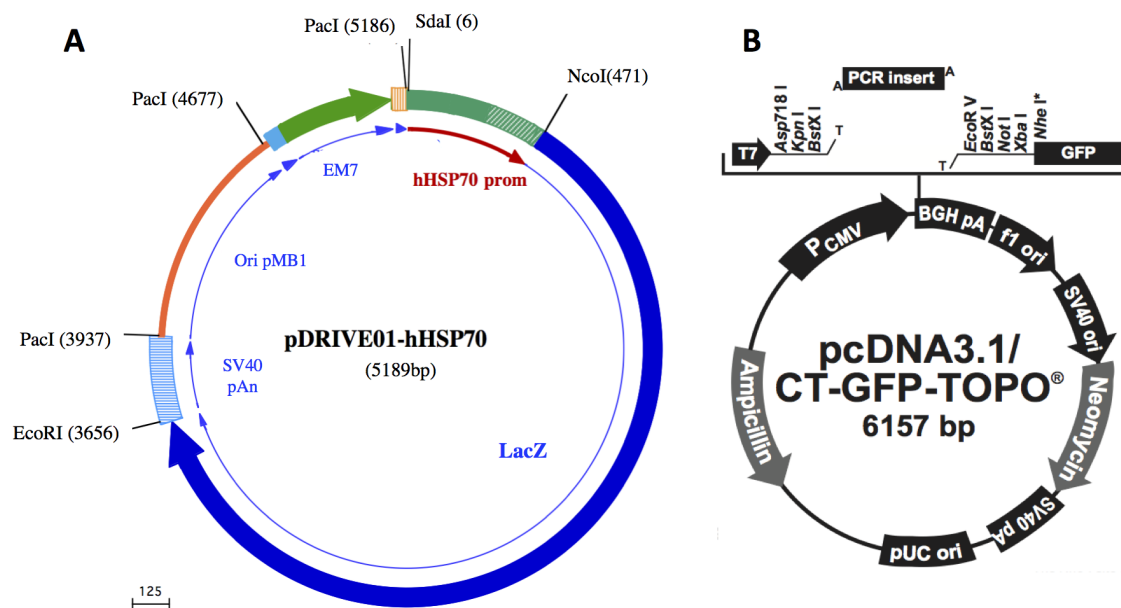


Figure 2.5: Maps of plasmid vectors used for molecular cloning of the pDRIVE-HSP70-GFP plasmid. (A) pDRIVE01-hHSP70 plasmid containing the 471bp human HSP promoter fragment (shown as the red arrow) that was amplified. (B) pcDNA3.1/CT-GFP-TOPO plasmid showing a linearised multiple cloning site into which the HSP promoter sequence was inserted.

2.4.5.2 Loading heat-inducible plasmid cargo onto MSNPs

Loading of pDRIVE-HSP70-GFP plasmid onto PEI-coated MSNPs was achieved via electrostatic interaction between the negatively-charged nucleic acid and the positively-charged cationic surface of functionalised MSNPs. To load particles with nucleic acids, non-fluorescent PEI-coated MSNPs with a size $>100\text{nm}$ were redispersed in 1.0ml of nuclease-free water (Millipore, UK) to 1.5-2mg/ml concentration. PEI-coated MSNPs were washed by centrifugation and redispersed in nuclease-free water (Ambion, UK). The optimum ratio with which to incubate the pDRIVE-HSP70-GFP plasmid and MSNPs for cargo loading was determined by incubating PEI-coated MSNPs and plasmid DNA together at different ratios of $\mu\text{gMSNPs}:\mu\text{gDNA}$ (10:1, 5:1, and 2:1) by mass for 24h at 4°C with gentle rotation. To evaluate the profile of plasmid DNA adsorption on the MSNPs, spectrophotometric quantification of free plasmid DNA concentration in the supernatant was carried out at hourly intervals during the first 6 hours of incubation, and then after 24

hours. Measurements were performed at 260nm in a minimum of three replicates (BiophotometerPlus, Eppendorf, UK). The optimum ratio for full cargo loading was determined to be incubation at 10:1 $\mu\text{gMSNP}:\mu\text{gDNA}$ for 24h and was used for MSNP plasmid loading for the subsequent experiments.

2.4.6 Cytotoxicity of laser treatment upon HEK293T cells

To evaluate the potential cytotoxic effects of infra-red laser treatment upon HEK293T cells, individual cells were laser-treated using the 400mW Saturn 5 Active laser. The laser pulse was delivered to the average centre of each cell, which was determined by locating the cross sections of middle of the longest cell length in both X and Y axes Figure 2.6. HEK293T cells treated with single laser pulses of 10mJ, 20mJ, and 30mJ were assessed for cell lysis and morphological changes. These energy levels were selected based on preliminary experiments conducted by the Coward lab (data unpublished, please refer to Appendix B Supplemental Data). A pulse of 30mJ was found to lyse the cells and therefore not used in subsequent investigations. Cells subjected to pulses of 10mJ and 20mJ were monitored for 24h, 48h, and 56h and assessed for cytotoxicity using PI and DAPI staining as described previously. Data was derived from three independent replications of 100 cells per treatment group. Since neither 10mJ nor 20mJ exhibited cytotoxicity, a pulse of 20mJ was selected for further investigation since the heat exclusion zone generated did not lyse the cells and because this energy has been used for other laser-based transfection methods in mammalian cells (Heinemann et al., 2013).

2.4.7 Laser-activation of plasmid delivered into HEK293T cells via MSNPs

Finally, after having optimised the MSNP plasmid loading conditions and confirming that neither the experimental MSNP concentrations nor laser treatments were cytotoxic to HEK293T cells, the effect of IR laser irradiation upon the induction of transgene expression was assessed. HEK293T cells containing PEI-coated MSNPs loaded with the pDRIVE-HSP70-GFP plasmid were laser-treated with 20mJ in

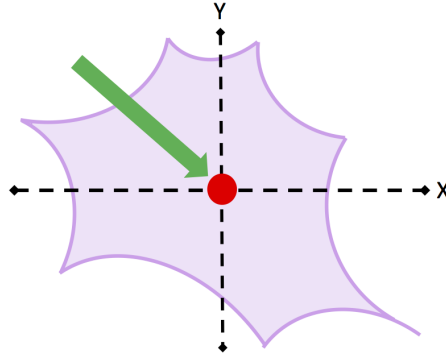


Figure 2.6: Diagram illustrating the laser location upon HEK293T cells. The HEK293T cell illustrated in purple. The length and width of the cell was measured and the point where the middle of the X-axis and Y-axis crossed was located (indicated by the green arrow) and used as the location for laser treatment.

order to assess plasmid expression. Prior to laser treatment, cells were incubated with plasmid-loaded MSNPs for 24h to allow MSNP entry and dispersion. Cells were then treated with 20mJ IR laser irradiation, as described in Section 2.3.3, and cultured for a further 32h. An additional group of cells incubated with plasmid-loaded MSNPs were not laser-treated, to act as controls. Cells were stained with PI and DAPI, then fixed and mounted for fluorescence imaging, as described in Section 2.6.1, in order to analyse the pDRIVE-HSP70-GFP expression. Data was derived from three independent replications of 100 cells per treatment group.

2.5 Imaging

2.5.1 Fluorescence imaging

Fluorescence microscopy was performed using filters for the following wavelengths: 330-380nm (blue for DAPI-nucleus); 465-495 (green for pDRIVE-HSP70-GFP; FITC-MSNPs; TUNEL-DNA fragmentation); and 540-580 (red for PI-plasma membrane integrity) using a Nikon Eclipse 80i microscope with a Nikon DS-Ri1 camera and NIS Elements v3.00 software (Nikon Instruments, UK).

2.5.2 Confocal imaging

Confocal fluorescent microscopy was performed under oil immersion at a magnification of 100X using a Becker and Hickl FLIM system mounted on an inverted IX81 Olympus FV1000 FluoView confocal microscope at the following wavelengths: 405nm (blue for DAPI-nucleus); 488nm (green for FITC-MSNP and pDRIVE-HSP70-GFP); and 559nm (red for PI-plasma membrane) at the Dunn School of Pathology (Oxford, UK).

2.6 Statistics

In all statistical analyses, the level of significance was set at 5%. Results are expressed as mean $\pm$ standard error of the mean ($\pm$ SEM). Data from each experimental condition, in triplicate, were managed using Microsoft Excel (Microsoft Office 2011, Microsoft Corporation, US), GraphPad Prism (GraphPad Software Inc.; California, US) and SPSS for Windows (SPSS Inc.; Version 21.0, Chicago Illinois, US). Representative graphs were constructed in GraphPad Prism v5.04 (GraphPad Software, La Jolla, CA, USA).

Data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). For mouse embryo experiments, a two-way analysis of variance (ANOVA) with the Tukey post hoc test was used for multiple comparisons to determine whether laser parameters differed significantly from the controls. For nanoparticle experiments, data were analysed by ANOVA or repeated measures ANOVA, followed by the Bonferroni post-hoc test for pair-wise comparisons. When data were non-normal, Friedman's test was performed as a non-parametric alternative to repeated measures ANOVA with a Wilcoxon matched-pairs test to evaluate differences between time points, and Kruskal-Wallis test was performed as a non-parametric alternative with Mann-Whitney matched-pairs test to evaluate differences between treatments within each time point.

2.7 Key messages

- This chapter presents a description of the general materials and methods used throughout this thesis
- Detailed information regarding specific experimental conditions are presented in the relevant experimental chapters

3

Safety of infra-red laser drilling upon mouse embryos at the molecular level

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3.1 Specialised background

When the use of lasers for ART was first proposed, there were understandable concerns regarding safety, due to its application at such a delicate stage of human development. Most concerns centred upon the risk of failed embryo development and the potential for congenital disorders (Montag et al., 2009). Thus far, safety studies have focused upon the effects of these lasers on blastomere lysis (Chatzimeletiou et al., 2001; Joris et al., 2003; Jones et al., 2006), blastocyst development (Wong et al., 2003; Lee et al., 2015), implantation rate (Montag et al., 1998; Iwayama et al., 2011), and pregnancy success (Han et al., 2003). Numerous studies report the success rates of achieving pregnancies following various laser applications in ART and the consensus of these studies was that laser treatment exhibited equal or superior success at ablating the zona pellucida, compared to the other available methods of mechanical drilling or Acid Tyrode's degradation (Joris et al., 2003; Hartshorne et al., 2005; Jones et al., 2006). However, there is a notable absence of literature pertaining to safety investigations regarding the potential detrimental effects of laser treatments upon embryos at the molecular level.

It has been shown that the temperature generated during zona pellucida ablation by a diode laser beam ranges from 130 to 160°C, which raises significant concern in relation to safety (Douglas-Hamilton and Conia, 2001). Studies have also shown that preimplantation embryos are sensitive to thermal changes and that DNA damage response in embryos is specific to developmental stage (Ealy et al., 1995; Adiga et al., 2007a; Adiga et al., 2007b). A few studies have investigated the effects of laser treatments upon preimplantation embryo membrane integrity (Chatzimeletiou et al., 2001), cytoskeletal structures (Chatzimeletiou et al., 2005) and heat-shock protein transcription (Hartshorne et al., 2005). Chatzimeletiou et al. (2005) showed no significant difference in embryo damage between Acid Tyrode's or laser drilling for embryo biopsy in human embryos, when comparing growth to the blastocyst stage, spindle deformities, or chromosomal abnormalities. These authors used a 1.48 μ m diode non-contact Research Instruments Saturn infra-red laser, the model preceding the one used in this thesis, and using a pulse length of 1.2ms, fired the

laser 6-8 times creating a line of approximately $45\text{-}50\mu\text{m}$ in length. The authors concluded that using laser drilling at a safe working distance did not cause adverse immediate or longer term damage to the development of biopsied human embryos. While these results were positive and encouraging, the authors did not monitor or report how far away the laser pulses were from the blastomere in each treatment and what would constitute a safe distance, and therefore safe or unsafe laser pulse locations cannot be extrapolated and used for other studies.

A study by Hartshorne et al. (2005) investigated whether infra-red laser drilling would cause a stress-induced heat shock protein response and result in altered levels of heat-shock protein (*hsp70i*) RNA levels in mouse embryos. Interestingly, these authors investigated individual blastomeres within cleavage stage embryos to assess whether there were changes in the blastomeres closest to the laser pulses when compared to the blastomeres further away. The authors concluded that laser drilling of the zona pellucida was safe since they used a greater number of pulses, and a higher pulse length, than is applied in clinical procedures and there was no significant increase in heat shock protein RNA transcripts. These results should be interpreted with caution however, because while the authors stated that they used either four to ten pulses of 1ms in length, or 8 pulses of 1.5ms in length, they did not precisely specify where in the zona pellucida the pulses were created (Hartshorne et al., 2005).

Another preliminary study by Chatzimeletiou et al. (2001) investigated different laser pulse lengths and distances from blastomere cells to ascertain whether damage was caused to the plasma membrane in mouse embryos. These authors concluded that a pulse length of 0.2ms should only be used at a distance $>2.7\mu\text{m}$ from the cell membrane, and that a pulse duration of 0.4ms should only be used at a distance $>5.0\mu\text{m}$ from the cell membrane. This study was very rudimentary however, with only 3, 4, or 5 individual embryos tested for each laser pulse and distance parameter. Therefore the results had no real statistical power and must be expanded upon before a reliable conclusion can be made (Chatzimeletiou et al., 2001).

Laser treatment is routinely used in ART clinics, particularly for embryo biopsy, and is continually the subject of investigative research to refine and develop novel

laser-assisted techniques. Effects at the molecular level could lead to downstream consequences upon embryo development or implantation potential, and existing laser data analysis might be confounded by the use of differing laser protocols, some of which might unknowingly be dangerous.

3.2 Chapter aims

This chapter aimed to address the paucity of literature regarding changes at the molecular level following laser treatment by expanding upon the preliminary study of Chatzimeletiou et al. (2001) and evaluating multiple hole sizes (correlating to different energy levels and respective pulse lengths), along with multiple distances from blastomeres, to specifically investigate the effects of these different conditions at the molecular level. This should permit the identification of unsafe parameters and allow refinement for safer methodology. For these investigations, the mouse model was utilised. The mouse possesses many favourable qualities as a preimplantation embryo model such as: similar development from zygote to morula to blastocyst stage, conserved gene order, and 99% homology with human genes (Niakan et al., 2012).

3.3 Specialised materials and methods

The average thickness of the human zona pellucida varies from 10-31 μm with reported mean values of 26 μm for women less than 30 years old, and 21 μm for women older than 35 years (Nieminen et al., 1992; Gabrielsen et al., 2000). Clinical laser ART procedures at Oxford Fertility (OF) routinely use hole sizes of 15 μm for embryo biopsy (personal communication, Tracey Griffiths, Senior Embryologist, OF), representing approximately 60-70% of the thickness of the zona pelludica, with laser treatments beginning with pulses near the outer edge of the zona pellucida, gradually moving inwards towards the blastomeres until a sufficiently sized hole is created (personal communication, Bianka Seres, Embryologist, OF). To refine safe working distances with which to apply laser treatments to embryos, four laser hole sizes (correlating to different laser energies and pulse lengths) were applied at

four distances away from blastomeres in 2-cell mouse embryos, at an orientation perpendicular to the first cleavage plane.

Figure 3.1 depicts a schematic of the investigations deployed to determine safe and unsafe laser applications. Each laser treatment was assessed for cytotoxicity immediately following laser-treatment using propidium iodide (PI) staining to reveal compromised plasma membrane integrity, and any parameters that showed significant damage were eliminated from subsequent investigations. Embryos were next assessed for development to the blastocyst stage, and blastocysts classified as being of a "good" grade (3-5AA and 3-5AB) were further investigated for DNA fragmentation via terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Gardner and Schoolcraft, 1999). Specific differential gene expression of a select cohort of developmentally important genes was investigated using real-time quantitative PCR (qPCR). Please refer to Table 2.1 in Section 2.3.8 for more information regarding the selected genes.

The average thickness of the mouse zona pellucida at the time of laser drilling was determined to be $15.98 \pm 1.14 \mu\text{m}$. To investigate hole sizes ranging above and below those presently applied clinically, hole sizes of $2 \mu\text{m}$, $4 \mu\text{m}$, $8 \mu\text{m}$, and $16 \mu\text{m}$ were tested, representing 12.5%-100% of the zona pelludica thickness. Each hole size was applied perpendicular to the first cleavage plane at distances of $1 \mu\text{m}$ (inner edge of the zona), $8 \mu\text{m}$ (middle of the zona), and $16 \mu\text{m}$ (outer edge of the zona) from the nearest blastomere. A laser pulse at a distance of $24 \mu\text{m}$ from the nearest blastomere, directly adjacent to the embryo but falling outside the boundary of the zona pellucida, served as a control for each hole size.

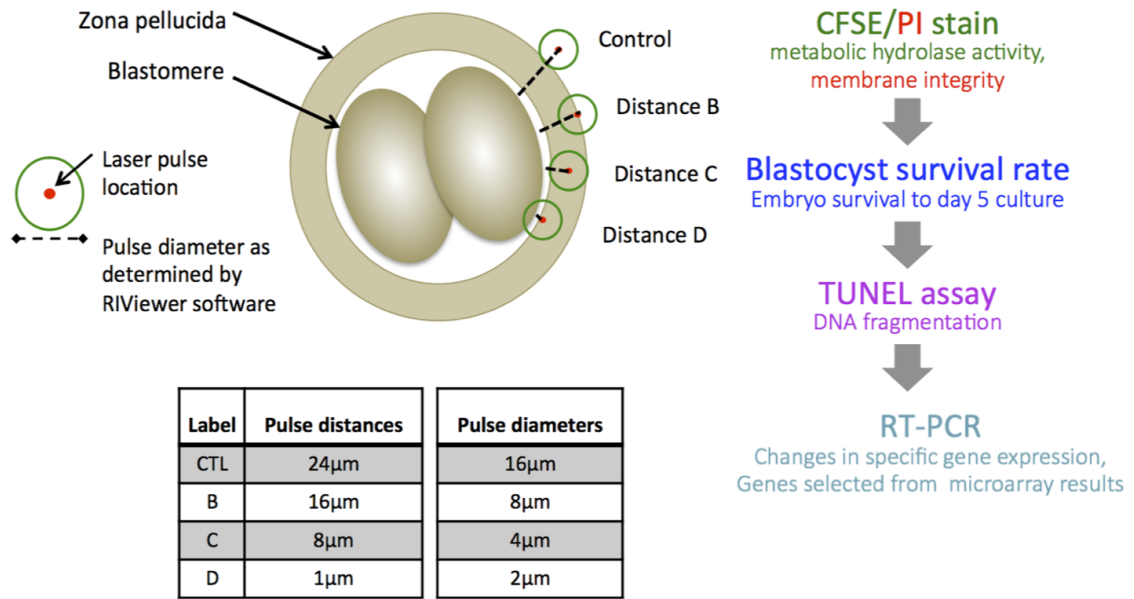


Figure 3.1: Schematic of exclusion zone experimental design using mouse embryos as an investigative model. The effects of four different laser energies (corresponding to different hole sizes) ablating the ZP were examined at four different distances to the nearest blastomere. These laser conditions were subjected to different tests with increasing sensitivity to determine any deleterious effects upon the embryo. CTL = control distance. CFSE = carboxyfluorescein succinimidyl ester. PI = propidium iodide.

3.4 Results

3.4.1 Viability and cytotoxicity staining

In order to assess the immediate cytotoxic effects of different laser treatments following laser treatment, mouse embryos were stained with propidium iodide (PI) to reveal damage to plasma membrane integrity, and treated with carboxyfluorescein succinimidyl ester (CFSE) to visualise healthy levels of hydrolase activity. PI staining is a gold standard method of assessing cell cytotoxicity, with positive staining resulting from the binding of PI to nuclei of cells whose plasma membranes have become permeable due to cell death (Nieminen et al., 1992). CFSE is a cell permeable dye which enters cells by diffusion and is cleaved by intracellular esterase enzymes in a hydrolysis reaction to produce a fluorescence product, indicating active metabolic hydrolase activity (Schorl and Sedivy, 2007). CFSE staining was used to confirm that all blastomeres not showing PI staining were healthy and metabolically

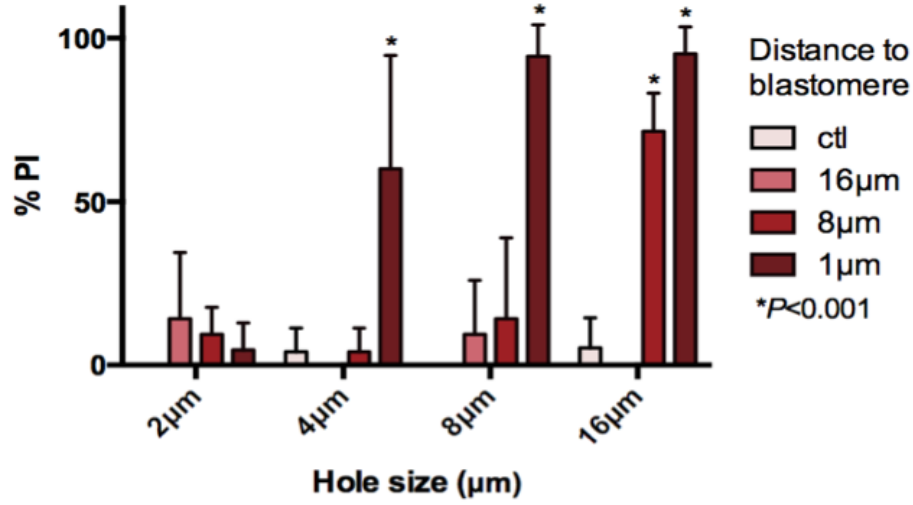


Figure 3.2: Mean proportion of mouse embryos showing cytotoxicity via positive propidium iodide (PI) staining (%±SEM). ctl = control pulse located at a distance of 24µm from the blastomere. Data derived from three independent replicates of 10 embryos each (* $P < 0.001$).

active cells, indicating that any PI staining observed was due to damage incurred by the laser. At a 1µm distance from the nearest blastomere, hole sizes of 4µm, 8µm and 16µm showed significant damage ($P < 0.001$) to the plasma membrane of the blastomere compared to their respective controls. The mean proportion of positive PI staining at a 1µm distance for each condition compared to respective controls (laser-treated *versus* control) for a hole size of 4µm was 60.12%±20.00 *versus* 4.16%±4.16 ($P < 0.001$), for a hole size of 8µm was 94.43%±5.57 *versus* 0.00%±0.00 ($P < 0.001$), and for a hole size of 16µm was 95.23%±4.77 *versus* 5.13%±5.13 ($P < 0.001$) (Figure 3.2). A laser hole size of 16µm also showed significantly higher levels of PI staining ($P < 0.001$) at 8µm from the nearest blastomere compared to the control (71.58%±6.73 *versus* 5.13%±5.13). Positive CFSE staining, observed in green, was seen in all distal blastomeres that were located on the opposite side of the embryo as the laser pulse, and in all proximal blastomeres near the laser pulse that were not positive for PI staining, confirming that the damage observed via PI staining was due directly to the laser treatment, suggesting that the damage observed via PI staining was due to the laser treatment (Figure 3.3).

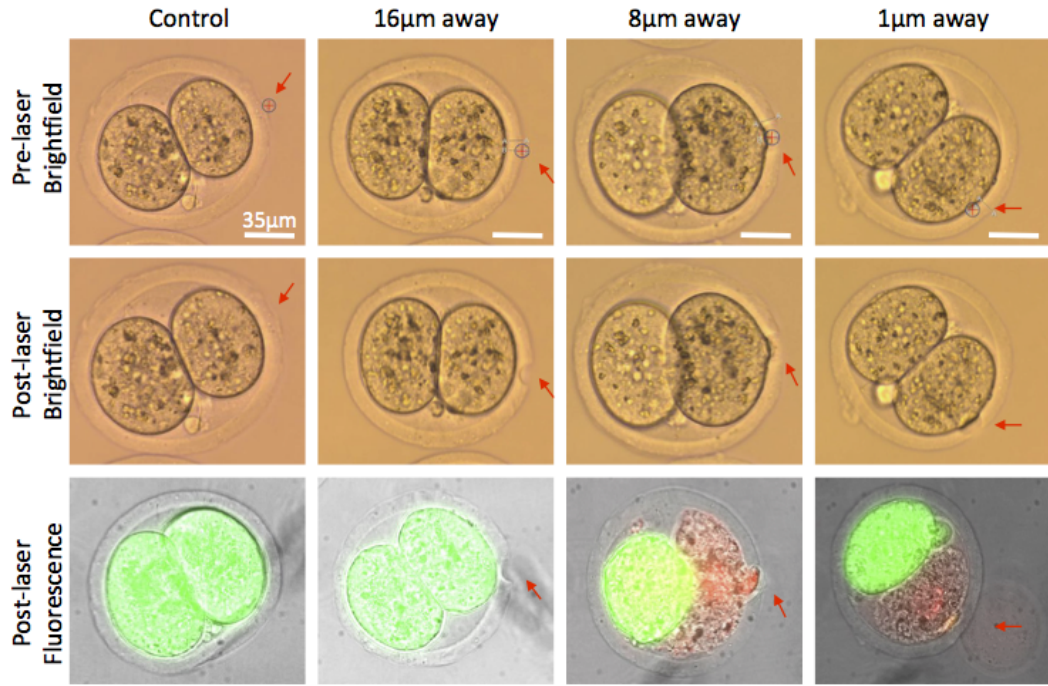


Figure 3.3: Representative fluorescent and brightfield images of mouse embryos laser-treated with $16\mu\text{m}$ holes at varying distances from the blastomere membrane and stained with propidium iodide (PI-red) and carboxyfluorescein succinimidyl ester (CFSE-green). Red arrows indicate the location of laser treatment, and the crosshair within the target represents the focal point of the laser pulse. Magnification 40X.

3.4.2 Development to the blastocyst stage

Subsequent investigations were carried out to assess any detrimental effects of laser treatment upon survival to the blastocyst stage of development. While laser manipulation of mouse embryos has previously been shown to perturb the compaction process, especially adjacent to the ablation area (Malter et al., 2001), this effect was not observed in the current study. Growth of embryos to "good" quality blastocysts (3-5 AA or AB as classified by Gardner grading (Gardner and Schoolcraft, 1999)), was significantly ($P < 0.05$) compromised in two of the conditions (Figure 3.4). A hole size of $8\mu\text{m}$ located $1\mu\text{m}$ from the blastomere showed a mean survival rate of $64.33\% \pm 2.73$ compared to $96.33\% \pm 3.67$ in the control. A hole size of $16\mu\text{m}$ located $1\mu\text{m}$ from the blastomere showed a mean survival rate of $41.87\% \pm 2.31$ compared to $95.83\% \pm 4.17$ control. Notably, two of the conditions also showed significant PI damage. While growth to blastocyst stage was significantly

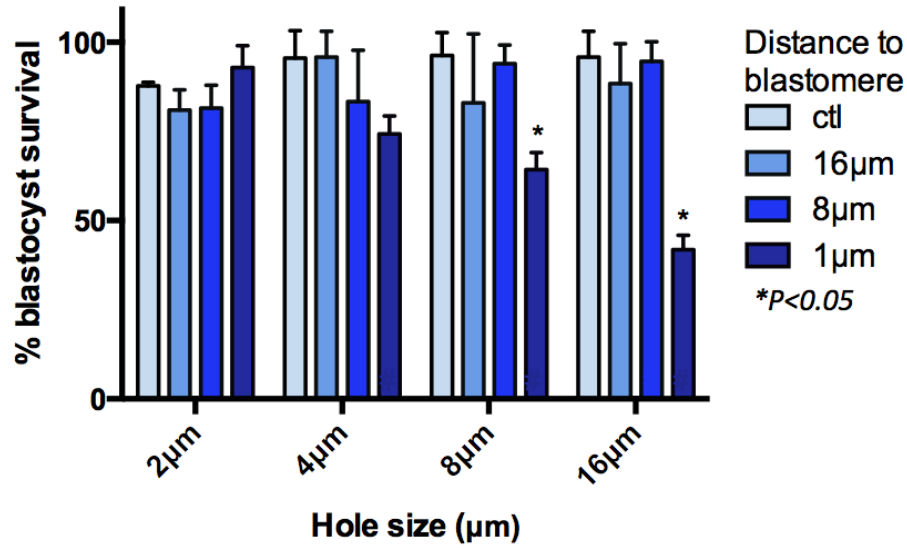


Figure 3.4: Mean proportion of mouse embryo survival to 3-5AA/AB grade blastocyst stage ($\% \pm \text{SEM}$), determined by Gardner grading (Gardner and Schoolcraft, 1999), following infra-red laser treatment. Results are from three independent replicates of 10 embryos each. ctl = control pulse located $24\mu\text{m}$ from the blastomere. ($*P < 0.001$).

lower in these two conditions, good quality embryos that were produced from all conditions were subsequently investigated for levels of DNA fragmentation.

Survival to the blastocyst stage is a common assessment point in investigations involving effects upon embryos. However, many studies complete their investigations at this stage, without addressing any further potential effects at the molecular level. It is entirely conceivable that different laser treatments could be imparting detrimental effects upon embryos which would not be discernible by the routine morphological assessment of growth to the blastocyst stage alone. Therefore, conditions that allowed for successful development to the blastocyst stage were further investigated for more in-depth molecular effects. These investigations are described below in Sections 3.4.3 and 3.4.4.

3.4.3 DNA fragmentation

Laser-treated blastocysts that were graded as "good quality" (3-5AA and 3-5AB by Gardner grading (Gardner and Schoolcraft, 1999)) following culture were further assessed for DNA fragmentation. The apoptotic index, or proportion (%) of DNA

fragmentation within each blastocyst, was determined from the ratio of nuclei exhibiting positive TUNEL staining and the total number of DAPI-stained cells (Figure 3.5). All four laser conditions which exhibited significant PI damage also showed significant levels of DNA fragmentation ($P<0.05$), even though only good-quality blastocysts were selected (Figure 3.6).

The mean proportion of positive PI staining at a $1\mu\text{m}$ distance for each condition compared to respective controls (laser-treated *versus* control) for a hole size of $4\mu\text{m}$ was $40.34\%\pm 5.35$ *versus* $3.49\%\pm 2.26$, for a hole size of $8\mu\text{m}$ was $39.58\%\pm 3.48$ *versus* $5.40\%\pm 0.30$, and for a hole size of $16\mu\text{m}$ was $48.93\%\pm 3.09$ *versus* $4.71\%\pm 2.80$. A laser hole size $16\mu\text{m}$ also showed significantly higher levels of PI staining at $8\mu\text{m}$ from the nearest blastomere compared to the control ($43.76\%\pm 5.70$ *versus* $4.71\%\pm 5.13$).

This suggests that although it might appear that embryos grow to morphologically good quality even after membrane damaging laser treatment, that at the molecular level, the embryos sustained damaged. All other conditions showed an increased trend for DNA fragmentation as the location of the hole approached the blastomere membrane, but this was not statistically significant.

3.4.4 Changes in gene expression

Changes in gene expression of a panel of genes was analysed by qPCR. The genes which were selected relate to ICM stem cells (*Nanog*), pro-apoptotic (*Bax*) and anti-apoptotic (*Bcl2l2*) pathways, DNA methylation (*Dnmt3*), and reactive oxygen species detoxification (*Sod1*, *Gpx1*). When performing laser treatments at a distance of $16\mu\text{m}$ away from the nearest blastomere, *Gpx1* (hole size of $16\mu\text{m}$), *Nanog* (hole size of $8\mu\text{m}$), and *Dnmt3a* (hole sizes of $8\mu\text{m}$ and $16\mu\text{m}$) relative expression was significantly increased as larger holes were used (Figure 3.7A). At a hole distance of $16\mu\text{m}$ and $8\mu\text{m}$, respectively, *Dnmt3a* expression significantly increased as hole size increased. *Dnmt3a* was also upregulated when a hole was administered $1\mu\text{m}$ from the blastomere (Figure 3.7A,B). Notably, as distance to the blastomere was reduced, changes in gene expression were observed. For example, when using a hole size of $2\mu\text{m}$, all genes except for *Nanog* exhibited significantly increased or decreased gene

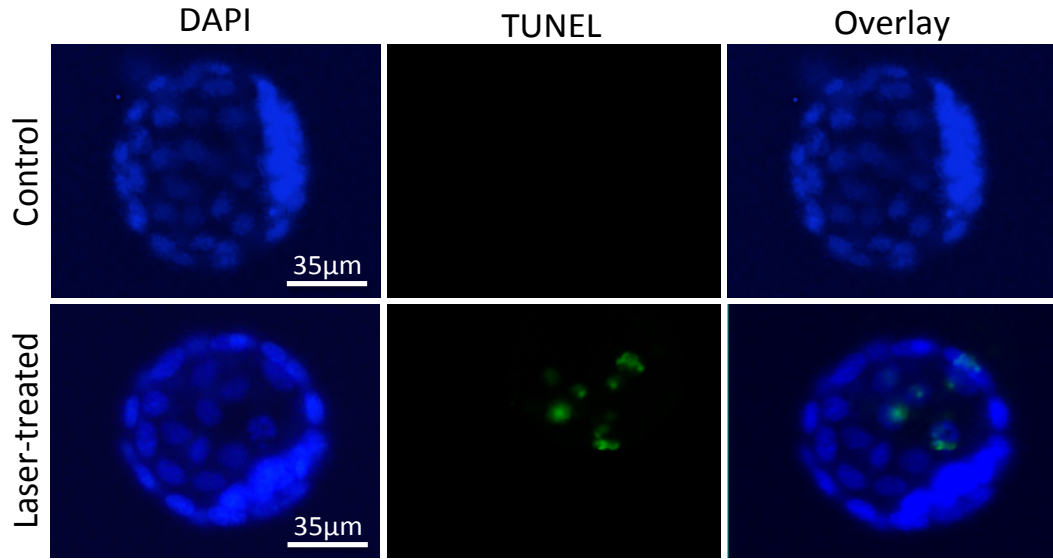


Figure 3.5: Representative fluorescence images of control and laser-treated ($16\mu\text{m}$ hole; $8\mu\text{m}$ distance) embryos following TUNEL assay showing DNA fragmentation in green, and total number of cell nuclei in blue. Composite images represent an overlay of nuclei stain (DAPI - blue) and fragmented DNA (TUNEL - green). DAPI = 4',6-diamidino-2-phenylindole, binds DNA; TUNEL = fluorescein-12-dUTP. Magnification at 40X.

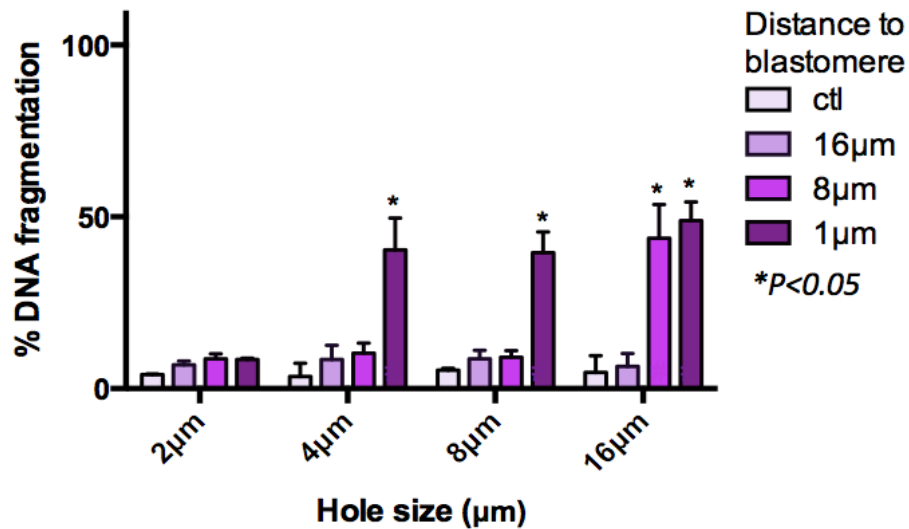


Figure 3.6: Effects of infra-red laser treatment upon DNA fragmentation. Mean proportion of DNA fragmentation ($\% \pm \text{SEM}$) assessed by TUNEL assay at the blastocyst stage following infra-red laser treatment. Results are from three independent replicates of 10 embryos each. ctl = control pulse located at a distance of $24\mu\text{m}$ from the blastomere. (* $P < 0.001$).

expression at $1\mu\text{m}$ away from the blastomere (Figure 3.7C), but no change in gene expression as distance was increased to $8\mu\text{m}$ or $16\mu\text{m}$. While an overall change in relative expression at a distance of $1\mu\text{m}$ was observed for all genes except *Nanog*, the change in expression was lower than the significant changes observed at distances of $8\mu\text{m}$ and $16\mu\text{m}$. Relative expression of *Bcl2l2* was significantly increased with a hole size of $8\mu\text{m}$ at a distance of $8\mu\text{m}$ from the membrane, and a hole size of $2\mu\text{m}$ at a distance of $1\mu\text{m}$ from the membrane. No significant changes in the relative expression of *Sod1* were observed with the laser treatments at a distance of $8\mu\text{m}$ or $16\mu\text{m}$, however compared to controls, it was higher when a hole was administered at a location of $1\mu\text{m}$ from the blastomere, even with a small hole size of $2\mu\text{m}$. The relative expression of *Gpx1* was significantly higher when a hole was administered at the furthest location ($16\mu\text{m}$), or with the smallest hole size ($2\mu\text{m}$).

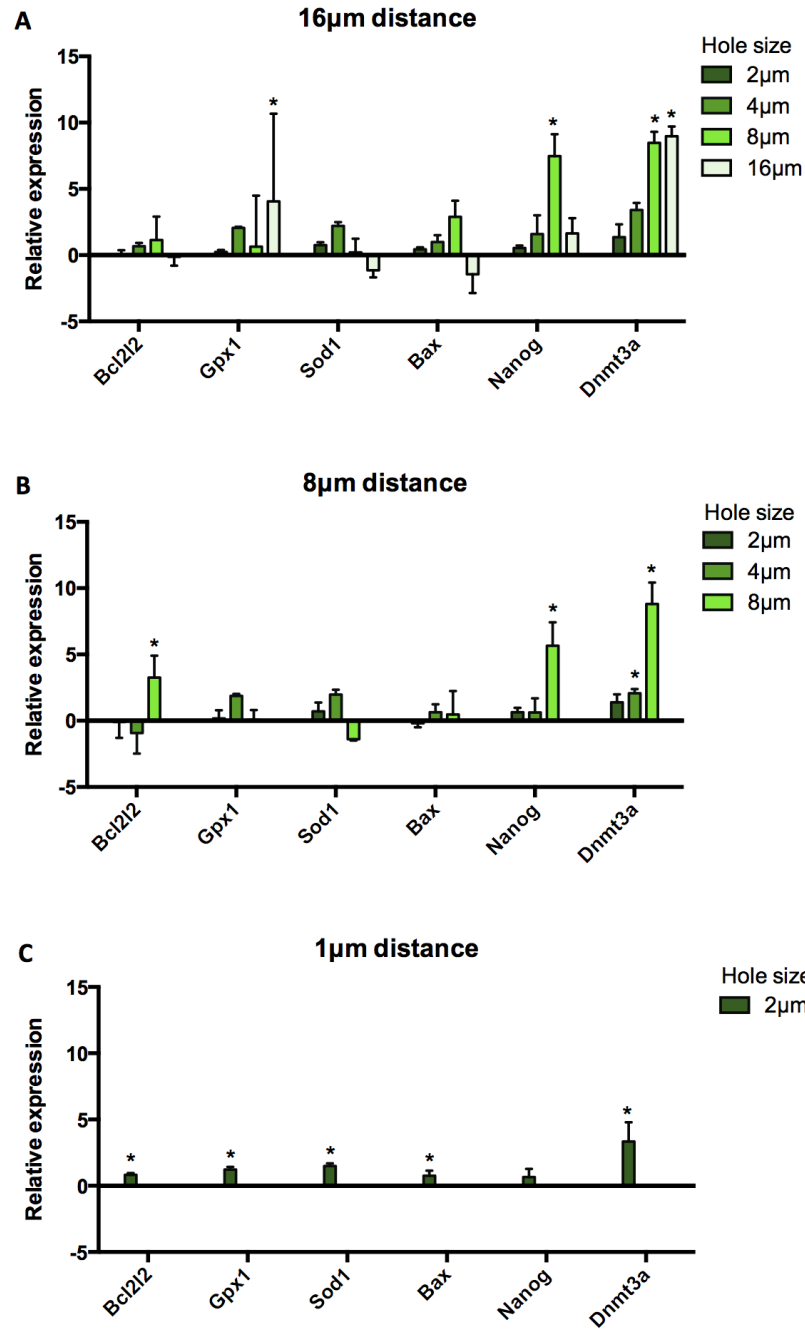


Figure 3.7: Relative changes in gene expression following laser treatment assessed by quantitative polymerase chain reaction (qPCR). Laser treatments involved a range of hole sizes administered at varying distances to the blastomere cell membrane. *Gapdh* and *Actb* were used as housekeeping genes and relative levels of gene expression were normalised to the respective gene expression level in control embryos. (A) changes in gene expression of four different hole sizes administered 16µm from the cell membrane. (B) changes in gene expression of three different hole sizes administered 8µm from the cell membrane. (C) changes in gene expression of one hole sizes administered 1µm from the cell membrane. Results are from three independent replicates of 10 embryos each. *denotes significant changes in gene expression (* $P < 0.05$).

3.5 Discussion

This present chapter evaluated the relationship between laser pulse energy (and thus hole size) and distance to blastomere cells in order to determine safe working distances for different energy pulses in ART and to investigate potential damage at the molecular level. Currently, laser application in the clinic appears to be up to the discretion of the embryologist, with no restrictions on what hole sizes or locations should or should not be administered. This chapter determined that laser pulses with hole sizes of $4\mu\text{m}$, $8\mu\text{m}$, and $16\mu\text{m}$ should not be applied at a distance of $1\mu\text{m}$ from the blastomere membrane, and a hole size of $16\mu\text{m}$ should not be applied at a location of $8\mu\text{m}$ from the blastomere membrane. Therefore, these laser parameters should be avoided in clinical and research applications. Many previous investigations involving laser-treated embryos have analysed only embryo morphology before deeming the embryo healthy for transfer for implantation. Such studies ignored potential effects upon molecular systems which could subsequently impact upon the crucial process of implantation. It is imperative that laser-assisted procedures, especially those for which potential benefit remains controversial, are refined to use safe conditions, and standardised to investigate the true effects of whether the procedure is beneficial for clinical implementation or not.

The first part of this study sought to identify potential damage immediately following laser treatment by assessing plasma membrane integrity with propidium iodide (PI), and hydrolase activity with carboxyfluorescein-diacetate succinimidylester (CFSE). PI staining is the gold standard method for assessing cell cytotoxicity, with positive staining resulting from the binding of PI to the nuclei of cells whose plasma membranes have become permeable due to cell death (Nieminen et al., 1992). CFSE is a cell permeable dye which enters cells by diffusion and is cleaved by intracellular esterase enzymes in a hydrolysis reaction to produce a fluorescence product, indicating active metabolic hydrolase activity (Schorl and Sedivy, 2007). Significantly higher levels of positive PI staining were observed when using hole sizes of $4\mu\text{m}$, $8\mu\text{m}$, and $16\mu\text{m}$ at a distance of $1\mu\text{m}$ from the blastomere membrane. Additionally, a hole size of $16\mu\text{m}$ at a distance of $8\mu\text{m}$ away from the nearest

blastomere showed significant PI staining (and thus membrane damage); this differs from data reported by Chatzimeletiou et al. (2001), who failed to detect damage using pulse sizes of 15-18 μ m at a distance of >8 μ m, although these authors did not specify exactly what distances greater than 8 μ m were used (Chatzimeletiou et al., 2001). The four laser conditions which were found to cause immediate significant membrane damage and were thus deemed "unsafe" laser parameters. Embryos are known to be able to "rescue" themselves following an injury and still produce morphologically-healthy looking blastocysts (Wang et al., 2013b), therefore all laser conditions were further investigated for any effect on the development of the embryos to the blastocyst stage.

Survival to the blastocyst stage is a common assessment point in investigations involving embryos, with many studies completing their investigations at this stage without addressing potential effects at the molecular level. It is difficult to perform molecular investigations on clinical human samples since the embryos must often be used for clinical transfer afterwards, and surplus embryos can be difficult to obtain. For example, Taylor et al., (2010) investigated the use of different laser pulse lengths when performing a biopsy on human embryos for IVF, however could only assess morphological outcomes such as blastomere lysis, fragmentation patterns, and growth to the blastocyst stage, and the authors stated their own limitations in not standardising the number or location of pulses (Taylor et al., 2010). In the present study, blastocysts were graded according to the Gardner grading system (Gardner and Schoolcraft, 1999). Blastocysts which reached a grade of 3-5AB were classified as having developed into a "good quality" blastocyst and were reported as the mean proportion of total cultured blastocysts within each laser condition. While growth to the blastocyst stage was statistically significantly impaired in two conditions (two of which had also shown significant PI damage), a proportion of blastocysts in all laser conditions did survive and grew to a "good quality" stage when assessed by morphology. It has been reported that in the C57BL/6 strain of mouse used in the present investigations, that 79% develop to the blastocyst stage when cultured in KSOM supplemented with amino acids (Sugawara and Ward, 2013); all

of the control and non-statistically significant laser conditions exhibited a blastocyst growth rate higher than this. While morphology is a commonly used indicator of embryo health, it is apparent that within groups of healthy looking embryos, variability exists, and it is entirely conceivable that different laser treatments could impart detrimental effects upon embryos that are not discernible by morphological observation alone (Chatzimeletiou et al., 2005; Rocha et al., 2016). In support of this notion, proteomic and metabolomic studies of embryos are beginning to be applied in order to provide information to distinguish healthier embryos from other morphologically-normal embryos. For example, Poli et al. (2015) investigated the quantification and role of attomole levels of proteins found in the blastocoel cavity and found correlations with aspects of embryo viability such as ploidy status, presenting a possible biomarker for embryo health.

To investigate more in-depth molecular effects of the laser parameters used in these experiments, embryos which were morphologically assessed as "good quality" blastocysts via Gardner grading were further analysed for DNA fragmentation using the TUNEL assay, a common tool for assessing apoptosis in studies involving embryos (Castillo-Martin et al., 2015b). Interestingly, all laser conditions which initially showed significant PI damage also showed significant DNA fragmentation at the blastocyst stage, regardless of the embryo growing to a morphologically "good-quality" blastocyst, suggesting that while embryos may morphologically appear healthy, detrimental effects still remain at the molecular level. It has been demonstrated that single blastomeres, damaged at the 2-, 4- and 8-cell stages, cease further cleavage, fail to compact into morula, and undergo apoptosis once they reach the blastocyst stage (Wang et al., 2013b). It has been reported that in-bred C57BL/6 mice exhibit an apoptotic index of up to 14.4% when cultured in KSOM media (Kamjoo et al., 2002). All control samples, and all laser conditions with apoptotic indices which had not increased significantly, exhibited apoptotic indices lower than this, suggesting these laser conditions were safe in terms of DNA fragmentation.

Of the remaining laser conditions which did not show significant DNA fragmentation damage, qPCR was utilised to investigate changes in gene expression

in a panel of key developmental genes in the preimplantation embryo. The gene transcripts investigated in this study were selected due to their role in embryo development, apoptosis, and stress. *Nanog* is an important ICM stem cell gene, widely recognised as being important for preimplantation development. Increased expression of *Nanog* has been associated with higher quality embryos (Castillo-Martin et al., 2015b), and none of the laser treatments in the present study showed any significant down-regulation.

Bax is a gene with pro-apoptotic function and *Bcl2l2* is a gene with anti-apoptotic function. Both are known to participate in the extrinsic apoptosis pathway (Brill et al., 1999). Decreased levels of *Bax* and increased levels of *Bcl2l2* are associated with stronger embryos (Castillo-Martin et al., 2015a). Relative expression of *Bcl2l2* was significantly increased in two of the laser parameters, suggesting effects on apoptotic pathways, even though no significant changes in apoptotic indexes were observed for either of these laser treatments. *Sod1* is a superoxide reactive oxygen species (ROS) related gene, and *Gpx1* is a peroxide ROS gene indicative of ROS detoxification (Castillo-Martin et al., 2014). Over-expression of *Sod1* in mice has been shown to protect embryos against oxidative damage and from malformation in a diabetic environment (Takahashi, 2012; Wang et al., 2013a). No significant changes in *Sod1* expression were observed at the further distances, but levels were higher at closer distances, even with smaller holes. Higher expression of *Gpx1* is associated with higher blastocyst survival following vitrification thawing (Castillo-Martin et al., 2014). The relative expression of *Gpx1* was significantly higher when a hole was administered at the furthest location (16 μ m), or with the smallest hole size (2 μ m). *Dnmt3a* is a gene involved in increased levels of *de novo* methylation. At a hole distance of 16 μ m and 8 μ m, respectively, *Dnmt3a* expression significantly increased as hole size increased. *Dnmt3a* was also upregulated when a hole was administered 1 μ m from the blastomere. Transcripts of *Dnmt3a* are not usually found in such early stage embryos, as transcript timing has been shown to be important and dysregulation can cause disease states or epigenetic modifications, for example through hypomethylation (Watanabe et al., 2002). Overall, none of

the significant changes in gene expression were indicative of a cause for concern regarding the laser treatments. Since the remaining laser conditions not showing significant DNA fragmentation did not show clear patterns in changes of gene expression, future studies could be carried out to elucidate any changes in gene expression in the laser conditions that did show significant DNA fragmentation. This would help generate for a clearer picture regarding the effects of damage on gene expression, allowing for comparison for the analysis of gene expression changes in conditions with unknown damage effects.

A new study by Wong and Mills (2017) investigated the thermal effects of laser drilling upon embryos in an attempt to automate laser zona drilling with computer algorithms. These authors created an algorithm which could analyse images of an embryo and deliver laser pulses at a location with the furthest distance from embryonic structures, in an effort to minimise thermal heating. Interestingly, the pulses that are delivered using the automated software are not the same as would be predicted, and leave parts of the zona pellucida intact, suggesting that these inner pulses could be causing too much heating near the blastomeres or perivitelline space (Wong and Mills, 2017). These findings substantiate the notion that further studies are needed to refine safe working distances. Findings such as those presented in this chapter could be used to inform and refine the creation of algorithms for future developments, or highlight standardised working conditions, which could readily be adopted and promoted by manufacturers and researchers for testing prior to clinical translation.

3.6 Limitations

The main limitation regarding experiments in this chapter is that clinical biopsy conditions were not mimicked exactly, as the laser pulses were performed on 2-cell embryos, not cleavage stage embryos. It was initially decided that the laser treatments should be applied at an earlier stage to try and avoid compaction, which starts at the 8-cell stage in mice, and thus avoid interference with cell-cell adhesion, gap junction and cytoplasmic polarisation (Sugawara and Ward, 2013). Compaction

in human blastocysts occurs around the 16-32 cell stage (Ziomek and Johnson, 1980) with embryonic genome activation initiated at the 4-8 cell stage (Telford et al., 1990). Since embryonic gene activation in mice occurs at the 2-cell stage (Bensaude et al., 1983), and compaction around the 8 cell stage, laser treatment around the 2-4 cell stage was thought to be representative of the 6-8 cell stage in humans. However, through discussions with colleagues, laser treatment at the 2-cell stage was highlighted as a potential cause for concern in the present experiments, as clinical conditions were not closely replicated. Since a decent body of results was already generated at this time (PI, Gardner grading, TUNEL), the remainder of the experiments (qPCR) in this chapter were carried out with laser treatment at the 2-cell stage to maintain consistency. The mouse embryo experiments in subsequent chapters were adapted to have laser treatments applied at the cleavage stage, to better emulate clinical biopsy conditions. Lastly, while the mouse model is one of the strongest preimplantation embryo models available, there are still important differences between these two species and thus ideally experiments would be validated and confirmed in human samples (Niakan et al., 2012).

3.7 Conclusions

The present investigations significantly strengthen the limited body of published literature and provides supportive evidence to define safe working distances for laser treatment which will not cause deleterious effects to the mouse embryo at the molecular level, at least with those parameters assessed herein. Overall, laser pulses with hole sizes of $4\mu\text{m}$, $8\mu\text{m}$, and $16\mu\text{m}$ should not be applied at a distance of $1\mu\text{m}$ from the blastomere membrane, and a hole size of $16\mu\text{m}$ should not be applied at a location of $8\mu\text{m}$ from the blastomere membrane. In clinics, laser pulses are generally not applied as close as $1\mu\text{m}$ from the membrane, however, pulses are often applied in different locations in the zona pellucida from the outside inwards, to ensure that the full zona is breached. When such pulses are applied and need to be applied $8\mu\text{m}$ or less from the blastomeres, a hole smaller than $16\mu\text{m}$ should be utilised. Laser treatment is routinely used in ART clinics and is

continually the subject of investigative research to refine and develop novel laser-assisted techniques. Establishing safe working parameters for laser application will allow for improved standardisation and facilitate more accurate analysis of the benefits of laser-assisted techniques in order to help minimise or prevent potentially damaging effects upon vulnerable embryos, which will ultimately improve infertility research and thus, treatment options.

3.8 Future directions

The continuation of studies investigating effects of laser treatment at the molecular level are paramount in establishing any system of standardisation for laser-assisted treatments which would benefit both clinical and research applications. Future investigations should explore further downstream molecular events, for example effects on implantation-related protein expression and how such proteins might interact with the endometrium. This will allow us to investigate how laser treatment might interfere with the interaction of proteins in the implanting embryo with the endometrium. Additionally, epigenetic changes following laser ART procedures have not yet been investigated, but could provide interesting insight into the effects of laser procedures, since it has been shown that the methylation pattern of imprinted genes can change in differing embryo culture conditions (Doherty et al., 2000).

3.9 Key messages

- This chapter investigated the deleterious effects at the molecular level of a range of laser hole sizes applied to the zona pellucida at different distances away from the nearest cell
- Blastocysts assessed as healthy via morphology grading were shown to have significant damage at the molecular level in terms of DNA fragmentation
- Laser pulses with hole sizes of $4\mu\text{m}$, $8\mu\text{m}$, and $16\mu\text{m}$ should not be applied at a distance of $1\mu\text{m}$ from the blastomere membrane, and a hole size of $16\mu\text{m}$ should not be applied at a location of $8\mu\text{m}$ from the blastomere membrane
- Results from this chapter have contributed to a research manuscript which will be submitted to *Human Reproduction*

4

Investigating the effects of altering laser power when treating preimplantation embryos

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4.1 Specialised background

Assisted reproductive technology lasers use heat generated by high energy light to dissolve the zona pellucida. The energy produced by the laser is influenced by three factors: the wavelength of the laser beam, the power of the laser, and the pulse length. The wavelength refers to the spectrum of light in which the laser operates, with current lasers operating in the infra-red (IR) range, to minimise mutagenic damage to DNA. The first IR laser used for ART operated with a power output of 120mW, however, a maximum of 55mW was available at the objective plane. In addition to lasers operating in the IR range being much safer, it was also possible to adjust the diameter of the hole created by the ablation simply by adjusting irradiation time (Rink et al., 1996). The laser systems currently used in fertility clinics are solid-state non-contact infra-red light lasers operating with a 300mW or 400mW power, with the power level remaining constant and determined by the manufacturer. Therefore, to alter the hole size produced, pulse length is adjustable, with a longer duration of the laser pulse positively correlating with a larger hole produced. The product of the laser power and the pulse duration (time) is equal to the amount of energy delivered ($\text{Power} \times \text{Time} = \text{Energy}$). An application note was released in 2012 by Research Instruments reported that employing a higher laser power required less energy to produce a specific hole size compared to a lower power laser system (Figure 4.1)(Thompson, 2012). It has been hypothesised that the overall application of less energy to the embryo is beneficial for embryo health, however this has not been investigated in detail and embryologists are currently manipulating the settings at their own discretion.

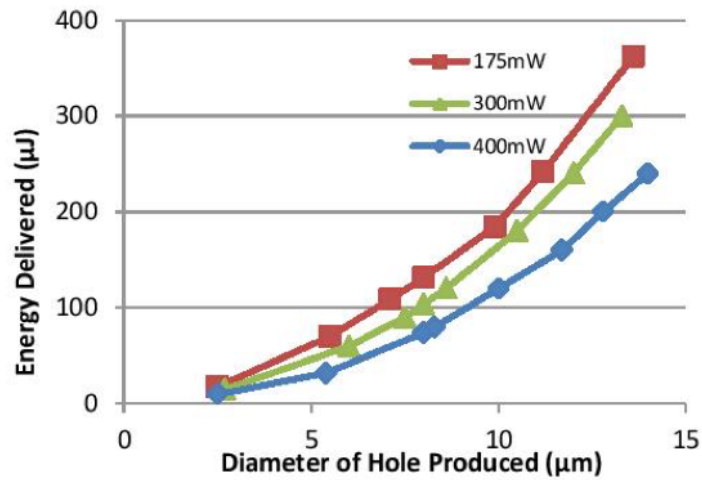


Figure 4.1: Comparison of the energy delivered and the diameter of the hole produced by 175mW, 300mW and 400mW power lasers. Adapted from the Research Instruments White Paper (Thompson, 2012).

4.2 Chapter aims

This study investigated the potential difference in damage to the cell membrane and DNA integrity caused by altering laser power to deliver a set amount of energy to preimplantation mouse embryos. The commercial 400mW Research Instruments Ltd clinical laser was compared to a custom-made 100mW laser manufactured by Research Instruments specifically for this study. The investigations aimed to determine which of the following hypotheses was true:

- The power of the laser does not matter - it is the amount of energy delivered that causes the damage, regardless of the length of time required to deliver the energy.
- The power of the laser does matter - if a higher power laser is used to deliver the same overall energy (over a shorter period of time), a smaller amount of damage is incurred.
- The power of the laser does matter - if a lower power laser is used to deliver the same overall energy (over a longer period of time), a smaller amount of damage is incurred.

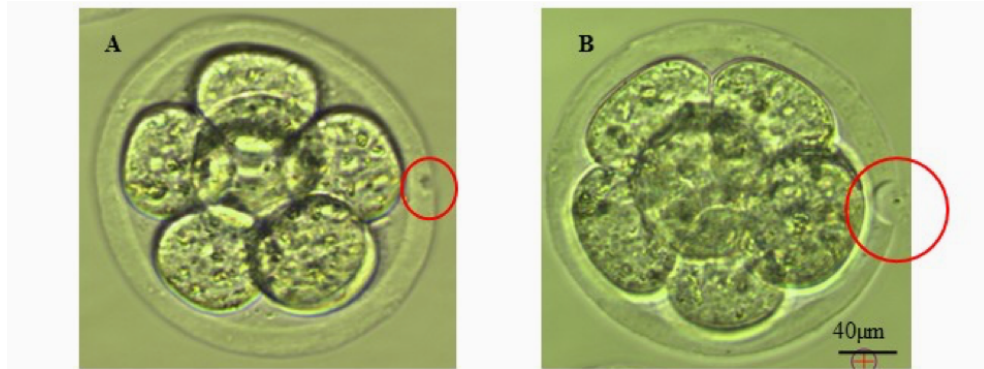


Figure 4.2: Brightfield image of different hole sizes created by a single 173.6mJ laser pulse emitted by the (A)100mW and (B)400mW laser. Red circle indicates the hole location.

4.3 Specialised materials and methods

Mouse embryos at the cleavage stage, with no morphological evidence of damage or developmental growth abnormalities, were selected and subjected to a single laser pulse to either the zona pellucida, or to the centre of a single blastomere, depending on the experimental conditions. Please refer to Chapter 2, Section 2.3.1 for the information regarding sourcing the mice and embryos. The non-contact infrared laser (Saturn 5 Active laser System; Research Instruments, UK) was attached to an inverted microscope (Nikon Eclipse TiS, Mirror module rev 1, Integra 3; Research Instruments, UK) fitted with a 40X objective and connected to a fixed power source of either 100mW or 400mW. Each experiment was performed in independent triplicates with a minimum of 10 embryos per replicate. In a droplet of culture media (M2 medium; Sigma Life Science, USA), overlaid with mineral oil, each embryo was subjected to a laser fired at a pulse length of 1.736ms or 0.434ms for the 100mW and 400mW, respectively, since each generated a total energy of 173.6 mJ (Figure 4.2). With a fixed energy, these values were calculated with the following equation; power X time = energy. The control group of embryos were not laser-treated. Hole sizes generated using the 100mW laser were all individually measured using the RViewer software measuring tool (Research Instruments, UK), since the software was initially calibrated using the 400mW laser, which produced different hole sizes at the respective pulse length durations.

4.4 Results

4.4.1 Preliminary studies exploring the 100mW laser

Preliminary studies were carried out to assess the functionality of each laser by determining the different hole sizes created at specific energies with both laser powers, as well as the pulse lengths required to emit such energies (Table 4.1). The mean thickness of the zona pellucida was determined to be $15.5\mu\text{m} \pm 0.38$, from three independent measurements of 16 embryos. The highest possible pulse length which could be delivered by the 100mW power laser was 2.0ms, therefore energies to be examined needed to use pulse lengths below this value. In deciding on which parameters to investigate, hole size was also considered to ensure that energies emitted by both lasers produced hole sizes that were observable. For the 400mW laser, the energy needed to produce a hole size of $24\mu\text{m}$ was 173.6 mJ, and a pulse length of 0.434ms. Using the 100mW power laser to deliver this same energy, a pulse length of 1.736ms was required, and created a hole size of $9.73\mu\text{m}$. Although the 100mW laser produced a relatively small hole size, it was clearly observable and all other lower energies delivered using the 100mW laser were difficult to observe and measure.

Next, it was determined whether it was the hole size or the total amount of energy delivered which should be kept constant when comparing the two laser systems. If the hole size was kept constant, instead of energy, the results could have been inaccurate since specific hole sizes created could vary depending on the thickness of the zona pellucida. Therefore, for the experiments requiring a single laser pulse, an energy of 173.6 mJ was kept constant for both lasers. By keeping the energy constant, it allowed for a more straightforward comparison of the effects of altering power or energy. Moreover, this energy level was selected since it presented the most considerable hole size for the 100mW laser. This preliminary study confirmed the trend described in the application note released by Research Instruments which suggested that the higher the power, the larger the hole size produced by the ablation delivered at the same energy (Thompson, 2012).

Table 4.1: Relationship between energy, pulse length, and hole size created by 100mW and 400mW lasers.

400mW			100mW	
Energy (mJ)	Pulse length (ms)	Hole size (μm)	Pulse length (ms)	Hole size (μm)
288.0	0.720	32	2.880	N/A*
173.6	0.434	24	1.736	9.73
86.0	0.215	16	0.860	7.02
41.2	0.103	8	0.412	3.57
23.6	0.059	4	0.236	Unmeasurable**

*laser power does not reach this pulse length; **hole too small to measure

4.4.2 400mW *versus* 100mW laser power in delivering a single pulse of 173.6mJ to cleavage stage embryos

In the following set of data, embryos at the cleavage stage were treated with a single pulse to the zona pellucida from either the 400mW or 100mW power laser delivering a total energy of 173.6mJ. Embryos were laser-treated on day 3 with a single pulse to the outer edge of the zona pellucida, at the location of the first laser pulse that would be administered when laser treating for clinical embryo biopsy. After laser treatment to the outer edge of the zona pellucida, as described in Chapter 2 Section 2.3.3, the embryos were stained immediately with CFSE and PI to assess blastomere viability. Embryos were also laser-treated and cultured to the blastocyst stage, Gardner graded (Gardner and Schoolcraft, 1999), then stained with CFSE and PI, or assessed for DNA fragmentation using the TUNEL assay, as described in Chapter 2 Sections 2.3.4, 2.3.5, and 2.3.6, respectively. Each experiment was performed in independent triplicates with a minimum of 10 embryos per measurement.

4.4.2.1 Immediate effects upon cell viability

PI staining was used to assess cytotoxic effects of laser treatments by assessing membrane integrity and CFSE was used to assess hydrolase metabolic activity (Figure 4.3). DAPI was used to counterstain the nuclei of the blastomeres. PI staining of the embryos immediately after laser treatment showed that the embryos in the control group had the lowest proportion of blastomere damage (9.65%) while

the 100mW group recorded the highest percentage of damage at 11.6% (Figure 4.4). No significance was observed between the controls or either laser power for PI staining ($P=0.8114$). CFSE staining was used to ensure that the cells in the embryo that were not showing PI staining were healthy and showing metabolic hydrolase activity. The 400mW laser exhibited the lowest proportion of CFSE staining (70.2%), while the control and 100mW exhibited 93.9% and 87.76%, respectively (Figure 4.5); however there were no significant differences between any of these values ($P=0.1653$).

4.4.2.2 Growth to blastocyst stage

No significant difference in terms of PI damage was observed immediately following laser treatment, therefore embryos were then subjected to the same laser treatments and then cultured to the blastocyst stage in KSOM media in three independent replicates. The Gardner grading system was employed to morphologically evaluate the embryos and determine how many, as a proportion, reached a 'good quality' stage. 'Good quality' embryos were those which were graded as 3-5AA or 3-5AB (see Chapter 2 Section 2.3.5). In the non-laser-treated control group, 74 of 113 embryos (65%) grew from cleavage stage to 'good quality' blastocysts. In the 100mW laser-treated group, 103 of 137 embryos (75%) grew from cleavage stage to 'good quality' blastocysts. In the 400mW laser-treated group, 101 of 138 embryos (73%) grew from cleavage stage to 'good quality' blastocysts. No significant differences were observed between these survival rates (Figure 4.6).

4.4.2.3 Viability at blastocyst stage

After laser-treating the embryo ZP at the cleavage stage, and then culturing the embryos to the blastocyst stage, the 'good quality' blastocysts were assessed for cytotoxicity and metabolic activity with PI and CFSE (Figure 4.7). The highest level of PI damage was observed in the group treated with the 400mW laser, exhibiting a mean of 16.1% cells showing damage. The control group and the 100mW laser treated group exhibited 9.3% and 9.2% damage, respectively (Figure 4.8). As laser power increased, a trend of increasing PI damage was observed, however there were no statistically significant differences between the values ($P=0.1525$).

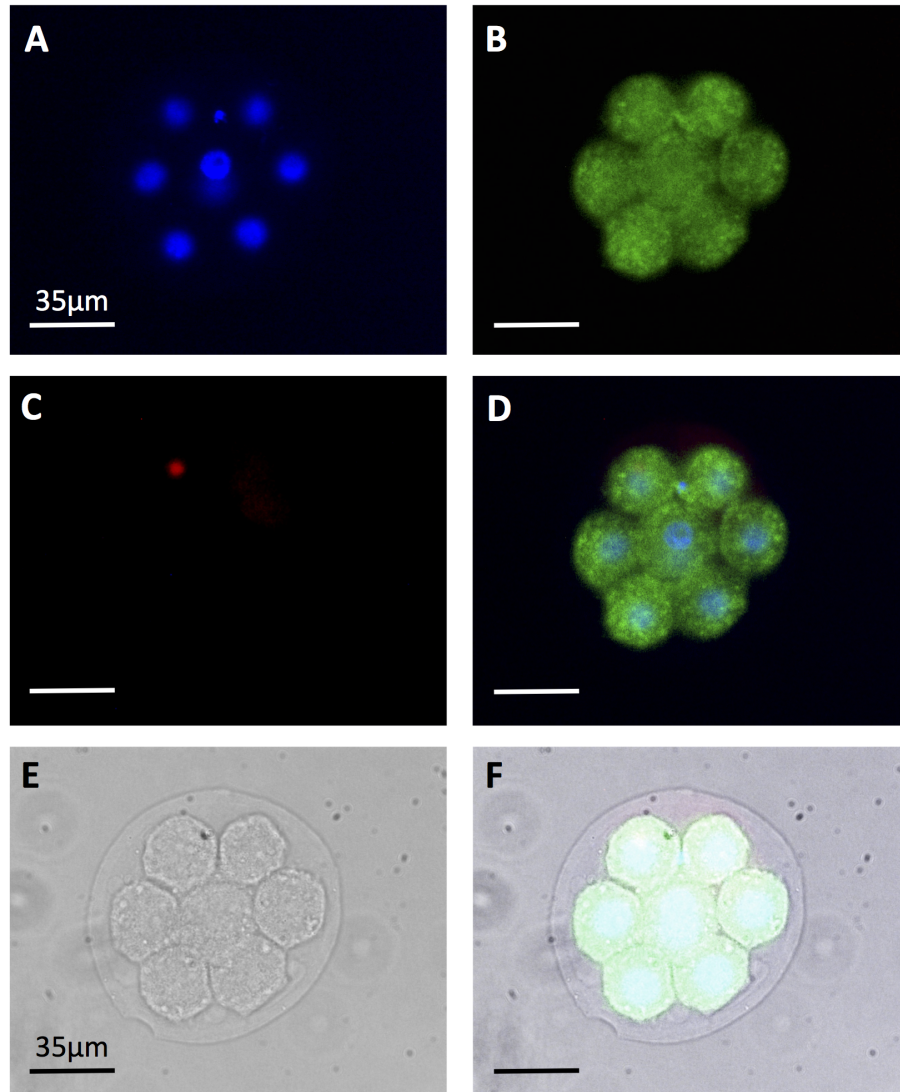


Figure 4.3: Representative images of cleavage stage mouse embryos laser-treated with 400mW and stained with (A) 4,6-diamino-2-phenylindole (DAPI-blue), (B) carboxyfluorescein succinimidyl ester (CFSE-green), and (C) propidium iodide (PI-red). (D) Composite overlay of fluorescent channels. (E) Brightfield image. (F) Composite overlay of all fluorescent channels and brightfield.

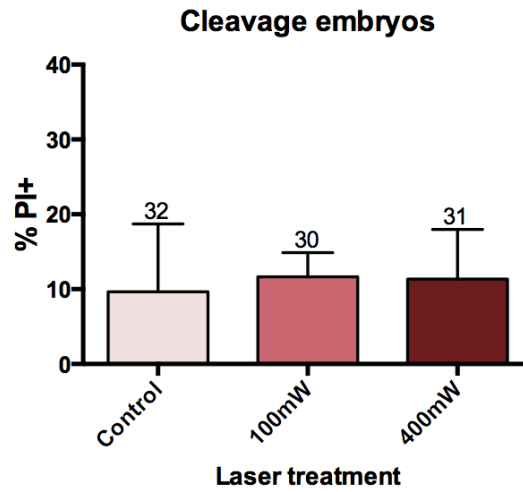


Figure 4.4: Mean proportion ($\% \pm \text{SEM}$) of cleavage stage mouse embryos showing cytotoxicity via positive propidium iodide (PI) staining immediately following laser treatment with 100mW or 400mW to the outer edge of the zona pellucida. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each.

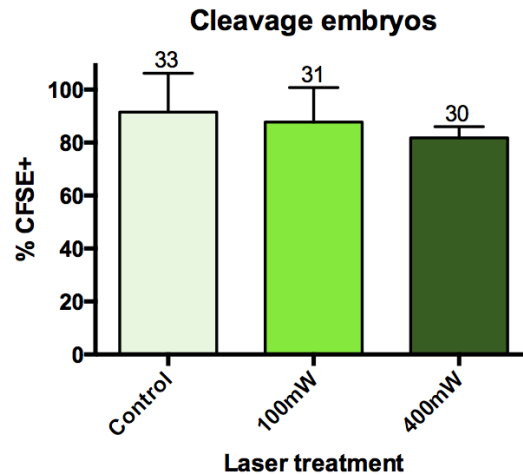


Figure 4.5: Mean proportion ($\% \pm \text{SEM}$) of cleavage stage mouse embryos showing metabolic activity via positive carboxyfluorescein succinimidyl ester staining (CFSE) immediately following laser treatment with 100mW or 400mW to the outer edge of the zona pellucida. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each.

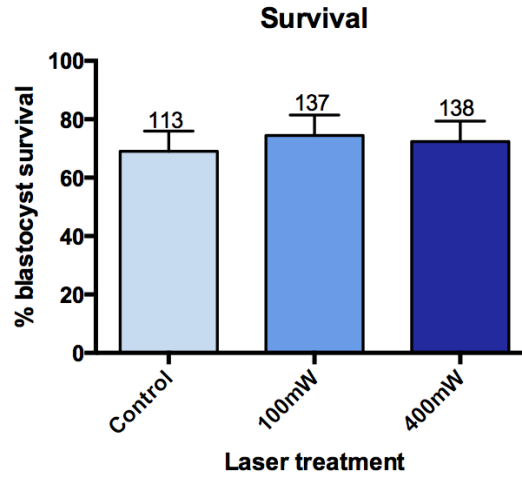


Figure 4.6: Mean proportion of mouse embryo survival to 3-5AA/AB grade blastocyst stage (%±SEM), determined by Gardner grading, following infra-red laser treatment with 100mW or 400mW. Data was derived from 113, 137, and 138 embryos for the control, 100mW, and 400 mW treatments, respectively.

The proportions of CFSE stained cells in blastocysts indicated healthy metabolic activity in the blastocyst cells with no significant differences between the controls and laser treated conditions (Figure 4.9).

4.4.2.4 DNA fragmentation at blastocyst stage

Following laser treatment at the cleavage stage and growth to the blastocyst stage, 'good quality' (3-5AA or 3-5AB) embryos were assessed for DNA fragmentation using the TUNEL assay as described in Chapter 2 Section 2.3.6. The apoptotic index was expressed as a percentage and determined as the number of TUNEL positive cells divided by the total number of DAPI stained cells (Figure 4.10). Interestingly, the control group exhibited the highest apoptotic index (19.2%), with the 100mW and 400mW groups exhibiting indices of 15.8% and 15.9%, respectively (Figure 4.11). Neither laser-treated group showed any significant differences compared to the control ($P=0.4679$).

4.4.3 Confirmation that both 100mW and 400mW laser treatment can cause damage

Interestingly, all of the investigations above, comparing 100mW and 400mW laser treatment, showed no significant differences when compared to each other or to

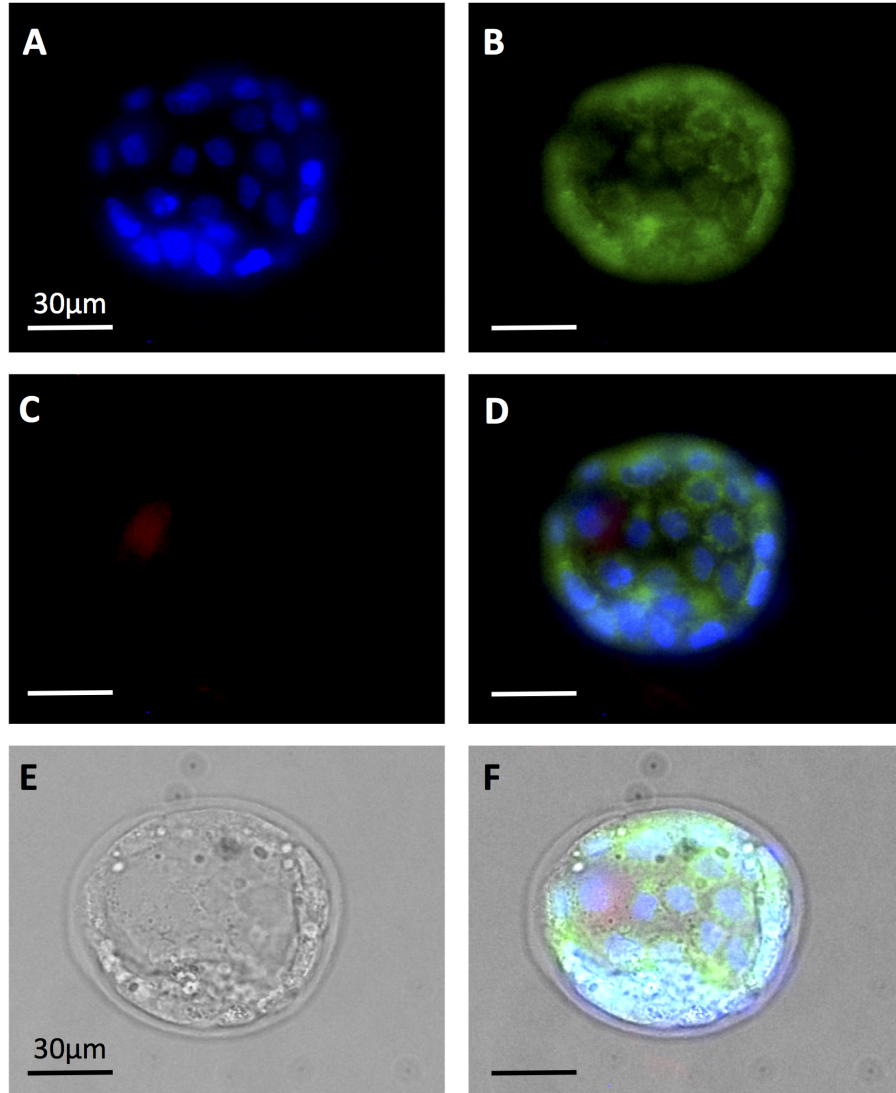


Figure 4.7: Representative images of blastocyst stage mouse embryos laser treated with 400mW at the cleavage stage then cultured and stained with (A) 4,6-diamino-2-phenylindole (DAPI-blue), (B) carboxyfluorescein succinimidyl ester (CFSE-green), and (C) propidium iodide (PI-red). (D) Composite overlay of fluorescent channels. (E) Brightfield image. (F) Composite overlay of all fluorescent channels and brightfield.

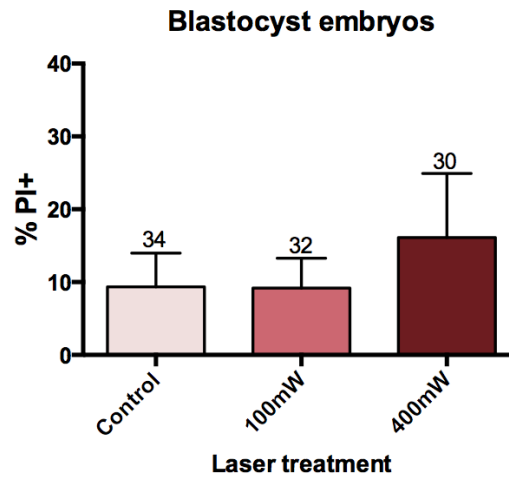


Figure 4.8: Mean proportion ($\% \pm \text{SEM}$) of mouse embryos cultured to the blastocyst stage showing cytotoxicity via positive propidium iodide (PI) staining following laser treatment with 100mW or 400mW to the outer edge of the zona pellucida at the cleavage stage. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each.

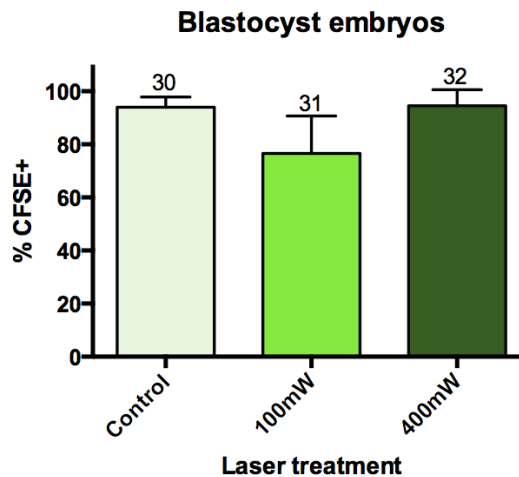


Figure 4.9: Mean proportion ($\% \pm \text{SEM}$) of mouse embryos cultured to the blastocyst stage showing metabolic activity via positive carboxyfluorescein succinimidyl ester staining (CFSE) following laser treatment with 100mW or 400mW to the outer edge of the zona pellucida at the cleavage stage. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each.

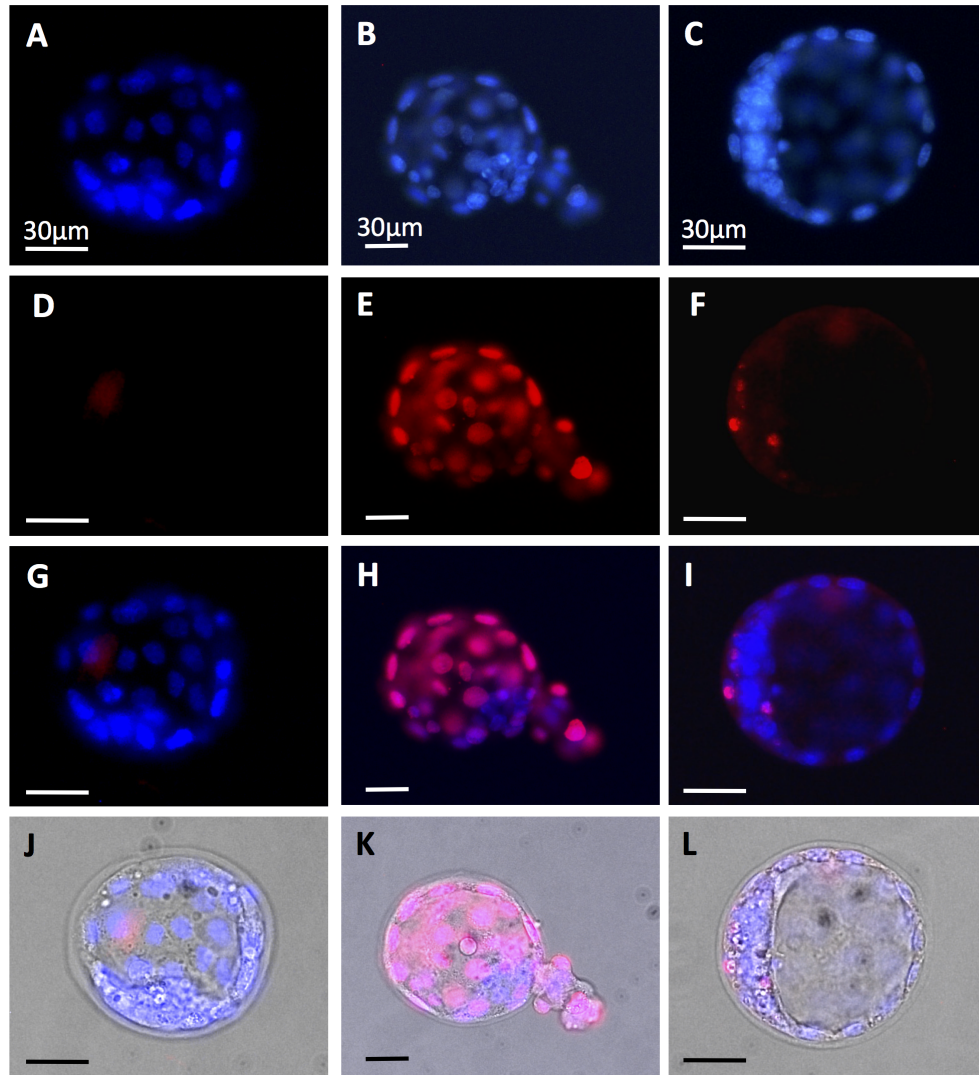


Figure 4.10: (A,D,G,J) Representative images of non laser-treated negative controls. (B,E,H,K) Representative images of DNase treated positive controls. (C,F,I,L) Representative images of blastocyst stage mouse embryos laser treated with 400mW at the cleavage stage then cultured to blastocyst. Embryos were stained with (A,C,B) 4,6-diamino-2-phenylindole (DAPI-blue), and (D,E,F) terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL-red) to assess DNA fragmentation. (G,H,I) Fluorescent channel composite images. (J,K,L), Composite images of fluorescent channels and brightfield image.

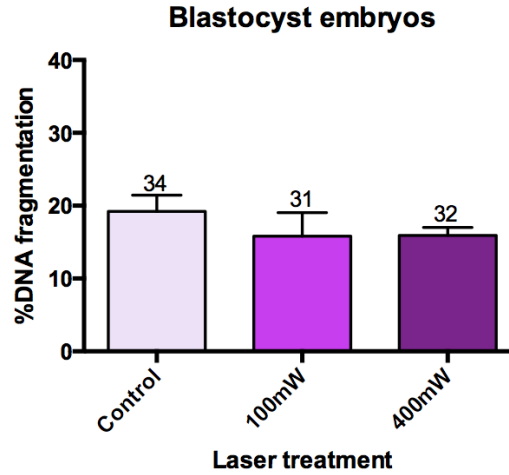


Figure 4.11: Mean proportion ($\% \pm \text{SEM}$) of blastocyst mouse embryos showing DNA fragmentation via TUNEL positively stained cells (apoptotic index) following laser treatment with 100mW or 400mW at the cleavage stage. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each.

controls. This could be due to the laser pulse being located towards the outer edge of the zona pellucida. As a proof of principle, before going forward with different laser location experiments, each laser was used to treat the centre of a blastomere cell, to investigate whether molecular damage indeed was observed. A single pulse of 173.6mJ (the same as used in the above investigations), was applied with each laser to respective embryo blastomeres, and immediately analysed for cytotoxicity and metabolic activity with PI and CFSE. The highest level of cytotoxicity observed was following treatment with the 400mW laser, with 43.2% of laser-treated embryos showing damage to the integrity of the plasma membrane. The 100mW laser-treated group displayed 20.8% of embryos with damage, and the control showed 9.7%. The level of damage caused by the 400mW laser was significantly higher than both the 100mW group and the controls ($P < 0.05$) (Figure 4.12). There was no significant difference in PI staining between the control and the 100mW laser-treated groups. The CFSE staining followed an opposite trend with the 400mW, 100mW, and control groups exhibiting 25.8%, 74.4%, and 91.5% staining, respectively. The 400mW treated values were significantly lower than both the 100mW and control groups ($P < 0.05$) (Figure 4.13). There were no significant differences in CFSE staining

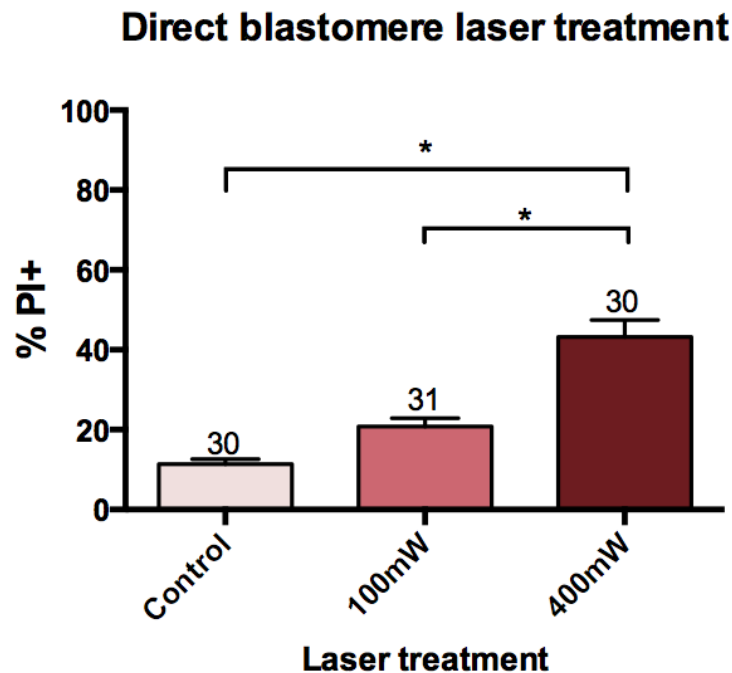


Figure 4.12: Mean proportion ($\% \pm \text{SEM}$) of cleavage stage mouse embryos showing cytotoxicity via positive propidium iodide (PI) staining immediately following laser treatment with 100mW or 400mW to the centre of a blastomere cell. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each. *denotes $P < 0.05$.

between the control and the 100mW laser-treated groups.

These results showed that laser treatment does cause direct damage to embryo cells, at least when directed to the centre of a blastomere, and that further laser treatments should be investigated for any significant effects. Since the initial experiments with a single pulse to the outer edge of the zona pellucida showed no significant differences, it was hypothesised that comparing laser treatment with multiple pulses, similar to the practices currently employed in clinic, might show more insights into differences in damage between the two powered lasers.

4.4.4 Investigating multiple pulses delivered by the 100mW and 400mW laser

Following discussion with the embryo biopsy specialist from Oxford Fertility, UK (personal communication, Mrs Tracey Griffiths, Senior Embryologist at Oxford

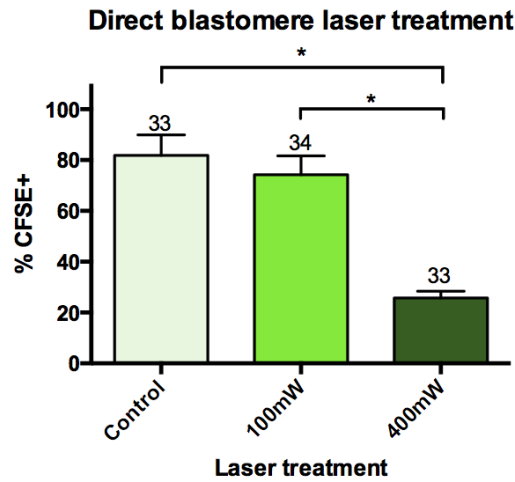


Figure 4.13: Mean proportion ($\% \pm \text{SEM}$) of cleavage stage mouse embryos showing metabolic activity via positive carboxyfluorescein succinimidyl ester staining (CFSE) immediately following laser treatment with 100mW or 400mW to the centre of a blastomere cell. Control embryos were not laser-treated. Data derived from three independent replicates of a minimum of 10 embryos each. *denotes $P < 0.05$.

Fertility), preliminary experiments were carried out investigating multiple hole treatments delivered similarly to day 3 treatment for trophectoderm biopsy. Since the single laser pulse comparisons to the zona pellucida did not show significant differences, it was hypothesised that mimicking clinical applications more closely, using multiple pulses, would better highlight the effects of these laser treatments. The hole sizes used for human biopsy size were scaled down to an appropriate size for mouse embryo size. A maximum of 3 pulses from the 400mW laser at a pulse length of 0.130ms emitting an energy of 52mJ per pulse, and creating a hole size of $7.5\mu\text{m}$ were delivered from the outside edge of the ZP inwards in cleavage stage embryos, until a hole traversing the whole ZP was created (Figure 4.14). Embryos were then cultured to the blastocyst stage to allow trophectoderm herniation through the created hole upon expansion, and assessed for DNA fragmentation. It was hypothesised that the 100mW laser may require more holes to be created to traverse the zona pellucida, since the holes produced would be smaller, and it was unknown whether an increased number of smaller hole created a different level of damage. This experiment could not be completed due to a technical malfunction of the 100mW laser, which was sent for repair. Unfortunately, the repair had not been

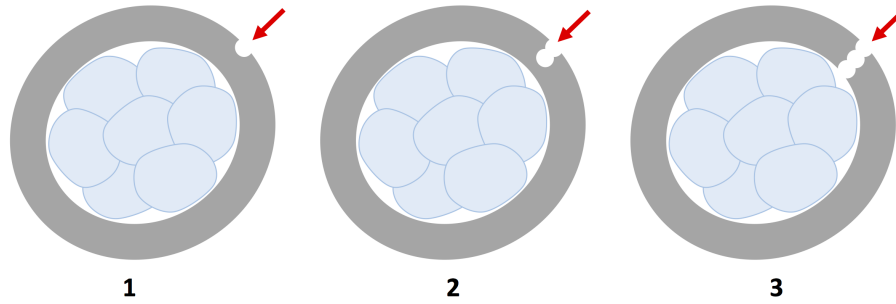


Figure 4.14: Diagram showing direction of multiple overlapping laser pulses to breach the zona pellucida at the day 3 cleavage stage prior to day 5 trophectoderm biopsy. The red arrow indicates the location and direction of laser pulses.

completed at the time of submission. Preliminary data from this experiment can be found in Appendix B Supplemental Data. Future directions involve the completion of this data set with the 100mW laser and a comparison with results from the controls and 400mW laser treated embryos.

4.5 Discussion

Current laser systems have evolved in an attempt to maximise safety to both the user and embryos, as well as fit a wide range of clinical applications to improve ART success rates. The application of laser systems in fertility treatment has become a globally accepted, and often preferred, method for clinical zona pellucida drilling. Nevertheless, there is still insufficient information regarding the safety and standardisation of this technology for embryos at the molecular level. The investigations herein focused upon laser power and energy, utilising a range which produced hole sizes that were clearly visible and measurable in the zona pellucida. Preliminary experiments confirmed that when an equal amount of energy was delivered using both powered lasers, that the 400mW laser will cause a larger ablation hole compared to the 100mW laser. Therefore, for practical implementation, more pulses will be required from a 100mW laser compared to the 400mW laser to create a specific hole size or to ablate an equal surface area of zona pellucida.

In this chapter, embryos were laser-treated on day 3 at the cleavage stage since in the clinic laser treatments for embryo biopsy (both blastomere and trophectoderm)

are performed on cleavage stage embryos. The fluorescent markers PI and CFSE were used to investigate cytotoxicity and metabolic activity immediately after laser treatment at the cleavage stage. No significant differences were observed in terms of cytotoxicity between the controls or either 100mW or 400mW laser conditions. Chatzimeleiou performed two studies involving laser drilling the zona pellucida and staining with CFSE and PI in 2001 and 2005. Interestingly, these authors compared their results from their 2001 study to the results they obtained in 2005. The authors state that in 2005, a higher powered laser was used than in 2001, and that less damage was observed with the higher powered laser, which is not congruent with my non-significant results (Chatzimeleitiou et al., 2001; Chatzimeleitiou et al., 2005). However, the authors did not state the specifications of either laser power, and these studies were performed so far apart, that one cannot reliably compare damage caused by different laser powers from these two studies. Additionally, comparing laser power was not the primary aim for either of these studies.

In the present investigations, over 65% of embryos in all three conditions survived to grow to 'good quality' blastocyst stage, with no significant difference between the laser-treated embryos and the controls, suggesting that neither of the powers did not impede further embryo growth. Conversely, a previous study by Malter et al. showed that laser-treated embryos exhibited a significant reduction in blastocyst development as determined by morphological assessment in terms of compaction, cavitation, and implantation in a mouse model (Malter et al., 2001). Additionally, many previous studies involving laser treatment investigations did not specify details of the laser hardware or administration procedures, therefore it is difficult to interpret and compare the present data with past reports. The embryos treated with the 400mW laser and then cultured to blastocyst stage exhibited the highest proportion of PI cytotoxicity when stained at the blastocyst stage, however these levels were not significantly different to either 100mW laser-treated embryos or non-laser-treated controls.

A report by Kamjoo and colleagues demonstrated that different strains of mice and different compositions of embryo culture medium can affect DNA fragmentation

rates in control embryos (Kamjoo et al., 2002). In the present chapter, there were no significant differences between the three groups in terms of DNA fragmentation, and all of the apoptotic indices were comparable to the value of 14.4% reported by Kamjoo et al. as the mean apoptotic index of healthy control C57BL6 mouse embryos cultured in KSOM medium (Kamjoo et al., 2002). It is possible that these results could indicate that the laser drilling was administered at a safe distance or that embryos are capable of recovering from transient damage incurred at the cleavage stage.

Since the investigations using one laser pulse from each laser to the outer edge of the zona pellucida demonstrated no significant differences, to confirm whether both laser systems could incur damage, investigations were carried out using each laser to laser treat a single blastomere cell in cleavage stage embryos. Each laser was used to deliver the same amount of energy to the centre of one blastomere cell on day 3 and assessed for PI and CFSE staining. Laser treatment with the 400mW laser caused a significantly higher level of PI staining compared to both the 100mW treatment and the controls, and caused a significantly lower proportion of cells showing CFSE staining compared to both 100mW and control conditions. The 100mW laser showed no significant difference in PI or CFSE staining compared to the controls. These results served as a successful proof of principle that laser treatment can incur damage to embryonic cells. Interestingly, at the energy of 173.6mJ, the 100mW laser exhibited a slightly higher amount of PI staining than the control, but this was not significant, suggesting that even direct laser treatment to an embryonic cell using the 100mW laser is safe, even though it causes a visible hole when ablating the zona pellucida, and is more safe than the 400mW laser.

Once the laser is fired, the pulse comes into contact with media in the culture dish; therefore heat is generated and transferred through the media creating temperature gradients around the embryo which may compromise further embryo development. The heat produced can be influenced by the three laser factors, and the shorter the duration of the laser beam, the less heat produced and the lower the temperature gradient. This chapter aimed to investigate potential damage caused by different

laser powers when drilling the zona pellucida and blastomere cells. A study by Taylor et al. investigated the effects of altering pulse lengths using a 400mW Research Instruments laser for embryo biopsy and the result on blastomere lysis during biopsy (Taylor et al., 2010). Interestingly, they found that altering pulse length (range of 0.604-1.010ms) did not result in any significant changes in blastocyst development. However, the three pulse lengths they investigated resulted in three different sized holes, and the authors did not state whether the same number of pulses were administered for each group. Moreover, the authors reported that the resulting hole sizes are in the nanometer range, when they actually should be in the micrometer range, and appear visible in representative brightfield images (Taylor et al., 2010).

4.6 Limitations

Many insights into mammalian embryo development have been initiated by studies in mice. However, limitations are presented by species-specific differences, which should be taken into consideration when interpreting data, such as patterns of gene expression and epigenetic modifications (Niakan et al., 2012). In addition, ideally, the mouse superovulation and reproductive tract dissection should have been carried out in the same laboratory as the embryo retrieval to avoid transportation of the reproductive tracts from one building to another outside of an incubator. Nonetheless, the mouse remains one of the most powerful models for mammalian preimplantation embryo development, and the present investigations serve as a strong foundation into exploring the effects of altering laser power when administering laser treatment to embryos.

4.7 Conclusions

The data presented in this chapter does not present conclusive evidence on the safety of different powered laser treatments on embryos for clinical procedures. The experiments do however provide evidence at the molecular level to contribute towards the establishment of standardised and safe parameter for laser application.

Results from this chapter demonstrate that the power of the laser does indeed matter in terms of causing damage to embryonic cells, as the 400mW laser caused significant damage to blastomeres while the 100mW did not. This needs to be explored further, as practically, the 100mW laser would require multiple pulses to create the same ablation hole size as the 400mW laser.

4.8 Future directions

These investigations, involving single pulses, allowed for simple controllable comparison between conditions, however, do not mimic clinical procedures for embryo biopsy, where multiple pulses are used to traverse the whole zona pellucida. Therefore preliminary studies were started to investigate potential effects of a more clinically relevant approach with multiple laser pulses (pilot data shown in Appendix B Supplemental Data). Day 5 trophectoderm biopsy involves an initial laser treatment at day 3 to breach the zona pellucida to create an opening through which trophectoderm cells can herniate out as the blastocyst forms and expands. Experiments comparing clinical biopsy methods between the two laser systems was started but not completed due to a technical hardware malfunction requiring the laser to be sent for repair. Further experiments should be continued carrying out full trophectoderm biopsy to complete the dataset to allow for comparison of biopsy procedures using both laser systems. Data derived from such experiments would provide a more full picture regarding the effects of altering power both at the initial laser application on day 3, and on day 5 when laser pulses are used to target the cell junction between trophectoderm cells. It would be ideal if the amount of total energy in these more complicated procedures could also be controlled and kept constant.

4.9 Key messages

- The lasers currently used in fertility clinics are non-contact infra-red light lasers operating with a 300mW or 400mW power
- Potential damaging effects via thermal excursion may be affected by the power of the laser used to deliver the required amount of energy for the desired hole size
- The power of the laser does matter - if a lower power laser is used to deliver the same overall energy (over a longer period of time), a smaller amount of damage is incurred

5

Global gene expression analysis of laser-treated preimplantation embryos using RNA sequencing

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5.1 Specialised background

To learn about the functional elements of the genome, the molecular constituents of cells, and to understand the development of disease, the transcriptome must be understood. Gene expression is often used as a proxy for protein abundance, but these are often not equivalent due to post-transcriptional events (Weil, 2015). The regulation of protein expression is orchestrated by trans-acting factors with cis-regulatory elements. Specific regions in the transcript are targeted by factors to elicit different outcomes. Post-transcriptional modifications, such as deadenylation, polyadenylation, transport, and storage can influence the subsequent protein expression (Weil, 2015). There have been different methods to explore and quantify transcriptomes, based on either hybridisation techniques or sequencing techniques. The first expression studies utilised hybridisation-based microarrays, however these techniques often led to poor quantification of low and high level expressed genes, exhibited high levels of background and cross hybridisation artefacts, and could be confounded by saturation of signal. Additionally, these approaches required prior knowledge of the genome sequence to be investigated, and could require complex normalisation procedures when comparing outputs from different experiments (Wang et al., 2009). Early sequencing methods involved using Sanger sequencing to directly determine the cDNA sequence, however the Sanger method was relatively low throughput and expensive. Conversely, the emergence of RNA sequencing methods, which uses next generation sequencing (NGS) to quantify levels of RNA in a biological sample at a specific time, have clear advantages over the other approaches (Wang et al., 2009; Tang et al., 2010; Kukurba and Montgomery, 2015).

RNA sequencing uses 'deep sequencing', which refers to sequencing the same genomic region multiple times, to use a single assay to allow researchers to detect both known and novel features of a transcriptome, for example: isoforms, gene fusions, single nucleotide polymorphisms, and allele-specific gene expression. It provides a more sensitive and accurate measurement of gene expression due to its broader dynamic range, and is not limited by requiring prior knowledge of the genome. Background signal, if any, is low due to the fact that the DNA sequences

can be unambiguously mapped to unique regions of the genome. Moreover, RNA sequencing can provide information regarding precise locations of transcriptome boundaries, for example to reveal how two exons are connected to a single base pair resolution (Wang et al., 2009; Tang et al., 2010; Kukurba and Montgomery, 2015).

To begin RNA sequencing, RNA must be isolated from the biological sample of choice. Following total RNA isolation, different populations of RNA can be selected for different examinations. For example, for a population of total RNA, no selection is needed, for coding RNA then polyA selection can be performed, for coding and non-coding RNA an rRNA depletion selection can be performed, and for targeted RNA an RNA capture selection can be utilised. RNA populations can also be selected based on size. The desired RNA population is fragmented and converted into cDNA, and sequencing adaptors are ligated to one or both ends. Each cDNA molecule is then sequenced in a high throughput manner to obtain short sequences from one or both ends. The sequence reads are then aligned to the genome and transcripts are assembled. Sequence reads may also be assembled into transcripts using *de novo* assembly approaches if required. The expression level of each gene is estimated by quantifying the number of reads that align to each exon or transcript (Martin and Wang, 2011; Kukurba and Montgomery, 2015). Figure 5.1 illustrates data generation for RNA sequencing with NGS and Figure 5.2 illustrates how the sequence reads obtained from NGS are analysed.

Some limitations of the procedure are that the cDNA library preparation can be complicated, and sometimes identical short reads can be obtained from cDNA libraries which may be PCR artefacts. Nevertheless, the emerging technology for RNA sequencing, and single-cell RNA sequencing, has provided the opportunity to study transcriptome-wide dynamics during preimplantation embryo development (Wang et al., 2009).

Recent studies have emerged in the last few years utilising RNA sequencing with mouse embryos to uncover new information regarding preimplantation embryo development. For example, a study by Shi et al. (2015) analysed the transcriptome of

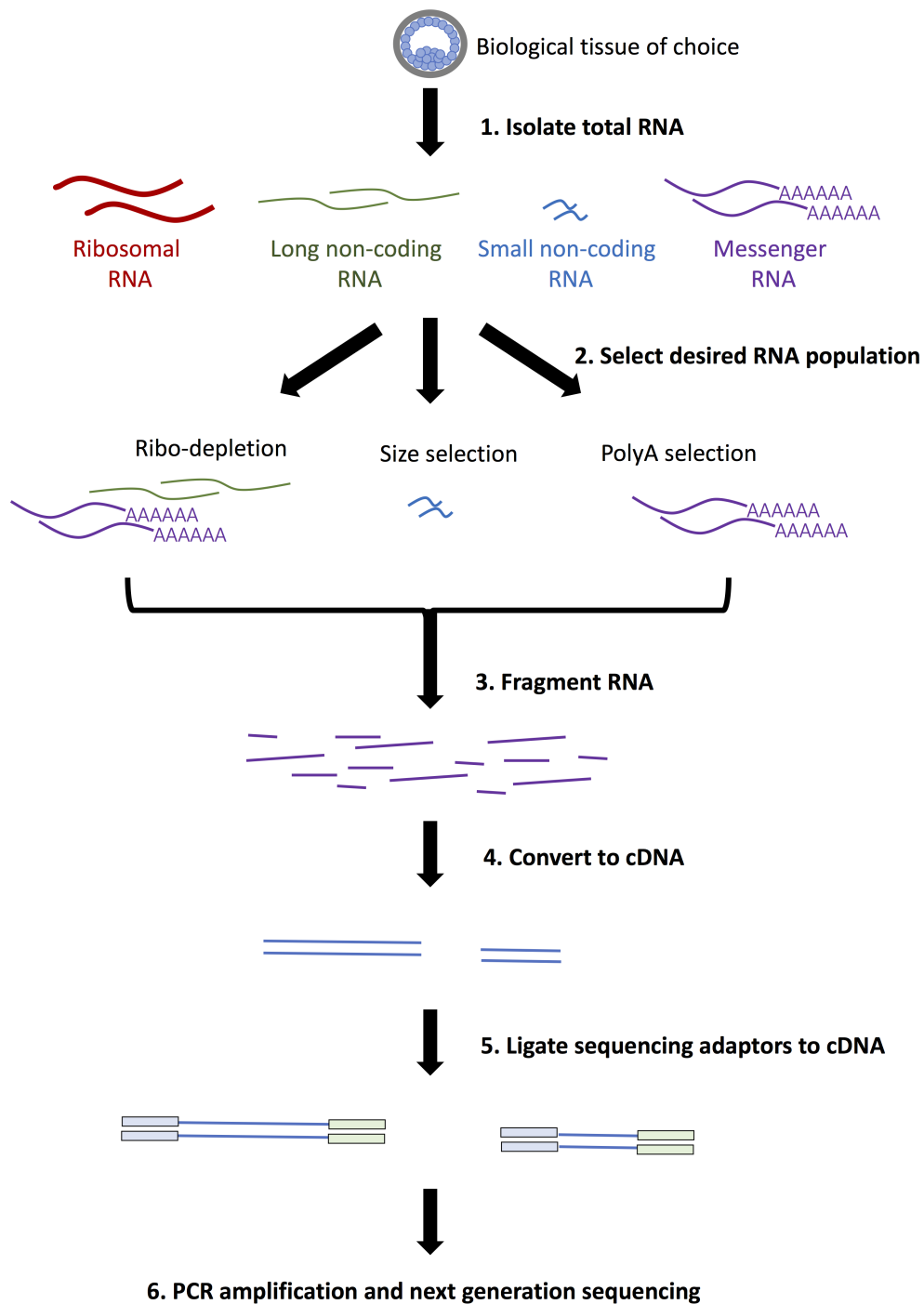


Figure 5.1: Schematic illustrating data generation for RNA sequencing. 1) RNA is isolated from the biological samples of interest. 2) If desired, a specific RNA population is selected from the isolated DNA. 3) RNA is fragmented. 4) RNA fragments are reverse transcribed into cDNA. 5) Sequence adaptors are ligated to one or both ends of cDNA fragments. 6) cDNA ends are sequenced using next generation sequencing. Figure created by author and adapted from Kukurba and Montgomery (2015).

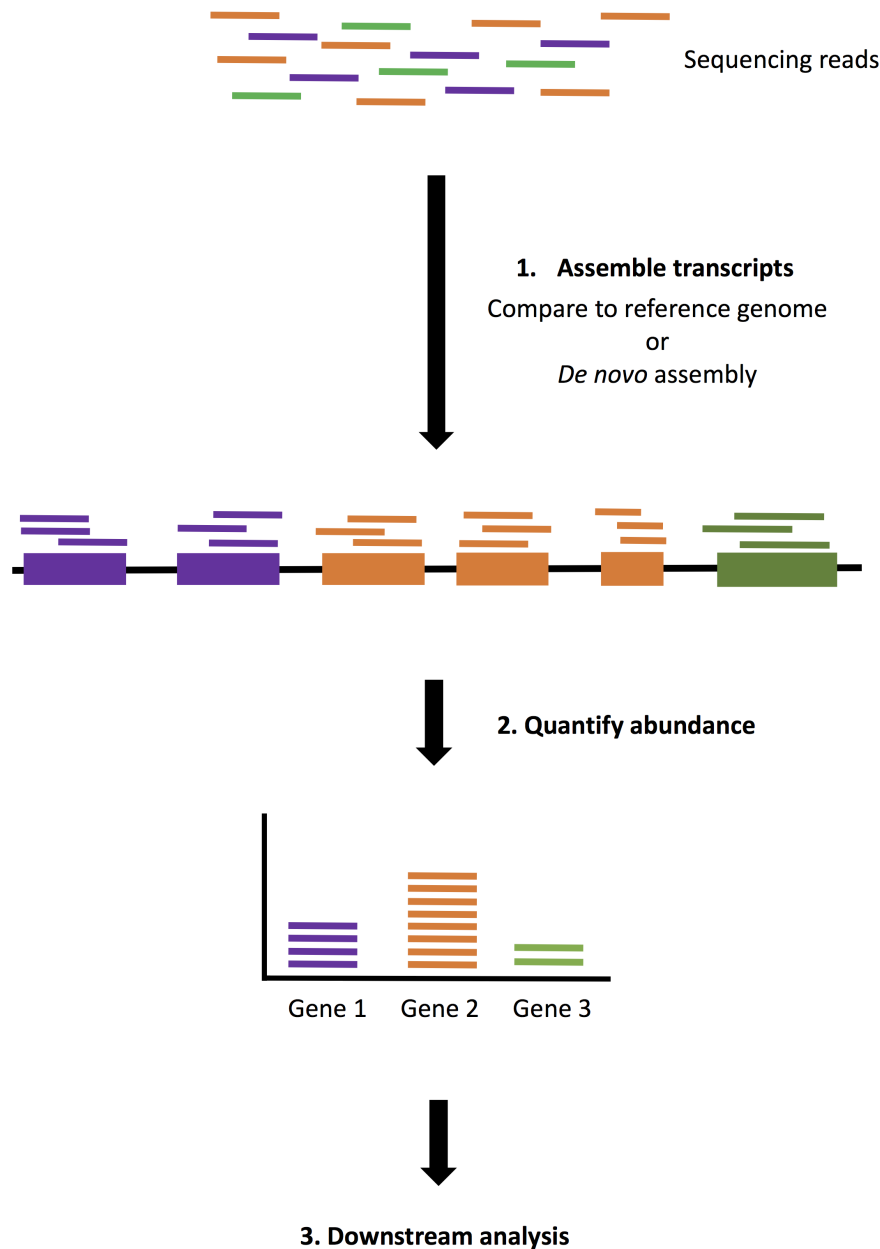


Figure 5.2: Schematic illustrating data analysis for RNA sequencing. 1) The sequencing reads obtained from next generation sequencing are assembled into transcripts either by comparison to a reference genome or by *de novo* assembly. 2) The abundance of gene transcripts is quantified by counting the number of reads aligning to each full-length transcript or exon. 3) Many downstream analyses on RNAseq data can be performed, such as testing for differential gene expression, and detecting allele-specific expression. Figure created by author and adapted from Kukurba and Montgomery (2015).

single cell blastomeres in mouse preimplantation embryos and found that blastomere-to-blastomere biases in symmetry and asymmetry emerge as early as the first embryonic cleavage division. The authors found that some genes showed asymmetry which continually increased between blastomeres, while other genes minimised asymmetry (Shi et al., 2015). Another study using RNA sequencing showed that expression patterns of protein coding genes were different at the 1-cell stage compared to other stages, but that no regions of interest of actively-expressed genes had elements specific to the 1-cell stage, suggesting that the unique gene expression pattern observed at the 1-cell stage was not governed by a specific promoter element (Yamamoto et al., 2016). Borensztein and colleagues used RNA sequencing to demonstrate that long non-coding RNA (lncRNA) Xist, expressed only from the paternal X chromosome, is essential for the initiation of imprinted X-chromosome inactivation in mouse embryos, with a lack of paternal Xist resulting in post implantation lethality (Borensztein et al., 2017). Posfai et al. (2017) used RNA sequencing to show that the trophectoderm transcription profile stabilises earlier than the ICM transcription profile, and prior to blastocyst formation, in mouse embryos, in response to *Hippo* signalling (Posfai et al., 2017). An interesting study employing RNA sequencing by Chen et al. (2015) found allele-specific expression of imprinted genes in both human and bovine embryos with large offspring syndrome. Bovine embryos are commonly used as an animal model for ART, and similar symptoms are seen in large offspring syndrome in ruminants as are seen in human loss of imprinting overgrowth Beckwith-Wiedemann syndrome. These overgrowth syndromes are also more prevalent in embryos created by ART. Chen and colleagues found a loss of allele-specific expression of imprinted genes in large offspring syndrome and differential transcript amounts of these genes between control and overgrown fetuses (Chen et al., 2015b).

5.2 Chapter aims

This chapter aimed to investigate global changes in gene expression in laser treated mouse embryos by performing RNA sequencing on a total RNA population. The

impact of laser treatment on embryos to the global transcriptome has not yet been investigated. Total RNA sequencing would provide the most complete view of the transcriptome and enable analysis of all forms of coding and noncoding RNA in a single experiment. It would allow for the analysis of thousands of genes in parallel, to help identify whether a specific cohort of genes are influenced by infra-red laser technology as applied in routine ART procedures. Data uncovered from such studies could be paramount in discovering and understanding the more intricate genetic profiles affected by laser ART treatments.

5.3 Specialised materials and methods

5.3.1 Isolating RNA for sequencing

Total RNA was isolated from batches of 12 pooled embryos using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, UK) according to the manufacturer's instructions. In the first set of optimisation experiments, pooled embryos were frozen in 3 μ l RNA later at -80°C, in the second set of optimisation experiments fresh pooled blastocysts were used immediately for RNA isolation without freezing. The embryos were transferred into 50 μ l of extraction buffer and incubated for 30 min at 42°C, then centrifuged at 800 $\times$ g for 2 min to collect the contents at the bottom of the tube. Samples were frozen at -80°C until all samples were collected.

RNA Purification Columns were pre-conditioned with 250 μ l conditioning buffer for 5 min at room temperature and centrifuged at 16 000 $\times$ g for 1 min. RNA samples were thawed and 10 μ l of 70% ethanol was added and mixed by pipetting up and down. The cell extract and ethanol mixture were loaded onto the pre-conditioned purification column. The column was centrifuged for 2 min at 100 $\times$ g to bind the RNA, immediately followed by a centrifugation at 16 000 $\times$ g for 30 s to remove flow through. The column was washed with 100 μ l wash buffer and centrifuged for 1 min at 8 000 $\times$ g.

The optional DNase treatment step was performed to ensure the degradation of any genomic DNA to prevent DNA contamination. It was reported by Norhazlin et al., (2015) that the inclusion of the DNase treatment when using mouse embryos

with the Pico Pure RNA Isolation Kit resulted in a higher quality of RNA (Norhazlin et al., 2015). The treatment was performed by adding 10 μ l DNase (2U/100 μ l in reaction buffer) to the column and incubating at 37°C for 10 min. One μ l of 0.5M ethylenediaminetetraacetic acid (EDTA) was added to protect the RNA and the DNase was heat-inactivated at 75°C for 10 min.

The column was washed twice more with 100 μ l wash buffer and centrifuged at 16 000 $\times$ g for 1min. The column was transferred to a fresh 0.5mL microcentrifuge tube and 11 μ l elution buffer was added directly onto the column membrane to elute total RNA. The column was centrifuged for 1 min at 1 000 $\times$ g to distribute the elution buffer in the column and then for 1 min at 16 000 $\times$ g to elute the RNA. From each sample, 1 μ l was analysed to determine RNA concentration and purity (A260/80 ratio) using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). The remaining sample volumes were stored at -80°C until being sent off for RNA sequencing with the 'High-throughput Genomics Centre' at the Wellcome Trust Centre for Human Genetics. Dr Sarah Lamble, my RNA sequencing project manager and main liaison with the Wellcome Trust, advised that we required a minimum concentration of 1.0ng/ μ l, and an A260/280 ratio of near 2.0.

5.4 Results

5.4.1 Preliminary testing of RNA isolation

RNA sequencing procedures are highly expensive, therefore before preparing experimental samples for analysis, preliminary experiments were conducted to test the isolation procedure of total RNA from pooled embryos. RNA was isolated according to the manufacturer's instruction, and the concentration and purity was assessed with a Nanodrop spectrophotometer. These experiments involved surplus cleavage stage embryos, as well as blastocysts. Nanodrop analysis revealed a high quantity of good quality RNA isolated from the blastocysts (Table 5.1). The ratio of absorbance at 260nm and 280nm is commonly used to assess the purity of nucleic acids, with a ratio of approximately 2.0 being considered accepted as "pure" for RNA (Glase, 1995).

Table 5.1: Nanodrop quality assessment of isolated RNA from pooled embryos in preliminary optimisation experiments.

Embryo stage	Concentration (ng/ μ l)	A260/280 ratio
Cleavage	4.89	1.51
Cleavage	6.14	1.61
Cleavage	10.51	1.48
Cleavage	13.95	1.45
Cleavage	5.63	1.54
Cleavage	5.65	1.42
Blastocyst	10.43	2.30
Blastocyst	10.89	1.88
Blastocyst	11.54	1.83

5.4.2 Isolating RNA from embryos for RNA sequencing

Following the positive results from these preliminary experiments, the same RNA isolation procedure was conducted on laser-treated embryos and control embryos. Eight groups of pooled embryos were laser-treated, and eight groups of pooled embryos were not laser-treated and served as controls. Each group of lasered embryos was paired with a group of control embryos that were harvested and cultured on the same day, pooled from a mix of embryos from three mice. Unfortunately, following the same RNA isolation procedure, analysis using the Nanodrop spectrophotometer did not show good quality RNA (Table 5.2). These nanodrop results were discussed with Dr Sarah Lamble, the Project Manager at the Wellcome Trust High-throughput Genomics Centre for my sequencing experiments, who said that we could run RNA sequencing with these samples, but that the quality was too low. Due to the expensive nature of RNA sequencing, I thought it was best to not run the sequencing on these samples, and to instead look for ways to improve the RNA quality obtained.

The first set of embryos isolated for RNA sequencing analysis used embryos that were frozen in RNAlater until all samples were collected. Since RNA quality is known to degrade with increased handling and freezing, in an attempt to obtain better quality RNA, new embryo samples were collected and the RNA was isolated immediately from fresh pooled blastocysts without freezing (Table 5.3)(Norhazlin

Table 5.2: Nanodrop quality assessment of isolated RNA from frozen pooled laser treated and control embryos.

Embryos	Concentration (ng/ μ l)	A260/280 ratio	A260/230 ratio
Control A	1.2	1.65	0.44
Control B	3.0	1.97	0.49
Control C	2.7	1.58	0.31
Control D	1.9	0.93*	0.38
Control E	3.3	1.30*	0.52
Control F	1.9	1.02*	0.35
Control G	11.3	1.74	0.48
Control H	6.2	1.73	0.22
Laser-treated A	6.0	1.28*	0.51
Laser-treated B	3.1	1.02*	0.21
Laser-treated C	11.7	1.44*	0.50
Laser-treated D	1.6	2.73	0.39
Laser-treated E	6.7	1.39*	0.55
Laser-treated F	2.0	2.10	0.51
Laser-treated G	5.2	1.80	0.41
Laser-treated H	6.9	1.69	0.51
*low quality RNA,		ideal ratio~2.0	ideal ratio~2.0

Table 5.3: Nanodrop quality assessment of isolated RNA from fresh pooled control embryos.

Embryos	Concentration (ng/ μ l)	A260/280 ratio	A260/230 ratio
Control A	12.07	1.54	0.11
Control B	13.45	1.49	0.12
*low quality RNA,		ideal ratio~2.0	ideal ratio~2.0

et al., 2015). Unfortunately, even using fresh embryos immediately, the quality of the isolated RNA was low, with A260/280 ratios of 1.54 and 1.49, when a ratio of near 2.0 is desired. While now outside of the scope of this thesis, due to time restrictions, future studies will be carried out using a fresh kit with fresh reagents, under aseptic conditions in a laminar flowhood. Additionally, the pipette tips and microcentrifuge tubes will be rested for RNA nuclease (RNase) contamination.

5.5 Discussion and future directions

The results from these experiments have been inconclusive thus far, as the actual RNA sequencing has not yet been performed. To be prudent and cost effective, RNA isolation will continue to be optimised to ensure that good quality RNA is sent for sequencing. Embryos utilised in this experiment were either frozen in RNAlater or used immediately in an attempt to protect RNA integrity, however both isolations resulted in poor quality RNA. Interestingly, the very first pilot experiment with the Arcturus PicoPure RNA Isolation kit, with control embryos frozen in RNAlater, did obtain good quality RNA. However subsequent experiments following the same protocol exhibited poor quality RNA in many samples. This could likely be due to contamination occurring while the experiments were being carried out. A specialised cabinet was used to conduct the RNA isolation, however the cabinet has also been used for qPCR preparation and could have been contaminated with RNases. Future attempts to isolate RNA for these experiments should use a laminar flowhood, have all equipment wiped down with a surface RNase decontaminant solution, test all reagents for RNase contamination, and use RNase free certified pipette tips and tubes. For example, lo-bind microcentrifuge tubes should be utilised to ensure they are free of RNase contaminants. Additionally, increasing the PCR cycling times from the manufacturer's suggestions should be explored as a means of potentially increasing RNA yield (Lorenz, 2012).

The Arcturus PicoPure RNA Isolation kit was selected for several reasons. First, it was advertised as a reliable kit to obtain RNA from a small amount of sample, which is often required for experiments involving embryos. Secondly, the kit utilises silica membrane columns for RNA extraction, which is a broadly used, efficient, and simple technique which does not require toxic organic solutions (Norhazlin et al., 2015). Thirdly, a recent study by Norhazlin et al. (2015) compared four different RNA isolation methods, with and without DNase treatment, and concluded that RNA isolation using the Arcturus PicoPure RNA Isolation kit with DNase treatment produced the best quality of RNA (Norhazlin et al., 2015).

Norhazlin et al. (2015) tested RNA isolation from 2-cell, 8-cell, morula stage, and blastocyst stage mouse embryos with and without DNase treatment using the following kits: TRIzol (Invitrogen, USA), RNeasyMini Kit (Qiagen, USA), NucleoSpin kit (Macherey-Nagel, USA), and the Arcturus PicoPure RNA Isolation Kit (Molecular Devices, USA). These authors used pools of 30 embryos, eluted the RNA in a 20 μ l fraction, assessed quality using a Nanodrop spectrophotometer, and validated the results with microarrays and qPCR. The Arcturus PicoPure RNA Isolation Kit utilised in my thesis (and by Norhazlin et al.) was specifically designed for low RNA content samples with low elution volumes. I followed the given protocol of eluting the RNA in 10 μ l, and used pools of 12 blastocysts. Norhazlin and colleagues pooled 30 embryos and eluted RNA in 20 μ l, which possibly could have contributed to increasing their quality of obtained RNA. It should also be noted, however, that the A260/280 ratios reported by Norhazlin and colleagues for their RNA obtained with the Arcturus PicoPure RNA Isolation Kit was only 1.62 and 1.83, which were similar to some of my obtained values, but still not close to 2.0. Additionally, Norhazlin et al. only tested two groups of 30 pooled embryos, whereas my experiment tested 16 groups of pooled embryos. While some of my samples indeed showed A260/280 ratios similar to those reported by Norhazlin, there were too many samples with much lower ratios to be considered good enough quality to utilise for subsequent RNA sequencing.

It is imperative in gene expression experiments to mitigate any misinterpretation of RNA concentration from genomic DNA contaminants (Tavares et al., 2011). The optional DNase treatment step was performed to remove genomic DNA contamination, and has been reported in several studies using the Arcturus PicoPure RNA Isolation Kit with early embryos (Vallee et al., 2009; Larman et al., 2011; Norhazlin et al., 2015). Nonetheless, the use of DNase is not completely without disadvantages, residual RNase from the DNase treatment can degrade RNA samples and affect their quality when stored long term (Bustin, 2002).

Since the initial pilot experiment testing the Arcturus PicoPure RNA Isolation kit on embryos frozen at -80°C in RNAlater produced good quality, it is highly likely

that the subsequent poor quality RNA was a result of contamination with RNases. RNases pose a constant threat of contamination and degradation to purified RNA since they are ubiquitous in nearly all cells and enzymatically degrade aqueous RNA (Seelenfreund et al., 2014). RNA samples are often stored at -20°C , -80°C or in liquid nitrogen to provide protection from these degradative reactions (Seelenfreund et al., 2014). The embryo samples used in my experiments were frozen immediately at -80°C in RNAlater, and following RNA isolation were frozen immediately at -80°C until Nanodrop spectrophotometer analysis. RNAlater is an aqueous tissue storage reagent which permeabilises tissues and inactivates RNases immediately to stabilise and protein cellular RNA. It has been used as an RNA protection solution for many different types of embryos used as ART models such as: pig embryos (Buemo et al., 2016), bovine embryos (Huang et al., 2010), feline embryos (Moro et al., 2014), sea urchin embryos (Ruocco et al., 2017), mouse blastocysts (Schulz and Roberts, 2011), human ICM cells (Adjaye et al., 2005), as well as mouse post-implantation embryos (Sun et al., 2011).

The Arcturus PicoPure RNA Isolation Kit utilised in these experiments was designed to isolate RNA from a small number of cells. The online description of the kit specifies that the kit is designed to provide consistent high-quality total RNA recovery from fewer than ten cells, which is much lower than the number of cells used with pooled blastocysts used in the experiments presented above. However, the manufacturer's quality control checks for each kit utilises samples of 100 cells, and the kit brochure specifications manual displays replicate samples of RNA successfully extracted from 1, 10, 100, and 1000 cells. Moreover, Norhazlin and colleagues (2015) obtained good quality RNA from an even larger pool of embryos than I used (30 blastocysts compared to 12).

After receiving poor RNA quality again using fresh embryos, I telephoned the kit manufacturing company. The manufacturer's said that in the past using a sample of over 1000 cells has been reported to not produce good quality RNA since the reagents can be oversaturated, and that other customers have called to inquire and report the same. Since my experiments pooled 12 blastocyst stage embryos,

it is likely that there was too much starting material for the kit. While it was advised by my RNA sequencing project manager (Dr Sarah Lamble at the Wellcome Trust High-throughput Genomics Centre) to pool embryos to increase total levels of RNA obtained, it is conceivable that the kit reagents in this specific kit became over saturated with too much sample from too many cells, and thus did not work efficiently. In the future, this kit should be investigated with a single blastocyst, to see if a reduction in starting cellular material allows the kit to function and produce better quality RNA. Additionally, the manufacturer's recommended a different kit to try, the RNAqueous-Micro Total RNA Isolation Kit (ThermoFisher Scientific), which is designed for starting material <500 000 cells.

Spectrophotometers take absorbance measurements and will include the absorbance of all molecules in a sample which absorb at the wavelength of interest. Nucleotides, single stranded DNA, double stranded DNA, and RNA absorb at a wavelength of 260nm and will contribute to the total absorbance measurement of the sample. Therefore the ratio of absorbance at 260nm and 280nm is used to assess the purity of DNA and RNA. A ratio of approximately 2.0 is considered 'pure' for RNA samples, and if a ratio is considerably lower, it could be indicative of the presence of contaminants in the sample, such as protein or any other molecules which absorb strongly at or near 280nm (Desjardins and Conklin, 2010). The kit specifications for the Arcturus PicoPure RNA isolation kit state that the RNA sample is eluted in a 'low ionic strength buffer', however the manufacturers do not state the components of this buffer, and whether they are acidic, basic or neutral. It has been reported that small changes in the pH of solutions can cause the 260/280 ratio to vary when conducting spectrophotometric assessment of nucleic acid purity, for example with a Nanodrop spectrophotometer, as used in my investigations (Wilfinger et al., 1997). Acidic solutions will under-represent the 260/280 ratio by 0.2-0.3, and basic solutions will over-represent the ratio by 0.2-0.3 (Wilfinger et al., 1997; Desjardins and Conklin, 2010). It is possible that if the elution buffer was slightly acidic then the quality of the RNA was better than quantified by the Nanodrop spectrophotometer.

Upon reflection of the Nanodrop spectrophotometer raw data, I analysed the A260/230 ratios, which were all considerably low. While the A260/230 ratios represent a secondary measure of purity, and often considered less sensitive as a measure of purity, the low values were strongly suggestive of contamination in the sample with something that absorbs near a wavelength of 230nm (Desjardins and Conklin, 2010). EDTA absorbs at 230nm and was used during the DNase treatment step. EDTA has been reported to help protect RNA in samples while DNase is being heat-inactivated, and was thus recommended by the manufacturer (Scientific, 2010). While the column was washed twice following the DNase treatment, before eluting the RNA, it is possible that EDTA remained on the column and contaminated the eluted RNA sample. Another common contaminant resulting in increased absorbance at 230nm is contamination with guanidine thiocyanate, which is often present in high concentrations in lysis buffer or extraction agents in many RNA purification procedures. Unfortunately, the Arcturus PicoPure RNA Isolation Kit does not specify the exact components of each of the kit's solutions, but it is conceivable that the lysis buffer or extraction buffer could also contain substance that would absorb at 230nm that were not sufficiently washed from the column prior to extraction (Scientific, 2010).

Another method to determine the integrity of RNA starting materials is to use the Agilent 2100 Bioanalyzer System to determine an RNA Integrity Number (RIN) number for each sample (Schroeder et al., 2006; Mueller et al., 2016). With this technology, samples are electrophoretically separated and detected with laser-induced fluorescence detection to determine a ribosomal ratio. In order to standardise RNA integrity interpretation, the RIN software algorithm was developed to take into account the entire electrophoretic trace and remove individual interpretation (Mueller et al., 2016). Future work to optimise RNA isolation from embryos should also measure the RIN number of the samples to gain a better view of the quality of the RNA.

Currently in ART, morphological assessment remains the method of evaluating embryo quality, however it is increasingly believed that morphological criteria are

insufficient and predicting embryo quality and pregnancy outcomes (Assou et al., 2010; Mastenbroek et al., 2011). As such, the expression of mRNAs in cumulus cells are being explored as a non-invasive biomarker to predict oocyte and embryo quality, whereby RNAs expressed in cumulus cells could be subjected to qPCR and used for the selection of oocytes to be fertilised or embryos to transfer for IVF (Assou et al., 2010; Assidi et al., 2011; Fragouli et al., 2012; Bouckenheimer et al., 2016). Moreover, RNA sequencing has highlighted that long non-coding RNAs (lncRNAs) change significantly during human early embryonic development, and could serve as a biomarker in the development of tests for oocyte or embryo quality (Bouckenheimer et al., 2016). It has been shown that knockdown of specific lncRNAs in bovine embryos affects embryo growth, and that silencing of a promoter associated with lncRNAs expressed at the 2-cell mouse embryo stage results in embryonic lethality (Hamazaki et al., 2015).

Recent advancements employing RNA sequencing with next-generation sequencing methods have identified novel gene expression patterns, alternative splicing, and new transcripts in a disease states and biological pathways (Mortazavi et al., 2008; Sultan et al., 2008; Ren et al., 2012). However, it should be noted that even when obtained from an apparently homogenous population, there can exist some significant variations and differences between individual cells, or cell subpopulations, in terms of gene and protein expression (Bengtsson et al., 2005; Raj and van Oudenaarden, 2009). Single cell RNA sequencing has allowed the global profiling of individual cells, capturing important transcriptional differences in cellular heterogeneity which could be masked in bulk RNA studies. Single cell RNA sequencing could allow the identification of novel genes or transcript variants which could be important during preimplantation embryo development (Petropoulos et al., 2016).

Clear global transcriptional signatures in different cell populations during the development of human and mouse preimplantation embryos have been investigated using single cell RNA sequencing (Guo et al., 2010; Xue et al., 2013; Yan et al., 2013; Blakeley et al., 2015; Petropoulos et al., 2016). For example, an inverse correlation between *Sox2* and *Id2* emerging in the 16-cell morula stage in the mouse

embryo is indicative of inner cell mass and trophectoderm initiation, and *Gata4* and *Sox2* expression in inner cell mass cells at the 32-cell stage are markers of primitive endoderm and epiblast, respectively (Guo et al., 2010). A study by Blakely et al. investigated gene expression and transcription factors between mouse and human preimplantation embryo lineages, highlighting further that there exist important differences between the mouse model and human embryos. While ELF5, EOMES and ID2 were highly expressed in the mouse, they were minimally expressed or absent in the human trophectoderm. Conversely, PLAC8, TRIML1 and CLDN10 were detected in human TE and not in the mouse. Additionally, in the primitive endoderm lineage, there were differences in GATA4, GATA6, SOX17, PDGFRA, COL4A1, SPARC and FOXA2 between mouse and human conserved genes (Blakeley et al., 2015). These investigations highlight the need to further explore gene and transcript expression in the developing embryo.

Identifying whether a specific cohort of genes in the early embryo are influenced by the use of infra-red laser technology, as applied in routine ART, will provide a crucial foundation for future investigations. For example, existing literature shows that a number of genes are co-regulated in both the embryo and endometrium during the process of implantation (Davidson and Coward, 2016). It is hypothesised that alterations induced by laser-treatment at the gene expression level could ultimately cause detrimental effects upon final protein activity, and thus interfere with the normal process of implantation. RNA sequencing data could uncover novel links into the effects of laser-technology upon gene expression in the implanting embryo. Future investigations should work to improve the quality of RNA isolated from samples and explore different RNA population selection methods for examples. For example, coding and noncoding RNA could be investigated by performing an rRNA depletion, or only coding RNA could be investigated by employing polyA tail selection, to investigate the global effects of laser treatment upon messenger RNA (Kukurba and Montgomery, 2015).

5.6 Conclusions

RNA sequencing was not able to be performed on the samples collected for these sets of experiments, due to the low quality of RNA isolated from the tissues. Ongoing work is being undertaken to improve the quality of the isolated RNA, with the hopes that the RNA sequencing can be carried out in the near future. The analysis of the result data will fall outside the scope of this thesis.

5.7 Key messages

- RNA sequencing uses next generation sequencing to quantify levels of RNA at a specific moment in time
- RNA sequencing would allow for the analysis of thousands of genes in parallel, to help identify if any novel genes are influenced by infra-red laser technology as applied in routine ART procedures.
- RNA sequencing was not able to be performed adequately on the samples collected for these sets of experiments, due to the low quality of RNA isolated from the tissues.

6

Development of a laser-activated mesoporous silica nanocarrier system for applications in molecular research

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6.1 Specialised background

One emerging application of lasers for research in the reproductive sciences lies in its pairing with nanotechnology. The use of nanocarriers for targeted gene transfer into reproductive tissues, gametes, and embryos may represent a powerful tool with which to manipulate and study the fine pathophysiological mechanisms underlying unexplained infertility. Nanomaterials are gaining power as research tools in reproductive biology to manipulate gene expression in offspring (Kim et al., 2010; Campos et al., 2011; Yang et al., 2011), mark and select specific populations of gametes (Fynnewever et al., 2007; Barchanski et al., 2011; Campos et al., 2011), and investigate molecular pathways in gametes and early stage embryos (Makhluף et al., 2008). The less invasive nature of gene or protein delivery using nanoparticles presents a promising approach to explore underlying mechanisms of infertility.

The risk of systemic and/or local toxicity of engineered nanomaterials is a particular concern in reproductive medicine, where the essential function of highly specialised cells and sensitive biological systems involved is to safely transmit genetic information to offspring. Mesoporous silica nanoparticles (MSNP) are a silica-based nanomaterial distinguished for their compatibility with biological tissue and their low toxicity thus representing an ideal candidate for prospective clinical applications (Table 6.1)(Tarn et al., 2013). MSNPs exhibit controllable structural features with an overall size ranging from 50-300nm in diameter, with numerous uniform hexagonal pores ranging from 2-50nm in size, allowing high loading capacity (Rosenholm et al., 2012). They exhibit both mechanical and thermal stability, are more resistant to pH fluctuations than polymer based nanoparticles, and can be customised by attaching a wide array of functional groups or ligands to the inner pores or outer surface via adsorption to silanol groups allowing interaction with specific target receptors (Slowing et al., 2008; Tarn et al., 2013). For example, polyethyleneimines (PEI) are synthetic cationic polymers that that can be attached to the surface of nanoparticles through covalent and electrostatic interactions to attract DNA and small interfering RNA (siRNA) into complexes for delivery. It has been shown that PEI-coated MSNPs can effectively deliver siRNA to knock

Table 6.1: Studies investigating the toxicity and biocompatibility of MSNP in the reproductive sciences.

Study	Findings
He et al. (2009)	Spherical MSNP of 190nm and 420nm showed significant toxicity above 25mg/ml. 120nm particles exhibited slight toxicity due to reduced endocytosis
Lu et al. (2009)	MSNP uptake of fluorescein-labelled MSNP by HeLa cells is size dependent. Of 30nm, 50nm, 110nm, 170nm and 280nm, 50nm exhibited maximum uptake
Tao et al. (2009)	Aminated MSNP exhibit less cellular injury than naked MSNP and reduced cytotoxicity
He et al. (2010)	Polyethylene glycol masking silanol groups on MSNP surface reduced excretion rate, increased circulation time and decreased MSNP distribution in the liver and spleen.
Lin and Haynes, (2010)	MSNP exhibited reduced cytotoxicity compared to non-porous particles
Nadrah et al. (2013)	Demonstrated the ability of MSNP systems to intrinsically control the rate and the amount of the released cargo using minor structural variations to gate-like structures on the surface. Demonstrated that the MSNP delivery system is non-teratogenic in the zebrafish model
Tarn et al. (2013)	Showed that intrinsic porosity of MSNP reduces hydrogen bonding or electrostatic interactions with cell membranes. Shows MSNP high dissolution into soluble silic acid species which are non-toxic
Teng et al. (2013)	Showed MSNP to be effective for delivery and bio-distribution of 2 cancer therapeutics for breast cancer
Barkalina et al. (2014)	FITC-labelled MSNP internalised and attached to surface of boar sperm with no adverse effects on motility and viability. Mouse oocytes able to associate MSNP after incubation and internalise following microinjection

down the expression of green fluorescent protein (GFP) in transduced HEPA-1 cells, and also to deliver anti-cancer drugs (Xia et al., 2009).

Genetic interference by exogenous DNA, RNA or morpholino delivery is a widely used approach to study gene function in developmental biology; however investigations are often hampered by the lack of temporal control during later stages

of embryogenesis. Despite their strong potential as a research tool, a limitation of current investigative nanocarriers is the unwanted delivery of cargo to non-target sites due to passive diffusion. Fickian diffusion, which governs the leakage of cargo from nanoparticles, is not specific to cells or tissues; therefore, more efficient delivery strategies are needed. Promising progress is being made to develop on-demand processes for tailored release of cargo with spatial, temporal, and dosage control. These tailored on-demand processes are becoming feasible through stimuli-responsive systems (Shao et al., 2011). The newest generations of nanoparticles have sophisticated coatings such as gate-keeping mechanisms that are responsive to certain micro-environments permitting the release of cargo only in the presence of specific exogenous stimuli, in order to restrict off-target release of cargo (Albanese et al., 2012; Rosenholm et al., 2012; Alvarez-Lorenzo and Concheiro, 2014). Numerous methods have been developed for gated nanoparticles, such as surface-located supramolecular nanovalves responsive to chemical triggers such as small redox molecules, antigens, enzymes, and oligonucleotides (Tarn et al., 2013; Dai et al., 2014). Physical stimuli such as light and temperature are also being investigated due to the attractive potential of allowing for remote activation.

Thermo-controlled nanocarrier systems include thermo-sensitive polymers, dendrimers, micelles, liposomes and hydrogels, however little work has been established for thermo-sensitive MSNPs. Recent advancements in the temperature-controlled release of molecules from mesoporous materials have been achieved using thermo-sensitive polymer derivatives. Aznar et al. (2011) reported paraffin-capped MSNPs whose caps melted at certain temperatures released loaded cargo in a temperature-mediated manner (Aznar et al., 2011). It has also been demonstrated that double stranded DNA sequences attached to MSNP pore openings can release encapsulated cargo upon heating to the specific DNA melting point (Schlossbauer et al., 2010). It was further demonstrated that MSNPs containing gold nanoparticle cores, along with cargo within the pores, could be heated causing the gold cores to unblock the pore and release preloaded cargo molecules (Croissant and Zink, 2012). Another investigation demonstrated that gold cores inside MSNP pores could be heated with

near infra-red (IR) light to transfer heat to liposome bilayer cargo to release encapsulated dye molecules (Volodkin et al., 2009). While thermo-controlled nanocarrier systems are increasingly being investigated, research into the laser-activation of molecular cargo on nanoparticles is in its infancy.

To successfully confer thermo-control to a nanocarrier system, a specific controllable mechanism must be established. Heat shock proteins (HSPs) are an evolutionally well-conserved protein family whose synthesis is induced in response to many different stress stimuli, such as environmental, pathophysiological, or even non-stressful signals (Figure 6.1)(Morimoto and Santoro, 1998). HSP promoters are valuable for developmental studies as they can regulate exogenous gene expression and control the induction of downstream gene expression. Moreover, their inducible nature allows unwanted effects from earlier stages in development to be circumvented, thus representing a major advantage for *in vivo* studies. HSP70 are a family of 70KDa HSPs found in a range of organisms ranging from bacteria to mammals. Expression is characteristically induced in response to heat-shock, ultraviolet (UV) and gamma radiation, and chemical exposure. Heat-inducible transcription of HSP70 is mediated by heat-shock transcription factors (HSF), which are synthesised constitutively, but remain dormant under unstressed conditions. In response to heat-shock, HSFs trimerise and bind with high affinity to heat-shock promoter elements (HSE) found upstream of all stress protein genes resulting in the transcription of HSPs (Figure 6.2)(Zou et al., 1998).

HSP promoters are widely used in transgenic research, for example to develop a heat-shock inducible transgenic zebrafish line, or to study transgene expression during embryonic and larval development of fish (Grabher and Wittbrodt, 2004; Wu et al., 2008). While HSP promoters have frequently been used for ectopic gene induction *in vivo* through global bulk heating, laser activation enables spatio-temporal control (Kimura et al., 2013). Previous studies have used laser irradiation to induce HSP-mediated expression in transgenic organisms with tissue-specific promoters. In 2007, Sato-Maeda et al. used a 400nm laser to induce heat-shock in transgenic zebrafish; however, DNA damage induced by the UV wavelength

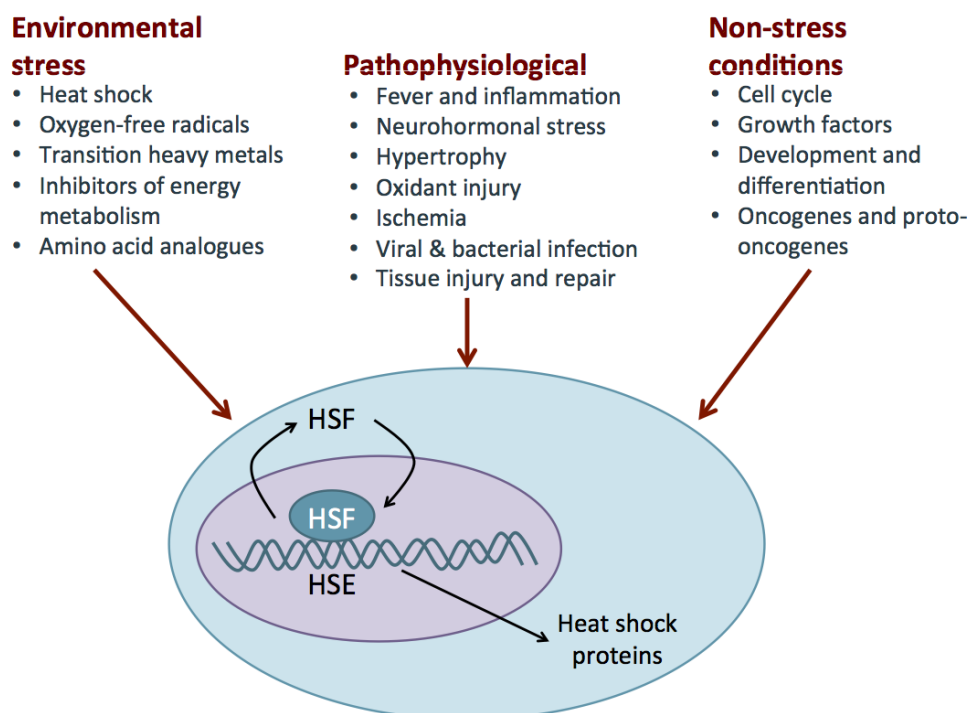


Figure 6.1: Stimuli that are capable of inducing heat-shock response. The expression of heat-shock genes is represented by the activation of heat-shock transcription factor (HSF) binding to heat-shock promoter element (HSE), resulting in elevated expression of HSPs such as HSP70. The heat-shock response helps in the homeostatic control of protein biogenesis by elevating the synthesis of molecular chaperones and proteases, repairing protein damage and helping the cell recover. Image created by Author; adapted from Morimoto and Santoro (1998).

laser irradiation was evident, posing a concern (Sato-Maeda et al., 2006). Studies are now focusing on IR wavelength ranges, which have superior ability to heat water and whose wavelengths are less likely to damage DNA (Kimura et al., 2013). Building from this, Deguchi et al. (2009) used an IR laser, along with HSP promoters and reporter genes, to demonstrate that IR irradiation could induce gene expression in various tissues of living medaka, zebrafish and *Arabidopsis thaliana* (Deguchi et al., 2009). Following these developments, Kimura et al. (2013) used transgenic zebrafish with HSP:mCherry to show that IR laser irradiation induced the expression of mCherry in single targeted endothelial cells with a maximum efficiency rate of 60% (Kimura et al., 2013).

The pairing of laser-activated HSP induction with nanoparticle delivery has yet to be explored. The development of HSP-induced molecular cargo for MSNPs

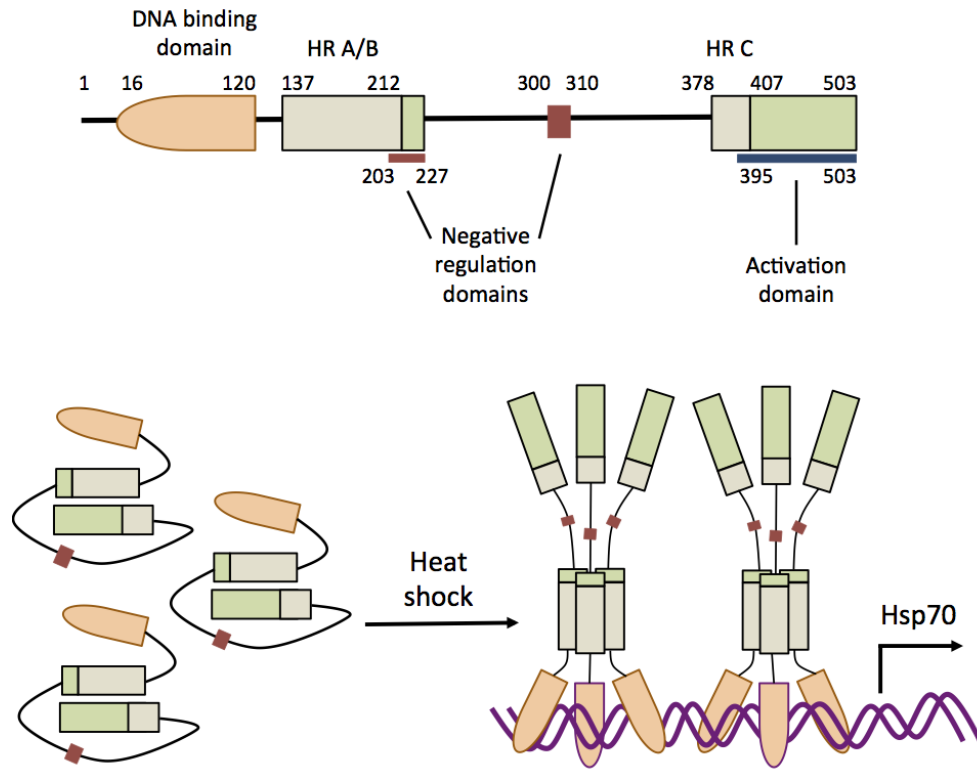


Figure 6.2: Schematic of heat shock transcription factor (HSF) trimer formation binding to heat shock elements (HSEs) to activate transcription of HSP70. Model representation of the general structure of HSF1, showing relative amino acid positions, including the DNA binding domain, hydrophobic heptad repeats (HR-A/B and HR-C), negative regulatory domains, and carboxy-terminal transcriptional activation domain. As proteins unfold with increasing temperature, HSFs become unrepressed, form active trimers and bind to HSEs to activate transcription of HSP70. The HSF have highly conserved N-terminal regions containing DNA-binding domains for HSEs. HSF binds to the promoter region and activates transcription through a potent heat activation domain in its C-terminus, which is negatively regulated in the absence of stress signals. Image created by Author; adapted from Morimoto and Santoro (1998).

that can be infra-red laser-activated represents a promising approach with which to increase delivery efficiency while allowing researchers to control temporal and spatial aspects of nanoparticle cargo delivery and activation. Development of such a system may provide the foundation from which to help elucidate causes of infertility and unknown molecular mechanisms in developmental and reproductive biology, and to perhaps extrapolate to the human gamete or embryo in the future. Eventually, this system could be applied in developmental biology research to help decipher

causes of unexplained infertility and overcome inefficiencies of ART.

6.2 Chapter aims

The Coward group have spent several years developing MSNPs as a novel nano-mediated delivery system for mammalian sperm (Barkalina et al., 2014a; Barkalina et al., 2014b; Barkalina et al., 2015b). The current project worked to extend this previous work by adding laser treatment as a means of controlling cargo expression, both temporally and spatially. Thermo-controlled activation of nanoparticle cargo via HSP promoters has not yet been explored, and could present a novel method of providing sensitive temporal, spatial and rate control of cargo release from nanocarriers. This chapter aimed to develop a specialised MSNP nanocarrier system whose genetic cargo was under the control of a heat-shock promoter and was activated by infra-red laser treatment to manipulate gene expression and molecular pathways (Figure 6.3). Firstly, a construct was engineered in-house by fusing a heat shock protein (HSP) promoter to the DNA encoding green fluorescent protein (GFP). Secondly, the construct (pDRIVE-HSP70-GFP) was electrostatically associated to MSNPs and delivered into HEK293T cells. Finally, infra-red laser treatment was used to investigate whether this strategy might induce and increase levels of pDRIVE-HSP70-GFP construct expression.

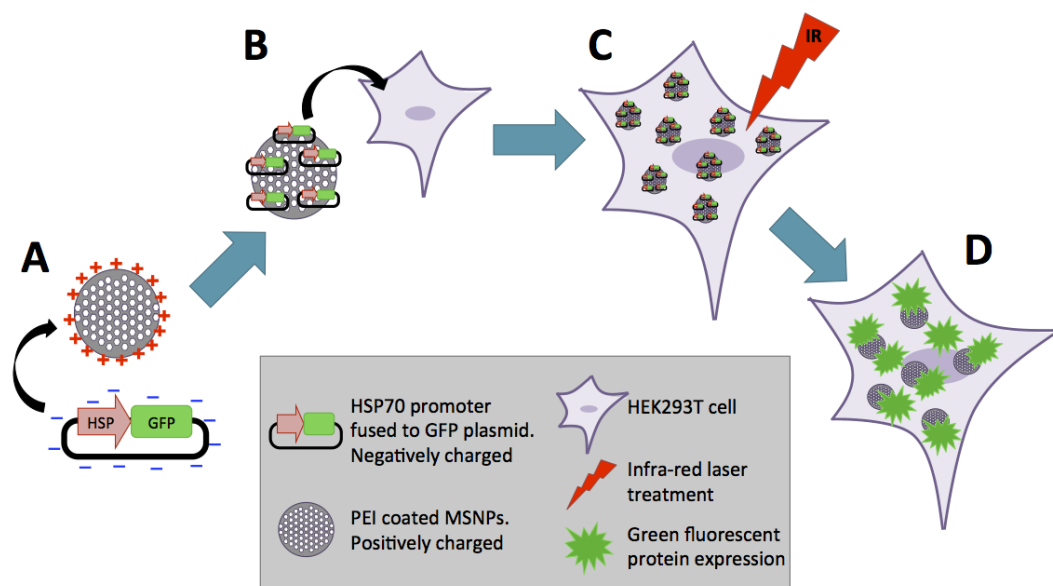


Figure 6.3: Schematic showing a novel developed thermo-controlled nanocarrier system activated by infra-red laser treatment. (A) A heat-shock promoter fused to green fluorescent protein (pDRIVE-HSP70-GFP) plasmid with overall negative charge was electrostatically associated to positively-charged polyethyleneimine coated mesoporous silica nanoparticles (MSNPs). (B) MSNPs loaded with the pDRIVE-HSP70-GFP plasmid were incorporated into HEK293T cells via incubation. (C) Treatment of HEK293T cells with a laser to induce a cellular heat-shock response. (D) Activation of the HSP promoter to drive the expression of the green fluorescent protein. Image created by author and reproduced from (Davidson et al., 2016).

6.3 Specialised materials and methods

6.3.1 Plasmid cloning, transformation, and purification

All cloning activities were performed under aseptic conditions. The cloning reaction was carried out using a GFP Fusion TOPO TA Expression Kit (Life Technologies, UK) according to the manufacturer's protocol. Figure 6.4 illustrated the vector from which the HSP promoter was amplified, and the GFP containing vector it was cloned into. The amplified HSP promoter PCR product was mixed with the pcDNA3.1/CT-GFP-TOPO plasmid for 5 minutes at room temperature to allow ligation of the HSP promoter to the GFP-containing plasmid (herein referred to as 'pDRIVE-HSP70-GFP'). *E. coli* bacteria (TOP 10 competent cells) (Life Technologies, UK) were incubated with pDRIVE-HSP70-GFP constructs on ice for 30 minutes and transformation achieved via heat-shock (42°C for 40 seconds). Transformed cells

were then plated onto sterile Luria-Bertani agar plates (Sigma, UK) containing 100 μ g/ml ampicillin to selectively propagate transformed bacteria. Following overnight incubation at 37°C, three bacterial colonies were randomly selected for each construct, inoculated into autoclaved Luria-Bertani broth containing 100 μ g/ml ampicillin and incubated overnight (37°C) with shaking at 220-250rpm. Three colonies were selected from the LB plate to allow propagation and purification of plasmid DNA.

Extraction and purification of plasmid DNA was performed using the QIAprep Spin Miniprep Kit (Qiagen, UK) according to the manufacturer's protocol. Purified DNA yield was increased using the Qiagen Plasmid Maxi Kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, bacterial cells were resuspended in 250 μ l of P1 buffer (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100 μ g/mL RNase A) and then mixed with 250 μ l P2 buffer (200mM NaOH, 1% SDS). Next, 350 μ l of N3 buffer (4.2M Gu-HCl, 0.9M potassium acetate, pH 4.8) was added and mixed by inverting the tube, followed by centrifugation for 10min at 13 000rpm. After the formation of a pellet through centrifugation, 800 μ l of the supernatant was applied to the QIAprep 2.0 spin column, and the column was centrifuged for 60s. The column was washed with 0.5ml PB buffer (5M Gu-HCl, 30% isopropanol) and centrifuged for 60s, then washed with 0.75ml PE buffer (10mM Tris-HCl, pH 7.5, 80% ethanol) and centrifuged for 60s. Flowthrough was discarded and the column was centrifuged for an additional 60s to remove residual wash buffer. The column was placed into a clean 1.5mL microcentrifuge tube and DNA was eluted with 50 μ l EB buffer (10mM Tris-Cl, pH 8.5) by incubating with EB buffer for 60s followed by centrifugation for 60s.

6.3.2 Restriction digest confirmation

To confirm insertion of the promoter into the vector plasmid, the plasmid was digested with either *Kpn1* only, *Not1* only, both restriction enzymes, or neither restriction enzymes. Both *Kpn1* and *Not1* enzymes create sticky ends, and both *Kpn1* and *Not1* restriction sites are present in the vector. Therefore, single and double digest with these restriction enzymes should result in predictable fragment

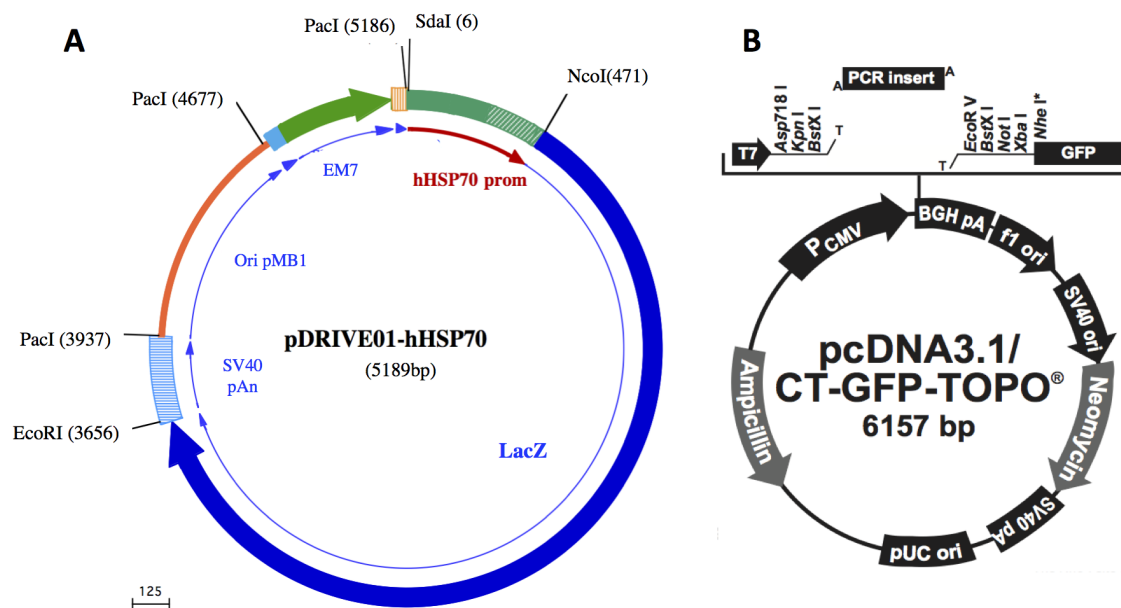


Figure 6.4: Maps of plasmid vectors used for molecular cloning of the pDRIVE-HSP70-GFP plasmid. (A) pDRIVE01-hHSP70 plasmid containing the 471bp human HSP promoter fragment (shown as the red arrow) that was amplified. (B) pcDNA3.1/CT-GFP-TOPO plasmid showing a linearised multiple cloning site into which the HSP promoter sequence was inserted.

sizes if the cloning was performed properly. Briefly, $1\mu\text{l}$ of plasmid ($1\mu\text{g}/\mu\text{l}$) was mixed with $2\mu\text{l}$ 10x buffer, $1\mu\text{l}$ restriction enzyme ($1\mu\text{l}$ each for double digestion), and made up to $20\mu\text{l}$ with sterile water. The reaction was incubated for 1h at 37°C . The digestion was stopped by heat inactivation at 65°C for 15 mins.

Analytical agarose (Promega, UK) was dissolved in 1X Tris-acetate-EDTA (TAE) buffer (Fisher Scientific, UK) and melted in a microwave to a final concentration of 2%, and ethidium bromide ($0.625\text{mg}/\text{ml}$, Fisher Scientific, UK) was added. DNA samples, treated with and without restriction enzymes, and a 50kbp DNA ladder (Fermentas, UK) were mixed with loading dye (Fermentas, UK), and the gel was run at 120V for 1 hour prior to visualisation using a GelDOC-IT TS imaging system (UVP, USA).

6.3.3 DNA sequencing

Cloned plasmids were also sequenced by Source BioScience (Oxford, UK), and the resultant sequences compared to the HSP70 sequence (pDRIVE01-hHSP70 sequence;

Invivogen, UK) and GFP sequence (pcDNA3.1/CT-GFP-TOPO sequence; Life Technologies, UK) using ClustalW Multiple Sequence Alignment (BioEdit Sequence Alignment Editor Software version 7.2.5). Only colonies showing positive insertion and verified sequence were selected for subsequent procedures.

6.3.4 Transfection procedure

To assess the functionality of the cloned plasmid, expression was examined following transfection into HEK293T cells. HEK293T cells are a mammalian cell line that are straightforward to culture and transfect, and are commonly utilised in gene expression studies. The 'T' variant of the HEK293 cell line possessed a SV40 large T antigen which allows episomal replication of transfected plasmids containing the SV40 origin of replication (Lin et al., 2014). The jetPEI transfection reagent (Polyplus transfection, France) was used to perform the transfection. HEK293T cells were maintained at 37°C with 5%CO₂ in a humidified chamber in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum (FBS), 1%v/v 5000U Penicillin, 5mg/ml streptomycin, 2mM L-glutamine and sodium pyruvate. For all experiments, cells were seeded at 30% confluency on detachable 8-well tissue culture chambers on a polyolefin slide (Sarstedt, Germany). The plasmid DNA was diluted (1µg DNA in 150mM NaCl to a final volume of 50µl) and the jetPEI reagent was diluted (2µg DNA in 150mM NaCl to a final volume of 50µl). The jetPEI solution was added to the plasmid DNA solution and incubated for 15min at room temperature. Drop-wise, 40µl of the mixture was added to HEK293T cells and the wells were gently swirled to distribute and mix the solution into the media. The plates were returned to the cell culture incubator and analysed with fluorescence microscopy for up to 56h, when the cells became too confluent to stay adhered to the slides.

6.4 Results

6.4.1 Creation of a thermo-controlled pDRIVE-HSP-GFP plasmid as nanoparticle cargo

The first step in developing this nanocarrier system was to create the thermo-controlled molecular cargo plasmid construct. The construct design was based upon the hypothesis that the GFP reporter gene was driven by the HSP promoter. Consequently, GFP reporter expression would only be expressed under heat-shock stimulus (induced via the laser). The human heat shock protein 70 promoter, HSPA1A promoter, was selected for this experiment, as the HSPA1A gene encodes chaperone proteins in response to environmental and pathophysiological stress stimuli (Morimoto and Santoro, 1998). The thermo-controlled plasmid was successfully created to act as a nanoparticle cargo by fusing the human HSP70 promoter to the GFP reporter gene (pDRIVE-HSP70-GFP). Successful pDRIVE-HSP70-GFP cloning was confirmed via restriction digestion analysis and Sanger sequencing showed 100% alignment between the cloned pDRIVE-HSP70-GFP (both forward and reverse sequences) and the sequences provided by the respective commercial suppliers.

6.4.1.1 HSP promoter amplification

The HSP70 promoter was successfully amplified from a commercial plasmid (pcDNA 3.1/CT-GFP-TOPO, InvivoGen, USA). Figure 6.5 shows a band (Band A) in lane 3 which corresponds to the amplified HSP70 promoter at a molecular weight of 471bp. The negative control in lane 5 showed no such band. Band A was duly cut out of the gel and the DNA was extracted for subsequent cloning into the pcDNA3.1/CT-GFP-TOPO plasmid. Following PCR amplification, the HSP promoter DNA concentration was determined to be $988.75\mu\text{g}/\text{ml}$ by absorbance assay at OD260nm.

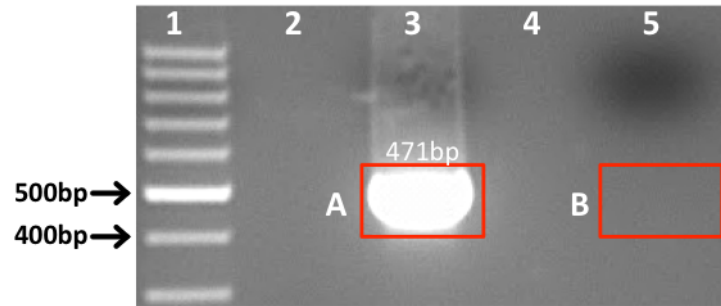


Figure 6.5: 2% agarose gel stained with 0.625mg/ml ethidium bromide to show the amplified HSP70 promoter. Lane 1 corresponds to a 50kbp molecular weight marker, lane 3 contains the HSP PCR product, lane 5 contains the negative control, and lanes 2 and 4 are empty. Band A corresponds to the amplified HSP70 promoter DNA fragment at the expected molecular weight. There was no corresponding band in the negative control (lane 5).

6.4.1.2 pDRIVE-HSP-GFP plasmid cloning and restriction digest analysis

The amplified HSP70 promoter was ligated into the linear pcDNA3.1/-CT-GFP-TOPO plasmid, transformed into competent cells, and grown on ampicillin-LB-agar plates overnight. Three colonies were selected from the plate to allow propagation and purification of plasmid DNA. Each resulting plasmid was digested with either *Kpn1* only (lanes 4, 8, 12), *NotI* only (lanes 5, 8, 13), or both restriction enzymes (lanes 6, 10, 14) (Figure 6.6). Lanes 3, 7, and 11 correspond to circular undigested pDRIVE-HSP-GFP plasmid. Lane 2 corresponds to the linear pcDNA3.1/CT-GFP-TOPO plasmid without restriction enzyme treatment. A faint band (A) was evident in lane 2 and corresponded to the linearised plasmid. Bands N, O, P correspond to the HSP70 promoter which had been excised for cloning; bands E, I, M correspond to the remaining portion of the pcDNA3.1/CT-GFP-TOPO plasmid after the HSP promoter insert had been removed via restriction enzymes. The N, O, and P bands observed in lanes 6, 10, and 14 correspond to the expected molecular weight (544bp) of the HSP70 promoter (471bp) and additional sequences from the pcDNA3.1/CT-GFP-TOPO multiple cloning site leading up to the restriction sites. The restriction digest analysis confirmed successful cloning of the pDRIVE-HSP70-GFP plasmid.

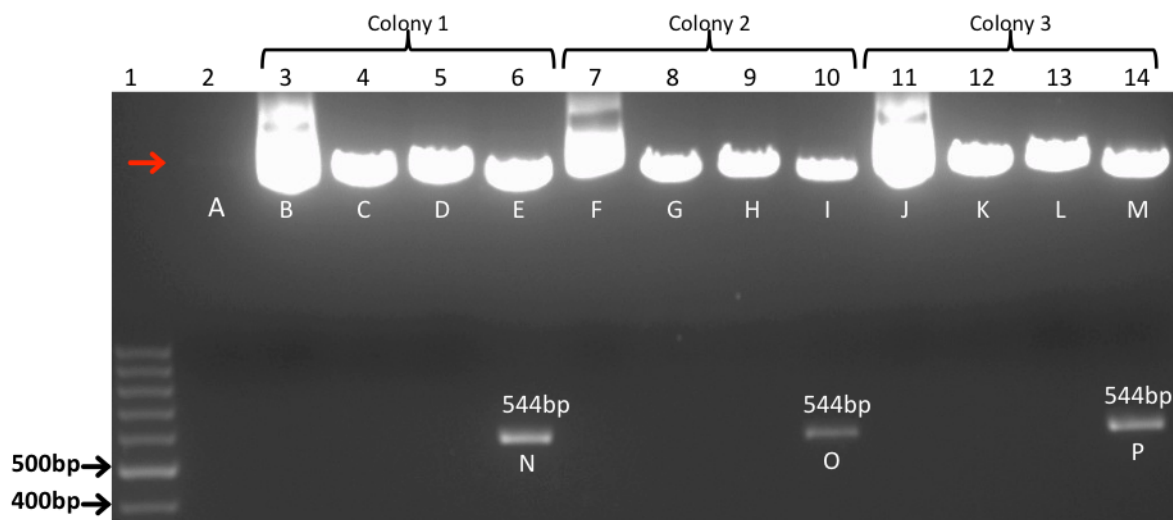


Figure 6.6: 2% agarose gel stained with 0.625mg/ml ethidium bromide to show restriction digestion products. Lane 1 contains a 50kbp molecular weight ladder marker. Lane 2 contains untreated pcDNA3.1/CT-GFP-TOPO plasmid; a faint band can be seen indicated by the red arrow. Lanes 3-6 correspond to colony 1, lanes 7-10 correspond to colony 2, lanes 11-14 correspond to colony 5. Bands B, F, J correspond to whole circular pDRIVE-HSP-GFP plasmid, bands C, G, K correspond to pDRIVE-HSP-GFP plasmid cut with *KpnI*, bands D, H, L correspond to pDRIVE-HSP-GFP plasmid cut with *NotI*, bands E, I, M correspond to the linear TOPO plasmid portion of the pDRIVE-HSP-GFP plasmid cut with both *KpnI* and *NotI*, bands N, O, and P correspond to the HSP70 promoter fragment inserted into the pDRIVE-HSP-GFP plasmid.

6.4.1.3 DNA sequencing confirmation of plasmid cloning

Plasmid sequencing was confirmed using ClustalW Multiple Sequence alignment of the cloned HSP70 promoter sequence from Geneservice Ltd (University of Oxford). The sequence alignment between the amplified HSP70 promoter (for both forward and reverse sequences) and the reference is shown below (Figure 6.7 and Figure 6.8). Results showed 100% similarity with the HSP promoter sequence provided by Invivogen, the commercial supplier. The "N" values at the beginning of the sequence correspond to a string of nucleotides unrecognised by the automated system, which is common at the beginning of all sequencing procedures. Asterisks denote a base pair match between the sample and reference sequences.

```

Colony 3 : Colony 3 Forward

      ....|....| ....|....| ....|....| ....|....| ....|....|
              10         20         30         40         50
colony 3  -----NNNN NNNNNNNNCT CAGGGTCCCT GTCCC-TCCA
commercial CCTGCAGGGC CGCCCACTCC CCCTTCCTCT CAGGGTCCCT GTCCCCTCCA
Match                                     ** *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              60         70         80         90        100
colony 3  GTGAATCCCA GAAGACTCTG GAGAGTTCTG AGCAGGGGGC GGCACCTCTG
commercial GTGAATCCCA GAAGACTCTG GAGAGTTCTG AGCAGGGGGC GGCACCTCTG
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              110        120        130        140        150
colony 3  CCTCTGATTG GTCCAAGGAA GGCTGGGGGG CAGGACGGGA GGCGAAAACC
commercial CCTCTGATTG GTCCAAGGAA GGCTGGGGGG CAGGACGGGA GGCGAAAACC
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              160        170        180        190        200
colony 3  CTGGAATATT CCGACCTGG CAGCCTCATC GAGCTCGGTG ATTGGCTCAG
commercial CTGGAATATT CCGACCTGG CAGCCTCATC GAGCTCGGTG ATTGGCTCAG
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              210        220        230        240        250
colony 3  AAGGGAAAAG GCGGGTCTCC GTGACGACTT ATAAAAGCCC AGGGGCAAGC
commercial AAGGGAAAAG GCGGGTCTCC GTGACGACTT ATAAAAGCCC AGGGGCAAGC
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              260        270        280        290        300
colony 3  GGTCCGGATA ACGGCTAGCC TGAGGAGCTG CTGCGACAGT CCACTACCTT
commercial GGTCCGGATA ACGGCTAGCC TGAGGAGCTG CTGCGACAGT CCACTACCTT
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              310        320        330        340        350
colony 3  TTTCGAGAGT GACTCCCGTT GTCCCAAGGC TTCCCAAGAG GAACCTGTGC
commercial TTTCGAGAGT GACTCCCGTT GTCCCAAGGC TTCCCAAGAG GAACCTGTGC
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              360        370        380        390        400
colony 3  GGCTGCAGGC ACCGGCGCGT CGAGTTTCCG GCGTCCGGAA GGACCGAGCT
commercial GGCTGCAGGC ACCGGCGCGT CGAGTTTCCG GCGTCCGGAA GGACCGAGCT
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              410        420        430        440        450
colony 3  CTTCTCGCGG ATCCAGTGTT CCGTTTCCAG CCCCCAATCT CAGAGCGGAG
commercial CTTCTCGCGG ATCCAGTGTT CCGTTTCCAG CCCCCAATCT CAGAGCGGAG
Match      *****

      ....|....| ....|....| ....|....| ....|....| ....|....|
              460        470        480        490        500
colony 3  CCGACAGAGA GCAGGGAACC CCATGGTTCT GGAAGGGCAA TTCTGCAGAT
commercial CCGACAGAGA GCAGGGAACC C-----
Match      ***** *

```

Figure 6.7: Multiple Sequence alignment of the forward DNA sequence of the cloned HSP70 promoter. 'colony 3' refers to the cloned plasmid, 'commercial' refers to the sequence provided by the commercial provider, 'match' denotes a base pair sequencing match with an asterix.

```

Alignment: Colony 3    reverse

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
           10      20      30      40      50
colony 3  -----NNNN NNNNNNNNNT GAGATTGGGG GCTGGAACG
commercial GGGTTCCTG CTCTCTGTCG GCTCCGCTCT GAGATTGGGG GCTGGAACG
Match                                     * *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
           60      70      80      90     100
colony 3  GAACACTGGA TCCGCGAGAA GAGCTCGGTC CTTCCGGACG CCGGAAACTC
commercial GAACACTGGA TCCGCGAGAA GAGCTCGGTC CTTCCGGACG CCGGAAACTC
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          110     120     130     140     150
colony 3  GACGCGCCCG TGCTTCGAGC CGCACAGGTT CGCTCTGGGA AGCCTTGGGA
commercial GACGCGCCCG TGCTTCGAGC CGCACAGGTT CGCTCTGGGA AGCCTTGGGA
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          160     170     180     190     200
colony 3  CAACGGGAGT CACTCTCGAA AAAGGTAGTG GACTGTGCGA GCAGCTCCTC
commercial CAACGGGAGT CACTCTCGAA AAAGGTAGTG GACTGTGCGA GCAGCTCCTC
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          210     220     230     240     250
colony 3  AGGCTAGCCG TTATCCGGAC CGCTTGCCCC TGGGCTTTTA TAAGTCGTCA
commercial AGGCTAGCCG TTATCCGGAC CGCTTGCCCC TGGGCTTTTA TAAGTCGTCA
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          260     270     280     290     300
colony 3  CGGAGACCCG CTTTTCCCT TCTGAGCCAA TCACCGAGCT CGATGAGGCT
commercial CGGAGACCCG CTTTTCCCT TCTGAGCCAA TCACCGAGCT CGATGAGGCT
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          310     320     330     340     350
colony 3  GCCAGTCCG GAATATCCA GGGTTTTCGC CTCCCGTCCT GCCCCCAGC
commercial GCCAGTCCG GAATATCCA GGGTTTTCGC CTCCCGTCCT GCCCCCAGC
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          360     370     380     390     400
colony 3  CTTCTTGA CCAATCAGAG GCCAGAGTGC CGCCCCCTGC TCAGAACTCT
commercial CTTCTTGA CCAATCAGAG GCCAGAGTGC CGCCCCCTGC TCAGAACTCT
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          410     420     430     440     450
colony 3  CCAGAGTCTT CTGGGATTCA CTGGAGGGGA CAGGGACCCT GAGAGGAAGG
commercial CCAGAGTCTT CTGGGATTCA CTGGAGGGGA CAGGGACCCT GAGAGGAAGG
Match *****

      ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
          460     470     480     490     500
colony 3  GGGAGTGGGC GGCCCTGCAG GATCCGAATT CAAGGGCAAT TCCACCACAC
commercial GGGAGTGGGC GGCCCTGCAG G----- -----
Match *****

```

Figure 6.8: Multiple Sequence alignment of the reverse DNA sequence of the cloned HSP70 promoter. 'colony 3' refers to the cloned plasmid, 'commercial' refers to the sequence provided by the commercial provider, 'match' denotes a base pair sequencing match with an asterix.

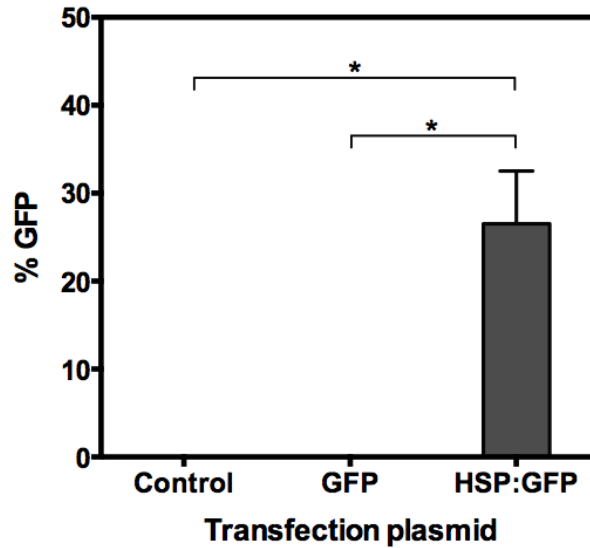


Figure 6.9: Mean proportion of transfected cells expressing green fluorescent protein driven by a heat shock promoter (pDRIVE-HSP70-GFP)(%±SEM). HEK293T cells transfected without DNA for control, 1 μ g linear GFP plasmid, or 1 μ g pDRIVE-HSP70-GFP cloned plasmid for 24h. (*) denotes $P < 0.05$. Data derived from three independent replications of 200 cells each. Image created by author and reproduced from Davidson et al. (2016).

6.4.1.4 Assessing the functionality of cloned plasmids by transfection

Next, the functionality of the engineered pDRIVE-HSP70-GFP plasmid was determined by transfection into HEK293T cells at 37°C. HEK293T cells were transfected with the engineered pDRIVE-HSP70-GFP plasmid, the linear GFP plasmid without the HSP promoter, and without any exogenous DNA treatment for controls. After 24h of transfection, GFP expression was only observed in cells that had been transfected with the pDRIVE-HSP70-GFP plasmid ($P < 0.05$) compared to both linear GFP plasmid and control transfections (Figure 6.9).

6.4.2 Assessment of MSNP cytotoxicity on HEK293T cells

It was next demonstrated that the MSNPs used were not cytotoxic to the HEK293T cell line used in subsequent studies. The MSNPs used in these experiments were coated with polyethylenimine (PEI) to impart a positive charge to which DNA can bind. Previous investigations in the Coward laboratory showed MSNP incorporation into HEK293T cells after 24 hours at a concentrations of 0.5-4.0 μ g/10⁶ cells with no

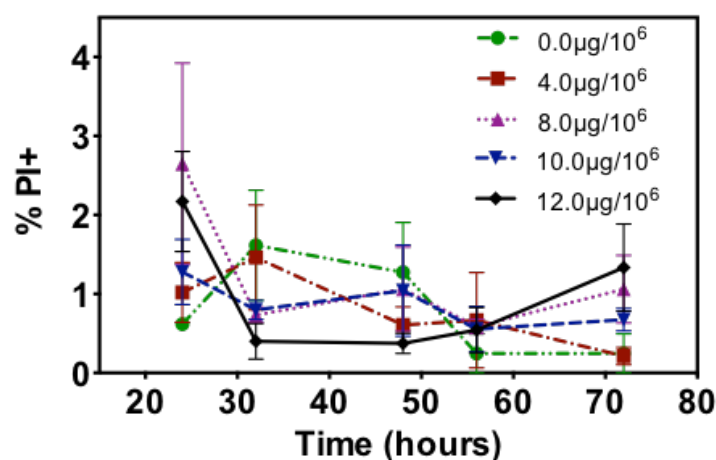


Figure 6.10: Mean proportion of cells showing cytotoxicity via positive propidium iodide (PI) staining ($\% \pm \text{SEM}$) following incubation with MSNPs. HEK293T cells were loaded with different MSNP concentrations and incubated for up to 72h. Following incubation, cells were stained with PI to assess cytotoxicity via non-intact plasma membrane. Data derived from three independent replications of 500 cells. Image created by author and reproduced from Davidson et al. (2016).

cytotoxicity via PI staining. Building from this, the present investigations tested higher MSNP concentrations for an extended period of time to assess if longer exposure at higher concentrations remained free of cytotoxic effects. HEK293T cells were incubated with increasing concentrations ($0.00\text{--}12.0\mu\text{g}/10^6\text{cells}$) for up to 72h. Following incubation, cells were stained with PI to identify whether if any concentrations of MSNPs displayed compromised plasma membrane integrity in HEK293T cells, and to determine any unsafe concentrations which should not be used in subsequent experiments (Figure 6.10). None of these MSNP concentrations showed cytotoxicity after up to 72h of incubation, indicating they were not cytotoxic to cells upon delivery and the highest concentration, $12.0\mu\text{g}/10^6\text{cells}$, was thus selected as the concentration to use in subsequent experiments.

6.4.3 Confirmation of MSNP internalisation into HEK293T cells

To assess whether MSNPs were successfully internalised into HEK293T cells via natural endogenous processes, cells were incubated by fluorescent FITC and

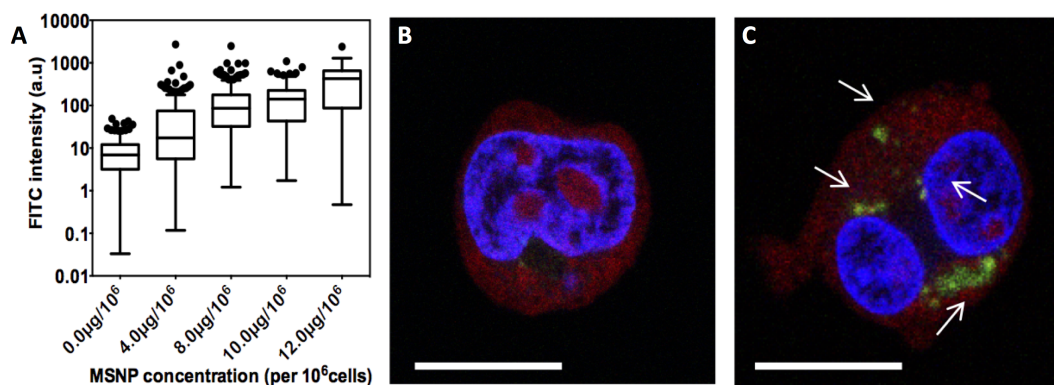


Figure 6.11: MSNP internalisation into HEK293T cells. (A) Variance of FITC-MSNP fluorescence intensity (arbitrary units) in HEK293T cells after incubation for 24h with increasing concentrations of nanoparticles. Data is depicted on a log10 scale. (B-C) Representative confocal images of HEK293T cells incubated without (B) and with (C) FITC-MSNPs (4.0 μgMSNP/10⁶cells). Images represent composite overlays of stains for nuclei (DAPI - blue), MSNP fluorescence (FITC - green), and plasma membrane integrity (PI - red). White arrows indicate FITC fluorescence. Scale bar = 20 μm. Image created by author and reproduced from Davidson et al. (2016).

PEI-coated MSNPs (FITC-MSNPs) and analysed with fluorescent and confocal fluorescent imaging. After 24h incubation with HEK293T cells, FITC-MSNP signals revealed a wide variance in fluorescent intensity at all concentrations, with a large number of outlier values evident in most conditions (Figure 6.11A). A trend, albeit non significant, for increased FITC intensity was evident with increasing nanoparticle concentration. The large variance in intensity measurements indicated that not all cells internalised the same amount of MSNPs, and that different-sized nanoparticle agglomerates were being formed. Confocal imaging confirmed the absence of fluorescent FITC-MSNP signals in control cells (Figure 6.11B), but fluorescence in different z-planes within the cells incubated with FITC-MSNPs (Figure 6.11B), thus confirming that MSNPs had been successfully internalised into cells. Internalisation was further demonstrated indirectly in subsequent experiments showing successful expression of the MSNP cargo.

6.4.4 Association of pDRIVE-HSP70-GFP with MSNPs

After successfully engineering the pDRIVE-HSP70-GFP construct and proving that it was functionally able to drive the expression of GFP via the HSP70 promoter,

I next tested the plasmid for potential as nanoparticle cargo. Polyethyleneimine-coated MSNPs (MSN-PEI) were exposed to the engineered pDRIVE-HSP70-GFP plasmid by incubation to permit the electrostatic association of DNA plasmid to the nanoparticles. Barkalina et al. demonstrated the effective loading of PEI-coated MSNPs with negatively charged nucleic acids at a ratio of 10:1 MSNP:DNA for 24h (Barkalina et al., 2015b). Following the protocol of Barkalina et al., I investigated whether high levels of plasmid could be loaded onto the MSNPs. To determine the optimum incubation ratio for plasmid loading, different ratios (MSN:DNA) of known amounts of MSNP-PEI and pDRIVE-HSP70-GFP plasmid were tested for up to 24 hours to create MSNP-PEI with associated pDRIVE-HSP70-GFP plasmid (Figure 6.12). Throughout the course of incubation, the concentration of free DNA in the supernatant was measured, thus indicating the amounts of DNA that had not bound to the nanoparticle. The higher incubation ratios (2:1, and 5:1) showed an increased association of negatively-charged plasmid to the positively charged nanoparticle over time, up to 10h incubation, after which it was seen to no longer be associating, and partially returning to free solution, and were therefore not used as loading conditions. When incubated at a ratio of 10:1, free DNA gradually decreased with all plasmid DNA associated with MSNPs after 24 hours, which is congruent with the results of (Barkalina et al., 2015b). Data thus confirmed the successful association of plasmid to PEI-coated MSNPs, indicating significant potential as nanoparticle cargo. These association conditions were therefore used for MSNP loading in subsequent experiments. pDRIVE-HSP70-GFP delivery via MSNPs.

6.4.5 pDRIVE-HSP70-GFP delivery using MSNPs

Following the successful loading of MSNPs with the pDRIVE-HSP70-GFP plasmid, their capability as a delivery vehicle into mammalian cells was explored. Using the same concentrations as used in the MSNP cytotoxicity investigations described above (section 6.4.3), increasing concentrations of MSNPs construct were incubated in HEK293T cells for up to 72h at a temperature of 37°C, however this time they were loaded with the pDRIVE-HSP70-GFP plasmid. Incubation at 37°C

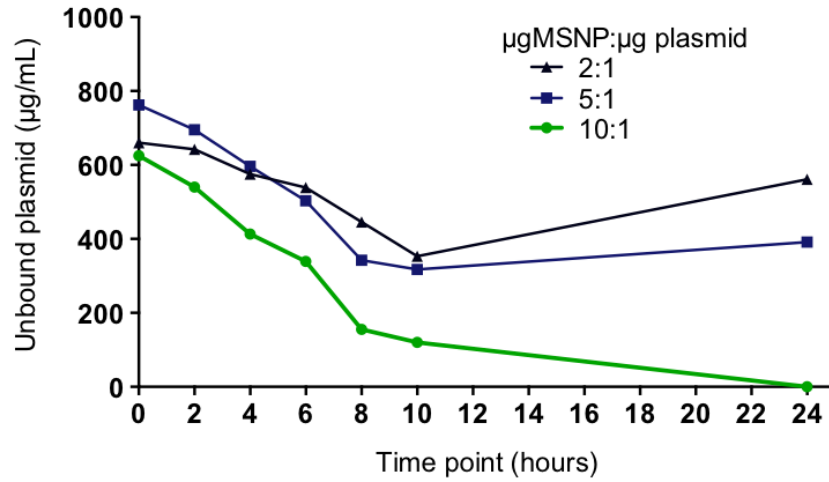


Figure 6.12: Concentration of free unbound pDRIVE-HSP70-GFP plasmid DNA un-associated with MSNP-PEI after incubation for 24h. Lower amounts of free DNA indicate more DNA associated to the nanoparticles. At 24h at a ratio of 10:1, all DNA appeared to be associated to the nanoparticles. Image created by author and reproduced from Davidson et al. (2016).

was hypothesised to mildly induce a heat shock response to generate pDRIVE-HSP70-GFP expression. After 48h, MSNP concentrations of $10.0\mu\text{g}/10^6\text{cells}$ and $12.0\mu\text{g}/10^6\text{cells}$ showed a significant level ($P<0.05$) of pDRIVE-HSP70-GFP expression compared to controls (Figure 6.13). Incubation with $12.0\mu\text{g}/10^6\text{cells}$ showed a significant increase ($P<0.05$) in plasmid expression at 48h and all subsequent time points, and was therefore used as the MSNP concentration for use in subsequent experiments.

6.4.6 Cytotoxicity of laser treatment on HEK293T cells

I next explored the potential for infra-red laser treatment to act as a thermo-control stimulus to the nanoparticle delivery system. First, I had to establish the effects of laser stimulus upon the viability of HEK293T cells. Previous investigations, in the Coward laboratory, optimised laser pulse conditions in HEK293T cells and found that a single pulse of 10mJ did not cause immediate cell lysis or cytotoxicity via PI staining (unpublished data, refer to Appendix B Supplemental Data). Therefore, the effects of 10mJ and 20 mJ laser pulses were further assessed for cytotoxicity over an extended culture period. HEK293T were laser-treated with a single 10mJ

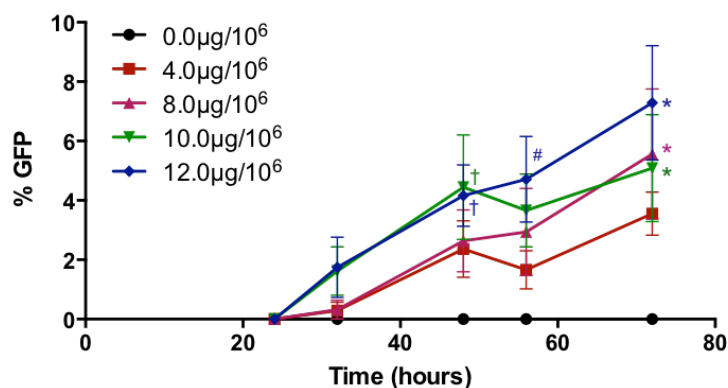


Figure 6.13: Mean proportion of cells showing pDRIVE-HSP70-GFP expression from MSNP delivery ($\% \pm \text{SEM}$). HEK293T cells were loaded with different pDRIVE-HSP70-GFP loaded MSNP concentrations and incubated for up to 72h. Following incubation pDRIVE-HSP70-GFP expression was assessed by fluorescence imaging. (†), (#), (*) denotes significantly different levels of GFP expression between nanoparticles and respective controls ($P < 0.05$) at 48h, 56h, and 72h respectively. Data derived from three independent replications of 500 cells each. Image created by author and reproduced from Davidson et al. (2016).

or 20mJ pulse to the centre of the cell, cultured for up to 56 hours, and stained with PI as an indicator of membrane damage. At each time point there was no difference in plasma membrane integrity (PI staining) compared to respective controls, suggesting membrane integrity competency as early as 24h after treatment (Figure 6.14). Application of 20mJ laser pulse treatment was therefore explored as a stimulus to increase pDRIVE-HSP70-GFP induction in subsequent experiments.

6.4.7 pDRIVE-HSP70-GFP expression following laser stimulus

HEK293T cells were incubated with pDRIVE-HSP70-GFP-loaded MSNPs at a concentration of $12.0 \mu\text{gMSNP}/10^6 \text{ cells}$ to investigate whether IR laser treatment would induce an elevated HSP response within cells and thus increase the amount of pDRIVE-HSP70-GFP cargo expressed. After 24h of incubation with loaded MSNPs to allow internalisation, cells were either treated with a 20mJ IR laser pulse or left untreated, and then left to incubate for a further 48h. HEK293T cells with unloaded MSNPs served as a control. HEK293T cells incubated with loaded MSNPs with and

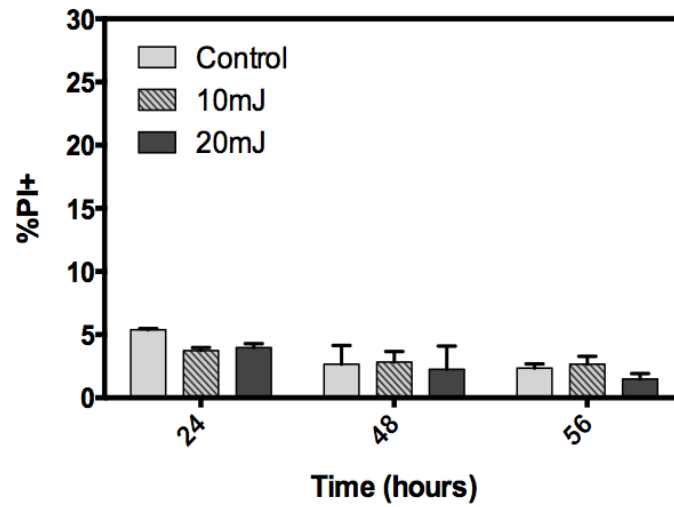


Figure 6.14: Mean proportion of cells showing no significant cytotoxicity when assessed by positive propidium iodide (PI+) staining ($\% \pm \text{SEM}$) following 10mJ or 20mJ infra-red laser treatment and subsequent incubation for 24, 48 or 56h. Data derived from three independent replications of 100 cells each. Image created by author and reproduced from Davidson et al. (2016).

without laser irradiation both exhibited pDRIVE-HSP70-GFP expression compared to controls ($P < 0.05$). Notably, while the highest level of pDRIVE-HSP70-GFP expression from loaded MSNP incubation alone was 7.29%, the level of pDRIVE-HSP70-GFP expression from laser-treated cells incubated with loaded MSNPs was 2-fold higher (14.50%; $P < 0.05$) (Figure 6.15; Figure 6.16). These results indicated that IR laser treatment elicited an elevated heat-shock response and thus increased the expression of nanoparticle cargo and efficiency of MSNP delivery.

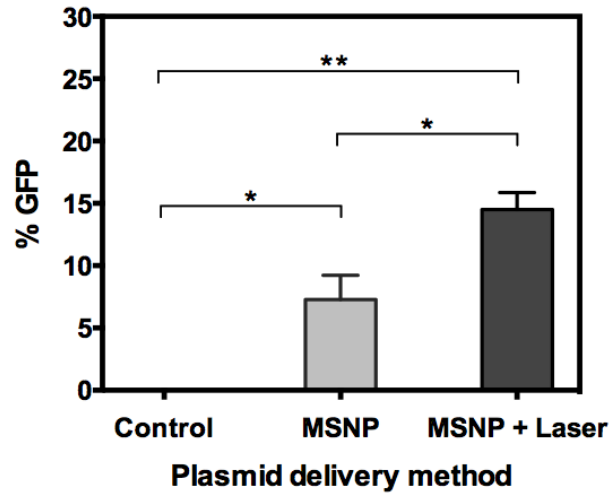


Figure 6.15: Mean proportion of cells expressing GFP ($\% \pm \text{SEM}$) following pDRIVE-HSP70-GFP loaded MSNP incubation ($12\mu\text{g}/10^6\text{cells}$) for 72h with or without laser stimulus (20mJ). (*) denotes $P < 0.05$, (**) denotes $P < 0.01$. Data derived from three independent replications of 100 cells each. Image created by author and reproduced from Davidson et al. (2016).

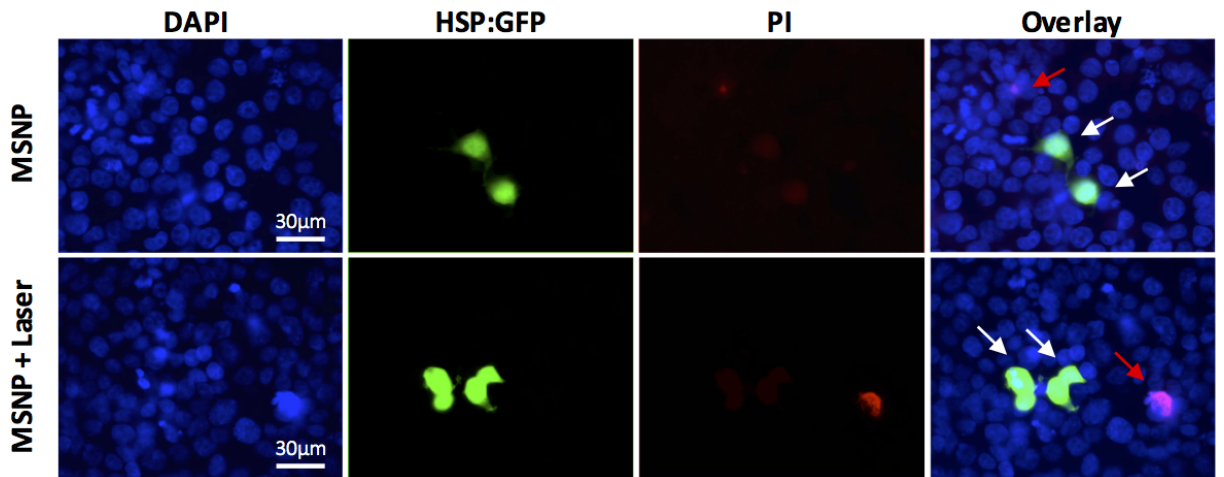


Figure 6.16: Representative fluorescence images of laser or non-laser treated HEK293T cells incubated with $12\mu\text{g}/10^6$ pDRIVE-HSP70-GFP-loaded MSNPs for 72h. Images are composite overlays of stains for nuclei (DAPI - blue), plasmid expression (pDRIVE-HSP70-GFP - green), and compromised plasma membrane (PI - red). White arrows represent cells expressing pDRIVE-HSP70-GFP. Red arrows represent cells with positive propidium iodide staining. Scale bars = $30\mu\text{m}$. 40X magnification. Image created by author and reproduced from Davidson et al. (2016).

6.5 Discussion

In this chapter an infra-red laser-activated MSNP delivery platform was developed and feasibility studies were carried out in mammalian cells. In order to incorporate a stimuli-responsive aspect into the delivery system, first a thermo-controllable molecular construct was developed with a heat-responsive characteristic via the incorporation of a heat-shock protein promoter. A functional HSP promoter was fused to a GFP construct and this construct successfully associated with non-toxic MSNPs, which were subsequently delivered into HEK293T cells. Finally, it was demonstrated that infra-red laser treatment could induce a heat-shock response in HEK293T cells containing loaded MSNPs, thereby increasing the expression efficiency of the delivered cargo. The data therefore describes a novel method for increasing nanoparticle cargo expression in a spatial manner due to the heat-responsiveness of the cargo itself. These original investigations provide a useful foundation for future research into laser thermo-controlled MSNP nanocarriers with increased efficiency and control.

Recent advances in the use of nanocarriers for gene transfer into reproductive tissues, gametes, and embryos are rapidly creating a powerful tool with which to manipulate and study pathophysiological mechanisms in the reproductive sciences (Kim et al., 2010; Campos et al., 2011; Yang et al., 2011). For example, our group (The Coward Laboratory) has demonstrated the ability of MSNPs to bind with mammalian sperm without negative effects upon key semen parameters (Barkalina et al., 2014a). In addition, customisation of these nanoparticles with a cell-penetrating peptide coating was shown to increase the rates of association with sperm (Barkalina et al., 2015b). Such developments in the utilisation of nanodevices in reproductive medicine are emerging as powerful research tools as well as platforms for potential simultaneous diagnostic and therapeutic applications. However, in order to permit more sophisticated research, these delivery systems now require further refinement. Current MSNP systems need to be more efficient in their association and internalisation rates. Moreover, it would be particularly useful to be able to control cargo expression and release in a spatial and temporal

manner, given the highly orchestrated series of events which occur when an egg is fertilised or when an embryo begins to develop.

The risk of systemic and/or local toxicity of engineered nanomaterials is a concern in many cells and tissues, but particularly for gametes in reproductive medicine, where the essential function of these highly specialised cells and sensitive biological systems involved is to safely transmit genetic information to offspring. Since MSNPs are distinguished for their low toxicity even in gametes, they represent a strong candidate for prospective clinical and research applications (Slowing et al., 2008; Tarn et al., 2013; Barkalina et al., 2014b). Nonetheless, an on-going challenge for MSNP delivery lies in the efficiency of cargo release. Recent advancements in the temperature-controlled release of molecules from mesoporous materials have been achieved using thermosensitive polymer derivatives. Paraffin (Aznar et al., 2011), lipid bilayers (Wu et al., 2013), complex acrylamide polymers (Baek et al., 2016), and acrylic acid co-polymers (Chen et al., 2015a), have been investigated as caps to mesoporous silica shells which are specifically designed to melt at certain temperatures. Moreover, double stranded DNA sequences (Schlossbauer et al., 2010), reversible single stranded DNA (Yu et al., 2014), and coiled peptide motifs (Martelli et al., 2013) have all been used as valves for MSNPs which denature at an increased temperature in order to release encapsulated cargo. Alternating magnetic fields have also been investigated to increase local temperature leading to pulsatile drug release from non-silica capped MSNPs (Croissant and Zink, 2012; Yu et al., 2014). A prominent limitation of current stimuli-enhanced MSNP systems is that they require complicated synthesis or assembly and non-silica components that do not possess the same levels of biocompatibility.

The development of a stimuli-responsive nanoparticle system with increased efficiency, which permits the spatial and temporal control of gene expression, would allow for more sensitive and in-depth analysis. Clinical assisted reproduction infra-red laser systems, such as the one used in this study, have been developed over decades of research and refinement to minimise risk to delicate reproductive tissues, rendering them an ideal candidate for research on biological tissue. These laser

systems are routinely used on clinical reproductive cells, and have been investigated as a research tool for many reproductive models, such as mice and zebrafish embryos.

The MSNPs used in these investigations were PEI-coated, a simple functionalisation known to be effective for electrostatically loading DNA as nanoparticle cargo, preventing nanoparticle agglomeration, and improving interaction with cells (Xia et al., 2009; Tarn et al., 2013). PEIs are synthetic cationic polymers that can be attached to the surface of nanoparticles via covalent and electrostatic interactions to compact DNA and small interfering RNA (siRNA) into complexes for delivery. The PEI-coated MSNPs in the present investigations were found to not have any significant cytotoxic effect upon the plasma membrane integrity, further supporting the large body of literature which characterise MSNPs as a particularly biocompatible nanomaterial with low toxicity with biological tissue (Slowing et al., 2008; Lin and Haynes, 2010; Nadrah et al., 2013). The current data further showed that the heat-responsive plasmid was successfully linked via electrostatic association to PEI-coated MSNPs after 24h of incubation, thus representing a highly suitable cargo for nanoparticle delivery. These results support previous studies reporting the effectiveness of coating MSNPs with PEI to bind and deliver nucleic acids (Xia et al., 2009; Kunzmann et al., 2011; Li et al., 2011). For example, PEI-coated MSNPs have been shown to effectively deliver siRNA to knock down the expression of green fluorescent protein (GFP) in transduced HEPA-1 cells, and also to deliver anti-cancer drugs (Xia et al., 2009). Our current investigations further demonstrated successful internalisation of loaded MSNPs by HEK293T cells, demonstrating cargo expression without additional intervention. This also concurs with a series of earlier studies (Mamaeva et al., 2011; Rosenholm et al., 2012; Wang et al., 2013a) which independently reported the association of MSNPs to HEK293T cells.

Evidence in the literature has shown the effectiveness of heat-shock promoters, particularly HSP70, at inducing gene expression in response to external stressors in a variety of different models (Grabher and Wittbrodt, 2004; Shoji and Sato-Maeda, 2008; Deguchi et al., 2009; Sajjadi et al., 2013). Previous reports have demonstrated the success of spatially-controlled laser-activated HSP promoter-induced genes in

transgenic models with global or tissue-specific promoters (Halloran et al., 2000; Ramos et al., 2006; Wilink et al., 2008). To the best of our knowledge, the data presented in this chapter, which has been published in *The Journal of Biomedical Optics*, is the first report describing the use of molecular cargo driven by an HSP response in order to improve cargo expression by MSNP delivery in a thermodynamic manner with laser activation (Davidson et al., 2016). The use of DNA cargo itself as the responsive element is highly beneficial due to its naturally high cellular uptake and biocompatibility, compared to the other responsive elements commonly associated with nanoparticles, such as gold spheres or chemical valves (Zandberg et al., 2012; Yu et al., 2014). It was hypothesised that an external laser stimulus would promote a cellular heat-shock response causing increased transcription of genes driven by a heat-shock promoter, and thus the expression of the plasmid cargo. In the present experiments, GFP expression from HEK293T cells incubated with pDRIVE-HSP70-GFP loaded MSNPs was 2-fold higher ($P < 0.05$) with the application of laser stimulus, compared to no laser treatment, indicating that the plasmid cargo being carried by the MSNPs were successfully infra-red laser activated, which had never before been shown. An explanation for this increased cargo expression is that the infra-red laser stimulus was successfully inducing a cellular heat-shock response. Heat-inducible transcription of HSP70 is mediated by heat-shock transcription factors (HSFs), which are synthesised constitutively, but remain mostly dormant under non-stressed conditions. In response to heat-shock, HSFs trimerise and bind with high affinity to heat-shock promoter elements found upstream of stress protein genes, resulting in the transcription of heat-shock proteins (Zou et al., 1998). The increased level of gene expression observed in our experiment is therefore likely due to more HSFs being activated by the laser stimulus. This represents a highly promising technique for increasing the gene expression of other molecular constructs in a thermo and spatially-controlled manner for molecular investigations and gene therapy.

To date, only a limited number of studies have reported the use of infra-red or near infra-red laser treatment with mesoporous silica; however, they require the

use of gold cores and the associated plasmonic resonance in response to the laser photons. For example, Volodkin et al. (2009) demonstrated that gold cores inside MSNP pores could be heated with near infra-red (IR) light to transfer heat to liposome bilayer cargo to release encapsulated dye molecules (Volodkin et al., 2009), while Mura et al. (2013) reported that an increase in temperature of near infra-red (NIR) treated gold nanoparticle cores caused a phase transition in polymers capping mesoporous silica shells, allowing leakage of a preloaded drug (Mura et al., 2013). Chen et al. (2014) further illustrated that mesoporous silica shells encapsulating gold nanorod cores provided mechanical support against thermal deformation following laser irradiation, compared to uncoated nanorods (Chen et al., 2014). While these reports used near-IR treatment with mesoporous silica components, it was the gold particles that were responsible for thermo-responsiveness through plasmon resonance and two-photon-activated therapy of photosensitisers. Such investigations are useful for strategies to eradicate or harm tissues, for example in cancer applications (Terentyuk et al., 2009), but are not suitable for applications in highly specialised reproductive cells and tissues which need to safely transmit genetic information to future generations (Barkalina et al., 2015a). It is known that the toxicity of gold nanomaterials varies widely, in a dose-dependent manner (Alkilany and Murphy, 2010). Additionally, gold nanostructures are known to irreversibly deform under even very low energy stimulus, resulting in a spectral shift of peak surface plasmon resonance absorption, thereby reducing the efficiency of any photon or laser-induced methods, and have also been shown to be cytotoxic in mammalian cells following laser-induced modifications (Abdelhamid et al., 2012; Chen et al., 2014). In contrast, the present study investigated the use of MSNPs alone with infra-red laser treatment to increase the expression of MSNP cargo naturally via normal cellular machinery, illustrating that a heat-stimulus response can be achieved without the use of two-photon-activation using gold cores, which is highly desirable for investigations in the reproductive sciences.

A study by Andersson et al. (2014) explored the potential use of a laser-induced heat-shock response to activate expression of a GFP plasmid with a heat shock

protein promoter, however, these authors did not use a nanoparticle platform to deliver these constructs. Instead they used transfection to deliver the construct into HeLa cells, and then injected the cells along with gold nanorods subcutaneously into the hind legs of mice. Near infra-red laser treatment was then applied to heat the area containing the gold nanorods to create increased localised heating and induce a heat-shock response to increase plasmid expression. While this study showed that a heat shock promoter driven plasmid could be induced via localised heating from infra-red laser stimulus, this earlier study presented key limitations. For example, the positive control failed to express in response to laser treatment, and the authors did not provide critical information in support of their claims in terms of sample size, variance, and statistical analysis (Andersson et al., 2014). Conversely, the results from our present investigation demonstrate that the use of gold nanorods to impart localised heating does not represent a necessary requirement when using laser stimuli, and that molecular constructs can be delivered using MSNPs rather than chemical transfection (Davidson et al., 2016).

The ultimate goal is to apply this platform to reproductive biomedicine for targeted gene transfer which could assist in the elucidation of mechanisms underlying unexplained infertility and ultimately provide novel options for diagnosis and therapy. This new system could allow the delivery of cargo to inhibit, biomark, target, augment or suppress pathways involved in the development of early embryos to study gene expression and development. In future, laser irradiation could be applied to manipulate gene function *in vivo*, where increased expression efficiency and spatial control are required. In addition to increased expression, finely-tuned spatial control imparted by laser stimulus could help solve persistent problems of gene expression in non-target cells which often occurs in existing techniques such as mosaic analysis (Deguchi et al., 2009).

6.6 Limitations

One limitation of this nanocarrier system in its present form is with temporal control, as plasmid expression was observed following the delivery of MSNPs but without

laser stimulus, albeit at a far lower level. Following our encouraging results, further investigations are now needed to optimise different MSNP loading and delivery parameters, laser settings, and exposure timings, to refine the use of laser-activation for MSNP cargo. Once optimised to activate expression in a predictable manner, this represents a promising technique for inducing the gene expression of other constructs such as siRNA or for gene therapy. Additionally, these studies only demonstrate validation of the current system in a cell model, which may not be representative of how the MSNPs, plasmid, and laser activation will function when used in a more sophisticated model, such as zebrafish embryos or mouse embryos.

6.7 Conclusion

The investigations described herein explored a novel means of inducing thermo-controlled MSNP delivery and cargo activation. Specialised nanoparticle cargo featuring a heat-shock protein fused to a GFP reporter gene was successfully created, electrostatically loaded onto MSNPs, and delivered into HEK293T cells. Infra-red laser stimulus to these HEK293T cells was shown to induce a heat-shock response and significantly increase cargo expression two-fold. The current findings provide a useful preliminary foundation for future investigations into novel laser thermo-controlled MSNP nanocarriers, with strong potential for eventual translation into more complex systems such as human gametes or human embryos. Thermo-controlled cargo release will allow for spatial and temporal control of the nanocarrier system, allowing for more target-specific delivery and sensitive manipulation of gene expression. An effective and finely controlled nanocarrier system with increased efficiency, which can be controlled in both a spatial and temporal manner, could have exciting implications for reproductive science. For example, this refined system could help elucidate causes of unexplained infertility, which are increasingly being associated with abnormal gene expression/genetic polymorphisms, but proving difficult to study due to inefficient methods of precisely manipulating gene expression.

6.8 Future directions

The use of nanomaterials for investigative applications in reproductive biology has strong potential for expansion in the coming years. Such methods would represent highly useful technology for research into gene expression during early embryogenesis by permitting the non-invasive delivery of cargo such as small interfering RNA (siRNA), morpholinos, or antisense therapies to augment, inhibit or biomark target pathways during development.

Following the encouraging results from the present study in HEK293T cells, which show a significant two-fold increase in expression following laser stimulus, it is now important to carry out more detailed studies to further improve this expression efficiency. While the present system was developed and tested in a mammalian cell line, and has potential for use in a variety of disciplines, our goal is to continue developing this system for use in the reproductive sciences. The impact of mesoporous silica species on mammalian embryos or embryonic development is unknown, and is something our group is currently working to establish. This would eventually pave the way to translating this specialised nanocarrier system to human gametes and embryos.

Investigations need to be explored *in vivo* to refine the use of laser-activated MSNP cargo in a model more suitable to studying reproductive development, such as the zebrafish or mouse embryo. Such studies are imperative since there are still challenges that need to be investigated comprehensively before practical applications *in vivo* or clinically. For example, most MSNP-based gene-delivery systems carry DNA or RNA on their outer surface, and studies have shown that this results in the exposure of cargo to enzyme-mediated degradation *in vivo* (Chen et al., 2013). Additionally, while MSNPs are known to biodegrade and excrete through the urinary system, the biological effect of un-degraded MSNP products over a long period are still unknown. However, bio-assays to determine such biodegradation properties are difficult due to the complexity of *in situ* detection of the degradation process *in vivo*, and the presence of natural silica species making it difficult to distinguish the degraded products (Chen et al., 2013; Barkalina et al., 2015a). Bio-safety assays

should extend from general toxicities against major organs and tissues to specific and specialised facets such as neuro- or reproductive toxicity.

6.9 Key messages

- Mesoporous silica nanoparticles (MSNPs) are a silica-based nanomaterial distinguished for their low toxicity and compatibility with biological tissue
- Nanocarrier investigations are often hampered by a lack of spatial and temporal control over delivery, as such, the development stimulus responsive nanocarriers are of interest to improve delivery efficiency
- This chapter presents the successful development of a MSNP delivery system with heat-inducible molecular cargo whose expression is increased with infra-red laser stimulus
- Results from this chapter have been published in the *Journal of Biomedical Optics* (Davidson et al., 2016)

7

Summary and Future Directions

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7.1 Chapter Summaries

Assisted reproductive technology (ART) has undergone tremendous advancements since the birth of the first test tube baby. While an increasingly complex array of laboratory skills and procedures have been developed for infertility treatments, the success rate of ART remains low. Consequently, innovative technologies continue to be developed to identify and manipulate the most promising gametes and embryos with which to generate a pregnancy. The use of laser technology reduces procedure times, and globally, has led to live births following sperm immobilisation and

selection, embryo biopsy, assisted embryo hatching, and cryopreservation (Montag et al., 2009). Nevertheless, there is a paucity of literature pertaining to the safe standardisation of such laser procedures with evidence at the molecular level. This thesis investigated potential deleterious effects of different laser treatment applications, both by the operator and hardware, and established a new method to utilise ART lasers as a research tool in the reproductive sciences.

7.1.1 Safety of infra-red laser drilling upon mouse embryos (Chapter 3)

Numerous studies report the success rates of achieving pregnancies following various laser applications in ART and the consensus of these studies was that laser treatment exhibited equal or superior success at ablating the zona pellucida, compared to the other available methods of mechanical drilling or Acid Tyrode's degradation; however, there is a notable absence of literature pertaining to safety investigations regarding the potential detrimental effects of laser treatments upon embryos, potentially at the molecular level.

The first set of experiments presented in this thesis (Chapter 3) sought to evaluate any deleterious effects at the molecular level of a range of laser hole sizes applied to the zona pellucida at different distances away from the nearest cell membrane. Mouse embryos were harvested from the reproductive tracts of super-ovulated pregnant C57BL6 mice, and cultured in KSOM medium supplemented with amino acids. The mouse preimplantation embryo model was selected for these investigations for two main reasons. First, due to the scarcity, ethical, and logistical challenges of obtaining human embryos, particularly for new investigations without previous pilot data. Secondly, since the mouse embryo plays a significant role in the field of ART as an ideal animal model, due to its size, prolificacy, ease of handling, and genetic power.

The first set of experiments examined four different hole sizes produced by the 400mW infra-red laser at four locations in the zona pellucida. Four of the specific conditions showed significant cell membrane integrity cytotoxicity to embryos, and

two of these conditions further showed a significant reduction in the quality of the blastocysts produced following culture. Interestingly, blastocysts assessed as healthy via morphology Gardner grading were shown to have significant damage at the molecular level in terms of DNA fragmentation. The changes in gene expression that were observed were not indicative of a cause for concern regarding the laser treatments. Results from Chapter 3 suggest that laser pulses with hole sizes of $4\mu\text{m}$, $8\mu\text{m}$, and $16\mu\text{m}$ should not be applied at a distance of $1\mu\text{m}$ from the blastomere membrane, and a hole size of $16\mu\text{m}$ should not be applied at a location of $8\mu\text{m}$ from the blastomere membrane. Therefore, there are clear dangers to the current lack of standardisation or guidelines with embryologists creating their own protocols. Damaging effects at the molecular level could lead to downstream consequences upon embryo development or implantation potential, and existing laser data analysis might be confounded by the use of differing laser protocols, some of which might unknowingly be dangerous.

In order to establish any form of standardisation for laser-assisted ART treatments, for both clinical and research applications, it is imperative that investigations at the molecular level be continued. Using laser parameters that did not show any damage at the molecular level as evidenced in this thesis, future studies could investigate how these treatments affect protein expression. For example proteins which are involved in embryo implantation and cross-talk with the endometrium (Davidson and Coward, 2016). Moreover, more subtle changes to the epigenome following laser treatment have not been explored, and could provide novel insights into the developing embryo.

7.1.2 The effects of altering laser power when treating embryos (Chapter 4)

The energy produced by a laser is influenced by three factors: the wavelength of the laser beam, the power of the laser, and the pulse length. The lasers currently used in fertility clinics are non-contact infra-red light lasers operating with a set power of either 300mW (if manufactured by Hamilton Thorne) or 400mW (if manufactured by

Research Instruments Ltd.). Potential damaging effects via thermal excursion may be affected by the power of the laser used to deliver the required amount of energy for the desired hole size. This study investigated the potential difference in damage caused by altering laser power to deliver a set amount of energy to preimplantation embryos. The commercial 400mW Research Instruments Ltd clinical laser was compared to a custom-made 100mW laser manufactured by Research Instruments Ltd. specifically for this thesis.

In Chapter 4, preliminary studies were conducted to assess the functionality of the bespoke 100mW laser, to determine what hole sizes were created with set amounts of energy, and how these compared to to 400mW laser. An energy level of 173.6mJ was selected to remain constant, as it created an easily measurable hole in the zona pellucida when delivered with both powered lasers. A single pulse to the zona pellucida applied at the cleavage stage was compared between both laser systems. Embryos were assessed immediately for cytotoxicity via PI staining and no significant differences were found. Following laser treatment, embryos were then cultured to the blastocyst stage and re-assessed for viability with PI and DNA fragmentation. There were not significant differences compared to controls in terms of survival to the blastocyst stage, cytotoxicity, or DNA fragmentation. It was hypothesised that the laser pulse location, to the outer edge of the zona pellucida, may not cause damage to the embryo with either laser power.

To determine whether either, or both, laser systems can cause damage to embryos, a more direct approach was undertaken, applying a single laser pulse, of constant energy, to the centre of a blastomere. It was found that the 400mW laser exhibited significant damage compared to both the controls and the 100mW laser, and that the 100mW laser did not cause any significant damage compared to the controls. Therefore it was shown that the power of the laser does matter - if a lower power laser is used to deliver the same overall energy (over a longer period of time), a smaller amount of damage is incurred.

Since no clinical procedure requires laser treatment directly to a blastomere, further studies are now needed to ascertain whether this result is exhibited in more

clinically relevant procedures. It is worth noting that some clinical procedures do require laser treatment almost directly to embryonic cells. Trophectoderm biopsy targets the cell junction between trophectoderm cells on day 5, and blastocoel collapse for vitrification involves directly laser treating the embryo through the trophectoderm and blastocoel cavity (Montag et al., 2009; Davidson et al., 2015a).

To mimic clinical applications, where multiple pulses are used to traverse the zona pellucida, investigations should be carried out involving multiple pulses and altering laser power. Day 5 trophectoderm biopsy involves an initial laser treatment on day 3 to traverse the zona pellucida to create an opening through which trophectoderm cells can herniate out as the blastocyst forms and expands, and again on day 5, targeting the cell junction between herniated trophectoderm cells to obtain the sample. Experiments comparing changing laser power could be performed with multiple pulses delivered in the same manner as the day 3 initial laser treatment, and in order to maintain a simple mode of comparison, ideally the overall amount of energy delivered by the multiple pulses should still remain constant. Additionally, full laser biopsy on day 3 and day 5 could be investigated. Data derived from such experiments would provide a more complete picture regarding the effects of laser power and energy delivery.

7.1.3 Global changes in gene expression following laser treatment (Chapter 5)

RNA sequencing uses next generation sequencing technologies to allow researchers to detect both known and novel features of a transcriptome. It provides a more sensitive and accurate measurement of gene expression due to its broader dynamic range, and is not limited by requiring prior knowledge of the genome. Chapter 5 aimed to investigate global changes in gene expression in laser treated mouse embryos by performing RNA sequencing on a total RNA population. Total RNA sequencing would provide the most complete view of the transcriptome and enable analysis of all forms of coding and non-coding RNA in a single experiment.

Preliminary experiments were undertaken on surplus cryopreserved embryos to assess the quality of RNA isolation before isolating RNA from the laser-treated samples which were frozen in RNA later. While the preliminary experiments showed a high concentration of good quality RNA, when the same procedure was carried out on the frozen laser treated experimental samples, the quality of the RNA was low. Due to expense, it was decided not to run these samples for RNA sequencing, and to work on optimising the RNA isolation. Current work is being undertaken to improve the quality of RNA isolated. RNA isolation was undertaken again, this time using fresh embryos immediately following culture. The concentration and quality of the RNA was assessed with a Nanodrop spectrophotometer, and unfortunately, the quality was not any better.

It is possible that using pooled blastocysts oversaturated the kit, since it is designed for RNA extraction from a small number of cells, however this is incongruent with previous reports that have used the Arcturus PicoPure kit to successfully isolate high quality RNA from pooled blastocysts (Norhazlin et al., 2015). Another feasible, and likely, consideration is that there was contamination with RNases, perhaps with a contaminated solution, or pipette tips, or microcentrifuge tubes. The protocol was carried out in a dedicated qPCR cabinet, but not in a laminar flowhood. Next time, the protocol should be carried out in a laminar flowhood under aseptic conditions. Further methods to optimise RNA isolation from embryos needs to be explored. For example, testing different commercial kits with different amounts of starting material. The quality of RNA isolated could also be tested by determining the RIN number in addition to the Nanodrop spectrophotometer in order to give a more reliable picture of the RNA concentration and quality. Once a method to isolate good quality RNA is identified and used on laser treated samples, RNA sequencing will be performed. The data generated from RNA sequencing of laser-treated mouse preimplantation embryos will help to provide a more comprehensive view of the effects of such laser treatments upon embryos, and could uncover novel changes in gene expression related to ART treatments.

7.1.4 A novel laser-activated mesoporous silica nanocarrier system (Chapter 6)

An emerging application of lasers for research in the reproductive sciences lies in its pairing with nanotechnology. The risk of systemic and local toxicity of engineered nanomaterials is a particular concern in reproductive medicine, where the essential function of highly specialised cells and sensitive biological systems involved is to safely transmit genetic information to offspring. The specific choice of MSNPs as a delivery vehicle for these investigations were founded on the novelty of this nanomaterial and its inherent features, which made it at a promising candidate for reproductive applications. MSNPs are distinguished for their biocompatibility in different cell types. MSNPs were consistently shown to exhibit strong delivery capacity, including the possibility to internalise nucleic acids into cells.

Nanocarrier investigations are often hampered by a lack of spatial and temporal control over delivery. Therefore, there is a strong impetus to develop stimulus responsive nanocarriers to improve delivery efficiency. Chapter 6 presents the successful development of a MSNP delivery system with heat-inducible molecular cargo whose expression is increased with infra-red laser stimulus. A heat-responsive nucleic acid construct was created by fusing an HSP promoter with the GFP reporter gene and was used as nanoparticle cargo. Incubation with PEI-coated MSNPs and infra-red laser treatment were both shown to not be cytotoxic, when applied respectively and together to HEK293T cells. The negatively-charged nucleic acid construct was electrostatically loaded onto the positively charged PEI-coated MSNPs and delivered into HEK293T cells. Infra-red laser stimulus to these HEK293T cells was shown to induce a heat-shock response and significantly increase cargo expression two-fold. The current findings provide a useful preliminary foundation for future investigations into novel laser thermo-controlled MSNP nanocarriers.

Thermo-controlled cargo release will allow for spatial and temporal control of the nanocarrier system, allowing for more target-specific delivery to manipulate and study the fine pathophysiological mechanisms underlying unexplained infertility. An effective and finely-controlled nanocarrier system with increased efficiency, which

can be controlled in both a spatial and temporal manner, could have exciting implications for reproductive science. Development of such a system may provide the foundation from which to help elucidate causes of infertility and unknown molecular mechanisms in developmental and reproductive biology, and to perhaps translate to the human gamete or embryo in the future. Eventually, this system could be applied in developmental biology research to help decipher causes of unexplained infertility and overcome inefficiencies of ART.

The use of nanomaterials for investigative applications in reproductive biology has strong potential for expansion in the coming years. Following the encouraging results from this thesis in the HEK293T mammalian cell line, which show a significant two-fold increase in expression following laser stimulus, further studies need to be conducted to improve the expression efficiency. Future experiments should aim to define the highest concentrations of MSNPs which can be used for delivery, and the highest amount of DNA which can be loaded onto the MSNPs. The system developed in this thesis has potential for use in a variety of disciplines, including use in the reproductive sciences. Investigations need to be explored *in vivo* in a reproductive model, such as the zebrafish embryo, or the mouse embryo.

7.2 Future directions

7.2.1 Implantation

For a successful pregnancy to occur, both naturally and through ART, the embryo must hatch out of the zona pellucida to make contact with a receptive endometrium and begin to grow, a process known as 'implantation'. In some cases the embryo is unable to hatch from the zona pellucida, thus preventing implantation and pregnancy. Hatching *in vivo* is dependent upon a myriad of embryonic and uterine factors, however the physiological course of hatching, and the mechanisms preventing normal hatching and implantation in some patients, is still poorly understood (Das and Holzer, 2012; Davidson and Coward, 2016). The physiological and molecular processes initiated during implantation are complex and highly organised. The uterus becomes receptive to an embryo only during a discrete temporal phase

known as the 'window of implantation'. During this phase, ovarian steroids induce a number of physiological changes in the endometrium to create a receptive and appropriate environment (Dey et al., 2004). The window of implantation is a limited period of time when blastocyst competency coincides with a receptive uterus. If this coordination is out of phase, implantation becomes defective and fails (Cha et al., 2012a; Davidson and Coward, 2016). Implantation is a pivotal step of reproduction, with complex signalling processes mediated by bi-directional crosstalk between the embryo and the endometrium ((Figure 7.1)(Simón et al., 2000; Nardo et al., 2009; Nimbkar-Joshi et al., 2009; Simon and Laufer, 2012; Davidson and Coward, 2016). Failure at the implantation stage is a major contributor to the low success rates of both spontaneous pregnancies and those resulting from ART. Due to the inaccessible nature of the implantation site *in vivo*, efforts to obtain samples of embryos at the site of implantation have been largely unsuccessful. Thus, there is a distinct lack of knowledge regarding the specific mechanisms underlying the early stage of implantation and the specific molecular and cellular mechanisms of embryo implantation remain poorly understood (Singh et al., 2011).

The first physiological contact between an implantation-competent blastocyst and the receptive uterus involves sequential stages of apposition, adhesion, and invasion, which together, steer the process of successful implantation (Norwitz et al., 2001; Dey et al., 2004). During apposition, a multitude of small protrusions, referred to as uterodomes (or pinopodes) develop on the apical surface of the luminal uterine epithelium and subsequently inter-digitate with the blastocyst via surface microvilli on the apical cytotrophoblast (Bentin-Ley et al., 1999; Lopata et al., 2002). Once the embryo has settled upon the endometrium, a series of interactions take place to provide more stable adhesion with the uterine epithelium. Blastocyst attachment with the luminal epithelium overlaps with decidualization of the endometrial stroma, which is required for successful implantation. Once adhesion is complete, the trophoblast differentiates into the syncytiotrophoblast and cytotrophoblast. Located on the endometrial side of the embryo is the inner cell mass which penetrates the syncytiotrophoblast via the uterine epithelium and the underlying basement

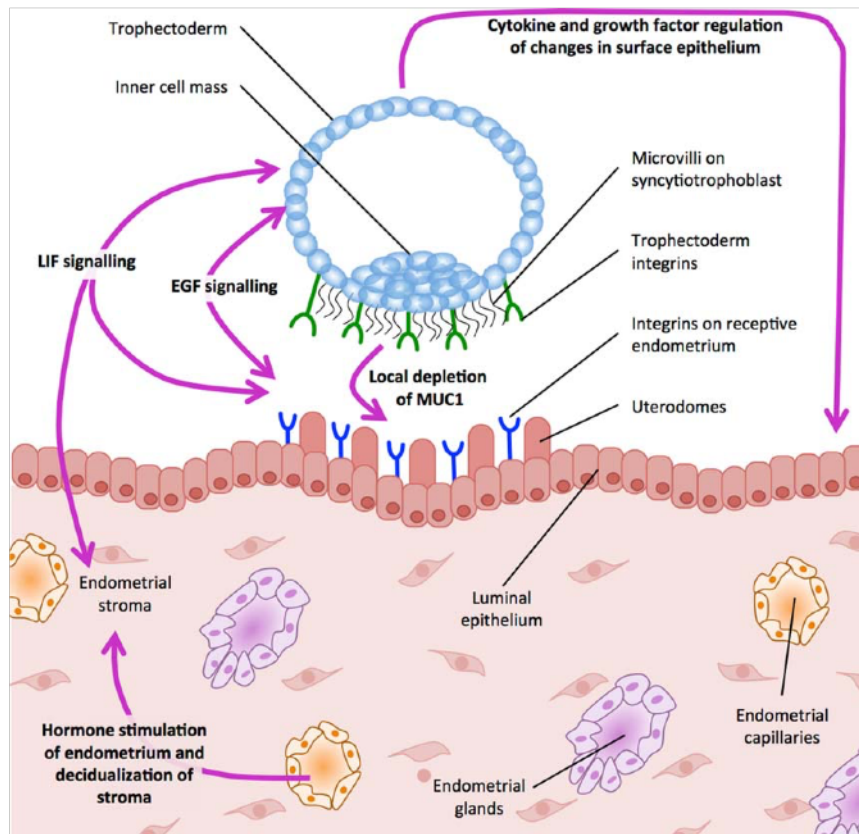


Figure 7.1: A schematic representation of early signalling between the embryo and endometrium preceding implantation. The blastocyst approaches the receptive endometrium, defined by the appearance of uterodomes and integrin profiles. Image reproduced from Davidson et al. (2016).

membrane. Eventually, the inner cell mass invades the endometrial stroma to interact with the maternal vasculature (Bentin-Ley and Lopata, 2000; Carson et al., 2000; Davidson and Coward, 2016). It is clearly evident from a mounting body of literature that numerous cell-cell and cell-matrix interactions are vital in modulating signal transduction to mediate embryo-endometrial interactions, uterine receptivity, and implantation. Such mechanisms are of huge importance as deficiencies are likely to manifest in implantation failure or infertility. However, specific molecular cross-talk between these pathways remains poorly understood, and much work is needed to elucidate and manipulate these transitions (Dey et al., 2004; Cha et al., 2012b). Unravelling the molecular foundation of implantation and implantation failure has significant implications for both understanding the underlying cause of infertility as well as determining patient prognosis (Davidson and Coward, 2016).

7.2.2 *In vitro* models of embryo implantation

Due to the multitude of technical and ethical restrictions involved in studying the precise site of human implantation *in vivo*, the majority of existing data regarding implantation are derived from animal studies (Mardon et al., 2007; Kang et al., 2014). Transgenic mouse models have helped to improve our understanding of the molecular basis of uterine receptivity and implantation in humans (Cha et al., 2012b), however there are still significant differences between the mouse model and the human processes (Davidson and Coward, 2016). As such, a variety of *in vitro* models have been developed in order to investigate interactions between the embryo and endometrium. Initially, *in vitro* models of human implantation have been developed using tissues acquired by hysterectomy or endometrial biopsy. These tissues can be co-cultured with trophoblast cells from embryos to investigate embryo positioning and invasion (Kliman et al., 1990; Landgren et al., 1996; Klemmt et al., 2009; Chillakuri et al., 2010). While endometrial tissue explants can be cultured in appropriate media to mimic the *in vivo* endometrial environment, tissue explant models are associated with the major problem of extensive tissue necrosis, thus prompting the use of cell lines as a more promising avenue (Davidson and Coward, 2016).

Multiple human cells lines have been investigated, however many often exhibit limitations such as lack of apical polarity, surface receptors, tight junctions, or adhesiveness to trophectoblast cells. Ishikawa cells appear to be a promising model with which to investigate the normal endometrial epithelium and study potential endometrial crosstalk with the embryo. The Ishikawa cell line, originally derived from endometrial 47 adenocarcinoma cells harvested from an Asian woman (39 years of age), expresses progesterone and estrogen receptors which are able to respond to steroid stimulation (Bentin-Ley et al., 1999). Moreover, this cell line exhibits a range of enzymes and proteins that are commonly found in the normal endometrium (Castelbaum et al., 1994; Mo et al., 2006). Ishikawa cells also express $\alpha1\beta1$ and $\alpha v\beta3$ integrins, which are also present in the normal endometrium, and appear to play key roles in endometrial receptivity and cell adhesion (Castelbaum et al.,

1997). Investigations have shown that Ishikawa cells are an exciting model for investigating endometrial integrins (Lessey et al., 1992; Lessey et al., 1992). They grow stably, form a monolayer, and maintain epithelial polarity, however can exhibit some morphological changes following long periods of culture (Singh et al., 2010).

The development of an *in vitro* model which completely mimics the complex *in vivo* endometrial environment has yet to be developed. Nonetheless, *in vitro* models using epithelial cells provide a useful means with which to investigate potential interactions that might occur *in vivo* and allow workers to systematically evaluate the complex network of biological pathways involved in implantation (Teklenburg and Macklon, 2009; Weimar et al., 2013; Berger et al., 2015; Davidson and Coward, 2016). An established *in vitro* model of implantation would allow further investigations into the effects of laser treatments on embryos, which currently are impossible to explore.

7.2.2.1 Laser-assisted hatching

While the exact causes of failed implantation are unknown, increased maternal age, reduced oocyte quality, and poor embryo morphology are all known to be contributing factors (Das and Holzer, 2012; Davidson and Coward, 2016). While the exact causes of failed hatching and implantation are unknown, increased maternal age, reduced oocyte quality, and poor embryo morphology, are thought to be contributing factors (Das and Holzer, 2012). It has been hypothesised that exposure of oocytes and embryos to the artificial conditions of *in vitro* culture may have negative effects upon the embryo's ability to undergo the normal hatching process. Consequently, an ART process known as 'assisted hatching' has been developed, which creates a controlled opening in the zona pellucida to facilitate embryo hatching before transfer to the patient's uterus. Assisted hatching can be performed mechanically, with acid degradation, or with various laser ablation pattern to the zona pellucida. However, the precise effects of these techniques still remain controversial, and a hatched embryo does not always implant. While, a Cochrane review demonstrated that assisted hatching offers a significantly increased chance of achieving a clinical pregnancy (Carney et al., 2012), a meta-analysis of randomised

controlled trials investigating the benefits of assisted hatching concluded that the majority results were not universally accepted due to the heterogeneity of the techniques being used (Sallam et al., 2003). This has lead to a widespread debate over the potential effects of assisted hatching, with recommendations suggesting the procedure only be used in patients with poor prognosis (ASRM, 2006). The discordant nature of the results in the available literature further defines the present debate of whether laser-assisted hatching, or assisted hatching in general, is a beneficial, neutral, or detrimental technique.

One reason for the incongruous results pertaining to laser-assisted hatching is the non-standardised use of lasers between different studies. It is evident in such studies that the specific details of laser ablation are rarely explained in detail and left up to the individual discretion of embryologists. It is conceivable that the laser conditions used in the published studies thus far have unknowingly been unsafe at the molecular level, thus confounding the results regarding the effects of the technique itself. The introduction of laser technology creates an option for safety standardisation and reproducible hatching for the first time, which is paramount for analysing results from multi-centre trials and comparing results from different studies. I hope that the determination of safe laser conditions will be more widely adopted and allow for more accurate comparison between studies using safe procedures to allow proper analysis to whether laser-assisted hatching is a beneficial technique.

I hypothesise that alterations induced by laser-treatment at the gene expression level could ultimately cause detrimental effects upon final protein activity, and thus interfere with the normal process of implantation. Future laser-based investigations could study the effects of laser treatment upon final protein translation and localisation in the developing mouse embryo, to investigate how laser treatment might interfere with the interaction of proteins in the implanting embryo with the endometrium. In order to do this, an established model of *in vitro* implantation involving endometrial stromal cells and matrigel cultured into a three-dimensional scaffold, as described above and in Kang et al. (2014), could be used (Figure 7.2). Following artificial 'implantation' of laser-treated and control embryos onto synthetic

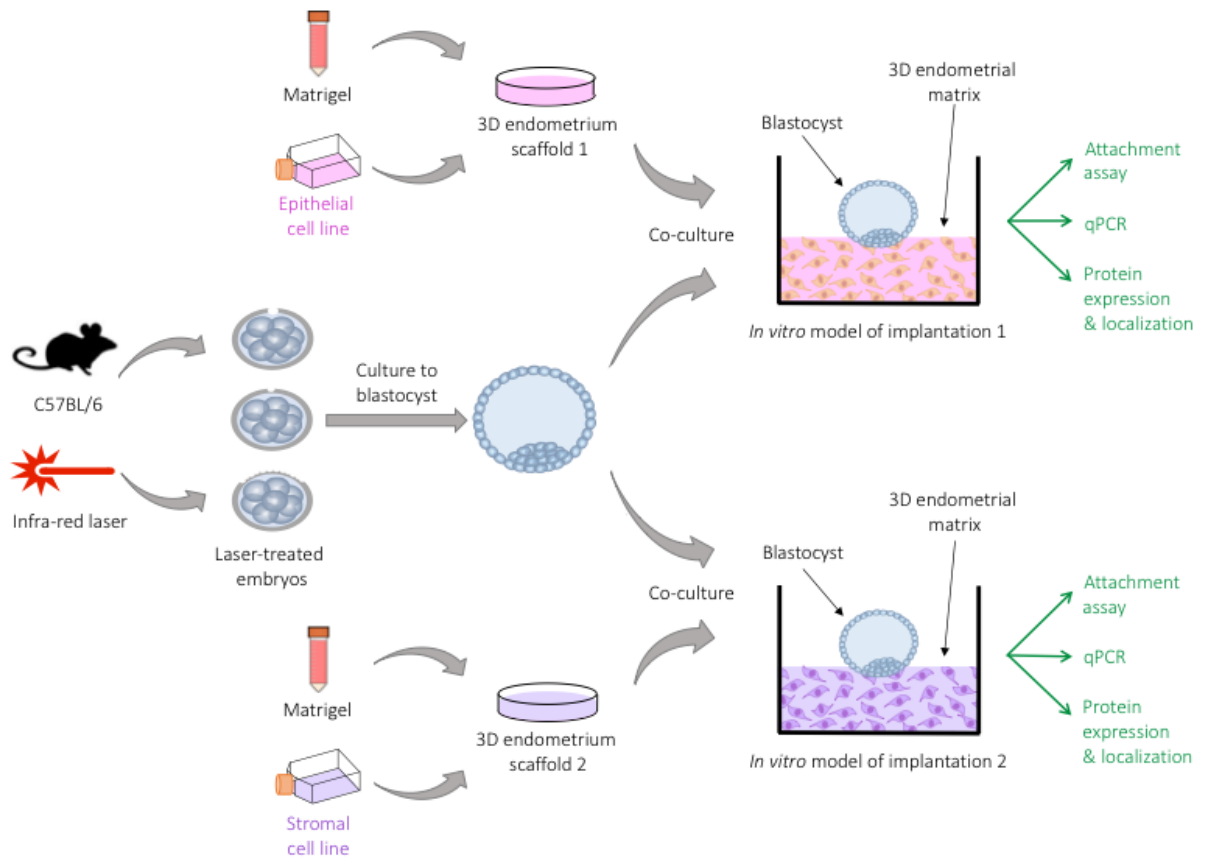


Figure 7.2: Schematic of an experimental plan to laser-treat embryos and co-culture on *in vitro* models of implantation based on endometrial epithelial cells (Ishikawa) and stromal (fibroblast) cells. Image created by author and adapted from (Carver et al., 2003).

matrigel, specific antibodies could be used to immunostain a select cohort of important proteins and thus investigate the effect of laser treatment upon embryo implantation, and how it may affect the complex molecular network involved in this critical process. Pending ethical approval, these investigations could be extended to human cryopreserved embryos that are surplus to clinical requirement, in order to analyse proteins of interest upon embryo attachment.

The development of new treatments to overcome infertility associated with implantation failure relies upon the identification, in functional models, of molecular targets with key functions in the human embryo implantation process. *In vitro* scaffolds created with endometrium surrogates co-cultured with embryos or embryo surrogates have great potential for expansion in the coming years (Weimar et al.,

2013). These *in vitro* systems will enable novel discoveries regarding the safety or iatrogenic effects of laser treatments and provide an in-depth spatial and temporal analysis of key molecular stages in the fragile and complex process of implantation. Moreover, human embryo self-organisation cultured past the implantation stage *in vitro* has only recently been reported (Shahbazi et al., 2016), and such studies could further inform future investigations regarding the effects of laser treatment upon the developing embryo. Robust investigations regarding laser treatments using *in vitro* models of embryo-endometrium implantation could represent a significant milestone in reproductive research, allowing the exploration of unseen mechanisms relating to implantation, to suggest key clues to solve problems of infertility and implantation pregnancy failure, and perhaps generate new prognostic or diagnostic biomarkers and therapeutic targets.

7.3 Concluding remarks

The field of reproductive science continues to benefit greatly from laser application for both clinical treatments and as an investigative tool to elucidate mechanisms underlying infertility with a hope of increasing our understanding and eventually developing new treatment options. It is clear that the continued success of ART lies in the dedication and skill of clinical embryologists and to the continued development and improvement of laboratory technologies. This is imperative to providing patients with the highest level of care and greatest chance of pregnancy. The manipulation of embryos using lasers represents a powerful tool to improve the efficiency and success rates of ART. Most notably, lasers are increasing the speed and efficiency of ART procedures by allowing embryologists to easily break through the zona pellucida with minimal force for procedures such as embryo biopsy, assisted hatching, and cellular microsurgery. While the manufacturing companies build the laser systems to operate at a set power, embryologists often tend to alter the pulse lengths, and thus energy and hole size delivered, using their own judgement and experience. This introduces an unknown factor into the system which could create protocol variation between each embryologist and between clinics. The continuation of studies investigating

effects of laser treatment at the molecular level are paramount in establishing any system of standardisation for laser-assisted treatments which would benefit both clinical and research applications. Future investigations should explore further downstream molecular events, for example effects on implantation-related protein expression and how such proteins might interact with the endometrium.

References

- Abdelhamid, S. et al. (2012). "Laser-induced modifications of gold nanoparticles and their cytotoxic effect". In: *J Biomed Opt* 17.6, p. 068001.
- Adiga, S. K. et al. (2007a). "Delayed and stage specific phosphorylation of H2AX during preimplantation development of gamma-irradiated mouse embryos". In: *Reproduction* 133.2, pp. 415–22.
- Adiga, S. K. et al. (2007b). "p21 provides stage specific DNA damage control to preimplantation embryos". In: *Oncogene* 26.42, pp. 6141–9.
- Adjaye, J. et al. (2005). "Primary Differentiation in the Human Blastocyst: Comparative Molecular Portraits of Inner Cell Mass and Trophectoderm Cells." In: *Stem Cells* 23, pp. 1514–1525.
- Adler, A. et al. (2014). "Blastocyst culture selects for euploid embryos: comparison of blastomere and trophectoderm biopsies". In: *Reprod Biomed Online* 28.4, pp. 485–91.
- Aktan, T. M. et al. (2004). "Use of a laser to detect viable but immotile spermatozoa". In: *Andrologia* 36.6, pp. 366–9.
- Albanese, A., P. S. Tang, and W. C. Chan (2012). "The effect of nanoparticle size, shape, and surface chemistry on biological systems". In: *Annu Rev Biomed Eng* 14, pp. 1–16.
- Ali, H. et al. (2013). "Nanomedicine for uterine leiomyoma therapy". In: *Ther Deliv* 4.2, pp. 161–75.
- Alkilany, A. M. and C. J. Murphy (2010). "Toxicity and cellular uptake of gold nanoparticles: what we have learned so far?" In: *J Nanopart Res* 12.7, pp. 2313–2333.
- Alvarez-Lorenzo, C. and A. Concheiro (2014). "Smart drug delivery systems: from fundamentals to the clinic". In: *Chem Commun (Camb)* 50.58, pp. 7743–65.
- Andersson, H. A. et al. (2014). "HSP70 promoter-driven activation of gene expression for immunotherapy using gold nanorods and near infrared light". In: *Vaccines (Basel)* 2.2, pp. 216–27.
- ASRM (2006). "The role of assisted hatching in invitro fertilisation: a review of the literature. A Committee opinion." In: *Fertility and Sterility* 86, S124–126.
- Assidi, M. et al. (2011). "Biomarkers of human oocyte developmental competence expressed in cumulus cells before ICSI: a preliminary study." In: *J Assist Reprod Genet* 28, pp. 173–188.
- Assou, S. et al. (2010). "Human cumulus cells as biomarkers for embryo and pregnancy outcomes." In: *Mol Hum Reprod* 16, pp. 531–538.
- Aznar, E. et al. (2011). "Finely tuned temperature-controlled cargo release using paraffin-capped mesoporous silica nanoparticles". In: *Angew Chem Int Ed Engl* 50.47, pp. 11172–5.
- Baek, S. M. et al. (2016). "Triple Hit with Drug Carriers: pH- and Temperature-Responsive Theranostics for Multimodal Chemo- and Photothermal-Therapy and Diagnostic Applications". In: *ACS Appl Mater Interfaces*.
- Barchanski, A. et al. (2011). "Golden perspective: application of laser-generated gold nanoparticle conjugates in reproductive biology". In: *Reprod Domest Anim* 46 Suppl 3, pp. 42–52.

- Barkalina, N. et al. (2014a). "Effects of mesoporous silica nanoparticles upon the function of mammalian sperm in vitro". In: *Nanomedicine* 10.4, pp. 859–70.
- Barkalina, N., C. Jones, and K. Coward (2014b). "Mesoporous silica nanoparticles: a potential targeted delivery vector for reproductive biology?" In: *Nanomedicine (Lond)* 9.5, pp. 557–60.
- Barkalina, N. et al. (2015a). "Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature". In: *Hum Reprod Update* 21.5, pp. 627–39.
- Barkalina, N. et al. (2015b). "Functionalization of mesoporous silica nanoparticles with a cell-penetrating peptide to target mammalian sperm in vitro". In: *Nanomedicine (Lond)* 10.10, pp. 1539–53.
- Barkalina, Natalia, Celine Jones, and Kevin Coward (2014c). "Mesoporous silica nanoparticles: a potential targeted delivery vector for reproductive biology?" eng. In: *Nanomedicine (Lond)* 9.5, pp. 557–560.
- Bengtsson, M. et al. (2005). "Gene expression profiling insingle cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA levels." In: *Genome Res* 15.10, pp. 1388–1392.
- Bensaude, O. et al. (1983). "Heat shock proteins, first major products of zygotic gene activity in mouse embryo". In: *Nature* 305.5932, pp. 331–3.
- Bentin-Ley, U. and A. Lopata (2000). "In vitro models of human blastocyst implantation". In: *Baillieres Best PRact Res Clin Obstet Gynaecol* 14, pp. 765–74.
- Bentin-Ley, U. et al. (1999). "Presence of uterine pinopodes at the embryo-endometrial interface during human implantation in vitro". In: *Human Reproduction* 14, pp. 515–520.
- Berger, C et al. (2015). "Effects of ulipristal acetate on human embryo attachment and endometrial cell gene expression in an in vitro co-culture system." eng. In: *Hum Reprod* 30.4, pp. 800–811.
- Blakeley, P. et al. (2015). "Defining the three cell lineages of the human blastocyst by single-cell RNA-seq". In: *Development* 142.18, pp. 3151–65.
- Blanchet, G B et al. (1992). "Laser micromanipulation in the mouse embryo: a novel approach to zona drilling." eng. In: *Fertil Steril* 57.6, pp. 1337–1341.
- Boada, M. et al. (1998). "Successful use of a laser for human embryo biopsy in preimplantation genetic diagnosis: report of two cases". In: *J Assist Reprod Genet* 15.5, pp. 302–7.
- Bogliotti, Y.S. et al. (2014). "Developmental outcomes and efficiency of two CRISPR/Cas9 microinjection methods in bovine zygotes." In: *Reproduction, Fertility, and Development* 27.1, pp. 94–94.
- Bogliotti, Y.S., M. Vilarino, and P.J. Ross (2016). "Laser-assisted cytoplasmic microinjection in livestock zygotes". In: *J Vis Exp* 116, e54465.
- Borensztein, M. et al. (2017). "Xist-dependent imprinted X inactivation and the early developmental consequences of its failure." In: *Nat Struct Mol Biol.* 24.3, pp. 226–233.
- Bouckenheimer, J. et al. (2016). "Long non-coding RNAs in human early embryonic development and their potential in ART." In: *Hum Reprod Update* 23.1, pp. 19–40.
- Bredenoord, A. L., G. Pennings, and G. de Wert (2008). "Ooplasmic and nuclear transfer to prevent mitochondrial DNA disorders: conceptual and normative issues". In: *Hum Reprod Update* 14.6, pp. 669–78.
- Brill, A. et al. (1999). "The Role of Apoptosis in Normal and Abnormal Embryonic Development". In: *J Assist Reprod Genet* 16.10, pp. 512–519.

- Brodie, D. et al. (2012). "Preimplantation genetic diagnosis for chromosome rearrangements - one blastomere biopsy versus two blastomere biopsy". In: *J Assist Reprod Genet* 29.8, pp. 821–7.
- Buemo, C.P. et al. (2016). "Embryo Aggregation in Pig Improves Cloning Efficiency and Embryo Quality." In: *PLoS One* 11.2, e0146390.
- Bustin, S A (2002). "Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems." eng. In: *J Mol Endocrinol* 29.1, pp. 23–39.
- Callaway, E. (2016). "UK scientists gain licence to edit genes in human embryos." In: *Nature* 530.7588.
- Campbell, K. H. et al. (2007). "Somatic cell nuclear transfer: Past, present and future perspectives". In: *Theriogenology* 68 Suppl 1, S214–31.
- Campos, V. F. et al. (2011). "NanoSMGT: transgene transmission into bovine embryos using halloysite clay nanotubes or nanopolymer to improve transfection efficiency". In: *Theriogenology* 76.8, pp. 1552–60.
- Cao, S. et al. (2014). "Retrospective clinical analysis of two artificial shrinkage methods applied prior to blastocyst vitrification on the outcome of frozen embryo transfer". In: *J Assist Reprod Genet* 31.5, pp. 577–81.
- Carney, S. K. et al. (2012). "Assisted hatching on assisted conception (in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI)". In: *Cochrane Database Syst Rev* 12, p. CD001894.
- Carson, D D et al. (2000). "Embryo implantation." eng. In: *Dev Biol* 223.2, pp. 217–237.
- Carver, Janet et al. (2003). "An in-vitro model for stromal invasion during implantation of the human blastocyst." eng. In: *Hum Reprod* 18.2, pp. 283–290.
- Castelbaum, A J et al. (1994). "Timing of the endometrial biopsy may be critical for the accurate diagnosis of luteal phase deficiency." eng. In: *Fertil Steril* 61.3, pp. 443–447.
- Castelbaum, A J et al. (1997). "Characterization of integrin expression in a well differentiated endometrial adenocarcinoma cell line (Ishikawa)." eng. In: *J Clin Endocrinol Metab* 82.1, pp. 136–142.
- Castillo-Martin, M. et al. (2014). "Supplementing culture and vitrification-warming media with l-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of GPX1 and SOD1 expression". In: *Cryobiology* 68.3, pp. 451–8.
- Castillo-Martin, M. et al. (2015a). "Addition of L-ascorbic acid to culture and vitrification media of IVF porcine blastocysts improves survival and reduces HSPA1A levels of vitrified embryos". In: *Reprod Fertil Dev* 27.7, pp. 1115–23.
- Castillo-Martin, M. et al. (2015b). "Effects of vitrification on the expression of pluripotency, apoptotic and stress genes in in vitro-produced porcine blastocysts". In: *Reprod Fertil Dev* 27.7, pp. 1072–81.
- Castro, R.J. (2016). "Mitochondrial replacement therapy: the UK and US regulatory landscapes". In: *J Law Biosci* 3.3, pp. 726–735.
- Cha, Jeeyeon, Xiaofei Sun, and Sudhansu K Dey (2012a). "Mechanisms of implantation: strategies for successful pregnancy." eng. In: *Nat Med* 18.12, pp. 1754–1767.
- (2012b). "Mechanisms of implantation: strategies for successful pregnancy." eng. In: *Nat Med* 18.12, pp. 1754–1767.
- Chatzimeletiou, K., H. M. Picton, and A. H. Handyside (2001). "Use of a non-contact, infrared laser for zona drilling of mouse embryos: assessment of immediate effects on blastomere viability". In: *Reprod Biomed Online* 2.3, pp. 178–187.

- Chatzimeletiou, K. et al. (2005). "Comparison of effects of zona drilling by non-contact infrared laser or acid Tyrode's on the development of human biopsied embryos as revealed by blastomere viability, cytoskeletal analysis and molecular cytogenetics". In: *Reprod Biomed Online* 11.6, pp. 697–710.
- Chen, Alice E et al. (2009). "Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines." eng. In: *Cell Stem Cell* 4.2, pp. 103–106.
- Chen, L. et al. (2015a). "Thermo-and pH dual-responsive mesoporous silica nanoparticles for controlled drug release". In: *J Control Release* 213, e69–70.
- Chen, N. T. et al. (2014). "Enhanced plasmonic resonance energy transfer in mesoporous silica-encased gold nanorod for two-photon-activated photodynamic therapy". In: *Theranostics* 4.8, pp. 798–807.
- Chen, S. U. et al. (2005). "Microsuction of blastocoelic fluid before vitrification increased survival and pregnancy of mouse expanded blastocysts, but pretreatment with the cytoskeletal stabilizer did not increase blastocyst survival". In: *Fertil Steril* 84 Suppl 2, pp. 1156–62.
- Chen, Y., H. Chen, and J. Shi (2013). "In vivo bio-safety evaluations and diagnostic/therapeutic applications of chemically designed mesoporous silica nanoparticles". In: *Adv Mater* 25.23, pp. 3144–76.
- Chen, Z. et al. (2015b). "Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction." In: *Proc Natl Acad Sci USA* 112.15, pp. 4618–4623.
- Child, T. (2013). *Textbook of Clinical Embryology*. Ed. by K. Coward and D. Wells. Cambridge University Press.
- Chillakuri, Chandramouli R, Celine Jones, and Helen J Mardon (2010). "Heparin binding domain in vitronectin is required for oligomerization and thus enhances integrin mediated cell adhesion and spreading." eng. In: *FEBS Lett* 584.15, pp. 3287–3291.
- Coco, R. (2014). "Reprogenetics: Preimplantation genetics diagnosis". In: *Genet Mol Biol* 37.1, pp. 271–284.
- Craig, J and K Turner (2013). *Cryopreservation in assisted reproduction*. In 'Textbook of Clinical Embryology'. New York, NY: Cambridge University Press.
- Croissant, J. and J. I. Zink (2012). "Nanovalve-controlled cargo release activated by plasmonic heating". In: *J Am Chem Soc* 134.18, pp. 7628–31.
- Cyranoski, D. and S. Reardon (2015). "Embryo editing sparks epic debate". In: *Nature* 520.7549, pp. 593–4.
- Dai, L. et al. (2014). "Redox-responsive nanocarrier based on heparin end-capped mesoporous silica nanoparticles for targeted tumor therapy in vitro and in vivo." In: *Langmuir* 8.26, p. 10.
- Danfour, MA and MS Elmahaishi (2010). "Human oocyte oolemma characteristic is positively related to embryo developmental competence after ICSI procedure". In: *Middle East Fertility Society Journal* 15, p. 4.
- Das, M. and H. E. Holzer (2012). "Recurrent implantation failure: gamete and embryo factors". In: *Fertil Steril* 97.5, pp. 1021–7.
- Davidson, L. M. and K. Coward (2016). "Molecular mechanisms of membrane interaction at implantation." eng. In: *Birth Defects Res C Embryo Today* 108.1, pp. 19–32.
- Davidson, L. M. et al. (2015a). "Beam me up, baby: exploring the use of lasers in assisted reproductive technologies". In: *Infocus* 38.

- Davidson, L. M., N. Barkalina, and K. Coward (2015b). "Development of nanoparticle-mediated delivery tools to investigate the role of molecular genetic mechanisms underlying male infertility." In: *Science Advances Today* 1, p. 25210.
- Davidson, L. M. et al. (2016). "Development of a laser-activated mesoporous silica nanocarrier delivery system for applications in molecular and genetic research." eng. In: *J Biomed Opt* 21.11, p. 115002.
- De Mouzon, J. et al. (2010). "Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE". In: *Hum Reprod* 25.8, pp. 1851–62.
- Debrock, S. et al. (2011). "The effect of modified quarter laser-assisted zona thinning on the implantation rate per embryo in frozen/vitrified-thawed/warmed embryo transfer cycles: a prospective randomized controlled trial". In: *Hum Reprod* 26.8, pp. 1997–2007.
- Deguchi, T. et al. (2009). "Infrared laser-mediated local gene induction in medaka, zebrafish and Arabidopsis thaliana". In: *Dev Growth Differ* 51.9, pp. 769–75.
- Desai, N et al. (2008). "Artificial Collapse of Blastocysts Before Vitrification: Mechanical vs. Laser Technique and Effect on Survival, Cell Number, and Cell Death in Early and Expanded Blastocysts." In: *Cell Preservation Technology* 6, p. 8.
- Desjardins, P. and D. Conklin (2010). "NanoDrop Microvolume Quantitation of Nucleic Acids". In: *J Vis Exp* 45, p. 2565.
- Dey, S K et al. (2004). "Molecular cues to implantation." eng. In: *Endocr Rev* 25.3, pp. 341–373.
- Dobson, Anthony T et al. (2004). "The unique transcriptome through day 3 of human preimplantation development." eng. In: *Hum Mol Genet* 13.14, pp. 1461–1470.
- Doherty, A. S. et al. (2000). "Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo". In: *Biol Reprod* 62.6, pp. 1526–35.
- Douglas-Hamilton, D. H. and J. Conia (2001). "Thermal effects in laser-assisted pre-embryo zona drilling". In: *J Biomed Opt* 6.2, pp. 205–13.
- Ealy, A. D. et al. (1995). "Developmental changes in sensitivity of bovine embryos to heat shock and use of antioxidants as thermoprotectants". In: *J Anim Sci* 73.5, pp. 1401–7.
- Ebner, T., M. Moser, and G. Tews (2005). "Possible applications of a non-contact 1.48 microm wavelength diode laser in assisted reproduction technologies". In: *Hum Reprod Update* 11.4, pp. 425–35.
- Edwards, R. G. (2005). "An astonishing journey into reproductive genetics since the 1950's". In: *Reprod Nutr Dev* 45.3, pp. 299–306.
- Egli, Dieter et al. (2017). "Inter-homologue repair in fertilized human eggs?" In: *bioRxiv*. eprint: <https://www.biorxiv.org/content/early/2017/08/28/181255.full.pdf>.
- Ehrmann, David A (2005). "Polycystic ovary syndrome." eng. In: *N Engl J Med* 352.12, pp. 1223–1236.
- El Danasouri, I et al. (1993). "Zona opening with 308 nm XeCl excimer laser improves fertilization by spermatozoa from long-term vasectomized mice." eng. In: *Hum Reprod* 8.3, pp. 464–466.
- El-Toukhy, T. et al. (2003). "Effect of blastomere loss on the outcome of frozen embryo replacement cycles". In: *Fertil Steril* 79.5, pp. 1106–11.
- Elhussieny, A et al. (2013). "Effect of laser-assisted hatching on outcome of assisted reproductive technology". In: *Open Journal of Obstetrics and Gynecology* 3, p. 5.

- Fasano, Giovanna et al. (2014). "A randomized controlled trial comparing two vitrification methods versus slow-freezing for cryopreservation of human cleavage stage embryos." eng. In: *J Assist Reprod Genet* 31.2, pp. 241–247.
- Feichtinger, W et al. (1992). "Photoablation of oocyte zona pellucida by erbium-YAG laser for in-vitro fertilisation in severe male infertility." eng. In: *Lancet* 339.8796, p. 811.
- Filliers, M. et al. (2011). "Laser capture microdissection for gene expression analysis of inner cell mass and trophectoderm from blastocysts". In: *Anal Biochem* 408.1, pp. 169–71.
- Fragouli, E. et al. (2012). "Alteration of a gene expression in human cumulus cells as a potential indicator of oocyte aneuploidy." In: *Hum Reprod* 27.8, pp. 2559–2568.
- Fynnewever, T. L. et al. (2007). "In vitro tagging of embryos with nanoparticles". In: *J Assist Reprod Genet* 24.2-3, pp. 61–5.
- Gabrielsen, A. et al. (2000). "Influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following in vitro fertilization treatment". In: *J Assist Reprod Genet* 17.6, pp. 323–8.
- Gardner, D. K., M. G. Larman, and G. A. Thouas (2010). "Sex-related physiology of the preimplantation embryo". In: *Mol Hum Reprod* 16.8, pp. 539–47.
- Gardner, DK and WB Schoolcraft (1999). *In vitro culture of human blastocysts*. Towards Reproductive Certainty: Fertility and Genetics Beyond 1999. UK: Parthenon Publishing London, p. 10.
- Geber, S. et al. (2011). "Laser confers less embryo exposure than acid tyrode for embryo biopsy in preimplantation genetic diagnosis cycles: a randomized study". In: *Reprod Biol Endocrinol* 9, p. 58.
- Gelbaya, T. A. et al. (2014). "Definition and epidemiology of unexplained infertility". In: *Obstet Gynecol Surv* 69.2, pp. 109–15.
- Gil, M.A. et al. (2017). "Developmental competence of porcine genome-edited zygotes." In: *Molecular Reproduction and Development* 9999, pp. 1–8.
- Glasel, J A (1995). "Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios." eng. In: *Biotechniques* 18.1, pp. 62–63.
- Grabher, C. and J. Wittbrodt (2004). "Efficient activation of gene expression using a heat-shock inducible Gal4/Vp16-UAS system in medaka". In: *BMC Biotechnol* 4, p. 26.
- Granne, Ingrid., Tim Child, and Geraldine Hartshorne (2008). "Embryo cryopreservation: evidence for practice." eng. In: *Hum Fertil (Camb)* 11.3, pp. 159–172.
- Greggains, G. D. et al. (2014). "Therapeutic potential of somatic cell nuclear transfer for degenerative disease caused by mitochondrial DNA mutations". In: *Sci Rep* 4, p. 3844.
- Guerif, F. et al. (2002). "Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal". In: *Hum Reprod* 17.5, pp. 1321–6.
- Guimaraes, F. et al. (2016). "Live births after polar body biopsy and frozen-thawed cleavage stage embryo transfer: case report". In: *JBRA Assist Reprod* 20.4, pp. 253–256.
- Guo, Guoji et al. (2010). "Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst." eng. In: *Dev Cell* 18.4, pp. 675–685.
- Gupta, T. and M. C. Mullins (2010). "Dissection of organs from the adult zebrafish". In: *J Vis Exp* 37.
- Haas, R. H. et al. (2007). "Mitochondrial disease: a practical approach for primary care physicians". In: *Pediatrics* 120.6, pp. 1326–33.

- Halloran, M. C. et al. (2000). "Laser-induced gene expression in specific cells of transgenic zebrafish". In: *Development* 127.9, pp. 1953–60.
- Hamazaki, Nobuhiko et al. (2015). "Gene activation-associated long noncoding RNAs function in mouse preimplantation development." eng. In: *Development* 142.5, pp. 910–920.
- Hammadeh, M. E., C. Fischer-Hammadeh, and K. R. Ali (2011). "Assisted hatching in assisted reproduction: a state of the art". In: *J Assist Reprod Genet* 28.2, pp. 119–28.
- Hammoud, I. et al. (2010). "Are zona pellucida laser drilling and polar body biopsy safe for in vitro matured oocytes?" In: *J Assist Reprod Genet* 27.7, pp. 423–7.
- Han, T. S. et al. (2003). "Laser-assisted human embryo biopsy on the third day of development for preimplantation genetic diagnosis: two successful case reports". In: *Fertil Steril* 80.2, pp. 453–5.
- Harper, J. et al. (2010). "What next for preimplantation genetic screening (PGS)? A position statement from the ESHRE PGD Consortium Steering Committee". In: *Hum Reprod* 25.4, pp. 821–3.
- Hartshorne, C, A Anshelevich, and L Wang (2005). "Laser zona drilling does not induce hsp70i transcription in blastomeres of eight-cell mouse embryo". In: *Fertility and Sterility* 84, p. 3.
- Heinemann, D. et al. (2013). "Gold nanoparticle mediated laser transfection for efficient siRNA mediated gene knock down". In: *PLoS One* 8.3, e58604.
- Hendriks, W. K. et al. (2014). "Cellular damage suffered by equine embryos after exposure to cryoprotectants or cryopreservation by slow-freezing or vitrification". In: *Equine Vet J*.
- Hiraoka, K., M. Kinutani, and K. Kinutani (2004). "Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts". In: *Hum Reprod* 19.12, pp. 2884–8.
- Hou, Y. et al. (2013). "Genome analyses of single human oocytes". In: *Cell* 155.7, pp. 1492–506.
- Hsieh, Y. Y. et al. (2002). "Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age". In: *Fertil Steril* 78.1, pp. 179–82.
- Huang, W., B.S. Yandell, and H. Khatib (2010). "Transcriptomic profiling of bovine IVF embryos revealed candidate genes and pathways involved in early embryonic development." In: *BMC Genomics* 11.23, doi:10.1186/1471-2164-11-23.
- Hur, Y. S. et al. (2011). "Effect of artificial shrinkage on clinical outcome in fresh blastocyst transfer cycles". In: *Clin Exp Reprod Med* 38.2, pp. 87–92.
- Hyslop, Louise A et al. (2016). "Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease." eng. In: *Nature* 534.7607, pp. 383–386.
- Iwayama, H., S. Hochi, and M. Yamashita (2011). "In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification". In: *J Assist Reprod Genet* 28.4, pp. 355–61.
- Jain, J. K. and R. J. Paulson (2006). "Oocyte cryopreservation". In: *Fertil Steril* 86.4 Suppl, pp. 1037–46.
- Jeong, J.K. et al. (2014). "Evaluation of reference genes in mouse preimplantation embryos for gene expression studies using real-time quantitative RT-PCR (RT-qPCR)." In: *BMC Research Notes* 7, p. 675.

- Ji, Guixiang et al. (2012). "Common variants in mismatch repair genes associated with increased risk of sperm DNA damage and male infertility." eng. In: *BMC Med* 10, p. 49.
- Jones, A. E. et al. (2006). "Comparison of laser-assisted hatching and acidified Tyrode's hatching by evaluation of blastocyst development rates in sibling embryos: a prospective randomized trial". In: *Fertil Steril* 85.2, pp. 487–91.
- Joris, H. et al. (2003). "Comparison of the results of human embryo biopsy and outcome of PGD after zona drilling using acid Tyrode medium or a laser". In: *Hum Reprod* 18.9, pp. 1896–902.
- Kahraman, S. et al. (2004). "The results of aneuploidy screening in 276 couples undergoing assisted reproductive techniques". In: *Prenat Diagn* 24.4, pp. 307–11.
- Kamjoo, M., D. R. Brison, and S. J. Kimber (2002). "Apoptosis in the preimplantation mouse embryo: effect of strain difference and in vitro culture". In: *Mol Reprod Dev* 61.1, pp. 67–77.
- Kang, Y. J. et al. (2014). "The role of the osteopontin-integrin alphavbeta3 interaction at implantation: functional analysis using three different in vitro models". In: *Hum Reprod* 29.4, pp. 739–49.
- Kanyó, K. and J. Konc (2003). "A follow-up study of children born after diode laser assisted hatching". In: *Eur J Obstet Gynecol Reprod Biol* 110.2, pp. 176–80.
- Kashir, J et al. (2012). "Magnifying human infertility: microscopy and assisted reproductive technology". In: *Infocus* 25, p. 18.
- Kim, D., Y. Y. Jeong, and S. Jon (2010). "A drug-loaded aptamer-gold nanoparticle bioconjugate for combined CT imaging and therapy of prostate cancer". In: *ACS Nano* 4.7, pp. 3689–96.
- Kimura, E. et al. (2013). "Application of infrared laser to the zebrafish vascular system: gene induction, tracing, and ablation of single endothelial cells". In: *Arterioscler Thromb Vasc Biol* 33.6, pp. 1264–70.
- Klemmt, P.A.B. et al. (2009). "Effects of gonadotrophin releasing hormone analogues on human endometrial stromal cells and embryo invasion in vitro." In: *Human Reproduction* 24.9, pp. 2187–2192.
- Kliman, H.J., R.F. Feinberg, and J.E Haimowitz (1990). "Human trophoblast-endometrial interactions in an in vitro suspension culture system." In: *Placenta* 11, pp. 349–367.
- Kochevar, I E (1989). "Cytotoxicity and mutagenicity of excimer laser radiation." eng. In: *Lasers Surg Med* 9.5, pp. 440–445.
- Kokkali, G. et al. (2007). "Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of beta-thalassaemia: a pilot study". In: *Hum Reprod* 22.5, pp. 1443–9.
- Kukurba, K.R. and S.B. Montgomery (2015). "RNA Sequencing and Analysis". In: *Cold Spring Harb Protoc* 13.11, pp. 951–969.
- Kuliev, A. et al. (2006). "Pre-embryonic diagnosis for Sandhoff disease". In: *Reprod Biomed Online* 12.3, pp. 328–33.
- Kunzmann, A. et al. (2011). "Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation". In: *Biochim Biophys Acta* 1810.3, pp. 361–73.
- Kutlu, P., O. Atvar, and O. F. Vanlioglu (2010). "Laser assisted zona thinning technique has no beneficial effect on the ART outcomes of two different maternal age groups". In: *J Assist Reprod Genet* 27.8, pp. 457–61.

- Landgren, B.M. et al. (1996). "A new method to study the process of implantation of a human blastocyst in vitro." In: *Fertility and Sterility* 65, pp. 1067–70.
- Lanphier, E. et al. (2015). "Don't edit the human germ line". In: *Nature* 519.7544, pp. 410–1.
- Lanzendorf, S. E. et al. (2007). "A randomized, prospective study comparing laser-assisted hatching and assisted hatching using acidified medium". In: *Fertil Steril* 87.6, pp. 1450–7.
- Larman, M G et al. (2011). "Analysis of global gene expression following mouse blastocyst cryopreservation." eng. In: *Hum Reprod* 26.10, pp. 2672–2680.
- Ledford, H. (2015). "CRISPR, the disruptor". In: *Nature* 522.7554, pp. 20–4.
- Lee, H. J. et al. (2012). "Ultrasmall superparamagnetic iron oxides enhanced MR imaging in rats with experimentally induced endometriosis". In: *Magn Reson Imaging* 30.6, pp. 860–8.
- Lee, Sun Hee et al. (2017). "Comparison of clinical outcomes between in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in IVF-ICSI split insemination cycles." eng. In: *Clin Exp Reprod Med* 44.2, pp. 96–104.
- Lee, Y. S. et al. (2015). "Effect of laser-assisted multi-point zona thinning on development and hatching of cleavage embryos in mice". In: *Clin Exp Reprod Med* 42.2, pp. 51–7.
- Leese, Henry J (2002). "Quiet please, do not disturb: a hypothesis of embryo metabolism and viability." eng. In: *Bioessays* 24.9, pp. 845–849.
- Lessey, B.A. et al. (1992). "Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle." In: *J Clin Invest* 90, pp. 188–195.
- Levin, I. et al. (2012). "Effects of laser polar-body biopsy on embryo quality". In: *Fertil Steril* 97.5, pp. 1085–8.
- Li, E, T H Bestor, and R Jaenisch (1992). "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." eng. In: *Cell* 69.6, pp. 915–926.
- Li, J. et al. (2009). "Human embryos derived by somatic cell nuclear transfer using an alternative enucleation approach". In: *Cloning Stem Cells* 11.1, pp. 39–50.
- Li, X. et al. (2011). "The packaging of siRNA within the mesoporous structure of silica nanoparticles". In: *Biomaterials* 32.35, pp. 9546–56.
- Liang, P. et al. (2015). "CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes". In: *Protein Cell* 6.5, pp. 363–72.
- Lin, Y. S. and C. L. Haynes (2010). "Impacts of mesoporous silica nanoparticle size, pore ordering, and pore integrity on hemolytic activity". In: *J Am Chem Soc* 132.13, pp. 4834–42.
- Lin, Y.C. et al. (2014). "Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations". In: *Nature Communications* 3.5, pp. 47–67.
- Liu, H. S. and P. L. Chu (2014). "Three-parent embryo: The therapeutic future for inherited mitochondrial diseases". In: *J Formos Med Assoc*.
- Liu, W. X. et al. (2005). "Effects of removal of necrotic blastomeres from mouse cryopreserved embryos on blastocyst formation and hatching". In: *Theriogenology* 64.5, pp. 1114–20.
- Liu, W. X. et al. (2007). "Effects of removal of necrotic blastomeres from human cryopreserved embryos on pregnancy outcome". In: *Cryo Letters* 28.2, pp. 129–36.
- Lopata, A., U. Bentin-Ley, and A. Enders (2002). "Pinopodes" and implantation". In: *Rev Endocr Metab Disord* 3, pp. 77–86.

- Lorenz, Todd C (2012). "Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies." eng. In: *J Vis Exp* 63, e3998.
- Lu, H. F. et al. (2015). "The outcomes of intracytoplasmic sperm injection and laser assisted hatching in women undergoing in vitro fertilization are affected by the cause of infertility". In: *Int J Fertil Steril* 9.1, pp. 33–40.
- Ma, Hong et al. (2017). "Correction of a pathogenic gene mutation in human embryos." eng. In: *Nature*.
- Mahajan, N. (2015). "Fertility preservation in female cancer patients: An overview". In: *J Hum Reprod Sci* 8.1, pp. 3–13.
- Makhluf, Shirly Ben-David et al. (2008). "Modified PVA-Fe₃O₄ nanoparticles as protein carriers into sperm cells." eng. In: *Small* 4.9, pp. 1453–1458.
- Malter, H. E., T. Schimmel, and J. Cohen (2001). "Zona dissection by infrared laser: developmental consequences in the mouse, technical considerations, and controlled clinical trial". In: *Reprod Biomed Online* 3.2, pp. 117–123.
- Mamaeva, V. et al. (2011). "Mesoporous silica nanoparticles as drug delivery systems for targeted inhibition of Notch signaling in cancer". In: *Mol Ther* 19.8, pp. 1538–46.
- Mantoudis, E. et al. (2001). "A comparison between quarter, partial and total laser assisted hatching in selected infertility patients". In: *Hum Reprod* 16.10, pp. 2182–6.
- Mardon, H., S. Grewal, and K. Mills (2007). "Experimental models for investigating implantation of the human embryo." In: *Semin Reprod Med* 25, pp. 410–417.
- Martelli, G. et al. (2013). "Coiled-coil peptide motifs as thermoresponsive valves for mesoporous silica nanoparticles". In: *Chem Commun (Camb)* 49.85, pp. 9932–4.
- Martin, J.A. and Z. Wang (2011). "Next-generation transcriptome assembly". In: *Nat Rev Genet* 12, pp. 671–682.
- Martins, W. P. et al. (2011). "Assisted hatching of human embryos: a systematic review and meta-analysis of randomized controlled trials". In: *Hum Reprod Update* 17.4, pp. 438–53.
- Mastenbroek, S. et al. (2011). "Preimplantation genetic screening: a systematic review and meta-analysis of RCTs." In: *Hum Reprod Update* 17.4, pp. 454–466.
- Merchant, R., G. Gandhi, and G.N. Allahbadia (2011). "In vitro fertilisation/intracytoplasmic sperm injection for male infertility." In: *Indian J Urol* 27.1, pp. 121–132.
- Min, S. H. et al. (2014). "Forced collapse of the blastocoel cavity improves developmental potential in cryopreserved bovine blastocysts by slow-rate freezing and vitrification". In: *Reprod Domest Anim* 49.4, pp. 684–92.
- Mo, Bilan et al. (2006). "ECC-1 cells: a well-differentiated steroid-responsive endometrial cell line with characteristics of luminal epithelium." eng. In: *Biol Reprod* 75.3, pp. 387–394.
- Montag, HM et al. (2009). "Application of non-contact laser technology in assisted reproduction." In: *Medical Laser Application*. 24, p. 7.
- Montag, M. et al. (1998). "Laser-assisted microdissection of the zona pellucida facilitates polar body biopsy". In: *Fertil Steril* 69.3, pp. 539–42.
- Montjean, D. et al. (2014). "Normal live birth after vitrified/warmed oocytes intracytoplasmic sperm injection with immotile spermatozoa in a patient with Kartagener's syndrome". In: *Andrologia*.
- Morimoto, R. I. and M. G. Santoro (1998). "Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection". In: *Nat Biotechnol* 16.9, pp. 833–8.

- Moro, L.N. et al. (2014). “212 INTERSPECIFIC CLONING AND EMBRYO AGGREGATION INFLUENCE THE EXPRESSION OF *oct4*, *nanog*, *sox2*, AND *cdx2* IN CHEETAH AND DOMESTIC CAT BLASTOCYSTS.” In: *Reproduction, Fertility and Development*. 27.1, pp. 196–196.
- Mortazavi, Ali et al. (2008). “Mapping and quantifying mammalian transcriptomes by RNA-Seq.” eng. In: *Nat Methods* 5.7, pp. 621–628.
- Mueller, O., S. Lightfoot, and A. Schroeder (2016). *RNA Integrity Number (RIN) - Standardization of RNA Quality Control*. Tech. rep. Agilent Technologies.
- Mukaida, T. et al. (2006). “Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts”. In: *Hum Reprod* 21.12, pp. 3246–52.
- Mura, S., J. Nicolas, and P. Couvreur (2013). “Stimuli-responsive nanocarriers for drug delivery”. In: *Nat Mater* 12.11, pp. 991–1003.
- Nadrah, P. et al. (2013). “Hindered disulfide bonds to regulate release rate of model drug from mesoporous silica”. In: *ACS Appl Mater Interfaces* 5.9, pp. 3908–15.
- Nardo, Luciano. G., Ingrid Granne, and Jane Stewart (2009). “Medical adjuncts in IVF: evidence for clinical practice.” eng. In: *Hum Fertil (Camb)* 12.1, pp. 1–13.
- Niakan, Kathy K et al. (2012). “Human pre-implantation embryo development.” eng. In: *Development* 139.5, pp. 829–841.
- NICE (2013). *Fertility: assessment and treatment for people with fertility problems*. second. National Collaborating Centre for Women’s and Children’s Health. Royal College of Obstetricians and Gynaecologists.
- Nieminen, A. L. et al. (1992). “A novel cytotoxicity screening assay using a multiwell fluorescence scanner”. In: *Toxicol Appl Pharmacol* 115.2, pp. 147–55.
- Nimbkar-Joshi, S. et al. (2009). “Embryo-induced alterations in the molecular phenotype of primate endometrium”. In: *J Reprod Immunol* 83.1-2, pp. 65–71.
- Nordhoff, V. (2015). “How to select immotile but viable spermatozoa on the day of intracytoplasmic sperm injection? An embryologist’s view”. In: *Andrology* 3.2, pp. 156–62.
- Norhazlin, J et al. (2015). “Effect of DNase treatment on RNA extraction from preimplantation murine embryos.” eng. In: *Genet Mol Res* 14.3, pp. 10172–10184.
- Norwitz, E R, D J Schust, and S J Fisher (2001). “Implantation and the survival of early pregnancy.” eng. In: *N Engl J Med* 345.19, pp. 1400–1408.
- Nyboe, A.A., E. Carlsen, and A. Loft (2008). “Trends in the use of intracytoplasmic sperm injection marked variability between countries.” eng. In: *Hum Reprod Update* 14.6, pp. 593–604.
- Oron, Efrat and Natalia Ivanova (2012). “Cell fate regulation in early mammalian development.” eng. In: *Phys Biol* 9.4, p. 045002.
- Palanker, D. et al. (1991). “Technique for cellular microsurgery using the 193-nm excimer laser”. In: *Lasers Surg Med* 11.6, pp. 580–6.
- Petropoulos, S. et al. (2016). “Single-cell RNA sequencing: revealing human pre-implantation development, pluripotency and germline development.” In: *J Intern Med* 280.3, pp. 252–264.
- Petros, R. A. and J. M. DeSimone (2010). “Strategies in the design of nanoparticles for therapeutic applications”. In: *Nat Rev Drug Discov* 9.8, pp. 615–27.

- Pierce, K. E. et al. (1997). "Preimplantation development of mouse and human embryos biopsied at cleavage stages using a modified displacement technique". In: *Hum Reprod* 12.2, pp. 351–6.
- Piliszek, A., G. S. Kwon, and A.-K. (2011). Hadjantonakis (2011). "Ex Utero Culture and Live Imaging of Mouse Embryos." In: *Methods in Molecular Biology* 770, pp. 243–257.
- Poli, M. et al. (2015). "Characterization and quantification of proteins secreted by single human embryos prior to implantation". In: *EMBO Mol Med*.
- Posfai, E. et al. (2017). "Position- and Hippo signaling-dependent plasticity during lineage segregation in the early mouse embryo." In: *Elife* 6, e22906.
- Poulton, J. et al. (2009). "Preventing transmission of maternally inherited mitochondrial DNA diseases". In: *BMJ* 338, b94.
- Primi, M. P. et al. (2004). "A European multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immunosuppressive/antibiotic treatment in different patient populations". In: *Hum Reprod* 19.10, pp. 2325–33.
- Punab, M et al. (2017). "Causes of male infertility: a 9-year prospective monocentre study on 1737 patients with reduced total sperm counts." eng. In: *Hum Reprod* 32.1, pp. 18–31.
- Raj, Arjun and Alexander van Oudenaarden (2009). "Single-molecule approaches to stochastic gene expression." eng. In: *Annu Rev Biophys* 38, pp. 255–270.
- Ramos, D. M. et al. (2006). "Temporal and spatial control of transgene expression using laser induction of the hsp70 promoter". In: *BMC Dev Biol* 6, p. 55.
- Reed, M. L., B. J. Woodward, and Swain J. A. (2011). "Single of group culture of mammalian embryos: the verdict of the literature". In: *J Reprod Stem Cell Biotechnol* 2.2, pp. 77–87.
- Ren, Shancheng et al. (2012). "RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent gene fusions, cancer-associated long noncoding RNAs and aberrant alternative splicings." eng. In: *Cell Res* 22.5, pp. 806–821.
- Richa, R. P. Sinha, and D. P. Häder (2015). "Physiological aspects of UV-excitation of DNA". In: *Top Curr Chem* 356, pp. 203–48.
- Rienzi, L. et al. (2001). "Laser-assisted intracytoplasmic sperm injection". In: *Fertil Steril* 76.5, pp. 1045–7.
- Rienzi, L. et al. (2002). "Laser-assisted removal of necrotic blastomeres from cryopreserved embryos that were partially damaged". In: *Fertil Steril* 77.6, pp. 1196–201.
- Rienzi, L. et al. (2005). "Developmental potential of fully intact and partially damaged cryopreserved embryos after laser-assisted removal of necrotic blastomeres and post-thaw culture selection". In: *Fertil Steril* 84.4, pp. 888–94.
- Rienzi, Laura et al. (2017). "Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance." eng. In: *Hum Reprod Update* 23.2, pp. 139–155.
- Rink, K et al. (1994). "1.48 um Diode Laser Microdissection of the Zona Pellucida of Mouse Zygotes". In: *SPIE* 2134, p. 10.
- Rink, K. et al. (1996). "Non-contact microdrilling of mouse zona pellucida with an objective-delivered 1.48-microns diode laser". In: *Lasers Surg Med* 18.1, pp. 52–62.
- Rocha, J. C. et al. (2016). "Methods for assessing the quality of mammalian embryos: How far we are from the gold standard?" In: *JBRA Assist Reprod* 20.3, pp. 150–8.

- Rosenholm, J. M. et al. (2012). "Nanoparticles in targeted cancer therapy: mesoporous silica nanoparticles entering preclinical development stage". In: *Nanomedicine (Lond)* 7.1, pp. 111–20.
- Ruocco, Nadia et al. (2017). "High-quality RNA extraction from the sea urchin *Paracentrotus lividus* embryos." eng. In: *PLoS One* 12.2, e0172171.
- Sajjadi, A. Y., K. Mitra, and M. Grace (2013). "Expression of heat shock proteins 70 and 47 in tissues following short-pulse laser irradiation: assessment of thermal damage and healing". In: *Med Eng Phys* 35.10, pp. 1406–14.
- Sallam, H. N., S. S. Sadek, and A. F. Agameya (2003). "Assisted hatching—a meta-analysis of randomized controlled trials". In: *J Assist Reprod Genet* 20.8, pp. 332–42.
- Sathananthan, H., J. Menezes, and S. Gunasheela (2003). "Mechanics of human blastocyst hatching in vitro". In: *Reprod Biomed Online* 7.2, pp. 228–34.
- Sato-Maeda, M. et al. (2006). "Sema3a1 guides spinal motor axons in a cell- and stage-specific manner in zebrafish". In: *Development* 133.5, pp. 937–47.
- Schlossbauer, A. et al. (2010). "A programmable DNA-based molecular valve for colloidal mesoporous silica". In: *Angew Chem Int Ed Engl* 49.28, pp. 4734–7.
- Schorl, C. and J. M. Sedivy (2007). "Analysis of cell cycle phases and progression in cultured mammalian cells". In: *Methods* 41.2, pp. 143–50.
- Schroeder, Andreas et al. (2006). "The RIN: an RNA integrity number for assigning integrity values to RNA measurements." eng. In: *BMC Mol Biol* 7, p. 3.
- Schulz, L.C. and M.R. Roberts (2011). "Dynamic changes in leptin distribution in the progression from ovum to blastocyst of the pre-implantation mouse embryo." In: *Reproduction* 141.6, pp. 767–777.
- Scientific, Thermo (2010). *T042 TECHNICAL BULLETIN NanoDrop Spectrophotometers, 260/280 and 260/230 Ratios*. Tech. rep. ThermoFisherScientific.
- Scott, R. T. et al. (2012). "Delivery of a chromosomally normal child from an oocyte with reciprocal aneuploid polar bodies". In: *J Assist Reprod Genet* 29.6, pp. 533–7.
- Seelenfreund, A. et al. (2014). "Long term storage of dry versus frozen RNA for next generation molecular studies". In: *PLoS ONE* 9.11, e111827.
- Shahbazi, Marta N et al. (2016). "Self-organization of the human embryo in the absence of maternal tissues." eng. In: *Nat Cell Biol* 18.6, pp. 700–708.
- Shao, P. et al. (2011). "The Application of Thermosensitive Nanocarriers in Controlled Drug Delivery". In: *Journal of Nanomaterials* ID 389640, p. 12.
- Shi, J. et al. (2015). "Dynamic transcriptional symmetry-breaking in pre-implantation mammalian embryo development revealed by single-cell RNA-seq." In: *Development* 142.20, pp. 3468–77.
- Shoji, W. and M. Sato-Maeda (2008). "Application of heat shock promoter in transgenic zebrafish". In: *Dev Growth Differ* 50.6, pp. 401–6.
- Simerly, C et al. (2004). "Embryogenesis and blastocyst development after somatic cell nuclear transfer in non-human primates: overcoming defects caused by meiotic spindle extraction." In: *Developmental Biology* 276, p. 5.
- Simon, A. and N. Laufer (2012). "Assessment and treatment of repeated implantation failure (RIF)". In: *J Assist Reprod Genet* 29.11, pp. 1227–39.
- Simón, C. et al. (2000). "Embryonic regulation of endometrial molecules in human implantation". In: *J Reprod Fertil Suppl* 55, pp. 43–53.
- Singh, H et al. (2010). "Early stages of implantation as revealed by an in vitro model." eng. In: *Reproduction* 139.5, pp. 905–914.

- Singh, M., P. Chaudhry, and E. Asselin (2011). "Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors". In: *J Endocrinol* 210.1, pp. 5–14.
- Slowing, I. I. et al. (2008). "Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers". In: *Adv Drug Deliv Rev* 60.11, pp. 1278–88.
- Solter, D and B B Knowles (1975). "Immunosurgery of mouse blastocyst." eng. In: *Proc Natl Acad Sci U S A* 72.12, pp. 5099–5102.
- Song, Wen-Yan et al. (2016). "Comparison of vitrified outcomes between human early blastocysts and expanded blastocysts." eng. In: *In Vitro Cell Dev Biol Anim* 52.5, pp. 522–529.
- Sugawara, A. and M. A. Ward (2013). "Biopsy of embryos produced by in vitro fertilization affects development in C57BL/6 mouse strain". In: *Theriogenology* 79.2, pp. 234–41.
- Sultan, Marc et al. (2008). "A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome." eng. In: *Science* 321.5891, pp. 956–960.
- Summers, M. C. and J. D. Biggers (2003). "Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues". In: *Hum Reprod Update* 9.6, pp. 557–82.
- Sun, L., M. Lee, and J.M. Veltmaat (2011). "A non-enzymatic microsurgical dissection technique of mouse embryonic tissues for gene expression profiling applications." In: *Int J Dev Biol* 55, pp. 969–974.
- Tachibana, M. et al. (2013). "Human embryonic stem cells derived by somatic cell nuclear transfer". In: *Cell* 153.6, pp. 1228–38.
- Tadir, Y. et al. (1989). "Micromanipulation of sperm by a laser generated optical trap". In: *Fertil Steril* 52.5, pp. 870–3.
- Tadir, Y et al. (1990). "Force generated by human sperm correlated to velocity and determined using a laser generated optical trap." eng. In: *Fertil Steril* 53.5, pp. 944–947.
- Tadir, Y et al. (1991). "Micromanipulation of gametes using laser microbeams." eng. In: *Hum Reprod* 6.7, pp. 1011–1016.
- Taft, R. A. (2008). "Virtues and limitations of the preimplantation mouse embryo as a model system". In: *Theriogenology* 69.1, pp. 10–6.
- Takahashi, Hideko and Chengyu Liu (2010). "Archiving and distributing mouse lines by sperm cryopreservation, IVF, and embryo transfer." eng. In: *Methods Enzymol* 476, pp. 53–69.
- Takahashi, M. (2012). "Oxidative stress and redox regulation on in vitro development of mammalian embryos". In: *J Reprod Dev* 58.1, pp. 1–9.
- Tang, F. et al. (2010). "RNA-Seq analysis to capture the transcriptome landscape of a single cell". In: *Nature Protocols* 5.3, pp. 516–535.
- Tarín, J. J. and A. H. Handyside (1993). "Embryo biopsy strategies for preimplantation diagnosis". In: *Fertil Steril* 59.5, pp. 943–52.
- Tarn, D. et al. (2013). "Mesoporous silica nanoparticle nanocarriers: biofunctionality and biocompatibility". In: *Acc Chem Res* 46.3, pp. 792–801.
- Tavares, Lucelia et al. (2011). "Comparison of different methods for DNA-free RNA isolation from SK-N-MC neuroblastoma." eng. In: *BMC Res Notes* 4, p. 3.
- Taylor, T. H. et al. (2010). "The effects of different laser pulse lengths on the embryo biopsy procedure and embryo development to the blastocyst stage". In: *J Assist Reprod Genet* 27.11, pp. 663–7.

- Teklenburg, Gijs and Nick S Macklon (2009). "Review: in vitro models for the study of early human embryo-endometrium interactions." eng. In: *Reprod Sci* 16.9, pp. 811–818.
- Telford, N. A., A. J. Watson, and G. A. Schultz (1990). "Transition from maternal to embryonic control in early mammalian development: a comparison of several species". In: *Mol Reprod Dev* 26.1, pp. 90–100.
- Terentyuk, G. S. et al. (2009). "Laser-induced tissue hyperthermia mediated by gold nanoparticles: toward cancer phototherapy". In: *J Biomed Opt* 14.2, p. 021016.
- Thompson, R. (2012). *A comparison of different power levels used by laser systems in the IVF laboratory*. White Paper released by Research Instruments Ltd.
- Thornhill, A R et al. (2005). "ESHRE PGD Consortium 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)'." eng. In: *Hum Reprod* 20.1, pp. 35–48.
- Vallee, Maud et al. (2009). "Revealing the bovine embryo transcript profiles during early in vivo embryonic development." eng. In: *Reproduction* 138.1, pp. 95–105.
- Van Landuyt, L et al. (2015). "A prospective randomized controlled trial investigating the effect of artificial shrinkage (collapse) on the implantation potential of vitrified blastocysts." eng. In: *Hum Reprod* 30.11, pp. 2509–2518.
- Vanderzwalmen, P. et al. (2002). "Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification". In: *Hum Reprod* 17.3, pp. 744–51.
- Vassena, Rita et al. (2011). "Waves of early transcriptional activation and pluripotency program initiation during human preimplantation development." eng. In: *Development* 138.17, pp. 3699–3709.
- Vizzello, G. et al. (2005). "[Laser assisted intracytoplasmic sperm injection: a more effective and faster technique of immobilization of spermatozoa than traditional one]". In: *Minerva Ginecol* 57.4, pp. 467–70.
- Volodkin, D. V., A. G. Skirtach, and H. Möhwald (2009). "Near-IR remote release from assemblies of liposomes and nanoparticles". In: *Angew Chem Int Ed Engl* 48.10, pp. 1807–9.
- Wan, C. Y. et al. (2014). "Laser-assisted hatching improves clinical outcomes of vitrified-warmed blastocysts developed from low-grade cleavage-stage embryos: a prospective randomized study". In: *Reprod Biomed Online* 28.5, pp. 582–9.
- Wang, J. et al. (2013a). "Increasing cellular uptake of mesoporous silica nanoparticles in human embryonic kidney cell line 293T cells by using Lipofectamine 2000". In: *J Biomed Nanotechnol* 9.11, pp. 1882–90.
- Wang, Q Tian et al. (2004). "A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo." eng. In: *Dev Cell* 6.1, pp. 133–144.
- Wang, Z. et al. (2013b). "Laser microbeam-induced DNA damage inhibits cell division in fertilized eggs and early embryos". In: *Cell Cycle* 12.20, pp. 3336–3344.
- Wang, Zhong, Mark Gerstein, and Michael Snyder (2009). "RNA-Seq: a revolutionary tool for transcriptomics." eng. In: *Nat Rev Genet* 10.1, pp. 57–63.
- Watanabe, D. et al. (2002). "Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis". In: *Mechanisms of Development* 118.1, pp. 187–190.
- Weil, T.T. (2015). "Post-transcriptional regulation of early embryogenesis". In: *F1000 Prime Reports* 7.31.

- Weimar, Charlotte H E et al. (2013). "In-vitro model systems for the study of human embryo-endometrium interactions." eng. In: *Reprod Biomed Online* 27.5, pp. 461–476.
- WHO (2010). *WHO laboratory manual for the examination and processing of human semen*. Fifth. World Health Organization Department of Reproductive Health and Research.
- Wilfinger, W.W., K. Mackey, and P. Chomczynski (1997). "Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity." In: *BioTechniques* 22, pp. 474–481.
- Wilmink, G. J. et al. (2008). "In-vivo optical imaging of hsp70 expression to assess collateral tissue damage associated with infrared laser ablation of skin". In: *J Biomed Opt* 13.5, p. 054066.
- Wong, BJ, CA Boyd, and SE Lanzendorf (2003). "Randomized control study of human zona pellucida dissection using the Zona Infrared Laser Optical System (ZILOS): evaluation of blastomere damage, embryo development and subsequent hatching". In: *Fertility and Sterility* 80, p. 5.
- Wong, C. Y. and J. K. Mills (2017). "Automation and Optimization of Multipulse Laser Zona Drilling of Mouse Embryos During Embryo Biopsy". In: *IEEE Trans Biomed Eng* 64.3, pp. 629–636.
- Wong, Connie C et al. (2010). "Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage." eng. In: *Nat Biotechnol* 28.10, pp. 1115–1121.
- Wong, Kai Mee et al. (2017). "Fresh versus frozen embryo transfers in assisted reproduction." eng. In: *Cochrane Database Syst Rev* 3, p. CD011184.
- Woods, E. J. et al. (2004). "Fundamental cryobiology of reproductive cells and tissues". In: *Cryobiology* 48.2, pp. 146–56.
- Wu, X. et al. (2013). "pH and thermo dual-stimuli-responsive drug carrier based on mesoporous silica nanoparticles encapsulated in a copolymer-lipid bilayer". In: *ACS Appl Mater Interfaces* 5.21, pp. 10895–903.
- Wu, Y. L. et al. (2008). "Development of a heat shock inducible gfp transgenic zebrafish line by using the zebrafish hsp27 promoter". In: *Gene* 408.1-2, pp. 85–94.
- Xia, T. et al. (2009). "Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of siRNA and DNA constructs". In: *ACS Nano* 3.10, pp. 3273–86.
- Xu, J. et al. (2014). "A simple and effective method for the isolation of inner cell mass samples from human blastocysts for gene expression analysis". In: *In Vitro Cell Dev Biol Anim* 50.3, pp. 232–6.
- Xue, Z. et al. (2013). "Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing". In: *Nature* 500.7464, pp. 593–7.
- Yamamoto, R. et al. (2016). "Characterization of gene expression in mouse embryos at the 1-cell stage." In: *J Reprod Dev.* 62.1, pp. 87–92.
- Yan, Liying et al. (2013). "Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells." eng. In: *Nat Struct Mol Biol* 20.9, pp. 1131–1139.
- Yang, P. T. et al. (2011). "In utero gene delivery using chitosan-DNA nanoparticles in mice". In: *J Surg Res* 171.2, pp. 691–9.
- Yu, Z. et al. (2014). "Temperature-responsive DNA-gated nanocarriers for intracellular controlled release". In: *Chem Commun (Camb)* 50.26, pp. 3494–7.

- Zakhidov, S.T. et al. (2010). "Gold nanoparticles disturb nuclear chromatic decondensation in mouse sperm in vitro." In: *Biochemistry Supplement Series A: Membrane and Cell Biology*. 4.3, pp. 293–296.
- Zakhidov, S.T. et al. (2012). "Effect of gold nanoparticles on mouse spermatogenesis." In: *Biology Bulletin* 39.3, pp. 229–236.
- Zandberg, W. F. et al. (2012). "Photothermal release of small molecules from gold nanoparticles in live cells". In: *Nanomedicine* 8.6, pp. 908–15.
- Zhao, M. D. et al. (2012). "Gene therapy of endometriosis introduced by polymeric micelles with glycolipid-like structure". In: *Biomaterials* 33.2, pp. 634–43.
- Zhou, H et al. (2014). "No adverse effects were identified on the perinatal outcomes after laser-assisted hatching treatment." In: *Reproductive BioMedicine Online*. 29, p. 6.
- Ziomek, C. A. and M. H. Johnson (1980). "Cell surface interaction induces polarization of mouse 8-cell blastomeres at compaction". In: *Cell* 21.3, pp. 935–42.
- Zou, J. et al. (1998). "Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1". In: *Cell* 94.4, pp. 471–80.

Appendices



Supplemental Methods

A.1 Mesoporous silica nanoparticle (MSNP) production

In a round-bottomed flask, 0.1g of cetyltrimethylammonium bromide (CTAB, >99%) was dissolved in a mixture of 48ml double distilled water and 0.35ml of 2M NaOH. The solution was heated in a silicone oil bath to 80°C with magnetic stirring. After the temperature had stabilised, 0.5ml of tetraethylorthosilicate (TEOS, >98%) was added to the reaction. After 15 minutes, 0.127ml of 3-(trihydroxysilyl)propyl methylphosphonate (3-THPMP, monosodium salt, 42 wt.% solution in water) was added, and the reaction stirred for 2 hrs at 80°C. The solution was then cooled to room temperature, and MSNPs were recovered and washed twice with methanol via centrifugation. Particles were redispersed in a mixture of 40ml of methanol and 2ml of 12.1M hydrochloric acid (HCL, 37.2 wt.% solution in water, 12.1M), and refluxed for 24 hrs at 80°C with magnetic stirring to remove CTAB. After refluxing, MSNPs were recovered and washed twice in absolute ethanol via centrifugation, and vacuum dried overnight.

A.1.1 Functionalisation of MSNPs with FITC

To synthesise fluorescent MSNPs, fluorescein isothiocyanate (FITC, >90%) was introduced into the silica framework conjugated with 3-aminopropyltriethoxysilane (APTES, >98%) during the main reaction, to form FITC-APTES. The conjugate

was prepared by mixing 100 μ l of APTES with 25mg of FITC in 5ml of absolute ethanol, and stirring magnetically for 12 hrs under a dry nitrogen atmosphere. To produce fluorescent particles, 50 μ l of FITC-APTES conjugate was added to the reaction 10 minutes after 0.5ml of TEOS had been introduced. After 5 minutes, 0.127ml of 3-THPMP was added, and the remaining steps were carried out in accordance with the standard procedure as described by Barkalina et al., 2014a.

A.1.2 Functionalisation of MSNPs with PEI

Functionalisation of the surface of MSNPs with the cationic polymer polyethylenimine (PEI, 50 wt.% solution in water, MW 1.3kD, Mn 1,200g/mol) aimed to reduce particle agglomeration, improve cell interaction and provide a positively charged surface for electrostatic binding of nucleic acids (Xia et al., 2009). To perform the coating, 5mg of dry MSNPs with a size >100nm was redispersed in a mixture of 75 μ l of PEI and 30ml of absolute ethanol in a round-bottomed flask, and stirred at RT for 1 hour. MSNPs were recovered and washed by centrifugation in absolute ethanol and sterile DDW, and vacuum-dried overnight.

B

Supplemental Data

B.1 Infra-red laser cytotoxicity on HEK293T cells

Previous studies conducted in the Coward lab investigated the effects of infra-red laser treatment on HEK293T cells by observing changes in cell morphology, and effects to the plasma membrane integrity using propidium iodide (PI) staining. Figure B.1 shows brightfield images taken before laser treatment, and 30 seconds after laser treatment with 10mJ, 20mJ, or 30mJ pulses. Pulses delivering 30mJ of energy exhibited changes in cell morphology, as indicated by the white arrows in Figure B.1. To assess cytotoxicity, laser-treated HEK293T cells were stained with DAPI and PI and imaged with fluorescence microscopy. Figure B.2 shows fluorescent images of laser-treated cells. The laser treatment delivering 30mJ showed red PI staining and changes to cell morphology appeared as possible blebbing or cytoplasmic leakage, as indicated by the white arrows. Due to these observations when delivering 30mJ laser pulses, for the laser treatment experiments in this thesis, I chose to investigate 10mJ and 20mJ pulses, as they did not show cytotoxicity in these preliminary assessments.

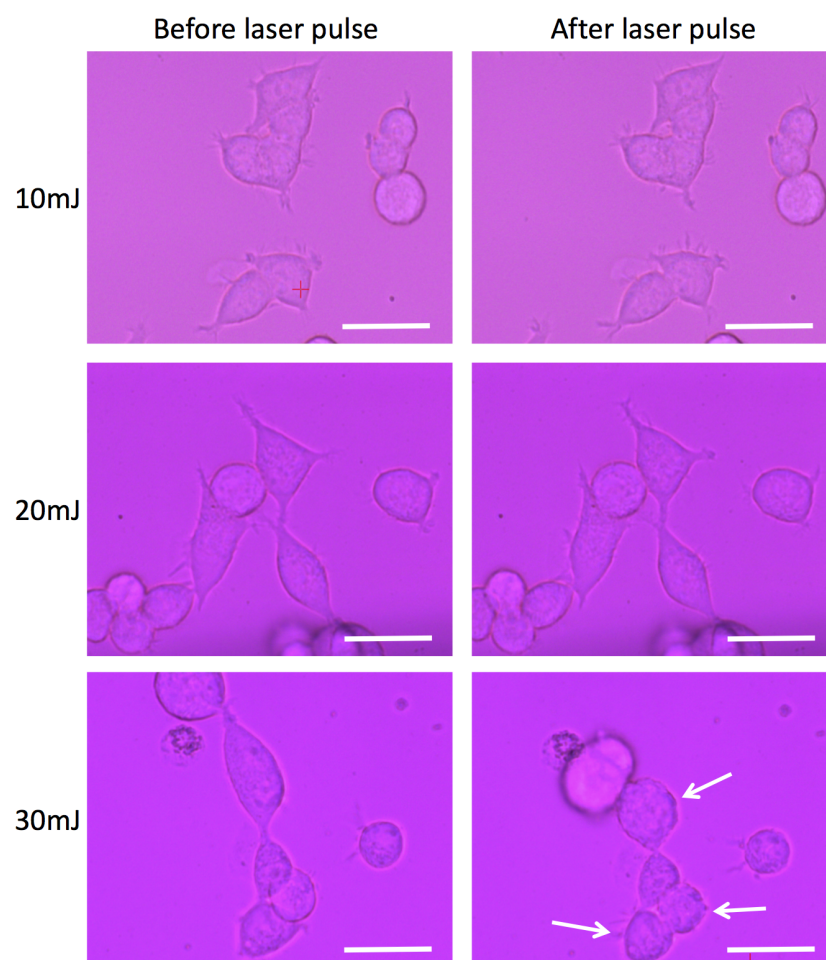


Figure B.1: Representative brightfield images of HEK293T cells before and 30 seconds after being treated with 10mJ, 20mJ, or 30mJ laser pulses. White arrows represent areas of change in cell morphology. Scale bars = $40\mu\text{m}$. 40X magnification.

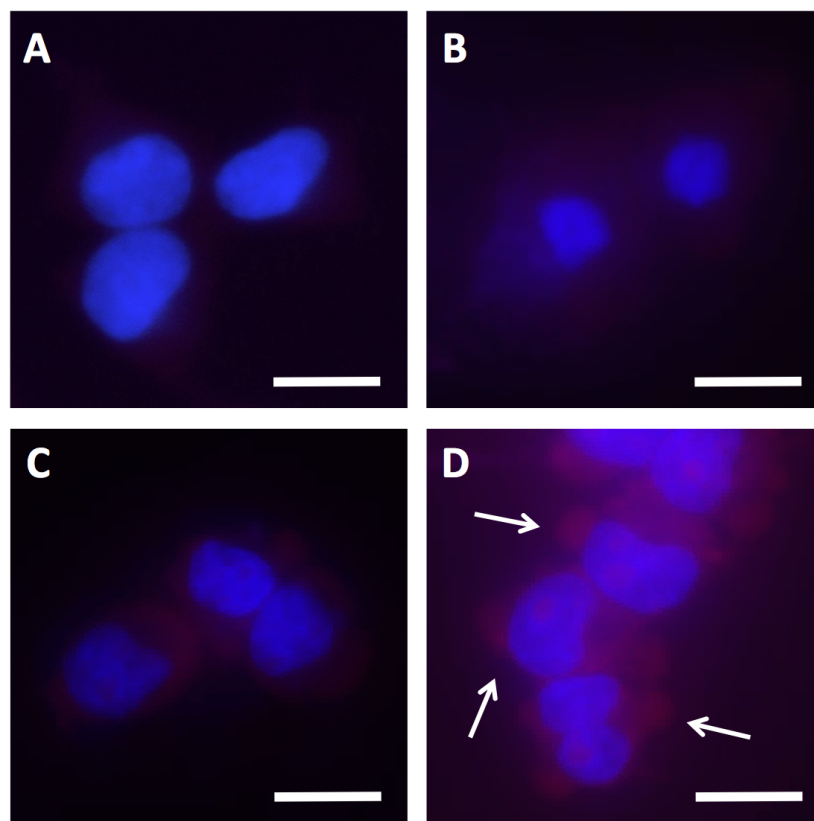


Figure B.2: Representative fluorescence images of HEK293T cells treated with (A) 0mJ, (B) 10mJ, (C) 20mJ, or (D) 30mJ laser pulses. Images are composite overlays of nuclei (DAPI - blue), and damaged plasma membrane (PI - red). DAPI = 4',6-diamidino-2-phenylindole, binds DNA; PI = propidium iodide, indicated non-intact plasma membrane. White arrows represent areas of change in plasma membrane morphology. Scale bars = 20 μ m. 40X magnification.

B.2 The effects of different laser power for trophoctoderm biopsy

For trophoctoderm embryo biopsy, multiple laser pulses are administered to the embryo on multiple days. On day 3, at the cleavage stage, multiple pulses are delivered to create a hole which traverses the full thickness of the zona pellucida, such that when the blastocyst forms and expands, trophoctoderm cells begin to herniate out through the hole. Then, on day 5, laser pulses are administered, as needed, to cell junctions between the cells which have herniated out of the zona pellucida, to separate trophoctoderm cells for biopsy. The investigations in this thesis comparing the 100mW and 400mW laser began with single pulses, however conditions with multiple pulses mimicking clinical conditions are now needed. Therefore, preliminary investigations were started, applying laser pulses mimicking trophoctoderm biopsy. Due to a technical fault requiring the 100mW laser to be sent for repair, these investigations were completed for the 400mW laser, but not for the 100mW laser. The preliminary data can be seen in Figure B.3.

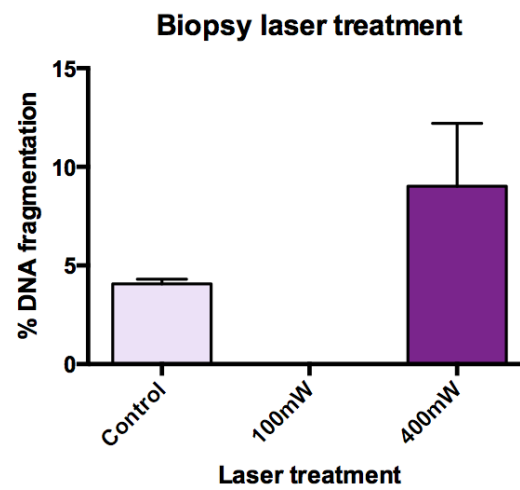


Figure B.3: Preliminary data representing the mean proportion ($\% \pm \text{SEM}$) of mouse embryos showing DNA fragmentation via TUNEL staining following multiple pulse laser treatment at the cleavage stage. Control embryos were not laser-treated.



Research Output

C.1 International peer-reviewed papers

Davidson, L.M., Jones, C., Millar, K. Fatum, M., and Coward, K. (2015). Deleterious effects of obesity upon the hormonal and molecular mechanisms controlling spermatogenesis and male infertility. *Human Infertility*. (24):1-10

Davidson, L.M., Barkalina, N., and Coward, K. (2016). Development of nanoparticle-mediated delivery tools to investigate the role of molecular genetic mechanisms underlying male infertility. *Science Advances Today* (1):25210

Davidson, L.M. and Coward, K. (2016). Molecular mechanisms of membrane interaction at implantation *Birth Defects Research Part C: Embryo Today*. 108(1):19-32

Davidson, L.M., Barkalina, N., Jones, C., and Coward, K. (2016). Development of a laser-activated mesoporous silica nanocarrier delivery system for applications in molecular and genetic research. *J Biomed Optic*. 21(11):115002

Davidson, L.M. and Coward, K. (2017). Safety of infra-red laser drilling parameters upon mammalian embryos at the molecular level. *Human Reproduction. Manuscript, In Preparation*.

C.2 Book chapters

Jones, C., Barkalina, N., Francis, S., **Davidson, L.M.**, and Coward, K. (2017) Applications of nanotechnology in reproductive medicine. In: Nanotechnology in Biology and Medicine (Tuan Vo-Dinh, Ed). *2nd Edition*. *CRC Press/Taylor and Francis, UK*

C.3 Popular science and magazine articles

Davidson, L.M., Jones, C., Griffiths, T., and Coward, K. (2015). Beam me up baby: Exploring the uses of lasers in assisted reproductive technology. *InFocus*, June

Davidson, L.M., and Coward, K. (2016). Think big with nanoparticles. *The Biologist*. 63(4):24-27

C.4 Conference and university presentations

2014 Oxford Developmental Biology Symposium [Poster]. Oxford, UK

2015 British Society for Cell Biology and Developmental Biology [Poster]. Warwick, UK

2015 Oxford Developmental Biology Symposium [Poster]. Oxford, UK

2016 University of Oxford Three Minute Thesis [Oral]. Oxford, UK

2016 Medical Sciences DPhil Day, University of Oxford [Oral]. Oxford, UK

2016 Applied Nanotechnology and Nanoscience International Conference [Oral]. Barcelona, Spain

D

Awards

D.1 Research grants

2016 Rosetrees Seedcorn Grant - *Rosetrees Trust*

Co-wrote and secured a £20 000 research grant to investigate the applications of lasers on embryo development in assisted reproductive technology.

D.2 Academic awards

2016 Graduate Scholar Award - *Trinity College, Oxford*

Awarded to the top 6 Distinction-level doctoral students in 2015/16

2015 Graduate Academic Prize - *Trinity College, Oxford*

For performing at Distinction level in 2014/2015

2014 Clarendon Studentship - *Clarendon Fund; Oxford Medical Sciences Graduate School*

A fully-funded scholarship to cover tuition, college fees and living expenses for the duration of the PhD degree.

D.3 Research awards

2016 Sir Alec Turnbull Scholarship - *Nuffield Department of Obstetrics and Gynaecology*

Departmental Award to present my work at an International NanoScience conference in Barcelona, Spain, 2016

2015 NDOG Departmental Research Poster Award - *Nuffield Department of Obstetrics and Gynaecology*

Second place research poster at the Departmental Away Day on the development of

heat-activated nanoparticle cargo controlled by infra-red lasers

2015 Postgraduate Research Poster Award - *Trinity College, Oxford*

Top postgraduate research poster in Trinity College on the safety of lasers in assisted reproduction

D.4 Other

Higher Education Academy accreditation

Associate Fellow Status of The Higher Education Academy in recognition of attainment against the UK Professional Standards Framework for teaching and learning in higher education (recognition reference PR107431). Designed and instructed a week-long laboratory practical to teach *in vitro* fertilisation using a mouse model to twenty MSc students for the University of Oxford MSc in Clinical Embryology.

2017 Stirling Boyd Prize - *Trinity College Oxford*

Awarded to the student 'whose attainments and influence on the life of the College are, in the opinion of the President and Fellows, of the highest value'

2016 Three Minute Thesis Winner - *Vitae Three Minute Thesis Competition*

1st place winner of the Medical Sciences Division; Runner-up at the University of Oxford Final

2015 Postgraduate Academic Grant - *Trinity College Oxford*

Financial endorsement from Trinity College to present my PhD research at the 50th British Society of Developmental Biology and British Society of Cell Biology joint meeting

2014 Britton Travel Bursary - *Trinity College Oxford*

Financial endorsement from Trinity College to attend the 2014 European Society of Human Reproduction and Embryology (ESHRE) conference in Munich, Germany